The Extracytoplasmic Function Sigma Factor SigV Plays a Key Role in the Original Model of Lysozyme Resistance and Virulence of *Enterococcus faecalis*

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**Abstract**

**Background:** *Enterococcus faecalis* is one of the leading agents of nosocomial infections. To cause diseases, pathogens or opportunistic bacteria have to adapt and survive to the defense systems encountered in the host. One of the most important compounds of the host innate defense response against invading microorganisms is lysozyme. It is found in a wide variety of body fluids, as well as in cells of the innate immune system. Lysozyme could act either as a muramidase and/or as a cationic antimicrobial peptide. Like *Staphylococcus aureus*, *E. faecalis* is one of the few bacteria that are completely lysozyme resistant.

**Results:** This study revealed that *oatA* (O-acetyl transferase) and *dlt* (D-Alanylation of lipoteicic acids) genes contribute only partly to the lysozyme resistance of *E. faecalis* and that a specific transcriptional regulator, the extracytoplasmic function SigV sigma factor plays a key role in this event. Indeed, the sigV single mutant is as sensitive as the *oatA/dltA* double mutant, and the sigV/oatA/dltA triple mutant displays the highest level of lysozyme sensitivity suggesting synergistic effects of these genes. In *S. aureus*, mutation of both *oatA* and *dlt* genes abolishes completely the lysozyme resistance, whereas this is not the case in *E. faecalis*. Interestingly SigV does not control neither *oatA* nor *dlt* genes. Moreover, the sigV mutants clearly showed a reduced capacity to colonize host tissues, as they are significantly less recovered than the parental JH2-2 strain from organs of mice subjected to intravenous or urinary tract infections.

**Conclusions:** This work led to the discovery of an original model of lysozyme resistance mechanism which is obviously more complex than those described for other Gram positive pathogens. Moreover, our data provide evidences for a direct link between lysozyme resistance and virulence of *E. faecalis*.

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**Introduction**

*Enterococcus faecalis* is a natural member of the gastrointestinal tract of humans and animals. This ubiquitous Gram positive bacterium is abundant in wastewater, on plants, and disseminated in various ecological niches where fecal contamination occurs [1,2]. It is also an important microorganism of dairy products and infections such as urinary tract infection, endocarditis, and surgical wound [3]. In addition, due to its innate and acquired resistances to several antibiotics including in some cases vancomycin, conventional therapies are insufficient to threat *E. faecalis* serious infections [6]. Although several genes for virulence factors in *E. faecalis* have been characterized, and their effects have been demonstrated in animal models or cultured cells [7], the mechanisms by which this peaceful commensal became a life-threatening pathogen are not well understood [4].

As for other pathogens, in order to survive and colonize a given host, *E. faecalis* must successfully overcome specific and non specific host defense mechanisms. One of the most important and widespread compounds of this defense system against invading microorganisms is lysozyme. This enzyme is found in a wide variety of body fluids, such as tears, breast milk, respiratory and saliva secretions, as well as in cells of the innate immune system, including neutrophils, monocytes, macrophages, and epithelial cells [8–10]. Lysozyme acts on bacteria by hydrolyzing the β-1,4 glycosidic bonds between N-acetylmuramic acid (MurNAC) and N-acetylglucosamine (Gluc-NAC), resulting in the degradation of the peptidoglycan (PG), and...
subsequent cell lysis. It has also a cationic anti-microbial peptide (CAMP) function that leads to bacterial death likely through the destabilization of the cytoplasmic membrane [11–13].

In contrast to the majority of bacteria, and like some important human pathogens such as Staphylococcus aureus or Neisseria gonorrhoeae, E. faecalis is completely resistant to lysozyme. Three main mechanisms involved in this resistance have been well described for different species of bacteria. Two of them counteract antibacterial activity of lysozyme: i) the modification of different sites of the PG structure by two kinds of enzymes such as the N-acetylglucosamine deacylase (PgdA) of Streptococcus pneumoniae [14], or the peptidoglycan-specific D-acetyltransferase (OatA) of S. aureus [15] prevents the binding of lysozyme to its substrate and contributes to the muramidase resistance; ii) the modification of the net negative charge of the bacterial cell surface by adding positively charged residues (D-alanine esterification through dlt genes) to teichoic and lipoteichoic acids helps bacteria to avoid being killed by antimicrobial peptides or CAMP activity of lysozyme [13,16]. The third mechanism to evade the killing action of lysozyme consists on the production of lysozyme inhibitors such as the streptococcal inhibitor of complement (SIC) in streptococci [17], the inhibitor of vertebrate lysozyme (Ivy) in E. coli [18], and the peptidylglycan-specific lysozyme inhibitor of c-type lysozyme (named PliC or MliC, in Salmonella enteritidis and E. coli, respectively) [19], which have protective function.

The availability of E. faecalis genome sequence [20] provides a tool for the identification of new virulence factors which could help understanding pathogenicity mechanisms and regulatory components, such as alternative sigma factors, that could be involved in their infection processes. Bacterial sigma factors are a class of proteins constituting essential dissociable subunits of RNA polymerase to direct the initiation of the transcription from specific promoter sequences [21,22]. In addition to the “housekeeping” sigma factor (i.e. RpoD or σ^70 of E. coli), most bacteria have multiple alternative sigma factors that they use to coordinately regulate the expression of genes whose products are involved in diverse functions, such as stress responses, iron uptake, virulence, morphological development, and chemotaxis [23,24]. Like those of Streptococcus pyogenes, S. pneumoniae, and Lactococcus lactis, the E. faecalis genome lacks a general stress response regulator homologous to σ^B of Bacillus subtilis [25]. However, in a previous work, we have shown that the extracytoplasmic function (ECF) sigma factor SigV contributes to survival following heat, acid and ethanol treatments, and is also likely involved in lysozyme resistance of E. faecalis [26]. In E. faecalis, this lysozyme resistance was only in part related to oatA gene [27].

This study is dedicated to the continuum of the story of E. faecalis lysozyme resistance. Based on in silico analyses and on previous studies [13,16,26,27], we analyzed in depth the role of some genes involved (oatA) or suspected to be involved (sigV, dlt and mprF) in the E. faecalis lysozyme resistance. We constructed their corresponding mutants and analyzed their behavior comparatively to the parental strain. Thus, we showed that in addition to the pleiotropic effects displayed by SigV, it also contributes importantly to the lysozyme resistance and virulence of E. faecalis.

Results

Identification of genes involved or suspected to be involved in lysozyme resistance

Based on preliminary results, we have already suggested that sigV (EF3186) gene encoding an ECF sigma factor is likely involved in lysozyme resistance of E. faecalis [26]. A further study revealed that among the two main genes pgdA-like (EF1843) and oatA (EF0783) able to confer this lysozyme resistance through the modification of different sites of the PG structure, only oatA has a significant role in E. faecalis [27].

In the context of cell envelope net charge that may have a role in lysozyme resistance, we included the analysis of the dlt operon (D-alanylation of lipoteichoic acids) for which evidence in lysozyme resistance mechanism was established in S. aureus [13]. The genetic organization of the dlt operon of E. faecalis as reported by Fabretti et al., [28] is composed by 4 genes dltA, B, C, and D (EF2749, EF2748, EF2747, and EF2746, respectively) where dltA encodes a putative D-alanine-activating enzyme. However, a careful analysis of the genome sequence of E. faecalis V583 strain revealed an additional gene dltX (EF2750), upstream of dltA which likely belongs to the dlt operon (Fig. 1). This gene encodes a putative short protein (49 amino acid residues), homologous to that described for S. aureus and highly conserved among Gram positive bacteria [29].

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**Figure 1. Structural organization of sigV, oatA, dltA and mprF loci.** Large arrows correspond to the indicated genes. Grey areas show the deleted region by double cross over event and that of sigV locus (harbouring an asterisk) represents the deletion carried out in SAS mutant strain. The solid triangle positioned on EF0031 shows the insertion site of the integrative pUCB300-mprF recombinant plasmid generating the mprF mutant. The putative promoters and terminators are indicated with P and T letters, respectively. Primers used to construct the different mutants are indicated by black arrows, and listed in Table 4.

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The *in silico* analysis of the V583 genome sequence has also revealed the presence of EF0031 gene, whose product shares 24% identity and 45% homology with MprF (multiple peptide resistance factor) of *S. aureus*. This protein contributes to the modification of the membrane net charge by incorporation of L-lysine to phosphatidylglycerol. This reduces attractive electrostatic interaction and thereby, the binding of antimicrobial peptides by bacteria [30]. In addition, Blast searches using different databanks did not lead to identification of any obvious *E. faecalis* homologue to lysozyme inhibitors such as Ivy, Sic, MbiC or PliC.

Thus, for this work, we focused on the analysis individually or in combination of *sigV*, *oatA*, *dltA*, and *mprF* genes whose structural organizations are represented in Fig. 1.

**Sensitivity to lysozyme**

Prior to lysozyme treatment, the parental *E. faecalis* JH2-2 strain and its isogenic derivative mutants were tested for their growth behavior on broth or solid media. Monitoring growth kinetics on GM17 broth revealed no significant difference for all mutants relatively to the parental JH2-2 strain (data not shown). Plating the strains on LB solid medium revealed also a similar result after 48 hours incubation at 37°C in absence of lysozyme (Fig. 2A).

The parental JH2-2 strain and its derivative mutants were tested for lysozyme sensitivity on LB plates containing large-scale range of lysozyme concentrations (0 to 20 mg/ml). The wild type JH2-2 strain was not affected in its growth at 20 mg/ml of lysozyme and even at higher concentrations (data not shown). Surprisingly, also the *mprF* insertional mutant was as resistant as the JH2-2 parental strain (Fig. 2A). This result showed that *mprF* (suspected to avoid CAMP activity) is not involved in lysozyme resistance mechanism of *E. faecalis*, and was no longer investigated in this work. In contrast, the other mutants tested do not grow at 20 mg/ml of lysozyme. Thus, for the histogram representation of the results (Fig. 2B), this concentration was chosen as the highest limit.

Both *oatA* and *dltA* single mutants were similarly more sensitive to lysozyme than JH2-2 parental strain, but inhibition of growth still needs at high concentration of lysozyme (about 10 mg/ml) (Fig. 2B).

![Figure 2. Susceptibility to lysozyme.](https://example.com/lysozyme.png)

**Figure 2. Susceptibility to lysozyme.** The lysozyme sensitivity of *E. faecalis* JH2-2 and its derivative mutants was tested on LB agar medium. Overnight cultures were adjusted to OD<sub>600</sub> = 1 in physiological water, and diluted up to 10<sup>-2</sup>. Equal volume of each of the 10<sup>-1</sup>, 10<sup>-2</sup>, and 10<sup>-3</sup> dilutions are plated on LB solid medium containing large scale range of lysozyme concentrations. (A) Photographs of LB plates after 48 hours incubation under the indicated lysozyme concentrations. (B) The overall results of the lysozyme sensitivity experiments are summarized in this histogram.

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This confirms previous results showing that oatA is not the main effector of *E. faecalis* lysozyme resistance [27], and that dltA is also involved in this mechanism. The oatA/dltA double mutant was more lysozyme sensitive (about 5 mg/ml) than each of the single mutants (Fig. 2B). This additive effect of both genes is in agreement with the dual activities (muramidase and CAMP) of lysozyme.

The sigV single mutant showed a sensitivity level (5 mg/ml) similar to that of oatA/dltA double mutant (Fig. 2A and 2B). Comparatively to sigV mutant, sigV/oatA/dltA and mainly sigV/dltA double mutants showed increased sensitivity to lysozyme, and sigV/oatA/dltA triple mutant is the most sensitive (starting from 0.5 mg/ml) among all the strains tested (Fig. 2A and 2B). The synergistic effect of sigV deficiency in the oatA/dltA double mutant highlights the key role of the ECF SigV sigma factor in the lysozyme resistance mechanism of *E. faecalis*.

In order to confirm the specificity of SigV in its involvement in lysozyme resistance, we undertook a complementation assay using JH2-2 parental strain and its derivatives sigV, SAS pMSP3535-sigV (expressing SigV), SASpMSP3535 (control). Growth on LB solid medium without (Fig. 3, panel A) or with 20 mg/ml of lysozyme (Fig. 3, panel B) showed that SigV was able to restore the lysozyme resistance phenotype even at a concentration of 20 mg/ml (Fig. 3, panel B).

**Are oatA and dltA genes under the control of SigV?**

Regarding the results reported above, we wondered if there is a link at the transcriptional level between sigV, dltA, and oatA genes. Since EF1843 gene (pgdA-like) shares the same promoter sequence than sigV [26], we included it as a positive control for RT-qPCR analyses. The results of Table 1 clearly showed that sigV and pgdA-like are overexpressed when cells were exposed to 3 mg/ml lysozyme treatment for 30 minutes. Indeed, sigV and pgdA-like were similarly induced in JH2-2 strain since they are 320 fold and 247 fold overexpressed with significant p values of 0.001 and 0.027, respectively whereas dltA and oatA genes can be considered as expressed at a basal level. Under the same conditions, expression of pgdA-like, dltA, and oatA genes was not significantly influenced by exposition to lysozyme in the sigV mutant (Table 1).

In the JH2-2 strain, sigV and rsV genes constituting a bicistronic operon are expressed in the same way [26] and the rsV mutant was as resistant as the parental JH2-2 strain (data not shown). Regarding that most ECF sigma factors are auto regulated, we wondered if the overproduction of SigV will lead to consistent expression of sigV and what will be the incidence on the expression of pgdA-like, dltA, and oatA genes. For this purpose, SigV was overproduced using SAS pMSP3535-sigV and SAS pMSP3535 (control) strains under appropriate condition of nisin (0.5 µg/ml) induction and we analyzed the transcription of these genes using RT-qPCR. The results reported in Table 2 revealed that only sigV and pgdA-like genes are drastically overexpressed (3983 fold and 1992 fold induced, respectively), whereas no enhanced expression of oatA and dltA genes was observed demonstrating that both genes are not under the control of SigV.

**Is sigV involved in the resistance to CAMP activity?**

Both lysozyme and nisin possess a cationic domain involved in membrane destabilization. To avoid CAMP activity, bacteria have evolved mechanisms through the reduction of the cell envelope net charge mainly mediated by dlt genes as it has been described for *S. aureus*, *E. faecalis* and many other bacteria [15,16,28]. In order to determine whether SigV contributes to the resistance towards CAMP activities, we monitored growth in the absence or presence of nisin (the representative model of CAMPs) of sigV and dltA (control expected to be affected by nisin activity) single mutants comparatively to *E. faecalis* JH2-2 parental strain. In the absence of nisin, all strains had similar growth on GM17 broth (Fig. 4). In the presence of nisin, the three strains showed a similar prolonged lag phase (3 hours), and subsequently only the dltA mutant demonstrated enhanced sensitivity towards nisin (Fig. 4). Interestingly, the sigV mutant behaved similarly to the parental JH2-2 strain and was able to grow on GM17 supplemented with 2 µg/ml of nisin after an adaptation period (Fig. 4). These results strongly suggest that SigV ECF sigma factor does not contribute to the resistance towards CAMP activity in the tested conditions.

**Triton X-100-induced autolysis assays**

Mechanisms affecting the modification of the membrane net charge or the PG structure play a role in the modulation of

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**Table 1. RT-qPCR values under lysozyme (3 mg/ml) treatment.**

| Orfs | Gene name | JH2-2 | ΔsigV |
|------|-----------|-------|-------|
| EF3180 | sigV     | +320  | nd    |
| EF1843 | pgdA-like | +247  | −1.21 |
| EF2749 | dltA     | +1.83 | +1.02 |
| EF0783 | oatA     | +2.4  | +1.26 |

*(a)Factor of activation or repression determined by RT-qPCR in JH2-2 wild-type strain and sigV mutant exposed to 3 mg/ml lysozyme for 30 minutes. Values >2 with p values <0.05 are considered as significant. nd, not determined. doi:10.1371/journal.pone.0009658.t001*

**Table 2. RT-qPCR values under nisin (0.5 µg/ml) treatment.**

| Orfs | Gene name | RT-qPCR*(a) | Reference 23S |
|------|-----------|-------------|---------------|
| EF3180 | sigV     | +3983       | +3191         |
| EF1843 | pgdA-like | +1992       | +1884         |
| EF2749 | dltA     | −1.25       | −1.5          |
| EF0783 | oatA     | −1.01       | +1.1          |

*(a)Factor of activation or repression determined by RT-qPCR when sigV is overexpressed in the SAS pMSP3535-sigV compared to SAS pMSP3535 (control). doi:10.1371/journal.pone.0009658.t002*
autolysins activity and thus, may have an impact on bacterial autolysis [31–34]. In this context, we evaluated the effect of autolysis induction by triton-X100 on oatA, sigV, dltA single mutants, sigV/oatA/dltA triple mutant, and JH2-2 wild-type strain. Over a period of 5 hours, there was a decrease in OD_{600} nm of about 50%, and reaching 65% after overnight incubation for all strains tested towards exposure to 0.05% or 0.1% of Triton X-100 (Fig. 5). This result suggests that oatA, sigV, and dltA genes are not involved in autolysis process as it was already reported for the dlt mutant of E. faecalis 12030 strain [28].

**SigV is involved in systemic and urinary tract infection models**

In order to assess the effects of oatA, dltA, and/or sigV genes deletion on the virulence of E. faecalis JH2-2, we compared the fate of the wild-type and its derivative mutant strains in two different infection mouse models.

At first, by using a well-established intravenous infection model [35], we compared tissue burdens in kidneys and livers of groups of infected mice. As shown in Fig. 6, only sigV and sigV/oatA/dltA mutants showed statistically significant reductions of tissue burden both in kidneys and livers. In particular, the sigV mutant exhibited a reduction of 1.51 logs (p = 0.0058) in kidneys and of 1.37 logs (p = 0.0153) in livers as compared to the JH2-2 wild-type strain. Similar results were obtained for the triple mutant (−1.77 logs in the kidney, p = 0.0053; and −1.60 logs in the liver, p = 0.0139), suggesting that sigV is involved in E. faecalis virulence. However, it is noteworthy that while non significant, oatA seems to display a slight reduction of tissue burdens in livers (−0.40 logs, p = 0.0546), following systemic infection in mice as it could be seen in Fig. 6B.

The same strains were also tested in a urinary tract infection (UTI) model as described elsewhere [36]. The resulting ID_{50} showed that only the sigV single and triple mutants required 0.82 and 1.03 log_{10} more cells (4.6 × 10^7 and 7.4 × 10^7, respectively) than did the JH2-2 wild-type strain (6.9 × 10^7) to infect 50% of the mice, thereby suggesting that in the UTI model only sigV was implicated in E. faecalis infection (Fig. 7). Thus, percentages of kidneys of mice infected for all the bacterial inoculates used were 84% for the JH2-2 wild-type strain, 58% for the sigV mutant (p = 0.0047), and 33% for the triple mutant strain (p = 0.0012). Also, the cumulative difference between the bladders infected with the two strains was similar to that of the infected kidneys (71% for the JH2-2 versus 40% for the ΔsigV, p = 0.0066; and 71% for the JH2-2 versus 34% for the triple mutant, p = 0.0015). The Fig. 7 shows the log_{10} CFU recovered from the kidney pairs of mice infected with 10^7 cells from JH2-2 or each of mutant strains. As for the systemic infection model, only the strains ΔΔsigV and triple mutant exhibited statistically significant reductions (−1.83 logs for the ΔΔsigV, p = 0.004; and −2.04 logs for the triple mutant, p = 0.0038) in kidneys tissue burden as compared to the wild-type strain (Fig. 7A). Similar results were obtained when analyzing bladders. In fact, in this case also, only sigV single and triple mutants showed statistically significant reductions (−2.42 logs, p = 0.0012; and −2.32 logs, p = 0.0009, respectively) in tissue burdens when compared to the wild-type strain (Fig. 7B).

**Discussion**

Environmental adaptation traits and stress resistance mechanisms have been linked to virulence, as bacterial survival in the host is often reliant on these factors. Despite its ability to adapt to many different environmental stresses, E. faecalis possesses a moderate number of regulatory genes [37]. Among these, the ECF sigma factor SigV was already shown to have a pleiotropic effect on the stress response of E. faecalis JH2-2 and was also suspected to be involved in its lysozyme resistance [26]. This constitutes a prominent concern since this antimicrobial component is considered as the first line of defense of the innate immune system, and also because E. faecalis is among the few bacteria that are completely lysozyme resistant.

Lysozyme can affect bacteria by its muramidase and/or CAMP activities. To overcome the killing action of lysozyme, bacteria
have developed different mechanisms among which some are well dissected. They are mainly based on the modification of the PG structure (pgdA and oatA for S. pneumoniae [14,38]) associated to the reduction of the cell surface net charge (oatA and dltA for S. aureus [13,15], or pgdA, oatA and dlt for L. lactis [39,40]). However, the wild-type strains of the E. faecalis related lactic acid bacteria S. pneumoniae R36A [41] and L. lactis MG1363 [39,40] have very low levels of lysozyme resistance (below 300 μg/ml). In terms of levels of lysozyme resistance, the most appropriate model for a comparative study is S. aureus, a human pathogen which also resists to high concentrations of lysozyme (50 mg/ml) [13,15]. Thus, according to our results, the comparison of the lysozyme resistance mechanism models between E. faecalis and S. aureus was summarized in Fig. 8.

In S. aureus, the mechanism of the lysozyme resistance is based on muramidase (through oatA gene) and predominantly CAMP activities (through dlt operon). Indeed, the interruptions of both gene structures (oatA/dltA double mutant) sensitize the bacterium almost entirely to lysozyme (30 μg/ml) [Fig. 8] [13]. In addition, these gene structures are under the control of the glycopeptide resistance associated two-component system (TCS) regulator GraRS which regulates positively the dlt operon and negatively the oatA gene [13]. This is not the case for E. faecalis (Fig. 8).

The lysozyme resistance mechanism of E. faecalis is widely different from those reported above and obviously more complex as shown in Fig. 8. In E. faecalis, oatA but not pgdA-like was shown to contribute to lysozyme resistance [27]. Here we demonstrate that another part of lysozyme resistance (similar level to that conferred by oatA gene) is due to dlt genes. Since the oatA/dltA double mutant is twofold more sensitive to lysozyme than the respective single mutants, the contribution of each system seems to be additive. However, oatA/dltA double mutant still demonstrates a relatively high resistance to lysozyme (5 mg/ml), indicating that oatA and dlt genes can not be considered as the major determinants of E. faecalis lysozyme resistance. These findings revealed also that E. faecalis may certainly have additional effectors involved in its lysozyme resistance.

In this work, in depth investigation showed that sigV single mutant is as sensitive to lysozyme as oatA/dltA double mutant. In addition, the sigV deletion associated to that of oatA or dltA lead systematically to greater lysozyme sensitivity and the lowest level of resistance (0.5 to 1 mg/ml) was observed for sigV/oatA/dltA triple mutant. These results demonstrate a synergistic effect of sigV, oatA, and dltA genes, and confirm the key role of SigV in the lysozyme resistance of E. faecalis.

The transcriptional results suggest that lysozyme induces specifically sigV and its potential regulon members as it was shown for pgdA-like gene. Moreover, the overexpression of sigV either under lysozyme treatment or nisin induction has no incidence on the expression of oatA and dltA ruling out both genes from the sigV regulon. Our analyses showed also that the sigV mutant is not affected by the CAMP activity of nisin and thereby likely by that of lysozyme. Moreover, neither dltA nor sigV or oatA do contribute to the autolysis of E. faecalis JH2-2 under moderate detergent treatment.

To our knowledge, this is the first study demonstrating that an ECF sigma factor is the most important effector of lysozyme resistance in a bacterium. SigV as a transcriptional regulator does not control neither oatA nor dlt genes but it regulates positively the pgdA-like gene which was already shown to be not involved in the

![Figure 6. SigV is required for the virulence of E. faecalis in a murine systemic model.](image)
lysozyme resistance of *E. faecalis* [27]. Taken together our results argue in favor of a complex and original model of resistance to lysozyme of *E. faecalis* (Fig. 8). It is based at least on three effectors, *oatA*, *dltA* and mainly SigV ECF sigma factor through one or more gene(s) under its control, which remain to be identified.

Since lysozyme is considered as the first line of defense of the innate immune system, the bacterial resistance towards this immunogenic compound could be associated to virulence of pathogens. Regarding elucidated lysozyme resistance mechanisms; there are evidences in favor of this claiming. Indeed, *pgdA* of *S.

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**Figure 7. SigV is required for the virulence of *E. faecalis* in a murine UTI model.** Enterococcal burdens of kidneys (A) and bladders (B) of Balb/c mice infected transurethrally with $10^6$ cells of *E. faecalis* JH2-2 wild-type (●) and its isogenic mutant strains Δ*dltA* (■), Δ*oat* (▲), Δ*sigV* (▲), and Δ*dltA-oat-sigV* (●). Kidney pair and bladders homogenates were obtained from groups of 15 mice that were sacrificed and necropsied 48 h after the transurethral challenge. Results, expressed as log$_{10}$ CFUs per gram of tissue, represent values recorded separately for each mouse. Horizontal bars represent the geometric means. A value of 0 was assigned to uninfected kidneys or bladders. *p* value of less than 0.05 was considered to be significant.

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**Figure 8. Comparison between the lysozyme resistance effectors of *S. aureus* and *E. faecalis*.** The lowest concentrations of lysozyme resistance are indicated in parenthesis (data for *S. aureus* are from Herbert et al. [13]) and the most relevant are shaded. The relationship to virulence of the *E. faecalis* lysozyme resistance effectors is also indicated. Arrows thickness is relatively proportional to the involvement of the considered gene products in lysozyme resistance or virulence. Blocked arrows correspond to no effect on the considered event. Signs + and − correspond to up and down-regulation, respectively.

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to that of oatA gene expressions (Fig. 8) arguing that hitherto unknown activities and mechanisms are responsible for this phenomenon in S. aureus. Indeed, we have shown in a previous study a significant sensitivity in macrophages survival of the oatA mutant relatively to the wild-type strain [27]. In the present work, the recovery of this oatA mutant seems only slightly affected in the intravenous infection model in mice. This part of virulence could be related to lysozyme resistance as already hypothesized [27] but independently from SigV. Based on the assumptions described for intravenous [35] and urinary tract [48] infections, these observations suggest that oatA may contribute to the inflammatory response and to the persistence in mouse peritoneal macrophages but likely not in promoting colonization and adherence to uroepithelium. In contrast, there is no effect on virulence attenuation of the E. faecalis dltA mutant comparatively to the parental JH2-2 strain, at least in the models studied.

Many ECF sigma factors have been described to be involved in virulence of different bacteria such as Mycobacterium tuberculosis, S. enterica and Pseudomonas aeruginosa (for review, see [23,24]). In this work, in addition to lysozyme resistance, the most striking role we have defined for SigV is its involvement in the virulence of E. faecalis. Using murine models of intravenous or UTI infections we demonstrated that in each of the tests applied, E. faecalis cells lacking a functional sigV gene were significantly impaired in their ability to establish and maintain infection for which a major hallmark is systemic dissemination, moving from the site of infection into the kidneys. In our analyses, the sigV and sigV/oatA/dltA mutants clearly showed a reduced capacity to colonize host tissues, as they are unambiguously less recovered from organs of mice subjected to intravenous or UTI infections than the wild-type JH2-2 strain. This demonstrates that SigV ECF sigma factor is certainly and more globally involved in virulence of E. faecalis. Interestingly, our results allowed also establishing through oatA and clearly sigV a direct link between lysozyme resistance and virulence of E. faecalis.

Unlike GraRS TCS regulator which controls the main effectors of the lysozyme resistance of S. aureus, SigV has no effect on dlt or oatA gene expressions (Fig. 8) arguing that hitherto unknown activities and mechanisms are responsible for this phenomenon in E. faecalis. On the other hand, the role of SigV could be paralleled to that of σ8, an ECF sigma factor of S. aureus recently described [49] as both are important components of the stress and pathogenic responses. In absence of a general stress response regulator in the genome sequence of E. faecalis, it appears that SigV can fulfill this role, at least in part, since it is not only involved in the response to harsh conditions [26] but also in the lysozyme resistance and virulence.

This work provides actual new insights that may help the investigation for new targets for antimicrobial drug development. The future purpose of our laboratory is to explore the mechanisms by which SigV constitutes an important regulatory component of the stress and pathogenic responses of E. faecalis in order to strengthen our knowledge about this potent opportunistic human pathogen.

Materials and Methods

Ethics statement

Marine work was performed under a protocol approved by the Institutional Animal Use and Care Committee at Universita Cattolica del S. Cuore, Rome, Italy.

Bacterial strains, plasmids, and culture conditions

Bacterial strains, plasmids, and oligonucleotide primers used in the present work are listed in Tables 3 and 4, respectively. E. faecalis JH2-2 strain and its derivatives were grown at 37°C without shaking, in M17 medium [50] supplemented with 0.5% glucose (GM17), or on otherwise specified media depending on requirements of the experiments. E. coli Top10F strain was cultured with vigorous shaking at 37°C in LB broth. When required erythromycin (100 μg/ml) or ampicillin (100 μg/ml) was added.

DNA manipulations

Restriction endonucleases and T4 ligase were obtained from Promega (Madison, Wi) and used in accordance with the manufacturer’s instructions. Plasmids and PCR products were purified using Nucleospin plasmid and nucleospin extract II kits (Macherey-Nagel, Düren, Germany). Molecular cloning and other standard techniques were preformed essentially as previously described [51]. E. coli and E. faecalis strains were transformed by electroporation using Gene Pulser Xcell (Bio-Rad Laboratories, Richmond, Ca, USA) as described by Dower et al., [52] and Holo and Nes [53], respectively.

Deletion mutagenesis

The construction of the JH2-2 derivative mutants was carried out with the pMAD plasmid by exploiting its property of thermo sensitive conditional replication [54]. Briefly, two fragments of approximately 900 bp corresponding to the flanking regions (including the 5’ and the 3’end parts, respectively) of the target genes were amplified by PCR using appropriate primers (Table 4). Then, they were purified, restricted with appropriate endonucleases and ligated into the pMAD vector in order to generate truncated allele of the gene of interest where the most part of the coding sequence (CDS) was deleted (60 to 80% of the CDS corresponding to the median part of the gene). The ligation mixture was transformed by electroporation into E. coli Top10F cells. After selection and verification, the generated recombinant plasmids were used to transform E. faecalis JH2-2 electrocompetent cells and gene replacement was performed via double cross over events as described previously [27].

Construction of the mprF insertional mutant

The mprF insertional mutant was constructed following another procedure. Briefly, an internal fragment of mprF gene was amplified by PCR with specific primers (Table 4), digested by EcoRI and PstI and ligated in the suicide vector pUCB300 [55] previously treated with the same enzymes. The ligation product was electroporated into E. coli Top10F cells. The subsequent recombinant plasmid (pUCB300-mprF) (Table 3) was transformed in E. faecalis JH2-2. The generated derivative mutant (mprF:pUCB300) (Table 3) was verified by PCR for the insertion of the recombinant plasmid pUCB300-mprF within the chromosome leading to inactivated mprF gene.

Lysozyme sensitivity

Lysozyme sensitivity assays were performed on LB medium plates containing different concentrations (0 to 20 mg/ml) of hen egg white lysozyme (HEWL) (Fluka, Buchs, Switzerland). Overnight cultures of the parental JH2-2 strain and its derivative mutants were adjusted to OD(600) of 1 in physiological water, and diluted up to 10⁻⁷. An equal volume (5 μl) of the 10⁻⁷, 10⁻⁸, and 10⁻⁹ dilutions was then spotted on LB plates with, or without
### Table 3. Bacterial strains and plasmids.

| Strains and plasmids          | Relevant characteristics                                                                 | Reference or source |
|-------------------------------|------------------------------------------------------------------------------------------|---------------------|
| **E. faecalis strains**       |                                                                                          |                     |
| JH2-2                         | Fus\(^1\), Rif\(^0\), plasmid-free wild-type strain                                      | [59]                |
| ΔsigV                         | JH2-2 isogenic derivative EF3180 mutant (S26)                                              | [26]                |
| ΔoatA                         | JH2-2 isogenic derivative EF0783 mutant                                                   | [27]                |
| ΔdltA                         | JH2-2 isogenic derivative EF2749 mutant                                                   | this study          |
| ΔsigV/ΔoatA                   | JH2-2 isogenic derivative EF3180 and EF0783 double mutant                                 | this study          |
| ΔsigV/ΔdltA                   | JH2-2 isogenic derivative EF3180 and EF2749 double mutant                                 | this study          |
| ΔoatA/ΔdltA                   | JH2-2 isogenic derivative EF0783 and EF2749 double mutant                                 | this study          |
| ΔsigV/ΔoatA/ΔdltA             | JH2-2 isogenic derivative EF3180, EF0783, and EF2749 triple mutant                        | this study          |
| SAS                           | JH2-2 isogenic derivative sigV-rsI\(^V\) double mutant                                   | [26]                |
| SAS pMSP3535                  | Em\(^R\), JH2-2 isogenic derivative sigV-rsI\(^V\) double mutant with pMSP3535          | [26]                |
| SAS pMSP3535-sigV             | Em\(^R\), JH2-2 isogenic derivative sigV-rsI\(^V\) double mutant with pMSP3535-sigV    | this study          |
| mprF-UCB300                   | pUCB300 carrying mprF internal fragment                                                   | this study          |
| SAS pMSP3535-csigV            | pMSP3535 carrying sigV gene                                                                | this study          |
| **E. coli strain**            |                                                                                          |                     |
| Top10F\(^{+}\)                | F\(^{-}\) lacI, Tn10 (TetR) mcrA Dimrr-hsdRMS-mcrBC f80lacZDM15 DlacX74 deoR recA1 araD139 D(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG | Invitrogen          |
| **Plasmids**                  |                                                                                          |                     |
| pMAD                           | 9.66 kb ori pE194\(^R\), Em\(^R\), Amp\(^R\), bga\(^8\)                                    | [54]                |
| pMAD-ΔsigV                    | pMAD carrying sigV deletion                                                               | this study          |
| pMAD-ΔdltA                    | pMAD carrying dltA deletion                                                                | this study          |
| pUCB300                       | 3.9 kb, lacZ\(^+\), Amp\(^R\), Em\(^R\)                                                   | [55]                |
| pUCB300-mprF                  | pUCB300 carrying mprF fragment                                                             | this study          |
| pMSP3535                      | 8.35 kb Em\(^R\), nisR nisK P\(_{max}\) (nisin inducible promoter)                      | [56]                |
| pMSP3535-csigV                | pMSP3535 carrying sigV gene                                                               | this study          |

Ery, erythromycin; Rif, rifampin; Fus, fusidic acid; Amp, ampicillin; bga\(^8\), β-galactosidase; nis, nisin.

\(^{+}\), resistant; \(^{0}\), sensitive; \(^{\text{Tm}}\), thermostable.
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### Table 4. Oligonucleotide primers used in this study.

| Primer name | Forward primer * | Reverse primer * | Operation |
|-------------|------------------|------------------|-----------|
| dltA 5–6    | CGACAACGGAATTCGCCTGTAAG (EcoRI) | ATTCTCCGTAATCGACATCGCTG (SalI) | pMAD Cloning |
| dltA 7–8    | GGAAGGGTATGGCGAACCACCTGTGGG (SalI) | ACGATCGATGGAATCCCTGAAAGAAT (SalI) | pMAD Cloning |
| sigV 2V-FT3 | TGGCACGAAATTCGCGAAAGTG (EcoRI) | GGATGGCCTGGAGGCTTCTTC (PstI) | pMAD Cloning |
| sigV 3V-4V  | AGATGGATCGATCCTGAAATGA (PstI) | CAATGACTTCATCTGGAATAC (BglII) | pMAD Cloning |
| pMSP V1-2   | CGCTCTATAAGGTCGCGGAA (PstI) | GTTGGGCGCTGCTTTGGAGAA (PstI) | pMSP3535 Cloning |
| Efo031 2-3r | GGTACCGCGATTCGCGGCTGTAAG (EcoRI) | GTTCCCATATTGGCGGAACATAGA (PstI) | pUCB300 Cloning |
| dltA RT 1-2 | AACCAAGCTTCAATGGA (TspI) | ACGAGGGTTAAAATTCTCA | RT-qPCR |
| LH 54-55    | GCATCGCGATTCGCTGTAATGA | ACCCTGGCCATCTGAGCTCAT | RT-qPCR |
| LH 56-57    | AACATCGATTCGCGAACCACAA (SalI) | GTTGGGCGCTGCTTTGGAGAA (PstI) | RT-qPCR |
| SVRT 1-2    | CGAAAGACAGATCGCTGGAT | AAGAACCACGCGCTCAAAATC | RT-qPCR |

* Underlined nucleotides stand for the restriction sites (indicated in parenthesis) inserted within the primer sequences and nucleotides in lowercase are different from those of the target sequence.
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lysozyme. The bacterial growth was evaluated after 48 hours of incubation at 37 °C and photographed.

Nisin sensitivity

In order to assess the effect of nisin on the kinetic growth of *E. faecalis* JH2-2 and its derivative mutants, strains were grown on GM17 to mid-log phase. At this step, 40 ml of fresh medium was inoculated in order to start the culture from OD<sub>600</sub> of 0.05 and divided into two parts. The first 20 ml culture was carried out without supplementation (control) and the second received 2 μg/ml of nisin (Sigma Chemical Co, St. Louis, Mo, USA). The growth kinetic of the two cultures was then monitored at OD<sub>600</sub> nm during 8 hours.

Trigon X-100-induced autolysis assays under non-growing conditions

Strains were grown to an OD<sub>600</sub> of 0.8 in GM17 medium and treated as previously described [34]. Briefly, cells were harvested by centrifugation (4000 g, 10 min at 4°C), washed with ice-cold sterile water, and then resuspended in the same volume of 50 mM Tris-HCl, pH 7.5, containing 0.05% or 0.1% Triton X-100. The cell suspensions were then transferred into 100-ml sterile micro plates and incubated at 37°C without shaking. Autolysis was monitored by measuring OD<sub>600</sub> of the cell suspensions every 30 min, with an automated incubator/optical density reader (Model 680, Bio-Rad Laboratories).

Construction of SAS pMSP3535-sigV strain

In order to overproduce the ECF sigma factor SigV, we cloned its corresponding gene into pMSP3535 plasmid [56]. Briefly, the entire gene with its own translation signal was amplified by PCR using the appropriate primers (Table 4) and then inserted under the control of the nisin inducible promoter (PnisC) of pMSP3535 plasmid. The subsequent recombinant plasmid (pMSP3535-sigV) (Table 3) previously obtained in *E. coli* TOP10F was transformed into electro-competent *E. faecalis* JH2-2 SAS cells generating the SAS pMSP3535-sigV strain (Table 3) which overproduces the SigV sigma factor.

RNA isolation and RT-qPCR experiments

In order to assess comparative transcriptional gene expression, we used JH2-2 wild-type strain and its sigV derivative mutant cultured on GM17 medium supplemented with 3 μg/ml lysozyme. On the other hand, to analyze and verify the effect of SigV overproduction on oatA, pgdA, dltA and on its own sigV gene expression, SAS pMSP3535 and SAS pMSP3535-sigV strains (Table 3) were used under nisin induction. Using RNeasy Midi Kit (Qiagen, Valencia, Ca, USA), three or two independent samples of total RNA were isolated for each condition, respectively. Prior to the extraction, the mid-log phase (OD<sub>600</sub> = 0.4) cultures were treated during 30 minutes with 3 μg/ml lysozyme or 0.5 μg/ml of nisin.

For reverse transcriptase-quantitative real-time PCR (RT-qPCR), specific primers were designed using the *E. faecalis* V583 genome sequence and the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primer pairs listed in Table 4 were designed in order to produce amplicons of equivalent length (100 bp). Two micrograms (2 μg) of total RNA were reverse transcribed with random hexamer primers and Omniscript enzyme (Qiagen). Quantification of 23S rRNA or gyrA (encoding the gyrase enzyme) levels was used as an internal control. Amplification (carried out with 5μl of cDNA dilution 1/100), detection (with automatic calculation of the threshold value), and real-time analysis were performed twice with cDNA samples using the iCycler iQ detection system (Bio-Rad Laboratories). The relative mRNA expression level of each gene in each sample was calculated using comparative cycle time as described previously [57].

Animal studies

Animal experiments were performed with the approval of the Institutional Animal Use and Care Committee at Università Cattolica del S. Cuore, Rome, Italy. Female BALB/c mice, 20 to 25 g, (Harlan Italy S.r.l) were housed in filter-top cages with free access to food and water at the Catholic University Unit for Laboratory Animal Medicine. In order to assess the virulence of the oat, dltA, sigV single-mutant strains and of the oat/dltA/sigV triple mutant strain with respect to the JH2-2 wild-type strain, two different mouse models were used.

In the intravenous infection model, experiments were performed according to Gentry-Weeks et al., [33]. Briefly, overnight cultures of the strains grown in brain-hearth infusion broth (BHI) supplemented with 40% heat-inactivated horse serum were centrifuged and the resulting pellets were resuspended in sterile PBS to achieve final concentrations of 1×10<sup>8</sup> bacteria/ml. Aliquots of 100 μl from each strain suspension were used to inject groups of 10 mice into their tail veins. Infections experiments were repeated three times. The mice were monitored with twice-daily inspections and they were sacrificed using CO2 inhalation at 7 days after infection. Kidneys and livers were then removed aseptically, weighed, and homogenized in 5 ml of PBS using a Stomacher 80 (Pbi International, Milan, Italy) for 120 s at high speed. Serial homogenate dilutions were plated onto Enterococcus selective agar (Fluka Analytical, Switzerland) for CFU determination.

In the urinary tract infection (UTI) model, we followed a previously described protocol [36]. Briefly, each bacterial strain was grown in 10 ml of BHI broth supplemented with 40% heat-inactivated horse serum for 10 h at 37°C under shaking. Cells were pelleted, resuspended in 10 ml of sterile PBS, and then adjusted to reach a concentration of 1×10<sup>9</sup> cells/ml. First, groups of five isoflurane-anesthetized mice per bacterial inoculum (10<sup>5</sup> to 10<sup>6</sup> CFU) were infected via transurethral catheterization using a polyethylene catheter, ~4 cm long; outer diameter, 0.61 mm; Becton Dickinson, Sparks, MD) with 200 μl of each strain suspension. Additionally, groups of 15 mice were infected with a sole inoculum of 10<sup>6</sup> CFU. Mice were sacrificed 48 h after transurethral challenge, bladders and kidney pairs were processed as described above. For each strain, the 50% infective dose (ID<sub>50</sub>) was determined as previously described [58]. The bacterial detection limits were 50 CFU/ml for kidneys and 10 CFU/ml for bladder homogenates. Differences between the total numbers of infected kidney pairs or bladders, obtained by combining all inoculum (10<sup>5</sup> to 10<sup>6</sup> CFU) groups were analyzed by Fisher’s exact test.

For both models, CFU counts were analyzed by unpaired t test. For all comparisons, a *p* value of less than 0.05 was considered to be significant.

Author Contributions

Conceived and designed the experiments: ALJ MS AB. Performed the experiments: ALJ RT JCG. Analyzed the data: ALJ MS AH YA AB. Wrote the paper: ALJ AB.
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