Contrbution of a PerR-like regulator to the oxidative-stress response and virulence of Enterococcus faecalis

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PerR is one of the most important transcriptional regulators involved in the oxidative-stress response in Bacillus subtilis. Here, the homologous gene in Enterococcus faecalis, ranked among the leading causes of nosocomial infection, was characterized and analysed. Phenotype analysis showed that the perR mutant was significantly more resistant to H2O2 challenge (P<0.05). Expression of eight genes with potential roles in the oxidative-stress response was determined in the wild-type and perR-mutant strains by real-time quantitative PCR. Surprisingly, low quantitative differences in the transcriptional activity of these genes in the mutant versus wild-type were observed. Likewise, this locus was not involved in survival within murine macrophages, but in the mouse peritonitis model, the perR mutant appeared less lethal than the JH2-2 wild-type strain. The combined results show that PerR affects E. faecalis virulence and that its implication in the transcriptional regulation in this bacterium deviates from the B. subtilis model.

INTRODUCTION

To survive in hostile environments, bacterial cells must possess mechanisms for defence against diverse stresses, such as reactive oxygen species (ROS). Consequently, bacteria have evolved specialized enzymes that enable them to detoxify these molecules, e.g. catalase, peroxidase and superoxide dismutase. In Bacillus subtilis, a model Gram-positive bacterium, the oxidative-stress response is managed by the metalloregulatory protein PerR (peroxide regulator), one of the three Fur-like (ferric-uptake regulator) proteins, along with Fur and Zur (zinc-uptake regulator) (Chen et al., 1995; Mongkolsuk & Helmann, 2002). Molecular evidence revealed that PerR is a manganese- and iron-dependent repressor of the genes encoding a catalase (KatA), an alkyl hydroperoxide reductase (AhpCF), a DNA-binding protein (MrgA, Dps homologue) and haem-biosynthesis enzymes (Bsat et al., 1998). As PerR was H2O2-responsive, it was considered as the analogue of OxyR, which is the main transcriptional regulator involved in the oxidative-stress response in Escherichia coli. Nevertheless, peroxide-stress activation of PerR seemed to be linked to the bound metal ion, whereas that of OxyR was via disulfide-bond formation (Herbig & Helmann, 2001, 2002; Storz & Imlay, 1999). PerR and PerR-like regulators have been found in other Gram-positive bacteria (Staphylococcus aureus, Streptococcus pyogenes and Listeria monocytogenes) (Horsburgh et al., 2001; King et al., 2000; Rea et al., 2004), as well as in the Gram-negative pathogen Campylobacter jejuni (van Vliet et al., 1999) and the spirochaete Borrelia burgdorferi (Posey & Gherardini, 2000). Moreover, PerR has been shown to be required for virulence in S. aureus, as the pathogenicity of a mutant is attenuated in a murine subcutaneous skin-abscess model of infection (Horsburgh et al., 2001). Interestingly, fur is a member of the PerR regulon in B. subtilis and S. aureus, but was not peroxide-inducible (Fuangthong & Helmann, 2003). These results show that not all members of the PerR regulon are members of the peroxide stimulon.

Enterococcus faecalis is a Gram-positive bacterium that is part of the commensal flora of humans and animals, but may also cause severe diseases and is one of the main causative agent of hospital-based infections (Gilmore et al., 2002). This hardy organism resists many kinds of environmental stresses and the emergence of multidrug-resistant strains has become a serious problem (Giard et al., 2003; Jett
et al., 1994). Not only may E. faecalis encounter peroxide during the infection of tissue, it has the ability to generate superoxide and H$_2$O$_2$ when cultivated in an oxygenated medium (Huycke et al., 2002). A survey of the E. faecalis genome sequence revealed that this bacterium possesses several genes encoding antioxidant enzymes, such as ahpCF, npr (encoding the NADH peroxidase), sodA (encoding the superoxide dismutase) and katA. Recently, we showed that a mutation in the ef2958 gene, named hypR, sensitized E. faecalis to H$_2$O$_2$ treatment and greatly affected the survival in murine peritoneal macrophages (Verneuil et al., 2004b). This gene encodes a regulator of the LysR family and, in spite of structural and functional differences, was the most closely related gene to oxyR of E. coli (Verneuil et al., 2004a). Transcriptional and DNA-shift analysis also revealed that expression of hypR and ahpCF was directly under HypR control, thus providing the first evidence of a new oxidative-stress regulon in E. faecalis (Verneuil et al., 2004b).

In this report, we characterized the PerR-like regulator in E. faecalis. We then analysed the effect of a perR mutation on the transcription of genes with putative roles in the oxidative-stress response. PerR does not seem to be either a major regulator of oxidative-stress genes or involved in survival within macrophages, but, according to the results obtained in the mouse model, is a factor important for virulence.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The E. faecalis strain used in this study was JH2-2 (Jacob & Hobbs, 1974; Yagi & Clewell, 1980). E. coli XL1Blue (Stratagene) was used as a recipient strain for internal fragment cloning and pUCB300 was used as a cloning and suicide vector (Frère et al., 1993). Cultures of E. faecalis were grown at 37°C without shaking in 20 ml glass tubes containing 10 ml semi-synthetic medium (Bacto Folic AOAC medium; Difco) supplemented with 0.2% (w/v) glucose. The Mn and Fe concentrations in the medium were 130 and 50 μM, respectively. For plate count, a sample was taken, diluted immediately in 0.9% (w/v) NaCl and poured in M17 (Terzaghi & Sandine, 1975) agar (1.5% w/v) (Difco) supplemented with 0.5% (w/v) glucose. Plates were incubated at 37°C for 48 h. E. coli strains were cultivated under vigorous agitation at 37°C in Luria–Bertani (LB) medium (Sambrook et al., 1989) with erythromycin (300 μg ml$^{-1}$) when required.

**H$_2$O$_2$ challenge conditions.** Wild-type and mutant cells (grown as described above) were harvested at an OD$_{600}$ of 0.5 by centrifugation and resuspended in 0.9% (w/v) NaCl with 20 mM H$_2$O$_2$. These cultures were placed into a 37°C water bath and, at the desired time point, samples were taken for plate count. The number of c.f.u. was determined after 48 h incubation at 37°C. The survival of the mutant and wild-type cells in the absence of peroxide stress has been determined and did not reveal any difference. Each point is the mean of at least three experiments with duplicate plating, and statistical comparison of means was performed by using Student’s t-test. Survival at any given time point was determined as the ratio of the number of c.f.u. after treatment to the number of c.f.u. at the zero time point.

**General molecular methods.** PCR was carried out in a reaction volume of 25 μl with 5 μg chromosomal DNA of E. faecalis JH2-2 by using PCR Master Mix (Eppendorf). The annealing temperature was 5°C below the melting temperature of primers and PCR products were purified by using a QIAquick kit (Qiagen). Plasmids were purified by using a QIAprep Miniprep kit (Qiagen). For Southern blotting, membrane-bound restricted chromosome was hybridized with 32P-labelled PCR probe. Membranes were then exposed to a storage phosphor screen (Packard Instrument Company) for 5 h. Genomic DNA extraction and other standard techniques were carried out as described by Sambrook et al. (1989).

**Construction of the perR (previously named ef1585) inser- tional mutant.** To construct an insertional mutant with a disruption in the E. faecalis perR gene, a 270 bp internal fragment (obtained by PCR amplification using the E. faecalis JH2-2 chromosome as template; see Table 1 for the sequences of primers used) was ligated into the suicide vector pUCB300 that had been digested.

**Table 1.** Primers used for real-time PCR and other amplifications

<table>
<thead>
<tr>
<th>Locus*</th>
<th>Forward primer (5′–3′)</th>
<th>Reverse primer (5′–3′)</th>
</tr>
</thead>
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<tr>
<td>ef0463</td>
<td>GGTCAAGTAGGGCTTGGTTTA</td>
<td>AAAACAGGTTGTGGCAGCCATC</td>
</tr>
<tr>
<td>ef1211</td>
<td>AGTGAACGCGATGATTTGC</td>
<td>TGGTACAGCATCTGTTGTC</td>
</tr>
<tr>
<td>ef1338</td>
<td>GAGCCTGCTTGGATGAAAC</td>
<td>TCGTATTTGAGCAATGTT</td>
</tr>
<tr>
<td>ef1585</td>
<td>CCAGGGTTAGAGCCGAGTTGA</td>
<td>TTTGGAATCTGGGACATAATCC</td>
</tr>
<tr>
<td>ef1586</td>
<td>ATCCACTTATCAAGCAATGC</td>
<td>ACCAGAGTTCCTGTTGTC</td>
</tr>
<tr>
<td>ef1597</td>
<td>GCTGTGGGAGATTTTGGTC</td>
<td>CATGATATGACGGAACAGC</td>
</tr>
<tr>
<td>ef2738</td>
<td>ATCAATCTGCGCCGTGAACA</td>
<td>TTGGTGCTGCAATCTTCG</td>
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<td>CCAATGAGTTGGAGCAACCT</td>
</tr>
<tr>
<td>ef3233</td>
<td>GGACCGCATATCCGAGAAC</td>
<td>CGCCTTCTTCCACGAAAAT</td>
</tr>
<tr>
<td>ef3270</td>
<td>CCAGACACAGCGATCAAATAG</td>
<td>GCCATTTGGGCTTGATTTT</td>
</tr>
<tr>
<td>23SrRNA</td>
<td>GGTCCCTAGAATTGTTGGA</td>
<td>CACTAGGGCAGCTTCGTC</td>
</tr>
<tr>
<td>ef1585int†</td>
<td>ATCGAAATATCATACACCC</td>
<td>TGTTAATTACTACGGCGCCA</td>
</tr>
<tr>
<td>ef1585comp‡</td>
<td>GATTTGCGAGCTGAAATTAG</td>
<td>TTAGACATTTCTCCGTTGCTG</td>
</tr>
</tbody>
</table>

*From the annotated sequence available at the TIGR web site (http://www.tigr.org).
†Internal fragment of ef1585.
‡Used for complementation experiments.
with Smal. The resulting plasmid obtained after transformation of E. coli XL1Blue was used to transform competent cells of E. faecalis JH2-2. Erythromycin-resistant colonies were selected on agar plates containing 150 µg erythromycin ml⁻¹. Integration was verified by PCR and Southern blot analysis. The stability of the insertion was determined by plate counts with and without antibiotic.

RNA isolation and mapping of the transcriptional start site. Total RNA of E. faecalis (grown in semi-synthetic medium as described above) was isolated from exponentially growing or stressed cells (30 min with 2-4 mM H₂O₂ in semi-synthetic medium) using an RNaseasy Midi kit (Qiagen). This stress condition does not affect growth behaviour, but has been shown to be the best adaptation condition for E. faecalis (Flahaut et al., 1998). Cells were broken by addition of glass beads (0-1 or 0-25 mm diameter) and by vortexing for 2 min. The transcriptional start point of perR was determined by using a RACE 5′/3′ kit (Roche) according to the manufacturer’s instructions.

Real-time quantitative PCR. Specific primers to produce amplions of equivalent length (100 bp) were designed by using the Primer3 software (available at http://frodo.wi.mit.edu/cgi-bin/primer3/primers3_www.cgi) and are listed in Table 1. The amplification efficiencies were between 95 and 100 %. Total RNA (2 µg) was reverse-transcribed by using Omniscript enzyme (Qiagen) according to the manufacturer’s recommendations. A 5 µl aliquot of the resulting cDNA-synthesis reaction mixture (dilution, 10⁻²) was used for subsequent PCR amplification with the appropriate forward and reverse primers (1 µM final concentration) and the QuantiTect SYBR Green PCR mix (Qiagen). As control, sham cDNA-synthesis reactions that lacked reverse transcriptase, followed by PCR amplification, were carried out to identify RNA preparations contaminated by residual genomic DNA. Quantification of 23S rRNA levels was used as an internal control, because its expression was not altered under oxidative-stress conditions. Amplification, detection (with automatic calculation of the threshold value) and real-time analysis were performed twice and in duplicate with two different RNA samples by using the Bio-Rad iCycler iQ detection system.

The value used for comparison of gene expression in various strains and environments was the number of PCR cycles required to reach the threshold cycle (Cₜ) (between 17 and 26). To relate the Cₜ value back to abundance of an mRNA species, Cₜ was converted to n-fold difference by comparing mRNA abundance in the JH2-2 wild-type strain (harvested in the middle of the growing phase or after 30 min in presence of 2-4 mM H₂O₂) with that obtained with the perR-mutant strain. The n-fold difference was calculated by the formula n = 2⁻ⁿ when Cₜ mutant < Cₜ JH2-2, and by n = 2ⁿ when Cₜ mutant > Cₜ JH2-2, with x = Cₜ mutant – Cₜ JH2-2. A value > 1 reflects a relative increase in mRNA abundance compared with the wild-type; a negative value reflects a relative decrease. Statistical comparison of means was performed by using Student’s t-test.

Survival assays in mouse peritoneal macrophages. Survival of E. faecalis in mouse peritoneal macrophages was tested by using an in vivo/in vitro infection model as described previously (Gentry-Weeks et al., 1999; Verneuil et al., 2004b). Brieﬂy, E. faecalis perR mutant and JH2-2 were grown aerobically at 37 °C in brain heart infusion (BHI) broth for 16 h. Then, the bacteria were pelleted and resuspended in an adequate volume of PBS for injection. Male BALB/c mice (10 weeks old) were infected with 10⁻⁷–10⁻⁸ cells of each strain by intraperitoneal injection. After a 6 h infection period, peritoneal macrophages were collected by peritoneal wash (2.5 ml PBS), centrifuged and suspended in Dulbecco’s modiﬁed Eagle’s medium containing 10 mM HEPES, 2 mM glutamine, 10 % (v/v) bovine fetal serum and 1 × non-essential amino acids, supplemented with vancomycin (10 µg ml⁻¹) and gentamicin (150 µg ml⁻¹). The cell suspension was dispensed into 24-well tissue-culture plates and incubated at 37 °C under 5 % CO₂ for 2 h. After exposure to antibiotics (i.e. 8 h post-infection) to kill extracellular bacteria, the infected macrophages were washed and triplicate wells of macrophages were lysed with detergent. After dilution with BHI broth, the lysates were plated on BHI agar to quantify the viable intracellular bacteria. The same procedure was performed at 24, 48 and 72 h post-infection. To assess their viability, macrophages were detached from tissue-culture wells with cell scrapers, stained with trypan blue and viable macrophages were counted with a haemocytometer. All experiments were performed five times and results were subjected to statistical analysis by using Student’s t-test.

Mouse peritonitis model. Testing of the JH2-2 and perR-mutant strains was performed as described by Teng et al. (2002). Briefly, the strains were incubated in BHI broth overnight at 37 °C under constant agitation. The cells were harvested by centrifugation, washed twice with ice-cold 0.85 % saline solution and resuspended in the same solution to reach a density of approximately 1·5×10⁹ c.f.u. ml⁻¹. The inoculum size was conﬁrmed by plating onto BHI agar. Dilutions (two- to 10-fold) of the bacterial suspension, prepared in chilled 0.85 % saline solution, were used as inocula, after 10-fold-diluting them in 25 % sterile rat-faecal extract (SRFE, from a single batch) (Pai et al., 2003). Outbred ICR female mice, 4–6 weeks old (Harlan Italy S.r.l.), were used. Mice were injected intraperitoneally with 1 ml of each bacterial inoculum made in 25 % SRFE, then housed ﬁve per cage and fed ad libitum. Mice were monitored every 3 h and the number of surviving mice was recorded. The LD₅₀ was determined as described by Reed & Muench (1938). Survival curves were obtained by the Kaplan–Meier method and compared by log-rank test using the GraphPad Prism software (GraphPad Software Inc.). Comparisons with P values <0.05 were considered to be signiﬁcant. All strains were tested more than once.

Complementation of the perR mutant. To complement the perR gene in trans, a PCR fragment containing perR and its promoter (see Table 1 for the sequences of primers used) was cloned into the pCU1 plasmid (Cm⁹) (Augustin et al., 1992). The resulting vector (pPER) was then transformed into the perR mutant. In order to compare the phenotypes of the complemented strain and the wild-type, plasmid pCU1 was also introduced into the JH2-2 and perR-mutant strains. Survival assays towards H₂O₂ in and mouse peritonitis model were carried out as described above.

RESULTS

Identification of PerR in E. faecalis

In B. subtilis, PerR, a member of the ferric-uptake regulator (Fur) family, was shown to be the major regulator of the H₂O₂ stress response. In silico analysis (BLASTP) using the E. faecalis V583 genome, provided by The Institute for Genomic Research (TIGR; http://www.tigr.org), was carried out in order to ﬁnd polypeptides homologous to this regulator. It appeared that E. faecalis possesses three genes encoding transcriptional regulators of the Fur family (ef1585, ef1525 and ef2417) (Table 2). Ef1585 shares 65 % identity at the protein level with PerR from B. subtilis (Table 2) and also 43 % with PerR from S. pyogenes. Ef1525 and Ef2417 share only 25 and 22 % identity to PerR of S. pyogenes, respectively. Like PerR, Ef1585 has in its N-terminal region a helix–turn–helix DNA-binding sequence and contained the two conserved CXXC motifs in its C-terminal part, corresponding to a putative metal-binding site (Mongkolsuk & Helmann, 2002). Note that S. pyogenes possesses only this
one Fur-family protein (King et al., 2000). Moreover, Ef1525 and Ef2417 were highly similar to Fur and Zur of *B. subtilis*, showing 69 and 47% identity, respectively (Table 2). Sequences of *ef1585* (*perR*) from *E. faecalis* V583 and JH2-2 are identical and the GenBank accession number for the latter sequence is DQ064645.

By using the rapid amplification of cDNA ends (RACE)-PCR technique, the transcriptional start point of the *E. faecalis* *perR* gene was mapped (data not shown). We concluded that the A 27 bp upstream of the ATG codon corresponds to the +1 of the transcript. A similar −10 box [TAT'TAT (mismatch underlined)] located 6 nt upstream of the transcriptional start point and a sequence near to the −35 box consensus [TTG'TCA (deviation underlined)] separated by 17 bp were identified (Fig. 1). Furthermore, the *perR* locus appeared to be followed by an inverted repeat (ΔG = −28.3 kcal mol⁻¹), which may be a Rho-independent transcription terminator (Fig. 1).

**Sensitivity of the perR mutant to H₂O₂ challenge**

Because PerR plays a crucial role in the oxidative-stress response in *B. subtilis*, the *E. faecalis* *perR* mutant was exposed to 20 mM H₂O₂ for 30 min. As shown in Fig. 2, this mutant appeared significantly more resistant to the H₂O₂ treatment [with a statistical significance (P) of <0.05] than the wild-type strain. After 30 min, 24 ± 9 and 68 ± 12% survival was observed with the wild-type and mutant cells, respectively. The survival of the complemented strain was reduced compared with the mutant carrying the pUC1 plasmid, showing that the addition of functional *perR* partially restored the wild-type phenotype (data not shown). Moreover, the growth rate of the *perR* mutant was identical to that of the wild-type strain and no difference was observed after stress caused by 155 mM t-butyl hydroperoxide (data not shown).

**Attempt to identify PerR-regulated genes in *E. faecalis***

In order to identify PerR-regulated genes, real-time quantitative PCR was carried out by using cDNA from *E. faecalis*

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**Table 2. Amino acid sequence identities between PerR protein paralogues from *B. subtilis* and *E. faecalis***

Values given are amino acid sequence identity (%). Values in bold type represent the highest identity values for each regulator.

<table>
<thead>
<tr>
<th><em>B. subtilis</em> regulator</th>
<th><em>E. faecalis</em> regulator</th>
</tr>
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<tbody>
<tr>
<td>Ef1585</td>
<td>Ef1525</td>
</tr>
<tr>
<td>Ef2417</td>
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<tr>
<td>PerR</td>
<td>65</td>
</tr>
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<td>Fur</td>
<td>32</td>
</tr>
<tr>
<td>Zur</td>
<td>33</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Mapping of the 5’ end of the *perR* mRNA by RACE-PCR analysis. The transcriptional start site is marked with an arrow. Potential −10 and −35 regions, ribosome-binding site (RBS) sequence and start codon are in bold type and underlined. Te indicates the putative transcriptional terminator. The GenBank accession number for the *E. faecalis* JH2-2 *perR* gene sequence is DQ064645.

**Fig. 2.** Survival of *E. faecalis* JH2-2 (○) and *perR*-mutant (■) growing cells after 15 and 30 min of challenge with 20 mM H₂O₂. One hundred per cent corresponds to the number of c.f.u. before H₂O₂ was applied (around 1·5 × 10⁸ c.f.u. ml⁻¹). Values are the means of results obtained from at least three independent experiments and SD is indicated.
JH2-2 and perR-mutant strains. Eight genes known or suspected to play a role in the oxidative-stress response in *E. faecalis* were selected from the annotated genome sequence available at the TIGR web site (http://www.tigr.org) and from genes listed by Paulsen *et al.* (2003) (Table 3). *perR* itself and *ef3233* (*dps*), genes regulated by PerR in *B. subtilis*, were included in our study. The latter encodes a DNA-binding protein with structural similarity to the iron-storage protein ferritin and has been shown to prevent DNA damage in *E. coli* through its capacity to bind Fe(II) and restrict the formation of hydroxyl radicals (Grant *et al.*, 1998; Zhao *et al.*, 2002). No significant differences were detected in internal control amplifications of 23S rRNA, regardless of the strain used or culture conditions. Comparisons were made between values obtained from amplification of RNA derived from *perR*-mutant and JH2-2 strains harvested in the exponential-growth phase or after 30 min in the presence of 2·4 mM H2O2.

Of the 75 genes derepressed in the *perR*-mutant strain of *B. subtilis*, three (*mgrA*, *zosA* and *katA*) are known to be directly under PerR control and showed a derepression level greater than fivefold (9·6-, 5·9- and 5·3-fold, respectively) (Helmann *et al.*, 2003). Moreover, in *E. faecalis*, the HypR transcriptional regulator controls the expression of four genes involved in the oxidative-stress response (*ef1597, ef3270, ef2738* and *ef2739*). The deregulation levels observed in the hypR mutant were between six- and 12·1-fold (Verneuil *et al.*, 2005). Comparatively, low quantitative differences in the transcripts examined in the *perR* mutant versus wild-type have been observed (Table 3).

Nevertheless, the increases in abundance of mRNA for the genes encoding the NADH peroxidase and NADH oxidase were 4·2- and 3·4-fold, respectively, and these values were statistically significant (Table 3). Therefore, PerR seems to exercise low transcriptional regulation of these genes. Note that the transcription of *perR* was detectable in the JH2-2 strain, but did not seem to be H2O2-inducible (data not shown).

### Effect of the *perR* mutation on survival within macrophages and on virulence of *E. faecalis*

It has been demonstrated that *E. faecalis* was able to persist for an extended period in mouse peritoneal macrophages (Gentry-Weeks *et al.*, 1999; Verneuil *et al.*, 2004b). In order to assess whether the *perR* gene affected the capability of *E. faecalis* to resist killing by macrophages, the intracellular survival of bacterial cells was monitored (Fig. 3). No significant difference in the number of viable cells was observed between wild-type and mutant strains.

To evaluate whether disruption of the *perR* gene affected the virulence of JH2-2, the *perR*-mutant strain was tested by a mouse peritonitis model, in comparison with the wild-type JH2-2 strain. Initial experiments showed that mice (10 per group) infected with the mutant strain lived significantly longer than mice infected with the wild-type strain (*P* = 0·0035) when the inoculum size of the *perR* mutant (6·9 × 107 c.f.u.) was the same or slightly greater than that of JH2-2 (6·2 × 107 c.f.u.) (Fig. 4). To confirm these results, the *perR* mutant was further examined in a larger-scale experiment by using different inocula (2·7 × 107 c.f.u. for JH2-2 and 3·1 × 107 c.f.u. for *perR* mutant) and 20 mice per group. The results again showed a higher survival rate of *perR*-mutant strains.

### Table 3. Change in abundance of *E. faecalis* mRNA of genes with a potential role in the oxidative-stress response in the *perR*-mutant strain relative to expression level in JH2-2 wild-type cells

<table>
<thead>
<tr>
<th>ORF</th>
<th>Function</th>
<th>Gene</th>
<th>Expo*</th>
<th>H2O2†</th>
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<td><em>ef0463</em></td>
<td>Superoxide dismutase</td>
<td><em>sodA</em></td>
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<td>−3·7</td>
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<td><em>ef3270</em></td>
<td>Glutathione reductase</td>
<td></td>
<td>1</td>
<td>−3·2</td>
</tr>
<tr>
<td><em>ef1211</em></td>
<td>NADH peroxidase</td>
<td><em>npr</em></td>
<td>+4·2$</td>
<td>−1·3</td>
</tr>
<tr>
<td><em>ef1586</em></td>
<td>NADH oxidase</td>
<td></td>
<td>+3·2</td>
<td>+3·4</td>
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<td><em>ahpF</em></td>
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<tr>
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<td>Ribosomal RNA</td>
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</table>

*RNA extracted from cells harvested in the exponential-growth phase.
†RNA extracted from cells harvested after H2O2 treatment (30 min with 2·4 mM H2O2).
‡Fold change relative to wild-type cultured under the same conditions.
$Values in bold type represent changes with statistical significance (*P* < 0·05).
Complementation of the mutant with an intact copy of the virulence of *E. faecalis* mutant-infected mice compared with JH2-2 (*P*= 0.0014) (data not shown). The LD_{50} values for JH2-2 and the *perR* mutant were 1.9 × 10^{7} and 8.1 × 10^{7}, respectively. These findings suggest that the *perR* gene contributes to the virulence of *E. faecalis* in the mouse peritonitis model. Complementation of the mutant with an intact copy of the *perR* operon restored the wild-type behaviour (Fig. 5). As a control, the *perR*-mutant and JH2-2 strains transformed with the pCU1 vector were used.

**DISCUSSION**

We looked through the genome sequence to investigate the mechanism of defence against toxic oxygen species in a bacterium responsible for hospital-based infection, *E. faecalis*. We showed that this micro-organism has homologues of the three main ion-dependent repressors, PerR, Fur and Zur, spread widely in Gram-positive prokaryotes. In *B. subtilis*, these paralogues regulate different sets of genes in response to distinct stimuli (oxidative stress and iron or zinc concentrations; Mongkolsuk & Helmann, 2002). As with other Gram-positive bacteria, such as *B. subtilis* (Bsat et al., 1998), *S. pyogenes* (King et al., 2000), *L. monocytogenes* (Rea et al., 2004) and *S. aureus* (Horsburgh et al., 2001), the *E. faecalis perR* mutant was significantly more resistant to the H_{2}O_{2} challenge than the wild-type. It should be noted that *E. faecalis* is able to produce superoxide and H_{2}O_{2} when cultivated in an oxygenated buffer (Huycke et al., 2002), but the concentrations reached are very low compared with the doses added exogenously in our experiments.

The PerR and Fur regulons of *B. subtilis* have recently been updated by transcriptome and proteome analysis, which confirmed that PerR plays a crucial role in the control of expression of key enzymes involved in the oxidative-stress response, such as *ahpCF* (encoding the alkyl hydroperoxide reductase), *katA* (encoding catalase) or *mrgA* (encoding a Dps-like protein) (Mostertz et al., 2004). In *B. subtilis*, it was suspected that the resistance of the *perR* mutant reflected the absence of repression of these genes (Bsat et al., 1998). This was obviously not the case for *E. faecalis*. Despite the identification of sequences nearly identical to the consensus sequence Per box in the promoter regions of *ahpCF* and *dps* in *E. faecalis*, none of them exhibited a conserved inverted-repeat structure (Fuangthong & Helmann, 2003). Expression of the *ahpCF* operon in *E. faecalis* cultivated in the presence of H_{2}O_{2} is known to be under the control of the recently discovered transcriptional regulator HypR, involved in survival within macrophages as well as resistance to H_{2}O_{2} challenge (Verneuil et al., 2004b). Therefore, our results seemed to exclude a dual regulation with PerR. In *S. pyogenes*, PerR seems able to bind specifically to a single site of the *ahpC* promoter, but transcriptional analyses revealed that *ahpC* regulation is independent of PerR (Brenot et al., 2005). This latter result has also been observed in our study. Another important characteristic that links *S. pyogenes* and *E. faecalis* and distinguishes them from the *B. subtilis* model is the absence of the general stress sigma factor σ^54_. Further investigation leading to the identification of members of the PerR regulon in *E. faecalis* and *S. pyogenes* will probably provide more insights about novel effectors of protection against H_{2}O_{2}.

In our study, we showed that the number of viable intracellular bacteria in infected murine macrophages was not reduced in the *perR* mutant over the 72 h infection period compared with the JH2-2 wild-type strain of *E. faecalis*. It was hypothesized that the oxidative-stress response leading to the production of enzymes that inactivate ROS generated by the oxidative burst may explain the ability of bacterial cells to survive inside macrophages (King et al., 2000; Verneuil et al., 2004b). The ability of the *perR* mutant to survive in murine macrophages may reflect its capacity to...
cope with the oxidative challenge. Nevertheless, our data obtained in the mouse peritonitis model demonstrated that PerR is absolutely required for full virulence potential. PerR has been shown to be also required for virulence of S. aureus strain 8325-4 in a murine skin-abscess model of infection (Horsburgh et al., 2001). In this bacterium, the ability of PerR to sense H$_2$O$_2$ and to regulate antioxidant defence and iron storage may be important for coordinating a survival response (Horsburgh et al., 2001). Furthermore, a perR mutant of L. monocytogenes was significantly affected in virulence for mice (Rea et al., 2004). Given these findings, together with the resistance against H$_2$O$_2$ challenge, it is likely that the virulence defect of the perR mutant is due to the alteration of a response other than oxidative stress, such as metallic-ion uptake or storage.

In conclusion, this report brings to light the fact that the B. subtilis model concerning the oxidative-stress response is different from that of E. faecalis and that, in the latter, the transcriptional regulator PerR is important for virulence in the mouse peritonitis model. Further work is being undertaken to characterize the precise role of the PerR regulator in the E. faecalis stress response.

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