Systematic and Applied Microbiology 34 (2011) 553–560

Contents lists available at SciVerse ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

Comparison of genotypic and phenotypic cluster analyses of virulence determinants and possible role of CRISPR elements towards their incidence in *Enterococcus faecalis* and *Enterococcus faecium*

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ARTICLE INFO

Article history:
Received 21 March 2011
Received in revised form 10 May 2011
Accepted 12 May 2011

Keywords:
Enterococcus faecalis
Virulence genes
CRISPR
MALDI-TOF-MS
RAPD

ABSTRACT

Enterococcus faecalis and *Enterococcus faecium* are human commensals frequently found in fermented foods or used as probiotics, but also recognized as opportunistic pathogens. We investigated 62 *Enterococcus* strains isolated from clinical, food and environmental origins towards a rationale for safety evaluation of strains in food or probiotic applications. All isolates were characterised with respect to the presence of the virulence determinants *fsrB, sprE, gelE, ace, efaAfs/fm, as, esp, cob* and the cytolysin operon. In addition RAPD-PCR was used to obtain genomic fingerprints that were clustered and compared to phenotypic profiles generated by MALDI-TOF-MS. The gelatinase phenotype (GelE) and the haemolytic activity (β-haemolysis) were analysed. *E. faecium* strains contained *esp* and *efaAfm* only, and none of them contained any CRISPR elements. The amenability of *E. faecalis* strains to acquisition of virulence factors was investigated along the occurrence of CRISPR associated (*cas*) genes. While distribution of most virulence factors, and RAPD versus MALDI-TOF-MS typing patterns were unrelated, 2 out of 5 RAPD clusters almost exclusively contained clinical *E. faecalis* isolates, and an occurrence of CRISPR elements versus reduced number of virulence factors was observed. The presence of the cytolysin operon, *cob* and *as* encoding pheromone and aggregation substance, respectively, significantly corresponded to absence of *cas*. As their production promote genetic exchange, their absence limits further gene acquisition and distribution. Thus, absence of the cytolysin operon, *cob* and *as* in a cas positive environment suggests itself as promising candidate for *E. faecalis* evaluation towards their occurrence in food fermentation or use as probiotics.

Introduction

Enterococci are ubiquitous, Gram-positive bacteria that are members of the healthy human intestinal microbiota. Within the genus *Enterococcus* the two species *Enterococcus faecalis* and *Enterococcus faecium* play a major role as commensals in humans and animals. Both are also found on plants, in soil and in several artisanal food products [40]. Strains of *E. faecalis* originating from the raw materials autochthonous microbiota take part in food fermentation processes, and are suggested to impact on the sensorial quality of foods, and are also used as probiotics [2]. However, they have been recognized as causative for endocarditis and are among the most common pathogens found in nosocomial infections, including bacteraemia, intra-abdominal and urinary tract infections [22,42]. Furthermore, *E. faecalis* was demonstrated to play a role in the development of inflammatory bowel disease (IBD) [1]. Important for the understanding of the wide spread of these organisms is the ability of enterococci to resist restrictive environmental conditions. They possess the capability to grow in a temperature range of 10 °C and 45 °C, at pH 9.6, in 6.5% NaCl broth and to survive at 60 °C for 30 min [7,8].

The ability of enterococci to acquire, accumulate, and share extrachromosomal elements encoding virulence traits or antibiotic resistance genes constitutes these organisms dual nature. Some virulence determinants are well characterised and have been described in enterococci, which are associated with infection-derived *E. faecalis* strains [27,32,41]. One of the best-investigated virulence factors is cytolysin, a toxin that can disrupt membranes of other bacteria, erythrocytes and other eukaryotic cells. This factor has been proven to enhance virulence in animal models [13,18,19].

Another confirmed extracellular virulence trait is the gelatinase (GelE). This secreted bacterial metalloproteinase catabolises many target molecules including gelatine, collagen, fibrinogen, casein and insulin [23]. As a result of the enzymatic hydrolysis of fibrinogen

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0723-2020/$ – see front matter © 2011 Elsevier GmbH. All rights reserved.
doi:10.1016/j.syapm.2011.05.002

the bacterial migration into host tissue could be enabled because of the disruption of enterocytes [43]. The gene encoding GelE occurs mostly combined in an operon-type structure together with a gene for a serine protease, sprE [32,38]. In animal models both have been suggested to play a major role in the infection process [37]. The expression of gelatinase and serine protease is controlled by a quorum sensing mechanism involving fsrB [31].

Some members of surface proteins such as collagen-binding protein (Ace), *E. faecalis* (EfaAfs) and *E. faecium* endocarditis antigen A (EfaAfm), and enterococcal surface protein (Esp) are assumed to be involved in mechanisms by which the enterococcal cells adhere to surfaces and furthermore to eukaryotic cells [27,35].

Another important enterococcal adhesion factor is the aggregation substance (AS, sometimes referred to as Agg) that induces the formation of cell–cell contact and mediates conjugative transfer of plasmids [10]. This may induce the transfer of virulence factors to harmless commensal strains, whose metabolic potential could be useful in starter, adjunct or probiotic cultures. The presence and expression of virulence factors in response to food-like conditions have been found in clinical as well as in food isolates [15,21].

Clustered, regularly interspaced short palindromic repeats (CRISPR) protect bacterial cells from infection with bacteriophages and plasmids [17,20,39]. The CRISPR loci operate as a diverse defence mechanism that withstands exposure to invading nucleic acids. CRISPR motives are typically located near CRISPR-associated (*cas*) genes, whose member's encode proteins that carry domains such as nucleases and helicases [14,16]. We found CRISPR elements and *cas* genes in the chromosome and plasmids of lactobacilli with very small genomes underlining their role in generally limiting DNA access to the cell and promoting genetic stability of strains (unpublished data). Recently, significant association between the absence of CRISPR and the presence of antibiotic resistance traits in clinical isolates, versus the presence of CRISPR elements in commensal strains was demonstrated [28]. While CRISPR elements are highly diverse and can only be detected in genome sequencing, *cas* genes exhibit homologies and may serve as PCR-detectable markers pinpointing to active CRISPR elements.

It was therefore the aim of this study to assess the distribution of virulence and *cas* genes among *Enterococcus* isolates from different origins and compare their occurrence in clusters generated by protein profiles (MALDI-TOF-MS) and genotypic fingerprints (RAPD-PCR).

Materials and methods

Bacterial strains

A total of 62 *Enterococcus* isolates from clinical, food and environmental origin were used in this study (see Table 1 for strains and origins of isolation). Out of these we tested 52 *E. faecalis* isolates and 10 *E. faecium*. All strains used in this study were verified as belonging to the species *E. faecalis* or *E. faecium* by sequencing the 815 bp amplification product of the 16S rRNA gene generated by primers 616 V 5′-AGAGTTTGATCCTGGCTCAG-3′ and 609R 5′-ACTACCAGGGTATCTAATC-3′. Additionally, this PCR-product was also used as a positive control for each strain analysed by PCR.

Gelatinase activity

Determination of gelatinase-producing strains was carried out on lysogeny broth (LB) agar containing 3% (wt/vol) gelatine. After incubation at 37 °C for 24 h plates were stored at 4 °C for 5 h according to Eaton and Gasson [5]. Zones of turbidity around colonies indicated gelatinase production.

Cytolysin activity

The phenotypic detection of the enterococcal strains for haemolytic activity was performed on Merckoplate® (Merck, Germany) containing 5% (vol/vol) sheep blood and additionally on LB agar supplemented with 5% (vol/vol) horse blood. According to Gaspar et al. [11] blood agar plates were incubated under aerobic and anaerobic conditions at 37 °C (Anaerocult®A, Merck, Germany). The production of cytolysin appeared in clear zones (β-haemolysis) around colonies.

DNA isolation

For DNA isolation all strains were grown in BHI broth (Sigma–Aldrich, Germany) at 37 °C. DNA isolation was done with DNA isolation kit (E.Z.N.A.® Bacterial DNA Kit, Omega bio-teck, USA) according to manufactures instructions.

Detection of virulence associated genes and cas genes

Specific primers for the following virulence associated and cas genes were used: *ace*, *as*, *cob*, cytolysin operon, *efaAfs*, *efaAfm*, *esp*, *fsrB*, *gelE*, *sprE*, CRISPR1-*cas csn1*, CRISPR2 loci, CRISPR3-*cas csn1*. The primers and their amplification products are listed in Table 2. Some primers were designed using the sequences found in the GenBank nucleotide sequence database under following accession numbers: AE016830.1 and AF454824.1. All primers were synthesised by Eurofins MWG-Operon (Ebersberg, Germany). The PCR reactions were performed in a total of 50 μl, using 80 pmol of each primer, 1.5 mM $MgCl_2$, 10 mM each of dNTPs, 2 U of Taq DNA, 1× Buffer mix (Qbiogene, USA) and sample DNA. Amplification conditions were as follows: an initial denaturation step of 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 1 min, annealing for 1 min (at an appropriate temperature) and extension at 72 °C for a duration adjusted according to PCR-product length, followed by a final elongation at 72 °C for 5 min. The PCR products were analysed on 1.2% agarose gels stained with dimidium bromide solution and visualized under UV light. All primers delivered fragments of the expected size.

The PCR screening for the complete cytolysin locus with an amplification product of 8364 bp [11] was performed using Phusion® High-Fidelity DNA Polymerase (Finnzymes, Finland) according to the recommendations of the manufacturer. All primers were tested before use with reference *E. faecalis* strains OG1RF and V583.

Analysis of genetic fingerprinting by RAPD-PCR

RAPD analyses were performed using the single primer M13 V (5′-GTTTTCCCAGTCACGAC-3′) as described by Ehrmann et al. [6]. The PCR cycling program was 3 cycles of: 94 °C for 3 min, 40 °C for 5 min, 72 °C for 5 min followed by 32 cycles of: 94 °C for 1 min, 60 °C, 72 °C for 3 min. The PCR products were electrophoretically separated on 1.5% TBE agarose gel and stained with dimidium bromide. A 3 kbp plus DNA Ladder (Fermentas, Canada) was used as a molecular weight marker. Generated RAPD-PCR fingerprints were analysed by BioNumerics™ software (version 3.0; Applied Maths BVBA, Belgium). The analysis of the similarities of the generated RAPD-PCR fingerprints was calculated on the basis of the Pearson product–moment correlation coefficient. A cluster analysis was derived from the matrix of similarities by the unweighted pair group method using arithmetic mean (UPGMA) cluster algorithm.

Table 1
Enterococcal strains used in this study. TMW, Technische Mikrobiologie Weihenstephan.

Species	TMW strain no.	Designation	Origin
E. faecalis	2.63	Food isolate	This study
	2.136	Environmental isolate	Isogenic with DSM 20060
	2.354	Environmental isolate	This study
	2.520	Environmental isolate	This study
	2.622	Environmental isolate	Isogenic with OG1RF, Barbara Murray, University of Texas, USA
	2.629	Food isolate	This study
	2.630	Food isolate	This study
	2.645	Clinical isolate	Isogenic with strain 10, Gehard Reuter, Freie Universität Berlin, Germany
	2.647	Food isolate	Isogenic with strain A/F2, Gerhard Reuter, Freie Universität Berlin, Germany
	2.648	Food isolate	Isogenic with strain 34.5, Gerhard Reuter, Freie Universität Berlin, Germany
	2.777	Environmental isolate	This study, Symbioflor®1
	2.798	Environmental isolate	This study
	2.815	Environmental isolate	This study
	2.852	Clinical isolate	isogenic with strain V583, Ingolf Nes, Norwegian University of Life Sciences, Norway
	2.900	Clinical isolate	Roger Vogelmann, TUM[a]
	2.901	Clinical isolate	Roger Vogelmann, TUM[a]
	2.902	Clinical isolate	Roger Vogelmann, TUM[a]
	2.903	Clinical isolate	Roger Vogelmann, TUM[a]
	2.904	Clinical isolate	Roger Vogelmann, TUM[a]
	2.905	Clinical isolate	Roger Vogelmann, TUM[a]
	2.906	Clinical isolate	Roger Vogelmann, TUM[a]
	2.907	Clinical isolate	Roger Vogelmann, TUM[a]
	2.908	Clinical isolate	Roger Vogelmann, TUM[a]
	2.909	Clinical isolate	Roger Vogelmann, TUM[a]
	2.910	Clinical isolate	Roger Vogelmann, TUM[a]
	2.911	Clinical isolate	Roger Vogelmann, TUM[a]
	2.912	Clinical isolate	Roger Vogelmann, TUM[a]
	2.913	Clinical isolate	Roger Vogelmann, TUM[a]
	2.914	Clinical isolate	Roger Vogelmann, TUM[a]
	2.915	Clinical isolate	Roger Vogelmann, TUM[a]
	2.916	Clinical isolate	Roger Vogelmann, TUM[a]
	2.917	Clinical isolate	Roger Vogelmann, TUM[a]
	2.918	Clinical isolate	Roger Vogelmann, TUM[a]
	2.919	Clinical isolate	Roger Vogelmann, TUM[a]
	2.921	Clinical isolate	Roger Vogelmann, TUM[a]
	2.922	Clinical isolate	Roger Vogelmann, TUM[a]
	2.923	Clinical isolate	Roger Vogelmann, TUM[a]
	2.924	Clinical isolate	Roger Vogelmann, TUM[a]
	2.925	Clinical isolate	Roger Vogelmann, TUM[a]
	2.926	Clinical isolate	Roger Vogelmann, TUM[a]
	2.927	Clinical isolate	Roger Vogelmann, TUM[a]
	2.928	Clinical isolate	Roger Vogelmann, TUM[a]
	2.929	Clinical isolate	Roger Vogelmann, TUM[a]
	2.930	Clinical isolate	Roger Vogelmann, TUM[a]
	2.931	Clinical isolate	Roger Vogelmann, TUM[a]
	2.932	Clinical isolate	Roger Vogelmann, TUM[a]
	2.933	Clinical isolate	Roger Vogelmann, TUM[a]
	2.934	Clinical isolate	Roger Vogelmann, TUM[a]
	2.935	Clinical isolate	Roger Vogelmann, TUM[a]
	2.936	Clinical isolate	Roger Vogelmann, TUM[a]
	2.937	Clinical isolate	Roger Vogelmann, TUM[a]
	2.947	Clinical isolate	Roger Vogelmann, TUM[a]
E. faecium	2.938	Clinical isolate	Roger Vogelmann, TUM[a]
	2.939	Clinical isolate	Roger Vogelmann, TUM[a]
	2.940	Clinical isolate	Roger Vogelmann, TUM[a]
	2.941	Clinical isolate	Roger Vogelmann, TUM[a]
	2.942	Clinical isolate	Roger Vogelmann, TUM[a]
	2.943	Clinical isolate	Roger Vogelmann, TUM[a]
	2.944	Clinical isolate	Roger Vogelmann, TUM[a]
	2.945	Clinical isolate	Roger Vogelmann, TUM[a]
	2.946	Clinical isolate	Roger Vogelmann, TUM[a]
	1.1045	Environmental isolate	This study

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Analysis of whole cell mass spectral profile by MALDI-TOF-MS

All isolates were grown for 20 h on Columbia sheep blood agar plates at 37 °C. Colonies were picked and resuspended in 300 μl of mutanolysin solution (20 u/ml) and incubated at 37 °C for 30 min. 900 μl of Ethanol abs. were added. The mixture was centrifuged at maximum speed for 2 min. After the supernatant was discarded, the pellet was centrifuged again. Residual Ethanol was completely removed by pipetting and the pellet was allowed to dry at room temperature. Subsequently 30 μl of formic acid (70%) were added and mixed with the pellet by vortexting. Next 30 μl of acetonitrile were added and mixed thoroughly. The solution was centrifuged at maximum speed for 2 min again and 1.5 μl of the supernatant were spotted on the MALDI target plate (Bruker Daltonics, Bremen, Germany) in two replicates. Direct after drying 1.5 μl of the matrix solution was added to each spot and allowed to air dry. The matrix used was a saturated solution of α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Bremen, Germany) dissolved in 50% acetonitrile

Table 2
PCR primers and products for detection of virulence determinants and CRISPR-associated genes.

Gene	Primer	Sequence (5′ to 3′)	PCR-Product length (bp)	Reference
ace	ace for ace rev	CCGAATTGAGCAAAAGTTC AGTGTAACGGACGATAA	746	This study
as	TE3 as rev	AAGAAAAAGAAGTAGACCAAC ACCTACAGCGTCCCAATCAC	923	[5] This study
cob	TE49 TE50	AACATTCAGCAAACAAAGC TTGTCATAAAGAGTGGTCAT	1405	[5]
cytolysin locus	cf cr	GGTTGCCATTGAAAAATATCTTCTAGTGGAGTATCCAGG GTGATTGATTGGCTTATTTCATCATCATCACTTTTGAGC	8364	[11]
efaAfs	TE5 TE6	GACAGACCCTCACGAATA AGTTCATCATGCTGTAGTA	705	[5]
efaAfm	TE 37 TE 38	AACAGATCCGCATGAATA CATTTCATCATCTGATAGTA	735	[5]
esp	esp for TE36	GAGTTAGCGGGAACAGGTCA GCGTCAACACTTGCATTGCCGAA	617	This study [5]
fsrB	fsrB for fsrB rev	GTTTGTCCCATCCATTGTCC TTTATTGGTATGCGCCACAA	346	This study
gelE	gelE for gelE rev	AATTGCTTTACACGGAACGG AGCCATGGTTTCTGGTTGTC	547	This study
sprE	sprE for sprE rev	CTTGTCTGCAAATGCAGAAG CGCCATTGGAATGAACACCA	660	This study
CRISPR1-*cas csn1*, homologue EFLG_01963	for rev	CAGAAGACTATCAGTTGGTG CCTTCTAAATCTTCTTCATAG	783	[28]
CRISPR1-*cas*	for rev	GCGATGTTAGCTGATACAAC CGAATATGCCTGTGGTGAAA	315	[28]
CRISPR2	for rev	CTGGCTCGCTGTTACAGCT GCCAATGTTACAATATCAAACA	Variable	[28]
CRISPR3-*cas csn1*, homologue EFKG_00787	for rev	GCTGAATCTGTGAAGTTACTC CTGTTTTGTTCACCGTTGGAT	258	[28]
CRISPR3-*cas*	for rev	GATCACTAGGTTCAGTTATTTC CATCGATTCATTATTCCTCCAA	224	[28]

(vol/vol) with 0.025% trifluoroacetic acid (vol/vol). Brukers Bacterial Test Standard (Bruker Daltonik GmbH, Bremen) was used as mass calibration standard. Samples were then processed in the MALDI-TOF-MS spectrometer (Microflex LT; Bruker Daltonics) with flex control software (Bruker Daltonics, Bremen, Germany). Each spectrum was obtained by averaging 240 laser shots acquired in the automatic mode at the minimum laser power necessary for ionization of the samples. The spectra were analysed in an m/z range of 2–20 kDa.

Data analysis was performed using LIMPIC for reduction of background noise and the baseline drift. Multiple spectra (6–8) of each strain were processed on the basis of the peak detection rate (PDR) of 0.6, expressed by the ratio between the number of spectra containing the considered peak and the total number of analysed spectra [25]. The obtained spectra were imported in BioNumerics™ and the cluster analysis was performed as described for the RAPD analysis.

Results

Occurrence of virulence determinants and CRISPR-cas genes

The virulence genes were detected in *E. faecalis* isolates only, with the exception of *esp*, the *Enterococcus* surface protein, which was present in 27 out of 52 *E. faecalis* isolates and also in 9 *E. faecium* strains. Ace, which encodes an adhesin protein in *E. faecalis* was found in 51 isolates and was the most frequent virulence gene found in the PCR tested strains. The gene encoding for endocarditis antigen EfaAfs was found in 34 *E. faecalis* strains whereas the equivalent of *E. faecium* (*efaAfm*) was present in all 10 *E. faecium* strain used in this study. The pheromone encoding gene *cob* was only present in 22 *E. faecalis* isolates and was one of the less frequent genes detected. The cytolysin operon was detected in 19 isolates, which all showed the phenotypic expression (β-haemolysis) on horse blood agar plates, only with one exception (TMW 2.928). The gelatinase gene (gelE) was present in 38 *E. faecalis* strains.

The distribution of CRISPR-associated (*cas*) genes was tested by the presence of following genes: CRISPR1-*cas csn1*, CRISPR3-*cas csn1* according to [28]. In parallel the absence of CRISPR1-cas and CRISPR3-cas loci was determined by the detection of the junction EF0672-0673 and EF1760-1759 [28]. The CRISPR2 locus was detected by primers targeting the conserved position. We detected a PCR-product in 47 *E. faecalis* strains.

Eighteen *E. faecalis* strains were *cas* positive (CRISPR1-*cas* and CRISPR3-*cas*) and negative for one of the junctions indicating the CRISPR loci appear in the locations between EF0672 and EF0673 (CRISPR1-*cas*) [3,28] or EF1760 and EF 1759 (CRISPR3-*cas*) as compared with the genome of V583 [28]. It was found that strains containing less virulence genes tended to have more frequently detected *cas* genes (see Table 3). However, a statistically significant correlation between the presence of CRISPR-cas loci and the absence of a virulence gene could only be determined for the cytolysin operon, *cob* encoding the pheromone, and *as* encoding the aggregation substance (p-value < 0.01; analysed by the Fisher exact test). According to Palmer and Gilmore [28] one CRISPR-*cas* locus was identified in three *E. faecium* genomes. However, this locus could not be detected in our ten *E. faecium* strains.

Gelatinase and cytolysin activity

A total of 24 strains showed a gelatinase phenotype that was only detected in isolates possessing *fsrB* and *gelE*. Two strains did not show a gelatinase phenotype although they possess *fsrB* and *gelE* indicating that these genes may be defective in their sequence (TMW 2.923, TMW 2.933). A distinct expression of a β-haemolytic activity was detected using horse blood agar plates under anaerobic conditions. Eighteen *E. faecalis* strains showed β-haemolysis.

RAPD-PCR analysis

The spectrum analysed by BioNumerics™ software, which uses UPGMA for comparative analysis derived five major groups of

Table 3
Summary of PCR screening results of virulence and CRISPR-associated genes. Strains are listed according to the number of detected virulence genes, from most present to less present virulence genes. Source of strains is marked: (a) clinical isolate, (b) environmental isolate and (c) food isolate (d) unknown. Strains marked with *, were tested *in silico* based on sequences published by Palmer and Gilmore (2010) with Broad Institute. For further explanations see text.

Strain	Virulence genes									CRISPR-*cas* and corresponding flanking regions			
	fsrB	*gelE*	*sprE*	*cytolysin operon*	*ace*	*efaAfs*	*as*	*esp*	*cob*	CRISPR 1-*cas*	CRISPR 1-*cas* flank	CRISPR 3-*cas*	CRISPR 3-*cas* flank
X98[b,*]	+	+	+	+	+	+	+	+	+	+			+
JH1[a,*]	+	+	+	+	+	+	+	+	+		+		+
2.929[a]	+	+	+	+	+	+	+		+		+		+
2.928[a]	+	+	+	+	+	+	+		+		+		+
2.927[a]	+	+	+	+	+	+	+		+		+		+
2.914[a]	+	+	+	+	+	+	+		+		+		+
2.915[a]		+	+	+	+	+	+	+	+		+		+
2.902[a]		+	+	+	+	+	+	+	+		+		+
T1[d,*]	+	+	+	+	+	+	+		+		+		+
Merz96[a,*]	+	+	+	+	+	+	+		+		+		+
HIP11701[a,*]		+	+	+	+	+	+	+	+		+		+
2.930[a]	+	+	+	+	+	+	+				+		+
2.908[a]	+	+	+	+	+	+	+				+		+
2.907[a]	+	+	+	+	+	+			+		+		+
2.926[a]	+	+	+	+	+		+		+		+		+
2.901[a]	+	+	+		+	+	+		+		+		+
2.630[c]	+	+	+		+	+	+	+		+			+
2.648[c]	+	+	+		+	+	+	+			+		+
2.924[a]			+	+	+	+	+	+	+		+		+
2.905[a]			+	+	+	+	+	+	+		+		+
2.922[a]		+	+	+	+	+	+	+			+		+
CH188[a,*]	+	+	+		+	+		+	+		+		+
T2[a,*]		+	+	+	+	+		+	+		+		+
T8[a,*]		+	+	+	+	+		+	+		+		+
T11[a,*]	+	+	+		+	+	+		+		+	+	
D6[b,*]				+	+	+	+	+	+	+			+
AR01/DG[b,*]	+	+	+		+	+	+		+		+		+
2.947[a]	+	+	+	+	+		+				+		+
2.354[b]	+	+	+		+		+		+		+		+
2.852[a]	+	+	+		+		+		+		+		+
2.815[b]	+	+	+		+		+		+		+		+
2.629[c]	+	+	+		+		+	+		+			+
2.921[a]	+	+	+		+	+		+		+			+
2.936[a]	+	+	+		+	+	+				+		+
2.798[b]	+	+	+		+			+	+	+			+
2.911[a]			+	+	+	+	+	+			+		+
2.932[a]		+	+	+	+		+	+			+		+
2.63[c]		+	+	+	+		+		+		+		+
2.900[a]		+	+		+	+		+	+		+		+
2.910[a]		+	+		+	+		+	+		+		+
2.925[a]	+		+		+	+		+	+		+		+
2.906[a]	+		+		+	+		+	+	+			+
2.937[a]	+		+		+	+	+	+		+			+
2.136[b]	+		+		+		+	+	+	+			+
T3[a,*]	+	+	+		+	+			+		+		+
E1Sol[b,*]	+	+	+		+	+			+	+			+
Fly1[b,*]	+	+	+		+	+			+		+		
2.912[a]	+	+	+		+	+				+			+
2.916[a]	+	+	+		+	+					+	+	
2.919[a]	+	+	+		+	+				+			+
2.923[a]	+	+	+		+	+				+			+
2.933[a]	+	+	+		+			+		+			+
2.931[a]		+	+		+	+		+		+			+
2.917[a]		+	+		+	+		+		+			+
2.918[a]		+	+		+	+		+		+			+
2.913[a]		+	+		+	+		+			+		+
2.934[a]			+	+	+		+	+			+		+
2.904[a]			+	+	+	+		+			+		+
2.903[a]			+		+	+	+		+		+		+
ATCC4200[a,*]		+	+			+		+	+	+			+
DS5(ATCC14508)[d,*]				+	+	+		+	+	+			+
2.622[b]	+	+	+		+					+			+
2.520[b]	+	+	+		+					+			+
2.647[c]		+	+		+		+			+			+
2.935[a]	+		+		+		+				+		+
2.645[a]			+		+		+	+			+		+
2.909[a]					+	+		+			+		+
2.777[b]							+				+		+

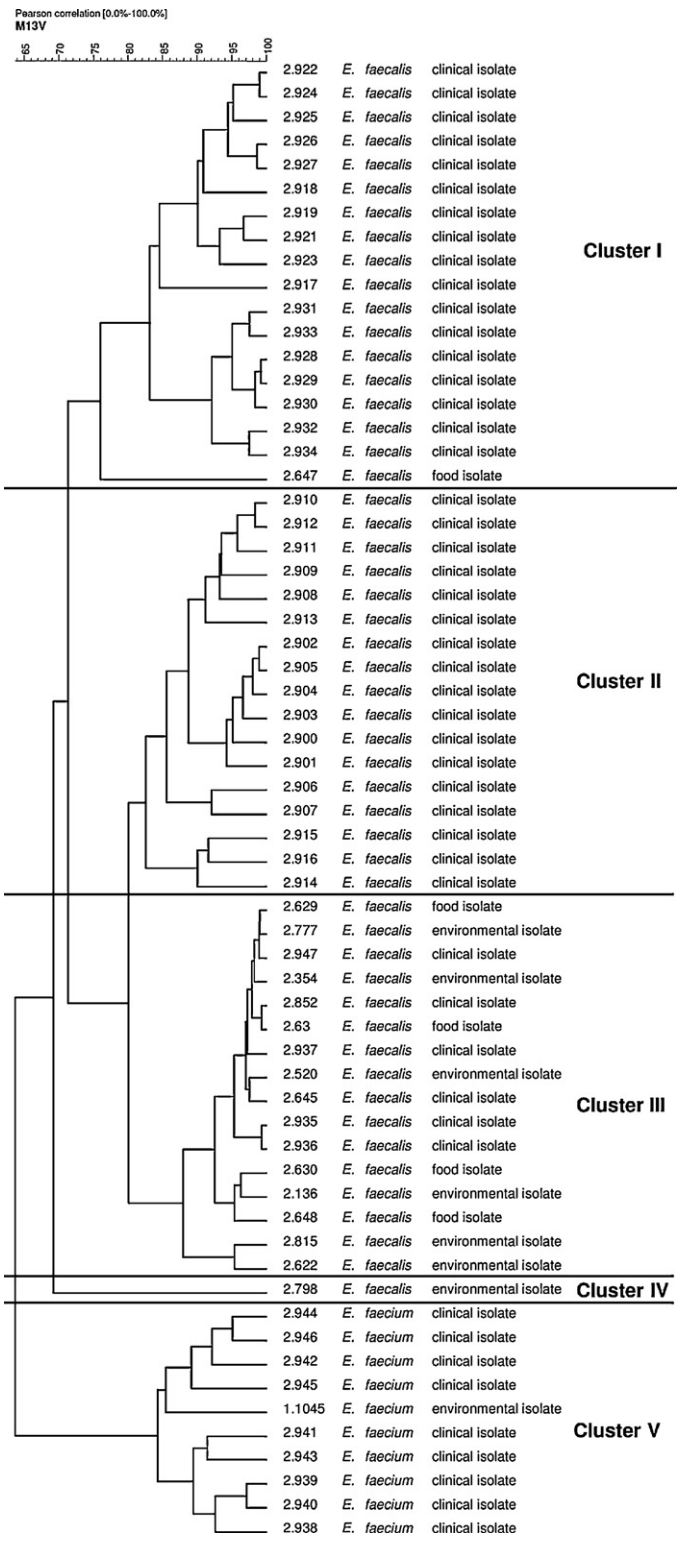

Fig. 1. Cluster analysis of genetic fingerprints of 52 *E. faecalis* isolates and 10 isolates of *E. faecium* generated by the use of RAPD-PCR. At a cut off level of 65% a species-specific clustering is shown (Cluster I–IV vs. Cluster V). Similarity coefficients were calculated using the method of Pearson, and the clustering was calculated using UPGMA approach.

enterococci (see Fig. 1I–V). At a cut-off level of 65% species-specific differentiation of *E. faecalis* and *E. faecium* was achieved. While cluster III contained strains of various sources of isolation, the RAPD-PCR fingerprint using the M13 V primer (cut-off level 70%) generated two clusters (I and II), which almost exclusively contained clinical isolates, with only one exception in cluster I. Further, one environmental isolate (TMW 2.798–designated as cluster IV) showed a RAPD-PCR fingerprint, which did not match any of the generated groups. Cluster V contained all *E. faecium* strains.

MALDI-TOF-MS analysis

The generated MALDI-TOF-MS spectra were analysed by BioNumerics™ software, which uses UPGMA for comparative analysis. For the analysis only peaks that had a PDR of 0.6 were included. This method clearly differentiated at strain level, and a general clustering in five main groups was only feasible under a cut-off level of 10% (Fig. S1). Within these groups (I–IV) the isolates showed neither a relation of their phenotype of gelatinase or β-heamolysis nor any relation to the origin of isolation. Even on species-specific basis one exception (TMW 2.942) appeared as an independent group (see Fig. S1: III).

Discussion

In this study, *E. faecalis* and *E. faecium* strains of diverse origin were typed by the use of RAPD-PCR, MALDI-TOF-MS, distribution of established virulence factors and presence of CRISPR elements/*cas* genes in search of a rationale for safety evaluation of strains to be used in food related applications. While distribution of most virulence factors, origin of isolation and typing patterns did not reveal any rationale for *E. faecalis* strain safety evaluation, the occurrence of CRISPR elements versus reduced number of (specific) virulence factors suggests itself to promising candidate.

The application of MALDI-TOF-MS analysis has been used to identify bacteria on genus, species [30] and strain level [29]. The identification and characterisation of *Enterococcus* isolates in accordance with their origin using the MALDI-TOF-MS has been subject of recent studies [12,33]. In contrast to those studies our clustering of the isolates according to their MALDI-TOF-MS profiles did not differentiate according to their origin of isolation. MALDI-TOF-MS profiles and RAPD fingerprints enabled distinction at species level, accounting for the validity of the method, and RAPD discriminated even close to strain level. The occurrence of virulence genes or the cas genes showed no distinct distribution neither within the groups of MALDI-TOF-MS clustering nor within the groups of RAPD-PCR clustering. While distribution of strains with various origins was random in MALDI-TOF-MS profiles, two RAPD clusters nearly exclusively contained strains of clinical origin. This may indicate some role of a diseased host in selecting for specific types or some genotypes causing clinical infections with an increased probability, while the biodiversity of *E. faecalis* is broader, also containing commensal strains. In this context it is interesting to note that these isolates clustering in together in RAPD analysis were isolated from patients suffering from different diseases (e.g. renal insufficiency, newborns with infection, patients with pneumonia, endocarditis, bacteraemia, sepsis, and patients with different kinds of cancer).

The tabulation of the sixty-two *Enterococcus* isolates showed a multiple number of detected virulence genes, mostly within *E. faecalis* strains, whereas the *E. faecium* isolates were devoid of these virulence genes except for *esp* and *efaAfm*. This finding corroborates the results of Eaton and Gasson [5] and Coque et al. [4] in which *esp* appears to be more frequent in *E. faecium* than in *E. faecalis*. Generally, studies have identified numerous virulence factors such as gelatinase, collagen-binding protein, enterococcal endocarditis antigen A or β-haemolysin, although there is no clear association of a single gene or origin of isolation with pathogenicity [5,15,26]. The most investigated and clearly proven virulence traits (not only

in enterococci) are gelatinase and β-haemolysin, which were phenotypically tested on corresponding agar plates in this study. The majority of positively tested phenotypes is comprised from clinical isolates, which is equivalent to other studies [24,34]. Still, the link to the source of isolation may not be as decisive as all isolates could also be simply considered as "faecal" or "intestinal" and virulence genes are a common trait in *Enterococcus* from any source.

The results of the β-haemolytic activity were more intense by the use of horse blood and the application of anaerobic incubation conditions. These results are in agreement with the data of Gaspar et al. [11], which verified a similar sensitivity of horse and human erythrocytes against cytolysin produced by enterococci. The incidence of this virulence trait could only be detected in *E. faecalis* (18 strains), which conforms to other studies that showed a higher incidence of β-haemolysin in this *Entercoccus* species [4,9]. Despite the fact that 17 out of the 18 positive strains were from clinical origin, a correlation could not be found in the clustering of RAPD or MALDI-TOF-MS.

The prevalence of gelatinase activity among all isolates was assessed, and 24 strains possessed this phenotype. Out of the six food isolates three showed a positive gelatinase phenotype, which was unexpected because of low numbers of food-associated strains in comparison to the number of the tested isolates from clinical origin. Mostly, gelatinase-positive food strains are originated from cheese, and possibly this is due to the protein-rich environment to utilize cheese protein as a source of amino acids [9].

Taken together, we suggest that any *E. faecalis* isolate should just be considered as intrinsically "faecal" irrespective of the specific source of isolation for a single strain, which appears as rather irrelevant in judging its virulence.

The CRISPR system is a diverse defence mechanism towards invading nucleic acids. CRISPR motives are typically located near CRISPR-associated (*cas*) genes [20,39].

No *cas* genes could be detected in the tested *E. faecium* isolates. *E. faecium* is generally judged as the less virulent species as compared to *E. faecalis*, and all strains of this species typically contain much less of the established *Enterococcus* virulence factors. This should preferably be attributed to a different lifestyle of this species or other mechanisms limiting virulence factor presence or acquisition may be involved.

Recently, a significant correlation of the absence of CRISPR-*cas* loci and the presence of antibiotic-resistance genes was reported for *E. faecalis* [28]. We could not find such a general inverse correlation between *cas* and genes encoding virulence traits, which is probably due to the fact that there is no selective force for the virulence traits as it can be expected from the application of antibiotics towards selection of antibiotic resistance. However, we found that the distribution of *cas* genes correlates inversely with the presence of the cytolysin operon, *cob* and *as*. The cytolysin operon, *cob* and *esp* are reported to reside on the same pathogenicity island, which was found in large, pheromone-responsive plasmids or in the chromosome [35,36], however, we could not find a respective correlation for *esp*. The *cob* and *as* genes promote pheromone production and aggregation promotion, respectively, and thus enhance the probability of genetic exchange. This negative correlation shows that counteraction of a CRISPR-*cas* defence system versus enhanced genetic flexibility in the presence of *cob* and *as* is minimized in these strains, and suggests that CRISPR-*cas* has a role in acquisition prevention of (parts of) the respective pathogenicity island. The absence of virulence factors and also CRISPR-*cas* probably results from the fact that some strains did not at all have the opportunity to pick up these virulence factors from others. While the molecular background is yet to be confirmed by demonstration of active CRISPR-*cas* systems, this view is further supported by the finding that *cob* and *as* negative strains generally have less virulence traits. Thus, testing for absence of the cytolysin operon, *cob* and *as* in a cas positive environment suggests itself as promising for strain evaluation towards minimizing risks in food fermentation or use as probiotics.

Acknowledgments

We thank Ulrich Busch, Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit, for the possibility to use the MALDI-facility in his lab, and Roger Vogelmann (Technische Universität München, Germany), Gerhard Reuter (Freie Universität Berlin, Germany), Barbara Murray (University of Texas, USA), and Michael S. Gilmore, Harvard University, Cambridge, USA for providing strains. We appreciate the sharing of information on CRISPR in the genomes of enterococci by Michael S. Gilmore prior to publication. This work was supported by GRK 1482 of the German Research Foundation (DFG).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.syapm.2011.05.002.

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