**Enterococcus faecalis** zinc-responsive proteins mediate bacterial defence against zinc overload, lysozyme and oxidative stress

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Two **Enterococcus faecalis** genes encoding the P-type ATPase EF1400 and the putative SapB protein EF0759 were previously shown to be strongly upregulated in the presence of high concentrations of zinc. In the present work, we showed that a Zn\(^{2+}\)-responsive DNA-binding motif (zim) is present in the promoter regions of these genes. Both proteins were further studied with respect to their involvement in zinc homeostasis and invasion of the host. EF0759 contributed to intramacrophage survival by an as-yet unknown mechanism(s). EF1400, here renamed ZntAEf, is an ATPase with specificity for zinc and plays a role in dealing with several host defences, i.e. zinc overload, oxidative stress and lysozyme; it provides E. faecalis cells with the ability to survive inside macrophages. As these three host defence mechanisms are important at several sites in the host, i.e. inside macrophages and in saliva, this work suggested that ZntAEf constitutes a crucial E. faecalis defence mechanism that is likely to contribute to the ability of this bacterium to endure life inside its host.

**INTRODUCTION**

Divalent metal cations are essential for all living cells, although they can be toxic in high concentrations. Tight control of the cellular concentration of divalent metals prevents the formation of toxic metal complexes and the occurrence of redox reactions that are noxious to the cell (Nies, 2007). One of these metals, zinc, is a component of many proteins, such as DNA polymerases, proteases and ribosomal proteins, and a co-factor of many enzymic reactions (Somerville & Proctor, 2009). Notwithstanding the importance of zinc, excess can be deleterious as it can compete with other metals for binding to the active centres of enzymes (Somerville & Proctor, 2009). Zinc concentrations in the human body are variable, with levels in saliva and lungs (Chicharro *et al.*, 1999; Versieck & McCall, 1985) being higher than those in serum or gastric juice (Powell *et al.*, 1992; Versieck & McCall, 1985). In particular, it has been shown that zinc accumulates in host immune cells such as macrophages upon infection, as a host defence mechanism (Wagner *et al.*, 2005). Zinc levels can also increase through antimicrobial therapy, as some antibiotics are formulated as zinc salts. That is the case for bacitracin, the salt of which has been demonstrated to induce the expression of zinc efflux systems in *Bacillus subtilis* (Mascher *et al.*, 2003). These efflux systems are essential for zinc resistance in bacteria (Blencowe & Morby, 2003). They either expel the metal ions from the cells or detoxify/sequester them so that the cells can grow in an environment containing high levels of zinc (Xiong & Jayaswal, 1998). Many efflux transporters involved in the expulsion of toxic amounts of zinc from the cell and in achieving zinc homeostasis are P-type ATPases. These enzymes constitute a large family of integral membrane transporters which generate and maintain crucial chemical gradients across cellular membranes (Bublitz *et al.*, 2011). Prokaryotic P-type ATPases can function as exporters or importers in the transport of a range of divalent metal cations, such as Cu\(^{2+}\), Ag\(^{2+}\), Cd\(^{2+}\) and Zn\(^{2+}\) (Agranoff & Krishna, 2004). The genes encoding the exporters of these metals are regulated in bacteria mostly by MerR-like activators or by ArsR-like repressors (Outten & O’Halloran, 2001).

In a recent genome-wide study, we identified transporter systems likely to be involved in zinc homeostasis in *Enterococcus faecalis* (Abrantes *et al.*, 2011). *E. faecalis* is an important nosocomial agent which is able to survive and colonize a diversity of environments. It is a human commensal Gram-positive bacterium naturally colonizing the human genitourinary tract and the oral cavity. However, in hospitalized patients, *E. faecalis* is a leading cause of antibiotic-resistant nosocomial infections of the urinary tract.
tract, bloodstream, intra-abdominal and pelvic regions, and surgical sites (Arias & Murray, 2012). Although some mechanisms involved in *E. faecalis* stress responses have been studied (Giard et al., 2001; Le Breton et al., 2003; Rincé et al., 2000, 2003; Yan et al., 2009), there is still a major lack in our knowledge of how these bacteria succeed in adapting to environments with different metal concentrations and maintain metal homeostasis. In particular, nothing is known about *E. faecalis* mechanisms for dealing with high zinc concentrations. In this study, we explored the specificity of the ATPase EF1400, the gene of which was previously shown to be highly overexpressed (44-fold change) in the presence of zinc stress (Abrantes et al., 2011), and investigated its role in biological processes such as intramacrophage survival and resistance to oxidative stress or lysozyme. This is the first report on the involvement of an *E. faecalis* P-type ATPase in biological processes relevant for bacterial infection and virulence; the work links zinc homeostasis to the outcome of host–pathogen interactions. The *ef0759* gene, which encodes a putative SapB protein, was also highly upregulated in the presence of zinc stress (49-fold change) and was also investigated for its role in these processes.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. *E. faecalis* was grown as standing culture at 37 °C in Difco M17 (Becton Dickinson) containing 0.5% glucose (GM17), or on brain heart infusion (BHI; Oxoid) or Luria–Bertani (LB; Sigma-Aldrich) agar plates. *Escherichia coli* cells were grown in LB in a shaking incubator at 37 °C. Strains with thermo-sensitive plasmids were grown at 30 °C. Chloramphenicol was used at a concentration of 30 μg ml⁻¹; tetracycline was used at concentrations of 15 μg ml⁻¹ for VE14192 and 10 μg ml⁻¹ for VE14089-derived strains. Kanamycin was used at 50 μg ml⁻¹ for VE14188 and VE14192. Erythromycin was used at 50 μg ml⁻¹ for SAVE27, SAVE32, SAVE33, SAVE34 and SAVE35. When necessary, 250 μg X-Gal ml⁻¹ (VWR International) was added to the growth medium.

**moods analysis.** MOODS software (Korhonen et al., 2009) was used on DNA microarray data obtained previously (Abrantes et al., 2011) in order to identify over-represented DNA-binding sites in the upstream DNA sequences of *E. faecalis* V583 genes.

**Mutant construction.** Single crossover insertion mutagenesis was performed to create strains SAVE28, SAVE29, SAVE30, SAVE31, SAVE36 and SAVE37 using the two-vector system essentially as described by Law et al. (1995). Plasmid pG′ host3 and the integrative plasmid pVE14218 were used in this strategy (Rigottier-Gois et al., 2011). Primers used for construction of the integration vectors are presented in Table S1 (available in the online Supplementary Material).

**Construction of a transcriptional lacZ fusion.** A transcriptional fusion to the *E. coli* lacZ gene was made in plasmid pLOR14 (Larsen et al., 2004). Primer pair EF1400-1/EF1400-2 (Table S1) was used to generate a PCR fragment spanning the upstream region of the *ef1400* gene, which was then cloned in the EcoRI/BamHI sites of pLOR14, resulting in pSAVE13. Confirmation of this construct was done by PCR with primers pLOR14-1 and pLOR14-2 (Table S1), and subsequent nucleotide sequencing. The plasmid was subsequently introduced in the various *E. faecalis* strains used in this study.

**β-Galactosidase assays.** Cells were grown in GM17 with erythromycin at 50 μg ml⁻¹, with and without added metal solution. Metals were used at the following (added) metal concentrations: ZnCl₂ 0.5, 2, 4 or 6 mM; MnCl₂ 0.2, 0.4, 0.6 or 2 mM; CuSO₄ 0.025, 0.05 or 0.075 mM; CoCl₂ 0.025, 0.05, 0.075 or 0.5 mM; FeCl₂ 0.05, 0.2 or 0.5 mM; NiSO₄ 0.05, 0.2 or 0.5 mM; MgCl₂ 0.005, 0.01, 0.05 or 0.5 mM; CdCl₂ 0.005 or 0.01 μM. Assays were performed as described previously (Abrantes et al., 2011). Activity of LacZ (in Miller units) was calculated according to Miller (1972). For each assay, three independent replicates were performed and the overall significance of the differences was determined by a two-tailed unpaired *t*-test.

**Zinc susceptibility assay.** Overnight cultures of the *E. faecalis* strains VE14089, SAVE36 (VE14089 *ef0759::tet*) and SAVE37 (VE14089 *zntAEf::tet*) were adjusted to OD₅₆₀ 1 in a 0.85% NaCl solution and diluted up to 10⁻⁶-fold. Subsequently, 20 μl of the 10⁻⁶ and 10⁻⁸ dilutions was spotted on BHI plates with and without added ZnSO₄ (0–20 mM). Experiments were performed in independent triplicates. Plates were incubated for 24 h at 37 °C and later photographed.

**Intramacrophage survival assay.** The macrophage survival assay was essentially performed as described previously (Bennett et al., 2007) with some modifications. The macrophage cell line J774.A1, established from a tumour that arose in a female BALB/c mouse (Ralph et al., 1976), was grown to confluent monolayers in Medium 1 [RPMI 1640 with l-glutamine (Invitrogen/Gibco), 10 % FBS (Invitrogen/Gibco), 1% penicillin/streptomycin (Invitrogen/Gibco)] at 37 °C and 5 % CO₂. Subsequently, the monolayers were infected with cultures of *E. faecalis*. Approximately 4 × 10⁶ bacteria were added to J774.A1 monolayers to yield a m.o.i. of ~10 bacteria per murine cell. The cell cultures were incubated at 37 °C in 5 % CO₂ atmosphere for 1 h to allow bacterial adherence and entry. Subsequently, 250 μg gentamicin ml⁻¹ and 60 μg penicillin G ml⁻¹ were added to the cultures, followed by incubation for 1 h to kill extracellular bacteria. Triton X-100 (Fluka Analytical) at 1% in PBS, pH 7.4 (Invitrogen/Gibco) was used to lyse macrophage cells at 0, 2 and 4 h post-infection. Lysates were then diluted and plated on BHI plates to count viable intracellular bacteria. These assays were performed in three independent replicates and results are reported as the intracellular survival index, i.e. the per cent (mean) of the internalized c.f.u. at 0 h post-infection that survived after phagocytosis. The overall significance of the differences was determined by a two-tailed paired *t*-test.

**Oxidative stress assay.** *E. faecalis* strains VE14089, SAVE36 (VE14089 *ef0759::tet*) and SAVE37 (VE14089 *zntAEf::tet*), grown in BHI until OD₅₆₀ 0.5, were submitted to 20 mM H₂O₂ stress (in 0.9 % NaCl) exactly as described by Verneul et al. (2004). Data points represented the mean of the data from three independent experiments performed in triplicate. The percentage of survival at a given point in time was calculated by determining the ratio between the number of c.f.u. at that time point after treatment and the number of c.f.u. at zero time.

**Lysozyme assay.** Dilutions of *E. faecalis* cultures were spot-plated on LB plates containing different concentrations (0–15 mg ml⁻¹) of egg white lysozyme (Sigma–Aldrich) according to the procedure described by Le Jeune et al. (2010). Plates were incubated for 24 h at 37 °C and photographed. Two independent experiments were performed.

**RESULTS AND DISCUSSION**

Zinc is a very important transition metal for humans and their (opportunistic) pathogens such as *E. faecalis*. It therefore seems surprising that there are no studies
The genes ef0759 and ef1400 encode two putative Cd²⁺, translocating P-type ATPases, whilst the gene ef0759 encodes a putative SapB protein. The two P-type ATPases have 32% homology and share certain similarities. Both have a multi-domain related to cation transport denominated ZntA that constitutes the biggest part of the proteins, an E1-E2 ATPase domain and a haloacid dehalogenase-like hydrolase domain (Fig. S1). EF1400, in particular, also shares 36% homology with E. coli ZntA and both contain a heavy-metal-associated domain at their N-terminal ends (for EF1400: GenBank accession number AAO81191.1). On the basis of these data and the results presented below, we propose to rename EF1400 as ZntAEf.

To explore the role of proteins with different putative functions in the cell, but encoded by genes highly responsive to added zinc, we chose to focus our study on one of the ATPases, ZntAEf, and on the putative SapB protein, EF0759. These proteins were further studied with respect to their possible roles in E. faecalis responses to zinc stress and to other host-related defences.

Zinc P-type ATPases are still to be explored in most bacterial species, particularly in Gram-positive bacteria. The few known P-type ATPases with a ZntA domain are

Table 1. Strains and plasmids used in this work

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>Enterococcus faecalis</strong></td>
<td>VE14089</td>
<td>V583 cured of its three plasmids</td>
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<tr>
<td></td>
<td>SAVE27</td>
<td>VE14089 (pSAVE13); Ery⁺</td>
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<td></td>
<td>SAVE412</td>
<td>VE14089 (pG⁺ host3); Cm⁺</td>
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<td></td>
<td>SAVE28</td>
<td>VE14089 with a Tet insertion in ef0421; Tet⁺</td>
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<td></td>
<td>SAVE29</td>
<td>VE14089 with a Tet insertion in ef0966; Tet⁺</td>
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<td>SAVE30</td>
<td>VE14089 with a Tet insertion in ef1699; Tet⁺</td>
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<td></td>
<td>SAVE31</td>
<td>VE14089 with a Tet insertion in ef2225; Tet⁺</td>
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<td></td>
<td>SAVE32</td>
<td>SAVE28 (pSAVE13); Tet⁺; Ery⁺</td>
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<td></td>
<td>SAVE33</td>
<td>SAVE29 (pSAVE13); Tet⁺; Ery⁺</td>
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<td></td>
<td>SAVE34</td>
<td>SAVE30 (pSAVE13); Tet⁺; Ery⁺</td>
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<td></td>
<td>SAVE35</td>
<td>SAVE31 (pSAVE13); Tet⁺; Ery⁺</td>
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<td></td>
<td>SAVE36</td>
<td>VE14089 with a Tet insertion in ef0759; Tet⁺</td>
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<td></td>
<td>SAVE37</td>
<td>VE14089 with a Tet insertion in zntAEf; Tet⁺</td>
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<tr>
<td><strong>Escherichia coli</strong></td>
<td>VE14188</td>
<td>GM1674 (dam⁻ dcm⁻); Kan⁺</td>
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<td></td>
<td>VE14192</td>
<td>GM1674 (pVE14218); Kan⁺</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td>pIIORI4</td>
<td>pIL252 carrying the MCS and promoterless lacZ of pORI13; Ery⁺</td>
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<td>pSAVE13</td>
<td>pIIORI4 with the promoter region of zntAEf cloned in the MCS (PzntAEf::lacZ)</td>
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<tr>
<td></td>
<td>pG⁺ host3</td>
<td>Thermo-sensitive replication; Cm⁺</td>
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<td></td>
<td>pVE14218</td>
<td>Derived from p3TETTery and pOrinew; integration vector</td>
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<tr>
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<td>pSAVE14</td>
<td>pVE14218 with ~80% of ef0421 in the MCS; Tet⁺</td>
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<td>pSAVE15</td>
<td>pVE14218 with ~80% of ef0966 in the MCS; Tet⁺</td>
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<td>pSAVE17</td>
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<td></td>
<td>pSAVE18</td>
<td>pVE14218 with ~80% of ef0759 in the MCS; Tet⁺</td>
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<td></td>
<td>pSAVE19</td>
<td>pVE14218 with ~80% of zntAEf in the MCS; Tet⁺</td>
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MCS, multiple cloning site.
mostly encoded by genes that are responsive to several metal ions, such as Zn$^{2+}$, Co$^{2+}$, Cd$^{2+}$ and Pb$^{2+}$ in *E. coli* (Sharma *et al.*, 2000). Our results point to zinc specificity of ZntAEf, but more functional studies on the protein would be needed to confirm these results. To the best of our knowledge, this is the first zinc P-type ATPase to be described in *E. faecalis*.

In order to evaluate the role of EF0759 and ZntAEf in *E. faecalis* resistance to zinc, *ef0759* and *zntAEf* mutants were constructed, and their growth on zinc-rich BHI plates was compared with that of the parent strain VE14089. The *ef0759* mutant grew as the WT strain (Fig. 2), suggesting that there is no role for this protein in survival in the presence of high concentrations of zinc. The *ef0759* gene is in an operon with that of the P-type ATPase EF0758. The *ef0758* gene is also highly upregulated in the presence of excess zinc and is putatively involved in cation transport (Abrantes *et al.*, 2011). Considering these observations, and as there are no reports of EF0759 homologues involved in resistance to zinc, we cannot discard the hypothesis that the overexpression of the *ef0758* gene in the presence of zinc excess is an indirect effect derived from the overexpression of the other member of its operon, the *ef0758* gene. Further studies on both genes would be necessary to validate this hypothesis. However, the *zntAEf* insertional mutant showed an increased susceptibility to high concentrations of zinc (20 mM; Fig. 2), as has been observed for an *E. coli* zntA mutant (Helbig *et al.*, 2008), which suggests that the ZntAEf ATPase might be involved in zinc expulsion, leading to increased resistance to zinc.

*E. coli* zntA is described to be regulated by ZntR (Brocklehurst *et al.*, 1999; Singh *et al.*, 1999). We used the *E. coli* ZntR protein sequence to search for homologues in the genome of *E. faecalis* V583. All *E. faecalis* ZntR homologues, i.e. EF0421, EF0966, EF1699 and EF2225, belong to the MerR family of regulators. *E. faecalis* strains carrying mutations in the genes encoding these MerR regulators were constructed in order to investigate their possible role in regulation of *zntAEf*. Plasmid pSAVE13 (*P$_{zntAEf}$::lacZ*) was introduced in the various mutants and the effect of zinc addition on the activity of *P$_{zntAEf}$* was tested. LacZ expression in the mutants still responded to zinc addition (data not shown), indicating that none of the predicted MerR regulators in the genome of *E. faecalis* V583 is involved in the regulation of *zntAEf* expression.

In order to reach distant body sites and infect the host, *E. faecalis* must be able to survive inside host defence cells. Phagocytic and antigen-presenting cells of the immune system, including macrophages, engulf bacteria into phagosomes, which then merge with lysosomes. Consequently,
engulfed bacteria are subjected to an onslaught of antimicrobial factors, such as reactive oxygen species, metal stress (Wagner et al., 2005) and lysozyme (Plüddemann et al., 2011). Most intracellular environments have negligible levels of free zinc. However, in macrophages the levels of this metal are likely to fluctuate, as zinc is essential for the normal function of these immune system elements. Amongst others, it contributes to intracellular pathogen killing (Prasad, 2009). Macrophages are likely to adapt zinc levels in order to inhibit pathogen growth. A recent study reported that Mycobacterium tuberculosis and E. coli use P-type ATPases in a strategy to avoid the toxic effects of zinc inside macrophages, in which a burst of free zinc occurs within a few hours following bacterial infection (Botella et al., 2011). The roles of ZntA Eff and EF0759 in E. faecalis survival inside macrophages, and in the resistance of the bacterium to oxidative stress and lysozyme, were thus investigated. As shown in Fig. 3, the E. faecalis zntA Eff and ef0759 mutants were significantly impaired in their capacity to survive inside macrophages when compared with their parent E. faecalis VE14089. The latter was even able to divide to some extent inside macrophages for the first 2 h and only later succumbed to macrophage defences, as reported previously (Kloosterman et al., 2008). Both proteins are, indeed, relevant for survival inside macrophages. However, it is unlikely that EF0759 contributes to this E. faecalis phenotype through increasing resistance to zinc, as shown earlier in this work.

To determine whether the decreased survival of the mutants inside macrophages was related to exposure to any of the host defences mentioned above, strain VE14089 and the mutants ef0759 and zntA Eff were tested for their tolerance to oxidative stress resulting from treatment with 20 mM H2O2 and to lysozyme. The ef0759 mutant did not behave differently from the WT strain VE14089, neither in terms of oxidative stress response nor lysozyme resistance (data not shown), suggesting that the EF0759 protein helps E. faecalis to survive inside macrophages in other ways than by protecting against zinc excess, oxidative stress or lysozyme exposure. The ef0759 gene encodes a putative SapB protein of unknown function. The protein contains an MgtC superfamily domain. MgtC family proteins are, in some bacterial species, involved in survival inside macrophages (Alix & Blanc-Potard, 2007; Günzel et al., 2006; Tao et al., 1995). The genes of these proteins are often found in an operon with a gene for a P-type ATPase (Alix & Blanc-Potard, 2007) and gene expression is modulated by zinc ions (Tao et al., 1998). At this stage, any attribution as to the actual function of EF0759 would be mere speculation. However, it is likely that this protein plays a role in E. faecalis pathogenicity, as the inactivation of its gene affected the survival inside macrophages (this work).

The zntA Eff mutant was killed faster by H2O2 than its parent strain (Fig. 4). It showed a >30-fold decrease in survival just 15 min after exposure and a 70-fold decrease in

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**Fig. 2.** E. faecalis tolerance to zinc. Two control strains [E. faecalis VE14089 and E. faecalis SAVE36 (VE14089 ef0759::tet)] and SAVE37 (VE14089 zntA Eff::tet) were grown overnight. Subsequently, the OD600 values were adjusted to 1, cultures were diluted up to 10^6-fold, and 20 μl was spotted on BHI plates with (a) 0 or (b) 20 mM ZnSO4 and incubated for 24 h at 37 °C.

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**Fig. 3.** Intramacrophage survival of E. faecalis. Percentage of survival of E. faecalis strains VE14089, SAVE36 (VE14089 ef0759::tet) and SAVE37 (VE14089 zntA Eff::tet) inside macrophages (J774.A1 cells) at the indicated time points after addition of the bacteria to a confluent layer of macrophages (m.o.i. 10). Bacterial survival was measured by plating appropriate dilutions on BHI agar plates. Results represent the mean of three independent experiments. *P<0.002; **P<0.0004.
survival after 30 min of exposure to 20 mM H₂O₂ relative to the WT. The zntAEF mutant was also markedly more susceptible to the presence of lysozyme (Fig. 5), suggesting that the decreased survival of the *E. faecalis* zntAEF mutant in macrophages may be linked to increased zinc, oxidative stress and lysozyme susceptibility. The fact that the zntAEF mutant displayed an increased susceptibility to zinc and a decreased sensitivity to both oxidative stress and to lysozyme suggests that the three phenotypes are linked. Such a connection between zinc resistance and higher tolerance to oxidative stress and lysozyme has, to the best of our knowledge, never been described previously. A zinc burst, described to occur inside macrophages (Botella *et al.*, 2011), could indirectly affect the mechanisms of oxidative stress response, as it is known that zinc is able to compete with manganese (Kloosterman *et al.*, 2008; McDevitt *et al.*, 2011), a crucial metal in oxidative stress response, in protein binding. McDevitt *et al.* (2011) found that extracellular zinc inhibits manganese acquisition in *Streptococcus pneumoniae* by competing for binding to the solute-binding protein PsaA. Similarly, we have previously reported that the operon ef0575–ef0577, which encodes a PsaA-like solute-binding protein (EF0577), is upregulated by zinc and downregulated by manganese (Abrantes *et al.*, 2011), revealing an *E. faecalis* manganese transporter that is modulated by zinc concentrations. Moreover, the *E. faecalis* regulator EfaR was recently shown to be involved in oxidative stress response and its activity was found to be impaired by zinc competing for manganese binding (Abrantes *et al.*, 2013). Furthermore, *E. faecalis* sodA expression is affected by EfaR (Abrantes *et al.*, 2013), indicating a role for manganese abundance in sodA expression. Collectively, these data strongly support a zinc toxicity mechanism whereby an increased zinc/manganese ratio is responsible for reduced tolerance to zinc overload and oxidative stress in *E. faecalis* zntAEF.

Lysozyme is present in several body fluids and plays a very important role in host defence against bacterial pathogens. *E. faecalis* is known for its high resistance to lysozyme (Le Jeune *et al.*, 2010) and several lysozyme resistance mechanisms have been identified. None of these, however, has an obvious link to metals, although manganese is known to be important in bacterial cell wall stabilization (Jakubovics & Jenkinson, 2001). If zinc toxicity is caused by reduced availability of manganese ions, the latter may explain the decreased resistance of the zntAEF mutant to lysozyme.

The results obtained with the zntAEF mutant indicate that ZntAEF plays a crucial role in *E. faecalis* survival inside macrophages, helping the cell to deal with the attack by host defences. Macrophages use at least three defence mechanisms to fight engulfed bacteria, i.e. oxidative burst, zinc overload and lysozyme production (Botella *et al.*, 2011; Gordon *et al.*, 1974). As ZntAEF is proven here to be involved in defending *E. faecalis* against all three host attacks, we propose that this efflux system is one of the main constituents that enables *E. faecalis* to endure life inside the host. A BLAST search revealed that ZntAEF is ubiquitous amongst all sequenced enterococcal genomes, strengthening the notion of its importance. This work is the first description of a Zn²⁺–specific P-type ATPase involved in such biological processes in *E. faecalis*. Saliva, one of the first host barriers, contains high concentrations of zinc and lysozyme, and the work presented here on ZntAEF could explain the success of *E. faecalis* as an orthodontia pathogen. Unravelling the exact mechanisms behind the link between zinc and lysozyme modes of action might provide novel insights to better fight pathogens such as *E. faecalis*.  

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**Fig. 4.** *E. faecalis* tolerance to oxidative stress. Survival of *E. faecalis* VE14089 (○) and its isogenic insertion mutant SAVE37 (VE14089 zntAEF::tetΔ) (△) at 15, 30 and 60 min after challenge with 20 mM H₂O₂. A value of 100% corresponds to the number of c.f.u. immediately prior to the submission to 20 mM H₂O₂ (0 min). The values represent the mean ± SD of three independent experiments. At 15 and 30 min, the two strains showed significant differences (P values of 4.356×10⁻⁹ and 1.628×10⁻⁶, respectively).

**Fig. 5.** *E. faecalis* tolerance to lysozyme. *E. faecalis* strains VE14089 and SAVE37 (VE14089 zntAEF::tetΔ) were grown overnight, adjusted to OD₆₀₀ 1 and diluted up to 1000-fold. Aliquots of 5 μl were spotted on LB plates with lysozyme at (a) 0 or (b) 10 mg ml⁻¹ and incubated for 24 h at 37 °C.
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