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Genetic and biochemical characterization of hyicin 3682, the first bacteriocin reported for *Staphylococcus hyicus*

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ABSTRACT

Hyicin 3682, the first bacteriocin reported for *Staphylococcus hyicus*, is a Bsa_{COL} variant produced by *S. hyicus* 3682, a strain isolated from bovine milk. Hyicin 3682 is found in the culture supernatant, is bactericidal and its producing strain exhibits a much broader spectrum of antimicrobial activity than the producing strain of Bsa_{COL} against several Gram-positive bacteria, which include foodborne pathogens, food-spoilage microorganisms and bacterial species of medical and veterinary importance. Sequencing of the genome of *S. hyicus* 3682 provided the nucleotide sequence of the entire gene cluster involved in hyicin 3682 production, which seems to be located on pRJ109, the single plasmid carried by this strain. This gene cluster is expressed and consists of 8525 bp and of eight genes (*hyiA*, *hyiB*, *hyiC*, *hyiD*, *hyiP*, *hyiF*, *hyiE* and *hyiG*) encoded on the same DNA strand. The mature lantibiotic exhibits 91% identity to Bsa_{COL} and its molecular mass was found to be ~26 Da higher due to two amino acid substitutions. *S. hyicus* 3682 proved to be only partially immune to its cognate bacteriocin up to 1024 AU/ml. Therefore, hyicin 3682, the first Bsa variant reported in coagulase-negative staphylococci, does exhibit antimicrobial and siblicidal activities.

1. Introduction

Bacteria are known to produce bacteriocins, ribosomally-synthesized antimicrobial proteins or peptides with either a narrow or a large spectrum of inhibitory activity generally against other bacteria (Heng et al., 2007).

Staphylococcins are bacteriocins produced by bacteria belonging to the genus *Staphylococcus* (Bastos et al., 2009). Most staphylococcins so far described are lantibiotics (Bastos et al., 2009), which are small, polycyclic peptides which contain post-translationally modified amino acids, such as lanthionine (Lan) and β-methyl-lanthionine (MeLan), among others (Bierbaum and Sahl, 2009; Dischinger et al., 2014). Many lantibiotics exhibit potent and broad-ranging antimicrobial activities against human and animal pathogens and, therefore, have potential applications in clinical settings and in food preservation (Cotter et al., 2013). Epidermin is a 22-amino acid lantibiotic of 2,164.6 Da, which is produced by *Staphylococcus epidermidis* Tü3298 (Schnell et al., 1988). Its biosynthesis is directed by plasmid pTü32 and its gene cluster was found to comprise 11 genes (Schnell et al., 1992). Epidermin and its variants, including Bsa (Bacteriocin of *Staphylococcus aureus*), are the most frequent lantibiotics encoded on the genome of staphylococci (Bastos et al., 2009; Daly et al., 2010).

Production of Bsa has not been reported in coagulase-negative staphylococci to date. It was first described in community-acquired *S. aureus* strains that were resistant to methicillin (CA-MRSA) and associated with human infections (Daly et al., 2010). Its gene cluster, which is composed of eight genes, is chromosomally encoded. Contradictory reports are found in the literature regarding to Bsa production. Daly and co-workers reported that the Bsa gene cluster encodes a functional lantibiotic of 2089–2091 Da with an antimicrobial activity of 64 AU/ml in cell-free supernatants (CFSs).

Abbreviations: aa, amino acids; AU, arbitrary units; Bac, bacteriocin; Bsa, Bacteriocin of *Staphylococcus aureus*; CFS, cell free supernatant; CoNS, coagulase-negative staphylococci; Imm, immunity; LAB, lactic acid bacteria; Lan, lanthionine; MeLan, methyl-lanthionine; PSM, phenol-soluble modulin.

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However, in a subsequent work, Joo et al. (2011) reported that the antimicrobial activity exhibited by the CA-MRSA strains studied by Daly and co-workers was not related to Bsa production but to proteolytically-processed derivatives of phenol-soluble modulins (PSMs) with molecular masses (from 2072 to 2090 Da) similar to those proposed for Bsa. Therefore, Bsa production in CA-MRSA strains is a controversial issue. However, recently, the production of a lantibiotic, named BacCH91 (2074 Da) and reported as related to epidermin, was described in a *S. aureus* strain of poultry origin. A bacteriocin structural gene homologous to *bacCH91* (allelic variant 2) has also been found on the genome of *S. aureus* strains of human origin (Wladyka et al., 2013). In the present work, BacCH91was shown to be more closely related to Bsa than to epidermin, being therefore a Bsa variant.

In a previous study, *Staphylococcus hyicus* 3682, a strain isolated from bovine milk, was shown to produce a bacteriocin-like inhibitory substance, which was named hyicin 3682. Hyicin 3682 proved to have a broad spectrum of activity, which included several bacterial pathogens (Fagundes et al., 2011). Therefore, hyicin 3682 has potential biotechnological applications in either prevention or control of bacterial infections. Based on the presence of an amplicon generated by PCR-based experiments performed with *S. hyicus* 3682 genomic DNA and a specific pair of primers which amplifies the Bsa structural gene (*bsaA2*), hyicin 3682 was predicted to be a Bsa variant and, consequently, to belong to the epidermin group (Fagundes et al., 2011). However, neither the bacteriocin nor its gene cluster was characterized in the previous study, and optimal exploitation of hyicin 3682 for biotechnological purposes requires knowledge on all gene products involved in its production. Therefore, in the present work, we describe the genetic and biochemical characterization of hyicin 3682, the first bacteriocin reported for *S. hyicus*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacterial strains and plasmids used in the present study are listed in Table 1. *Escherichia coli* strains were cultivated at 37 °C in Luria-Bertani medium (LB; Acumedia) supplemented with 75 μg ampicillin/ml (Sigma-Aldrich), when appropriate. *Staphylococcus* spp. and *Micrococcus luteus* ATCC 4698 were grown at 37 °C in brain heart infusion medium (BHI; Difco) or in trypticase soy broth (TSB; Difco). All media were supplemented with agar at 1.5 or 0.7% (w/v), when required.

The 47 bacterial strains used as targets in analysis of the bacteriocin inhibitory spectrum of activity (Tables Table 1 and S2) belonged to different species of Gram-positive bacteria, including lactic acid bacteria (LAB), foodborne pathogens, staphylococci and streptococci, and some of them were described in previous studies. With exception of LAB which were grown in de Man, Rogosa & Sharpe medium (MRS; Difco) at 32 °C, the remaining strains were grown in either BHI or TSB medium at 37 °C.

Bacteria were stored in their appropriate medium with 40% glycerol (w/v) at −20 °C until required. Working cultures of the selected strains were prepared by inoculating from stock cultures into 5 ml of the specific culture media and incubating for 18 h at either 32 or 37 °C.

2.2. DNA isolation and manipulations

Purification of *E. coli* plasmid DNA was done with QIAprep® Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. The same kit was used to purify plasmid DNA from *Staphylococcus* spp. In this case, the plasmid isolation was performed after treatment of the cells, for 30 min at 37 °C, with 100 μl of a lysis solution [50 mM Tris/HCl, 40 mM EDTA and 25% (w/v) sucrose, pH 7.8] supplemented with 20 mg lysozyme/ml (Sigma-Aldrich), 1 mg lysostaphin/ml (Sigma-Aldrich), 100 mg RNase A/ml (Sigma-Aldrich) and 2.5 mg SDS/ml. The genomic DNA of the staphylococcal strains was isolated as described by Pitcher et al. (1989) after treatment of the cells as described above. T4 DNA-ligase (Life Technologies do Brasil), *Eco*RI (Life Technologies do Brasil) and *Taq* DNA-polymerase (Thermo Fisher Scientific, Inc) were used as specified by the suppliers. Oligonucleotides were purchased from Life Technologies do Brasil. DNA and RNA preparations were quantified by using NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Inc). Agarose gel electrophoreses were performed by standard methods (Sambrook et al., 1989).

2.3. Plasmid curing experiments

Curing of pRJ109, the single plasmid carried by *S. hyicus* 3682, was attempted by the method described by Nascimento et al. (2012), except for the curing temperature, which was 42 °C.

2.4. Localization of the hyicin 3682 gene cluster

In order to test if the gene cluster involved in hyicin 3682 production was located on pRJ109, the plasmid DNA was isolated after two consecutive runs of purification from 0.7% (w/v) agarose gels using the Wizard® SV Gel and PCR Clean-Up System (Promega) and used as a template in PCR reactions for detection of the bacteriocin structural gene. As reported previously, hyicin 3682 is related to the lantibiotic Bsa (Fagundes et al., 2011). Therefore, primers BsaA2F and BsaA2R (Table 2), which amplify the Bsa structural gene, *bsaA2*, were used. The PCR mixtures consisted of 20 ng of plasmid DNA, 1 x reaction buffer, 0.5 U of *Taq* DNA-polymerase (Thermo Fisher Scientific, Inc), 1.25 mM $MgCl_2$, 0.2 mM of each dNTP (Promega) and 25 pmol of each primer in a final volume of 20 μl. The amplification was done in a Mastercycler Nexus (Eppendorf) and the thermal cycling consisted of an initial denaturation step at 92 °C for 3 min; 35 cycles at 92 °C for 1 min, 53 °C for 1 min and 72 °C for 1 min, followed by a final extension step at 72 °C for 5 min. The amplicons were analysed on 1.4% (w/v) agarose gels, using the 100-bp DNA ladder O'Range Ruler (Thermo Fisher Scientific, Inc) as size markers. The amplification reaction was repeated twice. The amplicons were cloned into pGEM-T-Easy (Promega), according to the manufacturer's instructions, and transformed into thermal competent *E.coli* DH5α (Sambrook et al., 1989). Four ampicillin-resistant transformants were chosen for plasmid DNA isolation. The presence of the insert in the recombinant plasmids was confirmed by digestion with *Eco*RI, which released the insert. The insert cloned in all four recombinant plasmids was sequenced using the ABI 3500xL Genetic Analyzer System (Life Technologies do Brasil). DNA sequencing was performed at "Laboratório Multiusuário de Genotipagem e Sequenciamento da UNICAMP", São Paulo, Brazil.

2.5. Genome sequencing and mining of the gene cluster involved in hyicin 3682 production

S. hyicus 3682 was grown for 18 h at 37 °C in TSB. The sequencing library was prepared using Nextera® XT DNA Sample Preparation Kit (Illumina) following the manufacturer's recommendations. Whole-genome sequencing was performed using the Illumina MiSeq system. De novo assembly of reads was conducted using the software A5-miseq pipeline (Coil et al., 2015). Genome annotation was performed by Rapid Annotation using Subsystem Technology (RAST) (Aziz et al., 2008). The search for plasmid replicons was done by searching all scaffolds for genes encoding plasmid Rep proteins. The bacteriocin gene cluster was then mined from scaf-