Frequency of *ace*, *epa* and *elrA* Genes in Clinical and Environmental Strains of *Enterococcus faecalis*

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Abstract Surface proteins play an important role in the pathogenesis of enterococcal infections. Some of them are candidates for a vaccine, e.g., the frequency of endocarditis in rats vaccinated with Ace protein was 75 % as opposed to 100 % in those who weren't. However, there are other components of enterococcal cells, such as Epa antigens or internalin-like proteins, which may be used in the prophylaxis of infections caused by them. However, also other virulence factors and resistance to antibiotics are important during enterococcal infection. Therefore, the relevance of *ace*, *epa*, *elrA*, other virulence genes, as well as resistance to antibiotics was investigated. 161 *Enterococcus faecalis* strains isolated from teaching hospitals in Lodz, cultured according to standard microbiological methods, were investigated for the presence of genes encoding surface proteins by PCR. Results were analyzed with χ^2 test. The *elrA* gene was found in all clinical and environmental strains, the *ace* gene was also widespread among *E. faecalis* (96.9 %). Both tested *epa* genes were found in the majority of isolates (83.25 %). There was correlation between the presence of *esp* and *ace* genes ($p = 0.046$) as well as between *epa* and *agg* genes ($p = 0.0094$; χ^2 test). The presence of the genes encoding surface proteins investigated in our study in the great majority of isolates implies that they would appear to be required during *E. faecalis* infection. Therefore, they could be excellent targets in therapy of enterococcal infections or, as some studies show, candidates for vaccines.

Keywords *Enterococcus faecalis* · Virulence factors · *ace* · *epa* · *elrA*

Introduction

Enterococci belong to the natural flora of human and animal intestines. Two species in particular, *E. faecalis* and *E. faecium*, have emerged as opportunistic pathogens causing a wide range of hospital-acquired infections [1]. Their resistance to several groups of antibiotics is responsible for treatment failure in many cases. Additionally, many important virulence factors have been identified in the enterococcal genome so far. They may play a role in the pathogenesis at different stages: in taking part in the initial attachment, in destroying tissues, in acquiring resistance to commonly used drugs as well as in avoiding immune system response. However, many of these processes are not fully understood.

One of the virulence factors of enterococci is Ace protein (Adhesin to collagen of *E. faecalis*), found commonly among *E. faecalis* strains [2]. It was shown that Ace mediates in the attachment of *E. faecalis* cells to human collagen type IV, bovine and rat collagen type I, laminin of mice and human dentin [3]. Expression of Ace was induced in vivo by the presence of serum or by compounds of extracellular matrix proteins (ECM) [4]. It was shown that diverse strains of *E. faecalis* synthesize Ace while infecting humans and Ace presented properties of an antigen in vivo. It is suggested that the Ace protein may be the target for drugs, because *E. faecalis* strains causing UTI often had the *ace* gene [5]. However, Ace protein was not necessary to cause this disease [6].

The other important virulence factors of *E. faecalis* strains are polysaccharide antigens [7]. They are encoded

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by the “*orfde1*” to “*orfde16*” genes found in the cluster of enterococcal polysaccharide antigen (*epa*) genes. It was suggested that *orfde4* (*epaB*) and *orfde6* (*epaE*), but not *orfde1* or *orfde2*, are necessary for polysaccharide synthesis [8]. *orfde4* (*epaB*), *orfde6* (*epaE*) mutants showed disturbed biofilm synthesis [9] and were vulnerable to attack by polynuclear neutrophils [10]. What is more, those mutants were also attenuated in a mice peritonitis model [8], and *E. faecalis* TX5179 mutants were also attenuated in a urinary tract infection model in mice [11].

Bacteria have evolved a plethora of molecular mechanisms to adhere to and invade host cells and tissues. Some important virulence factors, such as the proteins of *Listeria monocytogenes*, share a common leucine-rich repeat (LRR) domain which interact with host cell receptors and induce endocytosis and dissemination in tissues [12]. Some novel internalin-like proteins named Elr were found in *E. faecalis* strains. Inactivation of the *elrA* gene significantly reduced virulence in a mouse peritonitis model and dissemination within tissues as well as elicited the inflammatory interleukin-6 response [13].

The aim of our work was to evaluate the occurrence of *ace*, *elrA* and *epa* genes among clinical and environmental *E. faecalis* strains. Additionally, we checked the possible relationship between the occurrence of those genes and the presence of *agg*, *cylLS*, *esp*, *gelE*, *sprE* genes, as well as the susceptibility of enterococcal strains to antibiotics.

Materials and Methods

Enterococcus faecalis strains ($n = 161$) were isolated from operative wards in two hospitals of Lodz, Poland. Isolates were obtained from urology ward ($n = 66$), surgical ward ($n = 40$), internal medicine ward ($n = 31$), laryngology ($n = 11$), intensive care unit ($n = 8$) and outpatient clinic ($n = 5$). Strains were isolated from patient samples (wound swabs $n = 37$, urine $n = 54$, bile $n = 1$, blood $n = 1$, peritoneum exudates $n = 1$, throat swabs $n = 9$, groin swabs $n = 2$, swabs from catheters $n = 14$ and central venous catheters $n = 2$), swabs from healthcare staff (hands, white coats, nose, throat $n = 14$ strains), swabs from medical equipment and unanimated surfaces ($n = 25$ strains).

Enterococci were cultured according to standard microbiological methods. Bacteria were identified by API 20 Strep tests (bioMérieux), then identification was confirmed by PCR method for D-alanine-D-alanyl ligase (*ddl*) [14]. The presence of the *ace* gene was tested according Mannu et al. [15]. Reaction for *epa* genes was carried out in 25 μ l according to Teng et al. [10] with primers for *orfde5*, and for *orfde6*. The reaction mixture consisted of 0.5 U Hypernova polymerase (DNA Gdańsk), 600 nM of

primers (IBB, Warsaw) [10], 2 mM MgCl₂ (DNA Gdańsk), 2.5 μ l PCR buffer and 400 μ M dNTP (Fermentas). The presence of the *elrA* gene was checked with primers (for *E. faecalis* V583 EF_2686): 5' ACAGGTTGGACGA CTGTTCC 3' and 5' CTATTTCACCGCTGACGAT 3' (product size, 161 bp). The PCR profile used was as follows: denaturation 94 °C, 5 min; 38 cycles of denaturation 94 °C, 40 s; annealing 58 °C, 40 s; 72 °C, 40 s, and final elongation 72 °C, 5 min. The PCR profiles for *agg*, *cyl*, *esp*, *gelE*, *sprE* were described previously [14, 16]. PCR products were separated on 1 % agarose (Prona) in 1 $\times$ TAE (Fermentas), and stained with ethidium bromide (Sigma). A statistical analysis of the relationship between *ace* and *epa*, *agg*, *cylLS*, *esp*, *gelE*, *sprE* [16] and resistance to ampicillin, ciprofloxacin, gentamicin, erythromycin, linezolid and vancomycin was prepared by a χ^2 test.

Enterococcus faecalis ATCC 29212 and *Enterococcus faecium* ATCC 35667 strains were used as controls. Standard and clinical strains were stored in –70 °C for further investigations. Susceptibility testing was carried out by disc-diffusion method on Mueller–Hinton II Agar (bioMérieux) for ampicillin (10 μ g), ciprofloxacin (5 μ g), erythromycin (15 μ g), and gentamicin (120 μ g) [1] (Becton–Dickinson). Results were interpreted according to *Clinical and Laboratory Standard Institute* guideline (CLSI) [17] and National Institute of Health, Poland [18].

Results

Only five *E. faecalis* strains did not have the *ace* gene (3.1 %) and these were isolated from environmental samples ($n = 2$), wounds ($n = 2$) or urine ($n = 1$). There were no isolates resistant to ampicillin among strains without the *ace* gene. There was correlation between the presence of *esp* and *ace* genes ($p = 0.046$; χ^2 test).

Both *epa* genes were found in 136 enterococcal strains (84.5 %). Strains without the genes (*epa 5*, *epa 6*, or both) were obtained from urine ($n = 7$), wounds ($n = 2$), swabs from medical staff ($n = 1$), environmental swabs ($n = 2$), catheters ($n = 3$). Table 1 shows detailed results concerning *ace* and *epa* gene occurrence. All enterococcal isolates possessed the *elrA* gene.

All the investigated strains were susceptible to vancomycin and linezolid. Among enterococcal isolates without at least one *epa* gene all were also susceptible to ampicillin, but eight were resistant to ciprofloxacin, five moderately resistant to ciprofloxacin, seven resistant to erythromycin, three moderately resistant to erythromycin, two resistant to gentamycin. Table 2 presents data about the relationship of *ace* and *epa* gene frequency and resistance to antibiotics. There were seven strains isolated from urine that not possessed one *epa* gene but all isolates from urine had *ace*

Table 1 Occurrence of *ace* and *epa* genes

Sample	Number of isolates	Gene	
		<i>ace</i> (+) (n)	<i>epa</i> (+) (n)
Urine	54	53	47
Wound	37	35	35
Blood	1	1	1
Bile	1	1	1
Peritoneum exudate	1	1	1
Central venous catheters	2	2	2
Epidemiological swabs	28	28	25
Swabs from medical staff	14	14	13
Environmental swabs	25	23	23
Together	161	156	134

gene. Three strains from catheters were without one *epa* gene but had *ace* gene. Table 3 shows data about the presence of particular virulence genes. A correlation was found between the presence of *epa* and *agg* genes ($p = 0.0094$; χ^2 test).

Discussion

Enterococci may possess diverse genes, which make them more virulent [16, 19]. It was shown that clinical strains of *E. faecalis* are more likely to present virulence genes than *E. faecium* strains. Of these, *ace* is the gene which participates in the pathogenesis of enterococcal infections at the stage of adhesion to human cells [19]. It was shown that strains of *E. faecalis* with *ace* gene were often isolated from urinary tract infections (UTI) [20]. In the present study we found the *ace* gene in the majority of tested

strains of *E. faecalis* ($n = 156$; 96.9 %), both in clinical and environmental strains. Over 90 % of isolates obtained from urine had the *ace* gene, which supports its role in the pathogenesis of UTI.

Ace protein has often been found in the teeth roots of endodontic patients, but the frequency of isolation was diverse, probably because of the clinical and geographical background [21, 22]. In the present study, although there were no isolates from persistent endodontic infections, all strains isolated from the oral cavity ($n = 16$) had the *ace* gene, and all but one of them possessed the *epa* gene (6.25 %). Therefore the presence of enterococcal strains in the oral cavity which have genes crucial for adhesion could be associated with those infections. Our work strongly supports those results.

In our study, we checked the frequency of two genes from the *epa* cluster (*epa5*, *epa6*) among 161 strains of *E. faecalis*. Our results confirm the common presence of *epa* genes among enterococci, but in a lower number of strains when compared with the literature. Malathum et al. [23] showed that the *orfde4* to *orfde10* *epa* genes were in all the 12 genotypically and geographically diverse strains of *E. faecalis* investigated in their study. In our work, *epa* genes (at least one) were found in 136 enterococcal strains (84.5 %). The presence of these genes appears to facilitate infection by sheltering infectious agents from killing by polymorphonuclear neutrophils, probably as a result of resistance to phagocytosis, or increased ability to survive within phagosomes [10]. Our results show that most strains had at least one of the investigated genes, only two of them having none (1.24 %; environmental swab and swab from wound). Both of the isolates had no other virulence genes and were sensitive to all but one of the tested antibiotics. Those results seem to support the theory that *epa* genes are

Table 2 Occurrence of *ace*, *epa* genes and resistance to ampicillin, ciprofloxacin, gentamicin and erythromycin

	Ampicillin		Ciprofloxacin			Gentamicin			Erythromycin		
	S (n)	R (n)	S (n)	I (n)	R (n)	S (n)	I (n)	R (n)	S (n)	I (n)	R (n)
<i>Ace</i> (+)	146	10	29	61	66	80	6	70	45	30	81
<i>Ace</i> (−)	4	1	1	0	4	3	0	2	2	2	1
<i>epa</i> (+)	135	11	33	51	62	73	6	67	45	28	73
<i>epa</i> (−)	15	0	3	4	8	10	0	5	5	2	8

S sensitive strain, I intermediate sensitive strain, R resistant strain

Table 3 Occurrence of *ace*, *epa* genes and other genes

	Strains with <i>agg</i> gene (n)	Strains with <i>cylL</i> gene (n)	Strains with <i>cylS</i> gene (n)	Strains with <i>esp</i> gene (n)	Strains with <i>gelE</i> gene (n)	Strains with <i>sprE</i> gene (n)
Strains with <i>ace</i> gene (n)	100	82	83	112	134	130
Strains with <i>epa</i> gene (n)	98	78	78	103	122	121

required during infection. Isolates without *epa* genes could be noninvasive environmental strains. However that area of our study is limited and further investigation of expression of those genes is needed to confirm its role in human infections.

According to previous studies, the *elrA* gene is widespread among *E. faecalis* strains and its product is required during infection in a mouse peritonitis model. Therefore ElrA may play an important role in the pathogenesis of enterococcal infection. In our studies, the *elrA* gene was found in all strains investigated, which is in accord with previous studies [13] and means that that gene is necessary during enterococcal pathogenesis. However, further investigation is required to establish whether and how that protein is expressed by diverse clinical and environmental strains.

We also investigated some relationship between the occurrence of virulence genes among enterococcal strains and their resistance to antibiotics. *E. faecalis* isolates are usually sensitive to ampicillin [1]. In our study, there was no significant relationship between the occurrence of *epa* genes and resistance to ampicillin. Enterococci are also very often resistant to fluoroquinolones [1, 24]. Among investigated enterococcal strains resistant to ciprofloxacin ($n = 74$), there were only four without the *ace* gene (5.40 %). However, there was no correlation found between the presence of *ace* or *epa* genes and resistance to ciprofloxacin nor gentamicin which can be used in therapy of enterococcal infections. The only correlation ($p = 0.0124$) was shown for erythromycin and the presence of *epa* genes, which might suggest simultaneous transfer of those genes.

The possibility of horizontal transfer of genes very often is characteristic of hospital pathogens. Pathogenicity Islands (PAIs) discovered in *E. faecalis* isolates enable these bacteria to spread virulence genes as well as resistance genes among each other [25]. It would be interesting to see whether there is any correlation between occurrence of *epa* and *ace* genes and other virulence genes (for aggregation substance, cytolysin, Esp protein, gelatinase and serine protease). Therefore, it is suggested that these virulence factors may occur altogether and be transferred between *Enterococcus* spp. strains [26]. In our study, *agg* genes were found in 101 of 161 *E. faecalis* strains (62.7 %) [16] and there was significant difference between the occurrence of *agg* and *epa* genes ($p = 0.0094$) and *ace* and *esp* genes ($p = 0.046$), which may suggest their common transfer.

To summarize, the presence of the genes encoding surface proteins investigated in our study in the great majority of isolates implies that they would appear to be required during *E. faecalis* infection. As our and previous studies show, investigated genes are widespread. Therefore, they could be excellent targets in therapy of enterococcal infections or, candidates for vaccines. The

development of such a vaccine would be especially important in case of infections caused by multidrug resistant strains of *E. faecalis*.

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Conflicts of interest The authors declare that there are no conflicts of interest.

References

1. Łysakowska M, Denys A (2008) Susceptibility to antimicrobial agents and genotypic relatedness of enterococcal strains isolated from patients and hospital environment. *Med Dośw Mikrobiol* 60:19–26
2. Duh RW, Singh KV, Malathum K, Murray BE (2001) In vitro activity of 19 antimicrobial agents against enterococci from healthy subjects and hospitalized patients and use of an *ace* gene probe from *Enterococcus faecalis* for species identification. *Microb Drug Resist* 7:39–46
3. Nallapareddy S, Qin X, Weinstock GM, Hook M, Murray BE (2000) *Enterococcus faecalis* adhesin, ace, mediates attachment to extracellular matrix proteins collagen type IV and laminin as well as collagen type I. *Infect Immun* 68:5218–5224
4. Nallapareddy SR, Murray BE (2008) Role played by serum, a biological cue, in the adherence of *Enterococcus faecalis* to extracellular matrix proteins, collagen, fibrinogen, and fibronectin. *J Infect Dis* 197:1728–1736
5. Lebreton F, Riboulet-Bisson E, Serrero P, Sanguinetti M, Posteraro B, Torelli R, Hartke A, Auffray Y, Giard JC (2009) *ace*, Which encodes an adhesin in *Enterococcus faecalis*, is regulated by Ers and is involved in virulence. *Infect Immun* 77:2832–2839
6. Singh KV, Nallapareddy SR, Sillanpää J, Murray BE (2010) Importance of the collagen adhesin ace in pathogenesis and protection against *Enterococcus faecalis* experimental endocarditis. *PLoS Pathog* 6:e1000716. doi:10.1371/annotation/1ccae8f8-d274-4ff8-a295-815037ce9cc6
7. Teng F, Singh KV, Bourgonne A, Zeng J, Murray BE (2009) Further characterization of the *epa* gene cluster and *epa* polysaccharides of *Enterococcus faecalis*. *Infect Immun* 77:3759–3767
8. Xu Y, Singh KV, Qin X, Murray BE, Weinstock GM (2000) Analysis of a gene cluster of *Enterococcus faecalis* involved in polysaccharide biosynthesis. *Infect Immun* 68:815–823
9. Mohamed JA, Huang W, Nallapareddy SR, Teng F, Murray BE (2004) Influence of origin of isolates, especially endocarditis isolates, and various genes on biofilm formation by *Enterococcus faecalis*. *Infect Immun* 72:3658–3663
10. Teng F, Jacques-Palaz KD, Weinstock GM, Murray BE (2002) Evidence that the enterococcal polysaccharide antigen gene (*epa*) cluster is widespread in *Enterococcus faecalis* and influences resistance to phagocytic killing of *E. faecalis*. *Infect Immun* 70:2010–2015
11. Singh KV, Lewis RJ, Murray BE (2009) Importance of the *epa* locus of *Enterococcus faecalis* OG1RF in a mouse model of ascending urinary tract infection. *J Infect Dis* 200:417–420
12. Hamon M, Bierne H, Cossart P (2006) *Listeria monocytogenes*: a multifaceted model. *Nat Rev Microbiol* 4:423–434

13. Brinster S, Posteraro B, Bierne H, Alberti A, Makhzami S, Sanguinetti M, Serror P (2007) Enterococcal leucine-rich repeat-containing protein involved in virulence and host inflammatory response. *Infect Immun* 75:4463–4471
14. Kariyama R, Mitsuata R, Chow J, Clewell DB, Kumon H (2000) Simple and reliable multiplex PCR assay for surveillance isolates of vancomycin-resistant enterococci. *J Clin Microbiol* 38:3092–3095
15. Mannu L, Paba A, Daga E, Comunian R, Zanetti S, Dupre I, Sechi LA (2003) Comparison of the incidence of virulence determinants and antibiotic resistance between *Enterococcus faecium* strains of dairy, animal and clinical origin. *Int J Food Microbiol* 88:291–304
16. Łysakowska M, Śmigielski J, Denys A (2009) Occurrence of virulence genes among *Enterococcus faecalis* strains isolated from patients and hospital environment. *Med Dośw Mikrobiol* 61:125–132
17. Clinical and Laboratory Standard Institute (CLSI) (2009) Performance standards for antimicrobial susceptibility testing: eighteenth informational supplement. CLSI document M100-S18. Clinical and Laboratory Standard Institute, Wayne, PA
18. Hryniewicz W, Sulikowska A, Szczypa K, Skoczyńska A, Łuczak-Kadłubowska, Gniatkowski M (2006) Rekomendacje doboru testów do oznaczania wrażliwości bakterii na antybiotyki i chemioterapeutyki, 1st edn. National Institute of Public Health, Warsaw, pp 20–22
19. Abriouel H, Omar NB, Molinos AC, López RL, Grande MJ, Martínez-Viedma P, Ortega E, Cañamero MM, Galvez A (2008) Comparative analysis of genetic diversity and incidence of virulence factors and antibiotic resistance among enterococcal populations from raw fruit and vegetable foods, water and soil, and clinical samples. *Int J Food Microbiol* 31:38–49
20. Johansen TE, Cek M, Naber KG, Stratchounski L, Svendsen MV, Tenke P (2006) Hospital acquired urinary tract infections in urology departments: pathogens, susceptibility and use of antibiotics. *Int J Antimicrob Agents* 1(Suppl 1):91–107
21. Reynaud af Geijersstam AH, Ellington MJ, Warner M, Woodford N, Haapasalo M (2006) Antimicrobial susceptibility and molecular analysis of *Enterococcus faecalis* originating from endodontic infections in Finland and Lithuania. *Oral Microbiol Immunol* 21:164–168
22. Balaei-Gajan E, Shirmohammadi A, Abashov R, Agazadeh M, Faramazie M (2010) Detection of *Enterococcus faecalis* in subgingival biofilm of patients with chronic refractory periodontitis. *Med Oral Patol Oral Cir Bucal* 15:e667–e670
23. Malathum K, Singh KV, Weinstock GM, Murray BE (1998) Repetitive sequence-based PCR versus pulsed-field gel electrophoresis for typing of *Enterococcus faecalis* at the subspecies level. *J Clin Microbiol* 36:211–215
24. Piekarska K, Gierczyński R, Ławrynowicz-Paciorek M, Kochman M, Jagielski M (2008) Novel gyrase mutations and characterization of ciprofloxacin-resistant clinical strains of *Enterococcus faecalis* isolated in Poland. *Pol J Microbiol* 57:121–124
25. Shiono A, Ike Y (1999) Isolation of *Enterococcus faecalis* clinical isolates that efficiently adhere to human bladder carcinoma T24 cells and inhibition of adhesion by fibronectin and trypsin treatment. *Infect Immun* 67:1585–1592
26. Smedo T, Almeida Santos M, Martins P, Silva Lopes MF, Figueiredo Marques JJ, Tenreiro R, Barreto Crespo MT (2003) Comparative study using type strains and clinical and food isolates to examine hemolytic activity and occurrence of the *cyl* operon in enterococci. *J Clin Microbiol* 41:2569–2576