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Superoxide radical-generating compounds activate a predicted promoter site for paraquat-inducible genes of the *Chromobacterium violaceum* bacterium in a dose-dependent manner

J.E. Gabriel[1], E.P. Guerra-Slompo[2], E.M. de Souza[3], F.A.L. de Carvalho[1], H.M.F. Madeira[2] and A.T.R. de Vasconcelos[4]

[1]Colegiado de Ciências Biológicas, Centro de Ciências Agrárias, Universidade Federal do Vale do São Francisco, Petrolina, PE, Brasil
[2]Campus de Ciências Agrárias, Pontifícia Universidade Católica do Paraná, São José dos Pinhais, PR, Brasil
[3]Setor de Ciências Biológicas, Departamento de Bioquímica, Universidade Federal do Paraná, Curitiba, PR, Brasil
[4]Laboratório Nacional de Computação Científica, Petrópolis, RJ, Brasil

Corresponding author: J.E. Gabriel
E-mail: jane.gabriel@univasf.edu.br

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ABSTRACT. The purpose of the present study was to functionally evaluate the influence of superoxide radical-generating compounds on the heterologous induction of a predicted promoter region of open reading frames for paraquat-inducible genes (*pqi* genes) revealed during genome annotation analyses of the *Chromobacterium violaceum* bacterium. A 388-bp fragment corresponding to a *pqi* gene promoter of *C. violaceum* was amplified using specific primers and cloned into a conjugative vector containing the *Escherichia coli lacZ* gene without a

promoter. Assessments of the expression of the β-galactosidase enzyme were performed in the presence of menadione (MEN) and phenazine methosulfate (PMS) compounds at different final concentrations to evaluate the heterologous activation of the predicted promoter region of interest in *C. violaceum* induced by these substrates. Under these experimental conditions, the MEN reagent promoted highly significant increases in the expression of the β-galactosidase enzyme modulated by activating the promoter region of the *pqi* genes at all concentrations tested. On the other hand, significantly higher levels in the expression of the β-galactosidase enzyme were detected exclusively in the presence of the PMS reagent at a final concentration of 50 μg/mL. The findings described in the present study demonstrate that superoxide radical-generating compounds can activate a predicted promoter DNA motif for *pqi* genes of the *C. violaceum* bacterium in a dose-dependent manner.

Key words: *Chromobacterium violaceum*; Functional genome; Menadione; Paraquat-inducible genes; Phenazine methosulfate

INTRODUCTION

Chromobacterium violaceum is a Gram-negative, free-living beta-proteobacterium that dominates a variety of ecosystems in tropical and subtropical regions. Over the last decade, genome prospecting of this bacterium has revealed several refined mechanisms related to its remarkable and exploitable adaptability (Brazilian National Genome Project Consortium, 2003). Genome annotation analyses of *C. violaceum* have identified the presence of open reading frames (ORFs) with high similarity to sequences targeting paraquat-inducible genes (*pqi* genes) previously characterized in *Escherichia coli* (Farr and Kogama, 1991). The *pgi* genes are drastically modulated by the action of several oxidizing agents to minimize their deleterious effects on cellular homeostasis (Hungria et al., 2004). Bacterial cells respond to oxidative stress by inducing numerous regulons controlled by transcription factors responding either directly or indirectly to oxidizing agents.

Menadione (MEN) and phenazine methosulfate (PMS) substrates are characterized as important superoxide radical-generating compounds that generates reactive oxygen species through redox cycling, and high concentrations of these substrates trigger cell death (Loor et al., 2010). MEN has been implicated in the activation of the mitochondrial permeability transition pore as a mediator of cell death (Azevedo et al., 2014; Reed et al., 2014). Furthermore, PMS causes intracellular Ca^{2+} to rise and dramatically increases membrane permeability for K^{+} by activating Gardos channels, which results in the dehydration of circulating red blood cells (Gibson et al., 2003; Shcherbachenko et al., 2007).

Within this perspective, the purpose of the present study was to functionally evaluate the influence of the MEN and PMS compounds on the heterologous induction of a predicted promoter region of ORFs for the *pqi* genes identified during genome annotation analyses of the *C. violaceum* bacterium. The characterization of the functional proprieties of this regulatory DNA motif might provide insights into the biological responses of differing bacterial strains to the gene activation likely involved in the molecular mechanisms of environmental adaptation to oxidizing agents.

MATERIAL AND METHODS

Amplification of the *pqi* promoter and generation of *lacZ* fusion constructs

Assays to measure the expression of the β-galactosidase enzyme were carried out to evaluate the effects of superoxide radical-generating compounds on the predicted promoter region of the *pqi* genes of *C. violaceum*. First, specific primers were designed using a computational program PrimerQuest (Integrated DNA Technologies, http://www.idtdn.com/SciTools/SciTools.aspx) to amplify target sites situated between the ORFs CV2550 and CV2551 corresponding to the predicted promoter region of the *pqi* genes of *C. violaceum*. Forward (5'-CGT GAA TTC TAA TGG CAG ACC GAC ATC AG-3') and reverse (5'-GGT AGA TCT TTT CGT GCG GGT GCT GTT TC-3') primer sequences were constructed according to Sambrook and Russell (2001) to contain specific DNA cleavage sites for the restriction enzymes *Eco*RI and *Bgl*II (underlined bases), respectively. Genomic DNA of *C. violaceum* isolated using a standard saline solution and phenol-chloroform extraction method was amplified in Applied Biosystems® 2720 Thermal Cycler (Life Technologies, Gaithersburg, MD, USA) in the presence of PCR buffer 1X (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 0.4 mM of each dNTPs, 2.5 mM magnesium chloride, 1.2 mM specific primers, and 1 U Platinum® *Taq* DNA Polymerase (Life Technologies) at a final volume of 25 mL. The amplification reactions consisted of denaturation at 95°C for 30 s, annealing at 64°C for 45 s, and extension at 72°C for 45 s, for a total of 35 cycles. The resulting 388-bp amplicon was ligated into the conjugative vector pMP220 previously digested with restriction enzymes *Eco*RI and *Bgl*II. This broad host range conjugative vector contained the *E. coli lacZ* gene without a promoter to generate transcriptional *lacZ* gene fusions (Spaink et al., 1987). Competent *E. coli* S17 strains were electrotransformed with the amplicon for insertion into the conjugative vector carrying the promoter region of interest, as established by Sambrook and Russel (2001), followed by cellular growth of the bacterial isolates on Luria-Bertani agar containing 12.5 mg/mL tetracycline at 37°C.

Analysis of *pqi* promoter activation via the *lacZ* reporter gene

Heterologous induction of the promoter region of the *pqi* genes of *C. violaceum* was evaluated in the presence of superoxide radical-generating compounds by measuring the expression levels of the β-galactosidase enzyme in the presence of the ortho-nitrophenyl-β-D-galactopyranoside reagent (Sigma-Aldrich, St Louis, MO, USA), as proposed in detail by Miller (1972). The β-galactosidase enzyme expression assays were carried out using 100 µL saturated culture of transformed *E. coli* cells diluted in 4.9 mL Luria Broth medium containing 12.5 µg/mL tetracycline and maintained at 37°C under aeration conditions. To achieve an OD_{600nm} reading of 0.25 (approximately 2-h incubation), MEN (2-methyl-1,4-naphthoquinone) and PMS (N-methyldibenzopyrazine bromide) compounds commercially acquired from Sigma-Aldrich were added to the bacterial inoculums at the final concentrations of 50 and 100 µg/mL, with 5-h additional incubation under same conditions to trigger the induction of the promoter region. Two distinct *E. coli* isolates carrying promoter-*lacZ* gene fusions were designed *pqi* promoters 12 and 13 and were selected and tested. Alternatively, *E. coli* strains carrying *pqi* promoters 12 and 13 were maintained under the same experimental conditions without addition of the compounds, constituting the control groups.

Statistical analysis

Descriptive statistical analyses of the data were performed using the software STATISTICA/W statistical package version 10.0 (Statsoft, Tulsa, OK, USA) from three independent experimental assays. Results were presented as means, standard deviations, and standard errors of the means and were estimated by using hierarchical linear model and analysis of variance, with a P value <0.05 considered to be statistically significant.

RESULTS

The heterologous induction of a predicted promoter region for the *pqi* genes of the *C. violaceum* bacterium was evaluated by measuring the expression levels of β-galactosidase in the presence of different superoxide radical-generating compounds, as shown in Figure 1. The MEN reagent promoted a highly significant increase in the expression of β-galactosidase modulated by activating the promoter region of the *pqi* genes of *C. violaceum*, independent of concentration ($P < 0.05$), with 2-fold higher response to MEN than under control conditions (Figure 1A). In contrast, the induction of the *pqi* gene promoter was drastically affected by changing the final concentration of the PMS reagent, as significant increases in β-galactosidase expression in the presence of the PMS reagent were detected exclusively at a final concentration of 50 µg/mL ($P < 0.05$) (Figure 1B). No significant differences in the expression levels of the β-galactosidase enzyme were observed in the presence of the PMS reagent at a final concentration of 100 µg/mL ($P = 0.152$) (Figure 1B).

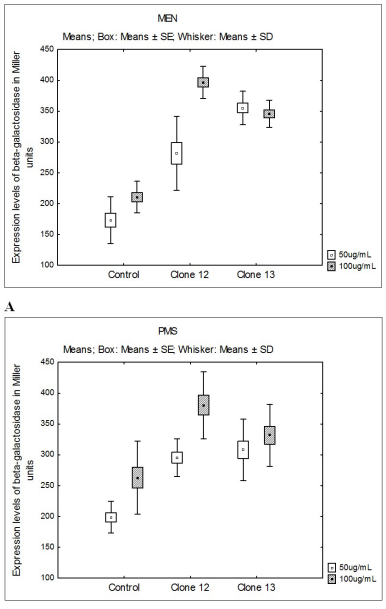

Figure 1. Expression levels of the β-galactosidase enzyme in response to activation of predicted promoter regions for paraquat-inducible genes of *Chromobacterium violaceum* exposed to MEN (**A**) or PMS (**B**) at final concentrations of 50 and 100 µg/mL. Results are reported as means, standard deviations (SD), and standard errors of the means (SE).

In addition, to verify the eventual intrinsic resistance of the *C. violaceum* bacterium in the presence of the compounds tested, colony-forming units (CFUs) per milliliter were calculated by monitoring the growth of the *C. violaceum* colonies on LB agar plates containing MEN and PMS compounds at final concentrations of 0 (control conditions), 25, 40, 60, 75, 100, 1000, and 2800 µg/mL. Bacterial growth of *C. violaceum* colonies was exclusively detected in the presence of MEN at a final concentration of 25 µg/mL (10^5 CFU/mL). On the other hand, no CFUs were detected in response to PMS at any concentrations tested. Approximately 10^7 CFU/mL of the *C. violaceum* bacterium were obtained under control conditions of bacterial growth on agar plates without addition of either compound.

DISCUSSION

The findings described herein demonstrate that different superoxide radical-generating compounds could induce a putative promoter region for the *pqi* genes of the *C. violaceum* bacterium in a dose-dependent manner (Figure 1). Recently, researchers have examined the heterologous activation of regulatory DNA motifs of the *C. violaceum* bacterium in response to superoxide radical-generating substrates. Within this perspective, Gabriel et al. (2015) have reported that the paraquat compound itself provoked significant increases in the expression levels of the β-galactosidase enzyme in *E. coli* strains carrying the predicted promoter sequence for the *pqi* genes of *C. violaceum* fused to the *lacZ* gene; in this study, reported values were 3.5 to 4-fold higher in response to paraquat than were those observed in the control group. These results suggested the intrinsic existence of regulatory DNA motifs in the genome of *C. violaceum* that would be potentially inducible by an oxidizing agent (Gabriel et al., 2015).

Over the past decades, some studies have examined the molecular mechanisms involved in the gene activation associated with the remarkable cellular response to environment adaptation seen in the *C. violaceum* bacterium (Hungria et al., 2004; Baraúna et al., 2011; Ciprandi et al., 2012). In fact, the free-living microorganism *C. violaceum* is exposed frequently to a series of abiotic factors such as different sources and abundance of nutrients, changes in temperature and pH, as well as toxic compounds and UV rays. The molecular characterization in this study of a *pqi* gene promoter sequence detected in the *C. violaceum* genome that is activated by different superoxide radical-generating substrates seems to provide supporting evidence for the great adaptability to a wide range of environments and strong protective systems triggered within these bacterial cells.

Furthermore, this study also clarified experimentally the functional properties of a regulatory DNA motif predicted previously in ORFs for *pqi* genes during genome annotation of the *C. violaceum* bacterium (Figure 1). With the advent of the genomic era, innumerable advances have arisen in basic science research, and extensive applications in a great variety of fields such as genetics and environment adaptation have been conducted. According to Feder and Mitchell-Olds (2003), the detailed elucidation of molecular, cellular, and organismal mechanisms is frequently essential to understanding their adaptive physiological aspects and evolutionary impact.

In conclusion, the significant effects of the MEN and PMS reagents on the heterologous induction of a predicted promoter region for the *pqi* genes in the *C. violaceum* genome seems to provide evidence for the functional aspects of the influence of superoxide radical-generating compounds on the activation of a potential molecular response to adaptive environments in this bacterium.

Conflicts of interest

The authors declare no conflict of interest.

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