Online journal ISSN - 1676-5680
GMR Genetics and Molecular Research
EVOLUTION AND TECHNOLOGY

Genetic analysis of violacein biosynthesis by *Chromobacterium violaceum*

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Genet. Mol. Res. 3 (1): 85-91 (2004)
Received October 13, 2003
Accepted January 12, 2004
Published March 31, 2004

ABSTRACT. *Chromobacterium violaceum* presents a distinctive phenotypic characteristic, the production of a deep violet pigment named violacein. Although the physiological function of this pigment is not well understood, the sequencing of the genome of this bacterium has given some insight into the mechanisms and control of violacein production. It was found that erythrose-4-phosphate (E4P), a precursor to aromatic amino acid biosynthesis, is produced by the non-oxidative portion of the hexose monophosphate pathway, since it lacks 6-phosphogluconate dehydrogenase. All genes leading from E4P plus phosphoenolpyruvate to tryptophan are present in the genome. Nevertheless, these genes are not organized in an operon, as in *E. coli*, indicating that other mechanisms are involved in expression. The sequencing data also indicated the presence and organization of an operon for violacein biosynthesis. Three of the four gene products of this operon presented similarity with nucleotide-dependent monooxygenases and one with a limiting enzyme polyketide synthase. As previously suggested, genes encoding proteins involved in *quorum* sensing control by N-hexanoyl-homoserine-lactone, an autoinducer signal molecule, are present in the bacterial genome.

These data should help guide strategies to increase violacein biosynthesis, a potentially useful molecule.

Key words: *Chromobacterium violaceum*, Tryptophan metabolism, Polyketide synthase, Tryptophan 2-monooxygenase, Violacein, N-acyl-homoserine synthase

INTRODUCTION

Since 1882, when *Chromobacterium violaceum* was first reported as an isolate from wet rice paste, its most notable characteristic has been the production of a deep violet pigment named violacein (Boisbaudran, 1882). The biological role of violacein in *C. violaceum*, as well as its biosynthesis pathway, has been under study since then. In early studies an increase in respiratory activity was observed when violacein extract was added to a non-pigmented cell suspension of *C. violaceum*, suggesting that violacein is a respiratory pigment (Friedheim, 1936). It was also suggested that violacein production is involved in the regulation of tryptophan production, which at high concentrations is toxic to bacteria (DeMoss, 1967). However, when grown on complex, complete medium, pigment production stops, showing that violacein is not required for *C. violaceum* growth and survival (Sivendra and Lo, 1975; Durán and Faljoni-Alario, 1980). The low solubility in water and the high molar extinction coefficient in methanol ($\varepsilon = 1.7 \times 10^4$ l mol^{-1} cm^{-1}, $\lambda = 577$ nm) lead us to suppose that violacein is involved in protection against visible radiation, since this bacterium is widely found in the water and soil of tropical and subtropical areas of the world. Although *C. violaceum* is able to grow under both aerobic and anaerobic conditions, violacein production occurs only in the presence of oxygen (DeMoss and Evans, 1959).

DeMoss and Evans (1959) reported that in addition to violacein, *C. violaceum* produces a less abundant pigment named deoxyviolacein (DeMoss and Evans, 1959). The elucidation of the structure of violacein (3-[1,2-dihydro-5-(5-hydroxy-1H-indol-3-yl)-2-oxo-3H-pyrrol-3-ylidene]-1,3-dihydro-2H-indol-2-one) began in 1958 through degradation and re-synthesis reactions of the compound (Ballantine et al., 1958). The data were confirmed by spectroscopic analysis in 1984 (Laatsch and Thomson, 1984).

Several efforts have been made to determine the violacein biosynthesis pathway through studies on the role of tryptophan and other indol derivatives in the stimulation of violacein biosynthesis (DeMoss and Evans, 1960; Hoshino et al., 1987a,b; Hoshino and Ogasawara, 1990; Durán et al., 1994). Momen and Hoshino (2000) grew the bacteria on a mixture of [2-^{12}C], [indole-3-^{13}C] and [indole-2-^{13}C] tryptophan, and found that all the carbon, hydrogen and nitrogen atoms of the violacein molecule come from tryptophan molecules. The hydroxylation of tryptophan with the production of an intermediate 5-hydroxytryptophan during the first steps of violacein biosynthesis suggested that molecular oxygen participates in an oxidation reaction in this synthesis, and confirmed that aerobic conditions are necessary for pigment production (Hoshino et al., 1987a,b; Hoshino and Ogasawara, 1990; Durán et al., 1994; Momen and Hoshino, 2000). The decarboxylation of one of the tryptophan molecules should occur later in the metabolic pathway, to yield violacein and related compounds (Durán et al., 1994; Momen and Hoshino, 2000).

Since tryptophan appears to be the only precursor molecule in violacein biosynthesis, its production is apparently essential for pigment production in *C. violaceum*. The recently published sequencing data have confirmed several of the functional features of *C. violaceum* metabolism (Vasconcelos et al., 2003).

RESULTS AND DISCUSSION

Aromatic amino acid biosynthesis starts with erythrose-4-phosphate (E4P), an intermediate hexose monophosphate pathway (HMP), with phosphoenolpyruvate, a glycolysis pathway intermediate, leading through several steps to the production of chorismate, which can be directed to a branch that starts with anthranilate, leading to a tryptophan pathway. Surprisingly the HMP is incomplete in *C. violaceum*, since it lacks genes encoding for 6-phosphogluconate dehydrogenase, although all the other enzymes of the pathway seem to be present. This observation suggests that E4P, for tryptophan biosynthesis, is provided by the linkage between the intermediates of glycolysis, fructose-6-phosphate and glyceraldehyde-3-phosphate, through the activity of the transketolases and transaldolases of the nonoxidative steps of the HMP. This characteristic should enable *C. violaceum* to convert a larger amount of glucose to aromatic amino acids than other organisms that have a complete HMP. Ikeda and Katsumata (1999) showed that HMP-defective mutants produce an increased amount of E4P, which is the limiting substrate for tryptophan biosynthesis.

As occurs in *E. coli*, tryptophan biosynthesis in *C. violaceum* starts with anthranilate synthesis; it is encoded by several genes (*trp*A, *trp*B, *trp*C, *trp*D, *trp*E, *trp*F, and *trp*G), but differently from *E. coli*, these genes are not organized into an operon (Table 1). However, they seem to compose clusters with genes not related to tryptophan biosynthesis.

The entire operon for violacein biosynthesis has been cloned and sequenced (August et al., 2000). Their results were confirmed by the *C. violaceum* genome sequence, which showed that the biosynthesis genes are in an operon constituted of *vio*D, *vio*C, *vio*B, and *vio*A genes (Figure 1, Table 1). These authors also suggest a biosynthesis pathway with attributes of the activity of each gene product in the pathway (Figure 2).

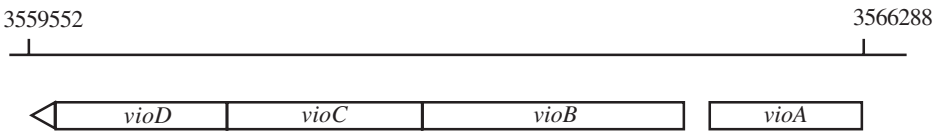


Figure 1. Schematic drawing of structural genes of the violacein biosynthesis operon.

The *vio*D, *vio*C and *vio*A products present similarity with nucleotide-dependent monooxygenases. While VioD seems to catalyze one tryptophan molecule hydroxylation, as suggested by most research on violacein biosynthesis, VioA catalyzes an oxidative deamination of a second tryptophan molecule, and VioC catalyzes intermediate violacein oxidation (August et al., 2000). The VioB protein is similar to polyketide synthase, an enzyme with a very interesting activity, since it is able to catalyze nonribosomal peptide bonds, and in violacein biosynthesis pathway it apparently catalyzes the condensation of two tryptophan derivative molecules, which are essential for pigment production (August et al., 2000).

Table 1. Main open reading frames (ORFs) found in *Chromobacterium violaceum* involved in violacein expression and metabolism. From Vasconcelos et al. (2003).

ORF number	ORF name	ORF product name	E.C. number
CV2173	trpD	anthranilate phosphoribosyltransferase	2.4.2.18
CV2179	trpE	anthranilate synthase component I	4.1.3.27
CV2712	trpC	indole-3-glycerol phosphate synthase protein	nd
CV2761	trpA	tryptophan synthase, alpha subunit	4.2.1.20
CV2762	trpB	tryptophan synthase, beta subunit	4.2.1.20
CV2763	trpF	phosphoribosylanthranilate isomerase	5.3.1.24
CV3271	vioD	vioD - hydroxylase	nd
CV3272	vioC	vioC - monooxygenase	nd
CV3273	vioB	vioB - polyketide synthase	nd
CV3274	vioA	vioA - tryptophan 2-monooxygenase	nd
CV4090	cviR	transcriptional activator, LuxR family of regulators	nd
CV4091	cviI	N-acyl homoserine synthase	nd

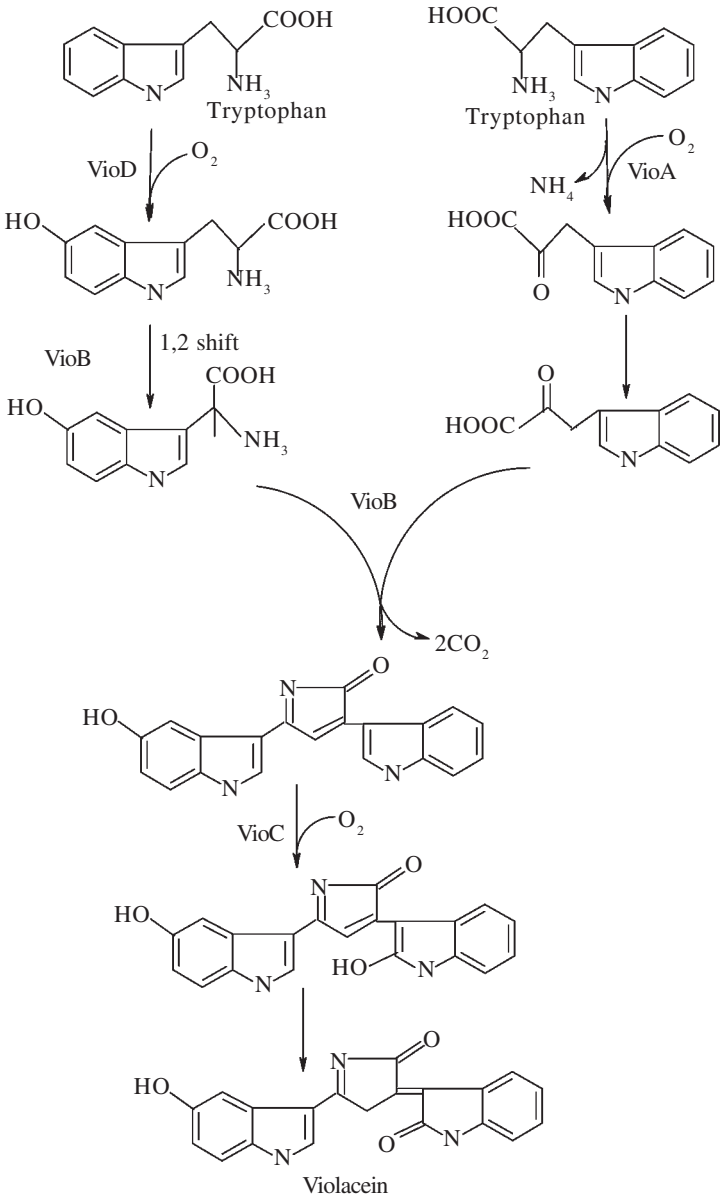
nd - not described.

Violacein biosynthesis is under the control of a diffusible signal molecule, generically named N-acyl-homoserine-lactone (AHL); AHL mediates physiological responses in Gram-negative bacteria, including cell differentiation, production of secondary metabolites and exoenzymes (Chernin et al., 1998). An AHL identified and characterized as N-hexanoyl-homoserine-lactone (HHL) has been found in *C. violaceum* (McClean et al., 1997). Two genes *cvi*I and *cvi*R have been identified in the *C. violaceum* genome (Table 1), encoding CviI and CviR, HHL synthase and a regulator protein, respectively, with structural and functional similarity with chemicals that are involved in the control of bioluminescence in *Vibrio fischeri*. There apparently is a similar secondary metabolic control in *C. violaceum.* Possibly, when *C. violaceum* growth is near the stationary phase, a large amount of constitutive CviI leads to an accumulation of HHL. This signal molecule binds to the *cvi*R operator, inducing its expression. The increase in CviR enables it to bind to a transcriptional regulator site of the violacein biosynthesis operon, increasing pigment production. CviR is able to stimulate the expression of some other genes in *C. violaceum*, such as chitinases and exoproteases (Chernin et al., 1998).

On the other hand, HHL seems to be not the only control mechanism in *C. violaceum* production, since even in densely populated cell cultures, produced with high glucose concentration and oxygen levels, violacein production is inhibited (Oliveira, C.G., Porto, L.M. and Antônio, R.V., unpublished data). Violacein production is also dependent on the carbon source and the concentration in the culture medium. These experimental observations suggest that a control similar to that mediated by cyclic AMP, through catabolic activator protein, may mediate violacein biosynthesis.

CONCLUDING REMARKS

The sequencing of the *C. violaceum* genome shows that violacein biosynthesis is dependent on the metabolism of carbohydrates, not only through the HMP pathway, but also through the glycolysis and the Entner-Dourduroff pathways, from which NADPH should be produced, since this bacterium lacks 6-phosphogluconate activity. The similarity of three of the



Violacein

Figure 2. Violacein biosynthesis, as proposed by August et al., 2000. VioA, VioB, VioC, and VioD are the gene products of the biosynthesis operon, encoding nucleotide-dependent monooxygenases and a protein similar to a polyketide synthase (VioB).

gene products of the operon of violacein biosynthesis to nucleotide-dependent monooxygenases, as well as the need for oxygen for pigment production, suggests that NADH or $FADH_2$ are not such nucleotides, or they could be shared with violacein biosynthesis, since under aerobic conditions they should also be directed to the respiratory chain. The reducing power of violacein biosynthesis is not clear and should be one of the limitations for pigment biosynthesis. A *quorum* sensing system mediated by HHL is also present, and it seems to be very similar in organization to that found in *Pseudomonas aeruginosa*.

ACKNOWLEDGMENTS

The study described here was undertaken within the context of the Brazilian National Genome Program (a consortium funded in December 2000 by the Ministério da Ciência e Tecnologia (MCT) through the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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