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Abundance and diversity of plasmid-associated genes among clinical isolates of *Enterococcus faecalis*

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ABSTRACT

Enterococcus faecalis, a normal compound of the human intestinal microbiome, plays an important role in hospital-acquired infections. Plasmids make a significant contribution to the acquisition of the novel traits such as antimicrobial resistance and virulence by this pathogen. The study investigated the plasmid content and the diversity of plasmid-associated genes in a group of 152 hospital isolates of *E. faecalis*. The majority of plasmids visualized by pulsed-field gel electrophoresis of S1 nuclease-digested DNA fell into the range of 50–100 kb. PCR-based screening allowed detection of genes of the *rep1*$_{pIP501}$, *rep2*$_{pRE25}$, *rep4*$_{pMBB1}$, *rep6*$_{pS86}$, *rep7*$_{pT181}$, *rep8*$_{pAM373}$, *rep9*$_{pAD1/pTEF2/pCF10}$, *rep10*$_{pIM13}$ and *rep13*$_{pC194}$ families in 29 different combinations. The *par* and ω-ε-ζ plasmid stabilization systems were ubiquitous (45 isolates, 29.6% and 88 isolates, 57.9%, respectively), while the *axe–txe* system was not found. The *asa1* gene homologues encoding aggregation substance characteristic for the pAD1 and related group of pheromone-responsive plasmids were present in 106 isolates. A variety of sequence variants, including novel ones, of genes associated with pheromone-responsive plasmids, such as *rep8*$_{pAM373}$, *rep9*$_{pAD1/pTEF2/pCF10}$, *par*, and *asa1* were observed. In conclusion, there is a big and only partially characterized pool of diverse plasmids in clinical *E. faecalis*.

1. Introduction

Enterococcus faecalis is a common and generally harmless commensal of the gastrointestinal tract of animals and humans. However, these bacteria cause serious hospital-associated infections (HAIs), such as bacteremia, endocarditis, urinary tract infections and infections of post-surgery sites in patients with underlying risk factors, such as hematological malignancies and post-transplantation immunosuppression (Hancock and Gilmore, 2000). The significance of *E. faecalis* as a hospital pathogen is additionally associated with selection of so-called enterococcal high-risk clonal complexes (HiRECCs) that evolve through acquisition of antibiotic resistance and virulence determinants by horizontal gene transfer (Moellering, 1992; Ruiz-Garbajosa et al., 2006; McBride et al., 2007; Leavis et al., 2006; Arias and Murray, 2012). Diverse mobile genetic elements (MGE) play an important role in this process, thus contributing to the recent spread of enterococci in the hospital environment (Hegstad et al., 2010). Among them, plasmids play special role in enterococcal evolution and adaptation (Weaver et al., 2002; Coque, 2008). They seem to be abundant in clinical isolates, in which several co-resident plasmids per one isolate are often detected (Rosvoll et al., 2010).

Generally, enterococcal plasmids are divided into conjugative and nonconjugative, where the first group includes pheromone-responsive plasmids and non-pheromone responsive plasmids, while the second group encompasses small rolling-circle replicating (RCR) and mosaic plasmids (Coque, 2008). Pheromone-responsive plasmids with their sophisticated mechanism of conjugation (Dunny et al., 1978), occur mainly in *E. faecalis* (Wardal et al.,

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2010). Several representatives of this group, such as pAD1, pCF10, pPD1, pTW9, pBEE99, pMG2200, pHKK701 and pAM373 are known. The aggregation substance (AS), which is involved in the transfer of pheromone-responsive plasmids, acts also as a virulence factor during host infection (Galli et al., 1990; Vanek et al., 1999) and encompasses a group of LPxTG adhesins that share over 90% homology with the exception of the central variable region, where their similarity is below 40% (Hendrickx et al., 2009). Apart from the genes responsible for maintenance and conjugation, plasmids from this group also carry various resistance determinants and genes encoding bacteriocins and operons specifying such virulence factors as cytolysin and pili (Ike and Clewell, 1984; Coburn et al., 2010; Nallapareddy et al., 2011). The group of conjugative, non-pheromone responsive plasmids includes *Inc18* plasmids and pMG1-type plasmids (Weaver et al., 2002; Coque, 2008). They are characterized by a low-copy number and the frequent presence of antibiotic resistance genes. Nonconjugative RCR plasmids are typically small, high-copy number and broad-host range plasmids that often carry antibiotic resistance genes (Khan, 1997). Only few representatives (e.g., pAMα1) of this group are known in *E. faecalis*. Mosaic plasmids, such as pRUM and pVEF1/pVEF2 in enterococci combine modules of various origins (Coque, 2008).

A recently developed classification and typing scheme for enterococci and other Gram-positive bacteria was based on the replication–initiation genes (*rep*) and distinguished 19 families and some unique *rep* genes (Jensen et al., 2010). The majority of these 19 families are further grouped into super-families, such as primases (*rep1*$_{pIP501}$, *rep2*$_{pRE25}$, *rep3*$_{pXO02}$ and *rep12*$_{pBMB67}$), topoisomerases I (*rep4*$_{pMBB1}$, *rep5*$_{pN315}$, *rep6*$_{pS86}$, *rep11*$_{pEF1071}$ and *rep18*$_{pEF418}$), RepL (*rep10* and *rep10b*), Rep_1 (*rep13*$_{pC194}$ and *rep16*$_{pSAS}$) and RepA_N (*rep8*$_{pAM373}$,*rep9*, *rep17*$_{pRUM}$, *rep15*$_{pSK41}$ and *rep19*$_{pUB101}$). Analysis of the RepA_N plasmid super-family revealed their narrow host range and co-evolution with the host (Weaver et al., 2009).

In the light of MGE-mediated genome plasticity, a multi-level approach encompassing clonal structure, MGE content and detailed clinical data analysis, seems necessary to obtain reliable results of epidemiological studies of enterococcal populations. The aim of our study was therefore to characterize the plasmid content and diversity of genes associated with these plasmids among hospital isolates of *E. faecalis*. For this purpose we used a collection of isolates that had been previously extensively characterized for their epidemiological characteristics, antimicrobial resistance, pulsed-field gel electrophoresis (PFGE)- and multilocus sequence typing (MLST)-types (Kawalec et al., 2007), multilocus VNTR analysis (MLVA)-types (Sadowy et al., 2011) and gelatinase phenotypes and genotypes (Strzelecki et al., 2011), and thus provided a very good basis for the study.

2. Materials and methods

2.1. Bacterial strains, total and plasmid DNA extraction

The previously characterized collection of 152 *E. faecalis* isolates (Kawalec et al., 2007) was used in the study. Among this group, originating from 61 centers in 27 cities, earlier studies discerned 51 sequence types (STs) and revealed the presence of three HiRECCs, CC2, CC9 and CC87 that included 44 isolates. Almost all studied isolates were of clinical origin and were derived from carriage (n = 42), non-invasive (n = 53) and invasive infections (n = 54). One isolate originated from a hospital environment and for two isolates the isolation source was not reported. Total (i.e., chromosomal and plasmid) DNA of isolates was extracted using genomic DNA Prep Plus kit (A&A Biotechnology, Gdansk, Poland), following the manufacturer's instructions. Additionally, as the above method may result in a low yield of small plasmids, plasmid DNA was specifically isolated using the alkaline lysis method described by Handwerger et al. (1995) with minor modifications. Briefly, bacteria from 5 ml of the overnight cultures in BHI broth were washed in 100 μl of TEG (50 mM Tris–HCl pH 8.0, 10 mM EDTA and 2% glucose), resuspended in 200 μl of TEG with 50 mg/ml of lysozyme and incubated for 30 min at 37 °C. Then 400 μl of 1% SDS, 0.2 M NaOH solution was added, followed by 300 μl of 3 M potassium acetate (pH 5.0) and 1–2 h incubation on ice. The mixture was extracted with phenol–chloroform–isoamyl alcohol (25:24:1) and precipitated with 96% ethanol.

2.2. Hemolytic test

Investigated isolates were examined for the production of cytolysin according to the hemolytic test of Lányí (1987). Bacterial cultures were streaked on THB agar with 5% horse blood and incubated 24–72 h in 5% CO_2 at 37 °C. The presence of clearing zones around the colonies was interpreted as beta-hemolysis.

2.3. Detection of plasmid-associated genes by PCR

The complete list of primers used in the study for PCR and sequencing is provided in Table 1. Plasmid DNA was used as template in PCR for all replication and addiction system genes. Additionally, PCR using total DNA of investigated isolates was carried out for *rep1*$_{pIP501}$, *rep2*$_{pRE25}$, *rep8*$_{pAM373}$, *rep9*$_{pAD1/pTEF2/pCF10}$, *rep17*$_{pRUM}$, *rep18*$_{pEF418}$, *rep*$_{pMG1}$, *rep*$_{pLG1}$, *axe–txe*, ω-ε-ζ and *par* (typically found on plasmids larger than 20 kb) to avoid missing the presence of the genes on megaplasmids that can escape from isolation by standard alkaline lysis method. Positive results for all *rep* families, genes of plasmid addiction systems, *asa*, *cyl* and *bee* were confirmed by sequencing of a few randomly chosen representatives that further served as positive controls.

2.4. Sequence analysis of selected plasmid genes and statistical analysis

Sequencing of *rep8*$_{pAM373}$ and *rep9*$_{pAD1/pTEF2/pCF10}$ PCR products was performed using primers designed by Jensen et al. (2010). The alignment of all *rep9* homologues available in the GenBank was used to design three pairs of primers, specific for (i) the *rep9* genes in pAD1 and pTEF1 (designated herein *rep9A*$_{pAD1}$), amplifying nt 497–694 of pAD1 *rep* gene, (ii) the *rep9* genes in pTEF2, pMG2200,