Negative Regulation of Violacein Biosynthesis in *Chromobacterium violaceum*

Giulia Devescovi\(^1\), Milan Kojic\(^2\), Sonia Covaceuszach\(^3\), Miguel Câmara\(^4\), Paul Williams\(^4\), Iris Bertani\(^1\), Sujatha Subramoni\(^1\)† and Vittorio Venturi\(^1\)*

\(^1\)International Centre for Genetic Engineering and Biotechnology, Trieste, Italy, \(^2\)Laboratory for Molecular Microbiology, Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia, \(^3\)Consiglio Nazionale delle Ricerche, Istituto di Cristallografia, U.O.S di Trieste, Trieste, Italy, \(^4\)Centre for Biomolecular Sciences, School of Life Sciences, University of Nottingham, Nottingham, UK

In *Chromobacterium violaceum*, the purple pigment violacein is under positive regulation by the N-acylhomoserine lactone CviI/R quorum sensing system and negative regulation by an uncharacterized putative repressor. In this study we report that the biosynthesis of violacein is negatively controlled by a novel repressor protein, VioS. The violacein operon is regulated negatively by VioS and positively by the CviI/R system in both *C. violaceum* and in a heterologous *Escherichia coli* genetic background. VioS does not regulate the CviI/R system and apart from violacein, VioS, and quorum sensing regulate other phenotypes antagonistically. Quorum sensing regulated phenotypes in *C. violaceum* are therefore further regulated providing an additional level of control.

**Keywords:** *Chromobacterium violaceum*, VioS, CviI/R quorum sensing, regulation, violacein, chitinase activity, protease activity

**INTRODUCTION**

Many Gram-negative bacteria regulate cell density dependent behavior by producing and sensing N-acylhomoserine lactone (AHL) signal molecules by a process called quorum sensing (QS; Fuqua et al., 1994). A canonical AHL-dependent QS system is composed of two proteins respectively belonging to the LuxI and LuxR protein families (Fuqua and Greenberg, 2002). Typically, AHLs are produced by an AHL synthase (LuxI homolog) and sensed at a threshold concentration due to increase in cell population density by an AHL-binding regulator (LuxR homolog) which then affects transcription of target genes (Fuqua and Greenberg, 2002). AHL QS regulates many phenotypes that impact on bacterial community or group behaviors including the expression of secreted enzymes, antibiotic and exopolysaccharide production, biofilm formation, conjugation, symbiosis, and virulence (Fuqua and Greenberg, 2002; Loh et al., 2002; Von Bodman et al., 2003; Waters and Bassler, 2005).

*Chromobacterium violaceum* is a betaproteobacterium found in a variety of soil and aquatic habitats causing infrequent but fatal mammalian infections (Brazilian National Genome Project, 2003). Two *C. violaceum* strains (ATCC31532 and ATCC12472) possess an AHL QS system and surprisingly they produce and respond to different AHLs (McClean et al., 1997; Morohoshi et al., 2008). The AHL QS system of *C. violaceum* ATCC12472 is encoded by the genetically linked *cviI* and *cviR* genes producing and responding with highest affinity to N-decanoyl-L-homoserine lactone (C10-HSL). CviR therefore binds to C10-HSL with highest affinity (Morohoshi et al., 2008; Swem et al., 2009) and the *cviI* AHL synthase is under positive feedback regulation by
C10-HSL-CviR (Stauff and Bassler, 2011). The CviI/CviR QS system of *C. violaceum* ATCC12472 is important for virulence as revealed by loss of pathogenicity in a *C. elegans* infection model in the presence of an antagonistic ligand for CviR instead of C10-HSL (Swem et al., 2009). In contrast, a much earlier report (McClean et al., 1997) demonstrated that the AHL signal produced by *C. violaceum* ATCC31532 is C6-HSL. However, cloning and genetic analysis of this *cviI/cviR* QS system has not been yet been reported in detail.

In *C. violaceum*, QS regulates (i) the *vioA* promoter of violaexen genes coding for the water insoluble purple pigment violacein (Lichstein and Van De Sand, 1946; McClean et al., 1997), (ii) genes coding for cyanide production and degradation (Durán and Menck, 2001), and (iii) multiple genes the products of which are chitinases (Chernin et al., 1998). Besides the *cviI* promoter, several other genes are directly regulated by CviR in *C. violaceum* ATCC12472 and these include genes coding for a putative transcriptional regulator (CV_0577), a guanine deaminase (CV_0578), a chitinase (CV_4240), and a type VI secretion system gene (CV_1432) (Stauff and Bassler, 2011). As in *C. violaceum* AHL QS regulates the production of the purple pigment violacein; this has allowed the convenient use of this bacterium as an AHL biosensor since the AHL-negative biosensor strain CV026 produces violacein only upon the addition of exogenous AHLs with from C4 to C8 acyl side chains (McClean et al., 1997; Steindler and Venturi, 2007).

Regulation of violaexin production by QS has been studied in more detail than the other phenotypes as it is an easily discernible and visible trait. Using a combination of mutagenesis-based analysis in *C. violaceum* ATCC31532 and experiments in a heterologous *Escherichia coli* host, the *vioA* promoter of *vioABCDE* operon has been shown to be under the direct positive regulation of CviR (McClean et al., 1997; Swem et al., 2009). Comprehensive mutational analysis of the *vioA* promoter has also enabled the identification of a CviR binding site (Stauff and Bassler, 2011). Interestingly, the level of violaexin produced by wild type *C. violaceum* ATCC12472 is much higher than that of wild type *C. violaceum* ATCC31532 (McClean et al., 1997). Furthermore, a violaexin repressor has been reported and inactivated by transposon mutagenesis in two independent studies in *C. violaceum* ATCC31532 giving rise to mutants with considerably higher violaexin production (McClean et al., 1997; Swem et al., 2009). In addition, the *Chromobacterium* AHL biosensor strain CV026 is a double transposon insertion mutant since single *Tn5* insertions in the putative AHL synthase failed to respond to exogenous AHLs unless a second transposon was introduced into the putative repressor locus (McClean et al., 1997). However, the mechanism of violaexin regulation by this putative repressor and its regulatory relationship with the *C. violaceum* AHL QS system are not known.

In this study we have examined the regulation of violaexin production in *C. violaceum* ATCC31532 and characterized its QS system as well as a repressor mutant of this strain with respect to violaexin production. We show that the expression of the *vioA* promoter of the *vioABCDE* operon is under negative regulation by this novel repressor which we have named VioS. VioS is also involved in the regulation of other AHL QS regulated phenotypes such protease and chitinolytic activity. Furthermore, we provide evidence for direct interference by VioS of QS mediated positive regulation of the *vioA* promoter in *C. violaceum* and in *E. coli*.

Finally, we show that VioS functions as a repressor of violaexin production in the closely related *C. violaceum* ATCC12472 when introduced in trans. We propose that VioS is a novel protein that functions to fine-tune the QS regulated phenotype of violaexin biosynthesis by regulating *vioA* promoter expression rather than modulating the regulation of *cviI/cviR* gene expression.

**MATERIALS AND METHODS**

**Bacterial Strains, Media, and Growth Conditions**

Wild type *C. violaceum* ATCC 31532, ATCC12472, and CV026 (McClean et al., 1997) and *Escherichia coli* strains DH5α and M15 were routinely grown at 30°C and 37°C, respectively, in Luria–Bertani (LB) broth medium (Miller, 1972). When required, antibiotics were added in the following concentrations: ampicillin 100 µg ml⁻¹, kanamycin 100 µg ml⁻¹, gentamicin 50 µg ml⁻¹, tetracyclin 40 µg ml⁻¹ for *C. violaceum* strains and, ampicillin 100 µg ml⁻¹, kanamycin 50 µg ml⁻¹, gentamycin 20 µg ml⁻¹ and tetracyclin 20 µg ml⁻¹ for *Escherichia coli* strains. AHLs used here were obtained from Sigma-Aldrich (St. Louis, MO, USA).

**Recombinant DNA Techniques**

DNA manipulations, including digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 DNA ligase, transformation of *E coli*, colony hybridization and radioactive labeling by random priming, were performed as previously described (Sambrook et al., 1989). Plasmids were purified using EuroClone columns (EuroClone S.p.A., Italy). Total DNA from *C. violaceum* was isolated with the sarkosyl-pronase lysis method (Better et al., 1983). Triparental matings to mobilize DNA from *E. coli* to *C. violaceum* were carried out with the helper strain *E. coli* (pRK2013) (Figurski and Helinski, 1979). PCR amplifications were performed on *C. violaceum* ATCC31532 genomic DNA using GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA).

**Plasmid Construction**

The plasmids used in this study are listed in Table 1.

The *gfp* reporter gene was chosen for studying the promoter activities in *C. violaceum* in order to reduce to the minimum, possible, interference by violaexin which can be an issue with the β-galactosidase assay. A *gfp* based reporter plasmid was constructed by amplifying the *gfp* gene, deprived of its promoter, from plasmid pBBR2-GFP (Passos da Silva et al., 2014) using the primers GFPEF and GFPFR. The amplified *gfp* was then cloned as an EcoRI/PstI fragment in pMP220 vector, generating pMPGFP.

Gene transcriptional fusion plasmids, based on the pMPGFP promoter probe vector, were constructed as follows: the promoter regions of *cviI, cviR, vioA*, and *vioS* genes were amplified from *C. violaceum* 31532 genomic DNA by using,
TABLE 1 | Strains, plasmids, and primers used.

| Strains /plasmids/ primer | Relevant features | References or sources |
|---------------------------|------------------|----------------------|
| **C. violaceum STRAINS**  |                  |                      |
| C. violaceum              | WT isolate       |                      |
| ATCC31532                |                 |                      |
| C. violaceum             | WT isolate       |                      |
| ATCC21472                |                 |                      |
| CVO26                    | Double transposon mutant of ATCC31532, violaeng and AHL negative | McClean et al., 1997 |
| MB8                      | visoS: Trs of C. violaceum ATCC31532; KmR | This study |
| MB11                     | visoS: Trs of C. violaceum ATCC31532; KmR | This study |
| 31532VIOS                | visoS: Km of C. violaceum ATCC31532; KmR | This study |
| 31532CviI                | cviR: Km of C. violaceum ATCC31532; KmR | This study |
| **PLASMIDS**              |                  |                      |
| pRK2013                  | Tra+ Mob+ ColEl replicon; KmR | Figurski and Heleni, 1979 |
| pGEM7T                   | Cloning vector; AmpR | Promega |
| pMP220                   | Promoter probe vector, IncP; TcR | Spank et al., 1987 |
| pQE30                    | Expression vector; AmpR | Qiagen |
| pBSK8S                   | Cloning vector; AmpR | Stratagenite |
| pBBRmcs5                 | Broad-host-range vector; GmR | Kovach et al., 1995 |
| pKNOCK-Km                | Conjugative suicide vector; KmR | Alexyev, 1999 |
| pKNOCK-Gm                | Conjugative suicide vector; GmR | Alexyev, 1999 |
| pSUP32021                | Trs delivery suicide plasmid; CviEl; KmR | Simon et al., 1983 |
| pLAF83                   | Broad-host-range vector; IncP; TcR | Staskiwicz et al., 1987 |
| pCVO7                    | pLAF83 containing C. violaceum 31532 DNA; TcR | |
| pBBSCVO7H                | pBSK8S carrying a HindIII 3 kb fragment from CVO7; AmpR | |
| pBCVO7XN                 | pBSK8S carrying a XhoI-Ndel 6.35 kb fragment from CVO7; AmpR | |
| pBBBmcs5                 | pBBRmcs5 containing VioS; GmR | |
| pKNOCKcvI                | Internal cvi fragment cloned in pKNOCK-Km | |
| pKNOCKcvII               | Internal cvi fragment cloned in pKNOCK-Gm | |
| pKNOCKvioS               | Internal vioS fragment cloned in pKNOCK-Km | |
| pMPGFP                   | pMP220 containing the GFPmu3 gene deprived of its promoter | |
| pPcviGFp                 | civ promoter cloned in pMPGFP | |
| pPcviRFGF                | civR promoter cloned in pMPGFP | |
| pPcviSFGF                | vioA promoter cloned in pMPGFP | |
| pPcviSGFP                | vioS promoter cloned in pMPGFP | |
| pPBBBmcs5                | vioS cloned in pBBBmcs5 | |
| pPCE30vioS               | vioS cloned in pPCE30 | |
| pPvioA220                | vioA promoter cloned in pMP220 | |
| pPvioS220                | pep promoter cloned in pMP220 | |
| pGFP50 vector expressing the B. cepacia cepR gene | Aguilar et al., 2003 |
| pMP77                    | Promoter probe vector; IncQ; CmR | Spank et al., 1987 |
| pMPcviRlacZ              | cviR translational fusion | |
| pMPvioAlacZ              | vioA translational fusion | |

**PRIMERS**

| Primers name | Sequence | Source |
|--------------|----------|--------|
| civB         | GAGATCCCCGGCAAGAAGAATCAA | Sources |
| civER        | GATCTCAGGCCATGTTGATGGCCCA | This study |
| civRFB       | GATGATCCGGCAAGGCATGTTGAAA | This study |
| civRER       | GATTTGCTGATGTTGATGGCCGAAT | This study |
| visABF       | GATGATCCGGCAAGGCATGTTGATGGCCGAAT | This study |
| visAER       | GATGATCCGGCAAGGCATGTTGATGGCCGAAT | This study |
| visSFR       | GATGATCCGGCAAGGCATGTTGATGGCCGAAT | This study |
| visSER       | GATGATCCGGCAAGGCATGTTGATGGCCGAAT | This study |
| GFPEF        | GATGATCCGGCAAGGCATGTTGATGGCCGAAT | This study |
| GFPR         | GATGATCCGGCAAGGCATGTTGATGGCCGAAT | This study |

(Continued)
first 49 amino acids was amplified by using the primers vioAPROMFXba and vioAR3BamHI, cloned in-frame upstream the lacZ gene and transferred to the pMP77 plasmid giving pMPVioAlacZ.

**Genomic Mutant Bank and Cosmid Gene Bank Construction and Screening**

A Tn5 genomic mutant library of C. violaceum ATCC31522 was created using pSUP2021, as previously described (Simon et al., 1983). Approximately 5,000 mutants were screened for the presence of violacein hyperproducer mutants by identifying colonies that showed purple coloration in contrast to the pale colonies of the C. violaceum ATCC31522 wild type. Two mutants were isolated and the genomic regions flanking the Tn5 insertions were amplified by arbitrary PCR technique (O’Toole and Kolter, 1998) and sequenced. The two mutants were designated as MB8 and MB11 respectively. A genomic bank (cosmid library) of C. violaceum ATCC31523 was constructed as follows. Briefly, C. violaceum 31532 genomic DNA was partially digested with EcoRI and ligated into pLAFR3 cosmids vector. The constructs obtained were introduced into E. coli cells using Gigapack III XL-4 packaging kit as recommended by the supplier (Stratagene-Agilent, Santa Clara, CA, USA). The genomic bank was then screened using the flanking DNA (obtained by arbitrary PCR on mutant colonies MB8 and MB11), as probes. Three cosmids were isolated which showed the same restriction pattern. Cosmid pCVO7 was chosen and subcloned in pBSIIKS generating two overlapping constructs: pBCVO7HI (containing a 3-kb HindIII fragment) and pBCVO7XN (containing a 6,350-bp XhoI-NorI fragment).

**Construction of 31532CVII, 31532CVIR, and 31532VIOS**

The three additional mutants, 31532CVII, 31532CVIR, and 31532VIOS were generated using the suicide vectors from the pKNOCK series (Alexeyev, 1999). To generate 31532cvil, an internal fragment (209-bp) of the cvil gene was PCR amplified using the primers KNcvivlBF and KNcvivlKR and cloned as a BamHI-KpfI fragment into the corresponding sites of pKNOCK-Km resulting in pKNOCKcvil. In order to generate 31532CVIR, an internal fragment of cvir (327-bp) was amplified with the primers KNcvivlRF and KNcvivlRR, blunted and cloned into pKNOCK Gm digested with the SmaI restriction enzyme, yielding pKNOCKcvirF. Finally, to obtain 31532VIOS, an internal fragment of vioS (187-bp) was amplified with primers KNvioSKF and KNvioSKR and cloned as a KpfI-BamHI fragment in the corresponding sites of pKNOCK-Km giving pKNOCKvioS. The pKNOCK constructs obtained were transferred to C. violaceum ATCC31532 via tri-parental mating and the knock-out mutants were verified by PCR analysis and sequencing. The 31532VIOS was altered in growth rate and behaved like the parent wild-type strain.

**Extraction and Quantification of AHLs**

C. violaceum strains were grown overnight in 20 ml of LB medium. The cells were pelleted at 5,000 g for 15 min. The cell free supernatants were filtered (using 0.45 µm filters; Millipore) and extracted twice with an equal volume of ethyl acetate containing 0.1% v/v acetic acid. The organic phases were collected, dried to completeness and re-suspended in 50 µl of ethyl acetate. To quantify the amounts of C6-HSL produced by the 31532 wild type strain, MB8, MB11, and 31532VIOS, the constructs pPvioA220 and pBBRcvir were used to constitute a CviR-based sensor regulating its target promoter vioA in the heterologous E. coli M15 system. In order to generate a calibration curve, different concentrations (0; 0.01; 0.05; 0.1; 0.5; 1 µM) of C6-HSL were added to 10 ml to each of the sensor strains. The cultures were grown for 6 h and β-galactosidase activity was determined. To quantify the AHls produced by each C. violaceum strain, the experiment was repeated by adding 10 µl of an AHL extract obtained from each strain to the sensor.

**β-Galactosidase and GFP Quantification Assays**

β-galactosidase activities were determined essentially as described by Miller (1972), with the modifications of Stachel (Stachel et al., 1985). Each experiment was performed in triplicate. GFP fluorescence in the stationary phase of the bacterial cultures was determined in a Perkin Elmer EnVision Multilabel Reader that was set to an excitation wavelength of 485 nm and an emission wavelength of 510 nm.

**Exoenzyme Activity**

To assess protease activity, C. violaceum strains were grown to stationary phase and 2 µl of culture was spotted onto M9 agar containing 2% dry milk, as the only carbon source. Zones of activity were measured after 36 h. For chitinase activity, the same protocol was followed and cultures were spotted onto M9 agar containing 0.2% colloidal chitin (Ahmadian et al., 2007).

**RESULTS**

**The AHL QS System of C. violaceum ATCC31522**

The unequivocal chemical identification of C6-HSL from culture supernatants of C. violaceum ATCC 31532 and the selection of a Tn5 transposon mutant with an insertion in a putative luxI orthologue demonstrated the presence of an AHL QS system in this organism (McClean et al., 1997). To isolate the locus encoding this system, a PstI genomic library of this strain was constructed in pUC18. The library was introduced into the AHL biosensor strain E. coli (pSB401) (Winson et al., 1998) and the recombinant colonies screened for the production of bioluminescence using a photon-imaging camera as previously described (Swift et al., 1997). A recombinant clone (pMW50) able to induce light production in the biosensor strain was identified as a highly bioluminescent colony. Expression of pMW50 in E. coli, was able to restore violacein production when cross-streaked against the AHL sensor strain C. violaceum CV026 (McClean et al., 1997) suggesting the presence of an AHL synthase in this recombinant clone. Sequence analysis of the
6 Kb PstI insert from pMW50 revealed the presence of two convergent open reading frames overlapping by 74 bp which were named cviR and cviI as their predicted amino acid sequences were homologous to the LuxI/LuxR family of QS genes. Solvent extraction of culture supernatants from E. coli harboring pMW50 followed by LC-MS/MS analysis revealed the presence of C6-HSL (data not shown). No other AHLs were detected from these extracts indicating that cviI is responsible for the synthesis of this AHL.

**Violacein Biosynthesis Is Negatively Regulated by VioS**

Violacein production by *C. violaceum* is regulated by QS via AHLs signal molecules (McClean et al., 1997; Morohoshi et al., 2008). We have previously shown that violacein production is stringently negatively regulated since we obtained a Tn5 insertion mutant that strongly overproduced violacein in the *C. violaceum* ATCC31532 genetic background (McClean et al., 1997; Table 1). This transposon was localized to a gene coding for a protein of unknown function homologous to CV_1055 of the sequenced genome of *C. violaceum* ATCC12472 demonstrating that violacein is very tightly regulated (Swem et al., 2009).

To further investigate the regulation of this phenotype and to make sure that no other loci was involved in this negative regulation, we constructed a Tn5 mutant library of *C. violaceum* ATCC31532 and screened for more mutants that overproduced violacein as described in the Materials and Methods. Two mutants, named MB8 and MB11 were identified in the screen and the location of the Tn5 insertion site in both mutants was also located in the CV_1055 gene homolog from *C. violaceum* ATCC12472 but in the putative promoter region; the Tn5 in mutant MB8 is located nearer to the ATG of the putative ORF whereas MB11 is further away (Figure 1A). We have now named the hypothetical protein encoded by this gene as VioS (Figure 1A). This predicted protein (138 amino acids; 15 kDa approximately) showed 91% identity and 94% similarity to a hypothetical protein from *Pseudogulbenkiana ferrooxidans* and 85% identity and 90% similarity to the hypothetical protein encoded by CV_1055 from *C. violaceum* ATCC12472 respectively. Conserved domain analysis of VioS amino acid sequence revealed the presence of a domain of unknown function annotated as DUF1484 spanning 32–138 amino acids (8.35e-03) that is exclusively found in bacteria belonging to the betaproteobacteria.

Both MB8 and MB11 transposon mutants exhibited increased violacein production in contrast to the pale white color of *C. violaceum* ATCC31532 wild type (Figure 1B). Mutant MB11 displayed a much stronger violet color compared with MB8 indicating that the transposon insertion in MB11 resulted in greater violacein production. As neither transposon insertion was located in the putative structural gene, an insertion mutant in the putative vioS ORF was generated (designated as 31532VIOS) as described in the Materials and Methods. This mutant showed violacein overproduction similar to MB8 (Figure 1B). Complementation of mutants MB8, MB11, and 31532VIOS with a plasmid construct containing full length vioS and flanking upstream DNA restored violacein production in all the mutants to wild type levels (Figure 1B). These results strongly suggest a role for VioS in the negative regulation of violacein biosynthesis in *C. violaceum* ATCC31532.

**VioS and CviR Regulate Violacein Biosynthesis in Opposite Ways**

Since the studies using the transposon insertion mutants described above clearly support a role for VioS in the negative
regulation of violacein production, which conversely is positively regulated by the CviI/R QS system, we sought to determine whether VioS interacted with the QS system. Consequently we investigated whether VioS influenced the expression of the CviI/R system which could then result in violacin de-regulation. We first determined the AHL levels produced by the wild type, MB8, MB11, and 31532VIOS strains as described in the Materials and Methods. Using a calibration curve derived by a CviI/R AHL biosensor constructed here, we found that all strains produced similar AHL levels production corresponding to a C6-HSL concentration of approximately 0.5 \( \mu \)M (data not shown).

The transcriptional levels of the QS genes using \textit{cviI}::\textit{gfp} and \textit{cviR}::\textit{gfp} plasmid transcriptional fusions were determined and the results showed that the \textit{cviI} and \textit{cviR} genes are expressed at comparable levels in the wild type, the \textit{vioS} mutants and complemented strains (\textit{Figures 2A,B}). To determine whether the CviI/R QS system modulated \textit{vioS} expression, assays were carried out to measure the levels of a plasmid-borne \textit{vioS:}\textit{gfp} transcriptional fusion in the wild type, \textit{cviI} and \textit{cviR} mutants. The expression of \textit{vioS} was similar in all of the strains examined (data not shown). These results indicate that VioS does not influence expression of the CviI/R QS system or vice versa. VioS furthermore does not significantly affect the levels of AHLS.

To further understand the opposing regulatory effects of VioS and CviR-AHL on violacein production we monitored the reporter activity of a plasmid \textit{vioA:}\textit{gfp} transcriptional fusion in the wild type, MB8, MB11, 31532VIOS, \textit{cviI}, and \textit{cviR} mutants (\textit{Figure 2C}). The \textit{vioA} promoter controls the expression of the operon (\textit{vioA-vioE}) encoding for the violacein biosynthesis genes (August et al., 2000; Antônio and Creczynski-Pasa, 2004; Sánchez et al., 2006). As expected, little expression of \textit{vioA:}\textit{gfp} was apparent in the \textit{cviI} and \textit{cviR} mutants compared with the wild type. On the other hand \textit{vioA:}\textit{gfp} fusion showed a drastic increase in expression in all three \textit{vioS} mutants, MB8, MB11, and 31532VIOS compared with the wild type.

**FIGURE 2** | CviI/R QS system is not influenced by VioS but VioS negatively regulates the expression of the \textit{vioA} operon. CviI promoter activity (A), \textit{cviR} promoter activity (B), and \textit{vioA} promoter activity (C) in \textit{C. violaceum} 31532, 31532 quorum sensing mutants, 31532VIOS mutants (MB8; MB11; 31532VIOS) and mutants complemented with pBBVioS, containing full length \textit{vioS}. Stationary phase bacterial cultures were monitored for GFP expression in a Perkin Elmer EnVision Multilabel Reader. The means plus standard deviations for five replicates are shown and 31532 (pMPCFP) represents the empty vector. In panels (D,E), \( \beta \)-galactosidase levels for the \textit{cviR} and \textit{vioA}\textit{lacZ} translational fusions are shown in \textit{C. violaceum} 31532 wild-type, in the \textit{vioS} mutant 31532VIOS and in the same mutant complemented with a plasmid—borne copy of \textit{vioS} gene.
strain. Complementation of the vioS mutants with a wild type copy of the vioS gene restored vioA::gfp expression to wild type levels (Figure 2C). These results demonstrate that VioS represses expression of the vio operon at the transcriptional level thus influencing violacein production in the C. violaceum ATCC31532 wild type strain in spite of presence of a functional CviI/R QS system.

To investigate whether VioS has an effect on the translational levels of cviI and vioA, we constructed cviR-lacZ and vioA-lacZ translational fusions as described in the Materials and Methods. As depicted in Figures 2D,E, VioS did not affect cviR translation. However, in the vioS mutant, the vioA-lacZ translational fusion displayed a 2-fold increase in β-galactosidase activity. These data indicate that VioS exerts a negative effect on the translation of vioA meaning that it could be acting at a post-transcriptional level; however this increase in translation could be due to the increase in transcription observed using the vioA transcriptional fusion (Figure 2C).

**VioS Is Sufficient to Antagonize CviR-Mediated Regulation of the Violacein Biosynthetic Operon in a Heterologous System**

To determine whether VioS is sufficient to antagonize CviR-mediated positive regulation of the vio operon, the entire system consisting of VioS, CviR, and the target promoter vioA::lacZ was reconstructed and introduced into a heterologous E. coli strain as described in Materials and Methods (Figure 3A). When the activity of vioA::lacZ fusion was monitored in E. coli in the presence of CviR and C6-HSL, the promoter showed high levels of expression consistent with CviR the positively regulating vioA in the presence of the cognate AHL signal.

The increased vioA::lacZ expression was not observed in the absence of C6-HSL. Upon expression of VioS in the same E. coli strain containing CviR and exogenously added C6-HSL, vioA::lacZ expression was reduced by over 6-fold indicating that VioS antagonizes the action of CviR, repressing vioA promoter activity. This observation in a heterologous system also indicates that VioS alone is sufficient to mediate the negative regulation of the vioA promoter.

It was also of interest to establish whether the negative effect of VioS on transcription of an AHL QS target gene was specific for the CviR regulated vioA promoter. Expression studies were therefore carried out using a different AHL QS system and target promoter. For this experiment we used the Burkholderia cepacia CepI/R system and the cepI target gene. The plasmid cepI::lacZ transcriptional fusion construct was introduced into E. coli harboring plasmids expressing either CepR or VioS. The expression of the cepI::lacZ fusion was determined with and without the exogenous addition of C8-HSL. In this experiment, the cepI promoter was upregulated in the presence of CepR and AHLS as expected but in contrast to the vioA promoter, it was not repressed in the presence of VioS (Figure 3B). Thus, the VioS mediated effect on the expression of a QS regulated promoter is likely to be specific for the CviI/R system.

**QS and VioS Antagonistically Modulate QS-Regulated Phenotypes in C. violaceum**

Since VioS negatively regulates violacein production, we investigated whether it plays a role in fine-tuning the expression of QS-regulated phenotypes. We observed that VioS antagonistically modulates the expression of QS-regulated genes in C. violaceum.
of other QS-regulated phenotypes in *C. violaceum*. Protease and chitinolytic activities are known to be positively regulated by the CviI/R QS system in *C. violaceum* (Chernin et al., 1998). In the cviR mutant of ATCC31532 both protease and chitinase activities were abolished when compared with the wild type. In contrast to this, the vioS mutant showed increased levels of both protease and chitinase activities which could be reduced back to wild type levels by providing VioS in trans (Figures 4A, B). This shows that VioS also acts as a repressor of these two CviI/R QS regulated phenotypes as well as of violacein production. VioS might therefore play a more general role in adjusting the expression of CviI/R QS target genes in a manner opposite to their regulation by CviI/R QS.

**DISCUSSION**

In this study we report the regulatory functions of VioS, a putative repressor protein that negatively controls violacein production without influencing expression of the CviI/R QS system. The repressor function of VioS on violacein production is dominant as it antagonizes positive regulation by CviR/C6-HSL in wild type *C. violaceum* ATCC31532. Other phenotypes positively regulated by CviR-AHL, including protease and chitinase production, were also negatively regulated by VioS. Our results have thus uncovered a novel repressor of *C. violaceum* QS and identified another layer of population dependent regulation in this bacterium.

*C. violaceum* is an environmental bacterium, found in soil and water, is generally non-pathogenic but occasionally extremely virulent to humans and animals (Brazilian National Genome Project, 2003). It has been shown that elimination of QS leads to loss of virulence of *C. violaceum* in a *C. elegans* model of infection suggesting that functions positively regulated by QS are important for infection (Swem et al., 2009). However, the phenotypes regulated by AHL-dependent QS can be energetically expensive such that constitutive expression of these shared traits is not likely to enable optimal utilization of available resources; it may also elicit stronger host defense responses. RsaL, a negative regulator of QS and QS-regulated genes in *Pseudomonas aeruginosa* has been reported to be important for optimum virulence as rsaL mutants are hypervirulent in a *Galleria mellonella* acute model of infection (Rampioni et al., 2009). Also, in a study involving dual-species co-culture of *C. violaceum* and *Burkholderia thailandensis*, it was reported that QS dependent antimicrobials like violacein can provide a competitive advantage in mixed microbial communities with limited nutrients (Chandler et al., 2012). Here, we have shown that VioS functions to fine-tune QS-regulated phenotypes and it is possible that it might play a role in providing optimum fitness to *C. violaceum* both in the environment and in host associations. Alternatively, it cannot be excluded that VioS responds to environmental stimuli or an unknown signal that results in de-repression and so promotes high levels of violacein production under certain circumstances.

Although the molecular mechanism of VioS-mediated repression in QS homeostasis is not known, it is possible that it belongs to a new class of regulators. Among the few characterized negative regulators of QS are RsaL, AlgQ, and a TetR-like transcriptional repressor of *P. aeruginosa*, all of which bind DNA (De Kievit et al., 1999; Ledgham et al., 2003; Rampioni et al., 2006; Venturi et al., 2011; Longo et al., 2013). RsaM of *P. fuscovaginae* as well as other repressor proteins with less sequence identity to RsaM including BcRsaM of *B. cenocepacia* and TofM of *B. glumae* are also QS repressors (Mattiuzzo et al., 2011; Chen et al., 2012; Michalska et al., 2014). However, BcRsaM is predicted to influence QS by an as yet unknown mechanism but not by binding to DNA (Michalska et al., 2014). The VioS amino acid sequence does not show similarity to any of these proteins and furthermore this study does not provide any direct evidence that VioS exerts its regulation at
the transcriptional level. Studies performed using translational fusions indicate that VioS had a negative effect on the translation of vioA. A comparison with Rsal of P. aeruginosa suggests that VioS exhibits some common and distinct features. The rsl gene is genetically linked to QS systems and its transcription is positively regulated by QS. However, RsaL negatively regulates expression of lasI coding for AHL synthase as well as some other QS regulated genes responsible for e.g., pyocyanin and HCN production (Schuster et al., 2004; Rampioni et al., 2006, 2007b). RsaL and LasR have been shown to bind to adjacent sites on the lasI promoter but the negative regulatory effect of RsaL is dominant over the activating effect of LasR-AHL (Rampioni et al., 2007a). In our study, the presence of VioS influences vioA promoter activity in a manner similar to RsaL-mediated repression of the lasI promoter because the repressor activity of VioS on vioA promoter is dominant over the activator effect of CviR-AHL. However, unlike the rslA system where the expression of the repressor is dependent on LasR-AHL, vioS expression is not linked to CviR-AHL and the mechanism of vioS expression and regulation requires further investigation. In addition vioS is found in a separate genomic location from the cviI and cviR genes and does not have any direct effect on their transcription but impacts at an as yet unknown level on CviR/QS target gene expression. Moreover, VioS appears to be sufficient and specific for CviR-AHL antagonism as it is not a general inhibitor of gene activation by other QS LuxR regulators in other bacteria, for example CepR-AHL from Burkholderia.

Sequence similarity searches with the predicted amino acid sequence of C. violaceum ATCC31532 VioS were undertaken to identify homologs of this protein in other bacteria. In our searches VioS homolog was identified only in the sequenced genomes of C. violaceum ATCC12472 strain and P. ferrooxidans. The exclusive presence of VioS in these two bacterial genera suggests that it may have specific functions in these bacterial species. In contrast, other QS repressors like Rsal and RsaM are present in multiple members of the proteobacteria (Venturi et al., 2011). Both C. violaceum and P. ferrooxidans produce the purple violacein pigment and it will be interesting to determine whether VioS also regulates pigment production in P. ferrooxidans. These two bacteria could share a similar niche(s) [P. ferrooxidans producing violacein has been isolated in a lake, ([Puranik, 2013 #736]) as well as profile and regulation of secondary metabolite production in order to survive in specific environmental conditions; this possibility is currently unknown. According to our experiments, the repressor function of VioS for violacein production is conserved in both C. violaceum ATCC31532 and the sequenced strain, C. violaceum ATCC12472 which however differ in the levels of violacein produced. We therefore decided to introduce the vioS gene of strain ATCC31532 in trans into the C. violaceum ATCC12472 wild type and this resulted in the transformation of the deep purple colony color to pale white color (Figure 1) indicative of violacein repression. Interestingly, C. violaceum ATCC12472 wild type has a gene homologous to vioS (CV_1055) and further experiments will be necessary to determine whether this gene codes for a functional protein or has lower expression levels than required to mediate its repressor effect in the presence of CviR-AHL. Interestingly, a very recent study has reported violacein production in the marine bacterium Pseudomonas salmiae and its regulation by AHL QS (Mireille Ayé et al., 2015). It would be interesting to determine whether VioS is present and regulates violacein production in this marine bacterium.

Our current understanding of VioS mediated regulation of violacein biosynthesis in C. violaceum is shown in the schematic model (Figure 5). Briefly, at high cell densities, the CviR protein binds AHLs to activate expression of vioA promoter in C. violaceum wild type. Expression of VioS under these conditions leads to repression of vioA promoter and consequently of violacein production and pale colonies of wild

![FIGURE 5](image_url) | Model for role of VioS in regulation of QS regulated phenotypes in C. violaceum. VioS negatively regulates the vio operon either directly or indirectly, which is positively regulated directly by the CviR/QS system. VioS negatively regulates chitinase and protease production, which are positively regulated by the CviI/R system.
type *C. violaceum* ATCC31532. A vioS mutant is relieved from this repression at the vioA promoter leading to violacin production which is clearly visible as purple-colored colonies. Future studies need to address whether the effect of VioS on the vioA promoter is due to a transcriptional, post-transcriptional control or possibly via protein-protein interaction with the CviR-AHL complex. In addition from this study it is important to determine the levels of VioS required to antagonize CviR-AHL and the conditions that regulate vioS expression in *C. violaceum*.

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GD, MK, SC, and IB performed experimental work whereas MC, PW, SS, and VV drafted the manuscript. All authors were involved in designing, discussing, and interpreting the results of the experiments.

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Devescovi et al. Regulation of Violacein in C. violaceum

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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