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Short Communication

Molecular analysis of the 21-kb bacteriocin-encoding plasmid pEF1 from *Enterococcus faecium* 6T1a

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Abstract

The complete 21,344-bp DNA sequence of the bacteriocin-encoding plasmid pEF1 from *Enterococcus faecium* 6T1a was determined. Thirty-four putative open reading frames which could code for proteins longer than 42 amino acids were found. Those included the structural genes encoding for the previously described bacteriocins enterocin I and J (also named as enterocins L50A and L50B). After comparison to sequences in public databases, analysis of the gene organization of pEF1 suggests a modular structure with three different functional domains: the replication region, the bacteriocin region and the mobilization plus UV-resistance region. This genetic mosaic structure most probably evolved through recombination events promoted by transposable elements. The hypothesis that the bacteriocin cluster on pEF1 could act as a functional plasmid stabilization module in *E. faecium* 6T1a is discussed.

Keywords: Bacteriocinogenic plasmid; Plasmid stability; *Enterococcus faecium*; Nucleotide sequence; Annotation

1. Introduction

Lactic acid bacteria (LAB) associated with foods and beverages have been generally recognized as safe (GRAS) microorganisms for a long period of time (Klaenhammer et al., 2005). Enterococcal species of LAB have been considered as harmless commensal gut microorganisms, although more recently strains of *Enteroccus faecium* and *Enterococcus faecalis* have been acknowledged as leading causes of hospital-acquired infections (Emori and Gaynes, 1993; Schwarz et al., 2001;Grady and Hayes, 2003). This is especially relevant as enterococci are intrinsically resistant to many antibiotics and can acquire further resistance through conjugative plasmids and transposons which can also donate to other bacterial species (Schwarz et al., 2001). Further competitiveness and resistance can be achieved by enterococci through bacteriocin production and immunity.

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Bacteriocins are ribosomally synthesized peptides or proteins usually displaying antagonistic activity against closely related species (Klaenhammer, 1993). Bacteriocins from enterococci are frequently associated to plasmids, as it is the case with the bacteriocin encoded by plasmid pAD1 (Ike et al., 1990), AS-48 from pMB2 (Martínez-Bueno et al., 1990), Bc-48 from pMB1 (Martínez-Bueno et al., 1992), enterocin 226NWC1 from pEF226 (Salzano et al., 1992), bacteriocin 21 from pPD1 (Fujimoto et al., 1995), bacteriocin 31 from pYI17 (Tomita et al., 1996), enterocin L50 and enterocin Q from pCIZ1 and pCIZ2, respectively (Cintas et al., 2000), enterocin SE-K4 from pEK4S (Doi et al., 2002), enterocin EJ97 from pEJ97 (Sánchez-Hidalgo et al., 2003), enterocins 416K1 and 388C from a 38 MDa enterococcal plasmid (Sabia et al., 2004), enterocins 1071A and 1071B from pEF1071 (Balla and Dicks, 2005), and bacteriocin 32 from pTI1 (Inoue et al., 2006) among others. In addition, most of these plasmids are conjugative.

In a previous work, it was shown that the ability to produce and be immune to a broad-spectrum, antilisterial bacteriocin named enterocin I by the strain *E. faecium* 6T1a was linked to a 23-kb plasmid (Floriano et al., 1998). This plasmid was designated pEF1, and only the 2.5-kb DNA sequence comprising the bacteriocin cluster was known at that time (EMBL Accession No. (Acc. No.) Y16413). This cluster included *orf2*, which could encode for a peptide 72.7 % identical to enterocin I. Cintas et al. (1998) described two enterocins produced by the strain *E. faecium* L50, isolated from Spanish dry-fermented sausages, named enterocin L50A and L50B, which were encoded by a 50-kb plasmid designated pCIZ1. At present, only a 3.5-kb fragment from pCIZ1 is available under the EMBL Acc. No. AJ223633. Enterocin L50A was identical to enterocin I, while enterocin L50B was identical to the putative product of *orf2* from pEF1. This product was later designated enterocin J. In this report, we describe the complete molecular analysis of plasmid pEF1.

2. Materials and methods

2.1. Bacterial strains, media, growth conditions and plasmids

Enteroccus faecium 6T1a, enterocins I and J producer, was isolated from an olive fermentation in southern Spain and described previously (Floriano et al., 1998). *E. faecalis* EFS-2 is an enterocin AS-48 producer. They were propagated in MRS broth (Oxoid, Basingstoke, England) at 37 °C. *Escherichia coli* DH5α was cultivated in LB broth (Sambrook et al., 1989), aerobically at 37 °C. Plasmid pEF1, conferring enterocins I and J production and immunity was described earlier (Floriano et al., 1998). Plasmid pUC18, used as cloning vector, was purchased from Stratagene (La Jolla, USA).

2.2. Bacteriocin assays

In order to test bacteriocin production and immunity, the strains *E. faecium* 6T1a and *E. faecalis* EFS-2 were streaked onto MRS-agar plates to obtain isolated colonies. The plates were incubated at 37 °C for 16 h and then overlaid with 4.5 ml MRS soft-agar (0.75% w/v agar) inoculated with ca. 10^5 cfu/ml of the indicator strain. *E. faecium* 6T1a and *E. faecalis* EFS-2 were alternatively used as indicator strains to check their sensitivity to the bacteriocin produced by the other strain. These plates were further incubated at 37 °C for 24 h, until lawns of the indicator strain were grown, allowing the detection of inhibition halos surrounding the isolated colonies. Alternatively, 10-μl drops of semi-purified AS-48 were spotted onto lawns of *E. faecium* 6T1a, prepared as described above. *E. faecalis* EFS-2 lawns were also used as negative controls. These plates were incubated at 37 °C for 24 h and resulting inhibition halos recorded.

2.3. DNA techniques

Total DNA was prepared from *E. faecium* as described previously (Cathcart, 1995), and plasmid purification was carried out by CsCl gradient centrifugation (Sambrook et al., 1989). Isolation of *E. coli* plasmid DNA and subsequent nucleic acid manipulations were carried out as described by Sambrook et al. (1989). Restriction enzymes and T4 DNA ligase were purchased from Roche Diagnostics (Penzberg, Germany) and used according to the manufacturer's instructions. Shrimp Alkaline Phosphatase (SAP) was purchased from USB Corporation (Cleveland, Ohio, USA) and used according to the manufacturer's instructions.

2.4. PCR amplifications

The Expand High Fidelity PCR System (Roche) was used to amplify the different DNA fragments from either pEF1 or the subsequent pUC18 subclones. Primers used in PCRs were synthesized by MWG Biotech (Ebersberg, Germany).

2.5. Sequencing of pEF1

Plasmid pEF1 was digested with *Hin*dIII. The resulting fragments were electrophoretically separated and individually collected by the freeze–squeeze technique (Sambrook et al., 1989). Each fragment was ligated to