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Original Article

Frequency of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) in non-clinical *Enterococcus faecalis* and *Enterococcus faecium* strains

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(With 1 figure)

Abstract

The fidelity of the genomes is defended by mechanism known as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) systems. Three Type II CRISPR systems (CRISPR1-*cas*, CRISPR2 and CRISPR3-*cas*) have been identified in enterococci isolates from clinical and environmental samples. The aim of this study was to observe the distribution of CRISPR1-*cas,* CRISPR2 and CRISPR3-*cas* in non-clinical strains of *Enterococcus faecalis* and *Enterococcus faecium* isolates from food and fecal samples, including wild marine animals. The presence of CRISPRs was evaluated by PCR in 120 enterococci strains, 67 *E. faecalis* and 53 *E. faecium*. It is the first report of the presence of the CRISPRs system in *E. faecalis* and *E. faecium* strains isolated from wild marine animal fecal samples. The results showed that in non-clinical strains, the CRISPRs were more frequently detected in *E. faecalis* than in *E. faecium*. And the frequencies of CRISPR1-*cas* and CRISPR2 were higher (60%) in *E. faecalis* strains isolated from animal feces, compared to food samples. Both strains showed low frequencies of CRISPR3-*cas* (8.95% and 1.88%). In conclusion, the differences in the habitats of enterococcal species may be related with the results observe in distribution of CRISPRs systems.

Keywords: Enterococcus faecalis, *Enterococcus faecium*, CRISPRs, food samples, fecal samples, wild marine animals.

Frequência das repetições palindrômicas curtas agrupadas e regularmente interespaçadas (CRISPRs) em cepas não-clínicas de *Enterococcus faecalis* e *Enterococcus faecium*

Resumo

A fidelidade dos genomas é defendida por mecanismos conhecidos como sistemas de repetições palindrômicas curtas agrupadas e regularmente interespaçadas (CRISPRs). Três tipos de sistemas CRISPR II (CRISPR1-cas, CRISPR2 e CRISPR3-cas) têm sido identificados em cepas de enterococos isolados de amostras clínicas e ambientais. O objetivo deste estudo foi observar a distribuição dos CRISPR1-cas, CRISPR2 e CRISPR3-cas em cepas não-clínicas de *Enterococcus faecalis* e *Enterococcus faecium* isoladas de amostras alimentícias e fecais, incluindo animais marinhos selvagens. A presenca dos CRISPRs foi determinada por PCR em 120 cepas de enterococos, sendo 67 *E. faecalis* e 53 *E. faecium*. É o primeiro relato da presença do sistema CRISPRs nas estirpes *E. faecalis* e *E. faecium* isoladas de amostras fecais de animais marinhos selvagens. Os resultados mostraram que em cepas não-clínicas, os CRISPRs foram mais frequentemente detectados em *E. faecalis* do que em *E. faecium*. E as frequências de CRISPR1-cas e CRISPR2 foram maiores (60%) em cepas de *E. faecalis* isoladas de fezes de animais, quando comparadas à amostras de alimentos. Ambas as cepas apresentaram baixas freqüências de CRISPR3-cas (8,95% e 1,88%). Em conclusão, as diferenças nos habitats das espécies de enterococos podem estar relacionadas com os resultados observados na distribuição dos sistemas CRISPRs.

Palavras-chave: Enterococcus faecalis, *Enterococcus faecium*, CRISPRs, amostras alimentares, amostras fecais, animais marinhos selvagens.

1. Introduction

Enterococci are important Gram-positive bacteria recognized as part of gut microbiota in humans and animals, and they are widely distributed in soil, water, plants and foods (Cassenego et al., 2013; Lebreton et al., 2014; Pieniz et al., 2015; Santestevan et al., 2015; Prichula et al., 2016; Medeiros et al., 2017). Their ubiquity reflects its ability to survive in a variety of environmental stressors (Byappanahalli et al., 2012). Another important characteristic is their intrinsic resistance to some antimicrobials agents commonly prescribed to treat Gram-positive cocci such as cephalosporin, lincomycin, and low levels of penicillin and aminoglycosides. Furthermore, they are also able to acquire a variety of resistance genes via transposons or plasmids. Epidemiological data suggest that enterococci are important reservoirs for the spread of antibiotic resistance to different species of bacteria (Lebreton et al., 2014).

The *Enterococcus* genus comprises more than 50 species, and *Enterococcus faecalis* and *Enterococcus faecium* are the most frequent species isolated from humans, animals and food samples (Lebreton et al., 2014). Moreover, they are the most prevalent species cultured from humans' infected sites, including bacteremia, surgical site infections, and urinary tract infections.

The genomes of *E. faecalis* and *E. faecium* have suffered rearrangements over hundreds of millions of years in evolution, given them selective advantages to survive and disperse in the environment. As a result, the presence of the *Enterococcus* spp. has been investigated and monitored in a variety of habitats (Van Tyne and Gilmore, 2014).

To defend the genome against parasitic DNA invasion, and to maintain the fidelity of the genomes in stable ecosystems, there is a mechanism known as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) systems (Sorek et al. 2008; Palmer and Gilmore, 2010; Magadán et al., 2012). The CRISPR systems provides a type of defense in prokaryotes, leading to a resistance to plasmid uptake and phage infections, and a barrier to horizontal gene transfection (Thomas and Nielsen, 2005; Wiedenheft et al., 2012). This immunity depends on the presence of specific target-derived spacer sequences, the intervening repeat palindromes short and highly conserved, and nuclease activity encoded by the *cas* genes (Haft et al., 2005; Makarova et al., 2006; Sorek et al., 2008).

Type II CRISPR-Cas loci consists of a CRISPR array, the type-specific *cas9* gene, and *cas1* and *cas2* genes. In enterococci strains, three Type II CRISPR-Cas loci have been identified, the CRISPR locus lacking *cas* genes, thus consisting solely of repeat-spacer arrays (orphan CRISPR2), and those carrying these *cas* genes (CRISPR1–Cas and CRISPR3–Cas) (Makarova et al., 2011). Palmer and Gilmore (2010) using comparative genomics found that pathogenic enterococci have multiple rearrangements that occurred during evolution and is followed by the loss of CRISPRs. In contrast, commensal strains have smaller genomes with fewer rearrangements, as demonstrated by whole-genome alignments. The same authors found an inverse relationship between the presence of a CRISPR-*cas* locus and acquired antibiotic resistance in *E. faecalis*, suggesting that antibiotic use inadvertently selects for enterococcal strains with compromised genome defense (Palmer and Gilmore, 2010).

CRISPR1-Cas and CRISPR3-Cas loci occur with variable distribution in *E. faecalis* and they are absent in multi-resistant strains isolated from clinical samples (Palmer and Gilmore, 2010). In addition, CRISPR2 locus is conserved across the species. Since the CRISPR system is reactive to the environment, it might play a critical role in the adaptation of the host to its surroundings and explains the persistence of particular bacterial strains in ecosystems where phages are present (Horvath et al., 2008).

The distribution of CRISPRs in clinical enterococci strains have been studied (Palmer and Gilmore, 2010; Lindenstrauß et al., 2011; Burley and Sedgley, 2012; Hullahalli et al., 2015), however, in non-clinical strains are still poorly studied (Katyal et al., 2013; Lyons et al., 2015). Therefore, the goal of this study was to determine the distribution and frequency of CRISPR1–c*as,* orphan CRISPR2 and CRISPR3–*cas* in *E. faecalis* and *E. faecium* strains isolated from food and fecal samples, including wild marine animals.

2. Materials and Methods

2.1. Enterococcus strains

A total of 120 enterococci isolated from food and animal fecal samples were used in this study (Table 1). Among these, 67 were identified as *E. faecalis* and 53 *E. faecium.* The enterococci were isolated from different foods samples (beetroot, potato, parsley, raw meat, buffalos milk and dairy products, such as mozzarella cheese), and animal feces (wild fur seals, chickens, wild Magellanic penguins and wild green turtles) in Southern Brazil, from 2009 to 2015. Strains were randomly selected from the culture collection of the Department of Microbiology, Universidade Federal do Rio Grande do Sul.

All strains were already identified as genus, species and virulence profile in previous studies (Frazzon et al., 2009; Riboldi et al. 2009; Cassenego et al., 2013; Prichula et al., 2013; Santestevan et al., 2015; Prichula et al., 2016). The strains were preserved in 10% (w/v) skim milk (Difco, Sparks, MD, USA) solution supplemented with 10% (v/v) glycerol (LabSynth®) frozen at -20 °C.

2.2. DNA isolation

For DNA isolation, an aliquot of frozen bacterial cells was recovered in Brain Heart Infusion agar (BHA, Himedia®, India), and incubated at 35 °C for 24 h. Genomic DNA was extracted using the method described by Donato (2007). All strains were confirmed to genus and species by polymerase chain reaction (PCR) assays according to the protocol established by Prichula et al. (2016). The primers sequences and their amplification products are listed in Table 2.

Table 1. *Enterococcus faecalis* and *E.faecium* used in this study.

Strains	Origin	n	Reference
E. faecalis	Vegetables[a]	11	Riboldi et al. (2008)
	Raw meat, milk and dairy products[b]	11	Riboldi et al. (2008), Frazzon et al. (2009), Prichula et al. (2013)
	Fecal samples of wild fur seals[c]	13	Santestevan et al. (2015)
	Fecal samples of wild green turtles[d]	09	Prichula et al. (2013)
	Fecal samples of wild Magellanic penguins[e]	05	Prichula et al. (2013)
	Fecal samples of chickens	18	Cassenego et al. (2013)
	Total	**67**	
E. faecium	Vegetables[a]	01	Riboldi et al. (2008)
	Raw meat and milk[b]	30	Riboldi et al. (2008), Frazzon et al. (2009), Prichula et al. (2013)
	Fecal samples of wild fur seal[c]	01	Santestevan et al. (2015)
	Fecal samples of wild green turtles[d]	06	Prichula et al. (2013)
	Fecal samples of wild Magellanic penguins[e]	15	Prichula et al. (2013)
	Total	**53**	

[a]beetroot, potato, parsley; [b]raw meat, buffalos milk and dairy products, such as mozzarella cheese; [c]South American fur seals (*Arctocephalus australis*) and Subantartic fur seals (*Arctocephalus tropicalis*); [d]Green turtles (*Chelonia mydas)*; [e]Magellanic Penguins (*Spheniscus magellanicus*).

Table 2. Primers used in the PCRs carried out in this study.

Primer	Sequence (5'- 3')	AT* (°C)	Product (pb)	Reference
Genus				
tuf-F	TACTGACAAACCATTCATGATG	54	112	Ke et al. 1999
tuf-R	AACTTCGTCACCAACGCGAAC			
Species				
EM1_A	TTGAGGCAGACCAGATTGACG	56	658	Cheng et al. 1997.
EM1_B	TATGACAGCGACTCCGATTCC			
DD13	CACCTGAAGAAACAGGC	54	475	Depardieu et al. 2004.
DD3-2	ATGGCTACTTCAATTTCACG			
CRISPR 1	GCGATGTTAGCTGATACAAC	50	783	Palmer and Gilmore, 2010
CRISPR1–cas F	CGAATATGCCTGTGGTGAAA			
CRISPR1–cas R				
CRISPR 2	CTGGCTCGCTGTTACAGCT	58	variable	Palmer and Gilmore, 2010
Orphan CRISPR F	GCCAATGTTACAATATCAAACA			
Orphan CRISPR F				
CRISPR 3	GATCACTAGGTTCAGTTATTTC	64	258	Palmer and Gilmore, 2010
CRISPR3–cas F				
CRISPR3–cas R	CATCGATTCATTATTCCTCCAA			

AT: anneling temperature.

2.3. Detection of CRISPR-associated (cas) genes and orphan locus lacking cas genes

Primers for CRISPR amplification reported by Palmer and Gilmore (2010) were used in PCR reaction. The primers and annealing temperatures used are listed in Table 2. The PCR was performed in a total volume of 25 µL containing: 1x PCR buffer (10 mM Tris-HCl [pH 9.0], (Invitrogen, Carlsbad, CA, USA), 1.5 mM of $MgCl_2$ (Invitrogen, Carlsbad, CA, USA), 200 µM of dNTPs (Ludwig Biotecnologia), 0.4 µM of each primer (Invitrogen, Carlsbad, CA, USA), 1 U of *Taq* polymerase (Invitrogen, Carlsbad, CA, USA), and 100 ng of genomic DNA. The reaction mixture was subjected to 30 cycles of denaturation at 94 °C for 1 min.; primer annealing for 1 min. (at appropriate temperature), and extension at 72 °C for 1 min., followed by a final extension at 72 °C for 10 min. The PCR products were analyzed on 1.2% agarose gels stained with SYBR safe (Invitrogen, Carlsbad, CA, USA) solution and visualized under UV light.

2.4. Sequencing of samples

In order to confirm the CRISPRs amplifications, PCR products amplified of CRISPR1–c*as*, orphan CRISPR2 and CRISPR3–*cas* genes were submitted to nucleotide

sequence analysis. The DNA fragments were purified using illustra GFX™ PCR DNA and gel band purification kit (GE Healthcare-Buckinghamshire, United Kingdom - UK). Sequencing was carried out with the Big Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems) in an ABI-PRISM 3100 Genetic Analyzer (ABI), according to the protocol of the manufacturer. The sequences obtained were compared with homologous nucleotide sequences deposited in GenBank).

3. Results

3.1. Detection of CRISPRs in enterococci isolates from food and animals fecal samples

The distribution and the frequency of the Type II CRISPRs (CRISPR1-*cas*, CRISPR2, and CRISPR3-*cas*) in non-clinical strains of *E. faecalis* and *E. faecium* are present in Figure 1. The CRISPRs were more frequently detected in *E. faecalis* than in *E. faecium* strains. In *E. faecalis* strains, the CRISPR1-*cas* and CRISPR2 were detected in 44.77% and 88.07%, respectively. On the other hand, in *E. faecium* the CRISPR1-*cas* were observed in 5.66% and CRISPR2 in 32.72% of the strains. Both strains showed low frequencies of CRISPR3-*cas* genes (8.95% and 1.88%).

The frequency of CRISPRs in *E. faecalis* and *E. faecium* strains and source of isolation were related. A higher frequency of CRISPR1-*cas* and CRISPR2 in non-clinical were observed in *E. faecalis* isolates from fecal samples, in contrast to food samples. In fecal strains, the CRISPR1-*cas* was observed in 60% of the *E. faecalis* strains, whereas in food strains in 13.66%. In relation to CRISPR2, it was positive in 97.11% and 68.18% of the *E. faecalis* isolated from fecal and food samples, respectively. Similar frequency of CRISPR3-*cas* gene was detected in both samples.

Identical proportions of CRISPR1-*cas* and CRISPR3-*cas* (3.22%) were observed in *E. faecium* isolated from food products, but a substantially high prevalence of CRISPR2 was observed in *E. faecium* (48.38%). In addition, equal frequency of CRISPR1-*cas* and CRISPR2 (9.095%) were observed in *E. faecium* isolated from animals fecal samples. The CRISPR3-*cas* amplification was not detected in *E. faecium* strains.

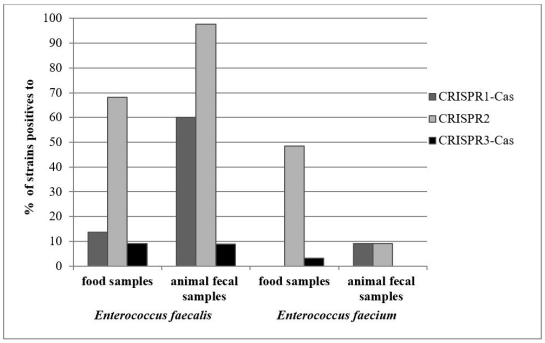

Figure 1. Distribution of CRISPR systems in non-clinical *Enterococcus faecalis* and *Enterococcus faecium* isolated from food and animal fecal samples.

3.2. Analysis of CRISPR sequences

The nucleotide BLAST of the PCR products from CRISPR1-*cas,* CRISPR2 and CRISPR3-*cas* genes showed that CRISPR1-*cas* gene had 99% identity with the DNA sequence of CRISPR1-*cas* of *E. faecalis* strain D32 isolated from pig feces in Denmark (GenBank: NC_018221), culture_collection *E. faecalis* strain OG1RF ATCC 47077 (GenBank: CP002621.1), and *E. faecalis* strain L12, isolated from suine in Brazil (GenBank: CP018102.1). The DNA sequence from CRISPR2 gene showed 91% of identity to CRISPR2 gene of *E. faecalis* strain L9, isolated from rectal swabs from suine in Brazil (GenBank: CP018004.1) and 88% to *E. faecalis* strain DENG1, isolated from sputum of human in China (GenBank: CP004081.1) and *Enterococcus* sp. strain 7L76 draft genome (GenBank: FP929058.1). The DNA sequence of CRISPR3-*cas* gene showed 98% of identity to the draft genome of *Enterococcus* sp. strain 7L76 (GenBank: FP929058.1).

4. Discussion

Differences in the distribution and the frequency of the Type II CRISPRs (CRISPR1-*cas*, CRISPR2, and CRISPR3-*cas*) in non-clinical *E. faecalis* and *E. faecium* were observed. These differences observed in CRISPRS distributions among enterococci species were reported before by Palmer and Gilmore (2010), Lindenstrauß et al. (2011), Katyal et al. (2013), Hullahalli et al. (2015) and Lyons et al. (2015). The incidence of CRISPR1-*cas* gene in *Enterococcus* species is recognized; sometimes *cas* sequences are distinctly different from each other, showing species-level evolution. CRISPR2 loci were detected in high frequency in all isolates evaluated, manly in animal fecal samples. Similar results were identified in other reports that evaluated *E. faecalis* strains (Palmer and Gilmore, 2010; Hullahalli et al., 2015). Hullahalli et al. (2015) showed that CRISPR2 locus is ubiquitous in *E. faecalis.* Until today, there are only a few reports evaluating the distribution of CRISPRs in non-clinical enterococci strains, isolated from food and animals fecal samples (Katyal et al., 2013; Lindenstrauß et al., 2011; Lyons et al., 2015). It is the first report of the presence of CRISPRs in *E. faecalis* and *E. faecium* isolates from fecal samples of wild marine animals. The high frequency of CRISPRs in *E. faecalis* strains isolated from animal fecal samples, especially in fecal samples of wild animal could be related with low frequency of antibiotic resistance genes in these strains (Santestevan et al., 2015; Prichula et al., 2016). To fecal strains, the results are partial in agreement with Lyons et al. 2015 and Katyal et al. 2003 that reported CRISPRs in *E. faecalis* isolated from fecal specimens. Although Lyons et al. (2015) described a high frequency of CRISPR1-*cas* in *Enterococcus* spp. isolates from animal fecal samples; CRISPR1-*cas* was not detected in *E. faecalis* strains. Instead, Katyal et al. (2013) reported a similar high frequency of CRISPR1-*cas* (52%) in *E. faecalis* isolates from pig feces; they have not reported the presence of

CRISPR3-*cas*. We observed for the first time the presence of this gene in animal fecal samples.

Our results demonstrated a low frequency of CRISPRs Type II in *E. faecium* strains isolated from animal and food samples. A similar result was observed to Palmer and Gilmore (2010) in clinical *E. faecium* strains. There is only one study which tested CRISPRs in environmental *E. faecium* from strains, and the authors did not detect any CRISPRs (Lindenstrauß et al., 2011). So, our data is the first report of the presence of CRISPR1-*cas* and CRISPR2 in *E. faecium* isolated from animal feces.

For some enterococcal lineages, CRISPR system has been recognized as a prokaryotic self-defense that provides a type of acquired immunity. Palmer and Gilmore (2010) observed an inverse correlation between CRISPRs presence and antibiotic resistance. In this study, we also observed that strains which contain less antibiotic resistant genes tended to have more *cas* genes (data not show). In previous studies from our group evaluating the same strains, was observed in food strains a large number of antibiotic resistance genes (data not published), and in animals strains, mainly wild animals, a low frequency of antibiotic resistance genes (Santestevan et al., 2015; Prichula et al., 2016).

In the conclusion, we detected different proportions and distributions of CRISPRs in *E. faecalis* and *E. faecium* isolates from food and fecal samples. The differences in the habitats of enterococcal species influenced this result. The CRISPRs genes were obtained more frequently between *E. faecalis* strains isolated from fecal samples of animals, highlighting to wild marine animals fecal strains. These strains isolated from wild animals are theoretically under an extremely small selective pressure when compared to strains isolated from food and clinical samples.

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