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Research review paper

Advances in *Chromobacterium violaceum* and properties of violacein-Its main secondary metabolite: A review

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ABSTRACT

Chromobacterium violaceum is important in the production of violacein, like other bacteria, such as *Alteromonas*, *Janthinobacterium*, *Pseudoalteromonas*, *Duganella*, *Collimonas* and *Escherichia*. Violacein is a versatile pigment, where it exhibits several biological activities, and every year, it shows increasing commercially interesting uses, especially for industrial applications in cosmetics, medicines and fabrics. This review on violacein focuses mainly on the last five years of research regarding this target compound and describes production and importance of quorum sensing in *C. violaceum*, mechanistic aspects of its biosynthesis, monitoring processes, genetic perspectives, pathogenic effects, antiparasitic and antimicrobial activities, immunomodulatory potential and uses, antitumor potential and industrial applications.

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1. Introduction

Chromobacterium violaceum has been extensively studied in the violacein production field, even though different yields and production conditions have been published for other bacterial strains (Durán and Menck, 2001; Durán et al., 2007, 2010, 2012; Choi et al., 2015a,b).

Violacein producers vary phylogenetically, and so the production of this pigment depends on the location where the bacteria have been isolated. Table 1 includes a selection of environments and different countries where violacen production had been reported. Perhaps the best known genus is *Chromobacterium* (Moss et al., 1978; Hoshino, 2011), which includes the species *C. violaceum* (Durán and Menck, 2001).

Table 1
Violacein production from various microorganisms.

Strains	Comments	References
Alteromonas luteoviolacea	Marine bactéria, Scotland	Laatsch and Thomson (1984)
Chromobacterium violaceum	Bacillus violaceum, USA	Tobie (1934)
Chromobacterium violaceum ATCC 553	Collection	De Moss and Happel (1959)
Chromobacterium violaceum	Lowland river, England	Moss et al. (1978)
Chromobacterium violaceum B78	From Amazon River, Manaus, Brazil	Riveros et al. (1989)
Chromobacterium violaceum CCT 3496	Collection	Rettori and Durán (1997), Rettori et al. (1998)
Chromobacterium violaceum CCT 3496	Collection	Mendes et al. (2001a, b)
Chromobacterium violaceum	Agricultural waste, Malaysia	Ahmad et al. (2012).
Chromobacterium violaceum (MTCC 2656)	Indian Collection	Chaudhari et al. (2014)
Citrobacter freundii/ pCOM10vio	Recombinant strains	Jiang et al. (2010), Yang et al. (2011)
Collimonas sp.	Costal water, Norway	Hakvag et al. (2009)
Duganella violaceinigra	Forest soil	Li et al. (2004)
Duganella violaceinigra str. NI28	Forest soil	Choi et al., 2015b
Duganella sp. B2	From China	Wang et al. (2009)
Duganella sp.	Agricultural soils (olive), Spain	Aranda et al. (2011)
Escherichia coli K12 DH5a	*E. coli* K12 cloning	Ahmetagic and Pemberton (2010)
Escherichia coli MG1655-Vio4	Recombinant strains	Rodrigues et al. (2013)
Escherichia coli BL21(DE3)/ pET32avio	Recombinant strains	Jiang et al. (2010)
Escherichia coli Bl21(DE3) B2/pED + pVio	Recombinant strains	Fang et al. (2015)
Janthinobacterium lividum S9601	Collection	Shirata et al. (1998)
Janthinobacterium lividum strain DSM1522	Collection	Pantanella et al. (2007)
Janthinobacterium lividum	Glacier, China	Lu et al. (2009)
Janthinobacterium svalbardensis	Glacier, Slovenia	Avgustin et al. (2013)
Pseudoalteromonas DSM 13623	Marine sediment bacterium Germany Patent	Tan et al. (2002)
Pseudoalteromonas sp.	Deep sea Waters, Japan	Yada et al. (2008)
Pseudoalteromonas luteoviolacea	Marine sponge, China	Yang et al. (2007)
Psychotropic bacterium RT102	Close to *J. lividum*	Nakamura et al. (2002, 2003)
Psychrotrophic bacterium, XT1	Close to *J. lividum*	Lu et al. (2009)
Pseudoalteromonas sp. 520P1	Pacific coast. Japan.	Dang et al. (2014)

Most recently, there have been different strategies for studying violacein producers in different parts of the world. Aranda et al. (2011), using different aspects such as microbiological, physiological and genetic analyses, isolated and identified *Duganella* spp. that was associated with the rhizosphere (plants) produced violacein. Seven isolated *Duganella* spp. strains produced high levels of violacein in vitro, contrary to previously published reports. Violacein showed growth-inhibitory activity against Gram-positive bacteria but not against Gram-negative bacteria and fungi. Different *Duganella violaceinigra* from Forest soil were isolated and characterized and showed excellent productivity of violacein (Li et al., 2004, Choi et al., 2015b). Ahmad et al. (2012) isolated *C. violaceum* from various plant waste sources, such as bagasse, fruit waste, molasses, and others, as an alternative to the rich medium normally used. Among them, sugar bagasse supplemented with L-tryptophan was the most efficient in the production of violacein when compared with common nutrients.

Janthinobacterium svalbardensis (JA-1 strain) was isolated from Norway glacier ice samples (Avgustin et al., 2013). The 16S rRNA gene sequences and DNA–DNA hybridization tests demonstrated that the JA-1 strain, although belonging to the genus *Janthinobacterium*, represented a novel lineage distinct from the two known species of this genus, *J. lividum* and *J. agaricidamnosum*. The isolate was a psychrotrophic Gram-negative bacterium, which, as rod-shaped with rounded ends, contained intracellular inclusions and one polar flagellum. On the basis of the results, authors proposed that strain JA-1 was the type strain of a novel species of *Janthinobacterium*, for which the name *Janthinobacterium svalbardensis* sp. nov. was given.

In the work of Rodrigues et al. (2013), systems-wide metabolic engineering was used to target *Escherichia coli*. The basic producer, *E. coli* dVio-1, that expressed the vioABCE cluster from *C. violaceum* under control of the inducible *araC* system, accumulated deoxyviolacein. Through intracellular metabolite analysis, bottlenecks in tryptophan supporting pathways were identified, which is the major product building block. This was used for understanding the engineering of serine, chorismate and tryptophan biosynthesis and the pathways of the non-oxidative pentose-phosphate process. The ultimate strain, *E. coli* dVio-6, accumulated deoxyviolacein in shake flask cultures. The created system of a high-flux tryptophan pathway was fulfilled by genomic integration of the vioD gene of *J. lividum* (exclusive production of violacein). Finally, in a fed-batch process, *E. coli* Vio-4 accumulated the violacein as the main product.

Chaudhari et al. (2014) showed that dimethyl sulfoxide (DMSO) enhanced violacein production in *C. violaceum* (MTCC 2656) in a dose-dependent manner, simultaneously exerting an inhibitory effect on bacterial growth. Thus, the effect of DMSO on violacein production by the cells was increased due to interference with the quorum sensing (QS) of *C. violaceum*.

It was known that in *Vibrio fischeri*, there are essential components in QS-regulated bioluminescence, such as N-acylhomoserine lactone (AHL) synthase (LuxI) and AHL receptor protein (LuxR), and their genes (*luxI*/*luxR*) have been determined (Lazdunski et al., 2004). The identification of these genes led to the understanding of the mechanisms of QS regulation and the nature of AHLs related to violacein production. Dang et al. (2014) sequenced the complete genome of *Pseudoalteromonas* strain 520P1 no. 412 (NBRC 107704) identifying the *luxI* and *luxR* genes. These authors reported a draft 5.25-Mb genome sequence of *Pseudoalteromonas* sp. 520P1 (marine violacein-producing bacterium) from the Pacific coast of Japan. BLAST searches (genome annotation) demonstrated the presence of one *luxI* and five *luxR* homologs. Subsequently, there was an important comparative study of the genomes with two other known violacein-producing *Pseudoalteromonas* (Thomas et al., 2008; Cress et al., 2013), which assisted to determine the protein components involved in the regulation of quorum-sensing in violacein synthesis.

An interesting strategy was followed by Fang et al. (2015) to enhance violacein production. Strains with a multivariate module for

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varied tryptophan productivities were first generated by combinatorial knockout of the trpR/tnaA/pheA genes and overexpression of two key genes, trpEfbr/trpD, from the upstream tryptophan metabolic pathway. Afterwards, the gene cluster of the violacein biosynthetic pathway was introduced downstream of the generated tryptophan pathway. After combination of these two pathways, maximum crude violacein production directly from glucose by *E. coli* B2/pED + pVio was achieved in flask culture, which was fourfold higher than that of the control without the tryptophan pathway up-regulation. In conclusion, the authors stated that metabolic pathway analysis using carbon labeling illustrated that the up-regulated tryptophan supply enhanced tryptophan metabolism from glucose, whereas the introduction of the violacein pathway drew more carbon flux from glucose to tryptophan, thereby contributing to the effective production of crude violacein in the engineered *E. coli* cell factory.

2. *Chromobacterium violaceum* and quorum sensing

The most important adaptive response of bacterial communities to the environment occurs by cooperative and coordinated metabolic arrangements produced by specific molecules, and is defined as QS. QS systems have been found in many organisms, and they are very well characterized in Gram-negative bacteria (March and Bentley, 2004). QS reflects the adaptation of the microbial community to the dynamic environmental conditions and provides significant benefits to the microbial colonies involving the production of defense molecules against competitors such as extracellular hydrolytic enzymes (e.g., proteases, esterases, chitinases and pectinases), biosurfactants, virulence factors and biofilm. QS involves the synthesis and release of small molecules into the environment to interact with receptors that modify directly or indirectly cellular processes and biosynthetic pathways interfering on mechanisms of transcription and translation. The main family of QS molecules identified includes the AHLs, also called autoinducers, with variable acyl chain length from 4 to 16 carbons with or without double bonds, and substituents such as hydroxyl or keto groups at the 3-position (Fig. 1).

The QS system of *Chromobacterium violaceum* ATCC 31532 comprise four main components: CviI synthase, AHLs diffusible molecule, a CivR-type signal receptor and some target genes which involve the activation of lytic activity such as chitinases, and exoproteases, virulence factor like type VI secretion related gene, and genes related to a transcriptional regulator, a guanine deaminase and *cviI* responsible for the AHL synthesis and the violacein operon (*vio*ABCDE) among others (Stauff and Bassler, 2011).

The mechanism of QS in *C. violaceum* involves a positive loop based on the RI-gene system (analogous to the Lux model), which contains *CviI* (homologous to LuxI) and CviR (homologous to LuxR) proteins (McClean et al., 1997). The protein, CviI synthase, converts *S*-adenosyl methionine and fatty acids into AHLs. The QS signal is triggered when the ALHs reach certain concentration levels in the medium and bind CviR, a constitutive protein, stimulating the transcription of the *vio*ABCDE operon (August et al., 2000, Sanchez et al., 2006) (Fig. 2). In fact, the production of violacein shows biocidal activity against many kingdoms (fungi, bacteria, nematodes, virus, etc.) during the microbial stationary growth phase, during which population density is very high

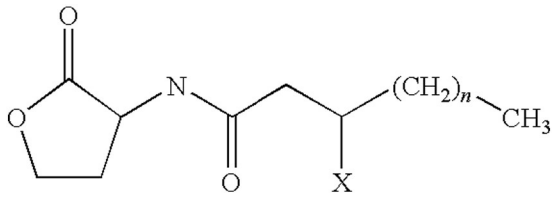

Fig. 1. Chemical structure of N-acyl-DL-homoserine lactone produced by *Chromobacterium violaceum*. Symbols: X-, hydroxyl- or keto-groups; and n from 4 to 14.

and nutrients are almost completely depleted (Durán et al., 2007). Thus, violacein production can be considered as a part of the competitive strategy to extend the life-span of the microbial colony.

The violacein biosynthetic gene cluster of *C. violaceum* contains the five contiguous genes *vioA*, *vioB*, *vioC*, *vioD and vioE*, which undergo transcription in the same direction. The enzymes VioA, VioC, and VioD were demonstrated to be FAD-dependent mono-oxygenases (Balibar and Walsh, 2006; Shinoda et al., 2007). VioB is a heme protein containing Fe^{2+}, considered to be a polyketide synthase (August et al., 2000). Inhibition of VioA or VioB leads to total depletion of violacein intermediates, while only violacein precursors are detected when VioC or VioD are inhibited. VioE is responsible for converting flavanone to isoflavone (phenyl ring shift and an indole nucleus 1,2-shift) produced only during violacein biosynthesis (Balibar and Walsh, 2006). In total, violacein biosynthesis involves six steps, five enzymatic reactions (VioA-E) and one non-enzymatic step of oxidative decarboxylation (Hoshino, 2011). Similar QS structure, function and regulation were reported for violacein biosynthesis by the Gram-negative *Pseudoalteromonas* sp. strain 520P1 (Wang et al., 2008; Zhang and Enomoto, 2011). Also, violacein has been detected in *Alteromonas luteoviolacea* (McCarthy et al., 1985), *Pseudoalteromonas luteoviolacea* (Yang et al., 2007), *Janthinobacterium lividum* (Pantanella et al., 2007), *Duganella* sp. B2 (Wang et al., 2008) and recently in other microorganisms. However, the regulation mechanism of violacein biosynthesis in most of the microbial producers has not been elucidated. On the contrary, violacein biosynthesis has been exhaustively analyzed in *C. violaceum*, and it is clear that AHLs play a role as QS inducers. In fact, the presence of AHLs not only plays a crucial role in the induction of QS bacterial response in *C. violaceum* cultures but also prompts drastic external morphological changes observed on the cell surface detected by atomic force microscopy and intracellularly in mesosomes observed by transmission electron microscopy (Rucinsky and Cota-Robels, 1974).

Besides, some regulatory discrepancies of AHLs were found in different strains. *C. violaceum* ATCC 12472 is able to produce several AHLs, but violacein production in strain VIR07 is induced by members of the C10-C16 acyl chain AHLs and particularly enhanced by C10-AHL. Meanwhile, violacein synthesis was found to be very low or undetectable in the presence of short-chain (C4–C8) acyl chain AHLs in the culture (Morohoshi et al., 2008, 2010). On the contrary, the biosynthesis of violacein in *C. violaceum* ATCC 31532 was induced only by the short-acyl chain (C4–C8) AHLs in the mutant CVO26 (HgR, *cviI*::Tn5 xylE, KanR, plus spontaneous StrR), and inhibited by C10–C14 long acyl-chain AHLs (McClean et al., 1997). Studies conducted with *C. violaceum* ATCC 31532 demonstrated that the affinity of AHL for the CviR protein to form the complex CviR-AHL was almost independent of N-acyl length, but the length of N-acyl chain played a crucial role in binding with RNA polymerase (Swem et al., 2009). These results clearly showed different sensitivity of violacein regulation to the AHLs in strains belonging to the same species. Also, the results could suggest possible additional missing regulatory steps in our present knowledge regarding violacein biosynthesis.

Recently, violacein biosynthesis from *C. violaceum* ATCC 12472 and some mutants (CV12472, VIR24) in the presence of antibiotics was studied (Liu et al., 2013). Violacein production was enhanced by 25% using sub-inhibitory concentrations of amikacin (1/4 MIC), erythromycin (1/8 MIC), gentamicin (1/2 MIC), kanamycin (1/6 MIC) and tetracycline (1/16 MIC) without bacterial growth inhibition. Gene expression analysis (real-time RT-PCR) showed upregulation of the AI synthase (*CviI*) and polyketide synthase (*VioB*) genes by kanamycin at sub-inhibitory concentrations ranging from 1/4 to 1/8 of minimal inhibitory concetrations (MIC) of those antibiotics (Liu et al., 2013). The authors suggested that antibiotics employed at sub-inhibitory concentrations trigger *CviI* synthase, the enzyme involved in the synthesis of AHLs and comprising the first step in violacein production.

The inducible biosynthesis of violacein in *C. violaceum* has been extensively used as a biosensor, since the bright violet color of the

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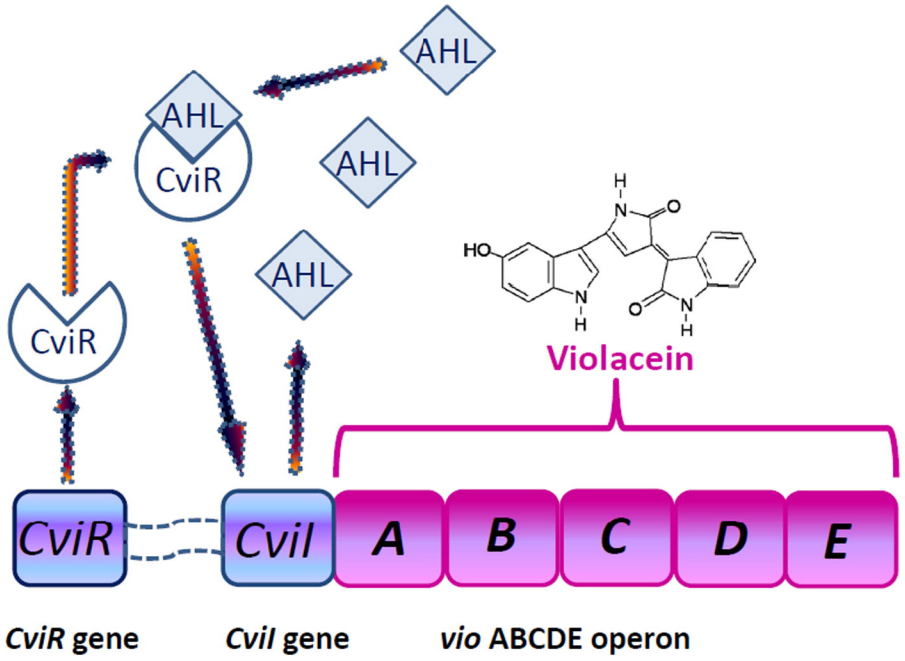

Fig. 2. Cartoon of violacein synthesis and regulation by quorum sensing of *Chromobacterium violaceum*.

molecule disappears in presence of QS-quenchers. Also, the discovery of novel QS quenchers could allow delving deeper into the QS mechanisms to better understand the network of multiple responses prompted by AHLs. Examples of QS quenchers of *C. violaceum* are widespread in several kingdoms and detected in plants extracts, fungi and bacteria. The mechanisms of QS inhibition are commonly based on:

1) Competitive inhibition of AHLs by natural or synthetic molecules interfering with the binding site of CviR protein forming inactive complexes unable to trigger the *CviI* gene. *C. violaceum* QS has been inhibited by various aqueous and organic plant extracts. However, the QS-quenching molecules have not been fully identified in more than 120 plant extracts, and only prospective inhibitors have been suggested, on the basis of previous chemical knowledge of the extracts and oils (Al-Hussaini and Mahasneh, 2009; Musthafa et al., 2016; Damte et al., 2003; Pellegrini et al., 2014). Besides, in some cases, *anti*-QS molecules were isolated and characterized from plants, such as 4-hydroxy-3 methoxybenzaldehyde (vanilla) from *Vanilla planifolia* Andrews, anacardic acids extracted from *Pimpinella anisum* (anise), *Anacardium occidentale* (cashew nuts) or *Amphypterygium adstringens* (Cuachalalate), or 6-gingerol, 6-shaol and zingerone from roots of *Zingiber officinale Roscoe* (ginger), or malabaricone C from *Myristica cinnamomea*, eugenol from *Syzygium aromaticum* (clove) drastically attenuated QS response (Choo et al., 2006; Chong et al., 2011; Castillo-Juárez et al., 2013; Zhou et al., 2013; Kumar et al., 2014). All these molecules have in common a phenolic ring containing mostly para- or meta-aliphatic side-chains of variable length mostly with some additional groups such as keto groups or double bonds, or an aromatic ring (Fig. 3).
Interestingly, in the photosynthetic bacterium *Rhodopseudomonas palustris*, N-(*p*-coumaroyl)-L-homoserine-lactone (pC-HSL) is biosynthesized instead of AHL as the QS signal, where fatty acids of the acyl-chain are replaced by *p*-coumaric acid (Schaefer et al., 2008). The structure of pC-HSL possesses two types of motifs: the lactone ring and also *p*-derivative of the phenolic ring (Fig. 4). pC-HSL could suggest dual biological activity as QS signal for *R. palustris* but also as QS-quencher for *C. violaceum*.
Similarly, a new class of synthetic antagonists of *C. violaceum* QS containing an aromatic ring plus the lactone motif has been reported (Swem et al., 2009). The structure of the inhibitor molecules resembles the structure of pC-HSL, but with slight structural changes in the molecules, e.g., replacement of O-lactone by S-lactone or OH by Cl, enhancing QS antagonist activity (Fig.5).
A similar chemical synthesis method was used to obtain a QS-antagonist library using N-sulfonyl homoserine lactones (SHL) as backbone (Zhao et al., 2013). The strategy was to insert a 4-aminobenzenesulfonyl moiety between the lactone ring and the acyl side chain (Fig. 6). The novel molecules were tested in violacein production in *C. violaceum* ATCC 31532 strain CV026. The SHL molecules containing phenyl groups substituted in the *ortho* position with fluoride or chloride showed the best inhibitory effect on violacein production at about 1.5 $\mu mol \cdot dm^{-3}$.
Another strategy to find QS quenchers was used in the study of volatile plant molecules able to trigger QS signals (Ahmad et al., 2014). Volatile secondary metabolites purified from plants, such as (+) enantiomers of limonene, carvone and borneol increased violacein production in *C. violaceum* ATCC 12472, while (−) enantiomers acted as QS antagonists. Particularly, α-terpineol and *Z*-3-nonen-1-ol were found to inhibit violacein synthesis by more than 90% (Ahmad et al., 2014). Alternatively, screening for QS quenchers in marine organisms was performed with relative success. Out of 78 marine samples including sponges, algae, terrestrial plants, fungi, tunicates and cyanobacteria, only 24% showed some QS antagonism using *C. violaceum* ATCC 31532 strain CV017. The most potent and abundant secondary metabolites with QS antagonism were microcolins A and B (1.5 and 15 $\mu mol \cdot dm^{-3}$), demethoxy encecalin (2.2 $\mu mol \cdot dm^{-3}$), hymenialdisin (40.2 $\mu mol \cdot dm^{-3}$), and kojic acid (36 $\mu mol \cdot dm^{-3}$) (Dobretsov et al., 2011). Similarly, *Halobacillus salinus* sp., which was isolated from a sea grass sample, showed QS inhibition in *C. violaceum* ATCC 31532 strain CV026, attributed to the production of secondary metabolites characterized as *N*-(2-phenylethyl)-isobutyramide and 3-methyl-*N*-(2-phenylethyl)-butyramide (Teasdale et al., 2009).

2) Biotransformation of AHL molecules by enzymes that attenuate and/or inactivate the bacterial QS response (Dong and Zhang, 2005). Biotransformations of AHLs are based on three types of enzymes: lactonases, acylases, and oxidoreductases. These enzymes were

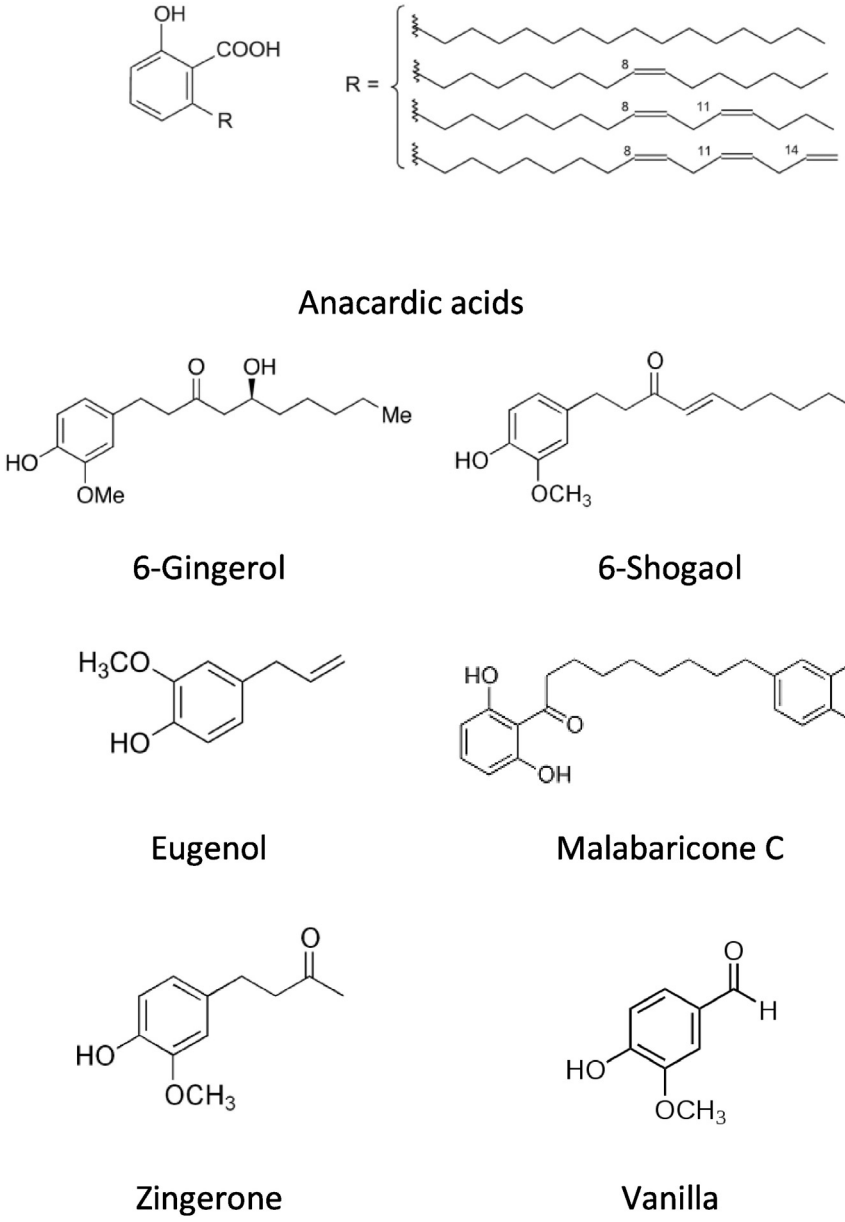

Fig. 3. Molecular structures of some natural QS-quenchers of *C. violaceum* obtaine.

reported in prokaryotes (*Firmicutes*, *Actinobacteria*, and *Proteobacteria*) and eukaryotes.

Lactonases break the ester bond of the lactone ring reducing the QS response (Fig. 7). N-acyl-L-homoserine lactone hydrolases (E.C. 3.1.1.81) have been detected in many Gram (+) and Gram (−) bacteria, mainly in the genus *Bacillus* (Dong et al., 2000). However, the substrate specificity for AHLs is very wide. As an example, the lactonase of *Bacillus thuringiensis* subsp. *kurstaki* HD263 was purified and challenged with N-acyl-L-homoserine lactone substrates with different acyl chain lengths. The *B. thuringiensis* lactonase showed the following decreasing specificity: C10 > 3-oxo-C8 > C8 > C6 > C4 > 3-oxo-C6 > C12 (Table 2) (Kim et al., 2005).

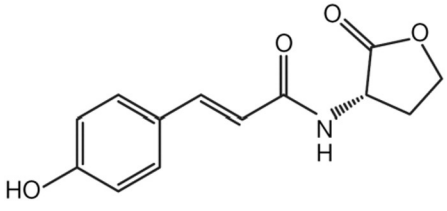

Fig. 4. N-(p-Coumaroyl)-L-homoserine-lactone synthesized by the photosynthetic bacterium *Rhodopseudomonas palustris*.

Acylases are biocatalysts able to cleave the amide bond of AHLs, releasing the L-homoserine lactone and the fatty acid which cause a substantial decrease in QS activity (Lin et al., 2003). AHL acylase activity is widespread in the bacterial kingdom showing moderate to high catalytic activity against a large number of substrates, ranging from short- to long-chain AHLs with or without 3-oxo or 3-hydroxy substitutions at C3 (Dong and Zhang, 2005).

The third group of enzymes involves oxidases and reductases, which specifically do not cleave the AHL molecules but just modify some functional groups; however, they are able to change their biological activities in QS. The oxidoreductase activity against AHLs was first detected in crude cell extracts of *Rhodococcus erythropolis* W2 (Uroz et al., 2005). The extract was able to reduce 3-oxo-C14-AHL into the hydroxyl-derivative, but no enzyme activity was detected against 3-

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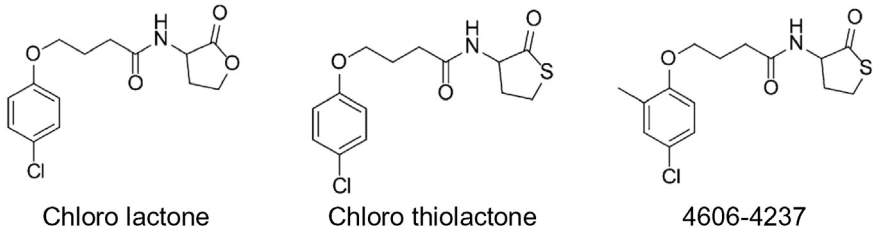

Fig. 5. Chemical structure of synthetic quorum sensing quenchers (modified from Swem et al., 2009).

hydroxyl-C14-AHL and unsubstituted AHLs. Another redox activity against AHLs by P450 BM3 monooxygenase was reported in *Bacillus megaterium* (Chowdhary et al., 2007). However, the specificity of the reaction is in doubt, since the enzymatic activity of CYP102A1 is normally related to fatty acids.

3. Mechanistic view of violacein biosynthesis

Violacein is a blue-violet pigment whose biosynthesis (Durán et al., 1994) is associated with a secondary metabolic pathway involving tryptophan, which has been under investigation for more than 80 years now, with the first reports dating back to 1934. Reasonable violacein yields can be achieved in a five-step biosynthetic pathway that involves the transformation two *L*-tryptophan residues, as shown in Fig. 2. This pathway requires the joint action of five enzymes, which enables the efficient biosynthesis of violacein (Alkhalaf and Ryan, 2015).

The first enzyme that starts violacein biosynthesis is called VioA and it is a flavin-dependent tryptophan 2-monooxygenase. VioA-catalyzed reaction results in tryptophan (Trp, W) oxidation to indole-3-pyruvic acid imine (IPA) and is accompanied by simultaneous co-factor FAD reduction to $FADH_2$ (Hoshino, et al., 1994; Balibar and Walsh, 2006). In the second reaction step, the VioB enzyme transforms IPA through a dimerization process, forming an imine-dimer that is very short-lived (Chang et al., 2013; Harrison and Ronczka, 2013). This short-lived compound undergoes the action of the VioE enzyme and is converted prodeoxy-violaceinic acid (PVA) (Hoshino, 2011). Finally, we can cite the coordinated action of two oxygenases, also flavin-dependent, designated VioC and VioD. VioD is able to hydroxylate PVA at the 5-position of the indole ring to yield proviolacein, and VioC can oxidize the other indole ring at the 2-position to generate the oxindole and finally to produce violacein (Jiang et al., 2010; Kamaeva et al., 2014; Onaka, 2009). Commonly, the 1,2-shift of the indole nucleus on the left side of the violacein structure (Fig. 8) is considered the most attractive biosynthetic mechanism in the entire proposed biosynthetic pathway because of the fact that no other pigment synthesis relies on this shift, nor does it occur in known natural product synthetic pathways (Alkhalaf and Ryan, 2015; Merlin et al., 2013).

All five enzymes, VioA-VioE, have been cloned and studied in vitro (Balibar and Walsh, 2006). Their molecular weights are 48 kDa (VioA), 111 kDa (VioB), 48 kDa (VioC), 42 kDa (VioD) and 22 kDa (VioE). The DNA fragment coding for these five enzymes is 7.3 kb in size and consists of five genes named *vio*A, *vio*B, *vio*C, *vio*D and *vio*E (Fig. 9), which are important for the production of violacein in *C. violaceum* cells as discussed above.

Once synthesized, the violacein molecule can be divided into three different moieties: (*i*) 2-pyrrolidone, (*ii*) 5-hydroxyindole and (*iii*) oxindole. If we investigate the biosynthetic origins of the elements in violacein, we find that almost all atoms are retained from the starting compounds, two Trp (W) moieties, during biosynthesis. On the other hand, oxygen atoms in violacein, unlike carbon, nitrogen and hydrogen atoms, come from molecular oxygen.

Although much biochemical information on the mechanism of violacein biosynthesis has been published (Sánches et al., 2006; Wang et al., 2012; Merlin et al., 2013), little is known about the chemical structures of the five enzymes involved in the synthesis of violacein, except for VioE (Ryan et al., 2008) and VioD. On the one hand, VioE (PDB entry: 2ZF3, Fig. 10) is a homodimer with opened baseball glove shape, where each monomer is on one side of this conformation (Hirano et al., 2008), and where three dimer units are organized as trimers (six chains being present in the monocrystalline), with none of the co-factors present in the protein.

Unlike VioE, VioD recombinant enzyme (*Escherichia coli*) is a monomeric protein (PDB entry: 3C4A, Fig. 10). It is an asymmetrical biomolecule that has a co-crystallized FAD molecule. VioD is a flavin-dependent oxygenase that is commonly responsible for the hydroxylation of prodeoxyviolaceinic acid (PVA) at the 5-position of the indole ring to yield proviolacein, and the structure this enzyme has been recently reported (Ran et al., 2015).

Thus, we can say that it is confirmed that all carbon and nitrogen atoms in violacein are derived from L-tryptophan, while one of the oxygen atoms comes from molecular oxygen, and that the complete biosynthetic process involves a multistep synthesis that ends as a 14-electron oxidation pathway (Balibar and Walsh, 2006). One or two of the enzymes involved in violacein synthesis are monooxygenases (VioC and VioD) that can either be NAD(P)H- or NADH-dependent.

One thing that still is not clear is how these multiple steps are coordinated in the synthesis of this violet pigment in vivo, since the structures of the involved enzymes have still not been elucidated. On the other hand, their biochemical characteristics are known (Balibar and Walsh, 2006).

4. Violacein-monitoring processes

It should be noted that the violacein concentrations reported in many of the articles are based upon the extinction coefficient as determined by the authors using spectrophotometric analyses, with extinction coefficient values ranging between 10.96 L/g-cm (ethanol 570 nm) (Wang et al. 2009), 56.0 L/g-cm (ethanol 575 nm) (Mendes et al., 2001a, b), 74.3 L/g-cm (ethanol 575 nm) (Rodrigues et al.,

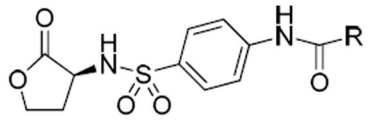

Fig. 6. Chemical formula of synthetic N-sulfonyl homoserine lactones. R: aliphatic or aromatic rings.

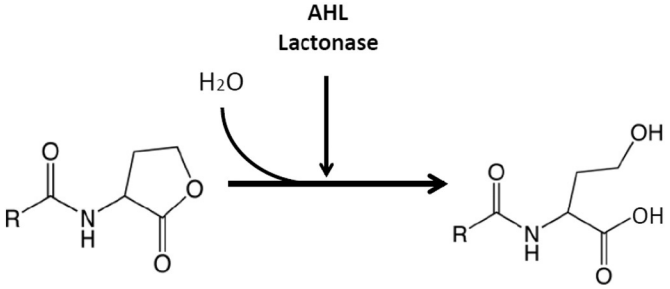

Fig. 7. Mechanism of N-acyl-L-homoserine lactone hydrolase (i.e. AHL lactonase).

Table 2
Relative activity of lactonase from *Bacillus thuringiensis* subsp. *kurstaki* HD263 on N-acyl-L-homoserine lactone of different acyl chain lengths (from Kim et al., 2005).

N-acyl-L-homoserine lactone	Lactonase activity (%)
C4	61.4
C6	100.0
C8	116.6
C10	217.1
C12	31.6
3-oxo-C6	49.5
3-oxo-C8	121.3

2012), 29.7 L/g-cm (Rettori and Durán, 1998) and the classic literature of 31.3 L/g-cm (acetone-water 565 nm) (De Moss, 1967). A recent article by Rodrigues et al. (2012) highlighted the discrepancy in spectrophotometric-based determinations of violacein and deoxyviolacein concentrations and stated that this could result in inflated violacein concentrations, by as much as 680%. This is not true for the HPLC pure violacein determination, since the extinction coefficient is calculated by Beer's law for different concentrations of pure HPLC-grade violacein, as demonstrated by Rettori and Durán (1998). It is reasonable to avoid any potential confusion for readers when a mixture of violacein and deoxyviolacein are present, and we are in agreement that the best method is really HPLC determination. But with estimation of concentration in bacterial cultures, it is convenient to use extinction coefficient, knowing previously the purity of violacein by HPLC.

Fig. 9. Illustration of the DNA fragments of 7.3 kb with genes *vio*A–*vio*E that codify for the key enzymes in the five-step violacein biosynthesis. The violacein operon contains promoters that affect the expression of the enzymes from the operon *vio*ABCDE codifing for the VioA-VioE proteins.

It is interesting to analyze different ways to monitor any violacein production. Actually, there are two interesting methods that are important to discuss. One is a new approach to obtain mechanistic information on violacein synthesis. The use of multi-wavelength fluorescence spectroscopy and parallel factor analysis (PARAFAC) was used. In this case, six fluorescent compounds were detected and their concentration and spectral profiles were resolved by this method. The main intermediate, tryptophan, was consumed during the bioprocess, corroborating the mechanism previously discussed in this review. The final product, violacein, was also identified by means of its concentration and spectral profiles. Some drawbacks were found since the identification of other fluorophores was not possible by comparison of spectra due to the lack of basic information for fluorescent natural products, where databases could be improved to aid the type of analysis proposed. An important conclusion was to show that the use of different instrumental settings was important to enable the visualization of fluorescence signals in the lower energy spectral region due to the wavelength-dependent sensitivity of the spectrofluorometer photomultiplier tube. The

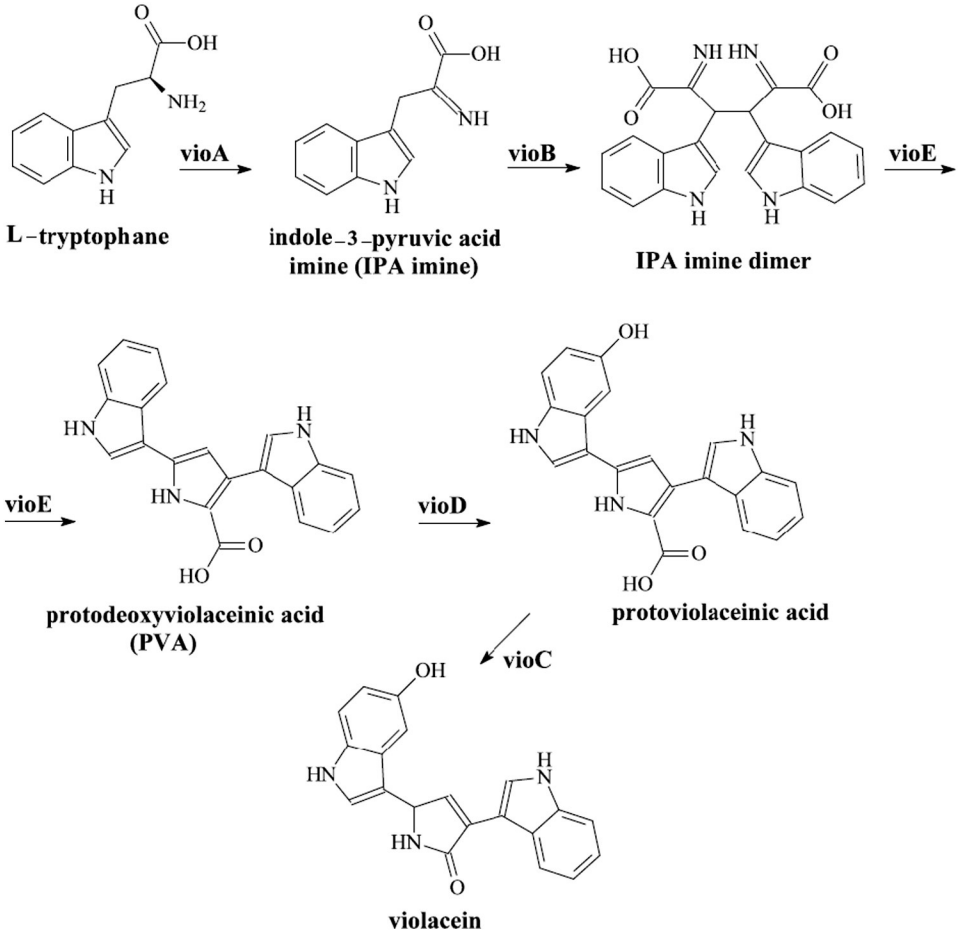

Fig. 8. Illustration of the violacein biosynthesis (Hoshino, 2011): The enzyme VioA is L-tryptophan oxidase that has FAD as a co-factor and is the first enzyme required for an IPA imine formation. This imine becomes then a substrate for VioB enzyme that forms a dimmer from it. VioB is an oxidase that uses heme moeity as the co-factor. It is believed that the key enzyme in the violacein biosynthetic pathway is VioE, the third enzyme that takes action in the proposed pathway. VioE uses the IPA imine dimmer as a substrate and transforms in to intermediates that are then used as substrates for VioD and VioC enzymes. The last two enzymes are classified as monooxygenases, both being FAD dependent, and responsible for violacein and deoxyviolacein production.

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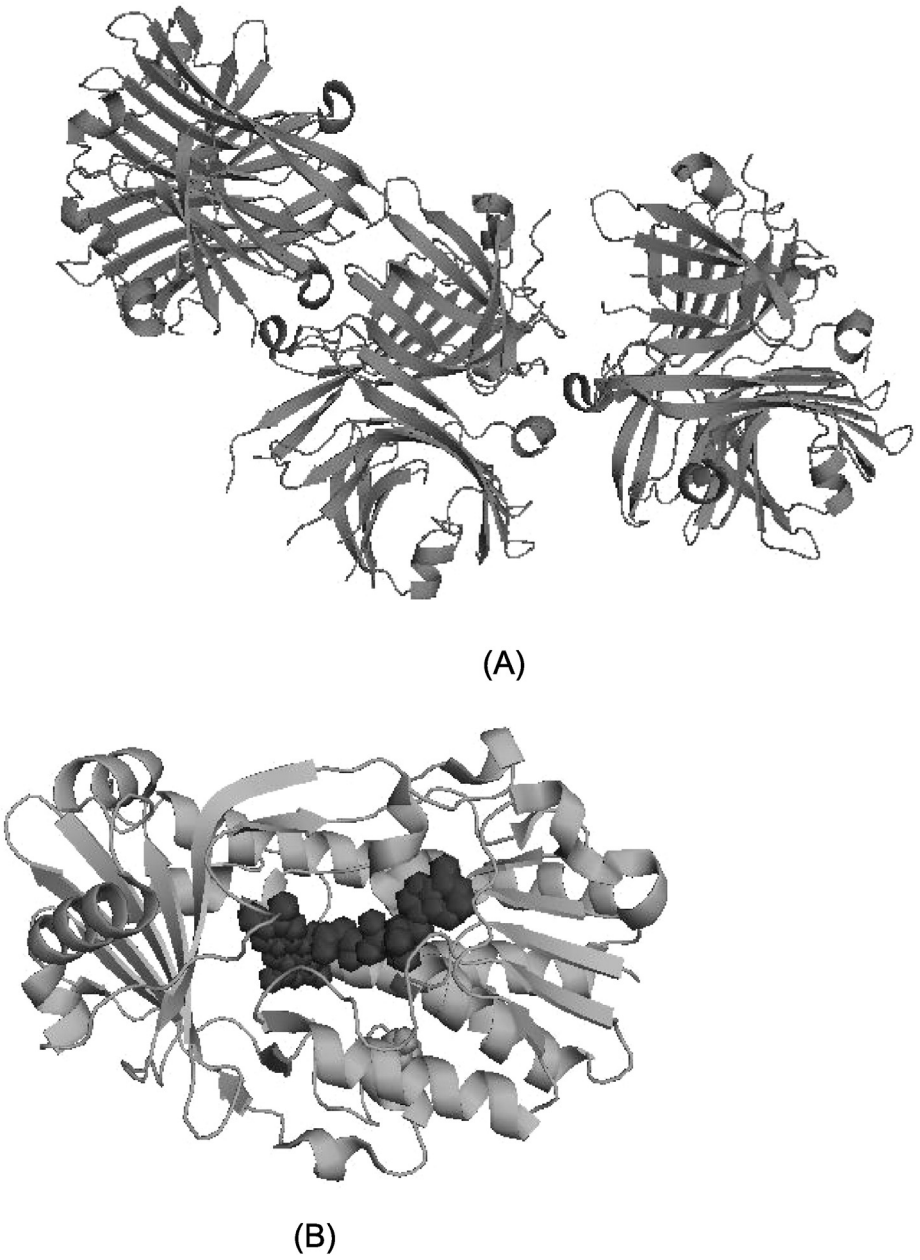

Fig. 10. Cartoon representations of the 3D structures of: (A) VioE (PDB entry: 2ZF3) and (B) VioD (PDB entry: 3C4A) enzymes. VioE and VioD are two very important enzymes in violacein biosynthesis in *Chromobacterium violaceum* (ATCC 1247). (A) The VioE structure is shown in grey color and is composed from six monomers organized in trimer of dimers. (B) The VioD structure is shown in grey with the FAD molecule being a part of this enzyme. Both enzymes had their structure resolved using X-ray diffraction.

authors believed that this method has an important potential for getting deeper insight into the biosynthesis of natural products (Dantas et al., 2012).

A hyperspectral imaging (HSI) system was used to recover the spectral signatures of pigment production from *C. violaceum* in a non-homogeneous medium with high spectral resolution and high sensitivity in vivo. A non-contact sensing technique to study temporal growth of a specific section in the bacterial colony was analyzed. At 580 and 764 nm, spatio-spectral time series was used to get quantitative information for kinetic parameters of violacein production and bacterial growth in a wild-type and mutant *C. violaceum* strains. Gallardo et al. (2014) used a real-time, non-invasive and simple process to quantify bacterial pigment production using an HSI system. This allowed getting new insights into the biosynthesis of violacein in a complex media. Also, since they detected the specific wavelengths that allowed the characterization of violacein production and growing process, the next step was to develop a filter-based imaging system aimed increasing the temporal resolution of the measurements.

5. Genetics of microbial producers of violacein

The Brazilian National Genome Sequencing Consortium sequenced the complete genome of *C. violaceum* ATCC 12472 strain in 2013. This strain contains a single circular chromosome of 4,751,080 bp with a G + C content of about 64.83% (Brazilian National Genome Project Consortium, 2003). The DNA sequences of this free-leaving bacterium revealed some characteristics that provided support for its versatility and adaptability to the environment.

C. violaceum showed a high proportion of open reading frames (ORFs) related to signal transduction, motility and secretion. These functions were correlated with bacterial adaptation to different environmental challenges. This microorganism also revealed 68 ORFs

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related to chemotactic capacity, and of these, 41 ORFs were for methyl-accepting chemotaxis proteins (Brazilian National Genome Project Consortium, 2003). In addition, genome of this bacterium showed an OmlA lipoprotein (CV1786), which promotes bacterial resistance under stress situations. This protein is also present in other pathogen opportunistic Gram-negative bacteria such as *Burkholderia cepacia* and *Pseudomonas aeruginosa* (Ochsner et al., 1999; Lowe et al., 2001). Also found in *C. violaceunm* genome were ORFs for the type III secretory system (T3SS or TTSS) similar to that in some genera of the family Enterobacteriaceae, such as *Salmonella enterica* serovar Typhimurium (Kimbrough and Miller, 2002) and *Yersinia pestis* (Tyler, 2002). T3SS genes are correlated with the invasion process in these bacteria by mediating secretion of protein effectors into the host eukaryotic cells. However, some important genes absent in *C. violaceum*, such as *invH*, could explain the low ability of this bacterium to infect humans (Brazilian National Genome Project Consortium, 2003). Additional genes encoding other virulence factors related to the adherence and invasion process, cytolytic proteins and synthesis of lypopolysaccharides (LPS), which are important in pathogenesis (Brazilian National Genome Project Consortium, 2003) were also found in *C. violaceum* genome, indicating an its virulence potential as discussed in the next section.

The production of biotechnological products is a remarkable attribute of *C. violaceum*. This characteristic was revealed by the presence of different ORFs releated to the production of biotechnological products found in the genome of ATCC 12472 strain (Table 3). As discussed previously, one of these products is violacein that is encoded by an operon consisting of five specific genes: *vio*A, *vio*B, *vio*C and *vio*D (August et al., 2000) and *vio*E (Asamizu et al., 2007), with a total sequence length of about 8 kb. Some studies using DNA-polymorphism techniques showed that *C. violaceum* isolated from the Brazilian Amazon have high genetic diversity and therefore a better adaptation to the environment (Hungria et al., 2005; Dall'Agnol et al., 2008). Therefore, future studies are necessary to better characterize these species and their full biotechnological potential.

Production of violacein is not restricted to *C. violaceum* as other species were also identified as violacein producers. For instances, *Janthinobacteria* are Gram-negative bacteria that also produce violacein, and the complete genome of *Janthinobacterium* sp. HH01 was sequenced and compared with *C. violaceum* (Table 4). The *Janthinobacterium* sp. HH01 genome shows a QS system with an autoinducer synthase gene-coding homologue of *Vibrio cholerae* and *Legionella pneumophilla*, which is associated with the regulation of violacein synthesis (Hornung et al., 2013). Other violacein-producer bacterium is *Pseudoalteromonas luteoviolacea* strain B (ATCC 29581) that had its genome sequencing recently described (Cress et al., 2013). Genomic sequence comparisons show differences in biosynthetic pathways and enzymes involved in bacterial metabolisms. General features of these genomes are listed in Table 4. Then, genetic studies of these microorganisms provide important data related to metabolism, pathogenicity, and interactions with host and environment, as well as synthesis of violacein.

Table 3
Some biotechnological products from *C. violaceum* with their ORFs and functions (described by Brazilian National Genome Project Consortium, 2003).

Biotechnological products	ORFs	Function
Violacein	CV3274, CV3273, CV3272, CV3271	Violet pigment with biological properties (antimicrobial, antitumor, anti-inflammatory)
Acid dehalogenase	CV0864	Environmental detoxification
Chitinases	CV2935, CV3316, CV4240	Biocontrol agents against insects, fungi and nematodes
Paraquat-inducible proteins	CV2547, CV2548	Bioengineering crops
Polyketide synthase	CV4293	Antibiotic synthesis
Phenazine	CV0931, CV2663	Antitumor
Hemolysins	CV0231, CV0513 CV1918, CV3342, CV4301	Anticoagulant
Cellulose	CV2675, CV2677, CV2678	Cellulose with different physicochemical properties

Table 4
General features of genomes of *Janthinobacterium* sp. HH01, *C. violaceum* ATCC12472 and *P. luteoviolacea* strain B ATCC 29581 (modified from Hornung et al., 2013).

Features	*Janthinobacterium* sp. HH01	*Chromobacterium violaceum* ATCC 12472	*Pseudoalteromonas luteoviolace* strain B ATCC 29581
Size (Mbp)	7.10	4.75	4.05
G + C content (%)	64.19	64.83	41.90
rRNA genes	20	25	7
tRNA genes	84	98	99
Protein-coding genes	5980	4407	3681

6. Pathogenic aspects of *C. violaceum*

All pathogenic effects were previously discussed in detail by Durán and Menck (2001), Durán et al. (2007) and Durán et al. (2010). Therefore, the present review will cover the period from 2010 to 2015.

As known, *C. violaceum* is a Gram-negative γ-protebacterium that grows in different ecosystems in tropical and subtropical areas, e.g., the waters and banks of the Rio Negro in the Amazon region of Brazil. Interestingly, the Rio Negro water is the main source of water for the surrounding population, and yet there is no widespread infection, indicating the low infectivity of this organism (Brito et al., 2004).

Despite low infectivity, *C. violaceum* is considered an opportunistic pathogen (Yang and Li, 2011), and its infection has a tremendous public health impact due to the mortality rate in humans (Rai et al., 2011) and animals (Liu et al., 2012), which justifies the study of its pathogenicity. In the last few years, there have been reports of clinical infection (Karthik et al., 2012) and environmental isolation of *C. violaceum* from different parts of India including the state of Kerala (Ambily et al., 2014). Infection is reported to be severe in malnourished and immunocompromised patients (Rashid et al., 2013). The symptoms include wound infection (Kumar, 2012), urinary tract infection (Swain et al., 2014) or sepsis and abscesses in various organs (e.g., liver, skin, lungs, lymph nodes and brain) (Chen et al., 2003; Kar et al., 2013; Orsetti et al., 2013). Infection occurs fast, and thus, a rapid diagnosis and antibiotic susceptibility profile determination are of paramount importance (Campbell et al., 2013).

In China, three cases of infection by *C. violaceum* were reported in patients varying from 42 to 81 year old, where the bacterial specimens were isolated from peritoneal fluid, wound infection area and urine (Ma et al., 2011). Also recently, the first case of *C. violaceum* infection in Cambodia was reported in a child, who recovered after antibiotic treatment (Ke et al., 2012), and cases of infection by this bacterium were also reported in Taiwan (Yang et al., 2011), Malaysia (Cheong, 2010) and Vietnam (Campbell et al., 2013). Recently, the first case of fatal adult bacteremia caused by *C. violaceum* was reported in Africa. In the Democratic Republic of the Congo, a 30-year old man with apparently no underlying disease was admitted with septic shock signs and blood and cerebrospinal fluid (CSF) cultures positive for *C. violaceum*. The patient died despite antibiotic treatment (Bottieau et al., 2015). *C. violaceum* infections were also reported in Europe, in a 14-year-old boy with cervical lymphadenitis (Arosio et al., 2011), and in the USA (Seigel et al., 2012), in a 14-year-old boy with necrotizing fasciitis.

The isolation of *C. violaceum* from water sampled in a hospital and the description of hospital-acquired infections indicated the potential of this bacterium to cause nosocomial infections (Umadevi et al., 2013; Hagiya et al., 2014). In the last few years, there have also been

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reports of *C. violaceum* infections in animals as well as humans, such as non-human primates (Baldi et al., 2010; Liu et al., 2012) and reptiles (Scheelings et al., 2012). Baldi et al. (2010) reported a *C. violaceum* infection in a wild adult monkey *Alouatta palliata* showing signs and symptoms of systemic infection. In a retrospective study at the Tulane National Primate Research Center (TNPRC) in the USA, between 2001 and 2010, a total of 13 cases of *C. violaceum* infection were described involving non-human primates, namely 8 pigtail macaques (*Macaca nemestrina*), 4 rhesus macaques (*Macaca mulatta*), and 1 baboon (*Papio papio*) (Liu et al., 2012). The work of Scheelings et al. (2012) suggested that *C. violaceum* must be considered a potential pathogen to reptiles.

The genome sequence of *C. violaceum* ATCC 12472 demonstrated the presence of different pathogenic factors (Brazilian National Genome Project Consortium, 2003). Miki et al. (2011) demonstrated that a type III secretion system (T3SS) encoded by genes present in the *Chromobacterium* pathogenicity islands 1 and 1a (Cpi-1/-1a) is involved with cytotoxicity in the murine model of infection. The CilA is a master regulator of the T3SS, and the effector protein CopE has been characterized as a guanine nucleotide exchange factor (GEFs) involved in the activation of Rac1 and Cdc42 and epithelial cell invasion (Miki et al., 2011). Two recent works (Ciprandi et al., 2013; Castro-Gomes et al., 2014) studied the protein secreted by *C. violaceum* into the culture medium, using mass spectrometry (MS). These works identified proteins potentially associated with virulence such as hemolysin, outer membrane proteins, collagenase, flagellar protein, metallopeptidases, and type VI secretion system (T4SS) effector protein. The role of these proteins in pathogenicity remains to be determined.

7. Violacein biological activities

Since *C. violaceum* characterization and violacein purification, this purple-colored indole derivative has been studied for its biological potential and has recently attracted the interest of the scientific community and, as a consequence, significantly increasing the number of studies in the literature.

Violacein exhibits several interesting biological activities with clinical relevance, some of which have already been previously reviewed (Durán and Menck, 2001; Durán et al., 2007, 2010, 2012; Soliev et al., 2011; Choi et al., 2015a). Early investigations reported its role as an antioxidant agent in the bacterium (Konzen et al., 2006), which was followed by the elucidation of its importance in two other violacein-producing bacterial species, *Pseudoalteromonas tunicata* and *J. lividum*, in which it has been suggested to be involved in defense mechanisms against eukaryotic predation and fungal diseases, respectively (Matz et al., 2008; Becker et al., 2009). These reports call attention to a still yet to be explored realm of violacein activities, which will uncover new knowledge on its role in ecological environments. On the other hand, more research has highlighted the clinical importance of violacein, including leishmanicidal, trypanocidal, antifungal, antiviral, antibacterial, antioxidant, antiprotozoal and antitumor activities, as well as its mechanisms of action, most of which have been previously reviewed (Durán and Menck, 2001; Durán et al., 2007, 2010, 2012). We describe here new features of this purplish secondary metabolite that point to its development as a chemotherapeutic agent.

7.1. Antiparasitic activity and mechanisms of action

Studies on the antinematode activity of violacein provide not only an evaluation of its potential in antihelminthic therapy but also an understanding of its toxicity with possible targets in multicellular eukaryotes (Ballestriero et al., 2014). The study was carried out using the marine bacterium *Microbulbifer* sp. D250 as a violacein producer. The direct toxicity caused by pure violacein purified from the bacteria and the increase in nematode survival induced by a violacein deficient mutant strain indicated that this indole derivative is responsible for the toxicity towards this multicellular organism. Indeed, the effective concentration of violacein against *Caenorhabditis elegans* ($LC_{50} > 30\ \mu mol \cdot dm^{-3}$) was in the range of several novel drug leads under investigation and other known chemotherapeutic agents, such as flavopiridol ($LC_{50} < 48.3\ \mu mol \cdot dm^{-3}$) (Ballestriero et al., 2014). Evaluation of the mechanisms involved in this action revealed an accumulation of the non-pathogenic *Escherichia coli* in the intestine of the worms in the presence of violacein, as well as, the induction of apoptosis. Finally, it was reported that the DAF-2/DAF-16 insulin/IGF-1 signaling pathway of innate immunity is involved in resistance against violacein, which would, in turn, determine the response of the antimicrobial peptide SPP-1 and the superoxide dismutase SOD-3 (Ballestriero et al., 2014).

Even though nematodes are more resistant than heterotrophic flagellates (Matz et al., 2004) and amoebae (Matz et al., 2008), a relatively low concentration of violacein is needed to kill *C. elegans*. Considering these results and its low toxicity in mice (Bromberg et al., 2010), it is conceivable to foresee the development of this indole derivative as an antiparasitic compound itself or to stimulate research based on the discovery of secondary metabolites from natural sources. In this respect, Rahul et al. (2015) tested the activity of violacein isolated from *C. violaceum* in combination with phytosynthesized silver (SNPs) and gold nanoparticles (GNPs) against two important neglected human diseases caused by parasitic protozoa, malaria and African trypanosomiasis.

Previous reports have shown in vitro activity of violacein against *Plasmodium falciparum* and in vivo activity against *Plasmodium chabaudi chabaudi* (Lopes et al., 2009). In addition to its antileishmanial activity (Leon et al., 2001), trypanocidal activity was reported against *Trypanosoma cruzi* (Durán et al., 2007), as well as antimicrobial activity of violacein-containing nanoparticles (Martins et al., 2010). In the study conducted by Rahul et al. (2015), the authors demonstrated that violacein shows low toxicity to *Trypanosoma brucei gambiense* bloodstream forms in vitro, with an IC_{50} value of 3.9 μg/mL, which was not altered when combined with SNPs or GNPs. Violacein and its combinations also inhibited *P. falciparum* similarly, with IC_{50} values around 52 μg/mL. Furthermore, violacein showed weak cytotoxicity to blood mononuclear cells and to HeLa cervical and MCF7 breast cancer cell lines, with IC_{50} values ranging from 98 to 158 μg/mL. Again, the presence of the nanoparticles did not affect cytotoxicity, supporting the safety of the compound (Rahul et al., 2015). This is the first time the violacein was reported to act against *T. brucei gambiense*.

7.2. Antimicrobial potential

Recently, the activity of violacein against pathogenic microorganisms of commercial interest was published. The ability of violacein to kill *Staphylococcus aureus* (*S. aureus*) and *E. coli* field strains isolated from bovine mastitis was investigated (Cazoto et al., 2011). These are the most frequent etiological agents of bovine mastitis, where they are responsible for serious economic losses in milk production, estimated in billions of dollars per year in the United States alone (Unnerstad et al., 2009). Evaluation of the antibacterial activity of violacein using *S. aureus* strains that display different degrees of resistance to antibiotics indicated sensitivity to violacein, exhibiting minimal inhibitory concentrations (MIC) ranging from 6.25 to 25 $\mu mol \cdot dm^{-3}$, supporting its use to combat *S. aureus*-induced bovine mastitis. Moreover, this purple derivative has synergistic effects when combined with other antibiotics, especially penicillin, against resistant strains, including those multidrug-resistant, with MIC values in the range of 0.04 $\mu mol \cdot dm^{-3}$. In contrast, no activity was detected against *E. coli* (Cazoto et al., 2011). Thus, it is reasonable to propose violacein as a possible antibacterial agent to be used for the treatment of bovine mastitis, either alone or in combination with other antibiotics.

To further increase bioavailability and toxicity of violacein against resistant *S. aureus*-induced bovine mastitis, poly(ε-caprolactone)/chitosan nanoparticles containing violacein were prepared (Berni et al.,

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2013). The MIC was reduced when encapsulated violacein was used to treat resistant strains (MIC = 25 μmol·dm^{-3} compared to MIC >-25 μmol·dm^{-3} for free violacein). Moreover, ecotoxicity tests indicated that the violacein nanosystem was more toxic to *Daphnia similis* than free violacein (EC_{50} between 0.3 and 1.1 μmol·dm^{-3} vs 3.3 and 5.0 μmol·dm^{-3}, respectively), which was related to the mucoadhesive property of chitosan. Indeed, a purple color could be seen in the organism's gut, indicating the increased retention time of violacein (Berni et al., 2013).

In a recent work by Subramaniam et al. (2014) the combined activity of violacein extracted from *C. violaceum* Bergonzini (*Neisseriaceae*) and commercial antibiotics was published. The authors established MIC values of 5.7 μg/mL for *S. aureus* and *Salmonella enterica* Typhi, 15.6 μg/mL for *Klebsiella pneumoniae*, 18.5 μg/mL for *Pseudomonas aeruginosa* and 20.0 μg/mL for *Vibrio cholerae*. Combination of violacein with gentamicin and cefadroxil decreased the MIC to 1.0 μg/mL against *S. aureus*. Furthermore, this work revealed significant synergism for violacein combined with azithromycin and kanamycin against *S. enterica* Typhi (Subramaniam et al., 2014).

Rajalakshmi et al. (2011) published an interesting study showing the isolation of a *C. violaceum* strain from spoiled vegetables. Although violacein production was not deeply investigated in this work, the morphological and biochemical characteristics, including the antibiotic susceptibility, of the isolated strain were similar to a standard *C. violaceum* (Rajalakshmi et al., 2011).

A new violacein-producing strain of an Antarctic *Janthinobacterium* sp. was also isolated by Asencio et al. (2014). The strain termed SMN 33.6 was defined morphologically, biochemically and phylogenetically, as well as, its ethanolic extract containing violacein was chemically characterized. The extract containing violacein was investigated against multi-resistant Gram-negative *Acinetobacter baumannii* and *P. aeruginosa*, both carbapenemase producers, *E. coli* and *K. pneumoniae* strains, extended-spectrum β-lactamase (ESBL)-producers and multi-resistant *Serratia marcescens* strains producing chromosomal AmpC beta-lactamase. MIC values ranging from 0.5 to 16 μg/mL were obtained, with significant effects against *P. aeruginosa* (MIC = 1 μg/mL) and two strains of *S. marcescens* (MIC = 0.5 and 2 μg/mL). The latter species is recognized as an important cause of morbidity in nosocomial infections (Asencio et al., 2014).

Recently, violacein was isolated from a new natural source, *Duganella violaceinigra* str. NI28, and its activity against *S. aureus* reported by Choi et al. (2015a, b). Violacein was capable of inhibiting the growth of different strains of *S. aureus*, including those multidrug resistants with a MIC value of 15 μmol·dm^{-3}. Moreover, this concentration of violacein was also bactericidal to the ATCC and clinical strains, leading to 69 and 78% decreased viability, respectively. Furthermore, tests with violacein isolated from *D. violaceinigra* demonstrated the same bioactivity compared with violacein extracted from *J. lividum* against these pathogens, confirming the potential of this purplish bisindole derivative to combat the resistance nature of *S. aureus*.

7.3. Immunomodulatory potential and applications

Despite the number of studies demonstrating the biological activities of violacein, it was only after 2010 that the first evidence of the immunomodulatory potential of violacein emerged (Antonisamy and Ignacimuthu, 2010; Antonisamy et al., 2014; Verinaud et al., 2015).

Antonisamy and Ignacimuthu (2010) reported various activities of violacein in rats, including analgesic, immunomodulatory and antipyretic activities. Violacein administered orally for 4 days at a dose of 40 mg/kg suppressed delayed-type hypersensitivity immune response elicited against sheep red blood cells, probably by modulating the inflammatory mediators. In addition, this compound administered orally for 11 days at the same dose was able to control type I IgE-mediated anaphylactic reaction. Violacein at a 40 mg/kg dose also induced central and peripheral antinociceptive response, which was in part inhibited by an opioid antagonist, naloxone, suggesting its action, at least in part, through spinal opioid receptors. Studies involving the analgesic activity of violacein include the formalin test, which evaluates its activity in an earlier phase of pain and a late inflammatory phase. Violacein exhibited effects on both phases, implying a direct effect on the nociceptor and its involvement in the synthesis and/or release of prostaglandins and other mediators. Violacein antipyretic activity further underscores its possible inhibitory action on prostaglandin (PG) biosynthesis (Antonisamy and Ignacimuthu, 2010). Indeed, this ability was confirmed in another study by this same group using the indomethacin-induced ulcer rat model (Antonisamy et al., 2014).

In a recent study Antonisamy et al. (2014) reported that violacein has significant gastroprotective effects against NSAID-induced gastric lesions at a single oral dose of 40 mg/kg. This activity was partially associated with an increase in PGE_2 and IL-10 levels, whereas levels of pro-inflammatory cytokines IL-1β, IL-6 and especially TNF-α, were decreased by violacein. Moreover, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) levels were increased by violacein, all of which have essential roles in angiogenesis, cyclooxygenase (COX) activation, mucin secretion and ulcer healing. In addition to its effects on these mediators, violacein also restores constitutive nitric oxide synthase (cNOS) to normal levels and implies NO in the gastroprotection. This study also indicates the involvement of the K^+-ATP channels in violacein activity. Interestingly, an antiapoptotic effect was found to contribute to reduce indomethacin-induced gastric lesions (Antonisamy et al., 2014).

Verinaud et al. (2015) also add to our knowledge on the immunomodulatory aspects of violacein activity. These authors used a mouse model (experimental autoimmune encephalomyelitis-EAE) of human multiple sclerosis, an inflammatory autoimmune disease. The effects of a single intraperitoneal dose of violacein at 3.5 mg/kg in mice injected with LPS suppressed acute inflammation by reducing the levels of pro-inflammatory cytokines and chemokines, and upregulating IL-10. Moreover, violacein also inhibited neutrophil migration to the peritoneal cavity. The effects of violacein in chronic inflammation were analyzed in EAE-inflicted mice treated with 3.5 mg/kg violacein i.p., initiated at the onset of the disease and continued for three days. The results revealed that violacein led to an improvement in the clinical course of the disease, which persisted until the 30th day, and a reduced incidence of EAE. Reduction of EAE was also achieved with adoptive transfer of violacein-elicited regulatory T (Treg) cells. These effects were related to modulation of dendritic cells and inflammatory mediators, including indoleamine 2,3-dioxygenase, IL-10 and IL-17, as well as induction of Treg cells (Verinaud et al., 2015). It is important to mention that no toxicity was reported in agreement with previous reports (Bromberg et al., 2005, 2010).

Taken together, these studies support further investigation of violacein immunomodulatory potential for future application in clinical approaches of autoimmune diseases.

7.4. Antitumor potential and mechanistic aspects

Several groups have demonstrated increased interest in the antitumor potential of violacein for the last few years and numerous studies have advanced our understanding of its mechanisms of action. In this respect, a key effort has been devoted to uncover the mode of violacein-induced cell death. It is well known that programmed cell death (PCD) in cancer is not well established for most chemotherapeutic agents and tumor cell types. In this respect, violacein is a model compound displaying lineage-specific cell death features and mechanisms of action. A previous study reported violacein-induction of apoptosis in myeloid leukemia HL60 cells (Ferreira et al., 2004). This was followed by studies in the less differentiated TF1 leukemia cells (Queiroz et al., 2012), which are known to be resistant to PCD (Bailly et al., 1995). Those results indicated that violacein clearly overcame TF1 resistance, inducing cell death with an IC_{50} of 2 μmol·dm^{-3}, as revealed by the

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MTT-reduction assay. Although direct necrosis and apoptosis were not involved in violacein-induced cell death, increased nuclear fragmentation was observed, which is in line with increased PARP cleavage. Noteworthy, TF1 cell death was induced by endoplasmic reticulum and Golgi linearization and 'horseshoe-shaped' nucleus, which led to organelle collapse and cell demise. Kinome profiling using peptide arrays indicates that this effect was mediated by inhibition of calpain and DAPK1, in contrast to the activation of PDK1, PKB and PDK. The authors stated that this was the first time the activation of a non-canonical mechanism of cell death by violacein in a resistant cell line (Queiroz et al., 2012). These aspects were important, since in the last few years violacein cytotoxicity has been studied in novel model cancer cell lines, as it is described in the next section.

Violacein cytotoxicity against breast cancer cells (MCF7 cell line), for example, was investigated by Platt et al. (2014), aiming to clarify its potential effect on cancer invasion and metastasis. Metastasis is dependent on the degradation of the extracellular matrix and its components by metalloproteinases (MMP), leading to invasion of the basement membrane and inflammation (Valastyan and Weinberg, 2011). In addition, inflammatory chemokines may also help in the metastatic process by inducing cell adhesion and migration. In this regard, Platt et al. (2014) reported the ability of violacein at a non-cytotoxic concentration ($1\ \mu mol \cdot dm^{-3}$) to downregulate the chemokine/receptor CXCL12/CXCR4 interaction in breast cancer cells. This chemokine axis is involved in the adhesion and migration of metastasis-initiating cancer cells and has been implicated in the pathogenesis of breast and pancreatic cancer and leukemias (Domanska et al., 2013). In addition, specific knockdown of MMP2 with siRNA revealed that violacien inhibits MMP2-mediated secretion of CXCL12. Furthermore, co-culture experiments indicated that this event triggered upregulation of MMP9 activity, which was inhibited when cells were treated with violacein. Thus, violacein could potentially inhibit early stages of inflammation by reducing MMP2 expression and later by abrogating MMP9 activation and CXCL12/CXCR4 interaction (Platt et al., 2014).

These results are of particular importance considering the roles of CXCL12/CXCR4 not only in cell-tumor microenvironment interaction, leading to tumor growth and metastasis, but also in angiogenesis. Interestingly, the study by Platt and coleagues (Platt et al., 2014) also reported a decrease in TNF-α and TGF-β-induced MMP2/9 expression by breast cancer cells. These pro-inflammatory cytokines mediate a number of effects in tumor cells, also acting as angiogenic stimulators. Antonisamy et al. (2014) have also shown important effects of violacein in angiogenic factors and pro-inflammatory cytokines, reinforcing the need to extend the study of violacein molecular mechanisms of action. Thus, these findings open new possibilities to explore violacein as a novel compound to improve anticancer treatment by acting on the communication of tumor cells with their surrounding microenvironment.

Mehta et al. (2015) recently reported the cytotoxicity and mechanisms of action of violacein in three different cells lines, namely glioblastoma (U87), breast cancer (MCF7) and lung cancer (A549). Breast and lung cancers are two of the most common cancer types in men and women, frequently showing a high incidence of brain metastasis. Thus, the authors also investigated the ability of violacein to inhibit migration of glioblastoma cells. Using a crystal violet proliferation assay, it was observed that $1\ \mu mol \cdot dm^{-3}$ violacein inhibited proliferation of all cell lines. Cell viability experiments indicated a significant sensitivity of glioblastoma and lung cancer cells to violacein, with an IC_{50} value around $1\ \mu mol \cdot dm^{-3}$. Analysis of the signaling kinases and some of their targets that might be activated in the mechanisms of violacein cytotoxicity revealed the involvement of p44/42 MAPK and cleaved PARP in U87 cell death. Of importance, reduction in glioblastoma migration induced by violacein without affecting adhesion was shown. This activity was attributed to the ability of violacein to disrupt the actin network, thus interfering with cell morphology and motility (Mehta et al., 2015). This study reinforced the previous work published by Platt et al. (2014) on the activity of violacein to prevent cancer metastasis.

In a work by Leal et al. (2015) conducted in the HeLa cervical cancer cell line, the mechanisms of violacein cytotoxicity were investigated. Cytotoxicity to HeLa cells seemed to be mediated predominantly by apoptosis. Cell death in two other non-tumor cell lines, MRC-5 (human fetal lung fibroblasts) and CHO-K1 (Chinese hamster ovary cells), occurs primarily by necrosis. In contrast to the effects observed in other cancer cell models, such as colon cancer (de Carvalho et al., 2006) and Ehrlich ascites tumor cells (Bromberg et al., 2010), no association between oxidative stress induction and cell death was observed. Interestingly, mitochondrial membrane hyperpolarization was demonstrated in violacein-treated HeLa and MRC-5 cells, suggesting cell-type specific induction of mitochondrial dysfunction that leads to a cell death (Leal et al., 2015).

In another study published by Hashimi et al. (2015), violacein antitumor activity was improved under hypoxia in several cell lines, with significant reduction in IC_{50} values, such as in MCF7 cells (4-fold), HCT 116 colon cancer cells (4.8-fold), HN5 head and neck squamous cell carcinoma cells (6.5-fold) and HT29 colon cancer cells (12.6-fold). Of importance, this derivative displayed in vivo activity in a subcutaneous model of HN5 tumor growth, thus providing additional evidence of violacein anticancer potential (Hashimi et al., 2015).

Collectively, these results highlight a key relevance of violacein antitumor activity, pointing out that there is still much research to be done to unravel the mechanisms involved in this effect. Additionally, they emphasize the importance of microbial secondary metabolites in anticancer drug development, with great opportunities of work in this field of research. In this respect, Menezes et al. (2013) described the cytotoxicity of crude extracts of five Brazilian isolates of *C. violaceum* sp. in several cell lines, including multidrug-resistant tumor cells. Besides other potential compounds with antitumor activity, chemical analysis of the metabolites identified violacein in all extracts (Menezes et al., 2013).

8. Potential industrial application of Violacein

The genome sequence of *C. violaceum* and also the knowledge of biosynthesis pathways poining to the important secondary metabolism, stress response and several metabolic intermediates provided important information related to the potential applications of this bacterium for many purposes, such as biotechnological and pharmaceutical applications (Kallmayer et al., 2007; Venil et al., 2015).

Much has been determined about the application of violacein over the last years, such as antibacterial and anti-Chagas, anti-cancer, antioxidant and leshmaniacidal uses, including its capability to induce apoptosis in colon cancer and leukemia cells. Besides all cited activities, violacein also showed anti-diarrheal and ulcer-protective effects, immunomodulator, analgesic and antipyretic agents in animal models could result in the development of new drugs in the future (Antonisamy et al., 2014). Violacein has other applications when it is biotransformed by basidiomycetes and bacteria. Studies demonstrated that 10% deoxyviolacein present in violacein showed activity against herpes and polioviruses, while at a higher concentration, it exhibited a weak inhibition of viral replication in HSV-1, *Poliovirus* type 2 and *Simian rotavirus* SA11 (Andrighetti-Frohner et al., 2003).

In the cosmetic industry, violacein is used as *trans*-hydroxy violacein, desoxyviolacein, deoxyviolacein or their derivatives, triacetylviolacein and diacetyl(di)methylviolacein and/or furan analogs, in combination with lipophilic and/or hydrophilic substances. Accordingly, cosmetics contain natural microbicides comprising violacein from *C. violaceum* in a medium containing salts, wool hydrolysate, anthranilic acid or containing yeast extract. Since violacein exhibits an important antibiotic activity against *S. aureus* and also an antioxidant effect on linoleic acid, a cosmetic lotion containing violacein has also been formulated (Kallmayer et al. 2007).

Another important area for commercial application of violacein is in commercial additives for solar protection of human skin, because of its

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antioxidant and antimicrobial activities. These characteristics were evident when violacein was used to supplement *Aloe vera* leaf extracts and *Cucumis sativus* (cucumber) fruit, which exhibit photoprotective activity. Violacein was also able to increase the sunscreen protection factors (SPF) of commercial sunscreens and some plants extracts. This pigment has potential for use as ingredient for a range of new products and represents a new paradigm for sunscreens based on biological materials. Violacein added to sunscreens was found to enhance UV-absorption in the range 290 to 320 nm, indicating that is able to increase SPFs when combined with other sunscreen ingredients. The information obtained from this evaluation indicates that violacein has a potential biotechnological role as a photoprotectant (Suryawanshi et al., 2015).

The textile industry also was interested in violacein application as it is a purple pigment. As example, we can point the lightfast dyed fibers that have been prepared by treating fibers dyed with violacein, deoxyviolacein, or mixtures of their aqueous solutions in the presence of thiourea, producing a woven silk fabric with bluish purple color (Durán et al., 2012).

Medical and epidemiological applications were widely discussed, but one of the goals for this pigment application is in the plant pathogens control, an important area for agricultural development. Violacein derived from *C. violaceum*, *Alteromonas luteoviolacea* or *Janthinobacterium lividum* can be applied for pest control. This pigment provides growth inhibition of plant pathogenic fungi and plant parasitic nematodes, thereby can be usefully used for the effective control of anthracnose, stem rot, pythium blight and damping-off of bean sprouts. Also, it is known that violacein has exhibited some interesting properties in insect prevention and antifungal activities. Therefore, an insecticide containing violacein or its derivatives was prepared showing effective prevention of plant mycosis, such as grass pythium blight, bean sprout seedling blight and sclerotinia stem rot and of plant parasitic nematode diseases such as watermelon *Meloidogyne* sp. diseases (Baek et al., 2007).

Bacterial pigments permit excellent opportunities due to their enhanced positive effects on the environment and superior performance characteristics. Violacein and other bacterial pigments offer benefits and advantages because of their broad range of activities. A study demonstrated that printed words could be highlighted using ink with violacein in the presence of citric acid and glycerol. This is a good indication that studies on pigments from bacteria need to be expanded to supply some industrial needs (Venil et al., 2013, 2015).

Other bluish-purple bacteria are capable of producing violacein, and one of them is *Janthiobacterium lividum*, which can produce violacein that is stable in acidic and alkaline solutions (pH 2–11) and upon heating in water bath for 1 h at dyeing temperature (55 °C), indicating the possibility of using it as a natural dye. Therefore, violacein pigment can be used to dye cotton fibers, providing good color tone and stability (Sawipak and Wichai, 2007).

Aranda et al. (2011) isolated a violacein producer from *Duganella* spp. associated with the rhizosphere (e.g., woody plants such as wild and cultivated olives) in Mediterranean environments. The yield of violacein was high, suggesting that *Duganella* could be useful in biological, medical, and industrial applications, since low violacein yields were always considered as one of the main limitations of extensive exploitation and violacein production. Another fact besides violacein production was that the pigmented strains from olives exhibited proteolytic and lipolytic activities and to a lesser extent siderophore production.

The use of synthetic pigments in the textile, cosmetic, food and pharmaceutical industries actually is not exploied more due to the toxicity problems. Nowdays, there is a great interest for using natural pigments for environmentally friendly consumer products (e.g., food, textile, and toy industries). However, high production costs often preclude the use of natural pigments on an industrial scale. But Aruldass et al. (2015) showed the feasibility for violacein cheap production using pineapple waste for growing the *C. violaceum* UTM5 either in a shake flask or in a 50 L bioreactor, thus introducing a low-cost growth medium that provide an excellent and scaled-up cultivation of violacein.

However, in many fields of industry, there is a great demand for natural blue dyes that are suitable for human digestion, which cannot be satisfied at present. It is worth to cite an invention and application of newly discovered marine sediment bacterium, the “Black Beauty” strain DSM 13623 of *Pseudoalteromonas* species in violacein synthesis. Compared to the conventional methods already described, this marine sediment bacterium showed a violacein yield that was about thirteenfold higher. Thus, an application of violacein in environmentally friendly consumer products, in particular in food, textile and toy industries (Tan et al., 2011) might soon be possible.

The Global Industry Analyst reported that demand for organic and natural colorants is expected to reach almost 10 million tons by 2017 (Venil et al., 2013). To compete with synthetic pigments, the development of bacterial strains using cheap and renewable substrates will be necessary. Recently, it was noted that there was a higher pigmentation of bacteria when there was increased UV radiation on the water surface. Also, protozoans have been found grazing in the presence of violacein, probably due to the putative antibiotic and/or antiviral activity of violacein.

Violacein can be used in a wide range of food industry products as a colorant in the food model systems, where yogurt and jelly have been selected to test the produced violet colorant. In the first food model system, powdered pigment was added to the chosen plain yogurt and sweetened yogurt, and color intensity increased in both samples. The samples were kept in the refrigerator at 4 °C for 30 days and aliquots were taken to evaluate color variation. Yogurt and jelly colored with this powder colorant produced foods of a vivid violet color and their color remained unchanged after one month of storage. These results are incentives for the development of a cost-effective natural colorant that appears as attractive to consumers (Venil et al., 2015).

9. Final remarks

The previous reviews and the current one clearly show that *C. violaceum* is an important bacterium in many aspects, such as a wellspring of knowledge in genetics and pathogenesis, and also as a producer of various secondary metabolites with different biological activities.

One of these important compounds is violacein, which appears to have important pharmacological activities for the treatment of many infectious diseases, such antiparasitic, antibiotic, antiviral, immunomodulatory, analgesic antipyretic and anticancer effects, where its greatest potential probably lies in the last cancer treatment. This review points out that violacein is potentially of economic importance for industrial uses, with a unique opportunity for applications in cosmetics, food (e.g., agroindustry) and fabrics.

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