RESEARCH ARTICLE

Distribution of antimicrobial resistance determinants, virulence-associated factors and clustered regularly interspaced palindromic repeats loci in isolates of Enterococcus faecalis from various settings and genetic lineages

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One sentence summary: High-risk clonal complexes of Enterococcus faecalis are limited to the hospital settings and play an important role in the burden of hospital infections caused by these bacteria.

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ABSTRACT

Enterococcus faecalis represents an important factor of hospital-associated infections (HAIs). The knowledge on its evolution from a commensal to an opportunistic pathogen is still limited; thus, we performed a study to characterise distribution of factors that may contribute to this adaptation. Using a collection obtained from various settings (hospitalised patients, community carriers, animals, fresh food, sewage, water), we investigated differences in antimicrobial susceptibility, distribution of antimicrobial resistance genes, virulence-associated determinants and phenotypes, and CRISPR loci in the context of the clonal relatedness of isolates. Bayesian Analysis of Population Structure revealed the presence of three major groups; two subgroups comprised almost exclusively HAI isolates, belonging to previously proposed enterococcal high-risk clonal complexes (HiRECCs) 6 and 28. Isolates of these two subgroups were significantly enriched in antimicrobial resistance genes, presumably produced a polysaccharide capsule and often carried the aggregation substance asa1; distribution of other virulence-associated genes, such as esp and cly, formation of a biofilm and gelatinase production were more variable.
INTRODUCTION

Enterococci commonly colonise the gastrointestinal tract of humans and animals, where they constitute a part of the natural microbiota and are found in plants, soil, water, sewage and food as well. However, these bacteria represent nowadays important opportunistic pathogens in hospital-associated infections (HAIs) (Tendolkar, Bagdhanay and Shankar 2003), with Enterococcus faecalis as the most common species responsible for both invasive (bacteraemia, endocarditis, peritonitis) as well as non-invasive infections, affecting the urinary tract and post-surgery wounds. The understanding of pathogenesis of E. faecalis is still far from being complete. The most studied virulence factors include enterococcal surface protein Esp, aggregation substance (AS), so-called MSCRAMMs (microbial surface components recognising adhesive matrix molecules), pili, and secretory proteins, such as cytolysin and gelatinase (Hendrickx et al. 2009; Sava, Heikens and Huebner 2010). Enterococci, as organisms adapted to various ecological niches, show remarkable ability to survive unfavourable conditions, such as high or low temperature, broad pH values, high salinity and presence of harmful compounds, e.g. disinfectants (Sherman 1937). Moreover, E. faecalis is naturally non-susceptible to multiple antimicrobial agents, such as cephalosporins, lincosamides, quinupristin-dalfopristin and low levels of aminoglycosides (Sood et al. 2008). All these features constitute predisposing factors that most likely facilitated establishing this species in the hospital settings. Additionally, enterococci tend to acquire additional resistance and virulence determinants by mutations and the horizontal gene transfer (HGT). In E. faecalis, acquired resistance phenotypes with the biggest clinical significance include resistance to glycopeptides, quinolones and high-level resistance to aminoglycosides (HLAR); acquisition of these determinants often results in multidrug resistance (MDR; Magiorakos et al. 2012).

The importance of enterococci in HAIs raises questions about their relationships with strains present in other settings, circulation of strains and existence of potential additional reservoirs of resistance and virulence genes. Population analyses performed for E. faecium, another enterococcal species of a clinical importance, revealed the existence of specific, hospital-adapted merocline of this pathogen (Willems et al. 2005), distributed globally. Studies on E. faecalis population with the use of multilocus sequence typing (MLST) also suggested the presence of distinct high-risk enterococcal clonal complexes (HiRECCs), such as CC6 (originally described as CC2), CC9 and CC28, circulating in hospitals and responsible for a significant proportion of HAIs (Ruiz-Garbajosa et al. 2006; Kawolec et al. 2007; Kuch et al. 2012). The acquisition of resistance and virulence determinants, often associated with mobile genetic elements (MGEs), was a crucial factor in adaptation of E. faecalis to nosocomial environment (McBride et al. 2007; Solheim et al. 2011). Moreover, gene acquisition by certain clones seems to be facilitated by the lack of the clustered regularly interspaced palindromic repeats (CRISPR)-Cas loci, considered prokaryotic immune system against incoming DNA. In E. faecalis two functional CRISPR-Cas loci, 1 and 3, and an orphan locus 2 were described (Palmer and Gilmore 2010).

Bayesian Analysis of Population Structure (BAPS; Corander et al. 2008) represents an alternative approach in the analysis of bacterial populations and while it further confirmed the existence of a distinct, hospital-associated subpopulation in E. faecium (Willems et al. 2005), it did not do so for E. faecalis (Tedin et al. 2015). Therefore, the question of the HiRECCs in E. faecalis requires further studies. Although several groups investigated the presence of particular resistance of virulence-associated genes in E. faecalis for isolates from various sources, still relatively little is known about the association of these factors with particular clones, obtained from distinct environments. Thus, our study aimed at a comparative characterisation of the content of resistance and virulence determinants, transposons and CRISPR loci in E. faecalis clones, derived from hospitalised patients, community carriers, animals, fresh food, waters and sewage over a limited time span in a single country.

Parts of this study were presented at the 25th European Congress of Clinical Microbiology and Infectious Diseases, Copenhagen, Denmark, 25–28 April 2015.

MATERIALS AND METHODS

Bacterial isolates, DNA isolation and antimicrobial susceptibility testing

The study included 325 strains of Enterococcus faecalis, isolated in the period 2006–2009 in Poland from hospitalised patients with invasive infections (25, 7.7%), non-invasive infections (23, 7.1%), hospital carriers (27, 8.3%), carriers in the community (23, 7.1%), animals (66, 20.3%), fresh food (35, 10.8%), hospital sewage (24, 7.4%), municipal sewage and marine water (102, 31.4%). For 98 human isolates, details concerning isolation materials, hospital centres, sequence types (STs) and antimicrobial susceptibility patterns were reported previously (Kuch et al. 2012). Animal isolates were obtained mostly from carriage in chicken (33 isolates) and other animals (4 isolates from a dog, a parrot and a fox) and from unchached chicken eggs and samples of chicken liver (20 isolates). For eight animal isolates, details were not reported. Sewage was sampled in winter (37 isolates) and in summer (54 isolates) in two treatment plants (Warsaw and Kalisz); additionally, 24 isolates were obtained from hospital sewage (Warsaw). Enterococci, isolated from marine water, were obtained in nine sampling points in seven harbour towns/villages at the Baltic Sea. Bacterial DNA was isolated using the Genomic DNA Prep Plus kit (A&A Biotechnology, Gdynia, Poland). Antimicrobial susceptibility testing was performed with the use of broth microdilution method in accordance with the Clinical and Laboratory Standard Institute (CLSI) methodology (CLSI 2016). The Etest method was used to determine MICs of daptomycin (bioMérieux, Marcy l’Etoile, France). The interpretation of results was performed according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST) available breakpoints (EUCAST...
2016); otherwise the CLSI breakpoint values were applied (CLSI 2016).

Clumping assay, cytolysin and gelatinase production and biofilm formation

Production of A5 was assessed by the observation of clumping of cells in the presence of sex pheromone (Franz et al. 2001) present in culture supernatant of the OG1X strain of E. faecalis. The cytolysin production was observed after 24–72 h as a clearing zone (β-haemolysis) around bacteria growing in the atmosphere of 5% CO₂ on Todd-Hewitt Agar (BD) with 5% horse blood (Lányi 1987). The gelatinase production was detected as decreased turbidity of Tryptic Soy Agar (BD) with 3% gelatine (Oxoid, Hampshire, England) (Su et al. 1991). Biofilm formation was investigated with the use of a modified quantitative adherence assay (Tryptic Soy Broth supplemented with 0.25% glucose) (Tkirkikonis et al. 2012) performed in triplicate and isolates were classified as non-producers, weak producers or strong producers depending on the observed OD₅₅₅ values (Baldassarri et al. 2001).

Analysis of resistance and virulence determinants, and CRISPR-Cas loci

Detection and analysis of resistance genes, transposon-specific genes and transposon structures, pathogenicity determinants and CRISPR loci was done by PCR and sequencing using primers listed in Table S1 (Supporting Information). DNA from previously described clinical isolates of E. faecium (Sadowy et al. 2013) and Streptococcus agalactiae (Sadowy, Matynia and Hryniewicz. 2010) were used as controls; when positive controls were not available, a few randomly chosen PCR products were sequenced to confirm PCR specificity. In the cases of discrepancy between observed phenotype and genotype, repeat testing was performed. The CRISPRfinder (Grissa, Vergnaud and Pourcel 2007) was used to identify the direct repeats (DR) and the spacers sequences in the CRISPR2 loci (http://crispr.u-psud.fr/Server/CRISPRfinder.php; 15 November 2016, date last accessed). The spacer sequences were used to query GenBank by blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for similar sequences (≥ 25bp/30 bp identity) as of the 21 November 2016.

MLST and data analysis

MLST was conducted following the reported seven-loci scheme (Ruíz-Garbajosa et al. 2006) using the MLST database (http://pubmlst.org/efaecalis/, 15 November 2016, date last accessed) to assign alleles and STs. The MLST data were analysed by the comparative eBURST analysis (http://eburst.mlst.net/enter_data/comparative/, 15 November 2016, date last accessed), by the geScreen in PHYLOVIZ software (Francisco et al. 2012) and by the BAPS (Corander et al. 2008), using the software 6.0 version (http://www.helsinki.fi/bsg/software/BAPS/, 10 February 2017, date last accessed). Differences in distributions were evaluated with chi-squared test (P-value ≤ 0.05 considered significant).

Nucleotide sequence accession numbers

Newly identified ebpC, bee2 and bee3 sequences were submitted to the GenBank (KJ710249–KJ710266, KJ756549–KJ756554, KJ756555–KJ756561, respectively).

RESULTS

Antimicrobial susceptibility of isolates, determinants of resistance and their MGE

Two-hundred twenty seven isolates from non-human sources were susceptible to penicillin, ampicillin, vancomycin, teicoplanin, tigecycline, linezolid and daptomycin. Some of these isolates showed high-level resistance to gentamicin and/or streptomycin (HLGR/HLSR; 1.3% and 9.2%, respectively) and were commonly non-susceptible to rifampin and tetracycline (81.9% and 53.7%, respectively) (summarised in Table 1A; details in Table S2, Supporting Information). In the whole analysed collection, the HLGR phenotype was conferred mainly by the aac(6′)-aph(2″) gene, specifying bifunctional aminoglycoside 6′-acetyltransferase/2″-phosphotransferase. This gene was carried on A, E and J types of Tn4001 (Sadowy et al. 2013). HLGR isolates sporadically carried the aph(2″)-lb gene of aminoglycoside 2″-phosphotransferase; the aph(2″)-lc and aph(2″)-ld genes were not found. The ant(6′)-lα gene, encoding streptomycin 6′-adenylyltransferase, was the sole detected determinant of the HLR phenotype; none of the isolates possessed ant(3′)-lα of streptomycin 3′-adenylyltransferase. Among the ant(6′)-lα-positive isolates, 48 (67.6%) also showed the presence of the sat4-aphA3 of the aminoglycoside-streptothricin resistance gene cluster. All tetracycline non-susceptible isolates carried from one up to three different tet genes, including tet(M), tet(O), tet(L) and tet(S); other tetracycline resistance genes tet(K), tet(T) and tet(W) were not found. The macrolide resistance gene erm(β) of 23S rRNA methylase was ubiquitous among human isolates, and mef(A), determining 14- and 15-membered macrolide efflux, was present in a single isolate. Among 143 ermB-positive isolates, 17 (11.9%) carried tnpA gene of Tn917, while 179 (96.8%) of tet(M)-positive isolates harboured intTn916. The tndX gene, characteristic for Tn5397 transposon was not detected.

Distribution and variability of virulence determinants and associated phenotypes

The CPS1 locus, specific for isolates deficient in the production of capsular polysaccharide (McBride et al. 2007), represented the most frequent (42%) type of the capsule locus (summarised in Table 1B; details in Table S2). Two other types, CPS2 and CPS5, were present in 35% and 18% of isolates, respectively, and two isolates were negative in this assay. The combined presence of the presumably functional CPS2 and CPS5 loci was significantly more frequent in isolates from hospitalised patients (P = 0.004). 12.9% of isolates were positives in clumping test and genes associated with this phenotype, asa1 and asa373, were prevalent in the collection. The majority (65.7%) of isolates positive for the cytolysin gene cylA showed β-haemolysis. PCR mapping, performed for 14 representatives of 24 cylA-positive and haemolysis-negative isolates, showed that the majority lacked some parts of the cyl operon, usually in its proximal region. Eleven isolates were negative for cylA and cylB, and among them one additionally lacked cylM, four lacked cylM and cylB and one lacked cyl. Two isolates carried solely cylA. Concomitantly, 15 β-haemolytic isolates lacked any parts of the cyl operon. asa1 and cylA were specific for human isolates (P = 0 in both cases). Among 255 gelE-positive isolates, 73% showed the gelatinase activity. The esp gene, encoding the enterococcal surface protein Esp, occurred mostly in the isolates of human origin (P = 0) and both the esp presence and the gelatinase production strongly correlated with the ability to form biofilm (P = 0 in both cases).
Table 1. Phenotypic and genotypic features of E. faecalis BAPS groups and subgroups.

### A. Distribution of phenotypes and determinants of antimicrobial resistance and MGE genes

<table>
<thead>
<tr>
<th>BAPS (n)</th>
<th>HLRG (%)</th>
<th>HLRG genes (n)</th>
<th>Tn4001 type (%)</th>
<th>HLSR (%)</th>
<th>HLSR genes (n)</th>
<th>sat4-aphA3 (%)</th>
<th>Tet NS (%)</th>
<th>intTn532 (n)</th>
<th>tet genes (%)</th>
<th>trnA_{Tn917} (n)</th>
<th>Rif NS (%)</th>
<th>Cip NS (%)</th>
<th>MDR (%)</th>
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<td>10 (16.9)</td>
<td>ant(6)'-Ia (10)</td>
<td>4</td>
<td>30 (50.8)</td>
<td>M (28)</td>
<td>L (13)</td>
<td>S (4)</td>
<td>5 (84.7)</td>
<td>7 (11.9)</td>
<td>12 (20.3)</td>
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<td>9 (15.8)</td>
<td>aac(6)'-aph(2') (4)</td>
<td>A (1) J (3)</td>
<td>14 (24.6)</td>
<td>ant(6)'-Ia (13)</td>
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<td>36 (63.1)</td>
<td>M (34)</td>
<td>L (4)</td>
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<td>L (1)</td>
<td>O (1) S (1)</td>
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<td>S (5)</td>
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<td>M (48)</td>
<td>L (6)</td>
<td>O (5) S (5)</td>
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<td>13 (100)</td>
<td>ant(6)'-Ia (13)</td>
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<td>12 (92.3)</td>
<td>M (12)</td>
<td>L (12)</td>
<td>S (2)</td>
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<tr>
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<td>11 (28.9)</td>
<td>ant(6)'-Ia (11)</td>
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<td>M (31)</td>
<td>L (1)</td>
<td>S (2)</td>
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<td>1 (2.6)</td>
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<td>M (4)</td>
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<td>S (1)</td>
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<td>0</td>
<td>0 (0)</td>
<td>M (185)</td>
<td>L (185)</td>
<td>S (185)</td>
<td>185 (77.2)</td>
<td>45 (13.8)</td>
<td>88 (27.1)</td>
</tr>
</tbody>
</table>

| All 325  | 44 (13.5) | aac(6)'-aph(2') (37) | A (22) E (12) J (3) | 72 (22.1) | ant(6)'-Ia (71) | 49             | 198 (60.9)  | M (185)     | L (185)       | S (185)         | 251 (77.2)  | 45 (13.8)  | 88 (27.1) |

| P-value  | 0        | nd             | nd              | 0          | nd             | nd              | 0.002       | nd          | nd           | nd              | 0.2         | nd         | nd       |
### Table 1 Continued.

#### B. Distribution of virulence-associated phenotypes and determinants, and CRISPR-Cas loci

<table>
<thead>
<tr>
<th>BAPS (n)</th>
<th>CPS (n)</th>
<th>Clumping (%)</th>
<th>Haemolysis (%)</th>
<th>gylA</th>
<th>Gel+ (%)</th>
<th>gelE</th>
<th>Biofilm (%)</th>
<th>esp (%)</th>
<th>bee23</th>
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<td>13 (22.0)</td>
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<td>14 (24.6)</td>
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<td>28</td>
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<td>35 (61.4)</td>
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**BAPS**, Bayesian Analysis of Population Structure; HLGR, high-level gentamicin resistance; HLSR, high-level streptomycin resistance; Tet NS, tetracycline non-susceptibility; Rif NS, rifampin non-susceptibility; Cip NS, ciprofloxacin non-susceptibility, with the interpretation following the current breakpoint of 4 mg/L (EUCAST 2016). MDR, multidrug resistance.

*a* One isolate with both acc(6')-aph(2") and aph(2")Ib.

*b* One isolate with mef(A).

*c* Calculated for presumably functional CPS 2 and 5 vs non-functional CPS 1.

*d* Calculated for presence of both CRISPR1 and 3 vs absence.

BAPS subgroups with presumable HiRECCs 6 and 28 in bold; nd, not determined; p calculations did not include BAPS (sub)groups 32, 35, 4 with fewer than 10 isolates.
most prevalent among hospital isolates ($P = 0.03$). The chromosomally located ebpC pilus gene was present in all isolates. Partial ebpC sequencing in 63 selected isolates revealed the presence of 21 variants (98.7%–99.9% identity; Fig. S1, Supporting Information), corresponding to 16 EbpC variants. The bee23 genes, specifying the second type of pilus, were present in 33 isolates (Table 1B; details in Table S2). Sequencing of bee2 and bee3 genes for seven representatives revealed seven different alleles for both bee2 and bee3 (98.7%–99.9% and 98.5%–99.9% identity, respectively), corresponding to five and seven Bee2 and Bee3 variants.

**Analysis of the CRISPR loci**

The csn genes of CRISPR1 and CRISPR3 loci were detected in 197 and 8 isolates, respectively (Table 1B; details in Table S2). PCR with primers specific to the genes adjacent to both these loci (absence assay) yielded expected products for 185 and 318 isolates, respectively. Thus, 57 and 2 isolates were reproducibly positive in both the presence and absence assays, likely due to a duplication of respective loci (Katyiel et al. 2013). Isolates lacking the functional CRISPR-Cas 1 and/or 3 systems significantly more frequently carried the bee operon, $aac(6\prime)-\text{aph}(2\prime \text{a})$, $\text{aph}(2\prime \text{b})$, tet(O) and tet(L) genes ($P$-values: 0.000057, 0.028, 0.048, 0.019 and 0.049, respectively); no significant differences were found for $\text{asa}1$ and cyl. The orphan CRISPR2 was present in almost all isolates (322 isolates, 99.1%), yielding PCR products of 0.6–2.2 kb in size. Sequencing performed for 64 representatives revealed 23 variants of the locus containing 1–15 spacers (Table 2) with 72 different sequences. Of these, 30 showed similarity (≥25 bp/30 bp) to known MGE sequences, including 27 spacers related to Gram-positive phages and 3 highly similar to enterococcal plasmids; 19 spacers had a partial identity to E. faecalis chromosomal sequences, including 2 spacers presumably originating from the ref358 gene of the VS83 strain of E. faecalis (Table S3, Supporting Information). Twenty-three of identified CRISPR2 spacers had no homology to any of the sequences deposited in the GenBank.

**Clonal composition of Enterococcus faecalis isolates**

Among 227 analysed non-human isolates, 72 STs were found. Thirty-one STs of 98 human hospital and community isolates, described previously (Kuch et al. 2012) were used in the current study for comparative purposes, resulting in 91 different STs in the whole analysed collection. The comparative eBURST analysis included 49 STs (specific for 234 isolates) into 27 CCs, and assigned 19 STs (52 isolates) as ‘doubletons’ (i.e. they formed a single-locus variant link with only one other ST in the database) and 23 STs (39 isolates) as singletons (Table 2). BAPS was performed on concatenated sequences corresponding to 754 STs deposited at the E. faecalis MLST database (accessed on 1 February 2017) and resulted in five groups (1–5). Three most numerous of these were re-analysed separately, yielding subgroups 1.1–1.3, 2.1–2.3 and 3.1–3.5. STs found in the current study belonged to the majority of thus defined groups and subgroups (Table 2). Among these, the subgroups 2.1 and 2.3 corresponded to the previously defined HiRECCs 6 and 28, respectively, and their isolates were obtained almost exclusively from the hospital settings, with the exception of STs 245 and 370, which were also included in BAPS 2.1. Non-random distribution of isolates among various origins was also seen for the BAPS subgroups 2.2, 3.1 and 3.3 while isolates from the BAPS subgroups 1.1, 1.2 and 1.3 were obtained in all sampled settings (Table 2 and Fig. 1). Thus, defined BAPS (sub)groups differed in the respect of the prevalence of antimicrobial resistance and virulence-associated phenotypes and genotypes (Table 1). In particular, isolates of the BAPS subgroups 2.1 and 2.3 were MDR and almost uniformly carried antimicrobial resistance genes such as $aac(6\prime)-\text{aph}(2\prime \text{a})$, located on the A-type of Tn4001, $\text{ant}(6\prime \text{a})$-Ia, tet(M) and $\text{erm}(B)$, while their content of virulence genes and associated phenotypic traits was more variable. Both subgroups were characterised by the presence of presumable functional CPS2/CFPS5 loci and $\text{asa}1$, but haemolysis, associated with the presence of cylA, lack of gelatinase activity and the presence of esp were typical for BAPS 2.3, while isolates from BAPS 2.1 were less often haemolytic, showed gelatinase production and almost all of them lacked esp. Both 2.1 and 2.3 subgroups were associated with only one ebpC variant while other subgroups showed more diversity of this gene (Fig. S1). Isolates belonging to CCs 6 and 28 were characterised by a low prevalence of CRISPR1-Cas system, the lack of CRISPR3-Cas, and uniformly carried the small A and S variants of CRISPR2, containing one and two spacers, respectively (Table 3), both homologous to ref358. CRISPR1-Cas was most prevalent in BAPS 1.1 and 3.1, while the less common CRISPR3-Cas system was associated with unrelated STs, from BAPS 1.3, 2.2 and 3.5. CRISPR2 loci among ‘ubiquitous’ CCs 16, 21, 25 and 40 were highly divergent and predominantly contained four or more spacers.

**DISCUSSION**

A rise of enterococci as nosocomial pathogens posed a question whether strains derived from infected patients represent their normal gastrointestinal flora or a different subgroup in the population that acquired specific adaptations to the hospital conditions. For a related species, E. faecium, extensive studies have demonstrated an existence of a distinct hospital merocline of this pathogen, responsible for the vast majority of HAIs, especially those associated with MDR and alarming phenotypes of resistance. Although several studies have already investigated phenotypic and genotypic features of E. faecalis from various settings, this pathogen remains relatively less studied, especially considering association of particular clones with ecological niches and specific genetic content of these clones. Therefore, we have undertaken a study on the country-wide collection of E. faecalis derived in a limited time span from a wide range of ecological settings, including hospitalised patients, human colonisation in the community, farm and other animals, food, community and hospital sewage, and marine water.

As could be expected, hospital isolates showed the highest levels of non-susceptibility to antimicrobials and associated determinants among all studied settings. The $\text{ant}(6\prime \text{a})$ and $aac(6\prime)-\text{aph}(2\prime \text{a})$ genes constituted main determinants of HLSR and HLGR, respectively, in accordance with previous reports (Chow 2000; Sood et al. 2008). The $\text{aph}(2\prime \text{a})$-Ib gene was detected in five HLGR isolates, exclusively of nosocomial origin and, to our knowledge, this is the first time when this gene was detected in enterococcal species other than E. faecium (Hammerum et al. 2012). Other HLAR determinants, $\text{aph}(2\prime \text{a})$-Ic, $\text{aph}(2\prime \text{a})$-Id and $\text{ant}(3\prime \text{a})$-Ia, described in enterococci (Clark et al. 1999; Ramirez and Tolmasky 2010), were absent in our collection. The streptomycin resistance gene $\text{ant}(6\prime \text{a})$-Ia often co-occurred with the sat4-aphA3 genes, consistent with the presence of the aminoglycoside-streptothricin resistance gene cluster, reported previously in E. faecium (Werner, Hildebrandt and Witte. 2001). Isolates non-susceptible to tetracycline carried up to three different tet genes, with tet(M) the most ubiquitous in all the settings. The high prevalence of this
Table 2. BAPS groups and ST in various settings.

<table>
<thead>
<tr>
<th>BAPS (n)</th>
<th>CCs (STs) and singleton/doubleton STs</th>
<th>Hospital infections (%)</th>
<th>Hospital carriage</th>
<th>Hospital sewage</th>
<th>Community carriage</th>
<th>Animals</th>
<th>Food</th>
<th>Municipal sewage, water</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 (59)</td>
<td>CC19 (19), CC21 (21, 202), CC30 (30, 217, 307), CC116 (116, 275, 374); STs: 134, 316</td>
<td>3</td>
<td>8</td>
<td>1</td>
<td>6</td>
<td>11</td>
<td>7</td>
<td>23</td>
<td>0.14</td>
</tr>
<tr>
<td>1.2 (57)</td>
<td>CC25 (25, 97, 133, 283), CC26 (26), CC40 (40, 233), CC55 (55, 218), CC88 (88, 215), CC206 (23, 206); STs: 27, 73, 197, 219, 301, 308, 367, 371, 375</td>
<td>10</td>
<td>7</td>
<td>0</td>
<td>6</td>
<td>4</td>
<td>9</td>
<td>21</td>
<td>0.053</td>
</tr>
<tr>
<td>1.3 (23)</td>
<td>CC4 (4, 32), CC21 (117), CC72 (1.68), CC81 (81); STs: 41, 86, 147, 216, 234</td>
<td>3</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>11</td>
<td>0.58</td>
</tr>
<tr>
<td>2.1 (12)</td>
<td>CC8 (6), CC404 (370), ST245</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0.000078</td>
</tr>
<tr>
<td>2.2 (93)</td>
<td>CC8 (8), CC34 (34), CC49 (49, 203), CC59 (59, 298), CC82 (82), CC121 (282); STs: 79, 84, 126, 151, 188, 211, 232, 235, 256, 290, 296, 299, 365, 368, 369, 373, 377</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>33</td>
<td>9</td>
<td>41</td>
<td>0.00000014</td>
</tr>
<tr>
<td>2.3 (13)</td>
<td>CC87 (28, 87)</td>
<td>12</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3.1 (38)</td>
<td>CC16 (16, 179, 302, 372), CC58 (58, 63, 315)</td>
<td>7</td>
<td>5</td>
<td>11</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>3</td>
<td>0.00000056</td>
</tr>
<tr>
<td>3.2 (1)</td>
<td>ST238</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>nd</td>
</tr>
<tr>
<td>3.3 (21)</td>
<td>CC141 (141); STs: 208, 237, 297, 314</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>3.4</td>
<td>(absent)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>3.5 (4)</td>
<td>CC256 (300), CC355 (36), ST165</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>nd</td>
</tr>
<tr>
<td>4 (4)</td>
<td>STs: 238, 366</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>nd</td>
</tr>
<tr>
<td>5</td>
<td>(absent)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>All (255)</td>
<td></td>
<td>48</td>
<td>27</td>
<td>24</td>
<td>23</td>
<td>66</td>
<td>35</td>
<td>102</td>
<td>–</td>
</tr>
</tbody>
</table>

BAPS subgroups with presumable HiRECCs 6 and 28 in bold.
Figure 1. MLST-based population structure of *E. faecalis* from various settings. MLST profiles were analysed by the goeBURST, allowing the visualisation of isolate origin. Numbers, STs; black circles, BAPS subgroups.

gene in enterococci, including *E. faecalis*, was also reported previously (McBride et al. 2007; Sadowsy et al. 2013). Among other tet genes, tet(L), tet(S) and tet(O) were identified less frequently, mostly in animals, municipal sewage and marine water. Such high prevalence of tetracycline resistance and the abundance of tet genes in various settings likely results from a high consumption of tetracyclines in the outpatient treatment (the fourth place, 10.5% in 2009; 2.47 of the defined daily dose per 1000 inhabitants per day, ESAC-Net data; http://ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/esac-net-database/Pages/database.aspx) and a very high use in veterinary practice, where tetracyclines are the most commonly used antimicrobials in Poland (36.2% of the total sales in 2011, https://hi.emsa.europa.eu/analyticsSOAP/saw.dll?PortalPages). The tet(M) gene is often associated with the Tn916-type conjugative transposons, identified and reported in *E. faecalis* (Franke and Clewell 1981; Mikalsen et al. 2015), and in our collection, the int gene specific for this transposon typically co-occurred with tet(M) in isolates from various settings, indicating the Tn916 presence. The int gene of Tn5397, other transposon responsible for tet(M) spread in enterococci (Agersø, Pedersen and Aarestrup 2006; Sadowsy et al. 2013), was not found. The macrolide resistance gene *erm(B)* was associated with human isolates, occasionally with *tnpA* of Tn917, which confirms a low frequency of this transposon in enterococci (Mikalsen et al. 2015). Another macrolide resistance gene *mef(A)* was detected in a single isolate, in agreement with its low prevalence in *E. faecalis* (Luna et al. 2002; Chouchani et al. 2012).

Hospital isolates, in comparison to isolates from other settings, were also significantly enriched in some, but not all, studied virulence-associated factors. Two functional CPS loci 2 and 5, responsible for biosynthesis of capsular polysaccharides C and D, respectively, were significantly overrepresented among HAI isolates, in agreement with the importance of capsule for providing protection from clearance by the host immune system (Thurlow et al. 2009). The aggregation substance gene, *asa1*, showed almost uniform presence in human isolates, from both hospital and community. Previously, among Polish hospital isolates of *E. faecalis* from 1996 to 2005, the prevalence of *asa1* reached
70% (Wardal et al. 2013). Other studies also showed a lower prevalence of asa1 among human isolates (66.2%) (Creti et al. 2004) than observed in our collection. In contrast, asa373 occurred rarely and only in non-human isolates, while this gene was previously reported among human isolates (Waar et al. 2002; Creti et al. 2004). Similarly to asa1, cylA showed significant association with human isolates and the prevalence of this gene in hospital settings showed an increase in comparison to the previous period from 30% to 52% (Wardal et al. 2013). The observed increasing prevalence of both asa1 and cyl may indicate further spread of certain types of pheromone-responsive plasmids, often carrying asa and cyl genes among hospital E. faecalis in Poland. The esp gene was also preferentially associated with isolates of human origin in our study. Hospital isolates carried this gene much more frequently (72%) than reported for such isolates (20.0%–56.2%) in other European countries and the USA (Eaton and Gasson 2001; Creti et al. 2004). In contrast to previous studies (Shankar et al. 1999; Creti et al. 2004; Upadhyaya, Lingadevaru and Lingegowda 2011), isolates from community carriers were even more frequently esp-positive (100%) than hospital isolates. On the other hand, the prevalence of esp among animal, food and municipal sewage/water isolates was low, as also observed by others for these settings (Eaton and Gasson 2001; Tsikrikonis et al. 2012; Medeiros et al. 2014, Papadimitriou-Olivgeris et al. 2015). In contrast, gelatinase-positive isolates showed the lowest prevalence in community (17%) and hospitals (33%). In the earlier period of 1996–2005, 53% of hospital isolates produced gelatinase (Strzelecki, Hryniewicz, and Sadowy 2011). This decrease may be due to the observed increased hospital prevalence of gelatinase-negative STs 28 and 88 (from 19% to 28%). Lack of gelatinase production typically results from the 23.9 kb deletion of the fsr regulatory region (Qin et al. 2000), characteristic for CC28 (Strzelecki, Hryniewicz, and Sadowy 2011). Importantly, lack of gelatinase increases adhesion of E. faecalis to collagen by abolishing the cleavage of collagen-binding protein Ace (Pinkston et al. 2011), and thus might contribute to the pathogenicity of gelatinase-negative strains in humans. Animal isolates were characterised by almost uniform gelatinase production, consistent with the results obtained for isolates from horses and poultry (Olsen et al. 2012; Kim et al. 2016). We observed the correlation between biofilm formation and both esp presence and gelatinase activity, which confirms previous findings (Toledo-Arana et al. 2001; Tsikrikonis et al. 2012). The chromosomally located ebp locus, encoding pili, is ubiquitous among E. faecalis strains (Nallapreddy et al. 2006, 2011) and in this study, the ebpC gene of ebp operon was present in all isolates, showing a high degree of conservation among isolates from different settings, as also observed for human and animal strains (Nallapreddy et al. 2011). Contrary to ebp, the plasmid-located bee genes of the second type pilus operon are sporadically (1.2%–6.6%) detected among E. faecalis isolates from hospitalised patients, community and animals (Tendolkar,
Baghdayan and Shankar 2006; Nallapareddy et al. 2011; Wardal et al. 2013. A higher prevalence of bee among Polish isolates (10.1%) may be caused by the spread of pheromone-responsive plasmids, associated with this operon (Coburn et al. 2010), similarly to asa1 and cyl.

CRISPR-Cas systems play a significant role in bacterial defence against invading DNA, such as phages or plasmids, and in E. faecalis two active CRISPR-Cas loci (CRISPR1 and CRISPR3) and one orphan CRISPR (CRISPR 2) locus were identified (Bourgogne et al. 2008). We observed the lowest prevalence of functional CRISPR-Cas loci in hospital-associated isolates, and isolates deficient in these systems were enriched in acquired antimicrobial resistance genes and bee but not asa1 or cyl. Lack of functional CRISPR-Cas systems, reducing the barrier for HGT, had been previously shown to be associated with the increased prevalence of resistance genes, such as tetM, ermB, aac6’-aph2’ and blaz (Palmer and Gilmore 2010), cyl and asa (Lindenstrauss et al. 2011). The orphan CRISPR2 locus was detected in almost all strains and showed a remarkable diversity, in agreement with previous observations (Palmer and Gilmore 2010; Hullahalli et al. 2015). Although the CRISPR2 locus is devoid of cas genes, it presumably may be complemented in trans by CRISPR1-Cas since the two loci share repeat sequences (Bourgogne et al. 2008; Horvath et al. 2009; Price et al. 2016), thus presumably allowing to add spacer sequences to CRISPR2. Most representatives of CCs 6 and 28 lacked CRISPR1-Cas locus and they all were characterised by small A and S variants of CRISPR2, containing one or two spacer sequences, both originating from the ref35B gene of E. faecalis V583. This gene was predicted to encode an antisense ncRNA (Fouquier d’Hérouel et al. 2011), although this function was not later confirmed (Innocenti et al. 2015). Thus, the role of these variants remains unclear but they appear conserved among CC6 and CC28 (Hullahalli et al. 2015) and probably may be considered a ‘molecular marker’ of such strains.

The current study demonstrated a high clonal diversity of collected isolates, typical for E. faecalis (Ruiz-Garbajosa et al. 2006; Kawalec et al. 2007; Tedim et al. 2015). Further analyses, with the use of eBURST and BAPS, allowed grouping STs into clonal complexes and BAPS groups and subgroups, respectively. BAPS turned out to be very useful in exploring bacterial recombining populations of e.g. E. faecium and Streptococcus pneumoniae (Willems et al. 2005; Hanage et al. 2009). This approach was also used for E. faecalis yielding three major and two minor groups; none of these groups showed significant association with the hospital environment (Tedim et al. 2015). In the current study, we further analysed the structure of observed major BAPS groups, which allowed us to link two resulting subgroups 2.1 and 2.3 to previously proposed HiRECCs 6 and 28 (Ruiz-Garbajosa et al. 2006; Kawalec et al. 2007; Kuch et al. 2012). Isolates of these CCs/BAPS subgroups were present almost exclusively in the hospital settings, were significantly enriched in resistance genes, presumably produced a polysaccharide capsule but differed in the virulence-associated gene content. Additionally, both 2.1 and 2.3 subgroups showed a low prevalence of CRISPR-Cas systems and presence of small CRISPR2 variants, compared to isolates from other complexes and doubletons/singletons. In genomic analyses, isolates of CC6 and CC28 formed well-defined separate clusters (Raven et al. 2016). Data reported to the MLST database also support strong hospital association of CC6 with hospital settings (116 out of 119 isolates from CC6 of known epidemiology; the whole database included 779 hospital isolates among 1429 of known epidemiology; http://pubmlst.org/efaecalis/, accessed 15 November 2016). We also observed a strong host specificity for CC82, belonging to BAPS subgroup 2.2. Isolates representing ST82 were mainly involved in chicken invasive infections, and showed a high prevalence of asa1, cylA, gelf and gelatinase activity. Isolates of this ST had been described earlier in association with chicken amyloid arthropathy (Petersen et al. 2009) and occasionally are also found in human invasive infections (Kawalec et al. 2007, this study).

In summary, our study demonstrated that certain CCs, corresponding to newly defined BAPS subgroups of E. faecalis, are limited to the hospital settings in Poland, supporting the existence of HiRECCs in this species. Although other clones, broadly distributed outside hospitals, are able to survive in the hospital conditions, the hospital-specific clones are important carriers of antimicrobial resistance determinants and factors associated with pathogenicity, resulting in a significant part of the burden of enterococcal infections. Data of this and other studies indicate that the survival of HiRECCs outside hospitals appears currently very limited. Specific properties of hospital-associated clones may have important implications for pathogenicity and treatment of enterococcal infections.

SUPPLEMENTARY DATA

Supplementary data are available at FEMS PD online.

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Conflict of interest. None declared.

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Sada...