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Original article

Expression of essential genes for biosynthesis of antimicrobial peptides of *Bacillus* is modulated by inactivated cells of target microorganisms

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Abstract

Certain *Bacillus* strains are important producers of antimicrobial peptides with great potential for biological control. Antimicrobial peptide production by *Bacillus amyloliquefaciens* P11 was investigated in the presence of heat-inactivated cells of bacteria and fungi. *B. amyloliquefaciens* P11 exhibited higher antimicrobial activity in the presence of inactivated cells of *Staphylococcus aureus* and *Aspergillus parasiticus* compared to other conditions tested. Expression of essential genes related to biosynthesis of the antimicrobial peptides surfactin (*sfp*), iturin A (*lpa-14* and *ituD*), subtilosin A (*sboA*) and fengycin (*fenA*) was investigated by quantitative real-time PCR (qRT-PCR). The genes *lpa-14* and *ituD* were highly expressed in the presence of *S. aureus* (inactivated cells), indicating induction of iturin A production by *B. amyloliquefaciens* P11. The other inducing condition (inactivated cells of *A. parasiticus*) suppressed expression of *lpa-14*, but increased expression of *ituD*. A twofold increase in *fenA* expression was observed for both conditions, while strong suppression of *sboA* expression was observed in the presence of inactivated cells of *S. aureus*. An increase in antimicrobial activity was observed, indicating that synthesis of antimicrobial peptides may be induced by target microorganisms.
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Keywords: Antimicrobial peptides; Biological control; *Bacillus*; Iturin; PCR

1. Introduction

Control of pathogenic agents by microorganisms that produce a variety of natural antimicrobial substances may be a valuable alternative to use of harmful chemicals and drugs [1,2]. Several species of *Bacillus* produce antimicrobial peptides that are considered safe for industrial use and products from *Bacillus* spp. are commercially available. Surfactin, iturin A, subtilosin A, subtilin and fengycin are some of the antimicrobial peptides described for this genus [3,4].

The lipopeptide surfactin has crucial biological functions related to biological control of bacteria and fungi [4], including the ability to stimulate immune-related responses in host tissues [5]. Production of surfactin is carried out by genes organized into a large operon in *Bacillus subtilis* and gene *sfp* encoding the enzyme 4′-phosphopantetheinyltransferase was characterized as essential to surfactin production [6,7].

Iturins produced by *Bacillus* spp. show broad antifungal activity, including mycotoxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* [8]. Iturin A production has been intensively investigated and the gene cluster involved in its synthesis has been elucidated [9,10]. Genes *ituD, ituA, ituB* and *ituC* are involved in iturin A synthesis, composing an operon that spans a region of about 38 kb. Disruption of a putative malonyl coenzyme A transacylase encoded by the

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ituD gene causes a specific deficiency in production of this peptide [9]. Another gene involved in iturin A synthesis is *lpa-14* encoding 4′-phosphopantheteinyl transferase required for maturation of the template enzyme of this antimicrobial peptide. Genes *ituD* and *lpa-14* play an important role in iturin A synthesis [9,11].

Gene cluster expression (*sboA-albABCDEFG*) is required for synthesis of subtilosin A, all transcribed from a promoter located upstream of the *sboA* structural gene. Immunity, secretion, post-translational modification and processing of presubtilosin are related to these genes, involved in the production of the antimicrobial peptide [12,13].

The fengycin synthetases *fenC, fenD, fenE, fenA* and *fenB* are involved in production of the non-ribosomal peptide fengycin [14,15], a decapeptide with a β-hydroxy fatty acid. Fengycins show antifungal activity mostly on filamentous fungi and also display unusual properties, such as the presence of ornithine in the peptide portion [16].

Some studies suggest that the presence of heat-inactivated cells of some microorganisms can increase the antimicrobial activity produced by lactic acid bacteria [17,18] and *Bacillus* species [19]. Components of the inducer cells can be exposed and/or solubilized during heating, causing an increase in production of antimicrobial activity [19]; in some cases, the inducing substance has been associated with the cell envelope [17].

Bacillus sp. P11 is a strain isolated from the teleost fish Piau-com-pinta that belongs to the *B. subtilis/B. amyloliquefaciens* cluster [20]. This strain has antibacterial activity [21] and antifungal activity against several fungi, including *Fusarium* spp., *Aspergillus* spp. and *Bipolaris sorokiniana* [22]. The aim of this study was to investigate the expression of genes related to biosynthesis of antimicrobial peptides by *Bacillus* sp. P11 in the presence of heat-inactivated cells of bacteria or fungi. The expression of genes *sfp, lpa-14, ituD*, *sboA* and *fenA* was monitored by quantitative real-time PCR (qRT-PCR).

2. Materials and methods

2.1. Bacterial cultures, media and growth conditions

Bacillus sp. P11 (GenBank DQ387864.1), previously isolated from the intestinal contents of the teleost fish Piau-com-pinta (*Leporinus* sp.), was the producer strain [20]. The strain was further characterized as *Bacillus amyloliquefaciens* using an API 50 CHB kit and APILAB Plus software (BioMérrieux, Marcy-l'Etoile, France). Bacteria were grown for 24 h at 30 °C in a rotary shaker at 125 rpm, in 20 mL of BHI broth (Oxoid, Basingstoke, UK) and then used to inoculate 50 mL of BHI broth with shaking under different induction conditions. Strains *Escherichia coli* ATCC 25922, *Staphylococcus aureus* subsp. *aureus* ATCC 25923, *Bacillus cereus* ATCC11788, *Listeria monocytogenes* ATCC 7644 and wild strains of *Fusarium oxysporum f. lycopersici, Fusarium graminearum* and *A. parasiticus* (Plant Health Department, UFRGS, Porto Alegre, Brazil) were used to induce production of antimicrobial activity by *B. amyloliquefaciens* P11. For detection of antimicrobial activity, *L. monocytogenes* ATCC 7644 was used as the indicator strain. All strains were stored at −21 °C in brain-heart infusion (BHI; Oxoid, Basingstoke, UK) containing 20% (v/v) glycerol and propagated in the same medium at 37 °C before use.

2.2. Antimicrobial activity assays

The agar disc diffusion method was used to determine antimicrobial activity. An aliquot of 20 μL of filtered supernatant was applied to sterile cellulose discs (6 mm) placed on BHI plates previously inoculated with a suspension of 10^8 cells/mL of the strain of *L. monocytogenes* ATCC 7644, used as the indicator microorganism. The plates were incubated at 37 °C for 24 h. The serial twofold dilution method was used to determine the antimicrobial titer [23]. Sample dilutions were performed in PBS. Activity was defined as the reciprocal of the dilution after the last serial dilution giving a zone of inhibition and expressed as activity units (AU) per milliliter. The pH of culture supernatants was checked and adjusted to 7.0 before testing. Samples were treated with 2 mg/mL proteinase K to verify the stability of antimicrobial activity against proteolysis [24]. The results were analyzed by comparing the averages of triplicates, using analysis of variance and the subsequent Tukey test using the software Statistica 7.0 (StatSoft Inc., Tulsa, OK), and differences were considered significant when $P < 0.05$.

2.3. Induction of antimicrobial activity

The inducing test for production of antimicrobial peptides by *B. amyloliquefaciens* P11 was originally performed by addition of heat-inactivated cells of *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *B. cereus* ATCC 9634, *L. monocytogenes* ATCC 7644, *F. oxysporum f. lycopersici, F. graminearum* or *A. parasiticus.* Cultures with an OD_{600} ranging from 1.76 to 1.95 were autoclaved at 121 °C for 30 min and then centrifuged at 10,000 *g* at 4 °C for 15 min to obtain a cell pellet of each inducer strain. Each pellet obtained was diluted in BHI to reach 5 mL and then added to a culture in BHI broth containing 1% pre-inoculum of the producing strain. This mixture was incubated at 30 °C for 40 h in an orbital shaker at 125 rpm.

For additional tests with the best inducers (*S. aureus* ATCC 25923 or *A. parasiticus*), heat-inactivated cells were centrifuged at 10,000 *g*, 4 °C for 15 min, the cell pellets were washed three times and suspended in 5 mL of BHI and added to 50 mL cultures of *B. amyloliquefaciens* P11. The cultures were incubated at 30 °C and tested for production of antimicrobial activity at different growth stages (time intervals of 0, 4, 8, 12, 24, 32, 40 and 48 h), under stirring.

Cell fractionation of *B. amyloliquefacienes* P11, *S. aureus* ATCC 25923 and *A. parasiticus* was carried out. Cultures were centrifuged at 10,000 *g* for 15 min at 4 °C and the supernatants were reserved. Cell pellets were washed three times and suspended in 20 mL of BHI. This suspension was autoclaved at

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