Involvement, and dissemination, of the enterococcal small multidrug resistance transporter QacZ in resistance to quaternary ammonium compounds

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Objectives: To investigate the role of a putative small multidrug resistance transporter, annotated in Enterococcus faecalis V583 genome as EFA0010 (we will refer to this gene as qacZ), in decreased susceptibility to biocides.

Methods: A derivative strain of V583, susceptible to erythromycin (V583ErmS), was complemented with pORI23 carrying the qacZ gene (strain EF-SA VE1). MICs of benzalkonium chloride, chlorhexidine and ethidium bromide were determined for the complemented strain and wild-type. RT–PCR and ethidium bromide efflux assays were performed in order to fully understand the role and specificity of the qacZ gene. The presence of qacZ in 73 enterococcal strains from different origins was investigated by PCR, and MICs of benzalkonium chloride and chlorhexidine were determined for the same strains.

Results: The complemented strain, EF-SAVE1, presented a higher MIC of benzalkonium chloride (8 mg/L) than V583ErmS (4 mg/L); the MICs of chlorhexidine and ethidium bromide were the same for both strains, 4 mg/L and 16 mg/L, respectively. Expression of qacZ was found to be higher in EF-SAVE1 and constitutive, i.e. not inducible by any of the three tested biocides. Overexpression of qacZ was not responsible for changes in ethidium bromide efflux. This gene was present in 52% of the enterococcal isolates studied and the MICs of benzalkonium chloride and chlorhexidine ranged between 2 and 8 mg/L.

Conclusions: We demonstrate the involvement of the qacZ gene in tolerance to the quaternary ammonium compound benzalkonium chloride, but not ethidium bromide. This work constitutes the first report of a biocide resistance mechanism in E. faecalis, and reveals its dissemination amongst the genus Enterococcus.

Keywords: Enterococcus, biocides, Qac

Introduction

In the past few decades, the use of biocides as antiseptics, disinfectants and preservatives has been growing, not only in the hospital environment, but also in the food industry and more recently in our homes. This practice has been suggested to contribute to selection of antibiotic-resistant bacteria, and may lead to a decrease in the number of solutions to control bacterial load in hospital settings, in the food industry and even at home. Thus, the need to understand the mechanisms responsible for biocide tolerance in bacteria causing severe infections is obvious and urgent. Enterococci are Gram-positive commensal bacteria that inhabit the gastrointestinal tract of humans and animals. Their robust nature allows them to disseminate in a variety of other habitats, namely fermented foods, water, soil and plants. Members of this genus have intrinsic and acquired resistance to several antibiotics and thus became the second most frequently reported cause of surgical wound and nosocomial urinary tract infections and the third most frequently reported cause of bacteraemia. However, and despite their relevance as nosocomial multiresistant pathogens, there are no tolerance mechanisms to biocides described so far for the genus Enterococcus. Among Staphylococcus isolates qac genes, encoding small multidrug resistance (SMR) transporters, mediate resistance to quaternary ammonium compounds (QACs). The Enterococcus faecalis V583 pTEF1 plasmid carries an open
reading frame, EFA0010, annotated as a putative SMR transporter. In this work we provide evidence that supports this activity of EFA0010 and we will thus refer to it as the qacZ gene.

Materials and methods

MIC determination

MICs, determined according to the CLSI (formerly the NCCLS), of benzalkonium chloride, a QAC, chlorhexidine, a biguanidine, and ethidium bromide, a dye, were compared for two strains: V583ErmS (a derivative of E. faecalis V583, susceptible to erythromycin due to deletion of the erm(B) gene (EFA0007), kindly supplied by Axel Hartke, from Caen, France); and EF-SAVE1 (a derivative of V583ErmS carrying the high-copy-number plasmid pSA VE1, constructed in the present study).

Construction of the EF-SAVE1 strain

For pSA VE1 construction, the qacZ gene was amplified from V583 using qac-IF (GAATCTCGATCCATCTTTAAAAAGGGC) and qac-ER (GAATCTTCTGCA-GATACATCAAACAAAGGGG) primers. qac-ER has a restriction site for BamHI and qac-ER has a restriction site for PstI, both underlined. The PCR product (480 bp) contained both the coding sequence and the ribosome binding site of qacZ, but excluded the promoter regions. The amplimer and the pORI23 plasmid\textsuperscript{6} were digested and ligated to construct the pORI23 plasmid. The ligation mixture was introduced into electrocompetent Escherichia coli DH5\textalpha{} cells. Transformants were selected on Luria-Bertani agar containing 500 mg/L erythromycin, and the presence of qacZ under pORI23 promoter regulation was confirmed by sequencing. The EF-SAVE1 strain was constructed by introducing pSA VE1 into electrocompetent V583ErmS cells and transformants were selected on brain heart infusion (BHI) agar containing 500 mg/L erythromycin.

RT–PCR

RT–PCR was performed in order to confirm whether a higher copy number of EFA0010 in V583ErmS led to higher expression of this gene and consequently a higher MIC of benzalkonium chloride. Total RNA was isolated from V583ErmS, and EF-SAVE1 and EF-SAVE2 (V583ErmS containing pORI23 without the amplicon) by means of the RNeasy Mini Kit (Qiagen). RNA was reverse transcribed using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche). RT–PCR was performed using qac-IF and qac-IR primers.

Ethidium bromide efflux assay

Ethidium bromide efflux assays were performed in accordance with Jonas et al.\textsuperscript{7} Briefly, overnight cultures of V583ErmS and EF-SAVE1 were washed with 20 mM HEPES buffer and resuspended in the same buffer. After that, cells were loaded with ethidium bromide (2.5 \mu{}M) by shaking at 37°C and adding carbonyl cyanide 3-chloro-phenylhydrazone (CCCP) at 40 \mu{}M. Cells were incubated for 1 h and after that they were washed with 20 mM HEPES buffer and resuspended in the same buffer. After that, cells were loaded with ethidium bromide (2.5 \mu{}M) by shaking at 37°C and adding carbonyl cyanide 3-chloro-phenylhydrazone (CCCP) at 40 \mu{}M. Cells were incubated for 1 h and after that they were

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**Figure 1.** Alignment of QacZ with homologous proteins in the database (BLASTP, NCBI), with identities >60%. QacZ has Glu-13 (E), which is conserved throughout the SMR family and is absolutely required for drug efflux activity. Putative transmembrane helix sequences of QacZ, a hydrophobic protein (SOU1 1.1), are represented by horizontal lines above the aligned sequences. Conserved amino acid residues are indicated by bold letters. NP_816940 is QacZ from *E. faecalis* V583, O87868 is QacH from *Staphylococcus saprophyticus*, NP_647561 is an ethidium bromide resistance determinant from *Staphylococcus epidermidis*, Q55339 is QacC from *Staphylococcus* sp. ST827, AAB47993 is an SMR efflux pump from *S. aureus*, N_783299 is a QAC resistance protein from *S. aureus* and O87866 is QacG from *Staphylococcus* sp. ST94.
Comparison of protein sequences
Protein sequences of EFA0010 and homologous proteins in Staphylococcus were obtained from the NCBI database and compared using NCBI BLASTP.

Screening of qacZ in enterococcal isolates
Seventy-three enterococcal isolates were randomly selected from the laboratory collection. All these isolates were already identified to the species level and typed by PFGE. None of the selected 73 isolates is a clone. Our selection focused on three different environmental sources, namely Portuguese milk and cheese, nosocomial infections (urinary, vagina, blood culture, pus, catheter and sputum) and animal infections (cats and dogs). The dissemination of qacZ in the 73 enterococcal isolates was addressed using PCR [primers qac-IF (ATATTGCTTACG-cats and dogs). The dissemination of qacZ in the 73 enterococcal isolates was addressed using PCR [primers qac-IF (ATATTGCTTACG-CAGTGG) and qac-IR (AGTGTGATGATCCGAATGTG)]]. MICs of benzalkonium chloride and chlorhexidine were also determined.

Statistical analysis
Microsoft Office Excel 2007 was used to process the data on the prevalence of qacZ and MICs obtained. The prevalence of the gene was compared using the χ² test to determine statistically significant differences. All tests were two-tailed and tested at a level of 0.05 for significance.

Results and discussion
MIC determinations for V583ErmS and EF-SAVE1 were repeated five times and gave consistent and reproducible values: 4 mg/L of chlorhexidine for both strains; 16 mg/L of ethidium bromide for both strains; 4 mg/L of benzalkonium chloride for V583ErmS; and 8 mg/L of benzalkonium chloride for EF-SAVE1. Although the increase in MIC of benzalkonium chloride was small (from 4 to 8 mg/L), it was consistent and reproducible.

Figure 2. Ethidium bromide efflux assays in V583ErmS and EF-SAVE1. Efflux was initiated by the addition of BHI. Assays were performed in triplicate. Fluorescence units are expressed as a percentage of the starting value. Ethidium bromide was used at a concentration of 2.5 μM and CCCP was used at a concentration of 40 μM.

qacZ is involved in resistance to chlorhexidine in enterococci

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None to declare.

Table S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).