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Violacein Biosynthesis and its Regulation in *Pseudoalteromonas* sp. 520P1

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DISSERTATION

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ABSTRACT

Violacein Biosynthesis and its Regulation in *Pseudoalteromonas* sp. 520P1

ZHANG Xi

Violacein is a violet pigment with several significant properties, such as antitrypanosomal and antitumoural activities. These bioactive properties provide a possibility to use violacein for pharmacological purpose or as a bio-dye due to its purple color. The violacein's broad pharmaceutical prospects have attracted increased interests in industrial applications. Violacein can be produced by several Gram-negative bacteria, including *Chromobacterium violaceum*, *Janthinobacterium lividum*, *Pseudoalteromonas luteoviolacea*, *Duganella*, *Collimonas* sp., *Pseudoalteromonas* sp. 520P1 and 710P1. Studies on the violacein biosynthetic pathway have been carried out to reveal the enzymes involved in the biosynthesis and their functions. It has been identified that five enzymes, VioA to VioE, is responsible for the violacein biosynthesis in *C. violaceum*. These five enzymes are coded by a gene cluster consisted of five enzyme genes, *vioA* to *vioE*. The enzymes catalyze the modification and condensation of two molecules of L-tryptophan to form violacein. VioE, the recently reported enzyme, is considered to play a key role to construct the molecular skeleton of violacein.

The violacein biosynthesis in *C. violaceum* has been identified to be under the regulation of quorum sensing mechanism via a diffusible signaling molecule, which is generally termed as *N*-acyl homoserine lactones (AHLs). It is supposed that the accumulated AHLs can bind to a receptor protein to form a complex and this complex interacts with a transcriptional regulator site of the violacein biosynthetic operon to activate the expression of the gene cluster leading to the production of violacein. The putative transcriptional promoter is located at the intermediate region between *vioA* gene and its upstream neighboring protein gene in *C. violaceum*. Although the regulatory site for quorum sensing is also supposed to be located in this upstream region, little evidence for the location of the regulatory site has been obtained. Moreover, following the first cloning of the gene cluster for violacein biosynthesis from the cosmid library of *C. violaceum*, several gene clusters for violacein biosynthesis have been isolated and sequenced in some other violacein-producing bacteria by different methods, including *J. lividum* (genomic sequence), *Pseudoalteromonas tunicata* (whole genome shotgun sequence), and *Duganella* sp. B2 (amplified from genomic DNA by PCR method). However, in most of violacein-producing wild strains other than *C. violaceum*, it is not clarified whether the production of violacein is regulated by quorum sensing or not. Therefore, it is valuable to utilize another violacein-producing bacterium whose violacein biosynthesis is also under the regulation of quorum sensing. We recently demonstrated that the production of violacein by *Pseudoalteromonas* sp. 520P1 was under the regulation of quorum sensing mechanism via AHLs. Therefore, in this study, we used the *Pseudoalteromonas* sp. 520P1 to promote the understanding of detailed

mechanisms of violacein biosynthesis and its regulation. To characterize the violacein biosynthetic pathway and its regulation mechanism, it is essential to identify three significant components: violacein gene cluster including its upstream region, where the promoter and regulatory binding site are supposed to be located; the *luxI* gene (AHL synthesizing enzyme gene) and *luxR* gene (AHL receptor protein gene), which are critical to intermediate the whole regulatory process of the violacein biosynthesis.

We have not identified *luxI* gene and *luxR* gene of strain 520P1 probably owning to the low homology of these two genes in strain 520P1 and other reported strains. In this study, we characterized the violacein gene cluster and its upstream region. We used a fosmid library which allows us to clone the gene cluster with its upstream region. The fosmid library that consisted of approximately 13,000 clones was constructed from the genomic DNA of strain 520P1. Five clones containing the violacein gene cluster were isolated. A cluster of five ORFs (*vioABCDE*) of a total length of 7383bp involved in violacein biosynthesis of strain 520P1 were obtained. This cluster was aligned in a single operon with 79.3% and 52.8% homology to those of *P. tunicata* D2 and *C. violaceum*, respectively. Phylogenetic analysis showed that the violacein genes in these five strains could be divided into three groups: namely a group of *J. lividum* and *Duganella* sp. B2, the second group of *C. violaceum* and the third group of *P.s tunicata* D2 and strain 520P1.

There was an uncoded region of 485 bp between *vioA* gene and its upstream neighboring protein gene. The DNA sequences in this region from strain 520P1 showed low homology (57.3%) to that from *P. tunicata* D2. However, in approximately 200 bp upstream of the violacein gene cluster, a highly conserved

region was found in strain 520P1, strain 710P1 and *P. tunicata* D2. In this region, possible promoter sequences, -10 and -35 box, were predicted. In strain 520P1, we discovered a palindromic sequence, 5'-AAC ATA TGT T-3' (10 bp) centered approximately 16 bp upstream (-21 to -12) from the putative start site of transcription. Moreover, another palindromic structure (5'-CCT ATT ATA GG-3', -32 to -22) was contiguous to this palindromic sequence. These features were shared by the corresponding sequences of strain 710P1 and *P. tunicata* D2. These sequences motioned above might be involved in the binding of LuxR-AHL complex. In the *lux* box (5'-ACC TGT AGG ATC GTA CAG GT-3')of *V. fischeri*, the most studied bacterium in the quorum sensing field, the CTG and CAG sequences (underlined) flanking 10 nucleotides were critical for regulation by LuxR homologue-AHL complex. However, we did not find such kind of special structure "5'-CTGN_{10}CAG-3" in the putative receptor binding site in strain 520P1.

To examine the ability of the gene cluster to synthesize violacein *in vivo*, heterologous expression of the cluster was performed in *E. coli* using a recombinant pET vector. Appearance of violet colonies of recombinant *E. coli* suggested a successful expression of violacein gene cluster from strain 520P1 in *E. coli*. Interestingly, we found that violacein production could occur when the recombinant *E. coli* was incubated at 20 °C, 50 rpm in absence of the inducer (IPTG). UV-VIS spectrum and HPLC analysis showed that the purple pigment produced by recombinant *E. coli* was identical to the violacein. Usually, a mixture of violacein and deoxyviolacein (a by-product of violacein biosynthesis) is produced by strain 520P1. However, the only one peak presented in the elution profile in HPLC indicated that

the recombinant *E. coli* can only produce violacein.

So far, though we have identified the sequences of violacein gene cluster and its upstream region, we still need more evidence to clarify the mechanism of regulation of the expression of the violacein gene cluster. On the other hand, a variant strain, isolated from the cultures of strain 520P1, allowed us to further study the AHLs involved in the regulation of violacein biosynthesis. Strain 520P1 was found to produce violacein under static culture conditions but hardly to produce violacein under an agitated culture condition. This variant showed an ability of highly stable production of violacein under agitated culture conditions. We did not find any difference in violacein gene cluster and its upstream region between strain 520P1 and the variant strain. As we have not yet identified the *luxI* gene and *luxR* gene, we examined the AHL produced by the variant strain. From the analysis of AHL produced by the variant strain, the production of violacein by this strain seemed to be regulated by quorum sensing. The relative amount of AHL produced by the variant strain was much higher than that produced by strain 520P1. TLC analysis of AHL showed only one kind of AHL produced by the variant strain, while two kinds of AHLs were produced by strain 520P1. These two differences of AHLs between strain 520P1 and the variant strain might be reasons for the ability of violacein production by the variant strain under agitated culture conditions. The differences of AHLs production may be caused by the mutation(s) of the *luxI* and *luxR* genes. LuxI enzyme is responsible for the AHL production. Therefore, the change of properties of the enzyme reaction may lead to the increased production of AHL(s) and/or the production of different kind of AHL(s). Similarly, the mutation in *luxR* gene might

change the specificity of LuxR receptor protein for AHLs, leading to the binding of different type of AHL(s). Correspondingly, it may change the specificity of the binding to the regulatory site on the target DNA. The mutation(s) mentioned above might ultimately affect the expression of the violacein gene cluster, leading to a different mode of violacein production.

The gene cluster and the variant strain isolated here could be used for further studies on violacein biosynthesis, quorum sensing regulation of the expression of the cluster genes, and the recombinant production of violacein.

Key Words: *Pseudoalteromonas*· violacein gene cluster · fosmid library · promoter prediction · heterologous expression · variant strain· acyl homoserine lactone (AHL)

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CHAPTER 1

Introduction

1.1 Introduction of violacein

Violacein, a blue-violet pigment, is a kind of secondary metabolite with various important bioactivities produced by several Gram-negative bacteria. Violacein production was firstly found in *Chromobacterium violaceum* in 19th century [1]. Violacein is an indole derivative synthesized from two molecules of tryptophan (**Fig. 1.1)**. Deoxyviolacein is a by-product accompanied with violacein. The crystal structures of violacein and deoxyviolacein have been identified by Lasstsch and Thomson [2].

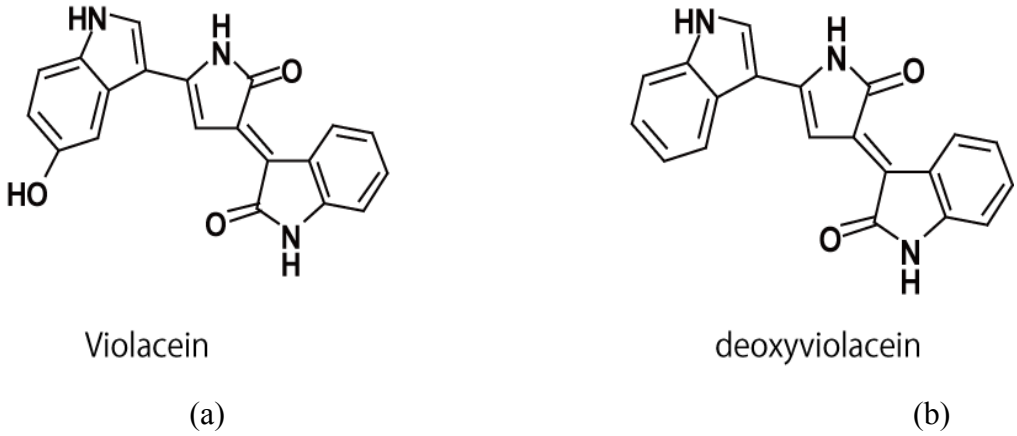


Fig. 1.1 Structures of vioacein and deoxyviolacein

(a): Structure of violacein;

(b): Structure of deoxyviolacein, a by-product in violacein biosynthetic pathway.

The antibacterial activity of violacein has been reported by several researchers [3]-[5]. Violacein possessed a strong inhibitory effect on most of the Gram-positive bacterial growth, but showed small or no effect on Gram-negative ones [6]-[8]. In addition, the activity of violacein against fungi such as *Rosellinia necatrix* (a plant pathogen that cause the root rot disease of mulberry tree) allows us to use violacein as a fungicide [9] [10]. Violacein has been also studied extensively as an antitrypanosomal agent to treat leishmania [11]-[13]. Violacein was also reported to show antiviral and antimalarial activities [14]-[16]. Recently, many studies reported the antitumor acitivity and cytotoxicity of violacein in several cell lines [17]-[20]. In many of tumor cell lines, violacein has been shown to exert its effect by inducing apoptosis of cells. However, no inhibitory effect of violacein was found in human normal cells, indicating violacein to be a potential medicine for the treatment of cancer. On the other hand, violacein is a good material for man-made dying of fabrics because of its stable color [9]. These broad pharmaceutical and industrial prospects of violacein have attracted increased interest in industrial applications.

1.2 Violacein-producing bacteria

Chromobacterium violaceum was the first bacterium that was found to produce violacein. Since then, several violacein-producing bacteria have been reported, such as *Janthinobacterium lividum* [7][9][21], *Pseudoalteromonas luteoviolacea* [22][23], *Pseudoalteromonas tunicate* [24], *Duganella* sp. [25], *Collimonas* sp. [26], and *Pseudoalteromonas* sp. 520P1 and 710P1 [27]. In the reported violacien-producing bacteria, *C. violaceum* is the most studied bacterium. However, *C. violaceum* was

found to be an opportunistic pathogen to animal and human beings and *C. violaceum* infection could lead to septicemia [28]-[32]. Therefore, *C. violaceum* is not an appropriate strain for a large scale production of violacein. On the other hand, relatively low, inefficient and unstable violacein production in other violacein-producing strains restricted the practical application of violacein [33][34]. To solve this problem, many studies on exploring new methods to produce violacein have been conducted using newly isolated strains and recombinant bacteria [26][35][36].

1.3 Violacein biosynthesis in *C. violaceum*

Early studies on the pathway of violacein synthesis proposed 5-hydroxy-L-trptpohan as a precursor in violacein biosynthesis [37]. However, it was found later that the oxygen atoms of violacein were originated from the molecular oxygen, and all the carbon, nitrogen and hydrogen atoms of violacein were derived from two molecules of L-tryptophan [13][37]-[40]. It is now evident that two molecules of tryptophan form a basic structure of violacein followed by the oxygenation of indole rings of the intermediate. Until recently, it was considered that four genes (*vioABCD*) were involved in violacein synthesis [41][42]. However, the complete genome sequence of *C. violaceum* determined in 2003 [43] suggested the presence of the fifth gene in the violacein gene operon. The violacein biosynthetic pathway was not fully identified until the discovery of the role of this newly found gene [44]-[46]. VioE, the product of the fifth gene, catalyzed the formation of protodeoxyviolacenic acid from compound X, an unidentified intermediate (**Fig. 1.2**). Thus, VioE was proved to be indispensable for the formation of the skeleton of violacein structure.

Detailed structure and reaction mechanisms of VioE have been reported [47] [48]. Five enzymes, VioA-VioE, responsible for violacein biosynthesis are encoded by a gene cluster consisting of five genes, *vioA–vioE*, aligned in a single operon. Balibar and Carl cloned and expressed the enzymes VioA-VioE *in vitro* and reported that L-tryptophan, not the 5-hydroxy-L-trptpohan, was the precursor of violacein [46]. The proposed biosynthetic pathway of violacein and deoxyviolacein is shown in **Fig. 1.2.**

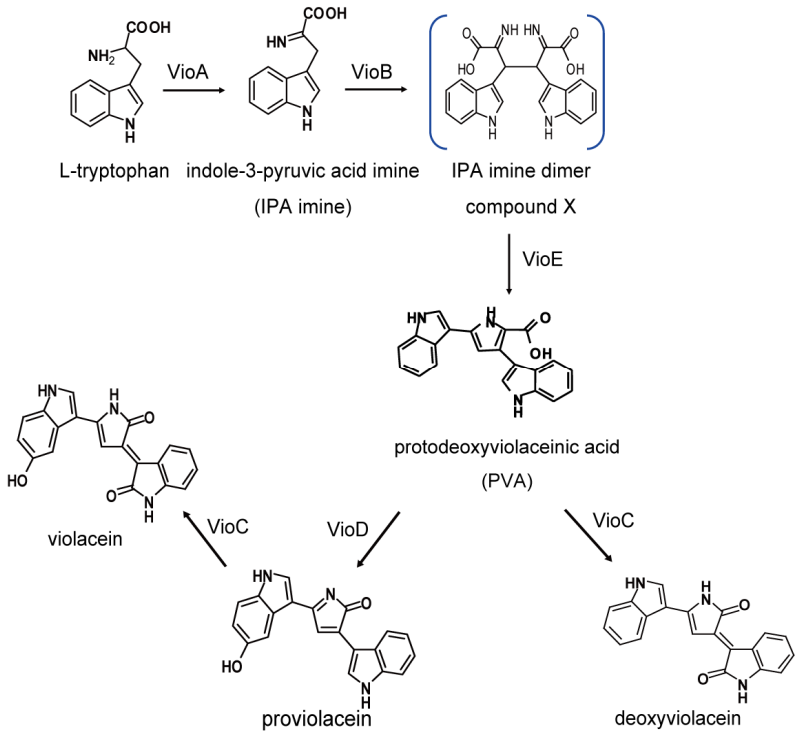


Fig. 1.2 Biosynthetic pathway of violacein and deoxyviolacein in *C. violaceum* (modified from Hirano et al., 2008)

VioA catalyzes the oxidation of tryptophan. VioB is supposed to be responsible for coupling two molecules of IPA imine to form an unknown intermediate X. VioE, a new enzyme recently found, produces protodeoxyviolaceinic acid. Subsequently, VioD and VioC catalyze the oxygenation at the5 and 2 positions of indole rings to produce violacein. Deoxyviolacein, the by-product of violacein, is produced by oxygenating at 2 position of indole ring with VioC from protodeoxyviolaceinic acid.

Following the first cloning of the gene cluster for violacein biosynthesis from the cosmid library of *C. violaceum* [49], several gene clusters for violacein biosynthesis have been sequenced. These are the gene clusters from uncultured microorganisms (VioABCD, from an environmental DNA cosmid library) [42] and some other violacein-producing bacteria, including *J. lividum* (genomic sequence) [50], *Pseudoalteromonas tunicata* D2 (whole genome shotgun sequence) [51], and *Duganella* sp. B2 (amplified from genomic DNA by polymerase chain reaction) [35]. The accumulated DNA sequences of violacein gene clusters from a variety of bacteria collected from soil, water and ocean suggested that the gene clusters share the evolutionary origin and expanded in Gram-negative bacteria through horizontal gene transfer.

1.4 Quorum sensing regulation of secondary metabolites

Violacein is a typical secondary metabolite. In *C. violaceum* and *Pseudoalteromonas* sp. 520P1, violacein production has been shown under the regulation of quorum sensing mechanism through autoinducer molecules secreted by the bacteria. Quorum sensing is a phenomenon that bacteria communicate with each other *via* some certain signaling molecules called autoinducer (**Fig. 1.3**) [52]-[55]. Many secondary metabolites production, such as the production of antibiotics, toxin, pigment, biofilm formation and bioluminescence, are all regulated by this quorum sensing system.

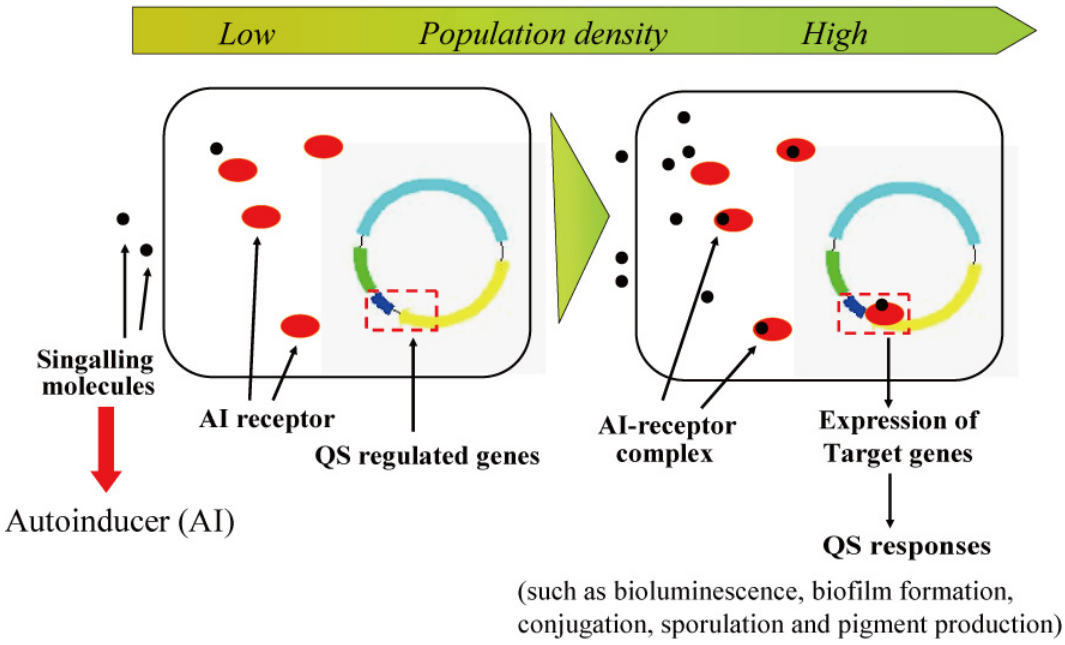


Fig. 1.3 Quorum sensing in Gram-negative bacteria

Gram-negative bacteria constantly secrete some certain signaling molecules, called autoinducers which are mostly acyl-homoserine-lactones (AHLs). At high cell densities, the high concentration of accumulated AHL promotes the receptor protein to form a complex with its cognate AHL. Then this complex actives the transcription of target genes.

Many Gram-negative bacteria use *N*-acyl homoserine lactone (AHL) as autoinducers to conduct this process. The structures of AHLs were shown in **Fig. 1.4**. Bacteria could produce a mixture of AHLs differing in the R group (the difference in the length of acyl side chains and the oxidation or hydroxylation at C3 position).

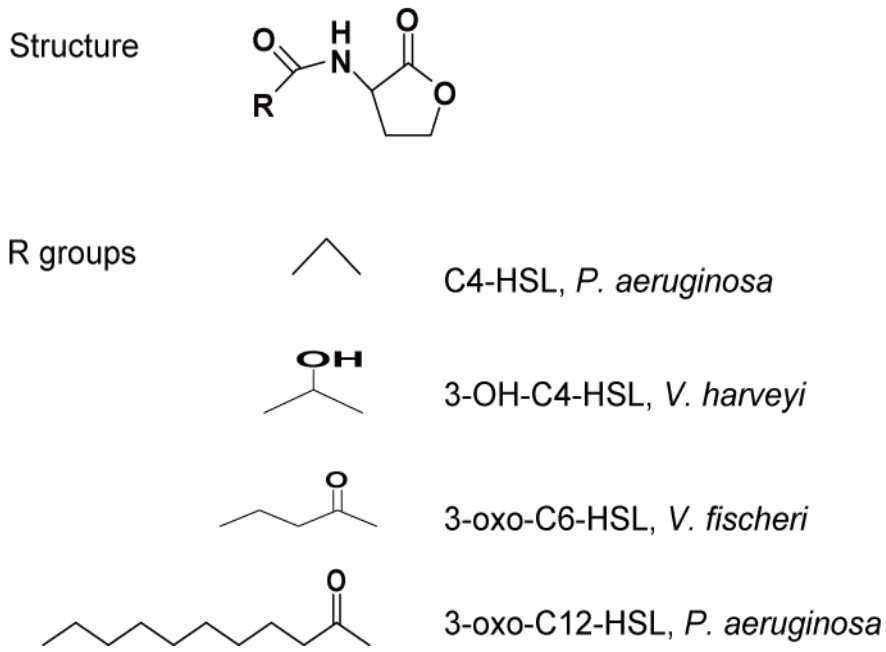


Fig. 1.4 Structures of AHLs

The general structure of AHL is shown on the top of the figure. Following structures are examples of R groups. The abbreviated names of the AHLs and the corresponding AHL-producing bacteria are given.

1.5 Mechanism of quorum sensing in violacein biosynthesis

McClean [56] demonstrated that violacein biosynthesis in *C. violaceum* is under the regulation of a quorum sensing mechanism *via N*-acyl homoserine lactones (AHLs) (**Fig. 1.5**). It is supposed that the accumulated AHL binds to a receptor protein to form a complex and this complex interacts with a transcriptional regulator site of the violacein biosynthesis operon to activate the expression of the gene cluster, thereby leading to the production of violacein.

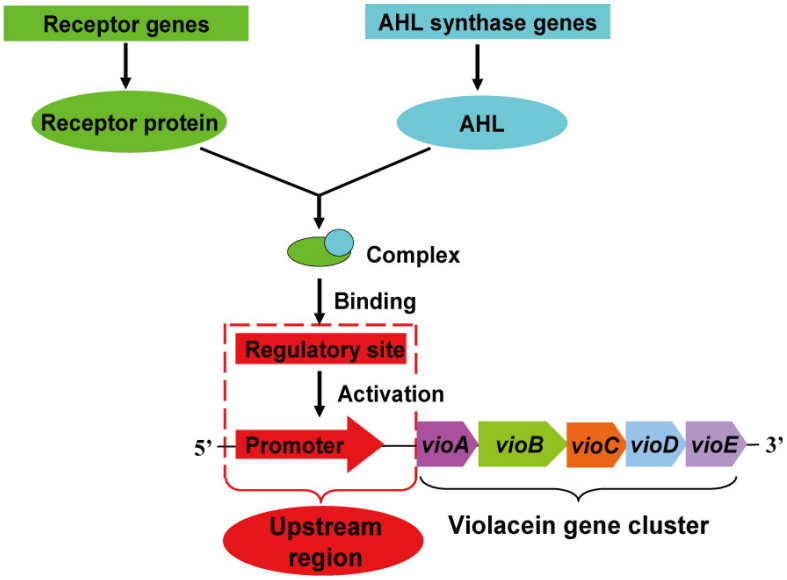


Fig. 1.5 Supposed mechanism of regulation of expression of violacein gene cluster in *C. violaceum*

Three significant elements involved in the regulation of violacein biosynthesis are colored. 1, AHL synthase gene and its product, AHL (in blue); 2, Receptor protein gene and AHL receptor protein (in green); 3, upstream region of violacein gene cluster (in red), the supposed location of the promoter and the regulatory site

The putative transcriptional promoter is located in an intermediate region between the vioA gene and its upstream neighboring protein gene in *C. violaceum* [41]. Although the regulatory site for quorum sensing is supposedly located in this upstream region, little evidence for the location of the regulatory site has been obtained. Moreover, in most violacein-producing strains other than *C. violaceum*, it has not been clarified whether the production of violacein is regulated by a quorum sensing mechanism or not.

1.6 *Pseudoalteromonas* sp. 520P1

Pseudoalteromonas sp. 520P1 is a Gram-negative bacterium isolated by our group from seawater, Cap Muroto, Japan [27]. Strain 520P1 shows ability of producing violacein only under static culture conditions. The previous results suggest that the production of violacein by *Pseudoalteromonas* sp. 520P1 was under the regulation of a quorum sensing mechanism *via* AHLs [57]. Therefore, to clarify the detailed mechanisms of violacein biosynthesis and its regulation, we attempted to clone and characterize a gene cluster for violacein biosynthesis from *Pseudoalteromonas* sp. 520P1, using a fosmid library that allows us to clone the gene cluster with its upstream region. Additionally, a variant strain (No. 4-2-3) capable of producing violacein under agitated culture conditions was isolated from the culture of strain 520P1 in this study. We investigated the mode of productions of violacein and AHLs by strain No. 4-2-3.

CHAPTER 2

Cloning and Heterologous Expression of a Gene Cluster for Violacein Biosynthesis in *Pseudoalteromonas* sp. 520P1

2.1 Introduction

Fosmid is a plasmid similar to cosmid, but uses the F-plasmid origin for replication. As the host cell can only contain a single fosmid cloning vector, the fosmid library with low copy number offers much higher stability than cosmid library with comparable high copy number. Fosmid vector can contain a 40 kb insert DNA fragment from randomly sheared genomic DNA of the target organism. Compared with the library containing insert DNA fragments resulting from partial restriction digest, fosmid library is constituted of much more unbiased DNA fragments. Fosmid system has been used for constructing stable libraries from complex genomes [58] and isolating genes [50] from the genomic DNA.

Five genes, responsible for violacein biosynthesis, are aligned together in a single operon to form a cluster. The expression of the violacein gene cluster is under the control of a single promoter. Several violacein gene clusters have been isolated and cloned from the violacein-producing bacteria using different experimental methods [35][49]-[51]. There are several cloning strategies to obtain the complete gene cluster from *Pseudoalteromonas* sp. 520P1. The efficiency and stability of the plasmid vectors are certainly essential for cloning. The selected plasmid vector should also

meet the requirements for the desired application. We desired to obtain the complete sequences of the violacein gene cluster as well as it upstream region where the promoter and the regulatory site are supposed to be located. The expected size of the violacein gene cluster in strain 520P1 is approximately 8 kbp. Moreover, little information of the sequences of the violacein gene cluster as well as its upstream has been known. Therefore, the fosmid system, which offers an unbiased library with insert DNA fragments of 40 kb, is an exactly suitable candidate for cloning the violacein gene cluster from strain 520P1.

The pET system is a fomous expression system and now widely used to produce many copies of a desired protein in a host cell. The pET vector contains several important elements: a *lacI* gene codes for the *lac* repressor protein; a T7 promoter, only specific to T7 RNA polymerase (not bacterial RNA polymerase); a *lac* operator blocks transcription; a polylinker; an f1 origin of replication resulting in producing a single-stranded plasmid; an ampicillin resistance gene for easy selection; and a ColE1 origin of replication. To examine the ability to synthesize violacein *in vivo*, the cloned gene cluster was subcloned in pET28a vector and then expressed in *E. coli*. We believe that the recombinantly engineered strain presented here can be used to produce violacein in industrial applications.

2.2 Materials and methods

2.2.1 Bacterial strains, growth conditions, plasmids

Pseudoalteromonas sp. 520P1 was cultured at 20°C in PPES-II culture mediumas

described previously [27]. *Escherichia coli* BL21 (DE3), DH5α and plasmid pET28a were purchased from Novagen (Darmstadt, Germany). All the *E. coli* strains were grown at 37°C on Luria-Bertani (LB) agar or in LB broth supplemented with the appropriate antibiotics. *E. coli* DH5α was used as the host for transformation with the recombinant plasmid pET28a-VGC containing the violacein gene cluster. *E. coli* BL21 (DE3) was used as the host for heterologous expression of violacein genes. Kanamycin (50 μg/ml) was used for selection of recombinants *E. coli* harboring the violacein gene cluster. Chloramphenicol (12.5 μg/ml) was used for the construction of fosmid library. All the relevant strains and plasmids used in this study were shown in **Table 2.1**.

Table 2.1 Strains and plasmids used in this study

Name	Description	Comments
E. coli DH5α	Transformation with recombinant plasmid pET28a-VGC	Strain
E. coli BL21 (DE3)	Heterologous expression of violacein genes	Strain
E. coli EPI300-T1^{R}	Construction of the genomic DNA library	Strain
pET28a	Cloning of the violacein gene cluster	Plasmid
pET28a-VGC	Recombinant plasmid with violacein genes	Plasmid
pCC2FOS	Construction of the genomic DNA library	Fosmid vector

Ex Taq and *LA Taq* were purchased from Takara (Otsu, Japan) for PCR

amplification. KOD plus DNA polymerase was purchased from Toyobo (Osaka, Japan). DNA marker, λ DNA mono cut mix, was purchased from New England Biolabs (Ipswich, MA, USA). Restriction enzymes *Nco*I, *Nde*I and *Bam*HI used in this study were purchased from Takara (Otsu, Japan).

2.2.2 Construction of a genomic DNA library

A fosmid DNA library for *Pseudoalteromonas* sp. 520P1 was constructed using a CopyControl Fosmid Library production kits (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions. The schematic diagram of the construction of the genomic DNA library was shown in **Fig. 2.1**.

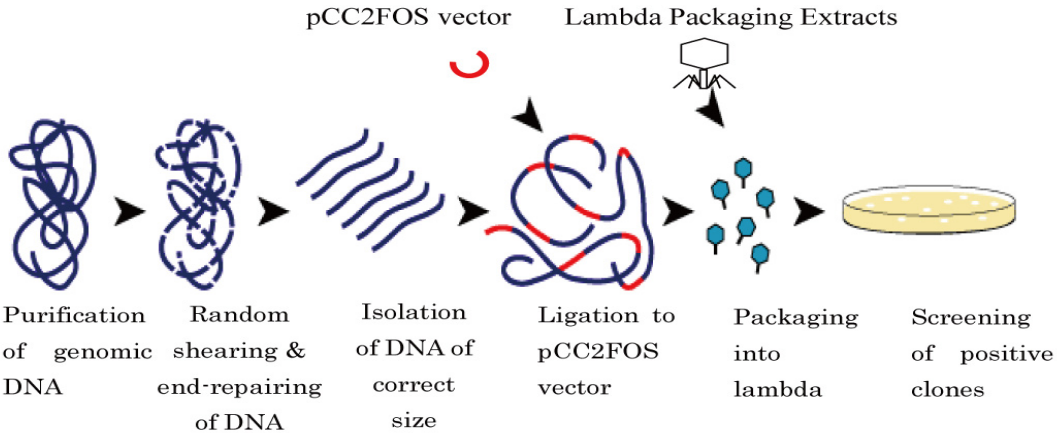


Fig 2.1 Schematic diagram of the construction of the genomic DNA library

Modified from manufacturer's instructions of CopyControl Fosmid Library production kits (Epicentre Biotechnologies, Madison, WI, USA).

Strain 520P1 was incubated on PPES-II plate medium and a single colony was picked and cultured in 5 ml PPES-II culture medium. After 16 h of incubation, the

genomic DNA of strain 520P1 was obtained with a genomic DNA isolation kit (Genomic-tip 100/G kit, Qiagen K.K., Tokyo, Japan) and then randomly sheared by passing through a sterile hypodermic needle (0.40 × 19 mm, Terumo, Tokyo, Japan). DNA fragments of 25–40 kb were isolated by pulse-field gel electrophoresis (PFGE) (Pharmacia Biotech, Uppsala, Sweden) using 1.0% low melting point agarose gel (SeaPlaque GTG Agarose, TaKaRa, Otsu, Japan) in 1 × Tris–acetate-EDTA (TAE) buffer for 16 h under the conditions of 6 V/cm, 5–30 s switching of the pulse. DNA fragments were treated according the manufacturer's instruction so that they were blunt-ended and cloned into the pCC2FOS fosmid vector linearized at the restriction enzyme site *Eco*72I and dephosphorylated. After ligation, the fosmid vector was packaged into lambda phages, and then the packaged DNA was transformed into *E. coli* EPI300-T1^{R}. The *E. coli* transformants were diluted and plated on LB agar containing chloramphenicol (12.5 μg/ml). About 250–300 colonies were observed on each plate after incubating at 37°C overnight. The genomic DNA library were divided into several clone pools, approximately 500 clones per pool, and stored at -80°C.

2.2.3 Isolation of the violacein gene cluster from strain 520P1

A partial *vioC* sequence was employed to screen fosmid clones containing the violacein gene cluster. Based on the sequence homology of violacein gene clusters from *J. lividum*, *C. violaceum* and *P. tunicata* D2 (GenBank Accession No. EF063591, AE016825 and AAOH00000000, respectively), several pairs of primers were designed (**Table 2.2**) and then employed to amplify the violacein genes using the genomic DNA of strain 520P1 as a template using *Ex Taq* DNA polymerase. The PCR

conditions were shown in **Table 2.3**.

Only one pair of primers, vioC-Fw1 and vioC-Rv1 (**Table 2.2**), were successful in amplifying a 1260 bp polymerase chain reaction (PCR) product using the genomic DNA of strain 520P1 as a template. Then the PCR product was extracted and purified using QIAquick Gel Extraction Kit (Qiagen K.K., Tokyo, Japan). The DNA sequence of extracted PCR product was determined in Bio Matrix Research (Nagareyama, Japan).

Table 2.2 Synthetic oligonucleotide primers used for PCR amplification of violacein genes of strain 520P1

Primers	Sequence
vioB-Fw1	5’-GGTAATAACCATTTTTCTTGGGAA-3’
vioB-Rv1	5’-TGCGTTCATTAAGCGCGAGCGTCG-3’
vioB-Fw2	5’-CTTTGGGGACACTACAACGACTA-3’
vioB-Rv2	5’-TAATGAATCATTTCTTCATGAGC-3’
vioC-Fw1	5’-ATTATCGTTGGTGGTGGCCTAGCAGG-3’
vioC-Rv1	5’-AATTTTGTACCAAACGTTTTGTTT-3’
vioC-Rv2	5’-TCAGCCATATCAGGGGGATAGA-3’
vioD-Fw1	5’-ATTCTTGTCATCGGTGCAGGTCCTGCC-3’
vioD-Rv1	5’-CATGGTGGTGCCATGGCCGATAGAGAA-3’
vioD-Fw2	5’-GGTTGGGGTGTGGTGCTGCCAGGT-3’
vioD-Rv2	5’-ATGCCCAGATTGCAGTGCATCGCC-3’

Table 2.3 PCR conditions for amplifying the violacein genes of strain 520P1

Temperature (°C)	Time	Cycles
95	1 min	1
95	15 sec	25
43	15 sec	
72	1 min	
72	5 min	1
4	∞	1

Another pair of primers, vioC-Fw3 and vioC-Rv3 (**Table 2.4**), complementary to the sequence of the PCR product were designed and used to screen clones from the fosmid DNA library. The screening was performed according to the method of Hrvatin [70]. Briefly, approximately 2,000 fosmid clones were divided into 20 clone pools (100 clones per pool). Each pool was examined by PCR using the primers described above. Clones in the positive group were divided into subgroups and examined by PCR until positive clones were obtained.

Table 2.4 Synthetic oligonucleotide primers used for PCR amplification

Primers	Sequence*	Comments
vioC-Fw1	5’-ATTATCGTTGGTGGTGGCCTAGCAGG-3’	Amplification of *vioC*
vioC-Rv1	5’-AATTTTGTACCAAACGTTTTGTTT-3’	Amplification of *vioC*
vioC-Fw3	5’-TCAAGGGCAATAGGTGTCAGTATG-3’	Screening of *vioC*

vioC-Rv3	5’-GTCTTCAAGAGCCATATTCATGCC-3’	Screening of *vioC*
pET28a-VGC-Fw	5’-CATGCCATGGGCTCGAATCAAGAAAATAC-3’	Subcloning into pET28a
pET28a-VGC-Rv	5’-GGGAATTCCATATGTTATTTATTTAAGGG-3’	Subcloning into pET28a

*Restriction enzyme sites are underlined.

2.2.4 Sequencing and analysis of the violacein gene cluster

The positive clones were cultured in LB liquid medium with 12.5 μg/ml of chloramphenicol for 16 h. The recombinant plasmids were extracted and purified using Qiaprep Spin Miniprep Plasmid Kit (Qiagen K.K., Tokyo, Japan). The extracted plasmids from the positive colonies were digested by restriction enzyme *Bam*HI. To determine the size of the insert DNA, the plasmids and the digested DNA fragments were performed on PFGE as described in **2.2.2**. The longest insert DNA from one of the positive clones was sequenced using vioC-Fw3, vioC-Rv3 along with the other primers listed in **Table 2.5**. DNA sequencing was carried out using the dye-terminator method in Bio Matrix Research (Nagareyama, Japan). The determined sequence of the violacein gene cluster with its upstream and downstream regions was deposited in the DDBJ under the accession number AB573101. Homology of the violacein gene cluster in strain 520P1 was compared with those of other bacterial strains using Genetyx version 8 software (Genetyx Corporation, Tokyo, Japan). The phylogenetic tree was constructed with the distance matrix using the neighbor-joining method [59]. The upstream sequence of the violacein gene cluster from *Pseudoalteromonas* sp. 710P1, a violacein-producing bacteria isolated by our group in a previous study [27],

was obtained by PCR with primers, 520P1-before-vioA-Fw2 and 520P1-vioA-Rv1 (**Table 2.5**). Promoter prediction was performed with BPROM software (SoftBerry, Mount Kisco, NY, USA).

Table 2.5 Synthetic oligonucleotide primers for sequencing the violacein genes

Primers	Sequence
520P1-before-vioA-Fw1b	5′—GAAGCTAATAGCTACATTACCCCTATC—3′
520P1-before-vioA-Fw2	5′—ACTATTTCAAGGTTTAGGTCGAGCTTTAC—3′
520P1-before-vioA-Fw3	5′—CGATGTAACATTAATATTAATTCAGGATAG—3′
520P1-vioA-Fw1	5′—TATATCCATTGTAGGGGCTGGAGTTTC—3′
520P1-vioA-Fw2	5′—ACCAACAGCAATGAGCAGGCTCAATTG—3′
520P1-vioB-Fw1	5′—TTTGATTTAAAACAACACCCAAGTAAATG—3′
520P1-vioB-Fw2	5′—ACAACCAGATGATACCTCTCAATTTTCACC—3′
520P1-vioB-Fw3	5′—GCTGACAAAGTATTTAGTATGGCCGATAAG—3′
520P1-vioB-Fw4	5′—TATTTTTAAACGAAATAATAAACCGTGC—3′
520P1-vioB-Fw5	5′—CGATCTCGATTAATGAATGCCGCTATAG—3′
520P1-vioC-Fw4	5′—AAGCCCAGTAATGATCTTATTAATGTGCG—3′
520P1-vioD-Fw1	5′—CATCATAATGACTCAGCCTTAACTAAAAC—3′
520P1-vioD-Fw2	5′—AAGCCTATAGCGATAATATTGTTTTACTAG—3′
520P1-vioE-Fw1	5′—TGAAGCTGCAGTATTAAATGATGAAATAG—3′
520P1-before-vioA-Rv1	5′—GTACATCAATGGTAAAAGTAATACATAG—3′
520P1-vioA-Rv1	5′—CTGTTCTTGAACTTTAATTGCATGCGCTC—3′

520P1-vioA-Rv1c	5′—TGTAATAAGCTCATAATCAAAATGAAACTC—3′
520P1-vioA-Rv1b	5′—TTCTTCAGCTTCAATCCGTTTTAGAAG—3′
520P1-vioB-Rv1a	5′—CTTATTAACAATGTACCCTGGGTTTAGCC—3′
520P1-vioB-Rv3	5′—CATAACTTTGATATGGAATGCTAATGGGC—3′
520P1-vioB-Rv2a	5′—CTTATCGGCCATACTAAATACTTTGTCAGC—3′
520P1-vioB-Rv2	5′—AAATTGTGGAGAATCAGCACTAGCGCCTTC—3′
520P1-vioC-Rv4	5′—CAGCCTCTATGCCACGGACAGTCATAC—3′
520P1-vioD-Rv1	5′—CCATTTGAAACAACAACTAAATCATATTTG—3′
520P1-vioD-Rv2	5′—TAGAGGAGGTAATTGGTTTCTGCGCG—3′
520P1-vioE-Rv1	5′—TTACTGACGCCTGTACTTTTGGATCTCC—3′
520P1-after-vioE-Rv1	5′—CCGGGTGTAGATGGTAAAGCTAAAATGTC—3′

2.2.5 Cloning and expression of the violacein gene cluster

The plasmid containing the violacein gene cluster was extracted and purified from the positive fosmid clone (No. 3) as previously described. Based on the sequences of the violacein gene cluster in strain 520P1, two primers, pET28a-VGC-Fw and pET28a-VGC-Rv (**Table 2.4**), were designed and employed to amplify the violacein gene cluster using *LA Taq* DNA polymerase. The PCR product containing restriction enzyme sites on both ends was cloned into the multiple cloning site of pET28a linearized with the restriction enzymes *Nco*I and *Nde*I. The schematic diagram of construction of recombinant vector was shown in **Fig. 2.2**. The recombinant vector was transformed into *E. coli* DH5α by electroporation and recombinant *E. coli* was

selected on an agar plate containing 50 μg/ml of kanamycin.

The positive colonies containing violacein gene cluster were selected by PCR using vioC-Fw3 and vioC-Rv3 as primers and then cultured in 5 ml of LB with 50 μg/ml of kanamycin at 37°C for 16 h. The recombinant plasmid was extracted and purified as previously described and then transformed into *E. coli* BL21 (DE3) by electroporation. A single colony BL21 (DE3) harboring the recombinant plasmid was picked up from the medium plate with 50 μg/ml of kanamycin, and then incubated in 5 ml of LB (50 μg/ml of kanamycin) at 37°C, overnight. Subsequently, 0.5 ml of the culture was transferred into 50 ml of fresh LB containing 50 μg/ml of kanamycin. After the OD_{600} of the culture reached 1.0 by incubation at 37°C for 2 h, 0.1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce the expression of the violacein gene(s). An aliquot of 1.0 ml of the broth was sampled after the incubation with IPTG (0.1 mM) at 20°C for 24 h and then sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to confirm the protein expression. The samples for SDS-PAGE were prepared as follows. Bacterial cells were pelleted by centrifugation at 12,000 rpm (T15A11, Hitachi, Japan) for 5 min at 4°C and resuspended in 100 μl of ultrapure water. A 100 μl aliquot of 2 × SDS-PAGE sample buffer was added into the suspensions. The mixed suspensions were heated for 5 min at 90°C. After the suspensions were cooled to room temperature, 20 μl of the mixture were subjected to SDS-PAGE.

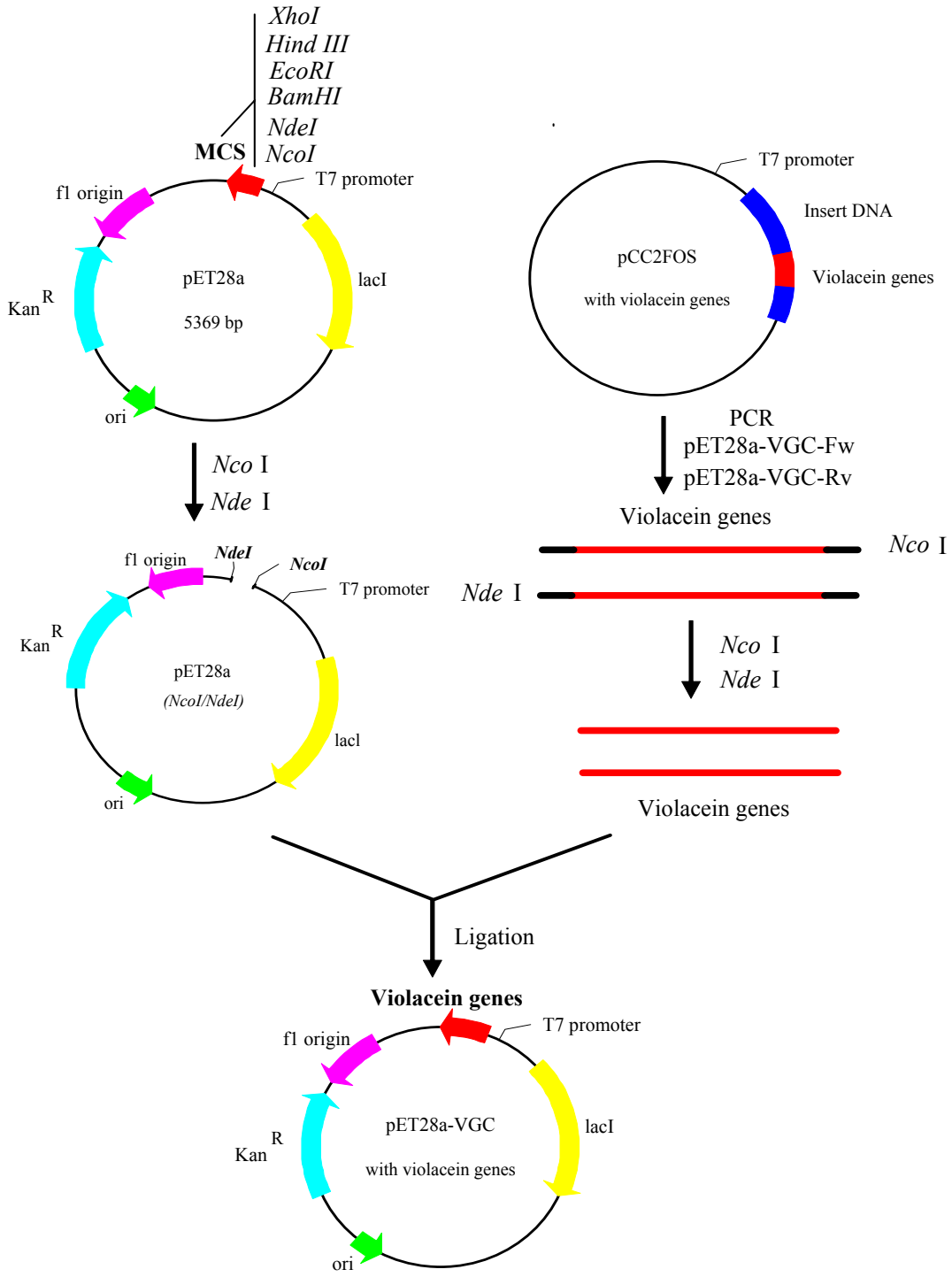


Fig. 2.2 Schematic diagram of construction of recombinant vector harboring the violacein gene cluster

2.2.6 Extraction of violacein

Violacein was extracted with ethanol from the recombinant *E. coli* as described previously [27]. In brief, 20 ml of the bacterial cultures was centrifuged at 8,000 rpm (11,800×g) for 15 min to harvest the bacterial cells. The obtained cell pellets were suspended in 2 ml ethanol and then centrifuged at 8,000 rpm (11,800×g) for 15 min to remove the cells. The supernatant containing violacein was recovered. The extraction procedure was repeated until the cells were completely whitened. All the extracted supernatants were mixed together and the amount of violacein in the extract was determined by measuring the absorbance at 575 nm (A_{575}, $\varepsilon = 2.8 \times 10^4$ $M^{-1}cm^{-1}$)[40] using UV-visible spectrophotometer (Hitachi U-3010, Japan).

2.2.7 HPLC analysis of violacein produced by the recombinant *E. coli*

Violacein was diluted with methanol to A575 = 1.0 and then analyzed by HPLC equipped with an ODS column (Capcell Pak C-18 MGII, 150 × 1.5 mm internal diameter, 5 μm particle, Shiseido) which was run isocratically at a flow rate of 100 μl/min using the mobile phase of 40% (v/v) acetonitrile (AcCN) aqueous solution and a temperature of 40°C. The monitoring wavelength was 575 nm.

2.2.8 Analysis of the expression conditions

E. coli BL21 (DE3) harboring the recombinant vector 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 (OD600) of the cultures reached 1.0. Then, violacein production was carried out at different temperatures (37°C, 20°C and 4°C)

and different rotation speeds (180 rpm and 50 rpm) in the presence and absence of 0.1 mM IPTG. Violacein was extracted and measured every 24 h as described below. Experiments were performed in duplicate.

2.3 Results

2.3.1 Construction of a genomic DNA library

To obtain the violacein gene cluster as well as its upstream and downstream regions, genomic DNA library of strain 520P1 was constructed using a fosmid vector. The genomic DNA was isolated and randomly sheared by a sterile hypodermic needle (0.40 × 19 mm). Due to the differences in concentration and purity of the prepared genomic DNA, the best sheared times by the needle used in this study varied from 30 to 50 times (**Fig 2.3**). The insert DNA fragments ranging from 25 kb to 40 kb were extracted and cloned into the fosmid vector. The library that consisted of 12,815 clones was constructed and screened to select the violacein gene cluster.

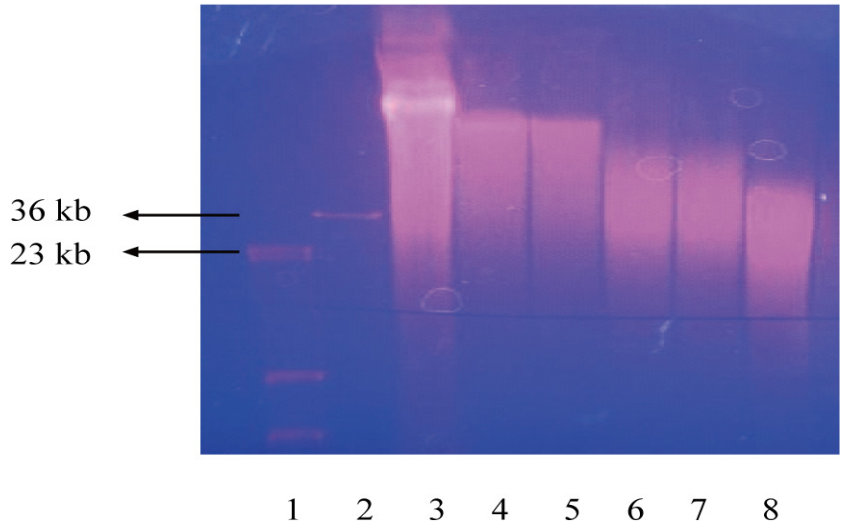


Fig. 2.3 Analysis of the randomly sheared genomic DNA of strain 520P1 by PFGE

Lane 1, Lambda DNA/ *Hin*d III marker; Lane 2, fomid control DNA (36 Kb); Lane 3, genomic DNA of strain 520P1; Lanes 4-8, genomic DNA of strain 520P1 randomly sheared for 10, 20, 30, 40 and 50 times.

2.3.2 Identification of partial violacein genes

To prepare the probe for screening clones containing the violacein genes, partial *vioC* sequence was determined by sequencing a 1260 bp PCR product amplified from the genomic DNA of strain 520P1 using vioC-Fw1 and vioC-Rv1 as primers (**Fig. 2.4**). The sequence of partial *vioC* was shown in **Fig. 2.5**.

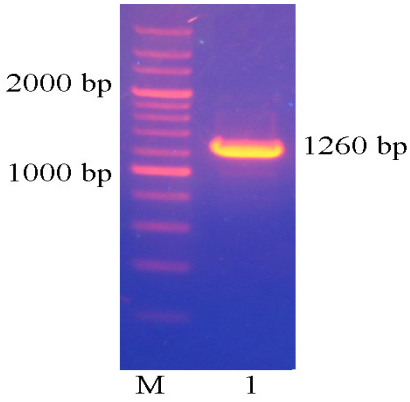


Fig. 2.4 Electrophoretic analysis of PCR product of partial *vioC* gene

vioC-Fw1 and vioC-Rv1 primers were employed for amplification of partial *vioC* gene using the genomic DNA of strain 520P1 as a template. Lane M, 200 bp DNA ladder; Lane 1, partial *vioC* gene amplified by PCR.

ATTGAAAAGCGAGGAAATCCTTTACTCGATCAAAGTGATTACATAGACCA

AGTTAGCTCAAGGCAATAGGTGTCAGTATGACTGTCCGTGGCATAGAGGC

TGTTGTTGAAGCTGGCATTCCACTTAAAGAGCTTCAAGCCTGTGGTATAG

AAGTATCAGGTATGTCTTTATTTGTTGCCGGTAAAAATAAAATAAGAGAA

CTCCCTCCATTAGATAAGTTAAAACCATTGTCTCTTAGTCGCAGTGCTTTT

CAGTTATTACTCAATAAATATGCAGAAAAAGCAGGCGTTAATTACCATTA

CAACCAGCGCTGCATTGAAGTTAATTTAAATAAATGTCACCTGTTAACTA

AAGATTTAAATGATAATTTTATTGAGCACTCAGGTGACTTATTAATTGGTG

CGGATGGCGCACGCTCTTGTGTAAGAGATGCCATGCAAACTCACTGTCGA

CGATTTGAATTTGAGCAAACATTTTTTAAACATGGGTATAAAACCTTAGTT

ATTCCAGATGCTAAAAAAGTCGGCTTAAGGCCTGATCTTTTACACTTTTTT

GGCATGGACTCTCATGGTCAATTTGCAGGTAGGGCAGCAACGATCCCTGA

TGGCAGTATCAGCTTCGCGGTTTGCCTACCCTTTAAAGGAAAAGTAAGTC

TACATACAGATGATAAAGTCGCCATGCGAGAATTTTTTGACCGATATTAC

TCTATGGTACCTAAACATATTCGCCAAGAGTTACTAGAACAATTTATGGTT

AAGCCGAGTAATGATCTTATTAATGTGCGCTCATCTACTTTTCACTATAAA

GATAAAGCCTTACTGATTGGCGACTCTGCGCATGCAACAGCGCCATTTTT

AGGTCAAGGCATGAATATGGCTCTTGAAGACGCTTATGTTTTATCATGCTT

ATTTGATAAATATGATGCTAATTTAAGTAAAATTTTACCTGACTT

Fig. 2.5 Nucleotide sequence of partial *vioC* gene

The sequences in red were used to prepare the new primers vioC-Fw3 and vioC-Rv3. The primers were used to screen the clones containing the violacein gene cluster from fosmid library of strain 520P1.

2.3.3 Screening of the clones containing violacein genes from fosmid library of strain 520P1

A fosmid library that consisted of approximately 13,000 clones was constructed from the genomic DNA of strain 520P1. After screening of clones by PCR amplification of the partial *vioC* sequence, five clones containing the violacein gene cluster were isolated (**Fig. 2.6**).

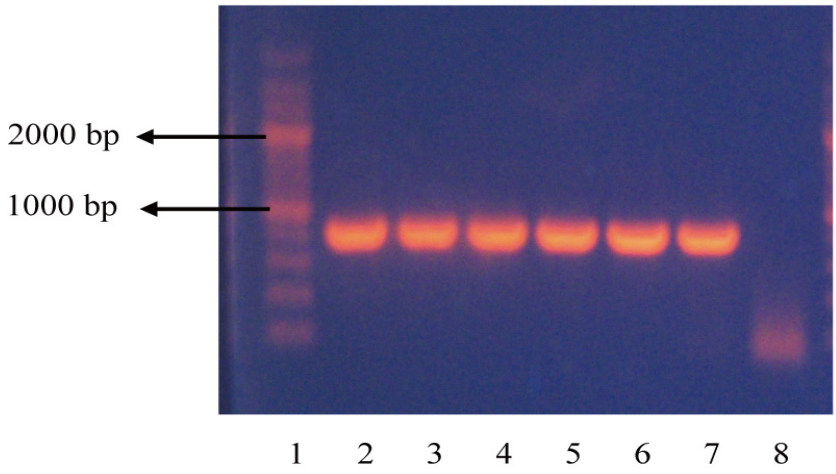


Fig. 2.6 Electrophoretic analysis of PCR product from positive fosmid clones

Amplification of partial *vioC* gene in fosmid clones was performed using vioC-Fw3 and vioC-Rv3 primers. Lane 1, 200 bp DNA ladder; Lane 2-6, positive clones contain partial *vioC*; Lane 7, positive control (genomic DNA of strain 520P1 as a template); Lane 8, negative control (no template DNA).

The plasmids from these five positive clones containing partial *vioC* gene were extracted and then analyzed on PFGE. The result of PFGE analysis was shown in **Fig 2.7**. The positive clone (No. 3) was shown to have the longest insert DNA fragments (approximately 30 kb).

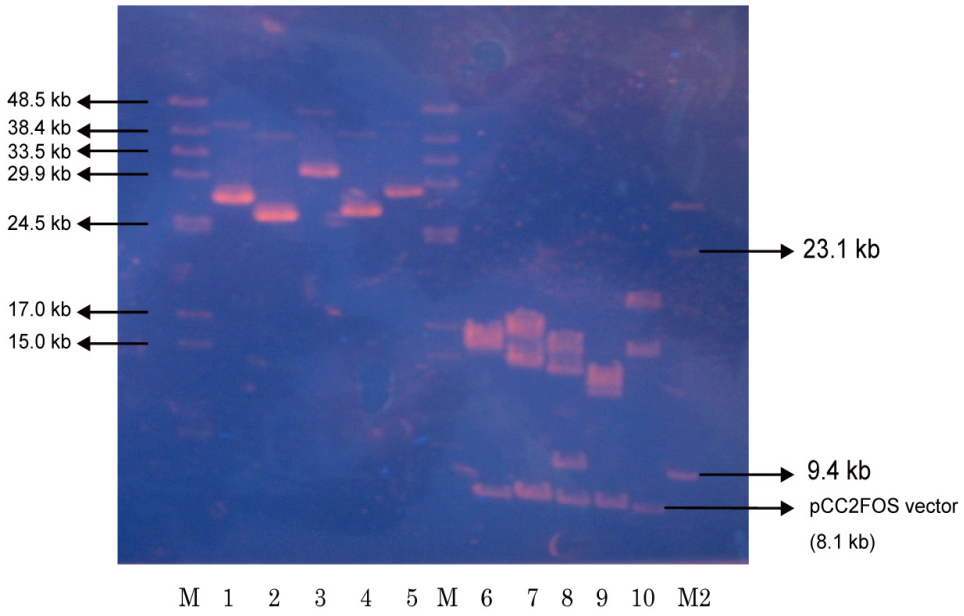


Fig. 2.7 Analysis of the positive clones containing partial *vioC* by PFGE

Lane M, λ DNA-Mono Cut Mix; Lanes 1-5, the positive clones contain partial *vioC*; Lane 6-10, the digested products by a restriction enzyme *Bam*HI. No. 3 positive clone (lane 3) containing the longest insert DNA was used for sequencing.

2.3.4 Sequence analysis of the violacein gene cluster from strain 520P1

The complete sequence of the violacein gene cluster with its upstream (1448 bp) and downstream (665 bp) were determined using the positive clone, No.3. vioC-Fw3, vioC-Rv3 along with other twenty seven primers were used for sequencing. The nucleotide sequence of the violacein gene cluster with its upstream and downstream regions was deposited in DDBJ (accession number AB573101) (**Appendix 1**). The sequence analysis demonstrated a cluster of five open reading frames (ORFs), *vioABCDE*, with a total length of 7383 bp presumably involved in the violacein biosynthesis of strain 520P1 (**Fig. 2.8**).

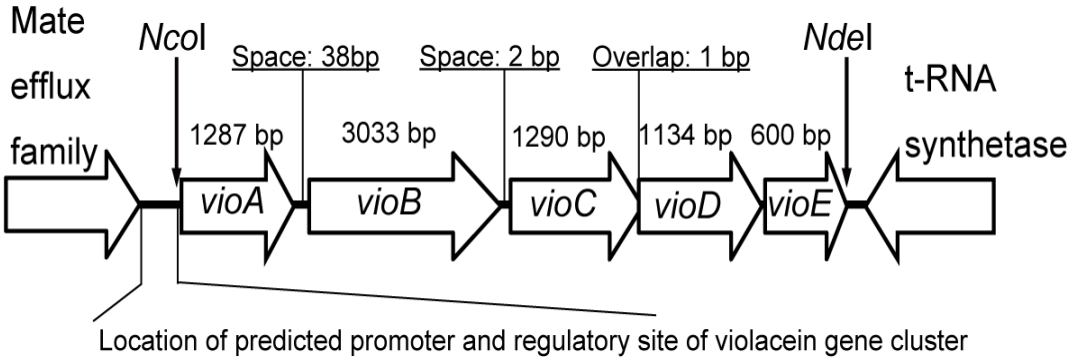


Fig. 2.8 Schematic diagram of the violacein gene cluster from strain 520P1

The violacein gene cluster sequenced from strain 520P1 is indicated by the arrows, which also denote the direction of transcription. The lengths of genes are shown above the arrows. The upstream (~1500 bp) and downstream (~700 bp) sequences encoding two putative proteins are shown on both sides of the violacein gene cluster.

The violacein genes were aligned in a single operon with 79.3% and 52.8% homology to those of *P. tunicata* D2 and *C. violaceum*, respectively. The homology of nucleotide and amino acid sequences of violacein genes in strain 520P1 were compared with those in *C. violaceum*, *J. lividum* and *P. tunicata* (**Table 2.6**).

The five ORFs encoded five proteins of 428, 1,010, 429, 377 and 199 amino acids with predicted molecular weights of approximately 49, 114, 48, 42 and 23 kDa, respectively. Amino acid sequences of VioA–VioE from strain 520P1 were compared with those from other reported strains (**Appendix 2a-2f**). As shown in **Appendix 2**, the proportions of fully conserved amino acids were 30.1% in VioA, 41.2% in VioB, 51.7% in VioC, 47.5% in VioD and 37.2% in VioE.

Table 2.6 Comparison of nucleotide and amino acid sequences of the violacein genes in strain 520P1 and other reported strains

	Homology to *C. violaceum* (%)		Homology to *J. lividum* (%)		Homology to *P. tunicata* D2 (%)	
	Nucleotide	**Amino acid**	**Nucleotide**	**Amino acid**	**Nucleotide**	**Amino acid**
vioA	45.2	40.5	46.6	41.3	83.1	89.4
vioB	50.5	50.6	51.8	52.2	76.1	80.4
vioC	56.4	63.6	55.5	59.2	82.8	90.4
vioD	53.1	55.4	56.6	56.2	81.3	85.4
vioE	48.3	45.2	52.5	46.2	77.3	76.4

Phylogenetic analysis of the nucleotide sequences in the violacein gene clusters from strain 520P1 and other violacein-producing strains was performed. As shown in **Fig. 2.9**, the phylogenetic tree suggests that the violacein genes in different strains might share the same evolutionary history. The violacein genes in these five strains could be divided into three groups: the first group would consist of *J. lividum* (EF063591) and *Duganella* sp. B2 (GQ266676), the second group would contain *C. violaceum* (AE016825) and the third group of *P. tunicata* D2 (AAOH00000000) and strain 520P1 (AB573101).

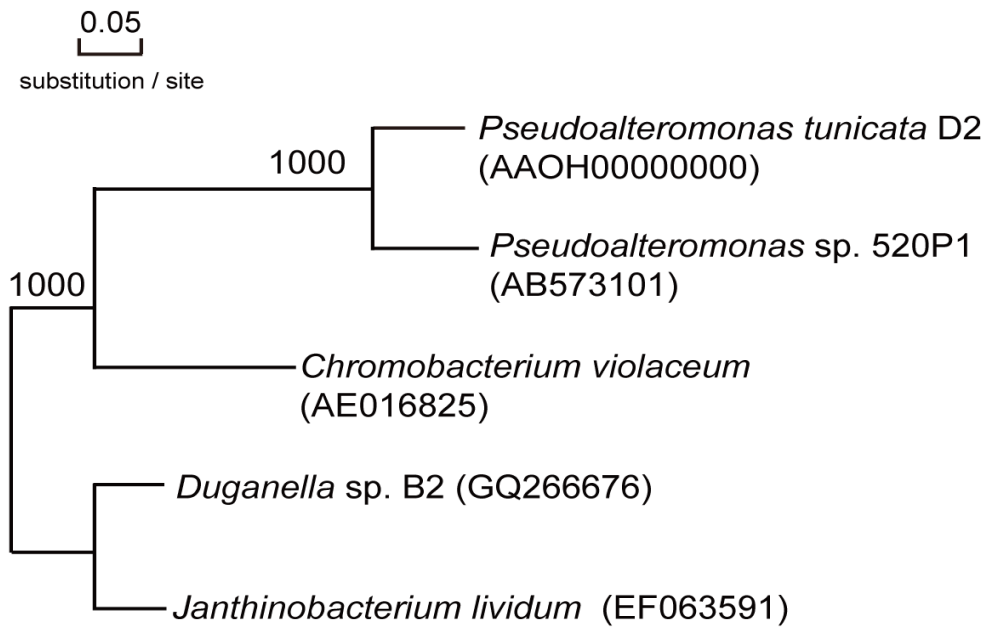


Fig. 2.9 Phylogenetic analysis of the nucleotide sequences of the violacein gene cluster from strain 520P1

Analysis was performed using the sequences of violacein gene clusters in violacein-producing bacteria obtained from DDBJ and GenBank. The tree was drawn with 1000 bootstrap trials. The bar shows the rate of nucleotide substitution. Accession numbers for the sequences are given in parentheses.

2.3.5 Sequences analysis of the upstream of the violacein gene cluster from strain 520P1

There was a non-coding region of 485 bp between *vioA* and its upstream neighboring protein gene. The corresponding upstream regions in strain 520P1 and *P. tunicata* D2 (379 bp) demonstrated 57.3% homology (**Fig. 2.10**).

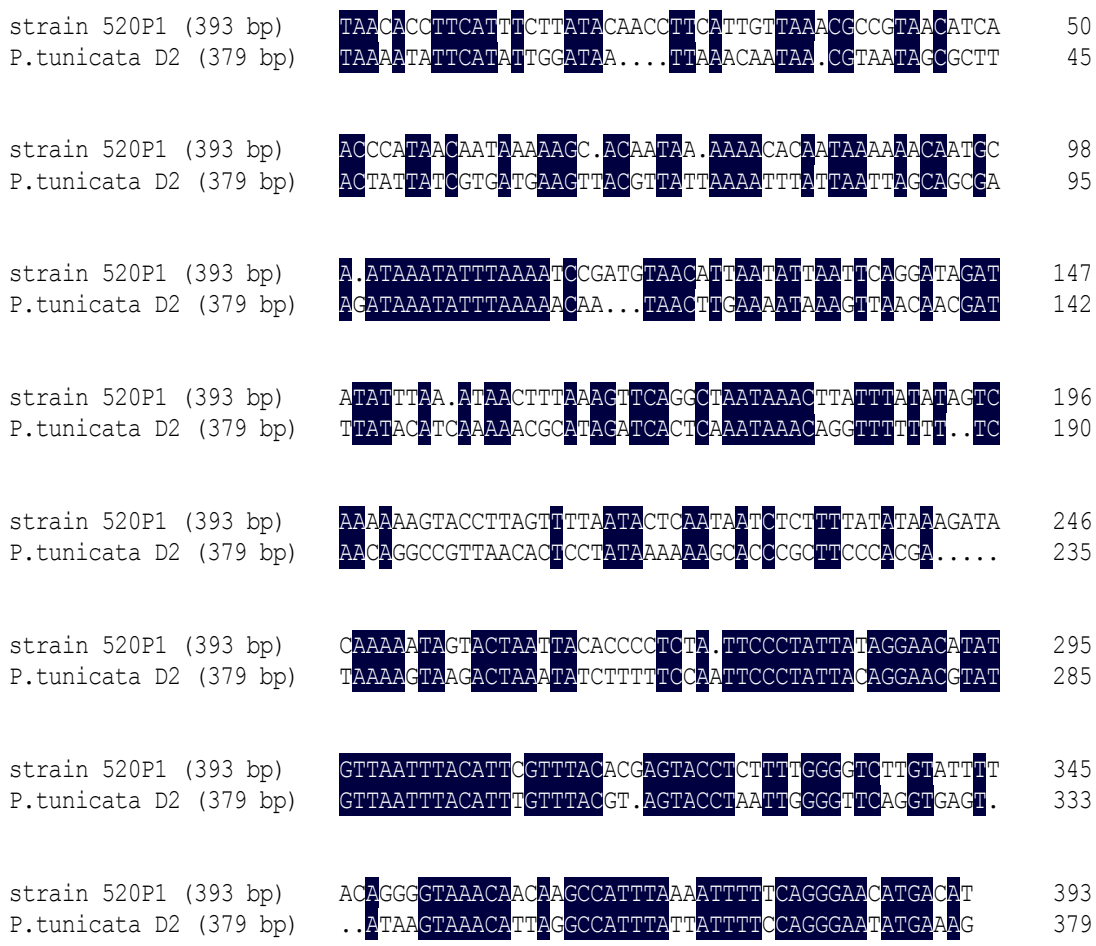


Fig. 2.10 Comparison of nucleotide sequences of the upstream of violacein gene clsuters in strain 520P1 (393 bp) and *P. tunicata* D2 (379 bp)

Fully conserved nucleotides are indicated in black and the gap was indicated with "●".

Under the same comparison conditions, an even lower level of homology (29.4%) was observed between strain 520P1 and *C. violaceum* (**Fig. 2.11**).

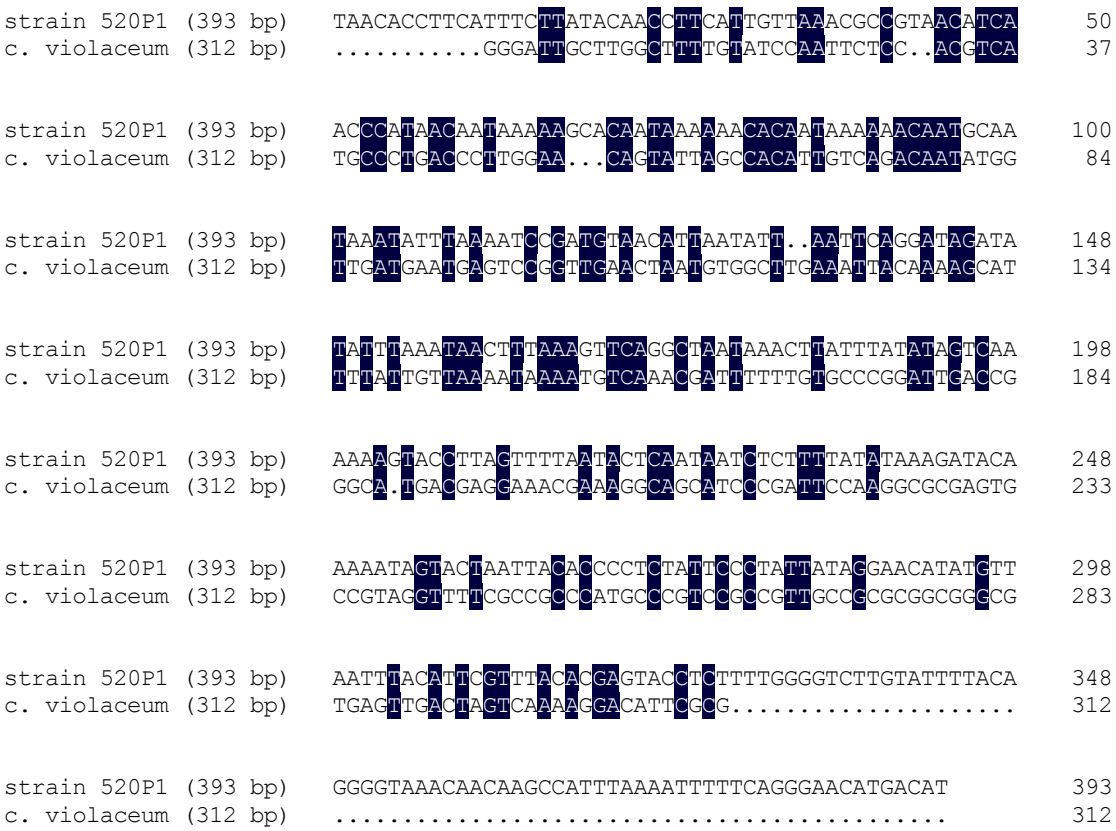


Fig. 2.11 Comparison of nucleotide sequences of the upstream of violacein gene clsuters in strain 520P1 (393 bp) and *C. violaceum* (312 bp)

Fully conserved nucleotides are indicated in black and the gap was indicated with "●".

An approximate 120 bp sequence near the start codon of *vioA* was highly conserved in *P. tunicata* D2, and strains 520P1 and 710P1 (**Fig. 2.12**). The upstream sequence of the violacien gene cluster in strain 710P1 were obtained by PCR method using 520P1-before-vioA-Fw2 and 520P1-vioA-Rv1 as primers and deposited in the DDBJ under the accession number AB583751. The determined upstream sequence in strain 710P1 was shown in **Appendix 3.**

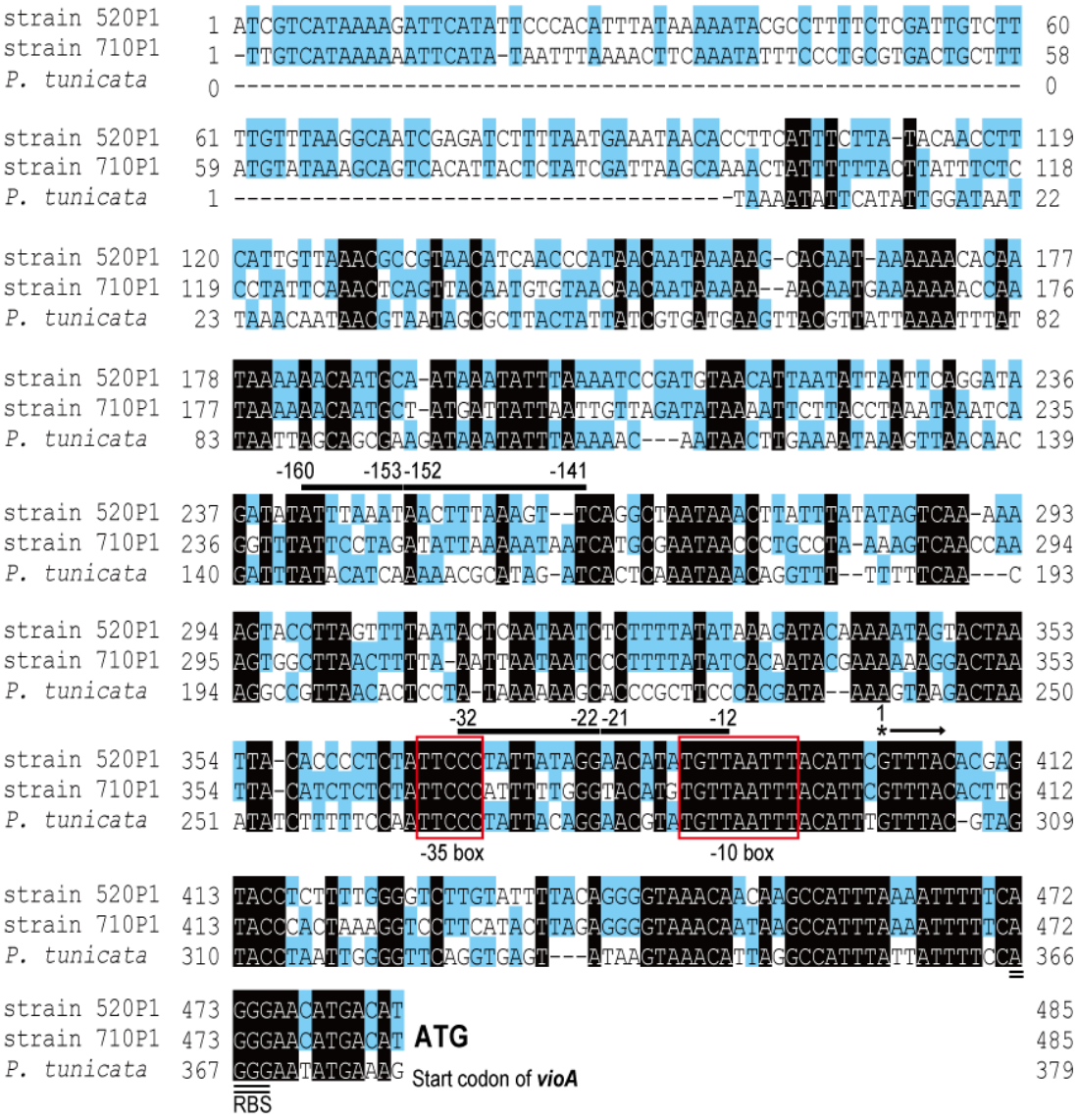


Fig. 2.12 Comparison of predicted promoter regions in strains 520P1, 710P1 and *P. tunicate* D2

Fully conserved nucleotides are indicated in black and nucleotides conserved in two strains are indicated in blue. The predicted starting site of transcription is marked by an asterisk (*). The direction of transcription is indicated by the arrow. Predicted -35 and -10 promoter sequences are boxed and the start codon of *vioA* is in boldface type. The putative ribosomal binding site (RBS) is double underlined. Palindromic sequences are marked by bars in boldface type.

In this region, putative promoter sequences, -10 and -35 boxes were predicted. Furthermore, in a region approximately 16 bp upstream from the predicted starting site of transcription, two contiguous palindromic sequences were found. The sequences were conserved in the upstream regions of *P. tunicata* D2 and strain 710P1 (**Fig. 2.13**). Those sequences were probably involved in the regulation of violacein gene expression.

Fig. 2.13 Palindromic sequences in the predicted promoter region of the violacein gene cluster from strain 520P1

Palindromic sequences of strain 520P1 in the predicted promoter region were compared with those of strain 710P1 (DDBJ accession number: AB583751) and *P. tunicata* D2. Conserved nucleotides are indicated in gray. The positions of nucleotides from the predicted starting site of transcription of the gene cluster are shown above the sequence of strain 520P1.

2.3.6 Expression of the violacein gene cluster from strain 520P1 in *E. coli*

To examine the ability of the gene cluster to synthesize violacein *in vivo*, heterologous expression of the cluster was performed in *E. coli* using a recombinant pET vector. **Figure 2.14** outlines the structure of the recombinant vector, designated pET28a-VGC containing the violacein gene cluster. The pET28a-VGC did not contain the upstream sequence of violacein genes.

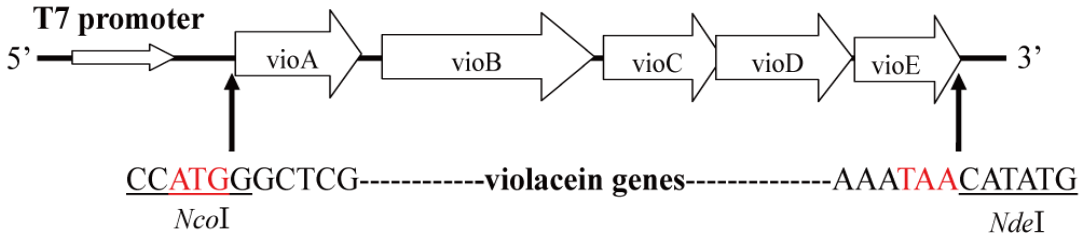
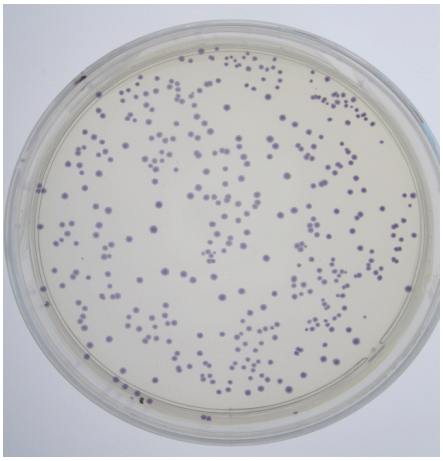


Fig. 2.14 Structure of the recombinant vector, designated pET28a-VGC

The cloning sites for the recombinant vector pET28a-VGC are marked with arrows. The restriction enzyme sites, *Nco*I and *Nde*I, were underlined and incorporated into the PCR primers. The start coden of *vioA* and stop coden of *vioE* are shown in red.

The violacein genes were expected to be expressed under the control of the T7 promoter contained in the pET vector. However, violet colonies formed on an agar plate in the absence of IPTG when the recombinant *E. coli* transformants (*E. coli* BL21 (DE3) harboring the recombinant vector pET28a-VGC) were incubated at 37°C for 16 h and subsequently placed at 4°C for 24 h, indicating the successful expression of violacein gene cluster of strain 520P1 in *E. coli* (**Fig. 2.15**).Violet colonies did not appear when the recombinant *E. coli* transformants were continuously incubated at 37°C. No violet pigment appeared when *E. coli* DH5α harvoring pET28a-VGC was incubated on a plate at 37°C for 16 h then at 4°C for 24 h.

Interestingly, when the recombinant *E. coli* BL21 (DE3) harboring with pET28a-VGC was incubated on an agar plate at 20°C for 24 h, violet color appeared both in the presence and absence of IPTG. The number of violet colonies on the agar plate in the presence of IPTG was less than that in the absence of IPTG, probably due to the toxic effect of IPTG to *E. coli* cells. Only the band of VioE could be observed by SDS-PAGE when IPTG was added as the inducer (**Fig. 2.16**).



Recombinant transformants *E. coli* BL21 (DE3) harboring the recombinant vector pET28a-VGC were incubated at 37°C for 16 h and subsequently placed at 4°C for 24 h. The violet colonies appeared on the agar plate in the absence of IPTG.

Fig. 2.15 Phenotype of the constructed recombinant *E. coli* containing the violacein gene cluster on the LB agar plate

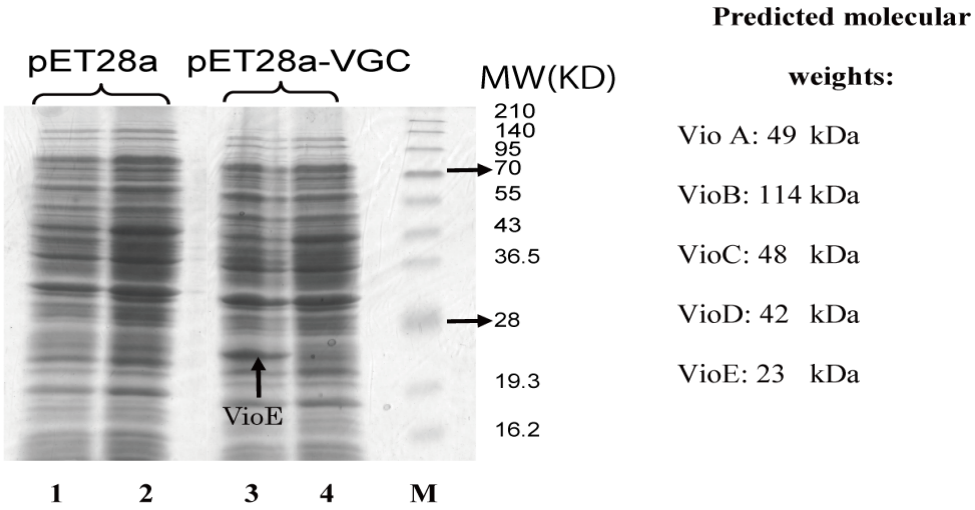


Fig. 2.16 SDS-PAGE analysis of the expressed proteins from the violacein gene cluster

The cultures were incubated at 37°C for 2 h until the OD_{600} reached at 1.0. After adding 0.1 mM IPTG, the cultures were incubated at 20°C, 180 rpm, for 24 h. *E. coli* BL21 (DE3) harboring pET28a in the presence of IPTG (Lane 1) and absence of IPTG (Lane 2); *E. coli* BL21 (DE3) harboring pET28a-VGC in the presence of IPTG (Lane 3) and absence of IPTG (Lane 4).

2.3.7 Characterization of the violet pigment produced by recombinant *E. coli*

Figure 2.17a shows the UV-visible spectrum of the violet pigment produced by recombinant *E. coli*. The maximum wavelength of absorbance was approximately 576 nm and the profile of the spectrum was identical to that of violacein. Strain 520P1 usually produces a mixture of violacein and deoxyviolacein. As shown in **Figure 2.17b**, peaks 1 and 2 represent violacein and deoxyviolacein, respectively, produced by strain 520P1. HPLC analysis demonstrated that the elution time of the violet pigment produced by the recombinant *E. coli* was identical to that of violacein (peak 1) produced by strain 520P1 (**Fig. 2.17c**). The absence of the second peak corresponding to deoxyviolacein indicated that no or little deoxyviolacein was produced by the recombinant *E. coli*.

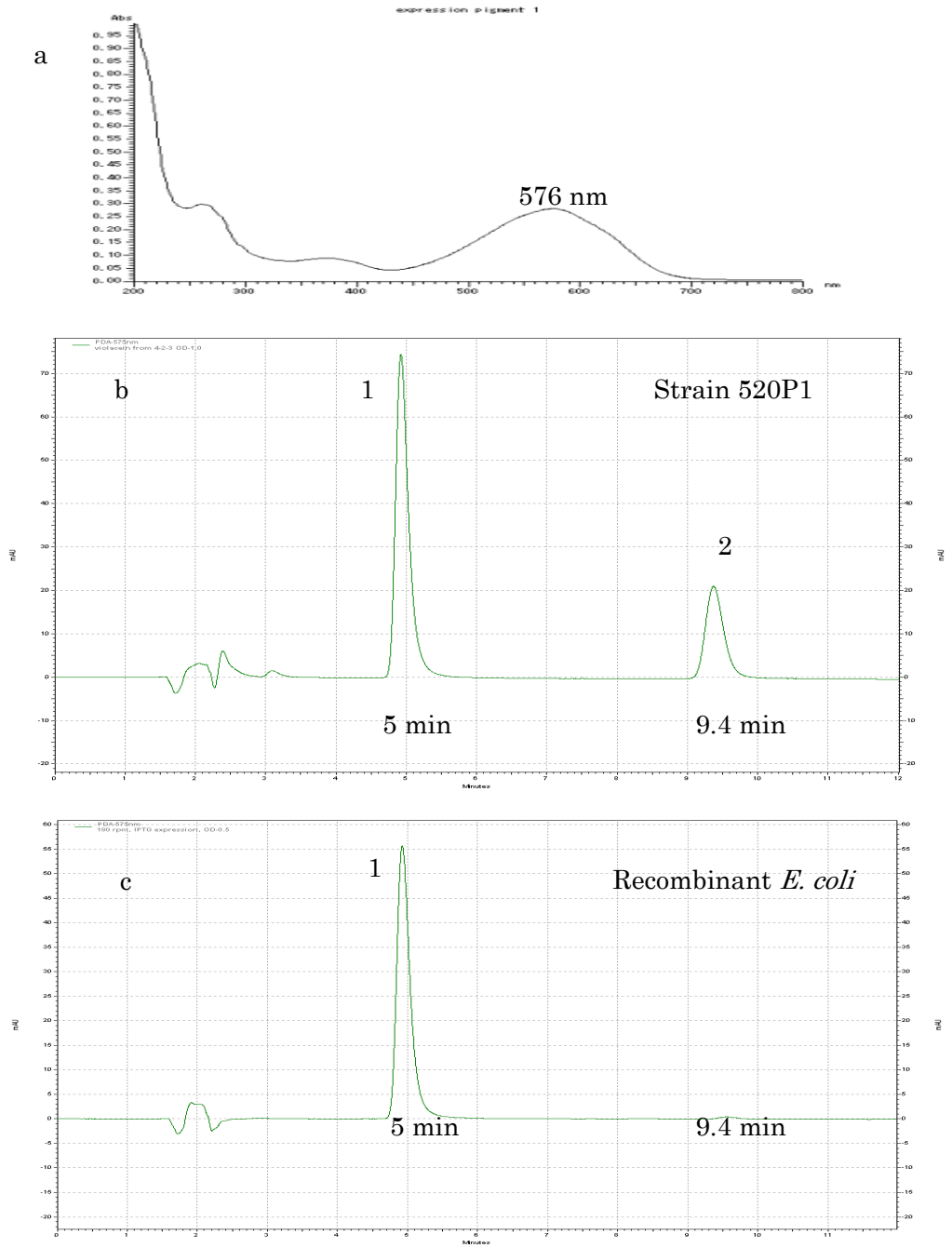


Fig. 2.17 Analysis of violacein produced by recombinant *E. coli*

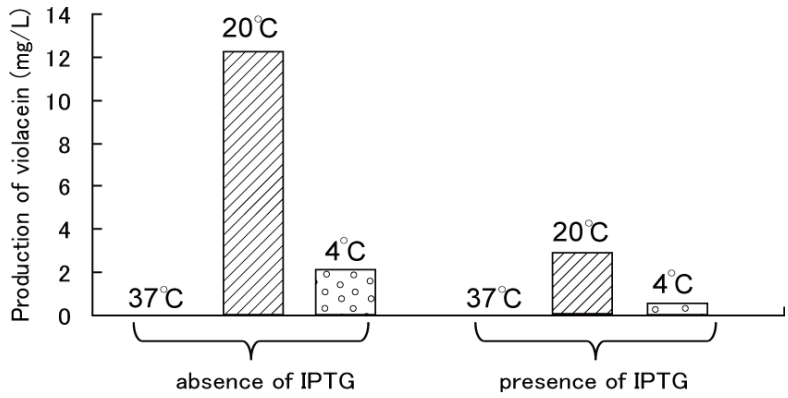
(a) UV-visible spectrum of the violet pigment produced by the recombinant *E. coli.*

(b) HPLC analysis of the culture extracts of strain 520P1. Peak 1: violacein. Peak 2: deoxyviolacein.

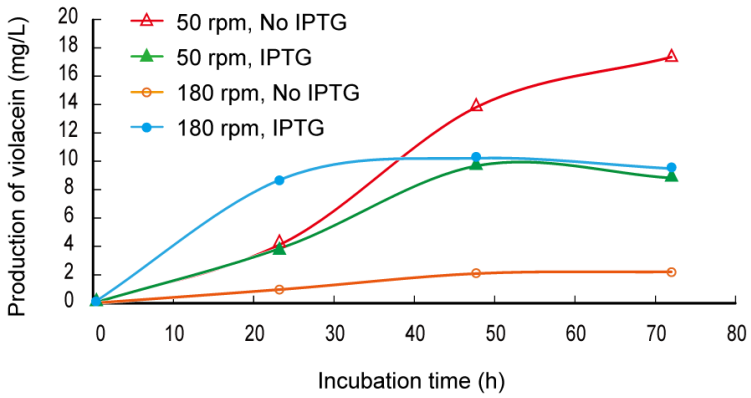
(c) HPLC analysis of the culture extracts of the recombinant *E. coli*.

2.3.8 Analysis of the expression conditions

Violet colonies formed on an agar plate in the absence of IPTG when the recombinant *E. coli* transformants were incubated at 37°C for 16 h and subsequently placed at 4°C for 24 h. Therefore, to examine detailed conditions for violacein gene expression, we cultured the recombinant *E. coli* at different temperatures and shaking speeds in the presence and absence of IPTG. We observed a violet pigment at 20°C and 4°C, 50 rpm **(Fig. 2.18a)**. No violet pigment was observed at 37°C both in the presence and absence of IPTG. As shown in **Fig. 2.18b**, violet pigment was produced in the presence of IPTG at 20°C/180 rpm, but only a small amount of the pigment was produced in the absence of IPTG. A considerable amount of violet pigment appeared when the recombinant *E. coli* was incubated in the absence of IPTG at 20°C/50 rpm (**Fig. 2.18b**). Production of the pigment with IPTG at 20°C/180 rpm and 20°C/50 rpm reached a plateau after 24 h and 48 h respectively, but the cultures grown without IPTG at 20°C/50 rpm continued to produce the pigment over 70 h (**Fig. 2.18b**). At 4°C/50 rpm, some amount of violet pigment was produced both in the presence and absence of IPTG (**Fig. 2.18a**). However, the time required for the production of this pigment was much longer compared with 20°C/50 rpm.



(a). Violacein production at 37°C/50 rpm, 20°C/ 50 rpm and 4°C/50 rpm for 72 h



(b). Violacein production at 20°C/180 rpm and 20°C/50 rpm

Fig. 2.18 Violacein production by the recombinant *E. coli* under different culture conditions

(a): Violacein was produced by the recombinant *E. coli* in the absence and presence of IPTG at 37°C/50 rpm, 20°C/ 50 rpm (stripes) and 4°C/50 rpm (cycles) for 72 h. (b): Violacein was produced at 20°C by the recombinant *E. coli* harboring the violacein gene cluster. The recombinant *E. coli* were cultured up to 72 h at 180 rpm in the presence (closed circles) or absence (open circles) of IPTG, and at 50 rpm in the presence (closed triangles) or absence (open triangles) of IPTG. Each point represents the mean value of duplicate cultures.

2.4 Conclusions and discussion

To characterize the violacein biosynthetic pathway and its regulation mechanism(s), it is essential to identify three significant components: the violacein gene cluster including the upstream region where the promoter and quorum sensing regulatory site are thought to be located; the *luxI* gene homolog (AHL synthesizing enzyme gene); and the *luxR* gene homolog (AHL receptor protein gene). These homologs are critical in regulating the whole process of violacein biosynthesis. In this part, we characterized the violacein gene cluster and its upstream region. The DNA sequence of the violacein gene cluster was obtained from a fosmid library of strain 520P1 and demonstrated highest homology to the gene cluster from *P. tunicata* D2, and lower levels of homology to *C. violaceum*, *J. lividum* and *Duganella* sp. B2. Therefore, it is believed that violacein gene clusters from strain 520P1 and *P. tunicata* D2 belong to the same group and possibly share a similar evolutionary history. Amongst the five enzymes in strain 520P1, VioC demonstrated the highest homology with other four strains (**Appendix 2d**). The high level of homology of VioC could be the reason why only one pair of primers succeeded in amplifying the *vioC* gene from the genomic DNA of strain 520P1.

The upstream regions of the violacein gene cluster are supposed to contain the promoter and the regulatory binding site for the LuxR homolog-AHL complex. However, the upstream regions of strain 520P1 and *P. tunicata* D2 showed a low level of homology (57.3%) (**Fig. 2.10**). An even lower level of homology (29.4%) was observed between strain 520P1 and *C. violaceum* (**Fig. 2.11**). Our data indicate that the binding site of the LuxR homologue-AHL complex in strain 520P1 might be

significantly different from that in *C. violaceum*. In *Vibrio fischeri*, which is the most studied bacterium in the quorum sensing field, the DNA binding site for the LuxR-AHL complex has been identified. It is a 20 bp palindromic sequence, designated the *lux* box, and positioned approximately 40 bp upstream from the start site of transcription in the operon for bioluminescence [60][61]. Therefore, palindromic sequences in the upstream region might be possible quorum sensing regulatory sites. In strain 520P1, we discovered a palindromic sequence, 5'-AAC ATA TGT T-3' (10 bp) centered approximately 16 bp upstream (-21 to -12) from the putative start site of transcription (**Fig. 2.12**). Moreover, another palindromic structure (5'-CCT ATT ATA GG-3', -32 to -22) was contiguous to this palindromic sequence. These features were shared by the corresponding sequences of strain 710P1 and *P. tunicata* D2 (**Fig. 2.13**). Therefore, it is likely that these sequences are involved in the regulation of violacein gene expression, although further study is necessary to confirm this. Furthermore, another two adjacent palindromic sequences (5'-ATT TAA AT-3', -160 to -153; 5'-AAC TTT AAA GTT-3', -152 to -141) were found in the upstream region. However, no sequence homology was found in strain 710P1 and *P. tunicata* D2. In the *lux* box of *V. fischeri* (5'-ACC TGT AGG ATC GTA CAG GT-3'), the CTG and CAG sequences (underlined) flanking 10 nucleotides were critical for regulation by LuxR-AHL [71]. A similar structure (5'-CTGN_{10}CAG-3') was also found in *C. violaceum* (5'-CTG ACC CTT GGA ACA G-3') (**Fig. 2.19**). However, we did not find a homologous structure for the putative receptor binding site in strain 520P1. In a previous study, we failed to identify the *luxI* and *luxR* gene homologs from strain 520P1 by PCR, based on the homology of the reported genes. This is probably due to

the low homology of these two genes between strain 520P1 and other reported strains. Taken together, we have supposed that the LuxR family and its regulatory site on the DNA in strain 520P1 might be different from other reported ones. This study should open the way to further detailed studies of the interactions of the LuxR transcriptional family with their target site of DNA. Although we obtained the putative -10 and -35 promoter sequences based on the highly conserved sequences, further study is still needed to characterize the promoter and the regulatory sites.

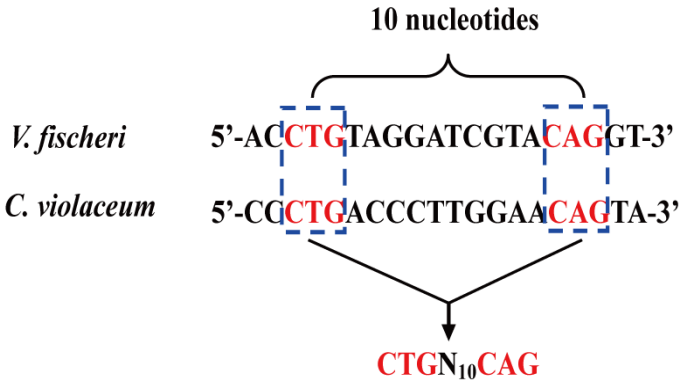


Fig. 2.19 The *lux* box and a similar structure in the upstream sequences of *V. fischeri* and *C. violaceum*

Heterologous expression is considered to be a tool to identify the particular gene(s) encoding functional proteins, understanding the exact synthetic pathway or improving the amount of desired metabolites. However, the most serious problem regarding the heterologous expression of the gene cluster is that biosynthetic enzymes may have no function or only weak activity due to the absence of the essential promoter, co-factors and/or regulatory factors. Our data demonstrated that the violacein gene cluster from strain 520P1 could be functionally expressed in *E. coli* BL21 (DE3). Violacein was

produced at 20°C and 4°C but not at 37°C, as lower temperatures were likely beneficial for protein folding and maintaining the activities of the expressed enzymes. It should be also noted that strain 520P1 does not survive at 37°C [27]. Interestingly, stable production of violacein occurred even in the absence of IPTG, when the recombinant *E. coli* was cultured at 20°C/50 rpm (**Fig. 2.18b**). No violacein was produced at 20°C/50 rpm in the presence and absence of IPTG when *E. coli* DH5α which does not contain T7 RNA polymerase gene was used as host cells. Therefore, the expression of violacein genes in *E. coli* BL21 (DE3) without IPTG is likely to be dependent on T7 promoter as well as the expression with IPTG. Lower temperatures and lower oxygen supply may have changed the metabolism of the host cells and finally suppressed the inhibitory action of the *lac* repressor on the expression of the gene cluster. Studier suggested that sporadic and unintended expression of genes under the control of T7 promoter was mostly caused by small amounts of lactose [62]. However, it remains elucidated why the expression of violacein genes occurred without any exogenously added inducer in this study.

We were able to obtain violacein using recombinant *E. coli* in a short time after induction. One of the features of the recombinant *E. coli* studied here is that it produced no or little deoxyviolacein. This property will make purification procedures of violacein much easier. However, the amount of violacein produced by the recombinant *E. coli* was similar to that produced by strain 520P1. To satisfy the requirements for industrial applications using recombinant *E. coli*, studies to optimize the culture conditions, such as incubation temperature, shaking speed and culture medium, as well as the development of efficient expression methods are still needed.

CHAPTER 3

Isolation of a Variant of *Pseudoalteromonas* sp. 520P1 Producing Violacein under Agitated Culture Conditions

3.1 Introduction

Pseudoalteromonas sp. 520P1, a Gram-negative marine bacterium, shows an ability of producing violacein only under static culture conditions. In the previous study, we reported that the production of violacein by strain 520P1 was regulated by quorum sensing and activated by *N*-(3-oxooctanoyl)-homoserine lactone (3-oxo-C8 HSL) [57]. However, we have not identified the genes for AHL synthesis and AHL receptor protein in strain 520P1. Identification of these genes is pivotal for understanding of regulatory mechanisms of quorum sensing and the nature of AHL(s) involved in the violacein production. In this part of study, we isolated a variant strain (No. 4-2-3), capable of producing violacein under agitated culture conditions, from the culture of strain 520P1. We suppose that there may be one or some mutation(s) in the variant No. 4-2-3. Therefore, the productions of violacein and AHLs by the variant No. 4-2-3 under both static and agitated culture conditions were investigated.

AHLs as signaling molecules play an important role in the quorum sensing system. The presence of AHL is detected by several bacterial biosensors, which do not produce AHLs by theirselves but respond to exogenous AHLs (**Fig. 3.1**).

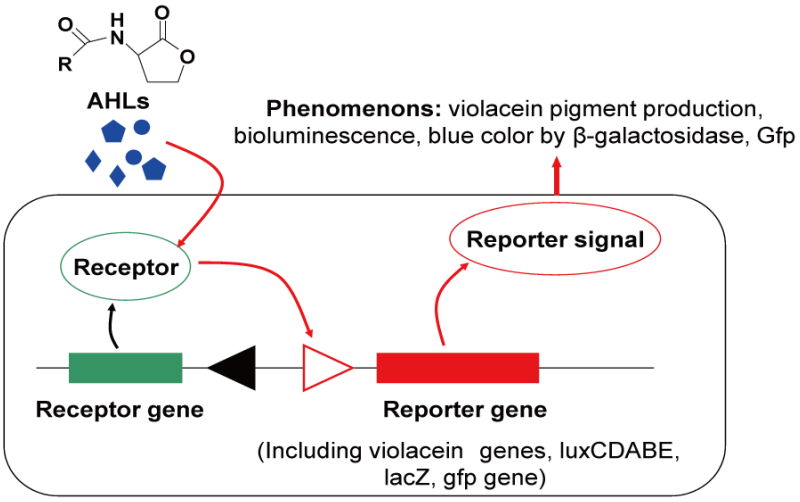


Fig. 3.1 Principle of detection of AHL by reporter strains

In some cases, a single bacterium produces multiple AHLs differing in the R group (including the length of the acyl side chain or the substitution at position C3 carrying an oxo- or hydroxyl group). The exogenously added AHL to the reporter strain interacts with a cognate receptor protein which is constitutively expressed from the promoter (closed triangle) inside of the cells. An AHL-receptor protein complex then activates the promoter (open triangle) of the reporter genes. The response to AHL can be detected in a variety of ways such as bioluminescence, development of blue color by β-galactosidase, green-flurescent protein and violacein pigment production.

Different biosensors respond to different AHLs in different sensitivities. **Table 3.1** shows representative AHL receptor strains. In TLC analysis, the AHLs migrate with specific mobility and form a spot detected by reporter strains. TLC analysis with corresponding reporter strain is considered to be a rapid and visual method of detecting the AHLs [63][64]. Some AHL biosensors could be used for quantifying AHL by measuring the level of the reporter signal. To quantify the concentration of the tested AHL, it is necessary to determine the minimum concentration of the AHL required by the reporter strain. Therefore, it is very important to choose a reporter

strain sensitive to the tested AHL.

Table 3.1 AHL reporter strains

Plasmid	Host	Phenotype	Best response	Good response	Ref.
None	*C. violaceum* CV026	Violacein pigment (lacks CviI)	C6 HSL	3-oxo-C6 HSL C8-HSL 3-oxo-C8 HSL C4 HSL	Mcclean et al. 1997 [56]
pSB401	*E. coli*	Bioluminescence (LuxI/R from *V. fisheri*)	3-oxo-C6 HSL	C6 HSL 3-oxo-C8 HSL C8-HSL	Winson et al. 1998 [65]
pZLR4	*A. tumefaciens* NT1	β-galactosidase (TraI/R from *A. tumefaciens*)	3-oxo-C8 HSL	All 3-oxo HSLs C6,8,10,12,14 HSL 3-OH-C6 HSL 3-OH-C8 HSL 3-OH-C10 HSL	Farrand et al. 2002 [66]
pAS-C8	Broad host range	Green fluorescence (CepI/R from *B. cepacia*)	C8 HSL	C10 HSL	Riedel et al, 2001 [67]

In this part, we employed *Agrobacterium tumefaciens* NTL4 (pZLR4) to respond to AHLs produced by strain No. 4-2-3. The variant strain reported here may help further understanding of the regulation of violacein production and also provides a rapid and efficient method of violacein production.

3.2 Materials and Methods

3.2.1 Isolation of a variant strain

Pseudoalteromonas sp. 520P1 was cultured at 20°C in PPES-II liquid culture medium under shaking culture conditions. From the culture which showed purple color, cultivation liquid was obtained and incubated at 20°C on a PPES-II agar plate for 4 days. Colonies of dark violet color were inoculated on agar plates separately for further incubation. Successive isolation of a single colony and the following incubation on an agar plate was repeated five times. A colony isolated from the final incubation on an agar plate was designated No. 4-2-3. Violet colonies with white edge, which were similar to original strain 520P1, were isolated from the same liquid culture and subjected to successive culture on agar plates. This strain was designated No. 4-1-2 and used as a control in the following experiments.

3.2.2 Production of violacein under static culture conditions

Overnight pre-cultures of strains No. 4-1-2 and No. 4-2-3 were inoculated to a 300 ml-flask containing 100 ml of PPES-II culture medium, respectively. The flasks were incubated at 20°C under static culture conditions for 10 days. To monitor the relative amount of violacein production, the incubated cultures were sampled every 24 h and violacein was extracted with ethanol as described in **Chapter 2**.

3.2.3 Production of violacein under agitated culture conditions

Strains No. 4-1-2 and No. 4-2-3 was cultivated for 36 h as described in **3.2.2**,

except that the cultivation were performed under agitated culture conditions at a rotary speed at 160 rpm. The bacterial growth and violacein production was monitored every 3 h by measuring the absorbance at 700 nm and 575 nm, respectively.

3.2.4 Sequencing of the upstream region of the violacein gene cluster in strain No. 4-2-3

The genomic DNA of strain No. 4-2-3 was isolated and purified as described in **Chapter 2.2.2**. Upstream sequence of strain No. 4-2-3 was amplified by PCR using primers 520P1-vioA-Fw1 and 520P1-vioA-Rv1 (**Table 2.5** in **Chapter 2**). The upstream sequence from strain No. 4-2-3 was determined and deposited in DDBJ under the accession number AB583752.

3.2.5 Extraction of AHLs

Overnight cultures of strains No. 4-1-2 and No. 4-2-3 were inoculated in 200 ml of PPES-II culture medium in 500 ml flasks, respectively. After 12h, 24h, 36h, and 48h of incubation at 20°C with shaking (180 rpm), the culture supernatants were harvested by centrifugation at 8,000 rpm (11,800×g) for 15 min and filtered using a 0.22-μm-pore size membrane filter (Advantec, Tokyo), respectively. The filtered supernatants were extracted with the equal volume of ethyl acetate (AcCN) for three times. Then, the combined extracts were evaporated to dryness and dissolved in 0.5 ml of ethanol. The extracts were stored at -20°C until use (**Fig. 3.2**).

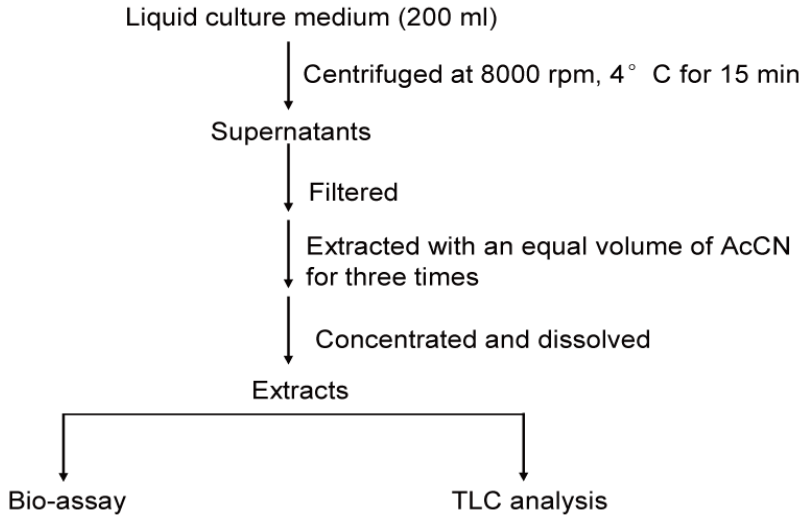


Fig. 3.2 Extraction of AHLs from strain No. 4-1-2 and No. 4-2-3

PPES-II culture medium was employed as negative control.

3.2.6 Bio-assay of AHLs

Reporter strain *Agrobacterium tumefaciens* NTL4 (pZLR4) was incubated in AB minimal medium (see **Appendix 5**) with gentamicin (5 μg/ml). AHL plate assay was performed according to the method of Shaw et al. with some modifications [63]. Soft agar containing 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and *A. tumefaciens* NTL4 (pZLR4) was overlaid on an AB minimal agar plate. Paper disks (8mm in diameter) containing AHL samples were placed on the plate. Assay plates were incubated overnight at 28°C, and the zone of blue pigment was measured. Standard synthetic AHLs, C6 HSL and 3-oxo-C8 HSL (Sigma-Aldrich K.K., Tokyo, Japan) were used as positive control as described above.

3.2.7 TLC assay of AHLs

AHL extracts and standard AHLs were spotted on reversed-phase ODS TLC plate (Darmstadt, Germany) and developed with 60% (v/v) methanol in water. AB minimal glucose medium containing 40 μg/ml X-gal and *A. tumefaciens* NTL4 (pZLR4) was overlaid on the air-dried plate and incubated at 28°C for 18 h. Standard synthetic C6 HSL and 3-oxo-C8 HSL were used as authentic standards.

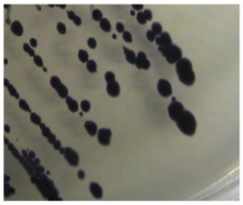
3.3 Results and discussion

3.3.1 Isolation and observation of strain No. 4-2-3

Colonies of strain No. 4-1-2 and strain No. 4-2-3 differed in their morphology. Strain No. 4-2-3 formed a circular, smooth, raised and dark violet-black colonies on a agar plate (**Fig. 3.3b**), while colonies of strain No. 4-1-2 showed similar shape but dark violet color with white edges (**Fig. 3.3a**). During five successive subcultures, neither the shape nor color of colonies of strain No. 4-2-3 changed, indicating that the characteristic properties of producing violacein was maintained stably from generation to generation (**Fig. 3.3c**).

a: strain No. 4-1-2

b: strain No. 4-2-3



c: fifth subculture of strain No. 4-2-3

Fig. 3.3 Morphology of strain No. 4-1-2 and No. 4-2-3

Strain No. 4-1-2 and No. 4-2-3 were isolated from the culture of original culture of strain 520P1. A single colony was inoculated and incubated at 20°C for 7 days on an agar plate. (a): Strain No. 4-1-2, similar to original strain 520P1; (b): Strain No. 4-2-3, the variant strain; (c): The fifth subculture of strain No. 4-2-3.

3.3.2 Violacein production by strain No. 4-1-2 and No. 4-2-3 under static culture conditions

When strains No. 4-1-2 and No. 4-2-3 were incubated at 20°C without shaking, both of them produced violacein (**Fig 3.4**). The time courses of violacein production by strain No. 4-1-2 and No. 4-2-3 were almost the same and there was no appreciable difference.

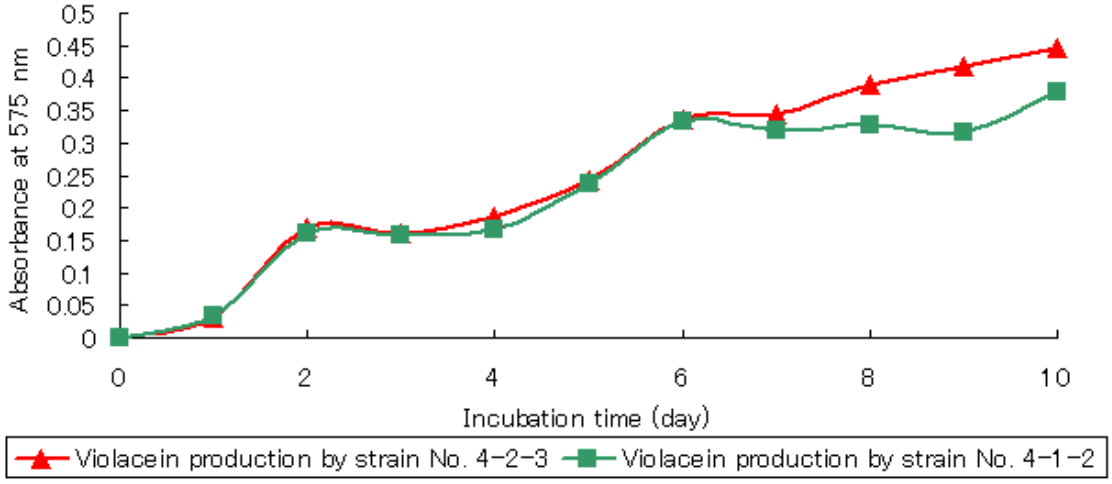
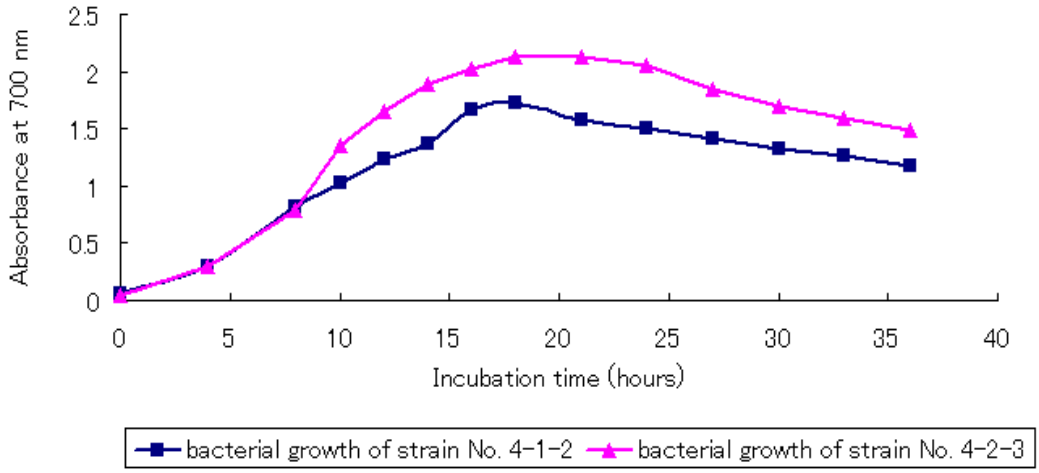


Fig. 3.4 Time courses of the violacein production by strains No. 4-1-2 and No. 4-2-3 under static culture conditions

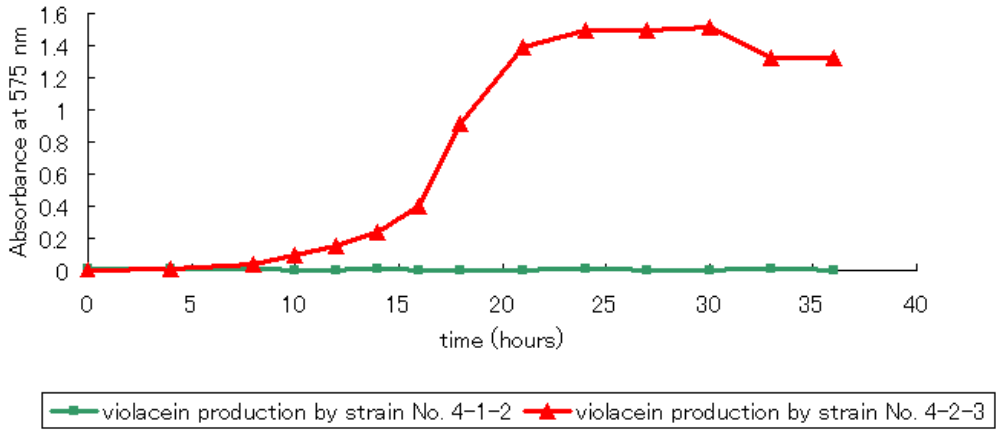
Strain No. 4-1-2 (square) and No. 4-2-3 (triangle) were cultured at 20°C without shaking for 10 days. Each point represents the mean value of duplicate cultures.

3.3.3 Violacein production by strain No. 4-1-2 and No. 4-2-3 under agitated culture conditions

When strains No. 4-1-2 and No. 4-2-3 were incubated at 20°C under shaking conditions (160 rpm), there was no significant difference of the bacterial growth between these strains (**Fig. 3.5a**). However, only strain No. 4-2-3 showed ability of producing violacein under agitated culture conditions (**Fig. 3.5b**). The violacein production by strain No. 4-2-3 started at around 6 h of cultivation and reached the maximum after 24 h of cultivation.



(a): Bacterial growth of strains No. 4-1-2 and No. 4-2-3



(b): Violacein production by strains No. 4-1-2 and No. 4-2-3

Fig. 3.5 Time courses of the bacterial growth and violacein production by strains No. 4-1-2 and No. 4-2-3 under agitated culture conditions

Strains No. 4-1-2 and No. 4-2-3 were cultured at 20°C/160 rpm for 36 h. Each point represents the mean value of duplicate cultures. (a): bacterial growth curves of strains No. 4-1-2 (square) and No. 4-2-3 (triangle); (b): violacein production of strains No. 4-1-2 (square) and No. 4-2-3 (triangle).

3.3.4 Analysis of upstream sequence of strain No. 4-2-3

We can consider several possible reasons for the violacein production by strain No. 4-2-3 under agitated culture conditions. One of them is a mutation in the upstream region of violacein gene cluster of strain No. 4-2-3. Therefore, we analyzed the upstream sequence in strain No. 4-2-3. The upstream sequence of the violacein gene cluster of strain No. 4-2-3 was obtained by PCR method as described in **3.2.7** and deposited in the DDBJ under the accession number AB583752 (**Appendix 4**). The nucleotide comparison of the upstream sequences between strain No. 4-2-3 and strain 520P1 is shown in **Fig. 3.6**. The upstream sequence of strain No. 4-2-3 was identical to that of strain 520P1, demonstrating that no mutation occurred in the upstream region of strain No. 4-2-3.

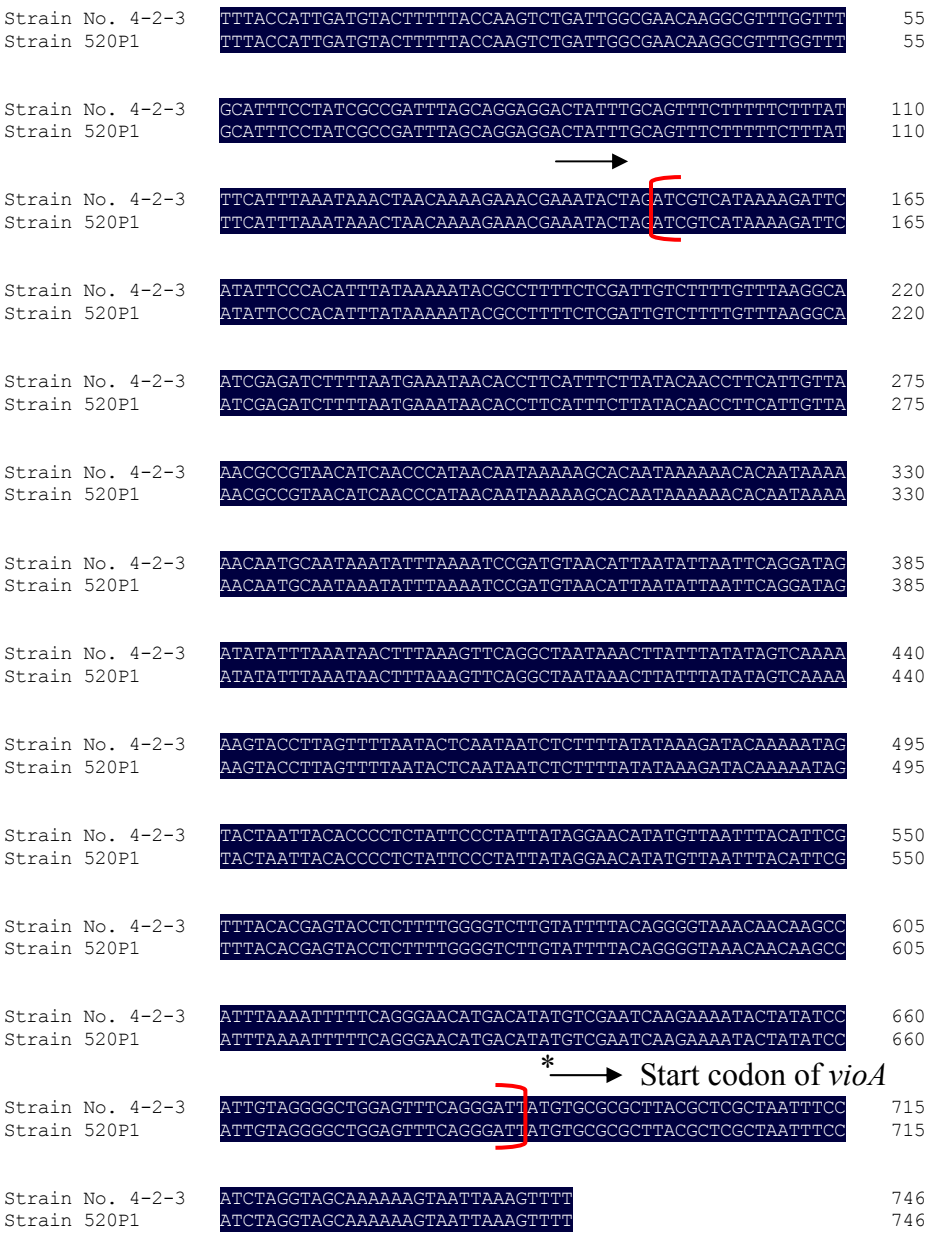


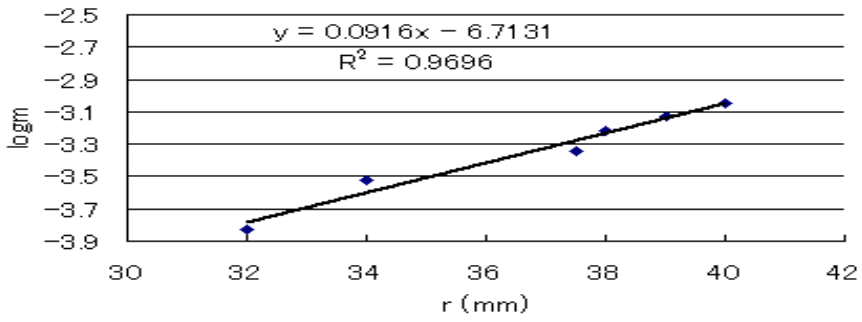
Fig. 3.6 Nucleotide comparison of the upstream sequences between strain 520P1 and strain No. 4-2-3

Upper and lower lines show the upstream sequences of strain No. 4-2-3 and strain 520P1, respectively. The sequences between vioA gene and the upstream protein gene are bracketed. The start codon of *vioA* and the stop codon of upstream protein gene are marked by asterisks (*) and triangle (Δ), respectively.

3.3.5 Analysis of AHLs produced by strains No. 4-1-2 and No. 4-2-3 under agitated culture conditions

Before measuring the activities of AHLs produced by strain No. 4-2-3, we constructed standard curves of synthetic AHLs, 3-oxo-C8 HSL (**Fig. 3.7a**) and C6 HSL (**Fig. 3.7b**), using the reporter strain *A. tumefaciens* NTL4 (pZLR4). The logarithm of the amount of the synthetic AHLs and the radii of the pigmented spots showed a good linearity. Therefore, we can assume the relative amount of AHLs by measuring the radius of the blue pigmented spots.

a



b

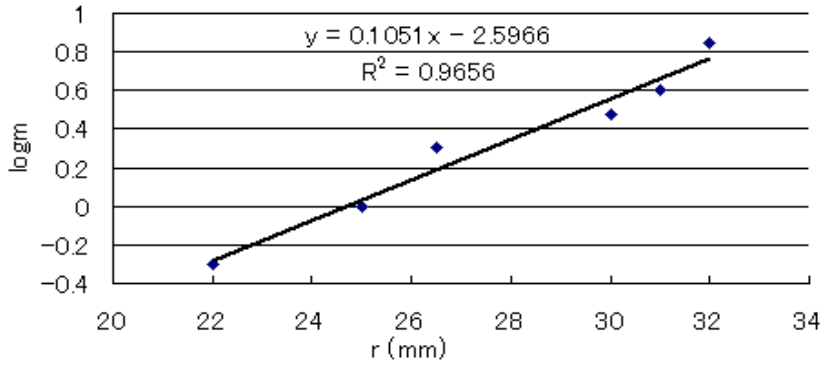


Fig. 3.7 Standard curves of synthetic 3-oxo-C8 HSL (a) and C6 HSL (b)

logm: logarithm of the amount of the synthetic AHL; r: radius of the blue pigmented spot. (a): Amounts of 3-oxo-C8 HSL (μg): 0.00015, 0.0003, 0.00045, 0.0006, 0.00075 and 0.0009; (b): Amounts of C6 HSL (μg): 0.5, 1, 2, 3, 4 and 7.

The reporter strain *Agrobacterium tumefaciens* NTL4 (pZLR4) showed different sensitivity to different synthetic AHLs. Compared with C6 HSL, this reporter strain responded to much smaller amounts of 3-oxo-C8 HSL. Therefore, 3-oxo-C8 HSL was used as the positive control in the following AHLs assays (bio-assay on agar plate and TLC analysis).

In the previous study, we found that the violacein production by strain 520P1 was regulated by quorum sensing [57]. 3-oxo-C8 HSL could activate the production of violacein in strain 520P1. Therefore, we supposed that there might be mutation(s) in AHL synthase gene and/or AHL receptor protein gene of strain No. 4-2-3. Since we have not yet identified the genes for these two molecules, we examined the activities of AHLs in strain No. 4-2-3 (**Fig. 3.8**).

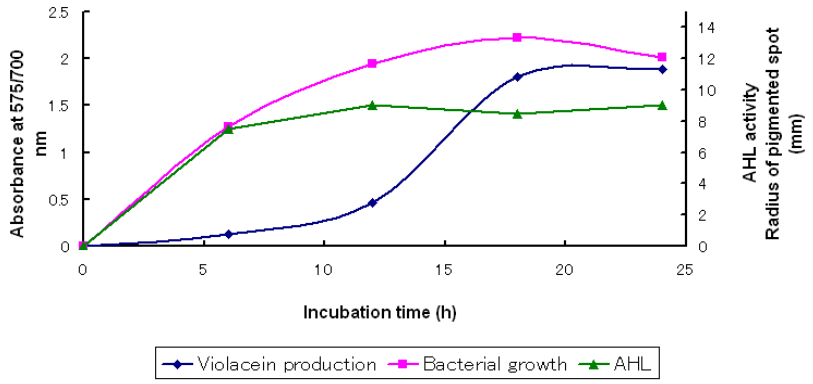


Fig. 3.8 Time courses of the bacterial growth, the production of violacein and *N*-acyl homoserine lactones (AHLs) in strain No. 4-2-3

The growth of the bacteria (square) was monitored by the turbidity of the incubation culture at 700 nm. The production of violacein (rhombus) was evaluated by measuring the absorbance of the extract at 575 nm. The AHL bioassay (triangle) was performed using a reporter strain, *Agrobacterium tumefaciens* NTL4 (pZLR4). The radii of the pigmented spots on assay plates were measured. All the points represent the mean values in duplicate.

The violacein production started at approximately 6 h of cultivation and reached the maximum after 18 h of cultivation. The production of AHL increased rapidly during the first 6 h of cultivation and reached the maximum after 12 h. It seemed that the production of violacein by strain No. 4-2-3 was also under the regulation of quorum sensing.

Fig. 3.9 shows the comparison of the activities of AHL extracts from strains No. 4-2-3 and No. 4-1-2. The AHL extract from strain No.4-2-3 obviously developed larger blue zone than that from strain No. 4-1-2. The results suggested a higher amount of AHL production by strain No. 4-2-3. However, we could not calculate the amounts of AHLs produced by strain No. 4-1-2 and No. 4-2-3 because we do not know the types of AHLs in the extracts and the reporter strain shows different sensitivity to different types of AHLs.

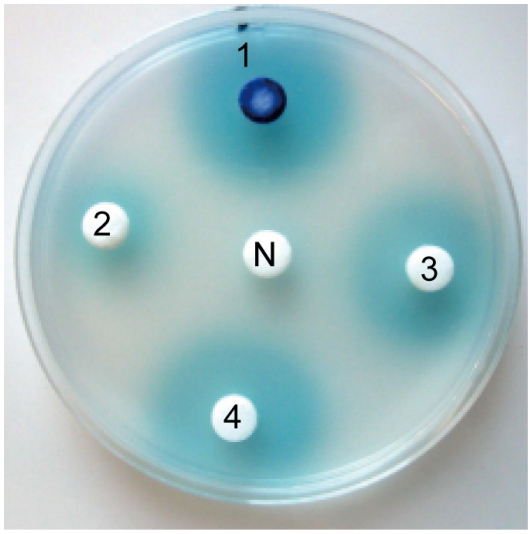
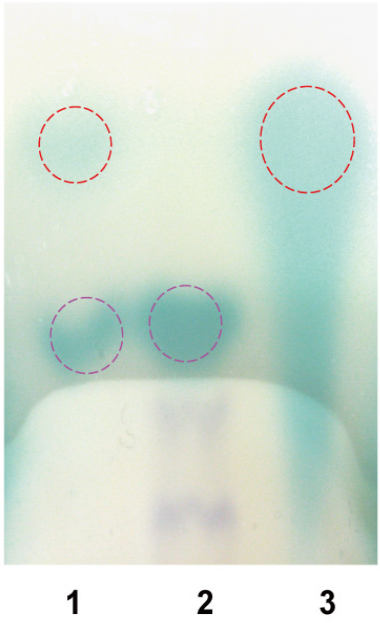


Fig. 3.9 Bio-assay of *N*-acyl homoserine lactones (AHLs) produced by strain No. 4-1-2 and No. 4-2-3

Spot 1: AHLs produced by strain No. 4-2-3 (100 µl of extract); Spot 2: AHLs produced by strain No. 4-1-2 (100 µl of extract); Spot 3: Standard 3-oxo-C8 (0.1 ng); Spot 4: Standard 3-oxo-C8 (0.01 ng); Spot N: DMSO (negative control, 100 µl).

To examine the types of AHLs in the extracts, AHL extracts from strains No. 4-1-2 and No. 4-2-3 were analyzed by TLC on C_{18} reversed phase plate. After chromatography, the TLC plate was overlaid with soft-agar containing the reporter strain and X-gal. Two spots were observed in the extract from strain No. 4-1-2 as previously shown by Wang et al. [57], while only one spot was detected in the extract from strain No. 4-2-3 (**Fig. 3.10**).



Lane 1: extract of strain No. 4-1-2 (100 µl)
Lane 2: extract of strain No. 4-2-3 (100 µl)
Lane 3: synthetic 3-oxo-C8-HSL (0.03 µg)

TLC plate: RP-18 F254
Mobile phase: CH_3OH/H_2O (60:40)
Reporter
strain: *Agrobacterium tumefaciens* NTL4 (pZLR4)

Fig. 3.10 TLC analysis of *N*-acyl homoserine lactones (AHLs) produced by strain No. 4-1-2 and No. 4-2-3

The mobility of the spot in the extract from strain No. 4-2-3 (Rf about 0.4) was similar to one of two spots in the extract from strain No. 4-1-2. The mobility of another spot in extract of strain No. 4-1-2 (Rf about 0.6) was similar to that of synthetic 3-oxo-C8 HSL. The results suggest that strain No. 4-2-3 produced at least one type of AHL, while strain No. 4-1-2 produced at least two kinds of AHL.

3.4 Conclusions and discussion

In this part of study, we isolated a variant strain No. 4-2-3 from the culture of the strain 520P1, which is capable of producing violacein under agitated culture conditions. Strain No. 4-2-3 showed stable and efficient ability of producing violacein

both under static and agitated culture conditions (**Fig. 3.3**, **3.4**, **3.5**). The property of violacein production under agitated culture conditions offers a rapid and efficient method to produce violacein.

As mentioned previously (in **Chapter 2**), there are three critical elements for violacein biosynthesis and its regulation mechanism: the upstream region of the violacein gene cluster, the AHL synthase gene and the AHL receptor protein gene. Here, we supposed the occurrence of mutation(s) in one or three of them. The identical upstream sequences between strains 520P1 and No. 4-2-3 demonstrated no mutation in the upstream region where the promoter and the quorum sensing regulatory site located (**Fig. 3.6**). Because we have not determined the AHL synthase gene and the receptor protein gene, we examined the activities of AHLs produced by the variant strain No. 4-2-3 using a reporter strain *Agrobacterium tumefaciens* NTL4 (pZLR4). The changes of the amount and the type of AHL produced by strain No. 4-2-3 might be one of reasons for the violacein production under agitated culture conditions (**Fig. 3.9, 3.10**). These differences of AHLs production may be caused by the mutation of the AHL synthase gene. AHL synthase is responsible for the AHL production. Therefore, the change of properties of the enzyme reaction may lead to the increased production of AHL(s) and/or the production of different kind of AHL(s). Similarly, the mutation in receptor protein gene might change the specificity of the receptor protein for AHLs, leading to the binding of different type of AHL(s). Correspondingly, it may change the specificity of the binding to the regulatory site on the target DNA. The mutation(s) mentioned above might ultimately affect the expression of the violacein gene cluster, leading to a different mode of violacein

production.

Considering the non-specificity and insensitivity of the reporter strain, we used another two reporter strains, *Chromobacterium violaceum* CV026 [56] and Violacein Inducible Reporter no. 24 (VIR24) [68] to substitute for *A. tumefaciens* NTL4 (pZLR4). However, reporter strains CV026 and VIR24 showed low levels of response to the AHLs produced by strain No. 4-2-3 and No. 4-1-2. We could not obtain desired results with those two reporter strains.

TLC analysis has been used to assume the putative structure of AHL, based on the comparison with authentic AHLs. However, AHL structure should be eventually determined by analyzing their spectroscopic characteristics such as analysis by MS and NMR [64]. In the previous study, we could not determine the structure of AHLs produced by original strain 520P1. One of the reasons of failure is that the amount of AHL was too low to identify their structures. Hence, high AHL production by strain No. 4-2-3 may provide a possibility to determine the AHL structure.

We also analyzed the AHL extract by HPLC. (The conditions of HPLC are shown in **appendix 6**). We found that a new peak (about 8.5 min) appeared when the extract from strain No. 4-2-3 was subjected to HPLC analysis. Standard synthetic 3-oxo-C8 HSL (0.001 μg) could not be detected by HPLC. At a high concentration (1.0 μg), the elution time of 3-oxo-C8 HSL was around 6.5 min. We could not observe the corresponding peak in the elution profiles of strains No. 4-1-2 and No. 4-2-3, probably due to the low concentration of 3-oxo-C8 in the extracts.

It should be noted that both strains No. 4-1-2 and No. 4-2-3 formed the biofilm when incubated under static culture conditions. In *C. violaceum*, the production of

violacein as well as the formation of the biofilm are all under the control of AHLs [56]. However, under agitated culture conditions, the biofilm was destroyed by shaking. It seemed that biofilm formation is not necessary condition for violacein production in strain No. 4-2-3 but it is an indispensable condition for strain No. 4-1-2.

One of the critical factors for violacein production is oxygen. In *C. violaceum*, aerobic conditions are necessary for violacein production [13], but high oxygen levels inhibited the violacein production (Oliveira et al, unpublished data). In *P. luteoviolacea*, the highest amount of violacein was obtained by culture under stagnant conditions [34]. Strain No. 4-2-3 is capable of producing violacein both under high or low oxygen concentrations. Our data indicate that the speed of violacein production by strain No. 4-2-3 but not the amount of violacein was affected by the concentrations of oxygen (**Fig. 3.4, 3.5**). We suppose that this effect of oxygen is achieved by regulating the speed of bacterial growth. In *Pseudomonas aeruginosa*, it seems that in addition to the dependency on cell density, iron and oxygen concentrations also strongly affect the expression of the regulatory protein LasR [69]. Strain No. 4-2-3 might share a similar regulatory mode as *Pseudomonas aeruginosa*. However, it remains elucidated why violacein production by strain No. 4-2-3 occurred under agitated culture conditions.

We believed that the study of variant strain No. 4-2-3 provides a short, convenient and efficient method to produce violacein as well as makes a contribution to further understanding of quorum sensing regulation of the expression of the violacein cluster genes.

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Table of APPENDIXES

APPENDIXES

Appendix 1

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```

Appendix 1 Nucleotide sequences of the violacein gene cluster with its upstream and downstream regions (DDBJ accession number AB573101)

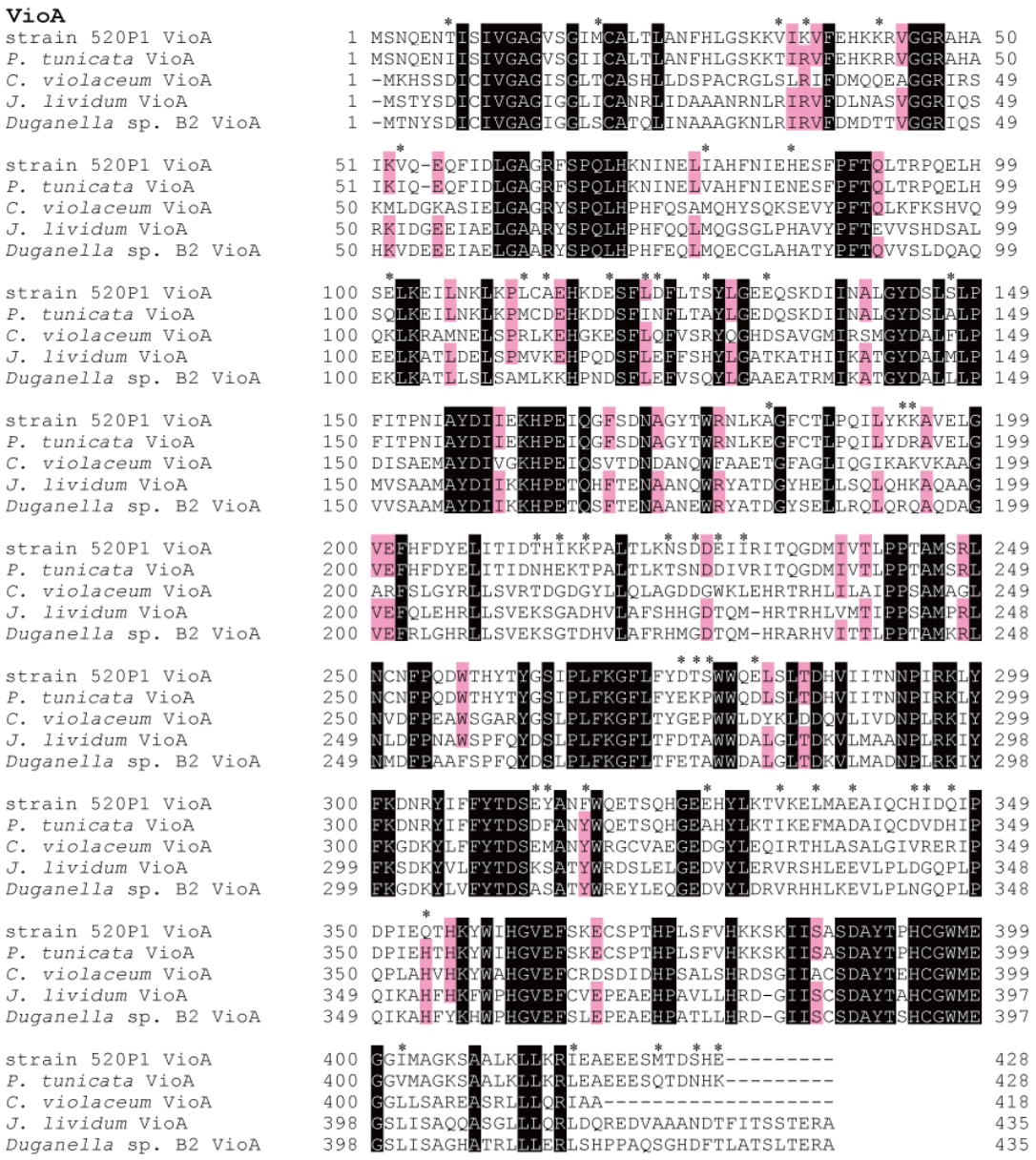
1449...2735: gene *vioA*, product: tryptophan 2-monooxygenase VioA

2774...5806: gene *vioB*, product: polyketide synthase VioB

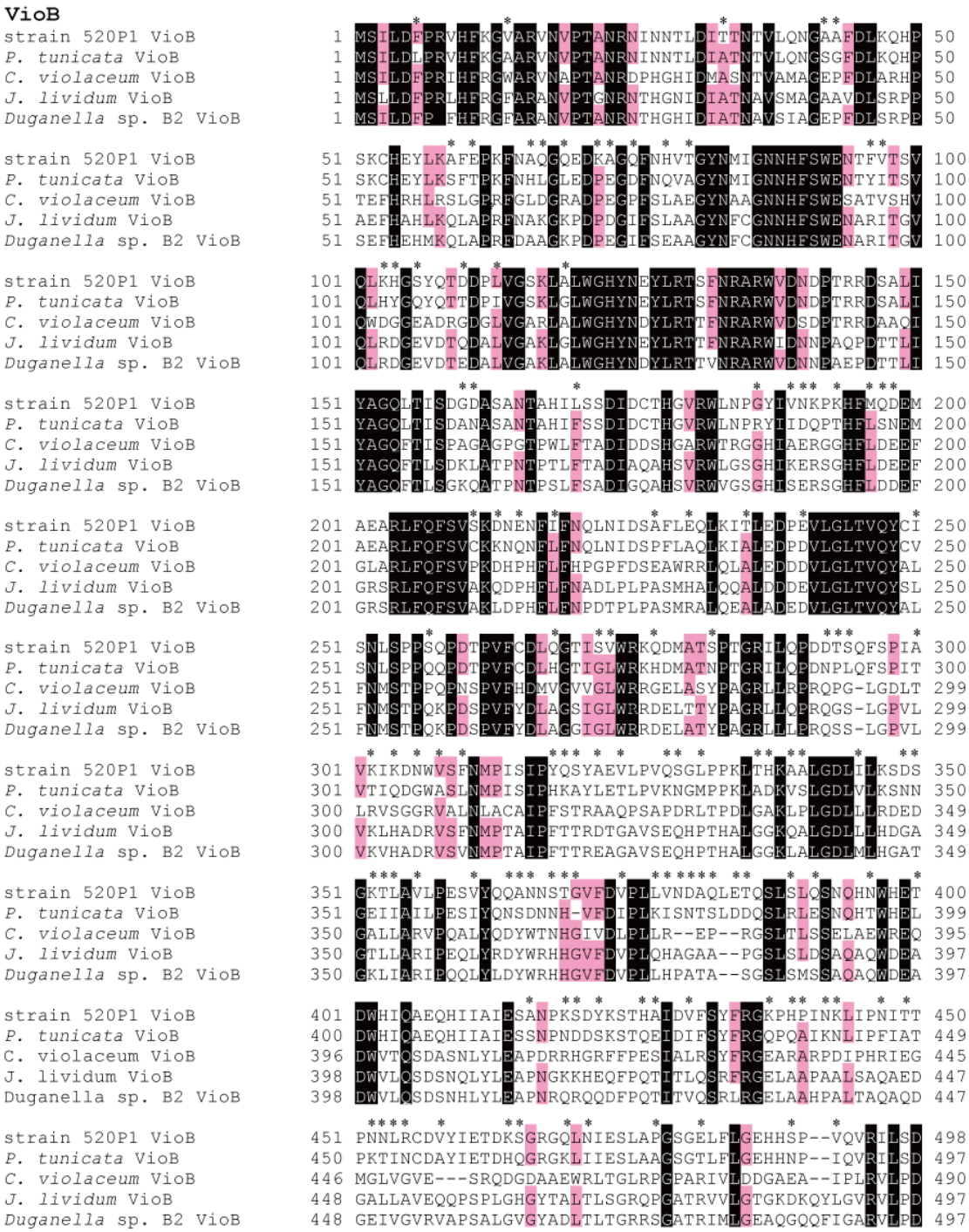
5809...7098: gene *vioC*, product: monooxygenase VioC

7098...8231: gene *vioD*, product: hydroxylase VioD

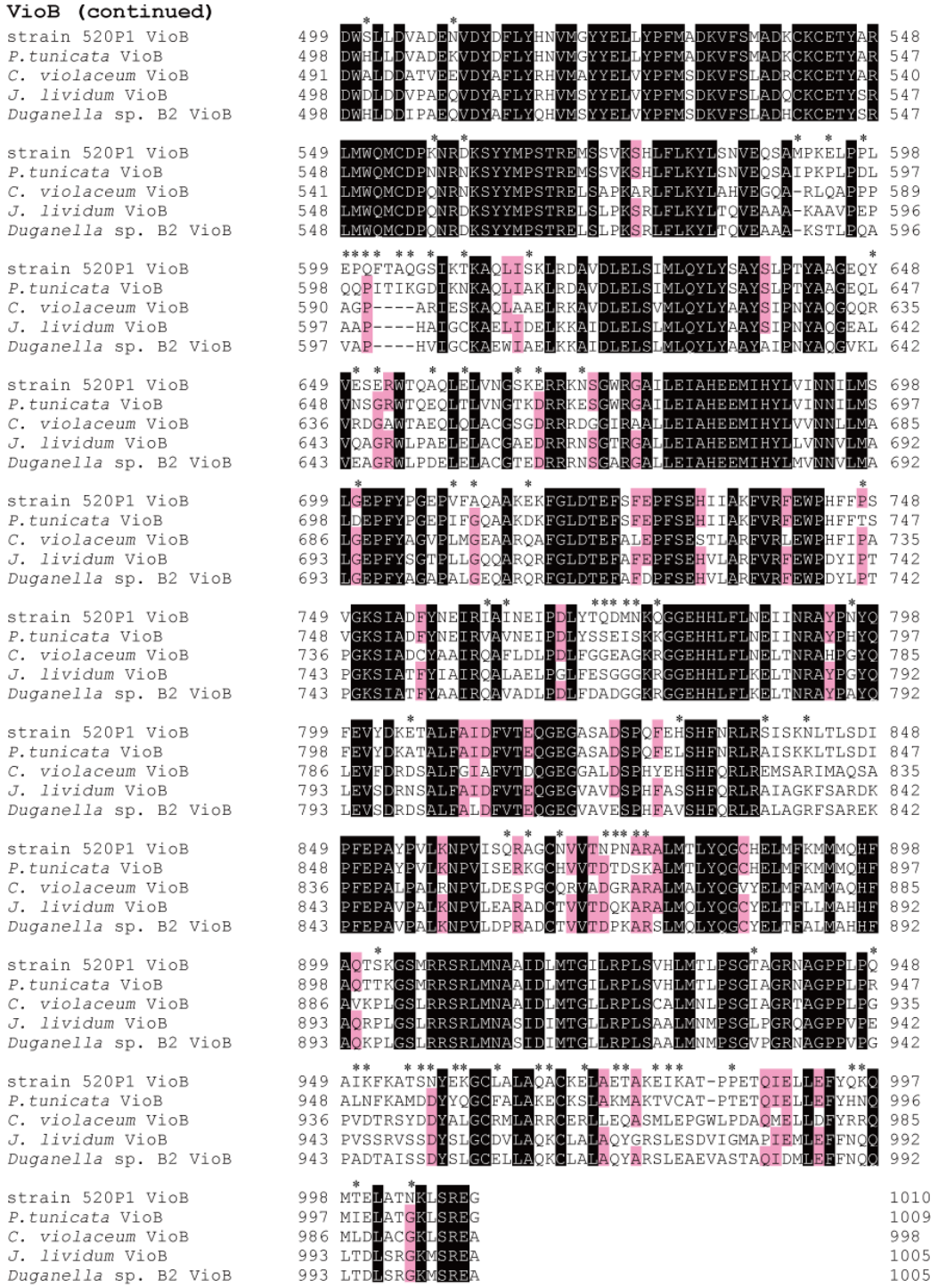
8232…8831: gene *vioE*, product prodeoxyviolacein synthase VioE,



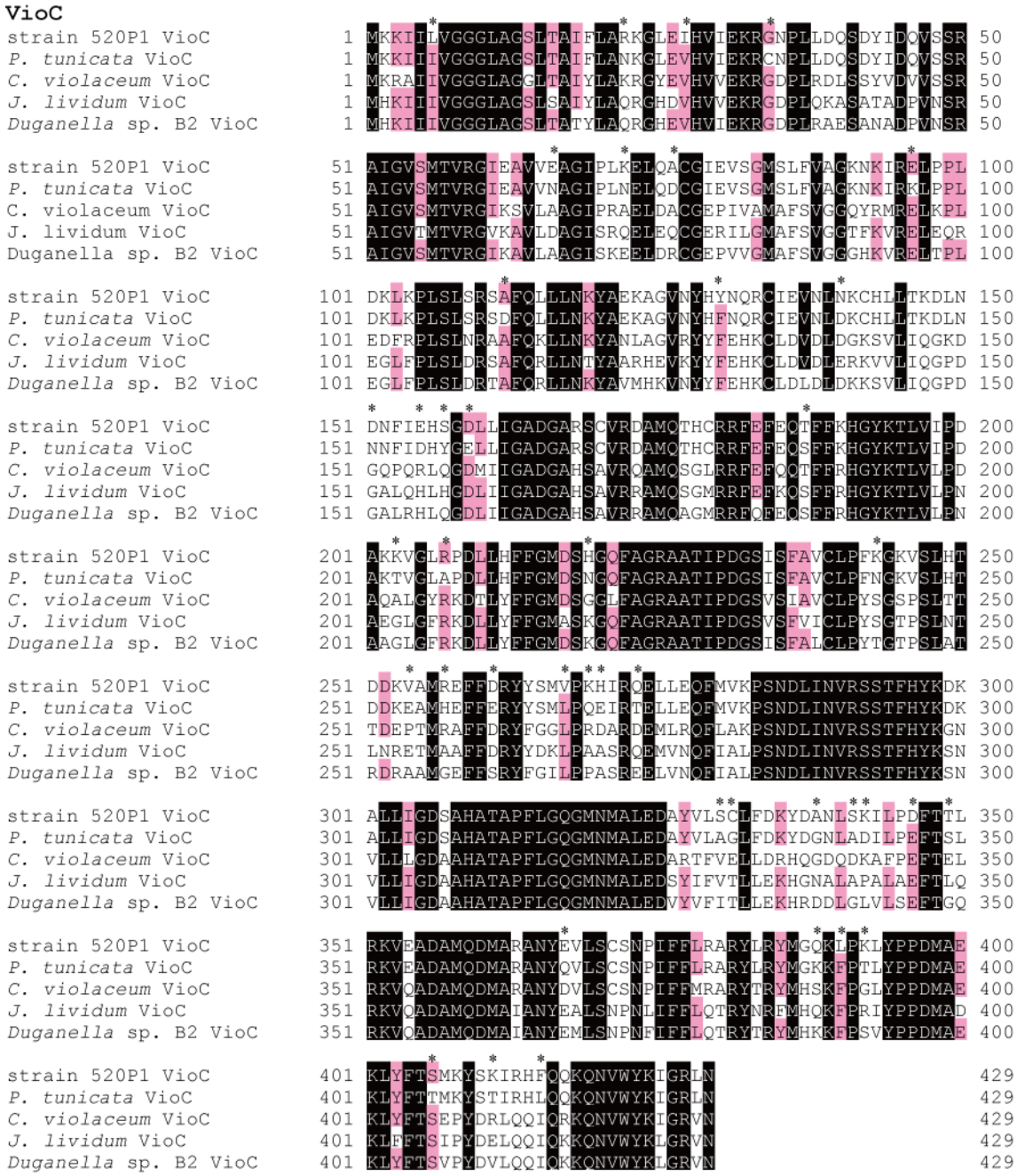
Appendix 2a Comparison of the amino acid sequences of VioA from strain 520P1 and other violacein-producing bacteria



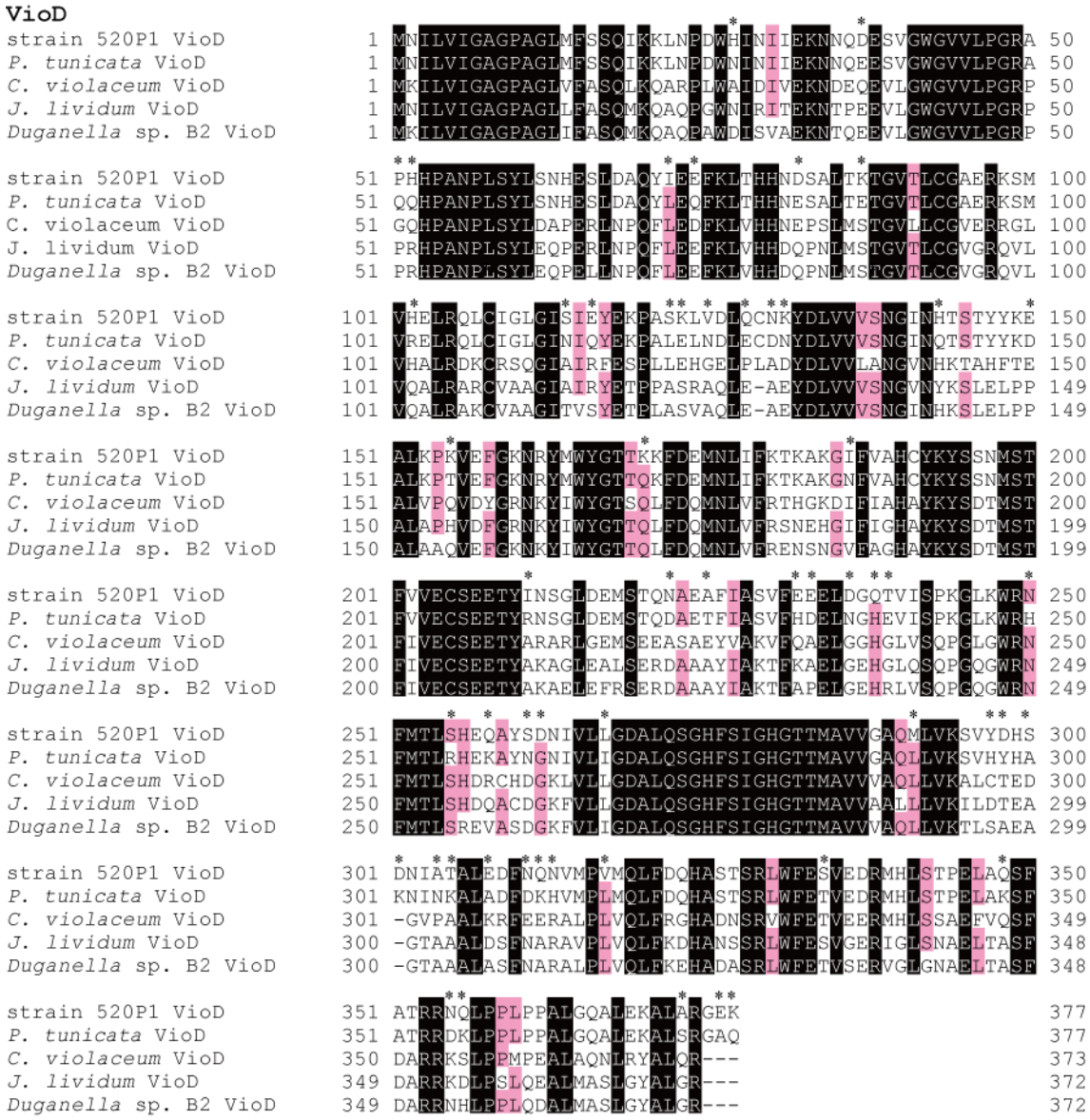
Appendix 2b Comparison of the amino acid sequences of VioB from strain 520P1 and other violacein-producing bacteria



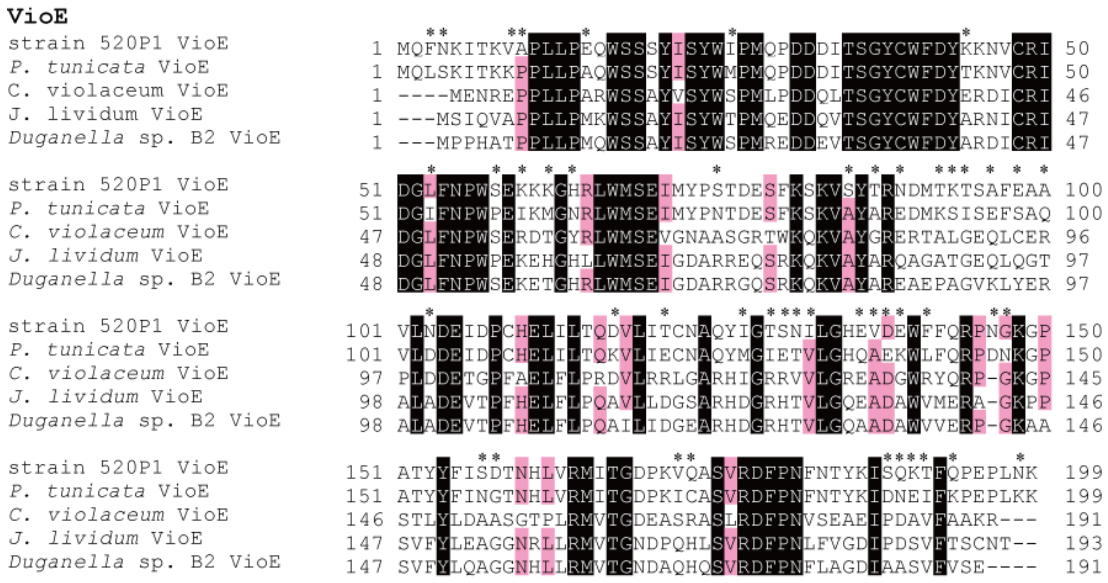
Appendix 2c Comparison of the amino acid sequences of VioB (continued) from strain 520P1 and other violacein-producing bacteria



Appendix 2d Comparison of the amino acid sequences of VioC from strain 520P1 and other violacein-producing bacteria



Appendix 2e Comparison of the amino acid sequences of VioD from strain 520P1 and other violacein-producing bacteria



Appendix 2f Comparison of the amino acid sequences of VioE from strain 520P1 and other violacein-producing bacteria

Appendix 2 Comparison of the amino acid sequences of violacein synthetic enzymes from strain 520P1 and other violacein-producing bacteria

Amino acid sequences of VioA (a), VioB (b, c), VioC (d), VioD (e) and VioE (f) of strain 520P1 were compared with those of other strains. Fully conserved amino acids are indicated in black and amino acids with homology greater than or equal to 75% are indicated in pink. Different amino acids between strain 520P1 and *P. tunicata* are marked with an asterisk (*). Amino acid sequences were deduced from the DNA sequences of strain 520P1 (DDBJ Accession number AB573101), *P. tunicata* D2 (AAOH00000000), *C. violaceum* (AE016825), *J. lividum* (EF063591) and *Duganella* sp. B2 (GQ266676).

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Appendix 3 Nucleotide sequences of upstream of violacein gene cluster in strain 710P1 (DDBJ accession number AB583751)

1…757 bp: upstream region of violacein biosynthetic gene cluster

162…646 bp: upstream region containing a putative promoter

647…>757 bp: tryptophan 2-monooxygenase VioA

Translation = “MSNQENTISIVGAGVSGIMCALTLANFHLGSKKTIRV”

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361    TAACATTAAT ATTAATTCAG GATAGATATA TTTAAATAAC TTTAAAGTTC AGGCTAATAA
421    ACTTATTTAT ATAGTCAAAA AAGTACCTTA GTTTTAATAC TCAATAATCT CTTTTATATA
481    AAGATACAAA AATAGTACTA ATTACACCCC TCTATTCCCT ATTATAGGAA CATATGTTAA
541    TTTACATTCG TTTACACGAG TACCTCTTTT GGGGTCTTGT ATTTTACAGG GGTAAACAAC
601    AAGCCATTTA AAATTTTTCA GGGAACATGA CATATGTCGA ATCAAGAAAA TACTATATCC
661    ATTGTAGGGG CTGGAGTTTC AGGGATTATG TGCGCGCTTA CGCTCGCTAA TTTCCATCTA
721    GGTAGCAAAA AAGTAATTAA AGTTTT
```

Appendix 4 Nucleotide sequences of upstream of violacein gene cluster in variant strain No. 4-2-3 (DDBJ accession number AB583752)

1…746 bp: upstream region of violacein biosynthetic gene cluster

149…633 bp: upstream region containing a putative promoter

634…>746 bp: tryptophan 2-monooxygenase VioA

Translation = “MSNQENTISIVGAGVSGIMCALTLANFHLGSKKVIKV”

Appendix 5 Media used in this study

<table>
<tr><th colspan="2">Name of medium</th><th colspan="2">Composition</th></tr>
<tr><td colspan="2" rowspan="3">Luria-Bertani medium (LB)
(pH 7.0)</td><td>Tryptone</td><td>10.0 g/L</td></tr>
<tr><td>Yeast Extract</td><td>5.0 g/L</td></tr>
<tr><td>NaCl</td><td>10.0 g/L</td></tr>
<tr><td colspan="2" rowspan="4">PPES-II medium
(pH 7.8)

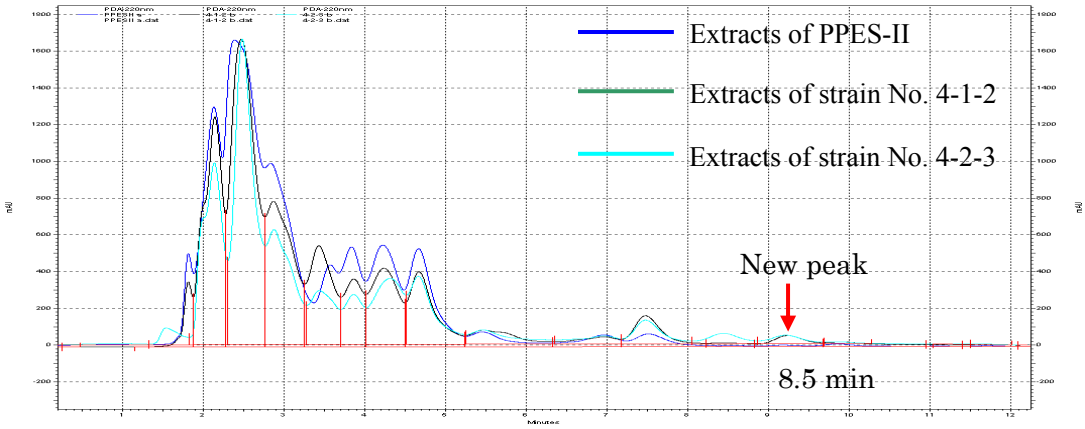
(Filtered seawater)</td><td>Polypeptone</td><td>2.0 g/L</td></tr>
<tr><td>Proteose Peptone No.3</td><td>1.0 g/L</td></tr>
<tr><td>Soytone Peptone</td><td>1.0 g/L</td></tr>
<tr><td>Yeast Extract
Ferric Citrate</td><td>1.0 g/L
0.1 g/L</td></tr>
<tr><td rowspan="6">AB minimal medium *

S1 50 ml
S2 50 ml
S3 10 ml
H_2O 890 ml

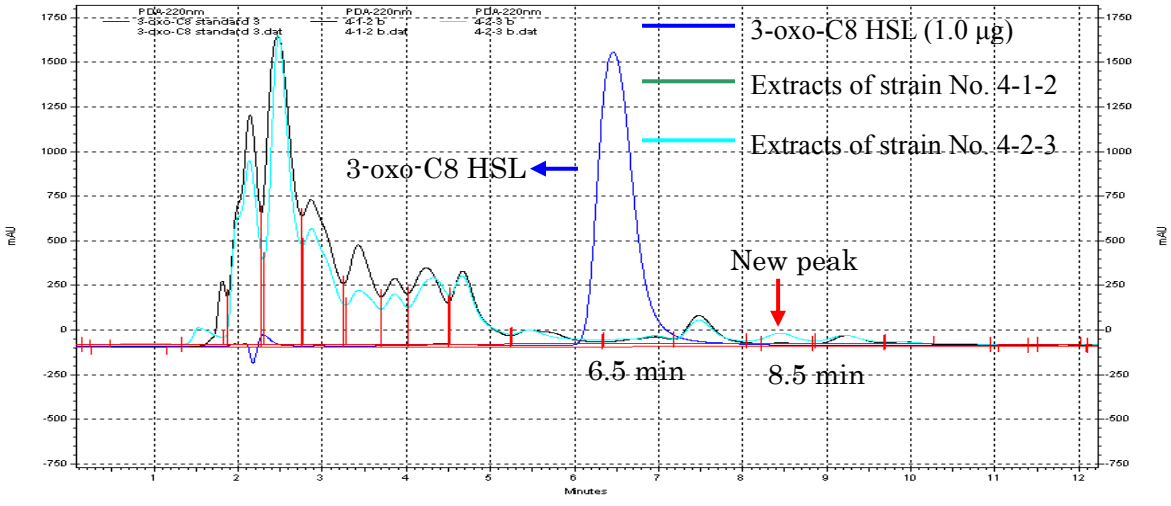
*: S1, S2 and S3 are autoclaved separately.</td><td>Solution 1 (S1)</td><td>K_2HPO_4
NaH_2PO_4</td><td>60.0 g/L
20.0 g/L</td></tr>
<tr><td rowspan="4">Solution 2 (S2)</td><td>NH_4Cl</td><td>20.0 g/L</td></tr>
<tr><td>$MgSO_4 \cdot 7H_2O$</td><td>6.0 g/L</td></tr>
<tr><td>KCl</td><td>3.0 g/L</td></tr>
<tr><td>$CaCl_2 \cdot 2H_2O$
$FeSO_4 \cdot 7H_2O$</td><td>0.2 g/L
0.05 g/L</td></tr>
<tr><td>Glucose (S3)</td><td>Glucose</td><td>20%</td></tr>
</table>

Appendix 6 Analysis of AHLs extracts by HPLC

Extracts were analyzed by HPLC equipped with an ODS column (Capcell Pak C-18 MGII, 150 × 1.5 mm, 5 µm, Shiseido) which was run at a flow rate of 100 µl/min using the mobile phase of 45% (v/v) methanol (MeOH) aqueous solution and a temperature of 40°C. The monitoring wavelength was 220 nm.



(a): Elution profiles of extracts of PPES-II (dark blue), strain No. 4-1-2 (green) and strain No. 4-2-3 (light blue).



(b): Elution profiles of extracts of synthetic 3-oxo-C8 HSL (1.0µg) (dark blue), strain No. 4-1-2 (green) and strain No. 4-2-3 (light blue).