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Chromobacterium violaceum: A Review of Pharmacological and Industiral Perspectives

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FOREWORD

This article presents the historical and actual importance of the *Chromobacterium violaceum* and focuses on the biotechnological and pharmacological importance of their metabolites. Although many groups in the world are working with this bacterium, very few reviews have been written in the last 40 years.[39,45,69]

ABSTRACT: Violet-pigmented bacteria, which have been described since the end of the 19th century, are occasionally the causative agent of septicemia and sometimes cause fatal infection in human and animals. Bacteria, producing violet colonies due to the production of a nondiffusible pigment violacein, were classified as a redefined genus *Chromobacterium. Chromobacterium violaceum* is Gram-negative, and saprophyte from soil and water is normally considered nonpathogenic to human, but is an opportunistic pathogen of extreme virulence for human and animals. The biosynthesis and biological activities of violacein and the diverse effects of this pigment have been studied. Besides violacein, *C. violaceum* produces other antibiotics, such as aerocyanidin and aerocavin, which exhibit *in vitro* activity against both Gram-negative and Gram-positive bacteria. 3,6-Dihydroxyindoxazene and Y-TO678h exhibit a selective activity against Gram-negative bacteria. Arphamenine A and B, and FR901228, that enhanced immunoresponse, and potentiators of β-lactam antibiotics and chelators such as ferrioxamine exhibit important clinical potential applications. Lipopolysaccharides and polyhydroxyesters together with several enzymes appear as important metabolites with biotechnological applications. Many of these metabolites were already studied at the genome level.

I. DISCOVERY AND CLASSIFICATION

In 1882 *Compt Rendues d' Academie du Science* published an article by Boisbaudran,[16] which described a violet coloration on rice flower preparations that the author attributed to a "small organism" referring to a microorganism. This observation was made around 1867, 15 years before the paper was published. This probably was due to a communication published by Gessard,[16] a contemporary of Boisbaudran, related to the blue material extraction from some pathological fluids, which motivated Boisbaudran to publish his observations.

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In an independent study in 1880, Bergonzini made an accidental discovery when he was working at Modena University in Italy. Near the end of March 1880, he was preparing ovoalbumin solutions to study the "action mechanisms that cause the retarding of putrefaction". After the experiments, he forgot to eliminate the control solutions and at the end of April he observed a singular aspect of the samples, which he described as fantastic. The Italian researcher reduced the volume by evaporation and obtained a solution covered by a fine, very dense film of violet color. Initially, he suspected *Cromococcus violaceus,* which was the only bacterium known that exhibited this coloration; however, this possibility was eliminated due to the insolubility of all components. Bergonzini after some tests concluded that this was a new bacterium that he named *Cromobacterium violaceum* (without ch), and published in 1881, the discovery of a "new colored bacterium".[12]

With respect to the "small organisms" and the pigment to which Boisbaudran mentioned, De Moss,[39] reported that they probably were *Chromobacterium violaceum* and violacein, respectively, based on the visible absorption spectra registered by Boisbaudran. In 1881, Zimmerman corrected the spelling of *Cromobacterium*, given by Bergonzini, to *Chromobacterium*.[9,18,186] Around that time, the name *Bacillus violaceus* was no longer used.[171] Today, the *Chromobacterium* genera, described by Sneath, is that found in Bergey's Manual of Systematic Bacteriology.[80]

In 1976, two different bacteria colonies: white and a violet were isolated in a water treatment station in Manaus city (Amazonas-Brazil). Prof. Wilson Chagas de Araujo, at Microbiology Institute of Universidade Federal de Rio De Janeiro, R.J., BRAZIL, identified the violet one as *Chromobacterium violaceum*. This it was the first time that this microorganism was isolated in Brazil.[21-23] The suspicion that violacein should be a solar protector for the bacterium was reported by Prof. R. Caldas (UFRJ), who originated a series of studies that demonstrated the photo therapeutic potential of violacein.[52]

II. PATHOGENICITY OF *CHROMOBACTERIUM VIOLACEUM*

C. violaceum is defined as a saprophyte bacteria, which is normally considered to be nonpathogenic for humans, and is a Gram-negative and facultative anaerobe found in soil and water samples of tropical and subtropical areas of several continents. Occasionally, it can act as an opportunist pathogen for animals and humans and cause fatal septicemia from skin lesions with many liver and lung abscesses.[121,146,147] Albeit rare, its potential pathogenicity was first described by Wooley in 1905,[182] who identified the organism as a cause of septicemia in water buffaloes in the Philippines.[80,139,182]

Some serious and in some cases fatal infections in humans were reported in Argentina, Australia, Brazil, Cuba, Nigeria, Singapora, Taiwan, United States, and Vietnam.[9,13,14,26,61,65,68,75,79,89,91,92,102,112,113,129,131,138,139,152,164-167,170,181] *C. violaceum* can also cause infections in animals and there are reported cases in pigs,[99,108,161,180] monkeys,[3,32,72,98,99,120] sheep,[24,173] and dogs.[70]

Petrillo et al.[138] pointed out that although *C. violaceum* is frequently found in nature and of causes serious infections in humans, the incidence is very low. Fourteen years later, Midadi and Rathore[121] reached practically the same conclusion. After an analysis of infected individuals, diagnosed in the United States, Petrillo et al.[138] stated that *C. violaceum* is a low-degree pathogenic agent capable of causing severe infections mainly in immunosuppressed patients. However, in agreement with recent studies, this generali-

zation is questionable. Bilton and Johnson[14] described a severe infection with *C. violaceum* in a 12-year-old child that did not present imunodepression.

There are also reports of chronic granulomatosis associated with *C. violaceum*[110,111] of adenite as a complication of chronic granulomatosis[166] and problems associated with *C. violaceum* in osteomielitis[172], cellulitis periorbital,[159] and in ocular infection.[62] All the cases mentioned up to now involve a pigmented bacteria, but there are also cases of infections originated by a nonpigmented strain (deficient in violacein synthesis).[122,162] This implies that pathogenicity of this bacterium is independent of violacein.

Comparisons of *in vitro* activity of Ciprofloxacin and 24 other antimicrobial were studied against clinical strains of C. *violaceum.*[1] Ciprofloxacin was the most active of the compounds tested, although norfloxacin and perfoxacin were highly active. No resistance was detected to meziocillin, piperacillin, apalcillin, imipenem, and aztreonam, while a single strain was resistant to ticarcillin. Among the cephalosporin/cephamycin group, only cefotetan showed good *in vitro* activity. Gentamicin was more active than amikacin and tobramycin. Good *in vitro* activity was also noted for chloramphennicol, doxycycline, and trimethoprim-sulfamethoxazole, while *C. violaceum* strain were highly resistant to rifampin and vancomycin. A high correlation was noticed among the tests between microdilution culture and disk diffusion susceptibility tests in predicting the susceptibility patterns of C. *violaceum.*

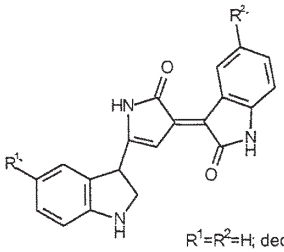
III. PIGMENTS, METABOLITES, AND ENZYMES

As mentioned since 1867, the color of the pigment produced by one of the strains of *C. violaceum*, the violacein, is a great attraction. Reilly and Pyne[140] were the first to study the chemical structure of the violet pigment in detail, starting from the beginning of the decade of 1930. Since that time, several proposals for the chemical structures of violacein appeared, which were all proven incorrect later. It was at the University of Liverpool in 1958, through degradation studies and by synthesis, that correct the structure of this compound was deduced (Figure1).[7,8]

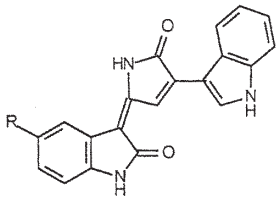
Detailed spectral studies of violacein were later accomplished.[100,148-151] In 1934, the studies on biosynthesis of the violacein began. Tobie[171] observed that when *C. violaceum* cultures were oxygenated, the violacein production was significantly reduced, which indicates the importance of oxygen in violacein synthesis.

At the end of the decade of 1950, De Moss and Evans[40] discovered that to synthesize violacein, the bacteria needed molecular oxygen and L-tryptophan. They also demonstrated that the synthesis does not start from D-tryptophan or from L-tryptophan in the absence of oxygen.[40] Using ^{14}C-labeled L-tryptophan (with in different positions, the two researchers showed that L-tryptophan was incorporated into violacein, except for the carboxylic carbon (C1), which is probably eliminated by a decarboxylation process during the biosynthesis (Figure 2).[41]

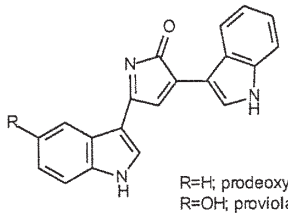
In 1986, using [2-^{13}C] and [3-^{13}C] labeled-tryptophan Hoshino et al.[82] discovered that the carbons of 2-pyrrolidone group of violacein were coming of the lateral chain of L-tryptophan (C1, C2, C3). Furthermore, they discovered the origin of O, N, and H of the 2-pyrrolidone group using the isotopes ^{18}O, ^{15}N, and ^{2}H.[83] Also in that period, intermediate compositions were proposed in the biosynthesis such as the chromopyrrolic acid,[84] proviolacein, prodeoxiviolacein, and pseudoviolacein,[85] and intramolecular rearrangements of the indolic ring on the 5-hydroxyindole side was observed.[123] Feeding experiments with the mixture of [2-^{13}C] - and [nature-3-^{13}C]-tryptophan (91:1 molar



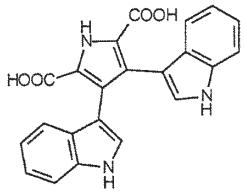
$R^1=R^2=H$; deoxyviolacein
$R^1=O$, $R^2=H$; violacein
$R^1=R^2=OH$; oxyviolacen



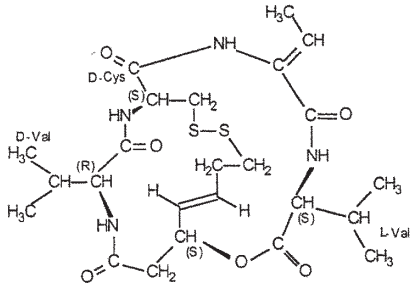
R=H; pseudodeoxyviolacein
R=OH; pseudoviolacein



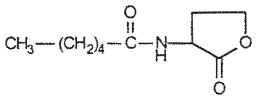
R=H; prodeoxyviolacein ≡ HS-1
R=OH; proviolacein



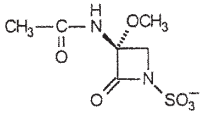
Chromopyrrolic acid



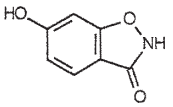
struture FR901228



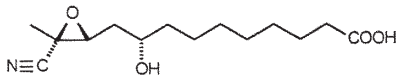
N-hexanoyl homoserine lactone



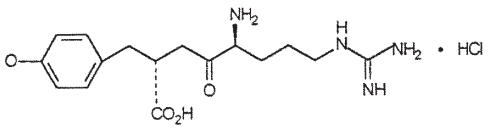
monobactam



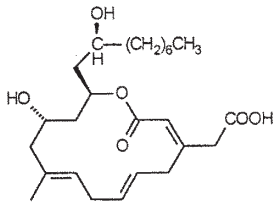
3,6 dihydroxyindoxazene



aerocianidine



arphamenine B



aerocavin

FIGURE 1. *Chromobacterium violaceum* metabolites.

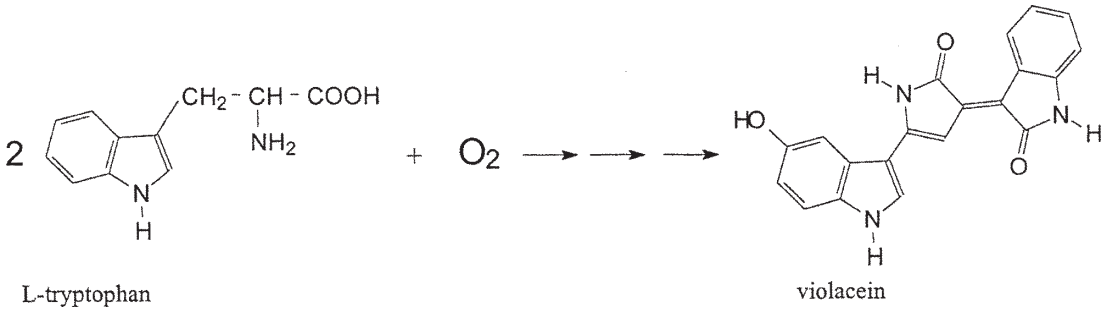


FIGURE 2. Violacein biosynthesis.

ratio) and other experiments have showed that the 1,2-shift of the nature ring occurred through an intramolecular process during the formation of the left part (5-hydroxyindole side) of the violacein skeleton and demonstrated that the C-C bond from C2 of the nature ring to C2 of the side chain was completely retained during formation of the right part (oxindole side) during the entire biosynthetic process (Figure 3).[123]

In the year 1989, studies began on other important parameters in the biosynthesis of violacein.[149] Durán et al.,[46] in a study with radioisotope, suggested that besides L-tryptophan, *C. violaceum* is capable of synthesizing violacein starting from indol-3-acetic acid as metabolite precursory of L-tryptophan, and proposed an amidic intermediate.[2] In that study, the production method and purification of violacein free deoxyviolacein were defined.[142-145]

Other studies revealed that *C. violaceum* presents a group of enzymes capable of synthesizing compounds structurally similar to violacein, as oxyviolacein, HS-1 (prodeoxyviolacein), and pseudodeoxyviolacein (Figure 1), starting from substrates other than L-tryptophan.[86-88,124] Acetylated *N*-(homoserine) lactones, that induce violacein production, have been described as genetic regulators in high-density populations in a variety of Gram-negative bacterial systems and is called *Quorum sensing*.[6,119] In *C. violaceum*, a number of phenotypic characteristics, including production of violacein, hydrogen cyanide, antibiotics, and exoproteases, are known to be regulated by the endogenous *N*-hexanoyl-L-homoserine lactone. Studies with mutants that do not produce violacein proved that the production of these compounds suggests that the *Quorum sensing* is a genetic regulatory mechanism in most Gram-negative bacteria.[25] The violacein production is specifically induced by *N*-hexanoyl homoserine lactone, and its extraction from *C. violaceum* allows a quantitative bioassay for the lactone.[15]

Studies have confirmed the importance of the *C. violaceum* metabolites. Some are of great biological interest, such as the ferrioxamine E, a growth accelerator of structure related to antibiotic of the sideromycine type,[66,125] which promotes growth of a great number among a variety of organisms, including bacilli, cocci, yeast, fungi and algae. Antibiotic potentiators such as β-lactamic glycopeptides SQ28,504 and SQ28,546,[33,34] which are glycopeptides that act specifically with β-lactamic antibiotics against Gram-negative bacteria, were described. Antibiotics such as aerocyanidin[134] bearing an isonitrile group and aerocavin[107,160] exhibit *in vitro* activity against both Gram-negative and Gram-positive bacteria. 3,6-Dihydroxy-indoxazene (also called Y-TO678H or 6-hydroxy-3-oxo-1,2-benzisoxazole)[73,183] inhibits growth of Gram-negative bacteria. Monobactam SB-26.180,[31,179] which after its discovery has opened up a new era of antibiotic research with important aspects of *C. violaceum* metabolites. An antitumoral depsipeptide,

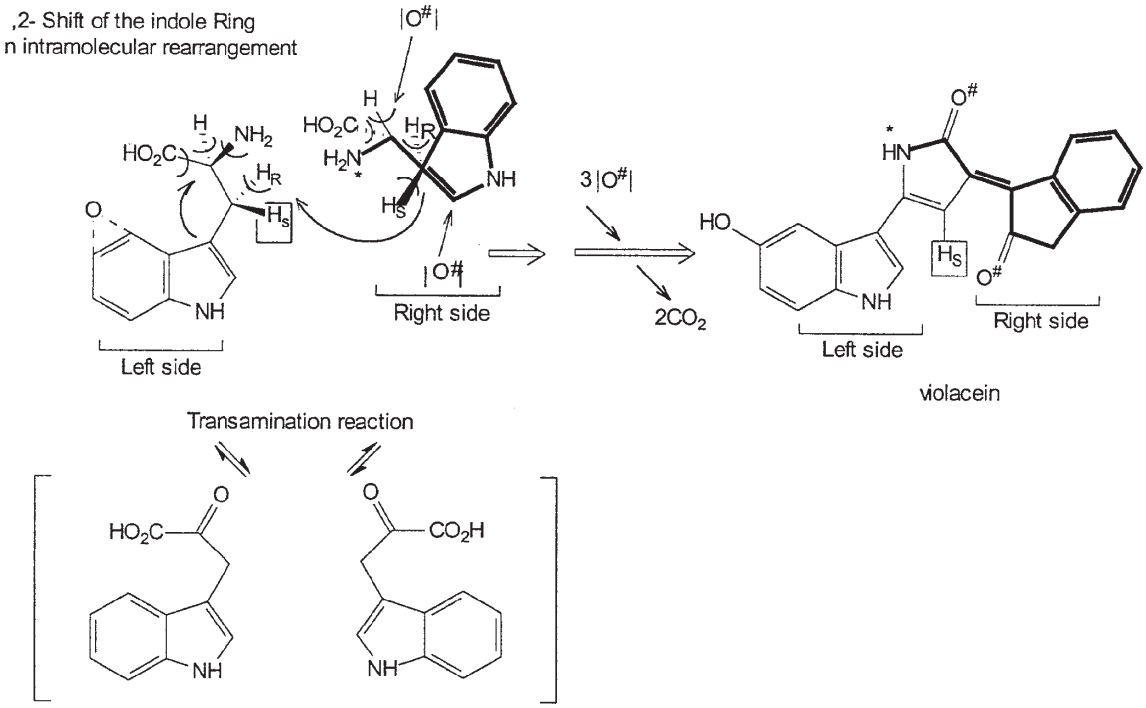


FIGURE 3. Summarized scheme for Violacein biosynthesis. (Modified from Ref. 123.)

FR901228,[155,174,175,104] which was an antibiotic and antitumoral extracted from *C. violaceum* WB968 strain[64,78] was tested against *Schizosaccharomyces pombe, Aureobasidium pollulans,* and *Aspergillus niger* with toxic effects. This compound also showed high cytotoxicity for human tumor cell lines A549 (lung cancer), MCF-7 (breast cancer), and SW 480 (colon cancer), and murine leukemia. The antitumoral activity indicated that FR901228 is a new inhibitor of histone deacetylase and has a different structure than known inhibitors as trichostatins and trapoxins.[127] This metabolite shown a great therapeutic potential,[20,30] when compared with the trichostatin, a specific inhibitor of histone acetylation, and is a selective agent against chronic leukemia of the lymphocytic cells in clinical assays.[19,126,153,154] A carboxypeptidase and aminopeptidase inhibitors, as intermediates of the analgesic arphamenine B, were isolated.[130,185] A carboxypeptidase and aminopeptidase inhibitors, as intermediates of the analgesic arphamenine B, were isolated from *C. violaceum*.[130,185]

C. violaceum has also been used in studies on the production of unusual sugar compounds such as extracellular polysaccharides.[69] The tough membranous aspect of some *C. violaceum* colonies is the result of the latter proposed saccharide. Their role in the formation of soil aggregates has been suggested.[69] More recently, the studies of the O-specific polysaccharides of lipopolysaccharides from *C. violaceum* were reported.[176] In an early investigation, the presence of D-glycero-D-galacto-heptose, together with L-rhamnose and D-glucosamine in *C. violaceum* was reported. Later, the lipopolysaccharides (LPS) was isolated and its lipidA moiety structurally characterized. The latter report[176] proved that D-glycero-D-galacto-heptose was a constituent of this LPS region.

The homopolyesters produced by the bacteria could be used in the formulation of biodegradable polymers and in view of their qualities[158] may be important as a source of biodegradable plastics from renewable sources (e.g., BIO-POL).[158] In the bacterial

growth, 70% of the dried biomass (40 g per liter of fermentation) corresponded to the polymer (3-hydroxyvaleric acid).[106, 118,168,169]

Some enzymes from *C. violaceum* were also isolated and characterized. For instance, a cell-free extract from a *C. violaceum* strain that vigorously produces cyanide and converts into γ-cyano-α-aminobutyric acid has been shown to catalyze the synthesis of the cyanoamino acids. An enzyme γ-cyano-α-aminobutyric acid synthase has been purified from the extract and a possible intermediate for this reaction, γ-thiocyano-α-aminobutyric acid, a sulfur amino acid has been synthesized. Thus, this enzyme catalyzes the synthesis of γ-cyano-α-aminobutyric and thiocyanate.[141] Tryptophan hydroxylase that functions in *C. violaceum* and produces a precursor for the characteristic pigment produced by this species, violacein. The enzyme is inducible to a greater extension by phenylalanine than by tryptophan. The hydroxylation requires O_2 and a reduced pteridine, and furthermore requires the addition of a sulfydryl compound.[103] A strain of *C. violaceum* isolated from a fatally infected patient produced a β-lactamase. Production of this β-lactamase appeared to be mediated for chromosomal genes.[60] *C. violaceum* forms cyanide as a secondary metabolite with glycine as the precursor and methionine as a stimulator. Since the metabolism of glycine and methionine are interrelated by the provision of C1 units for the tetrahydrofolate pool by the activities of serine hydroxymethyl transferase and glycine lyase, the regulation of function and activity of these enzymes were studied. Results in that study suggested that nearly all the C1 units for the tetrahydrofolate pool were supplied by the activity of serine hydromethyl transferase, which could lead to an excess formation of glycine and may explain the role of cyanogenesis. [157] Cyanide is formed and rapidly lost from the medium, and β-cyano-alanine is formed from L-cysteine by the β-cyano-alanine synthetase. Inhibition studies suggested that this enzyme was typical of pyridoxal phosphate-containing enzyme.[109] L-Tryptophan-2', 3'-oxidase,[67] which is an amino acid α,β-dehydrogenase isolated from *C. violaceum*, catalyzes the formation of a double bond between the Cα and Cβ carbons of various tryptophan derivatives provided that they posses a L-enantiomeric configuration, an α-carbonyl group, and an unsubstituted and unmodified indole nucleous. The stereochemistry of the dehydro product was determined to be a Z-configuration from H-NMR assigment.[67] A novel approach to the synthesis of deuterium- and tritium-labeled peptides through the catalytic asymmetric reduction of (Z)- α, β-dehydrotryptophan (DzTrp)-containing peptides, using rhodium complexes with chiral diphosphine ligands as the catalysts, was described. Ac-DzTrp-L-Phe-OMe is used as a model substrate to study this new route. The dehydropeptide was produced by L-tryptophan 2',3'-oxidase from *C. violaceum* in a single-step reaction. Diastereomeric excesses up to 98% have been obtained with (R,R)-dipamp as ligand in the catalyst. Extremely high stereoselectivities for producing the L,L- or D,L-isomer could be achieved by using the appropriate chiral ligands. This method has good potential for stereospecific deuteration or tritiation of peptides.[74] Indole oxygenase, a redox flavoprotein from *C. violaceum*, has been purified and crystallized in the presence and absence of NADH.[27] It is known that *C. violaceum* produces a set of chitinolytic enzymes (chitinase[29]) whose production is regulated by *N*-hexanoyl-L-homoserine lactone. A pleiotropic mini-Tn5 mutant of *C. violaceum* that is defective in lactone production and other quorum sensing-regulated factors was also found to be completely deficient in chitinase activity.[29] Essential amino acids involved in the catalytic role of the intracellular cytosine deaminase from *C. violaceum* were determined by

chemical modification studies. The results suggested that cysteine and methionine residues may be located in or near the active site of the enzyme, while tryptophan, histidine, and serine residues may be indirectly involved in the enzyme activity.[93,183] At present, these studies have been shown a significant diversity of enzymes, although not yet exhaustively studied, except for phenylalanine hydroxylase,[28,128] which is also called phenylalanine 4-monooxygenase.[63,136] A gene encoding phenylalanine hydroxylase has been cloned from *C. violaceum* and expressed in *E. coli*. The iron-oxy species was postulated to react with phenylalanine in the hydroxylation process.[28]

IV. BIOLOGICAL ACTIVITIES OF *CHROMOBACTERIUM VIOLACEUM* METABOLITES

Researchers such as Kidder and Stuart, in 1939, and Burbank, in 1942, reported, according to De Moss,[39] their observations on ciliated protozoa that died quickly when exposed to cultures of *C. violaceum.* At first, violacein was suspected to be responsible for this phenomenon, but later they believed that cyanide, a metabolite usually formed by *C. violaceum* in common media, was responsible.

According to Lichstein and Van de Sand,[105] Singh reported in 1942 that when crude extract of violacein was added to bacterial suspensions, these microorganisms were not ingested by terrestrial amebas. At the same time, deaths by septicemia due to *C. violaceum,* were occurring. In all the cases, no other bacteria were noticed in the infection region other than *C. violaceum* indicating a possible antibiotic activity of violacein. Lichstein and Van de Sand[105] made tests with 51 bacteria strains, totaling 21 species, and they showed that the violacein possessed outstanding inhibitory effect on Gram-positive bacteria growth and a small effect on the Gram-negative ones.[39,105] Subsequent studies by Durán et al.[45,50] on antibacterial activities with purified violacein showed that violacein is equally efficient against the two groups. Violacein displayed an antimicrobial activity against phytopathogenic fungi like *Rosellinia necatrix*, which cause white root rot of mulberry and it could also be used as a fungicide.[156]

The violacein exhibited *in vitro* antimycobacterial activity against *Mycobacterium tuberculosis* (H37Ra) with a minimum inhibitory concentration (MIC) of 64 μg/mL and minimum bactericidal concentration (MBC) of 128 μg/mL. Those values are comparable to those described in the literature for pyrazinamide, chemotherapeutic used in tuberculosis.[42,59]

Since that time, in addition to the bactericidal activities, other papers appeared describing violacein trypanocidal activity.[22,23,51,53,58,115] Violacein was active against *Trypanosoma cruzi* (Tulahuen strain, Chile) and the Y strain (Brazilian), and the latter was shown to be three times more resistant than Tulahuen. When albino mice (18 to 20 g) were inoculated intraperitoneally with 5×10^4 trypomastigotes of *T. cruzi* strain Y and then injected for 7 days with 100 mg/kg of the violacein and derivatives, only 4% reduction of the parasitemia was obtained. Unfortunately, that result reveals a low trypanosomal activity *in vivo* of violacein and their derivatives.[77]

The toxicity and cytotoxicity studies of violacein, with the fibroblast V79 cell lines,[77,15,116] following the methodology of MTT reduction, nucleic acid content, and uptake of neutral red, showed a high cytotoxic activity, which was an induction to study the potential use of this compound the antitumoral in collaboration with the National Cancer Institute at the United States. The obtained results were excellent in cultures of leukemia cells, lymphoma, lung, and colon

(GI50 in the region of 10^{-8} *M*),[54,117] and in lymphoma related to the AIDS.[56,57] The cytotoxicity of violacein in fibroblast V79 in the region of IC_{50} in the range of 5 to 12 μ*M* was evaluated that occurred through apoptosis and not necrosis, as demonstrated by the method of Tunnel and the Feulgen reaction coupled to the image analysis. The morphologic changes observed in the nucleus of those cells include chromatins condensation and a decrease of the content of nucleic acids.[117] Similar results for the apoptotic cytotoxic effect of the inhibitor of histone diacetylase FR901228 from *C. violaceum* on malignant lymphoid cells was published recently.[126]

The *in vitro* effect of a potent histone deacetylase inhibitor on human leukemia/lymphoma cells and cell lines was studied by comparison with normal hematopoietic cells. This novel agent may be useful in the treatment of lymphoid malignances, because the concentration used in the order of nanomoles are clinically achievable *in vivo* according to a recent clinical study.[126] Attempts to increase the solubility and increase of the biological activity using violacein inclusion complexes with β-cyclodextrin or sugar derivatives were published[35,36,48] and these studies revested antiulcerogenic and antioxidant activities.[37,38] Another strategy that is being persued is to synthesize derived of violacein with glycosidic groups[49,55] or through biotransformations of the violacein by oxidatives enzymes.[17] A preliminary test showed that violacein biotransformed with peroxidase exhibited fourfold less cytotoxicity to Chinese hamster V79 cell at 20 μ*M* concentration than violacein itself.[47]

Violacein (with 10% de deoxyviolacein) also presented activity against with Herpes Simplex Virus (HSV) and Polioviruses after infection of HeLa cells.[114] In the region of 0.25 μg/mL, violacein inhibited 62% of the HSV and in 0.063 μg/mL, inhibited 56% of poliovirus-infected HeLa cells. These results indicate antiviral activity for this compound.

The biotechnological potential of *C. violaceum* has not been studied in all its extension, but indications exist that this area deserves more profound studies.

Besides the interesting biological aspects of the bacteria, its capacity to hydrolyze plastic films of cellulose, probably due to its hydrolases action, was described.[71] More complex processes were also published, such as the denitrification[10] and solubilization of gold.[163] In the latter process, the solubilization reaches 215 parts per million of gold from 1000 parts per million of pure metal after 40 days of incubation. It is believed that the cyanide, produced by enzymatic processes, is one of the elements responsible for the extraction of gold. Apparently, this method would avoid the use of mercury and the consequent environmental contamination. Recently, it was found that 53% of the gold could be extracted from materials of low gold content.[101] Patents now exist for this application.[94]

V. GENETIC ASPECTS OF *CHROMOBACTERIUM VIOLACEUM*

With the announcement of the project for the sequencing of the complete genome of *C. violaceum* by a of Brazilian Laboratories Consortium, Brazil is included among the groups that accomplish genetic cloning and sequencing of this bacterial DNA. In fact, some regions of the *C. violaceum* genome, with related biotechnological interest, have been cloned and sequenced since 1989. Among the growing list are the genes involved in the violacein biosynthesis,[4,5,81,137] the phenylalanine-4-hydroxylase (phenylalanine-4-monooxygenase), [11,13,28,133,177,178] *orfD* (a *SoxR* homolog),[96] the acid polyhydroxyalkylic acid synthase,[90,95] and the related 3-ketothiolase,[95,97] and sequences of the DNA encoding for the ribosomal RNAs 16S and 23 S.[43,44,76,173] The cloning of entire genomic fragments of those targeted genes accidentally carried untargeted interesting

ones, among them part of a putative isomerase[97] and a putative hemolysin,[137] which were first annotated as unknown proteins.

It is interesting to point out that in those studies there are already experiments that can be considered post-genomic, including with data concerning the functional analysis of such proteins. In biotechnological terms, the knowledge of the gene sequence allows one to unmask the stages involved in the metabolic processes and to guide eventual improvements in the metabolites productivity. This knowledge also opens the possibility for designing new pharmaceutical or inhibitors of biochemical processes proper to control pathogenicity. Obviously, due to the commercial interest of those scientific data, the scientific information needs to be protected by patents. This has already been applied to the sequence data described for *C. violaceum* for almost all the metabolites with biotechnological or pharmaceutical importance related to this bacterium.

The violacein biosynthesis pathway is actually in an advanced state of analysis. The cloning of such genes in *E.coli* was performed with a clever and simple strategy.[132] The heterologous expression of these genes in *E.coli* results the production of the violet pigment, which facilitates the selection work, as the bacterial colonies stain violet naturally. The violacein biosynthesis proteins of *C. violaceum* are codified by one cluster of only four genes, denominated *vioABCD*, found in a fragment of DNA of about 8 kbp, probably arranged in a single operon. The use of such DNA fragment resulted in the production of high amounts of violacein in *E.coli* and in several other Gram-negative bacteria.[4,5,81,137]

There is still much to be discovered related to the sequence of reactions and the genetic correlation of the processes that result in the synthesis of that pigment. The analysis of the putative proteins obtained from the gene sequence indicates that *vioA*, *vioC*, and *vioD* codify for monooxygenases dependent of nucleotides (FAD). Moreover, VioA protein belongs to the family of amino oxidases and VioC is very similar to a human protein reported to be a kynurenine monooxygenase. Transposon mutagenesis resulted in a number of bacterial colonies with different phenotypes that varied from the absence of the pigment production (white colonies) to the formation of green or blue pigments (and the same colony coloration). These phenotypes depend on the position of transposon inserts: the inactivation of the *vioA* or *vioB* gene completely block the formation of the pigment, while *vioC* or *vioD* inactivation results in formation of violacein precursors.[4] Figure 4 illustrates a hypothetical route for violacein biosynthesis from tryptophan by evaluating the homology of the four genes required for violacein biosynthesis and the intermediates produced from mutant genes. The symbol (*) indicates incorporation pattern with stable ^{13}C into violacein from tryptophan.[4] Another ORF is found close to this pathway that was accidentally cloned in the same fragment. Curiously, the analysis of such sequence reveals it is related to beta hemolysin by similarity with homogous genes from other bacteria. This protein has a phospholypase-C activity and is in fact an exotoxin, potentially related to *C. violaceum* pathogenicity, thus deserving further investigation.

The gene of the phenylanine hydroxylase (PHA; phenylalanine 4-monooxygenase) of *C. violaceum* was also entirely sequenced and expressed in *E. coli*.[11,13,133] Surprisingly, the deduced protein of the sequence of DNA presents high similarity to mammals liver enzymes, which indicates a high degree of conservation of these genes. However, PHA of *C. violaceum* is a monomeric enzyme that contains one copper mol^{-1} in the active site, while the mammalian enzymes are active as a tetramer and contain one iron/subunit mol^{-1}.[177,178] However, Chen and Frey[28] described a dif-

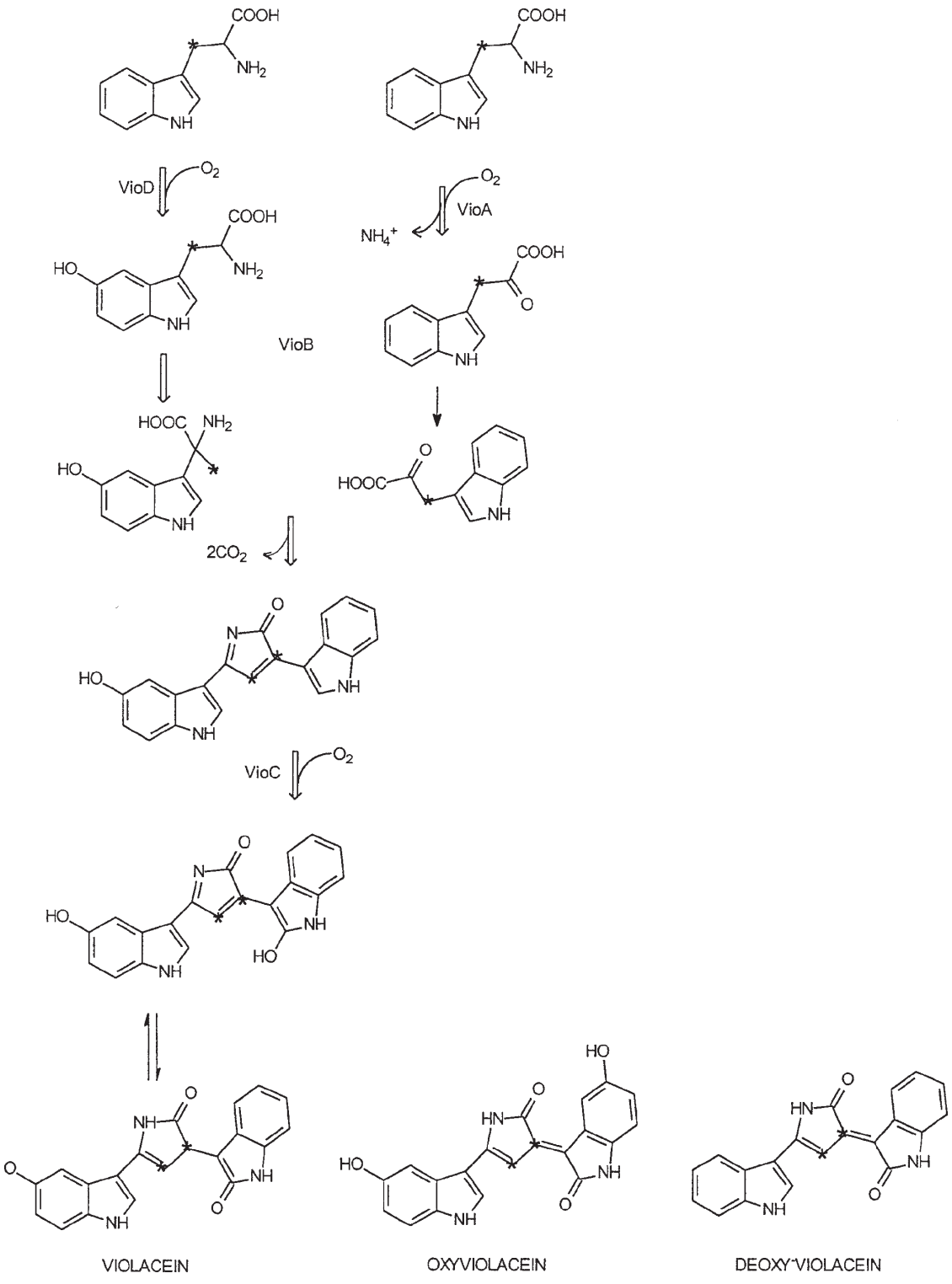


FIGURE 4. Hypothetical route for violacein biosynthesis. (Modified from Ref. 4.)

ferent gene encoding for a phenylalanine hydroxylase, compared with that described previously by Onishi et al.[133] indicating that *C. violaceum* may carry two different phenylalanine hydroxylases in its genome, which remains to be confirmed.

The polyhydroxylalkylic acids are carbon and energy resources produced in some bacteria, when the sources of nutrients are limited. Some of those polyacids and their polyesters have similar physical properties to those of polypropylenes and may become important as a source of biodegradable plastics from renewable sources (for instance, the BIO-POL).[158] Recently, the first detailed work of the gene cloning that code for polyhydroxyalkanoate synthase ($phaC_{CV}$) and to 3-ketothiolase ($phaA_{CV}$) from C. *violaceum* was reported. Both genes are arranged in a single operon, possibly responsible for the poly(3-hydroxybutyrate) or poly(3-hydroxybutyrate-co-hydroxyvalerate) accumulation in that bacterium.[97] The heterologous expression of that operon in *E. coli* allows the efficient production of those complex polyacids. Recently, a strain of *E. coli* was built containing this operon inserted in its chromosome, resulting in the formation of homogeneous product and highly efficient: 82% of the polyacid by weight.[91]

Recently, a fragment of DNA from *C. violaceum* was cloned and found to contain a ORF homolog to the transcriptional activator *soxR*, which is involved in the induction of several genes related to oxidative damage protection pathways in several bacteria.[96]

Finally, the 16S ribosomal RNA gene was sequenced totally 12 years ago,[44] and recently the 23S rDNA was also sequenced.[76] These genes are very important in the determination of the correct phylogeny and the taxonomic group of *C.violaceum*, confirming its classification as a beta proteobacteria, from the Neisseriaceae family.

VI. CONCLUSION AND FUTURE PROSPECTS

The genome of *C. violaceum* is relatively small (estimated as approximately three million base pairs) and one foresees the success of the Brazilian Laboratories Consortium for the complete sequence of its genome. The information to be generated should reveal many aspects of biotechnological interest of this bacterium. An important aspect, with promising perspectives in areas still not explored, is the biosynthesis of antibiotics isolated from *C. violaceum* (aerocianidin, aerocavin, and others mentioned above) in addition to the antitumoral depsipeptide FR901228, which is profiled with great potential in the treatment of cancer, but the genes responsible for its synthesis are still not identified. These studies can also help to understand the pathogenicity mechanisms of this bacterium, and, eventually, the knowledge of the genome can reveal the best infection control of this bacterium. Another interesting aspect, but with a more academic vision, is that this bacterium has been found free in the environment. The contact with other species of bacteria may have resulted in genetic transfers among them. Hence, the genetic code of *C. violaceum* may reveal aspects not only of the evolutionary history of this free-living bacterium as a whole, but also the specific evolution of its genes. Finally, the significant number of patents is a suggestive point of the importance of biotechnological, industrial, and clinical importance of *C. violaceum* metabolites.

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