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Biosynthesis and characterization of violacein, deoxyviolacein and oxyviolacein in heterologous host, and their antimicrobial activities

Haisheng Wang[a,*], Fengzhong Wang[c], Xiaofei Zhu[a], Yanchun Yan[a], Xianghong Yu[a], Peixia Jiang[b,d], Xin-Hui Xing[b,**]

[a] Graduate School of Chinese Academy of Agricultural Sciences, Beijing 100081, People's Republic of China
[b] Institute of Biochemical Engineering, Department of Chemical Engineering, Tsinghua University, Beijing 100084, People's Republic of China
[c] Institute of Agro-food Science & Technology, CAAS, Beijing 100193, People's Republic of China
[d] Department of Industrial Microbiology and Biotechnology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, People's Republic of China

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ABSTRACT

Violacein is a natural bisindole pigment produced by bacteria, which has attracted much attention recently for its antibacterial, antiviral, antitumor, and antioxidant activities. To better understand the bioactive properties of violacein derivatives, deoxyviolacein and oxyviolacein were biosynthesized by genetically engineered strains of *Citrobacter freundii*(pComvio) and *C. freundii*(pComvioΔd), respectively, using L-tryptophan or 5-OH-L-tryptophan as the precursor, respectively. The structure of oxyviolacein was confirmed based on HPLC–MS and NMR analysis. Deoxyviolacein and oxyviolacein were evaluated for their effectiveness against bacteria and phytopathogenic fungi. The two pigments exhibited strong activities against all Gram-positive bacteria tested except *Streptococcus* sp. Oxyviolacein showed a surprisingly high activity against *Phytophthora capsici*, a devastating pathogen of vegetable crops, and also showed strong antifungal activity against *Fusarium oxysporum*, *Botrytis cinerea*, and *Verticillium dahliae* at 0.5 mg mL^{-1}. Deoxyviolacein showed strong antifungal activity against *Rhizoctonia solani* at concentrations exceeding 2 mg mL^{-1}. The presence of a hydroxyl group appeared to be essential for their bioactivities. These findings provide important information for the further exploitation of violacein and its derivatives as fungicides.

1. Introduction

With the growing global threat of multidrug resistant (MDR) microbes, as well as the increasing desire for natural products, it is imperative to develop alternative platforms for the development of new antimicrobial agents. Bisindole alkaloids have attracted considerable attention because of their broad range of biological properties [1]. Researchers have studied bisindole-based biopigments to determine how these compounds provide a survival advantage for microorganisms in their natural environments [2].

The bisindole alkaloid violacein (Vio, Fig. 1b) is a natural blue-purple pigment isolated from Gram-negative bacteria including *Chromobacterium violaceum* [3], *Collimonas* sp. [4], *Duganella* sp. [5,6], *Iodobacter fluviatile* [7], *Janthinobacterium lividum* [8,9], and *Microbulbifer* sp. [10]. Vio exhibits broad-spectrum antimicrobial, antiviral, antiprotozoal, and antioxidant activities [11], and exerts cytotoxic effects against several tumor cell lines [12,13]. These biological and pharmacological activities of Vio have made it attractive for biotechnology research [14]. It is reported that intraperitoneal doses of Vio up to 1 mg kg^{-1} are not toxic in mouse blood, kidneys, or liver, which provides evidence for the *in vivo* use of Vio as a therapeutic compound with few side effects [15].

Vio could be formed by the condensation of two L-tryptophan molecules under the enzymes of Vio biosynthetic pathway which has been identified in bacteria [16]. Deoxyviolacein (Fig. 1b) is produced in the wild strains as a by-product of Vio biosynthesis. Compared to Vio, it has one less oxygen atom at the 6 position of the indole ring. Vio produced by wild strains usually contains up to 10% deoxyviolacein. Oxyviolacein (Fig. 1b) is another structural analog of Vio, with one more oxygen atom at the 20 position of the indole ring than Vio. Oxyviolacein could be produced using exogenous 5-hydroxy-L-tryptophan (5-HTP) as the precursor due to the molecular recognition and substrate specificity are not strict for enzymes involved in Vio biosynthesis [17]. Though the incorporation of 5-HTP into oxyviolacein is identical to the incorporation of L-tryptophan into Vio by the same metabolism pathway enzymes (Fig. 1a), the incorporation rate from 5-HTP to oxyviolacein was higher than that from 5-HTP to violacein. However, due to the

* Corresponding author. Tel.: +86 10 82109694.
** Corresponding author. Tel.: +86 10 62794771.
E-mail addresses: wanghaisheng@caas.net.cn (H. Wang), xhxing@tsinghua.edu.cn (X.-H. Xing).

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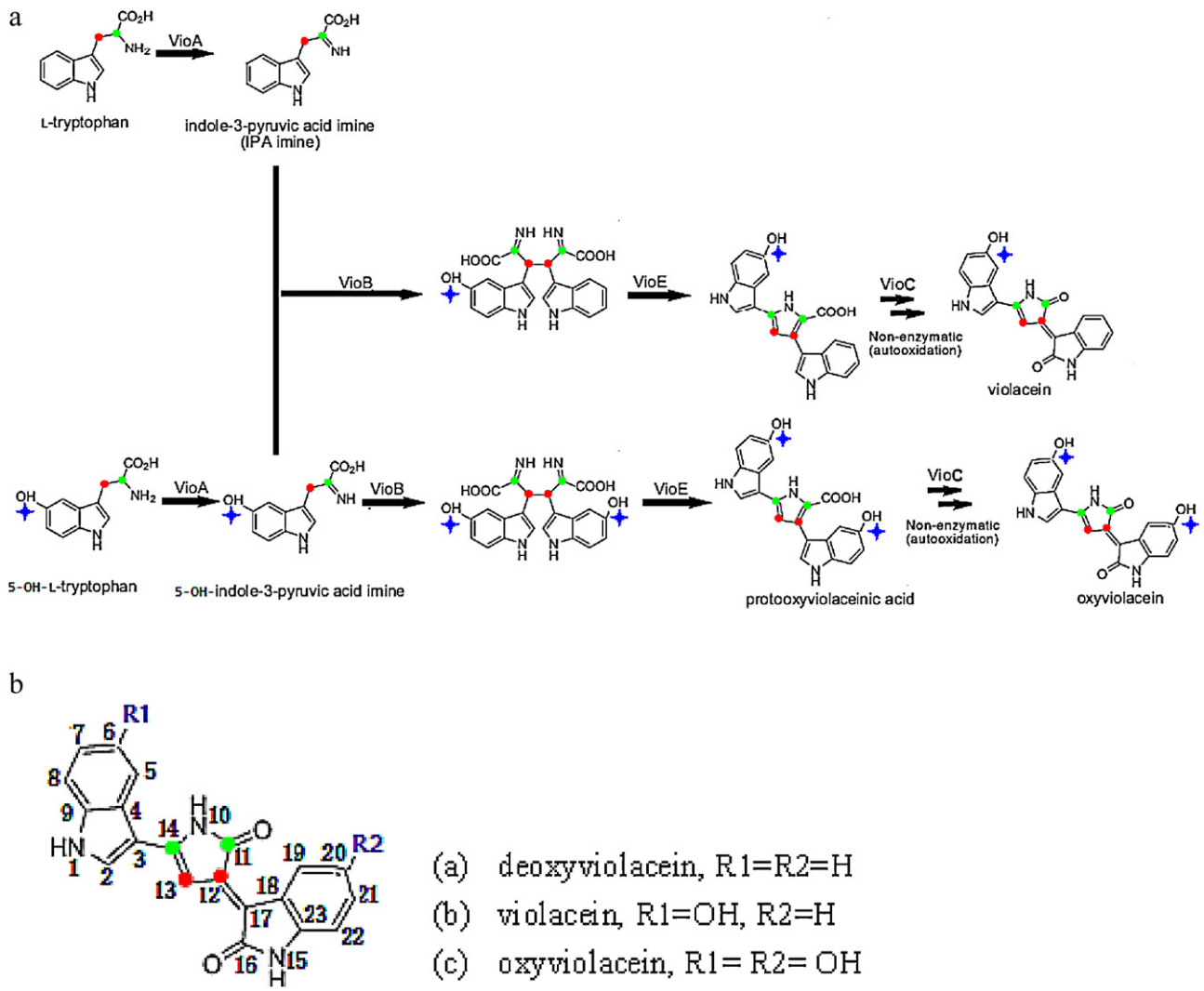

Fig. 1. (a) The production of Vio and oxyviolacein from extracellular 5-HTP by *vioD* deleted Vio biosynthesis pathway. (b) The structures of Vio and its derivatives. (a) Illustrates the production of Vio using extracellular 5-HTP and intracellular L-tryptophan as the precursors, and the production of oxyviolacein using extracellular 5-HTP as the precursor. The production of deoxyviolacein using L-tryptophan as precursor was shown in Ref. [21]. Symbol (+) is denoted as the hydroxyl group of 5-HTP.

difficulties in isolating and purifying of deoxyviolacein and oxyviolacein from crude pigments, it has been challenging to obtain sufficient quantities for their function analysis. Few definitive and direct evidences for the functional characteristics of deoxyviolacein and oxyviolacein have been reported to date.

It is well known that the position or number of hydroxyl groups in the compounds have vital effects on their bioactivities [18,19]. So, it is interesting to investigate the bioactivities of the three pigments differing only in the position or number of hydroxyl groups. In our previous research, *Citrobacter freundii* was genetically engineered by introducing a plasmid (pComvio) bearing the Vio biosynthetic gene cluster from *Duganella* sp. B2. This engineered strain showed rapid growth in flask culture and was able to produce 1.6 g L^{-1} crude Vio using glycerol as the sole carbon source [20]. In addition, a recombinant plasmid bearing *vioabce* (pComvioΔd generated by deleting *vioD* from the *vioabcde* operon) was demonstrated to synthesize pure deoxyviolacein in *C. freundii* [21]. Using these engineered strains, Vio and its derivatives could be produced for the studies on their bioactivities.

In this study, Vio, deoxyviolacein, and oxyviolacein were synthesized by wild or recombinant strains, respectively, and their structure was confirmed by HPLC–MS and ^{1}H NMR analysis. The bioactivities of crude pigment against various bacteria and phytopathogenic fungi were evaluated by the disc diffusion and mycelium growth rate methods. This is the first report describing the production of oxyviolacein by a genetically engineered strain and its antimicrobial activities. Results from this work will provide information for the investigation of the structure–activity relationship of Vio and its derivatives.

2. Materials and methods

2.1. Microorganism strains and culture conditions

Duganella sp. B2 (DSM 19531) was isolated as a Vio-producing bacterium [5]. *C. freundii*(pComvio) [20] or *C. freundii*(pComvioΔd) [21] were used for the heterologous biosynthesis of oxyviolacein or deoxyviolacien. The test strains shown in Tables 2 and 3 were obtained from the Agricultural Culture Collection of China.

All bacteria were grown in nutrient agar (NA) medium, and all fungi were grown in potato dextrose agar (PDA) medium. *C. freundii*(pComvio) and *C. freundii*(pComvioΔd) were grown in E2 medium [20] in a shaker at 200 rpm. L-tryptophan or 5-HTP (0.4 g L^{-1}) was added to the medium to produce Vio, deoxyviolacein, and oxyviolacein, as determined by preliminary optimization