



The University of
Nottingham

UNITED KINGDOM · CHINA · MALAYSIA

Devescovi, Giulia and Kojic, Milan and Covaceuszach, Sonia and Cámara, Miguel and Williams, Paul and Bertani, Iris and Subramoni, Sujatha and Venturi, Vittorio (2017) Negative regulation of violacein biosynthesis in *Chromobacterium violaceum*. *Frontiers in Microbiology*, 8 . 349/1-349/37. ISSN 1664-302X

Access from the University of Nottingham repository:

http://eprints.nottingham.ac.uk/40663/1/251601_Venturi_Manuscript.pdf

Copyright and reuse:

The Nottingham ePrints service makes this work by researchers of the University of Nottingham available open access under the following conditions.

This article is made available under the Creative Commons Attribution licence and may be reused according to the conditions of the licence. For more details see: <http://creativecommons.org/licenses/by/2.5/>

A note on versions:

The version presented here may differ from the published version or from the version of record. If you wish to cite this item you are advised to consult the publisher's version. Please see the repository url above for details on accessing the published version and note that access may require a subscription.

For more information, please contact eprints@nottingham.ac.uk

Negative regulation of violacein biosynthesis in *Chromobacterium violaceum*

Giulia Devescovi¹, Milan Kojic², Sonia Covaceuszach³, Miguel Camara⁴, Paul Williams⁴, Iris Bertani¹, Sujatha Subramoni¹, Vittorio Venturi^{1*}

¹International Centre for Genetic Engineering and Biotechnology, Italy, ²Laboratory of Molecular Microbiology, Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Serbia, ³Istituto di Cristallografia, Consiglio Nazionale delle Ricerche, Italy, ⁴School of Life Sciences, Centre for Biomolecular Sciences, University of Nottingham, United Kingdom

Submitted to Journal:
Frontiers in Microbiology

Specialty Section:
Systems Microbiology

Article type:
Original Research Article

Manuscript ID:
251601

Received on:
30 Dec 2016

Revised on:
08 Feb 2017

Frontiers website link:
www.frontiersin.org

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

Author contribution statement

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.

Keywords

Chromobacterium violaceum, VioS, Cvil/R quorum sensing, regulation, Violacein, Chitinase activity, protease activity

Abstract

Word count: 108

In *Chromobacterium violaceum*, the purple pigment violacein is under positive regulation by the N-acylhomoserine lactone Cvil/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 Cvil/R system in both *C. violaceum* and in a heterologous *Escherichia coli* genetic background. VioS does not regulate the Cvil/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.

Funding statement

We thank ICGEB for support.

Ethics statements

(Authors are required to state the ethical considerations of their study in the manuscript, including for cases where the study was exempt from ethical approval procedures)

Does the study presented in the manuscript involve human or animal subjects: No

1 **Negative regulation of violacein biosynthesis in *Chromobacterium***
2 ***violaceum***

3

4 **Giulia Devescovi^a, Milan Kojic^b, Sonia Covaceuszach^c, Miguel Cámara^d, Paul Williams^d, Iris**
5 **Bertani^a, Sujatha Subramoni^{a¶} and Vittorio Venturi^{a*}**

6

7 ^aInternational Centre for Genetic Engineering and Biotechnology, Padriciano 99, 34149 Trieste,
8 Italy.

9 ^bLaboratory for Molecular Microbiology, Institute of Molecular Genetics and Genetic
10 Engineering, University of Belgrade, Vojvode Stepe 444/a, 11010 Belgrade, Serbia.

11 ^cIstituto di Cristallografia, Consiglio Nazionale delle Ricerche, U.O.S di Trieste, Area Science Park
12 – Basovizza, S.S. n° 14 Km 163.5, I-34149 Trieste, Italy.

13 ^dSchool of Life Sciences, Centre for Biomolecular Sciences, University of Nottingham,
14 Nottingham NG7, 2RD, UK.

15

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

18 **Running title:** Regulation of violacein in *C. violaceum*

19 **Number of words:** 7784

20 **Number of figures:** 5

21

22 * Address correspondence to Vittorio Venturi, venturi@icgeb.org

23 International Centre for Genetic Engineering and Biotechnology, Padriciano 99, 34149 Trieste,
24 Italy.

25

26 ¶ current address: Singapore Centre for Environmental Life Sciences Engineering, Nanyang
27 Technological University, 60 Nanyang Drive, SBS-01N-27 Singapore 637551

28 **Abstract**

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

38

39 1. Introduction

40 Many Gram-negative bacteria regulate cell density dependent behaviour by producing
41 and sensing *N*-acylhomoserine lactone (AHL) signal molecules by a process called quorum
42 sensing (QS; (Fuqua et al., 1994)). A canonical AHL-dependent QS system is composed of two
43 proteins respectively belonging to the LuxI and LuxR protein families (Fuqua et al., 1996).
44 Typically, AHLs are produced by an AHL synthase (LuxI homolog) and sensed at a threshold
45 concentration due to increase in cell population density by an AHL-binding regulator (LuxR
46 homolog) which then affects transcription of target genes (Fuqua and Greenberg, 2002). AHL
47 QS regulates many phenotypes that impact on bacterial community or group behaviours
48 including the expression of secreted enzymes, antibiotic and exopolysaccharide production,
49 biofilm formation, conjugation, symbiosis and virulence (Fuqua and Greenberg, 2002;Loh et al.,
50 2002;Von Bodman et al., 2003;Waters and Bassler, 2005).

51 *Chromobacterium violaceum* is a betaproteobacterium found in a variety of soil and
52 aquatic habitats causing infrequent but fatal mammalian infections (Brazilian National Genome
53 Project, 2003). Two *C. violaceum* strains (ATCC31532 and ATCC12472) possess an AHL QS
54 system and surprisingly they produce and respond to different AHLs (McClellan et al.,
55 1997;Morohoshi et al., 2008). The AHL QS system of *C. violaceum* ATCC12472 is encoded by the
56 genetically linked *cvil* and *cviR* genes producing and responding with highest affinity to *N*-
57 decanoyl-L-homoserine lactone (C10-HSL). CviR therefore binds to C10-HSL with highest affinity
58 (Morohoshi et al., 2008;Swem et al., 2009) and the *cvil* AHL synthase is under positive feedback
59 regulation by C10-HSL-CviR (Stauff and Bassler, 2011). The Cvil/CviR QS system of *C. violaceum*
60 ATCC12472 is important for virulence as revealed by loss of pathogenicity in a *C. elegans*

61 infection model in the presence of an antagonistic ligand for CviR instead of C10-HSL (Swem et
62 al., 2009). In contrast, a much earlier report (McClellan et al., 1997) demonstrated that the AHL
63 signal produced by *C. violaceum* ATCC31532 is C6-HSL. However, cloning and genetic analysis of
64 this *cviI/cviR* QS system has not been yet been reported in detail.

65 In *C. violaceum*, QS regulates (i) the *vioA* promoter of violacein *vioABCDE* genes coding
66 for the water insoluble purple pigment violacein (Lichstein and Van De Sand, 1946;McClellan et
67 al., 1997), (ii) genes coding for cyanide production and degradation (Duran and Menck, 2001)
68 and (iii) multiple genes the products of which are chitinases (Chernin et al., 1998). Besides the
69 *cviI* promoter, several other genes are directly regulated by CviR in *C. violaceum* ATCC12472
70 and these include genes coding for a putative transcriptional regulator (CV_0577), a guanine
71 deaminase (CV_0578), a chitinase (CV_4240) and a type VI secretion system gene (CV_1432)
72 (Stauff and Bassler, 2011). As in *C. violaceum* AHL QS regulates the production of the purple
73 pigment violacein; this has allowed the convenient use of this bacterium as an AHL biosensor
74 since the AHL-negative biosensor strain CV026 produces violacein only upon the addition of
75 exogenous AHLs with from C4 to C8 acyl side chains (McClellan et al., 1997;Steindler and Venturi,
76 2007).

77 Regulation of violacein production by QS has been studied in more detail than the other
78 phenotypes as it is an easily discernible and visible trait. Using a combination of mutagenesis-
79 based analysis in *C. violaceum* ATCC31532 and experiments in a heterologous *Escherichia coli*
80 host, the *vioA* promoter of *vioABCDE* operon has been shown to be under the direct positive
81 regulation of CviR (McClellan et al., 1997;Swem et al., 2009). Comprehensive mutational analysis
82 of the *vioA* promoter has also enabled the identification of a CviR binding site (Stauff and

83 Bassler, 2011). Interestingly, the level of violacein produced by wild type *C. violaceum*
84 ATCC12472 is much higher than that of wild type *C. violaceum* ATCC31532 (McClellan et al.,
85 1997). Furthermore, a violacein repressor has been reported and inactivated by transposon
86 mutagenesis in two independent studies in *C. violaceum* ATCC31532 giving rise to mutants with
87 considerably higher violacein production (McClellan et al., 1997; Swem et al., 2009). In addition,
88 the *Chromobacterium* AHL biosensor strain CV026 is a double transposon insertion mutant
89 since single Tn5 insertions in the putative AHL synthase failed to respond to exogenous AHLs
90 unless a second transposon was introduced into the putative repressor locus (McClellan et al.,
91 1997). However, the mechanism of violacein regulation by this putative repressor and its
92 regulatory relationship with the *C. violaceum* AHL QS system are not known.

93 In this study we have examined the regulation of violacein production in *C. violaceum*
94 ATCC31532 and characterized its QS system as well as a repressor mutant of this strain with
95 respect to violacein production. We show that the expression of the *vioA* promoter of the
96 *vioABCDE* operon is under negative regulation by this novel repressor which we have named
97 VioS. VioS is also involved in the regulation of other AHL QS regulated phenotypes such
98 protease and chitinolytic activity. Furthermore, we provide evidence for direct interference by
99 VioS of QS mediated positive regulation of the *vioA* promoter in *C. violaceum* and in *E. coli*.
100 Finally, we show that VioS functions as a repressor of violacein production in the closely related
101 *C. violaceum* ATCC12472 when introduced *in trans*. We propose that VioS is a novel protein that
102 functions to fine-tune the QS regulated phenotype of violacein biosynthesis by regulating *vioA*
103 promoter expression rather than modulating the regulation of *cvil/cviR* gene expression.

104 **2. Materials and Methods**

105 **2.1. Bacterial strains, media and growth conditions**

106 Wild type *C. violaceum* ATCC 31532, ATCC12472 and CV026 (McClellan et al., 1997) and
107 *Escherichia coli* strains DH5 α and M15 were routinely grown at 30°C and 37°C respectively in
108 Luria–Bertani (LB) broth medium (Miller, 1972). When required, antibiotics were added in the
109 following concentrations: ampicillin 100 $\mu\text{g ml}^{-1}$, kanamycin, 100 $\mu\text{g ml}^{-1}$, gentamicin 50 μg
110 ml^{-1} , tetracyclin 40 $\mu\text{g ml}^{-1}$ for *C. violaceum* strains and, ampicillin 100 $\mu\text{g ml}^{-1}$, kanamycin, 50
111 $\mu\text{g ml}^{-1}$, gentamicin 20 $\mu\text{g ml}^{-1}$ and tetracyclin 20 $\mu\text{g ml}^{-1}$ for *Escherichia coli* strains. AHLs used
112 here were obtained from Sigma-Aldrich (St. Louis, MO, USA).

113 **2.2. Recombinant DNA techniques**

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

123 **2.3. Plasmid construction**

124 The plasmids used in this study are listed in Table1.

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

131 Gene transcriptional fusion plasmids, based on the pMPGFP promoter probe vector, were
132 constructed as follows: the promoter regions of *cviI*, *cviR*, *vioA* and *vioS* genes were amplified
133 from *C. violaceum* 31532 genomic DNA by using, respectively, the primers *cviIBF* and *cviIER*
134 (*cviIPROM*; 337-bp), *cviRBF* and *cviRER* (*cviRPROM*; 277-bp), *vioABF* and *vioAER* (*vioAPROM*;
135 328-bp), *vioSBF* and *vioSER* (*vioSPROM*; 196-bp). The amplified fragments were cloned in
136 pGEM-T Easy vector (Promega, Madison, WI, USA), sequenced and then excised as
137 *BamHI/EcoRI* fragments and cloned into the *BglII/EcoRI* sites in pMPGFP obtaining pP*cviIGFP*
138 pP*cviRGFP*, pP*vioAGFP* and pP*vioSGFP* constructs. The *vioA* promoter was also amplified with
139 primers *vioA220KF* and *vioA220XR* and cloned as a *KpnI/XbaI* fragment into the corresponding
140 restriction sites of the promoter probe vector pMP220, obtaining pP*vioA220* construct. The *cepI*
141 promoter was amplified with primers *cepI220EF* and *cepI220XR* and cloned as a *EcoRI/XbaI*
142 fragment in pMP220 giving pP*cepI220*. The *vioS* gene with its promoter was cut out from the
143 pBSCVO7H construct as a *SnaBI/XbaI* fragment and cloned into the corresponding restriction
144 sites of pBBRmcs5 to generate pBBR*vioS*. The *vioS* gene was also amplified from *C. violaceum*
145 31532 genomic DNA using the primers *VioSBFw* and *VioSHR* and cloned into the *BamHI/HindIII*

146 restriction sites of pQE30 vector to generate pQE30VioS. The *cviR* gene was amplified from *C.*
147 *violaceum* 31532 genomic DNA using primers cvRHF and cvRXR and inserted downstream of the
148 *lac* promoter in pBBRmcs5 linearized with *Hind*III and *Xba*I restriction enzymes. The fidelity of
149 all of the constructs described was verified by DNA sequencing (Macrogen, Europe).
150 Translational fusions were constructed as follows: the 5' region of the *cviR* DNA sequence,
151 containing the promoter and coding sequences for the first 98 amino acids was amplified from
152 *C. violaceum* 31532 genomic DNA by using the primers cviRPROMFXba and cviR2RBgIII. The
153 amplified fragment was then cloned in frame upstream from the *lacZ* gene and then the whole
154 construct was transferred into the pMP77 vector generating pMPCviRLacZ. Similarly, the 5'
155 region of the *vioA* gene, containing the promoter and coding sequences for the first 49 amino
156 acids was amplified by using the primers vioAPROMFXba and vioAR3BamHI, cloned in-frame
157 upstream the *lacZ* gene and transferred to the pMP77 plasmid giving pMPVioAlacZ.

158 **2.4. Genomic mutant bank and cosmid gene bank construction and screening**

159 A Tn5 genomic mutant library of *C. violaceum* ATCC31532 was created using pSUP2021, as
160 previously described (Simon et al., 1983). Approximately 5,000 mutants were screened for the
161 presence of violacein hyperproducer mutants by identifying colonies that showed purple
162 coloration in contrast to the pale colonies of the *C. violaceum* ATCC31532 wild type. Two
163 mutants were isolated and the genomic regions flanking the Tn5 insertions were amplified by
164 arbitrary PCR technique (O'Toole and Kolter, 1998) and sequenced. The two mutants were
165 designated as MB8 and MB11 respectively. A genomic bank (cosmid library) of *C. violaceum*
166 ATCC31532 was constructed as follows. Briefly, *C. violaceum* 31532 genomic DNA was partially
167 digested with *Eco*RI and ligated into pLAFR3 cosmid vector. The constructs obtained were

168 introduced into *E. coli* cells using Gigapack III XL-4 packaging kit as recommended by the
169 supplier (Stratagene-Agilent, Santa Clara, CA, USA). The genomic bank was then screened using
170 the flanking DNA (obtained by arbitrary PCR on mutant colonies MB8 and MB11), as probes.
171 Three cosmids were isolated which showed the same restriction pattern. Cosmid pCVO7 was
172 chosen and subcloned in pBSIIKS generating two overlapping constructs: pBCVO7H (containing
173 a 3-kb *HindIII* fragment) and pBCVO7XN (containing a 6350-bp *XhoI-NotI* fragment)

174 **2.5. Construction of 31532CVII, 31532CVIR and 31532VIOS**

175 The three additional mutants, 31532CVII, 31532VCIR and 31532VIOS were generated using the
176 suicide vectors from the pKNOCK series (Alexeyev, 1999). To generate 31532cvil, an internal
177 fragment (209-bp) of the *cvil* gene was PCR amplified using the primers KNcvilBF and KNcvilKR
178 and cloned as a *BamHI-KpnI* fragment into the corresponding sites of pKNOCK-Km resulting in
179 pKNOCKcvil. In order to generate 31532CVIR, an internal fragment of *cvir* (327-bp) was
180 amplified with the primers KNcvirF and KNcvirR, blunted and cloned into pKNOCK Gm digested
181 with the *SmaI* restriction enzyme, yielding pKNOCKcvir. Finally, to obtain 31532VIOS, an
182 internal fragment of *vioS* (187-bp) was amplified with primers KNvioSKF and KNvioSBR and
183 cloned as a *KpnI-BamHI* fragment in the corresponding sites of pKNOCK-Km giving pKNOCKvioS.
184 The pKNOCK constructs obtained were transferred to *C. violaceum* ATCC31532 via tri-parental
185 mating and the knock-out mutants were verified by PCR analysis and sequencing. The
186 31532VIOS was altered in growth rate and behaved like the parent wild-type strain.

187 **2.6. Extraction and quantification of AHLs**

188 *C. violaceum* strains were grown overnight in 20 ml of LB medium. The cells were pelleted at
189 5000 *g* for 15 min. The cell free supernatants were filtered (using 0.45µm filters; Millipore) and
190 extracted twice with an equal volume of ethyl acetate containing 0.1% v/v acetic acid. The
191 organic phases were collected, dried to completeness and re-suspended in 50 µl of ethyl
192 acetate. To quantify the amounts of C6-HSL produced by the 31532 wild type strain, MB8,
193 MB11 and 31532VIOS, the constructs pPvioA220 and pBBRcviR were used to constitute a CviR-
194 based sensor regulating its target promoter *vioA* in the heterologous *E. coli* M15 system. In
195 order to generate a calibration curve, different concentrations (0; 0.01; 0.05; 0.1; 0.5; 1 µM) of
196 C6-HSL were added to 10 ml to each of the sensor strains. The cultures were grown for 6 h and
197 β-galactosidase activity was determined. To quantify the AHLs produced by each *C. violaceum*
198 strain, the experiment was repeated by adding 10µl of an AHL extract obtained from each strain
199 to the sensor.

200 **2.7. β Galactosidase and GFP quantification assays**

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

206 **2.8. Exoenzyme activity**

207 To assess protease activity, *C. violaceum* strains were grown to stationary phase and 2 µl of
208 culture was spotted onto M9 agar containing 2% dry milk, as the only carbon source. Zones of

209 activity were measured after 36 h. For chitinase activity, the same protocol was followed and
210 cultures were spotted onto M9 agar containing 0.2% colloidal chitin (Ahmadian et al., 2007).

In review

211 **3. Results**

212 **3.1. The AHL QS system of *C. violaceum* ATCC31532**

213 The unequivocal chemical identification of C6-HSL from culture supernatants of *C. violaceum*
214 ATCC 31532 and the selection of a Tn5 transposon mutant with an insertion in a putative *luxI*
215 orthologue demonstrated the presence of an AHL QS system in this organism (McClellan et al.,
216 1997). To isolate the locus encoding this system, a *PstI* genomic library of this strain was
217 constructed in pUC18. The library was introduced into the AHL biosensor strain *E. coli* (pSB401)
218 (Winson et al., 1998) and the recombinant colonies screened for the production of
219 bioluminescence using a photon-imaging camera as previously described (Swift et al., 1997). A
220 recombinant clone (pMW50) able to induce light production in the biosensor strain was
221 identified as a highly bioluminescent colony. Expression of pMW50 in *E. coli*, was able to
222 restore violacein production when cross-streaked against the AHL sensor strain *C. violaceum*
223 CV026 (McClellan et al., 1997) suggesting the presence of an AHL synthase in this recombinant
224 clone. Sequence analysis of the 6Kb *PstI* insert from pMW50 revealed the presence of two
225 convergent open reading frames overlapping by 74bp which were named *cviR* and *cviI* as their
226 predicted amino acid sequences were homologous to the LuxI/LuxR family of QS genes. Solvent
227 extraction of culture supernatants from *E. coli* harbouring pMW50 followed by LC-MS/MS
228 analysis revealed the presence of C6-HSL (data not shown). No other AHLs were detected from
229 these extracts indicating that *cviI* is responsible for the synthesis of this AHL.

230 **3.2. Violacein biosynthesis is negatively regulated by VioS**

231 Violacein production by *C. violaceum* is regulated by QS via AHLs signal molecules
232 (McClellan et al., 1997; Morohoshi et al., 2008). We have previously shown that violacein
233 production is stringently negatively regulated since we obtained a Tn5 insertion mutant that
234 strongly overproduced violacein in the *C. violaceum* ATCC31532 genetic background [(McClellan
235 et al., 1997); Table 1]. This transposon was localized to a gene coding for a protein of unknown
236 function homologous to CV_1055 of the sequenced genome of *C. violaceum* ATCC12472
237 demonstrating that violacein is very tightly regulated (Swem et al., 2009). To further investigate
238 the regulation of this phenotype and to make sure that no other loci was involved in this
239 negative regulation, we constructed a Tn5 mutant library of *C. violaceum* ATCC31532 and
240 screened for more mutants that overproduced violacein as described in the Materials and
241 Methods. Two mutants, named MB8 and MB11 were identified in the screen and the location
242 of the Tn5 insertion site in both mutants was also located in the CV_1055 gene homologue
243 from *C. violaceum* ATCC12472 but in the putative promoter region; the Tn5 in mutant MB8 is
244 located nearer to the ATG of the putative ORF whereas MB11 is further away (Figure 1a). We
245 have now named the hypothetical protein encoded by this gene as VioS (Figure 1a). This
246 predicted protein (138 amino acids; 15 kDa approximately) showed 91% identity and 94%
247 similarity to a hypothetical protein from *Pseudogulbenkiana ferrooxidans* and 85% identity and
248 90% similarity to the hypothetical protein encoded by CV_1055 from *C. violaceum* ATCC12472
249 respectively. Conserved domain analysis of VioS amino acid sequence revealed the presence of
250 a domain of unknown function annotated as DUF1484 spanning 32-138 amino acids (8.35e-03)
251 that is exclusively found in bacteria belonging to the betaproteobacteria.

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

263 **3.3. VioS and CviR regulate violacein biosynthesis in opposite ways**

264 Since the studies using the transposon insertion mutants described above clearly
265 support a role for VioS in the negative regulation of violacein production, which conversely is
266 positively regulated by the Cvil/R QS system, we sought to determine whether VioS interacted
267 with the QS system. Consequently we investigated whether VioS influenced the expression of
268 the Cvil/R system which could then result in violacein de-regulation. We first determined the
269 AHL levels produced by the wild type, MB8, MB11 and 31532VIO strains as described in the
270 Materials and Methods. Using a calibration curve derived by a Cvil/R AHL biosensor constructed
271 here, we found that all strains produced similar AHL levels production corresponding to a C6-
272 HSL concentration of approximately 0.5 μ M (data not shown). The transcriptional levels of the
273 QS genes using *cvil::gfp* and *cviR::gfp* plasmid transcriptional fusions were determined and the

274 results showed that the *cvil* and *cvrR* genes are expressed at comparable levels in the wild type,
275 the *vioS* mutants and complemented strains (Figure 2A and 2B). To determine whether the
276 Cvil/R QS system modulated *vioS* expression, assays were carried out to measure the levels of a
277 plasmid-borne *vioS::gfp* transcriptional fusion in the wild type, *cvil* and *cvrR* mutants. The
278 expression of *vioS* was similar in all of the strains examined (data not shown). These results
279 indicate that VioS does not influence expression of the Cvil/R QS system or vice versa. VioS
280 furthermore does not significantly affect the levels of AHLs.

281 To further understand the opposing regulatory effects of VioS and Cvir-AHL on violacein
282 production we monitored the reporter activity of a plasmid *vioA::gfp* transcriptional fusion in
283 the wild type, MB8, MB11, 31532VIOs, *cvil* and *cvrR* mutants (Figure 2C). The *vioA* promoter
284 controls the expression of the operon (*vioA-vioE*) encoding for the violacein biosynthesis genes
285 (August et al., 2000; Antonio and Creczynski-Pasa, 2004; Sanchez et al., 2006). As expected, little
286 expression of *vioA::gfp* was apparent in the *cvil* and *cvrR* mutants compared with the wild type.
287 On the other hand *vioA::gfp* fusion showed a drastic increase in expression in all three *vioS*
288 mutants, MB8, MB11 and 31532VIOs compared with the wild type strain. Complementation of
289 the *vioS* mutants with a wild type copy of the *vioS* gene restored *vioA::gfp* expression to wild
290 type levels (Figure 2C). These results demonstrate that VioS represses expression of the *vio*
291 operon at the transcriptional level thus influencing violacein production in the *C. violaceum*
292 ATCC31532 wild type strain in spite of presence a functional Cvil/R QS system.

293 To investigate whether VioS has an effect on the translational levels of *cvil* and *vioA*, we
294 constructed *cvrR-lacZ* and *vioA-lacZ* translational fusions as described in the Materials and
295 Methods. As depicted in Figures 2D and 2E, VioS did not affect *cvrR* translation. However in the

296 *vioS* mutant, the *vioA-lacZ* translational fusion displayed a 2-fold increase in β -galactosidase
297 activity. These data indicate that VioS exerts a negative effect on the translation of *vioA*
298 meaning that it could be acting at a post-transcriptional level; however this increase in
299 translation could be due to the increase in transcription observed using the *vioA* transcriptional
300 fusion (Figure 2C) .

301 **3.4. VioS is sufficient to antagonize CviR-mediated regulation of the violacein biosynthetic** 302 **operon in a heterologous system**

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

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

314 It was also of interest to establish whether the negative effect of VioS on transcription
315 of an AHL QS target gene was specific for the CviR regulated *vioA* promoter. Expression studies
316 were therefore carried out using a different AHL QS system and target promoter. For this

317 experiment we used the *Burkholderia cepacia* CepI/R system and the *cepl* target gene. The
318 plasmid *cepl::lacZ* transcriptional fusion construct was introduced into *E. coli* harboring
319 plasmids expressing either CepR or VioS. The expression of the *cepl::lacZ* fusion was
320 determined with and without the exogenous addition of C8-HSL. In this experiment, the *cepl*
321 promoter was upregulated in the presence of CepR and AHLs as expected but in contrast to the
322 *vioA* promoter, it was not repressed in the presence of VioS (Figure 3b). Thus the VioS
323 mediated effect on the expression of a QS regulated promoter is likely to be specific for the
324 Cvil/R system.

325 **3.5. QS and VioS antagonistically modulate QS-regulated phenotypes in *C. violaceum***

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

336 **4. Discussion**

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

344 *C. violaceum* is an environmental bacterium, found in soil and water, is generally non-
345 pathogenic but occasionally extremely virulent to humans and animals (Brazilian National
346 Genome Project, 2003). It has been shown that elimination of QS leads to loss of virulence of *C.*
347 *violaceum* in a *C. elegans* model of infection suggesting that functions positively regulated by
348 QS are important for infection (Swem et al., 2009). However, the phenotypes regulated by AHL-
349 dependent QS can be energetically expensive such that constitutive expression of these shared
350 traits is not likely to enable optimal utilization of available resources; it may also elicit stronger
351 host defense responses. RsaL, a negative regulator of QS and QS-regulated genes in
352 *Pseudomonas aeruginosa* has been reported to be important for optimum virulence as *rsaL*
353 mutants are hypervirulent in a *Galleria mellonella* acute model of infection (Rampioni et al.,
354 2009). Also, in a study involving dual-species co-culture of *C. violaceum* and *Burkholderia*
355 *thailandensis*, it was reported that QS dependent antimicrobials like violacein can provide a
356 competitive advantage in mixed microbial communities with limited nutrients (Chandler et al.,
357 2012). Here, we have shown that VioS functions to fine-tune QS-regulated phenotypes and it is
358 possible that it might play a role in providing optimum fitness to *C. violaceum* both in the

359 environment and in host associations. Alternatively, it cannot be excluded that VioS responds to
360 environmental stimuli or an unknown signal that results in de-repression and so promotes high
361 levels of violacein production under certain circumstances.

362 Although the molecular mechanism of VioS-mediated repression in QS homeostasis is
363 not known, it is possible that it belongs to a new class of regulators. Among the few
364 characterized negative regulators of QS are RsaL, AlgQ and a TetR-like transcriptional repressor
365 of *P. aeruginosa*, all of which bind DNA (de Kievit et al., 1999;Ledgham et al., 2003;Rampioni et
366 al., 2006;Venturi et al., 2011;Longo et al., 2013). RsaM of *P. fuscovaginae* as well as other
367 repressor proteins with less sequence identity to RsaM including BcRsaM of *B. cenocepacia* and
368 TofM of *B. glumae* are also QS repressors (Mattiuzzo et al., 2011;Chen et al., 2012;Michalska et
369 al., 2014). However, BcRsaM is predicted to influence QS by an as yet unknown mechanism but
370 not by binding to DNA (Michalska et al., 2014). The VioS amino acid sequence does not show
371 similarity to any of these proteins and furthermore this study does not provide any direct
372 evidence that VioS exerts its regulation at the transcriptional level. Studies performed using
373 translational fusions indicate that VioS had a negative effect on the translation of *vioA*. A
374 comparison with RsaL of *P. aeruginosa* suggests that VioS exhibits some common and distinct
375 features. The *rsaL* gene is genetically linked to QS systems and its transcription is positively
376 regulated by QS. However, RsaL negatively regulates expression of *lasI* coding for AHL synthase
377 as well as some other QS regulated genes responsible for e.g. pyocyanin and HCN production
378 (Schuster et al., 2004;Rampioni et al., 2006;Rampioni et al., 2007b). RsaL and LasR have been
379 shown to bind to adjacent sites on the *lasI* promoter but the negative regulatory effect of RsaL
380 is dominant over the activating effect of LasR-AHL (Rampioni et al., 2007a). In our study, the

381 presence of VioS influences *vioA* promoter activity in a manner similar to RsaL-mediated
382 repression of the *lasI* promoter because the repressor activity of VioS on *vioA* promoter is
383 dominant over the activator effect of CviR-AHL. However, unlike the *rsaL* system where the
384 expression of the repressor is dependent on LasR-AHL, *vioS* expression is not linked to CviR-AHL
385 and the mechanism of *vioS* expression and regulation requires further investigation. In addition
386 *vioS* is found in a separate genomic location from the *cviI* and *cviR* genes and does not have any
387 direct effect on their transcription but impacts at an as yet unknown level on CviI/R QS target
388 gene expression. Moreover, VioS appears to be sufficient and specific for CviR-AHL antagonism
389 as it is not a general inhibitor of gene activation by other QS LuxR regulators in other bacteria,
390 for example CepR-AHL from *Burkholderia*.

391 Sequence similarity searches with the predicted amino acid sequence of *C. violaceum*
392 ATCC31532 VioS were undertaken to identify homologs of this protein in other bacteria. In our
393 searches VioS homolog was identified only in the sequenced genomes of *C. violaceum*
394 ATCC12472 strain and *P. ferrooxidans*. The exclusive presence of VioS in these two bacterial
395 genera suggests that it may have specific functions in these bacterial species. In contrast, other
396 QS repressors like RsaL and RsaM are present in multiple members of the proteobacteria
397 (Venturi et al., 2011). Both *C. violaceum* and *P. ferrooxidans* produce the purple violacein
398 pigment and it will be interesting to determine whether VioS also regulates pigment production
399 in *P. ferrooxidans*. These two bacteria could share a similar niche(s) [*P. ferrooxidans* producing
400 violacein has been isolated in a lake, {Puranik, 2013 #736}] as well as profile and regulation of
401 secondary metabolite production in order to survive in specific environmental conditions; this
402 possibility is currently unknown. According to our experiments, the repressor function of VioS

403 for violacein production is conserved in both *C. violaceum* ATCC31532 and the sequenced
404 strain, *C. violaceum* ATCC12472 which however differ in the levels of violacein produced. We
405 therefore decided to introduce the *vioS* gene of strain ATCC31532 *in trans* into the *C. violaceum*
406 ATCC12472 wild type and this resulted in the transformation of the deep purple colony colour
407 to pale white colour (Figure 1) indicative of violacein repression. Interestingly, *C. violaceum*
408 ATCC12472 wild type has a gene homologous to *vioS* (CV_1055) and further experiments will be
409 necessary to determine whether this genes codes for a functional protein or has lower
410 expression levels than required to mediate its repressor effect in the presence of CviR-AHL.
411 Interestingly, a very recent study has reported violacein production in the marine bacterium
412 *Pseudomonas ulvae* and its regulation by AHL QS (Mireille Aye et al., 2015). It would be
413 interesting to determine whether VioS is present and regulates violacein production in this
414 marine bacterium.

415 Our current understanding of VioS mediated regulation of violacein biosynthesis in *C.*
416 *violaceum* is shown in the schematic model (Figure 5). Briefly, at high cell densities, the CviR
417 protein binds AHLs to activate expression of *vioA* promoter in *C. violaceum* wild type.
418 Expression of VioS under these conditions leads to repression of *vioA* promoter and
419 consequently of violacein production and pale colonies of wild type *C. violaceum* ATCC31532. A
420 *vioS* mutant is relieved from this repression at the *vioA* promoter leading to violacein
421 production which is clearly visible as purple-coloured colonies. Future studies need to address
422 whether the effect of VioS on the *vioA* promoter is due to a transcriptional, post-transcriptional
423 control or possibly via protein-protein interaction with the CviR-AHL complex. In addition from

424 this study it is important to determine the levels of VioS required to antagonize CviR-AHL and
425 the conditions that regulate *vioS* expression in *C. violaceum*.

426 **Conflict of Interest**

427 The authors declare no conflict of interest.

428 **Authors Contribution Statement**

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

432 **Funding Statement**

433 We thank ICGEB for support.

In review

434 **References**

- 435 Aguilar, C., Bertani, I., and Venturi, V. (2003). Quorum-sensing system and stationary-phase sigma factor
436 (*rpoS*) of the onion pathogen *Burkholderia cepacia* genomovar I type strain, ATCC 25416. *Appl*
437 *Environ Microbiol* 69, 1739-1747.
- 438 Ahmadian, G., Degrassi, G., Venturi, V., Zeigler, D.R., Soudi, M., and Zanguinejad, P. (2007). *Bacillus*
439 *pumilus* SG2 isolated from saline conditions produces and secretes two chitinases. *J Appl*
440 *Microbiol* 103, 1081-1089.
- 441 Alexeyev, M.F. (1999). The pKNOCK series of broad-host-range mobilizable suicide vectors for gene
442 knockout and targeted DNA insertion into the chromosome of gram-negative bacteria.
443 *Biotechniques* 26, 824-826, 828.
- 444 Antonio, R.V., and Creczynski-Pasa, T.B. (2004). Genetic analysis of violacein biosynthesis by
445 *Chromobacterium violaceum*. *Genet Mol Res* 3, 85-91.
- 446 August, P.R., Grossman, T.H., Minor, C., Draper, M.P., Macneil, I.A., Pemberton, J.M., Call, K.M., Holt, D.,
447 and Osburne, M.S. (2000). Sequence analysis and functional characterization of the violacein
448 biosynthetic pathway from *Chromobacterium violaceum*. *J Mol Microbiol Biotechnol* 2, 513-519.
- 449 Better, M., Lewis, B., Corbin, D., Ditta, G., and Helinski, D.R. (1983). Structural relationships among
450 *Rhizobium meliloti* symbiotic promoters. *Cell* 35, 479-485.
- 451 Brazilian National Genome Project, C. (2003). The complete genome sequence of *Chromobacterium*
452 *violaceum* reveals remarkable and exploitable bacterial adaptability. *Proc Natl Acad Sci U S A*
453 100, 11660-11665.
- 454 Chandler, J.R., Heilmann, S., Mittler, J.E., and Greenberg, E.P. (2012). Acyl-homoserine lactone-
455 dependent eavesdropping promotes competition in a laboratory co-culture model. *ISME J* 6,
456 2219-2228.
- 457 Chen, R., Barphagha, I.K., Karki, H.S., and Ham, J.H. (2012). Dissection of quorum-sensing genes in
458 *Burkholderia glumae* reveals non-canonical regulation and the new regulatory gene *tofM* for
459 toxoflavin production. *PLoS One* 7, e52150.
- 460 Chernin, L.S., Winson, M.K., Thompson, J.M., Haran, S., Bycroft, B.W., Chet, I., Williams, P., and Stewart,
461 G.S. (1998). Chitinolytic activity in *Chromobacterium violaceum*: substrate analysis and
462 regulation by quorum sensing. *J Bacteriol* 180, 4435-4441.
- 463 De Kievit, T., Seed, P.C., Nezezon, J., Passador, L., and Iglewski, B.H. (1999). RsaL, a novel repressor of
464 virulence gene expression in *Pseudomonas aeruginosa*. *J Bacteriol* 181, 2175-2184.
- 465 Duran, N., and Menck, C.F. (2001). *Chromobacterium violaceum*: a review of pharmacological and
466 industrial perspectives. *Crit Rev Microbiol* 27, 201-222.
- 467 Figurski, D.H., and Helinski, D.R. (1979). Replication of an origin-containing derivative of plasmid RK2
468 dependent on a plasmid function provided in *trans*. *Proc Natl Acad Sci U S A* 76, 1648-1652.
- 469 Fuqua, C., and Greenberg, E.P. (2002). Listening in on bacteria: acyl-homoserine lactone signalling. *Nat*
470 *Rev Mol Cell Biol* 3, 685-695.
- 471 Fuqua, C., Winans, S.C., and Greenberg, E.P. (1996). Census and consensus in bacterial ecosystems: the
472 LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annu. Rev. Microbiol.* 50, 727-
473 751.
- 474 Fuqua, W.C., Winans, S.C., and Greenberg, E.P. (1994). Quorum sensing in bacteria: the LuxR-LuxI family
475 of cell density-responsive transcriptional regulators. *J Bacteriol* 176, 269-275.
- 476 Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A., Roop, R.M., 2nd, and Peterson, K.M.
477 (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying
478 different antibiotic-resistance cassettes. *Gene* 166, 175-176.

479 Ledgham, F., Soscia, C., Chakrabarty, A., Lazdunski, A., and Foglino, M. (2003). Global regulation in
480 *Pseudomonas aeruginosa*: the regulatory protein AlgR2 (AlgQ) acts as a modulator of quorum
481 sensing. *Res Microbiol* 154, 207-213.

482 Lichstein, H.C., and Van De Sand, V.F. (1946). The Antibiotic Activity of Violacein, Prodigiosin, and
483 Phthiocol. *J Bacteriol* 52, 145-146.

484 Loh, J., Pierson, E.A., Pierson, L.S., 3rd, Stacey, G., and Chatterjee, A. (2002). Quorum sensing in plant-
485 associated bacteria. *Curr Opin Plant Biol* 5, 285-290.

486 Longo, F., Rampioni, G., Bondi, R., Imperi, F., Fimia, G.M., Visca, P., Zennaro, E., and Leoni, L. (2013). A
487 new transcriptional repressor of the *Pseudomonas aeruginosa* quorum sensing receptor gene
488 LasR. *PLoS One* 8, e69554.

489 Mattiuzzo, M., Bertani, I., Ferluga, S., Cabrio, L., Bigirimana, J., Guarnaccia, C., Pongor, S., Maraite, H.,
490 and Venturi, V. (2011). The plant pathogen *Pseudomonas fuscovaginae* contains two conserved
491 quorum sensing systems involved in virulence and negatively regulated by RsaL and the novel
492 regulator RsaM. *Environ Microbiol* 13, 145-162.

493 Mcclean, K.H., Winson, M.K., Fish, L., Taylor, A., Chhabra, S.R., Camara, M., Daykin, M., Lamb, J.H., Swift,
494 S., Bycroft, B.W., Stewart, G.S., and Williams, P. (1997). Quorum sensing and *Chromobacterium*
495 *violaceum*: exploitation of violacein production and inhibition for the detection of N-
496 acylhomoserine lactones. *Microbiology* 143 (Pt 12), 3703-3711.

497 Michalska, K., Chhor, G., Clancy, S., Jedrzejczak, R., Babnigg, G., Winans, S.C., and Joachimiak, A. (2014).
498 RsaM: a transcriptional regulator of *Burkholderia* spp. with novel fold. *FEBS J* 281, 4293-4306.

499 Miller, J.H. (1972). *Experiments in molecular genetics*. Cold Spring Harbor, N.Y.

500 Mireille Aye, A., Bonnin-Jusserand, M., Brian-Jaisson, F., Ortalo-Magne, A., Culioli, G., Koffi Nevry, R.,
501 Rabah, N., Blache, Y., and Molmeret, M. (2015). Modulation of violacein production and
502 phenotypes associated with biofilm by exogenous quorum sensing N-acylhomoserine lactones in
503 the marine bacterium *Pseudoalteromonas ulvae* TC14. *Microbiology* 161, 2039-2051.

504 Morohoshi, T., Kato, M., Fukamachi, K., Kato, N., and Ikeda, T. (2008). N-acylhomoserine lactone
505 regulates violacein production in *Chromobacterium violaceum* type strain ATCC 12472. *FEMS*
506 *Microbiol Lett* 279, 124-130.

507 O'toole, G.A., and Kolter, R. (1998). Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365
508 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol* 28, 449-
509 461.

510 Passos Da Silva, D., Castaneda-Ojeda, M.P., Moretti, C., Buonauro, R., Ramos, C., and Venturi, V. (2014).
511 Bacterial multispecies studies and microbiome analysis of a plant disease. *Microbiology* 160,
512 556-566.

513 Rampioni, G., Bertani, I., Zennaro, E., Polticelli, F., Venturi, V., and Leoni, L. (2006). The quorum-sensing
514 negative regulator RsaL of *Pseudomonas aeruginosa* binds to the lasI promoter. *J Bacteriol* 188,
515 815-819.

516 Rampioni, G., Polticelli, F., Bertani, I., Righetti, K., Venturi, V., Zennaro, E., and Leoni, L. (2007a). The
517 *Pseudomonas* Quorum-Sensing Regulator RsaL Belongs to the Tetrahelical Superclass of H-T-H
518 Proteins. *J Bacteriol* 189, 1922-1930.

519 Rampioni, G., Schuster, M., Greenberg, E.P., Bertani, I., Grasso, M., Venturi, V., Zennaro, E., and Leoni, L.
520 (2007b). RsaL provides quorum sensing homeostasis and functions as a global regulator of gene
521 expression in *Pseudomonas aeruginosa*. *Mol Microbiol* 66, 1557-1565.

522 Rampioni, G., Schuster, M., Greenberg, E.P., Zennaro, E., and Leoni, L. (2009). Contribution of the RsaL
523 global regulator to *Pseudomonas aeruginosa* virulence and biofilm formation. *FEMS Microbiol*
524 *Lett*.

525 Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular cloning: a laboratory manual, 2nd ed.* Cold
526 Spring Harbor, N.Y.

527 Sanchez, C., Brana, A.F., Mendez, C., and Salas, J.A. (2006). Reevaluation of the violacein biosynthetic
528 pathway and its relationship to indolocarbazole biosynthesis. *Chembiochem* 7, 1231-1240.

529 Schuster, M., Urbanowski, M.L., and Greenberg, E.P. (2004). Promoter specificity in *Pseudomonas*
530 *aeruginosa* quorum sensing revealed by DNA binding of purified LasR. *Proc Natl Acad Sci U S A*
531 101, 15833-15839.

532 Simon, R., Priefer, U., and Puhler, A. (1983). A broad host range mobilization system for *in vivo* genetic
533 engineering: transposon mutagenesis in Gram negative bacteria. *Nat Biotech* 1, 784-791.

534 Spaink, H.P., Okker, R.J.H., Wijffelman, C.A., Pees, E., and Lugtemberg, B.J.J. (1987). Promoter in the
535 nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1J1. *Plant. Mol. Biol.* 9, 27-
536 39.

537 Stachel, S.E., An, G., Flores, C., and Nester, E.W. (1985). A Tn3 *lacZ* transposon for the random
538 generation of *b*-galactosidase gene fusions: application to the analysis of gene expression in
539 *Agrobacterium*. *Embo J* 4, 891-898.

540 Staskawicz, B., Dahlbeck, D., Keen, N., and Napoli, C. (1987). Molecular characterization of cloned
541 avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. *J Bacteriol* 169,
542 5789-5794.

543 Stauff, D.L., and Bassler, B.L. (2011). Quorum sensing in *Chromobacterium violaceum*: DNA recognition
544 and gene regulation by the CviR receptor. *J Bacteriol* 193, 3871-3878.

545 Steindler, L., and Venturi, V. (2007). Detection of quorum-sensing *N*-acyl homoserine lactone signal
546 molecules by bacterial biosensors *FEMS Microbiol Lett* 266, 1-9.

547 Swem, L.R., Swem, D.L., O'loughlin, C.T., Gatmaitan, R., Zhao, B., Ulrich, S.M., and Bassler, B.L. (2009). A
548 quorum-sensing antagonist targets both membrane-bound and cytoplasmic receptors and
549 controls bacterial pathogenicity. *Mol Cell* 35, 143-153.

550 Swift, S., Karlyshev, A.V., Fish, L., Durant, E.L., Winson, M.K., Chhabra, S.R., Williams, P., Macintyre, S.,
551 and Stewart, G.S. (1997). Quorum sensing in *Aeromonas hydrophila* and *Aeromonas*
552 *salmonicida*: identification of the LuxRI homologs AhyRI and AsaRI and their cognate *N*-
553 acylhomoserine lactone signal molecules. *J Bacteriol* 179, 5271-5281.

554 Venturi, V., Rampioni, G., Pongor, S., and Leoni, L. (2011). The virtue of temperance: built-in negative
555 regulators of quorum sensing in *Pseudomonas*. *Mol Microbiol* 82, 1060-1070.

556 Von Bodman, S.B., Bauer, W.D., and Coplin, D.L. (2003). Quorum sensing in plant-pathogenic bacteria.
557 *Annu Rev Phytopathol* 41, 455-482.

558 Waters, C.M., and Bassler, B.L. (2005). Quorum sensing: cell-to-cell communication in bacteria. *Annu Rev*
559 *Cell Dev Biol* 21, 319-346.

560 Winson, M.K., Swift, S., Fish, L., Throup, J.P., Jorgensen, F., Chhabra, S.R., Bycroft, B.W., Williams, P., and
561 Stewart, G.S. (1998). Construction and analysis of *luxCDABE*-based plasmid sensors for
562 investigating *N*-acyl homoserine lactone-mediated quorum sensing. *FEMS Microbiol Lett* 163,
563 185-192.

564

565 **Figure legends**

566 Figure 1(A) Genomic organization of the *vioS* locus. The Tn5 insertions in mutants MB8 and
567 MB11 are indicated. (B) Production of violacein in *C. violaceum* wt strains ATCC31532 and
568 ATCC12472, 31532VIO5 mutants (MB8;MB11;31532VIO5) and mutants complemented with
569 pBBVioS, containing full length *vioS* .

570 Figure 2. CviI/R QS system is not influenced by VioS but VioS negatively regulates the
571 expression of the *vioA* operon. CviI promoter activity (A), cviR promoter activity (B) and *vioA*
572 promoter activity (C) in *C. violaceum* 31532, 31532 quorum sensing mutants, 31532VIO5
573 mutants (MB8; MB11; 31532VIO5) and mutants complemented with pBBVioS, containing full
574 length *vioS*. Stationary phase bacterial cultures were monitored for GFP expression in a Perkin
575 Elmer EnVision Multilabel Reader. The means plus standard deviations for five replicates are
576 shown and 31532 (pMPGFP) represents the empty vector. In panels D and E, β -galactosidase
577 levels for the *cviR* and *vioA lacZ* translational fusions are shown in *C. violaceum* 31532 wild-
578 type, in the *vioS* mutant 31532VIO5 and in the same mutant complemented with a plasmid –
579 borne copy of *vioS* gene.

580 Figure 3. VioS antagonizes CviR mediated activation of the *vioA* promoter in a heterologous *E.*
581 *coli* strain and this inhibition is specific. (A) *vioA* promoter activity in the presence of CviR alone
582 or together with VioS, in the absence/presence of C6-HSL (1 μ M). (B) *cepI* promoter activity in
583 the presence of CepR alone or together with VioS, in the absence/presence of C8-HSL (1 μ M). β -
584 gal activities were measured after 12 h of growth. Experiments were performed in triplicate and
585 means plus standard deviations are plotted.

586 Figure 4. Effect of *vioS* and *cviR* mutations on (A) protease activity and (B) chitinase activity.
587 The halos of cleared zones in milk agar plates were measured after 36 h growth. The halos of
588 cleared zones in colloidal chitin plates were measured after 3 days of incubation. Experiments
589 were performed in triplicate and means plus standard deviations are presented.

590 Figure 5. Model for role of VioS in regulation of QS regulated phenotypes in *C. violaceum*. VioS
591 negatively regulates the *vio* operon either directly or indirectly, which is positively regulated
592 directly by the Cvil/R QS system. VioS negatively regulates chitinase and protease production,
593 which are positively regulated by the Cvil/R system.

In review

594 Table 1. Strains, plasmids and primers used

595

| Strains /plasmids/primer | Relevant features | Reference or source |
|-------------------------------|--|-------------------------------|
| <i>C.violaceum</i> STRAINS | | |
| <i>C. violaceum</i> ATCC31532 | WT isolate | |
| <i>C. violaceum</i> ATCC12472 | WT isolate | |
| CV026 | Double transposon mutant of ATCC31532, violacein and AHL negative | (McClellan et al., 1997) |
| MB8 | <i>vioS</i> ::Tn5 of <i>C. violaceum</i> ATCC31532; Km ^R | This study |
| MB11 | <i>vioS</i> ::Tn5 of <i>C. violaceum</i> ATCC31532; Km ^R | This study |
| 31532VIOS | <i>vioS</i> ::Km of <i>C. violaceum</i> ATCC31532; Km ^R | This study |
| 31532CVII | <i>cviI</i> ::Km of <i>C. violaceum</i> ATCC31532; Km ^R | This study |
| 31532CVIR | <i>cviR</i> ::Gm of <i>C. violaceum</i> ATCC31532; Gm ^R | This study |
| PLASMIDS | | |
| pRK2013 | Tra ⁺ Mob+ColE1 replicon; Km ^R | (Figurski and Helinski, 1979) |
| pGEM2T | Cloning vector; Amp ^R | Promega |
| pMP220 | Promoter probe vector, IncP; Tc ^R | (Spaink et al., 1987) |
| pQE30 | Expression vector; Amp ^R | Qiagen |
| pBSIIKS | Cloning vector; Amp ^R | Stratagene |
| pBBRmcs5 | Broad-host-range vector; Gm ^R | (Kovach et al., 1995) |
| pKNOCK-Km | Conjugative suicide vector; Km ^R | (Alexeyev, 1999) |
| pKNOCK-Gm | Conjugative suicide vector; Gm ^R | (Alexeyev, 1999) |
| pSUP2021 | Tn5 delivery suicide plasmid; ColE1; Km ^R | (Simon et al., 1983) |
| pLAFR3 | Broad-host-range vector, IncP; Tc ^R | (Staskawicz et al., 1987) |
| pCVO7 | pLAFR3 containing <i>C. violaceum</i> 31532 DNA; Tc ^R | This study |
| pBSCVO7H | pBSIIKS carrying a HindIII 3 kb fragment from CVO7; Amp ^R | This study |
| pBCVO7XN | pBSIIKS carrying a XhoI-NotI 6.35 kb | This study |

| | | |
|-------------|--|------------------------|
| | fragment from CVO7; Amp ^R | |
| pBBVioS | pBBRmcs5 containing VioS; Gm ^R | This study |
| pKNOCKcvil | Internal <i>cvil</i> fragment cloned in pKNOCK-Km | This study |
| pKNOCKcviR | Internal <i>cviR</i> fragment cloned in pKNOCK-Gm | This study |
| pKNOCKvioS | Internal <i>vioS</i> fragment cloned in pKNOCK-Km | This study |
| pMPGFP | pMP220 containing the GFPmut3 gene deprived of its promoter | This study |
| pPcvilGFP | <i>cvil</i> promoter cloned in pMPGFP | This study |
| pPcviRGFP | <i>cviR</i> promoter cloned in pMPGFP | This study |
| pPvioAGFP | <i>vioA</i> promoter cloned in pMPGFP | This study |
| pPvioSGFP | <i>vioS</i> promoter cloned in pMPGFP | This study |
| pBBRcviR | <i>cviR</i> cloned in pBBRmcs5 | This study |
| pQE30VioS | <i>vioS</i> cloned in pQE30 | This study |
| pPvioA220 | <i>vioA</i> promoter cloned in pMP220 | This study |
| pPcepI220 | <i>cepI</i> promoter cloned in pMP220 | This study |
| pScR2 | pQF50 vector expressing the <i>B. cepacia</i> <i>cepR</i> gene | (Aguilar et al., 2003) |
| pMP77 | Promoter probe vector; IncQ; CmR | (Spaink et al., 1987) |
| pMPCviRLacZ | <i>cviR</i> translational fusion | This study |
| pMPVioALacZ | <i>vioA</i> translational fusion | This study |

PRIMERS

| Primers name | Sequence | Source |
|--------------|-----------------------------|------------|
| cvilBF | GGATCCCCGTAGGCAAAGAACTAA | This study |
| cvilER | GAATTCTGTGTCTGAACGCCA | This study |
| cviRBF | GGATCCCCGAAACTCATCCAAAAA | This study |
| cviRER | GAATTCGTTGATGGGTTTCGAGAT | This study |
| vioABF | CGGATCCGTGTTGCATTTCTCAAATGG | This study |
| vioAER | GGAATTCGAAGAGTGCTTCATCACGA | This study |

| | | |
|---------------|-----------------------------|------------|
| vioSBF | GGATCCGCCCAAAGCCAGACTA | This study |
| vioSER | GAATTCTGAACGGCAGGATTGA | This study |
| GFPEF | GGAATTC AAGAGGAGAAATTAAGATG | This study |
| GFPPR | ACTGCAGTCAGCTAATTAAGCTTATT | This study |
| vioA220KF | AGGTACCGTGTTCATTTCTC | This study |
| vioA220XR | GTCTAGAGAAGAGTGCTTCAT | This study |
| cepl220EF | GAATTCTCGCTTACGTGACGGTCG | This study |
| cepl220XR | TCTAGAGCATGGTGTCTCGGATT | This study |
| cviRPROMF_Xba | TCTAGAGCCGAAACTCATCCAAAA | This study |
| cviR2R_BglII | AGATCTGGGCGTAGTTTTCTCATGT | This study |
| vioAPROMF_Xba | GTCTAGAAAATGGAAAGCCTGTCACT | This study |
| vioAR3_BamHI | AGGATCCTCTGCATGTCGAAAAT | This study |
| VioSBFw | AGGATCCCCTTGCATCACCCGAGT | This study |
| VioSHR | GAAGCTTTTACGAGGCGGGTTTAGA | This study |
| cviRHF | CAAGCTTCAAGGAAGACTCGCTCAT | This study |
| cviRXR | GTCTAGATCATTTCGTTTCGCTACGGT | This study |
| KncviiBF | AGGATCCAGGCTATTGGTGCC | This study |
| KNcviiKR | AGGTACCAGCCGGCGGTACAT | This study |
| KNcviRF | CCAGAACCAGATCCAGCG | This study |
| KNcviRR | GATGGACAGGATGCTGCCG | This study |
| KNvioSKF | AGGTACCCGGCTGCACGAAGC | This study |
| KNvioSBR | AGGATCCCAGGCAAGCCAGC | This study |

Figure 1.TIFF

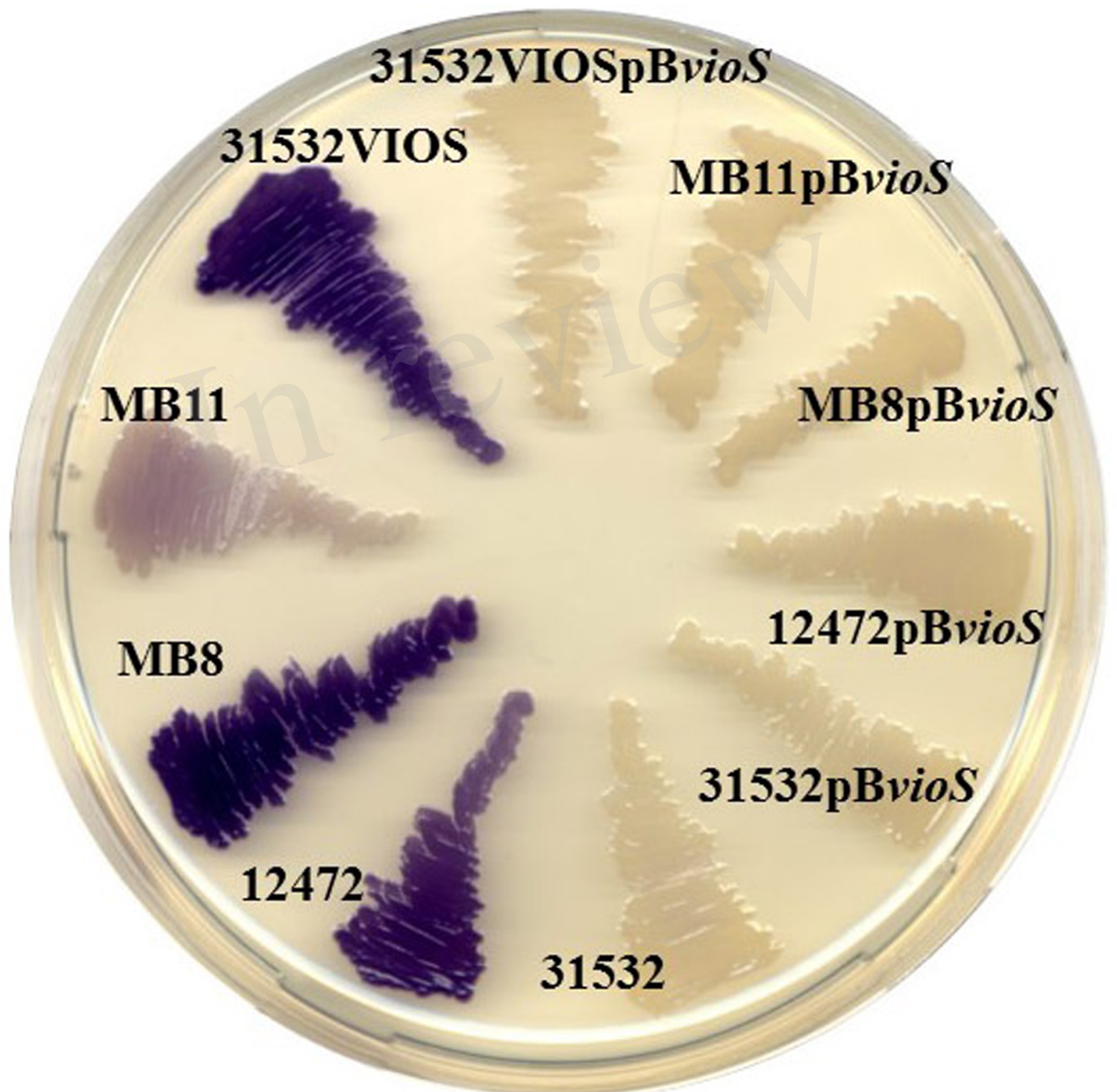
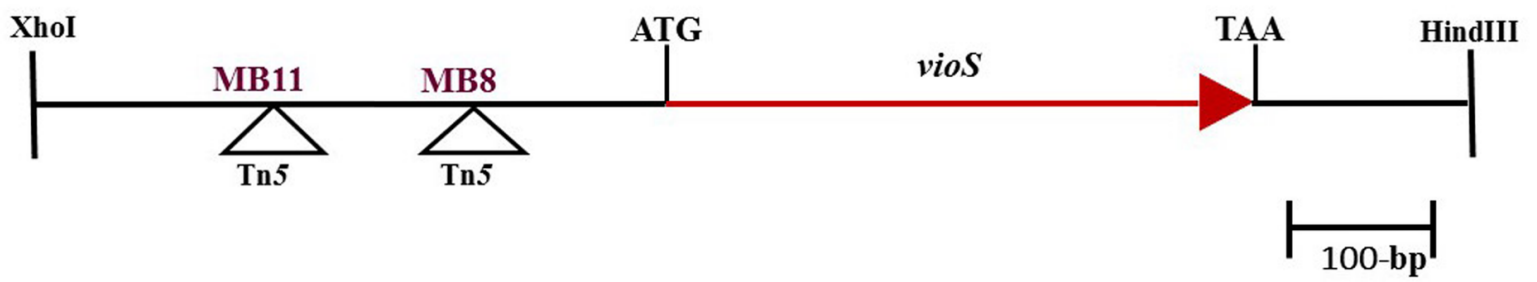


Figure 2.TIF

fig 2

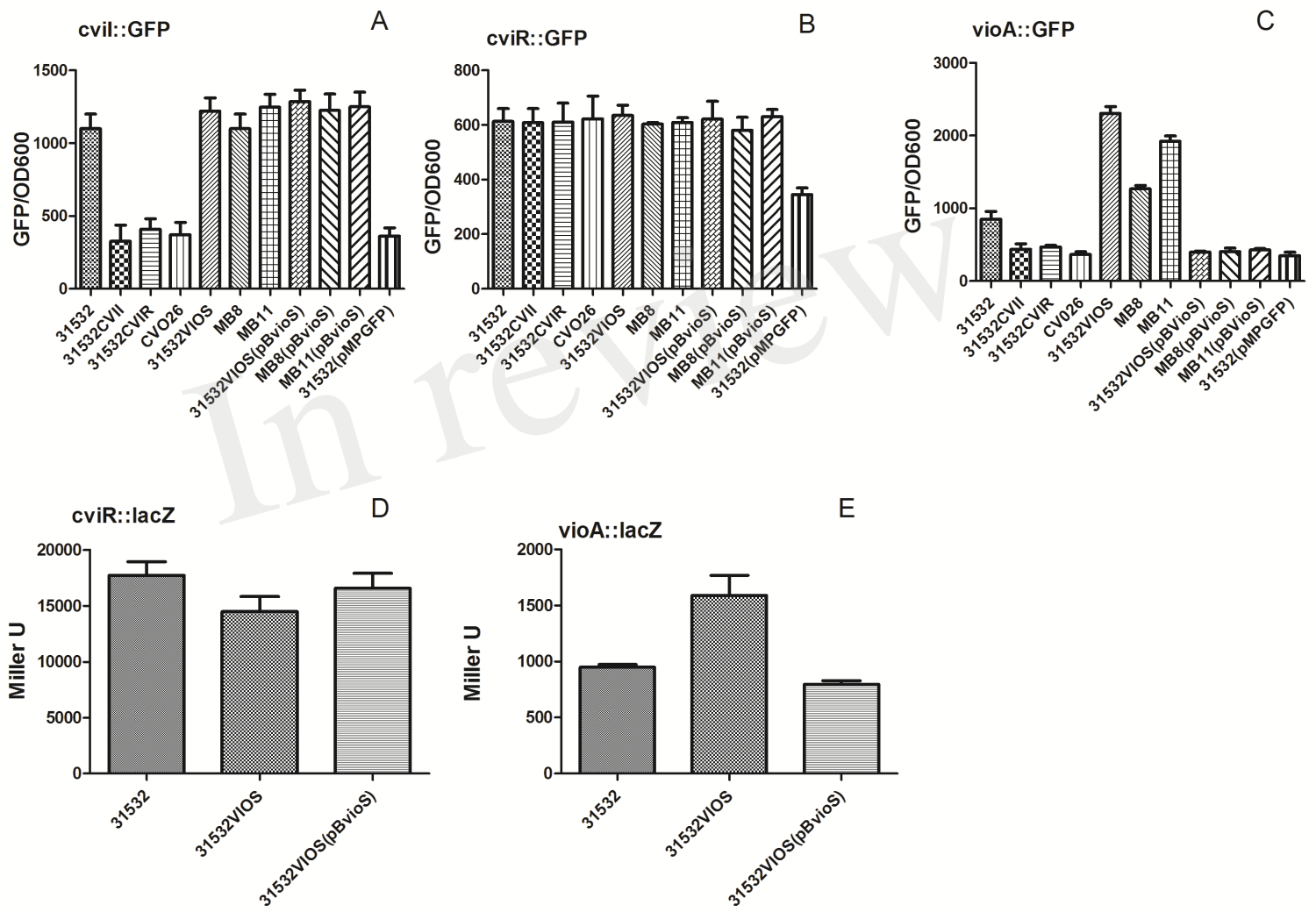


Figure 3.TIF

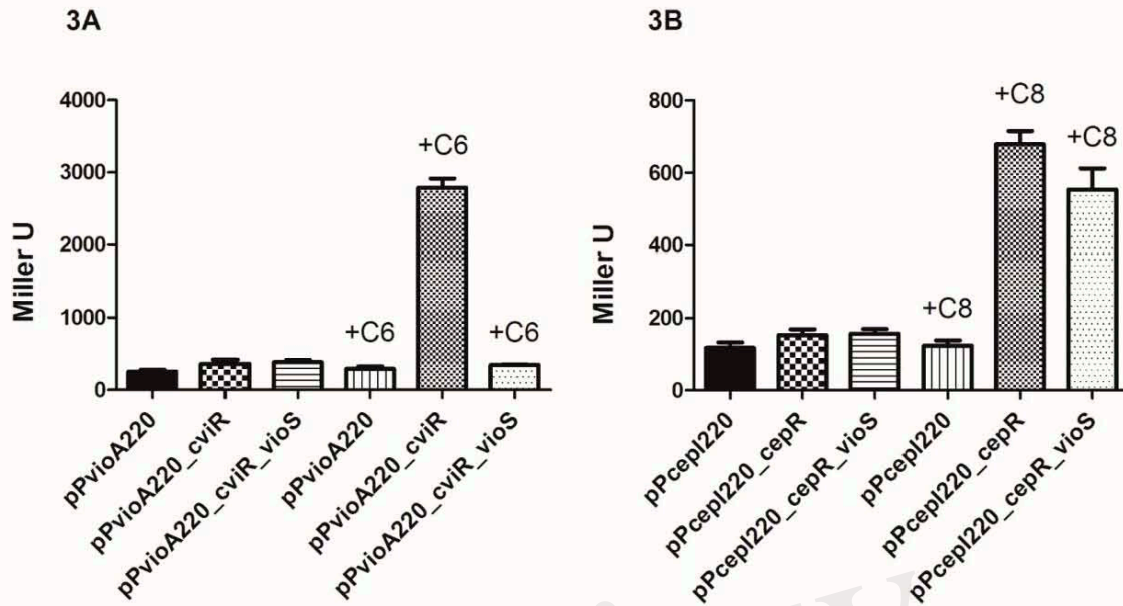


FIG.3 VioS antagonizes CviR mediated activation of VioA promoter in a heterologous *E.coli* strain and this inhibition is specific. (A) VioA promoter activity in the presence of CviR alone or together with VioS, in the absence/presence of C6 1 uM. (B) CepI promoter activity in the presence of CepR alone or together with VioS, in the absence/presence of C8 1uM. β -gal activities were measured after 12 hours of growth. Experiments were performed in triplicates and means plus standard deviations are plotted.

Figure 4

Figure 4.TIF

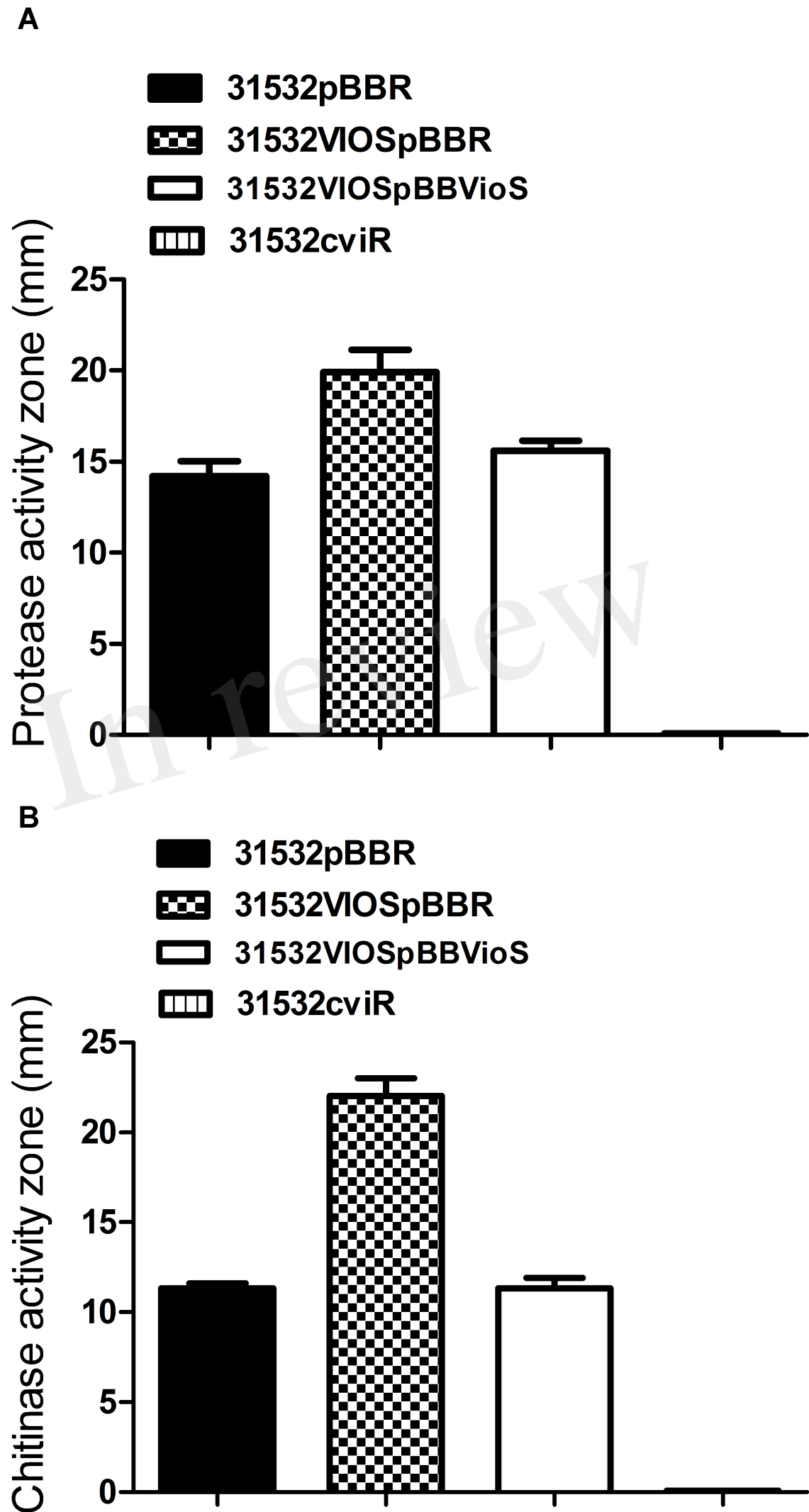


Figure 5.TIF

