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Transcriptomic response of *Enterococcus faecalis* V583 to low hydrogen peroxide levels

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Abstract :

Enterococcus faecalis is a Gram-positive commensal bacterium inhabiting the gastrointestinal tracts of human and other mammals, but is also increasingly recognized as an opportunistic human pathogen. Oxidative stress is one of the major challenges encountered by enterococci, both in their natural environment and during infection. In this paper, we evaluated the transcriptomic response of E. faecalis to oxidative stress, and showed that transcript abundance was reduced for 93 genes and increased for 39 genes during growth in medium containing 1.75 mM H2O2. The presence of hydrogen peroxide affected several metabolic pathways, including a large decrease in ethanolamine utilization and methylglyoxal metabolism, and an increase in transcript abundance for several transport systems. In particular, four operons encoding iron transporters appeared highly induced. By contrast, in our experimental conditions, the expression of most of the genes known to be involved in the enterococcal response to oxidative stress, did not appear significantly altered.

1. Introduction

Enterococcus faecalis is a Lactic Acid Bacterium naturally inhabiting the gastrointestinal tracts of humans and other mammals, and used as a constituent of some probiotic food supplements [17,18,30]. However, *E. faecalis* also can cause life-threatening infections in humans, especially in the hospital environment [35,46]. Oxidative stress is one of the major challenges encountered by *E. faecalis* during its life cycle [23,34,50]. Nearly two decades of research have revealed that this bacterium possesses genes encoding antioxidant enzymes such as *sodA* (Superoxide dismutase, [41]), *katA* (Catalase, [14]), *gor* (Glutathione reductase), *nox* (NADH oxidase), *trx* (Thioredoxine reductase), *dps* (DNA-binding protein), *msrA* (Peptide methionine-S-sulfoxide reductase) [39], *npr* (NADH peroxidase, [44]), *ohr* (Organic hydroperoxide resistance protein, [43]), *ahpC* (Alkyl hydroperoxide reductase, [57]), *ahpF* (Peroxiredoxine reductase), and several transcriptional regulators involved in the oxidative stress response, as *hypR* [55], *perR*, *fur*, *zur* [56], *soxS* [54].

Global experimental approaches that allow monitoring of gene and protein expression (i.e., transcriptomics and proteomics, respectively) in response to a particular environmental condition, constitute powerful tools to understand the metabolic and regulatory networks involved in stress adaptation on a genome-wide scale. To date, only one proteomic study examined changes in expression of 23 proteins in *E. faecalis* cells exposed to hydrogen peroxide (H_2O_2) [12]. In this paper, we report global changes in transcript abundance in *E. faecalis* V583 during growth in the presence of H_2O_2.

2. Materials and methods

2.1. Bacterial strains and growth conditions

In this work, we used *E. faecalis* V583 ery^S, an erythromycin sensitive derivative of the clinical isolate *E. faecalis* V583 [45]. Cells were cultivated with shaking in M17 medium supplemented with 0.5% (w/vol) glucose (GM17), supplemented with ±1.75 mM H_2O_2

2.2. Microarray experiments

V583 ery^S growth was monitored spectrophotometrically at 600 nm. When the culture reached mid-exponential phase (Fig. 1), cells were harvested and total RNA was extracted, purified, and residual DNA enzymatically removed as described previously [59]. Two independent experiments were performed for each condition. cDNA preparation, fragmentation, labeling and hybridization were performed as described in the Affymetrix manual ("GeneChip Expression Analysis Technical Manual: Procaryotic Target preparation" P/N 702232 Rev 2). Washing and scanning were performed using a GeneChip® Fluidics Station 450. The arrays were read at 570 nm with a resolution of 1.56 µm using an Affymetrix GeneChip Scanner 3000 7G (Affymetrix). Results were analyzed and compared using the GeneChip Operating Software (GCOS) version 1.4. Data were also analysed using R (version 3.0.0; R core team, 2013) and the Bioconductor software (version 2.12; [15]), to perform a t-test with a Benjamini and Hochberg multiple testing correction cut-off of $P < 0.05$ (data not shown). In all, we considered differences in transcript abundance significant if by GCOS they exhibited both a p-value below 0.05, and an absolute expression ratio significantly greater than 2 fold.

2.3. Real-Time quantitative PCR

Real-Time qPCR was performed exactly as described by Giard *et al.* [16]. Specific primers were designed using the Primer3 software available at the web site http://frodo.wi.mit.edu/cgibin/primer3/primer3_www.cgi (Table 1) with the following

parameters: amplicon length (99 to 101 bp), primer length (19 to 21 nucleotides), and primer melting temperature (Tm from 59 to 61 °C). Total RNAs, treated with DNase I (GE Healthcare) and purified using the DNA-free kit (Ambion), were reverse transcribed using the Omniscript enzyme (Qiagen) and random hexamer primers according to the manufacturer's recommendations. RT qPCR was performed using the QuantiTect SYBR Green RT PCR kit (Qiagen). Quantification of 23S rRNA transcript levels was used as an internal control. All experiments were performed twice, and in duplicate with two different RNA samples, using the BioRad iCycler iQ detection system (Bio-Rad Laboratories, Richmond, CA). The value used for the comparison of genes expressions in stressed and unstressed cells was the number of PCR cycles required to reach the threshold cycle (C_T). To relate the C_T value to the abundance of an mRNA species, C_T was converted to "*n*-fold difference" by comparing mRNA abundance in the control cells to that obtained with the H_2O_2 stressed cells. The *n*-fold difference was calculated by the formula ($n = 2^{-x}$) when the $C_{T\ mutant} < C_{T\ V583}$ and ($n = -2^{x}$) when $C_{T\ mutant} > C_{T\ V583}$, with $x = (C_{T\ mutant} - C_{T\ V583})$.

3. Results and discussion

3.1. Growth of E. faecalis V583 ery^S *under oxidative stress.*

E. faecalis cells were cultured in the presence of increasing concentrations of H_2O_2, from 1.5 mM to 2 mM to identify a condition where H_2O_2 had a detectable effect on growth, but where cells were also able to successfully respond to the challenge. Results presented in the Fig. 1 showed that the inhibitory effect of H_2O_2 was mainly observable on the duration of the Lag phase, which increased from approx. 30 min to 3 h in the presence of 1.5 mM to 2 mM H_2O_2. We aimed at determining the effects of low doses of H_2O_2 on the global genes expression profiles of exponentially growing *E. faecalis* cells, which were previously shown to display differences in stress tolerance [12]. Therefore, total RNAs were extracted from

mid-exponential growth phase cells (Fig. 1), and then processed for transcriptome analyses.

3.2. Transcriptome changes in response to H_2O_2 exposure.

Whole genome expression profiling of *E. faecalis* V583 was performed using affymetrix chips, using total RNAs extracted from both unstressed- and stressed- (1.75 mM H_2O_2) cultures at an OD_{600nm} of 0.6 (reached two and four hours after inoculation, respectively, Fig. 1). Comparative analysis showed that 132 genes were significantly differentially expressed in response to 1.75 mM H_2O_2, considering a ratio threshold of two fold (Tables 2 and 3). We selected 25 of the mostly deregulated genes, and subjected them to RT-qPCR (Table 4). It is noteworthy that we showed a very strong correlation ($r^2 = 0.86$) between the changes in genes expression observed with the whole genome transcriptomic- and the qPCR- experimental approaches.

Among the 132 genes for which transcript abundance changes significantly, 93 showed reduction and 39 were found to be increased. We classified these 132 genes into 9 groups according to the categories described in the comprehensive microbial resource of The Institute of Genome Research (http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl/). Figure 2 shows the number of differentially regulated genes in each functional class. The main observation is the large dominance of genes encoding proteins for which the function is annotated as unknown (60), and the abundance of genes encoding (i) transport and binding proteins (22 genes increased and 3 reduced), (ii) proteins involved in biosynthesis and metabolism (4 increased and 24 reduced), and (iii) proteins involved in regulation and cell signaling (8). Transcripts for only two genes annotated as involved in stress adaptation and virulence were found to be changed.

3.3. Classification of genes by their regulation patterns.

Table 2 and Table 3 identify genes associated with increased and decreased levels of mRNA, respectively, as a result of exposure to 1.75 mM H_2O_2. The most highly affected transcripts

are changed by about 10 fold. For example, the *ef3082-ef3085* operon encoding the 4 components of an iron compound ABC transport system, displayed increased transcript abundance from 9 to 11 fold, while the greatest decreases all related to ethanolamine metabolism, and displayed a reduction in transcript abundance reaching almost 12 fold.

To understand these changes in the global context of cellular metabolism, we searched for functional association networks using the STRING program ([13], available at http://string-db.org/). Results presented in Fig. 3 show that of the 132 genes for which significant transcript changes were observed, only 14 were disconnected from any node, and only 22 belonged to networks that included less than 4 partners (thus showing that 73% of the genes affected by H_2O_2 stress belonged to larger networks).

Gene ontology enrichment analyses showed that the main biological processes and molecular functions associated with global transcriptional change, again were those related to metal ion transport and ethanolamine metabolism P-values < 10^{-5} for each).

3.3.1. Metabolism

Ethanolamine metabolism

Both microarray and real time q-PCR (Table 3 and Table 4) showed that H_2O_2 decreased the the abundance of transcripts for 18 genes belonging to the *eut* operon involved in ethanolamine metabolism. Ethanolamine (EA) is an abundant compound in the human intestinal tract [24,26] and in processed food [7], and can be used as a source of carbon, nitrogen, and energy. However, growth of the V583 strain, and of RR17 (*ef1633*) a null mutant of *ef1633*, showed that EA provided with CoB12 allowed *E. faecalis* to grow, albeit poorly, only under anaerobic conditions, and the RR17 response regulator was required for this effect. A promoter upstream of RR17 is induced by EA-CoB12 in anaerobiosis [38]. The observation that most of the genes from EF1617 (*eutQ*) to EF1638 (*eutP*) were highly repressed by H_2O_2 in this study, could stem from a negative impact of H_2O_2 on either EA

and/or CoB12 in the medium, and reflects an important modulation of metabolism in enterococcal cells in response to oxidative stress. It is noteworthy that the Eut system was also shown to be negatively deregulated in (i) a microarray study of the Fsr system [5], and (ii) in *relA* mutants unable to produce (p)ppGpp [59,60] and unpublished data), showing that the regulation of the *Eut* operon is governed by many inputs. This new data adds to the understanding of the role of this system in the physiology of bacteria subjected to changing environments.

Methylglyoxal metabolism

The production of MG (methylglyoxal) in bacterial cells is maintained in balance with the capacity for detoxification and protection against this toxic electrophile, which can react with the nucleophilic centres of macromolecules such as DNA, RNA and proteins [4,11]. Table 3 and Table 4 show that the abundance of transcripts for 3 genes encoding glyoxalase family proteins (among the 9 genes identified in the genome with related function) is significantly reduced during the growth in the presence of H_2O_2. It should be noted that all other glyoxalase genes, and the *ef0939* gene encoding the methyl glyoxal synthase, were also negatively impacted, but by differences < 2 fold (Table 5). This indicates an important role for methylglyoxal metabolism under this stress. In enteric bacteria, three methylglyoxal detoxification routes have been identified, *i.e.* the glutathione-dependent glyoxalase I-II system [19], (ii) glutathione-independent glyoxalase III [31], and (iii) methylglyoxal reductases and dehydrogenases [19,32]. The 5 glyoxalase genes for which mRNA abundance decreased in the presence of H_2O_2 share sequence identity with genes of the glyoxalase I-II system. Although methylglyoxal is considered as an extremely toxic electrophile, potentially leading to the death of bacterial cell, several studies suggest that this compound is important for bacterial homeostasis. Indeed, methylglyoxal is thought to be important in bacteria for growth regulation, D-lactate production [8], the uncoupling of anabolism and catabolism [49],

191 the prevention of accumulation of phosphorylated intermediates [6,21], virulence [3],
192 programmed cell death [22,37], and oxidative stress [58]. Therefore, the reduction in the
193 abundance of transcripts encoding cellular glyoxalases suggests that tuning of methylglyoxal
194 intracellular content in H_2O_2 stressed cells is important in *E. faecalis* oxidative stress
195 adaptation.

196 *3.3.2. Transport*

197 Iron uptake

198 Table 2 and Table 4 show a notable increase in mRNA encoded by 10 genes belonging to 4
199 putative operons, with functions related to iron transport. Iron is an essential element for most
200 microbes, since many enzymes use iron as a cofactor [1]. During pathogenic processes, iron
201 acquisition systems are often found to be highly expressed to compensate for low iron
202 availability within the host [47]. Interestingly, the greatest increase in mRNA encoding an
203 iron transporter in our study (ef3082-3085), was also shown to be affected by growth in blood
204 [53]. Conversely, the expression of the *mntH* gene (ef1057), that was recently shown to be
205 strongly repressed in response to iron excess in *E. faecalis* OG1-RF [29], was not modified in
206 our experimental conditions. There is also an intimate relationship between iron metabolism
207 and oxidative stress. First, through the Fenton/Haber-Weiss reaction, iron can promote the
208 formation of hydroxyl radicals, which indiscriminately damage all cellular components [9,51].
209 Second, iron acts as a co-factor for several enzymes, including some involved in oxidative
210 stress defence, such as KatA or Dps.

211 The induction by H_2O_2 of genes with functions in iron uptake was also observed in *Bacillus*
212 *subtilis*, and could reflect an adaptive response to iron limitation [33]. Taken together, our
213 results suggest that H_2O_2-stressed cells encounter an intracellular iron limitation that could
214 result from iron recruitment by certain oxidative defence proteins (*e.g.* catalase), necessitating
215 increased transport capacity to meet the competing intracellular iron needs.

Stress related transporters

We observed in Table 2 and Table 3 that hydrogen peroxide resulted in increased abundance of transcripts of *kdpA, kdpB, kdpC, kdpD,* encoding the high affinity ABC potassium transport system Kdp, and *ef0575-ef0576* (cationic ABC transporter). At the same time, H_2O_2 exposure resulted in reduced levels of *cadA* (cadmium-translocating P-type ATPase), *ef0986* (cation transporter) and *nhaC* (Na^+/H^+ antiporter) mRNA. Select changes were verified by RT-qPCR (Table 4). All bacterial *kdp* operons investigated so far are repressed during growth in media of high external K^+ concentration ($[K^+]e$) [2]. Increased abundance of mRNA encoding the *kdp* operon under our experimental condition, suggests that H_2O_2 led to an intracellular K^+ limitation. Thus, the Kdp-ATPase is an efficient K^+ scavenging system that isexpressed when other K^+ transporters cannot support cellular requirement for K^+, which not only plays a vital role in bacterial osmotic adaptation [10,40] but is also important for pH regulation, gene expression and activation of cellular enzymes [42]. The most notable deficiency observed in the *nhaC* deletion strain of *Bacillus firmus* was its poor growth at pH 7.5 and Na^+ concentrations up to 25 mM [20], suggesting its contribution in osmotic stress. The cadmium cation is toxic to most microorganisms, probably by binding to essential respiratory proteins [52] and through oxidative damage by production of reactive oxygen species [48]. One of the best-characterized bacterial cadmium resistance mechanisms is determined by the cadmium-transporting ATPase encoded by *cadA* found in *Staphylococcus aureus* [36] and *Listeria monocytogenes* [27,28]. Various stresses (NaCl, heat, ethanol, acidity and alkalinity) induced weak or strong H_2O_2 cross-protection in *E. faecalis* ATCC19433 [12]. Combined results suggest that the pre-treatment of H_2O_2 could increase the bacterial osmotolerance and resistance to antibiotic, and decrease the bacterial resistance to cadmium.

3.3.3. Genes involved in the adaptation to oxidative stress

Expression of other genes clearly involved in the adaptation to oxidative stress did not

241 significantly change in GM17 medium supplemented with 1.75 mM H_2O_2 (Table 6). These
242 genes may already be expressed at a high level in laboratory growth. Interestingly, although
243 not reaching the criteria used for significance in this study, the abundance of transcripts
244 encoding *msrB* (Peptide methionine-S- sulfoxide reductase [25]) was reduced 1.65 fold by
245 H_2O_2. The repression of *msrB* in 2.5 mM H_2O_2 adaptation for 30 minutes and the resistance of
246 Δ*msrB* double crossing over mutant to the lethal challenge of 7 mM H_2O_2 for 6 hours
247 observed in *E. faecalis*, suggests that *msrB* contributes negatively to adaptation to oxidative
248 stress [61]. It therefore was not surprising that when *E. faecalis* is facing the oxidative stress
249 of H_2O_2, a reduction in transcripts for *msrB* was observed.

250 *3.4. Concluding remarks*

251 Global changes in the abundance of mRNA for all genes in exponentially growing *E. faecalis*
252 cells, cultured in the presence of a low dose (1.75 mM) of H_2O_2, was measured. At this time
253 (4 h), *E. faecalis* had almost completely adapted as evinced by restoration of its growth rate
254 (Fig. 1). Analysis of changes in transcript abundance in response to this stress revealed that (i)
255 the expression of most of the well-known oxidative stress genes of *E. faecalis* was not
256 significantly altered, possibly because they already were highly expressed in rapid growth in
257 laboratory conditions, or because they may be regulated post-transcriptionally to minimize the
258 time delay associated with response to a potentially lethal condition. Among the responses we
259 did observe, we found that under H_2O_2 stress, *E. faecalis* restricted the metabolism of
260 ethanolamine, and limited the metabolism of methylglyoxal, but increased its use of transport
261 systems to satisfy the specific demand for essential metal ions, such as iron. Concomitantly,
262 we observed decreased expression of some genes known to contribute to adaptation to other
263 stresses (*e.g. nhaC*, *cadA*). Future work will aim at determining whether and how (i)
264 metabolic changes in ethanolamine and methylglyoxal metabolism, and iron transport,
265 impact the adaptation of *E. faecalis* to these specific conditions; and (ii) how changes in

expression of genes clearly involved in the adaptation to other stresses affect cross adaptation. It will also be of importance to determine the role of the 60 genes encoding hypothetical proteins that were shown to be deregulated in these conditions.

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Legends to figures

Fig. 1. Growth of V583 ery^S with shaking in GM17 medium or supplemented with 1.5mM, 1.75mM and 2mM H_2O_2. The arrows indicate the time at which RNA was extracted.

Fig. 2. Functional classification of genes with statistically significant increases (■) and decreases (□) in mRNA level upon 4 h exposure to hydrogen peroxide (total of 213 genes). The total number of genes associated with increased/decreased mRNA abundance as the result of H_2O_2 exposure in each functional class is shown.

Fig. 3. Network analysis of genes shown to be associated with significant transcript changes (built using STRING v9.1 [13]).

447 Table 1. Oligonucleotide primers used for real-time quantitative PCR in this study.

0188L: TCGCAGCTTGTGGTAATACG	0188R: ACTCGCAATAATCCGTTTGG
0191L: GGAACACCGCAAGAAGTGAT	0191R: GCCGCTGTGTCTGACTATGA
0475L: TCGAATTGCGCTGACAGATA	0475R: TAGCGCGTTCTCCAACTTTT
0567L: CGCTATGCACTGAATGTGCT	0567R: ACCTCCCGGTAAAAAGTGCT
0570L: GCTGGTGTTGGGAAAACCTA	0570R: GGACGATCATGAGGTTCGAT
0575L: AGGCTTTGGAAAAGGTCGAT	0575R: AAATCCGCATTTTGAGCAAG
0630L: AGTCAACCACGTGGGGATTA	0630R: TTCCACCAATTTCATCACGA
0666L: TCTTGAGCAAGCGGGTATCT	0666R: AGCGCCATTTGCGTATTATC
1120L: TTAAAGTGATGGGCGTTTCC	1120R: TGCGAAGGATAAGCAGAAGC
1617L: ACCCGAAATCGACGTAACAG	1617R: GGCGCGGACTTTCTTCTAA
1620L: TGAAGGTAAGCCGGTATTGG	1620R: GTCCACGTTTTCCTGAATCG
1621L: CAACCTGGACAGTTCGTTTGT	1621R: TGTGATTCGCTACGTTCAGG
1624L: AAATTGGCACCGATTATTGC	1624R: ATTGTATGACCCGCACCTTC
1627L: CACGTCACTGAAACGACAGAA	1627R: CAGGATCTACGGCATTTTTCA
1629L: ATCCATCGCTGGTTCAGAAA	1629R: AGCTGCTTGACCACTGTGAA
1633L: TTAGGCGCACTGGGTTATTT	1633R: TAACAGCTGCGTTTGTTTGC
1635L: CAGGGATGGCGTTTAATGTT	1635R: TTGCATTCATTCGTCCATGT
1638L: ATAAAACACAGGCGGTGGAA	1638R: GCCGCTGTGACGTTTAATG
1669L: GCCCAAGTCGTTTTGGTAGA	1669R: TGATTTACGATCCGCTAGGC
3085L: AACCGATAGTGCAAGGTTGG	3085R: CCGCTTAGCGCAAAAATAAA

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Table 2. Genes of *Enterococcus faecalis* V583 ery^S associated with increased transcript abundance as the result of exposure to H_2O_2.

Locus	Descriptions (Gene symbol)	M*	SD
Biosynthesis and metabolism			
EF0693	1-phosphofructokinase (*fruK-1*)	1.1	0.3
EF1222	adenine deaminase (*ade*)	1.4	0.2
EF1511	mandelate racemase/muconate lactonizing enzyme family protein	1.1	0.4
EF2999	allantoinase, putative	1.1	0.1
Transport and binding protein			
EF0188	iron compound ABC transporter, substrate-binding protein	2.0	0.6
EF0191	ferrichrome ABC transporter, ATP-binding protein	1.1	0.3
EF0192	ferrichrome ABC transporter, permease protein	1.0	0.1
EF0193	ferrichrome ABC transporter, permease protein (*fhuG*)	1.0	0.3
EF0475	ferrous iron transport protein A (*feoA*)	1.3	0.0
EF0476	ferrous iron transport protein B (*feoB*)	1.6	0.1
EF0567	potassium-transporting ATPase, subunit A (*kdpA*)	1.2	0.4
EF0568	potassium-transporting ATPase, subunit B (*kdpB*)	1.2	0.4
EF0569	potassium-transporting ATPase, subunit C (*kdpC*)	1.2	0.1
EF0575	cationic ABC transporter, ATP-binding protein	1.0	0.2
EF0576	cation ABC transporter, permease protein	1.1	0.1
EF0694	PTS system, fructose-specific family, IIBC components	1.0	0.6
EF1053	ABC transporter, ATP-binding protein	1.0	0.5
EF1117	amino acid ABC transporter, permease protein	2.2	0.4
EF1118	amino acid ABC transporter, permease protein	2.3	0.2
EF1119	amino acid ABC transporter, amino acid-binding protein	2.0	0.1
EF1120	amino acid ABC transporter, ATP-binding protein	1.8	0.1
EF1220	spermidine/putrescine ABC transporter, ATP-binding protein	1.0	0.2
EF3082	iron compound ABC transporter, substrate-binding protein	3.1	0.8
EF3083	iron compound ABC transporter, ATP-binding protein	3.2	0.6
EF3084	iron compound ABC transporter, permease protein	3.2	0.7
EF3085	iron compound ABC transporter, permease protein	3.3	0.6
Regulation and signal			
EF0570	sensor histidine kinase (*kdpD*)	1.0	0.1
EF0578	helix-turn-helix protein, iron-dependent repressor family	1.3	0.1
Cell envelope and cell division			
EF0887	glycosyl transferase, group 2 family protein	1.5	0.1
EF3314	cell wall surface anchor family protein	1.5	0.1
Stress and Virulence			
EF2068	multidrug resistance protein, putative	1.2	0.1
Unknown			
EF0477	hypothetical protein	1.7	0.8
EF0886	hypothetical protein	1.4	0.2
EF0888	conserved hypothetical protein	1.0	0.1
EF0889	conserved hypothetical protein	1.2	0.4
EF1223	chlorohydrolase family protein	1.2	0.6
EF1512	conserved hypothetical protein	1.1	0.3
EF1808	agaS protein	1.1	0.4
EF2067	conserved hypothetical protein TIGR00481	1.1	0.0

*M is the mean Log2(fold ratio); SD is the standard deviation.

454 Table 3. Genes of *Enterococcus faecalis* V583 ery^S associated with transcript reductions as
455 the result of exposure to H_2O_2.

Locus	Descriptions (Gene symbol)	M*	SD
Biosynthesis and metabolism			
EF0098	L-serine dehydratase, iron-sulfur-dependent, beta subunit (*sdhB-1*)	-2.4	0.2
EF0099	L-serine dehydratase, iron-sulfur-dependent, alpha subunit (*sdhA-1*)	-2.7	0.6
EF0630	glyoxalase family protein	-1.0	0.1
EF0867	glyoxalase family protein	-1.2	0.1
EF1358	glycerol dehydrogenase, putative	-1.6	0.5
EF1617	conserved hypothetical protein (*EutQ*)	-2.6	0.5
EF1618	ethanolamine utilization protein (*EutH*)	-2.7	0.6
EF1619	carbon dioxide concentrating mechanism protein CcmL, putative (*EutN*)	-2.8	0.3
EF1620	hypothetical protein (*EutX*)	-2.9	0.5
EF1621	conserved hypothetical protein (*EutY*)	-2.8	0.5
EF1622	conserved domain protein (*EutZ*)	-3.0	0.3
EF1623	microcompartment protein (*EutK*)	-2.6	0.4
EF1624	aldehyde dehydrogenase, putative (*EutE*)	-3.1	0.1
EF1625	microcompartment protein family (*EutM*)	-3.0	0.0
EF1626	ethanolamine utilization protein (*EutL*)	-3.1	0.1
EF1627	ethanolamine ammonia-lyase small subunit (*EutC*)	-3.3	0.1
EF1629	ethanolamine ammonia-lyase large subunit (*EutB*)	-3.4	0.1
EF1632	sensor histidine kinase (*HK*)	-1.1	0.1
EF1633	response regulator (*RR*)	-1.1	0.1
EF1634	propanediol utilization protein PduU (*EutS*)	-1.1	0.1
EF1635	propanol dehydrogenase PduQ, putative (*EutG*)	-2.1	0.0
EF1637	ATP:cob(I)alamin adenosyltransferase, putative (*EutT*)	-1.7	0.0
EF1638	propanediol utilization protein PduV (*eutP*)	-1.3	0.1
EF1669	glyoxylase family protein	-1.0	0.1
Protein biosynthesis and fate			
EF0100	seryl-tRNA synthetase (*serS-1*)	-3.4	0.1
EF0650	lipoate-protein ligase A (*lplA-1*)	-1.4	0.1
DNA metabolism			
EF2114	adenine methyltransferase, putative	-1.7	0.1
Transport and binding protein			
EF0636	Na+H+ antiporter (*nhaC-2*)	-1.1	0.2
EF0986	cation transporter	-1.0	0.1
EF2623	cadmium-translocating P-type ATPase (*cadA*)	-2.4	0.4
Regulation and signal			
EF0097	regulatory protein pfoR, putative	-2.0	0.3
EF0110	transcriptional regulator, ArsR family	-1.2	0.1
EF0143	transcriptional regulator, CroCI family	-1.2	0.5
EF0923	transcriptional regulator, LysR family	-1.2	0.2
EF1668	transcriptional regulator, MarR family	-1.4	0.1
EF2141	transcriptional regulator, CroCI family	-1.3	0.1
Cell envelope and cell division			
EF0139	FtsKSpoIIIE family protein	-1.1	0.0
EF0153	cell wall surface anchor family protein	-1.1	0.2

Stress and virulence			
EF0149	aggregation substance, putative	-1.4	0.2
Mobile and extrachromosomal element functions			
EF0158	conjugal transfer protein, putative	-1.3	0.4
EF2096	tail protein	-1.5	0.1
Unknown			
EF0083	hypothetical protein	-1.1	0.6
EF0109	ThiJPfpI family protein	-1.1	0.1
EF0131	conserved domain protein	-1.5	0.2
EF0132	hypothetical protein	-1.4	0.2
EF0134	hypothetical protein	-1.1	0.1
EF0135	conserved hypothetical protein	-1.0	0.1
EF0136	hypothetical protein	-1.1	0.1
EF0138	conserved domain protein	-1.1	0.0
EF0142	conserved hypothetical protein	-1.0	0.3
EF0144	conserved domain protein	-1.2	0.3
EF0151	hypothetical protein	-1.2	0.4
EF0152	hypothetical protein	-1.0	0.6
EF0154	conserved hypothetical protein	-1.7	0.1
EF0155	conserved hypothetical protein	-1.6	0.4
EF0415	conserved hypothetical protein	-1.4	0.4
EF0519	hypothetical protein	-1.0	0.1
EF0971	conserved hypothetical protein	-1.1	0.1
EF1327	BadFBadGBcrABcrD ATPase family protein	-1.4	0.5
EF1359	conserved hypothetical protein	-1.3	0.3
EF1360	dihydroxyacetone kinase family protein	-1.2	0.4
EF1361	dihydroxyacetone kinase family protein	-1.4	0.2
EF1362	conserved domain protein	-1.1	0.1
EF2014	coenzyme F420 hydrogenase domain protein	-1.0	0.1
EF2065	conserved hypothetical protein	-1.0	0.1
EF2094	hypothetical protein	-1.1	0.1
EF2095	hypothetical protein	-1.1	0.3
EF2097	hypothetical protein	-1.5	0.1
EF2098	hypothetical protein	-1.4	0.2
EF2099	hypothetical protein	-1.6	0.1
EF2100	hypothetical protein	-1.5	0.1
EF2101	hypothetical protein	-1.6	0.2
EF2102	hypothetical protein	-1.7	0.4
EF2103	hypothetical protein	-1.6	0.3
EF2104	hypothetical protein	-1.7	0.1
EF2105	hypothetical protein	-1.7	0.1
EF2106	conserved domain protein	-1.6	0.1
EF2107	hypothetical protein	-1.7	0.1
EF2108	hypothetical protein	-1.6	0.3
EF2109	conserved domain protein	-1.8	0.0
EF2110	hypothetical protein	-1.6	0.0
EF2111	hypothetical protein	-1.7	0.1
EF2112	hypothetical protein	-1.7	0.1
EF2113	conserved hypothetical protein	-1.6	0.3
EF2120	conserved hypothetical protein	-1.0	0.6
EF2125	hypothetical protein	-1.0	0.3
EF2127	hypothetical protein	-1.0	0.2

EF2133	hypothetical protein	-1.0	0.3
EF2134	hypothetical protein	-1.0	0.6
EF2137	hypothetical protein	-1.2	0.3
EF2140	conserved hypothetical protein	-1.2	0.2
EF2606	conserved hypothetical protein	-1.0	0.1
EF2792	conserved hypothetical protein	-1.0	0.0

*M is the mean Log2(fold ratio); SD is the standard deviation.

Table 4. Comparison of gene expression analyzed by real-time quantitative PCR and Microarray.

Locus	***(Gene symbol)* Function**	**H_2O_2/GM17**	
		RT qPCR	Microarray
ef0188	iron compound ABC transporter, substrate-binding protein	9.24±1.95	3.94±1.20
ef0191	ferrichrome ABC transporter, ATP-binding protein	3.33±0.70	2.20±0.44
ef0475	(feoA) ferrous iron transport protein A	3.16±0.99	2.48±0.03
ef0567	(kdpA) potassium-transporting ATPase, subunit A	2.72±1.27	2.27±0.34
ef0570	(kdpD) sensor histidine kinase KdpD	1.67±0.38	1.99±0.12
ef0575	cationic ABC transporter, ATP-binding protein	2.37±0.77	1.92±0.25
ef1120	amino acid ABC transporter, ATP-binding protein	6.44±0.25	3.52±0.03
ef3085	iron compound ABC transporter, permease protein	18.1±7.18	11.2±3.81
ef0630	glyoxalase family protein	-3.92±0.91	-2.00±0.14
ef0867	glyoxalase family protein	-2.67±0.40	-2.33±0.12
ef1617	(eutQ) conserved hypothetical protein	-8.16±0.48	-6.10±1.41
ef1620	(eutX) hypothetical protein	-8.62±0.50	-7.50±1.41
ef1621	(eutY) conserved hypothetical protein	-3.41±0.95	-7.00±1.41
ef1624	(eutE) aldehyde dehydrogenase, putative	-9.18±0.60	-8.60±1.07
ef1627	(eutC) ethanolamine ammonia-lyase small subunit	-10.1±0.46	-9.80±1.07
ef1629	(eutB) ethanolamine ammonia-lyase large subunit	-7.76±0.45	-10.6±1.07
ef1633	(RR17) response regulator	-4.13±0.24	-2.10±1.07
ef1635	(eutG) propanol dehydrogenase PduQ, putative	-11.7±0.85	-4.30±1.07
ef1638	(eutP) propanediol utilization protein PduV	-2.82±0.26	-2.50±1.07
ef1669	glyoxylase family protein	-2.90±1.15	-2.04±0.04

Values are given as means of the fold ratios ± standard deviation (SD).

463 Table 5. Expression of all the genes encoding glyoxalase family protein analyzed by

464 Microarray.

Locus	**Function**	**Level of gene expression**				**Ratio**
		GM17	**H_2O_2**	**GM17**	**H_2O_2**	**H_2O_2/GM17**
ef0358	glyoxalase family protein	330.7	164.6	408.5	224.2	-1.92±0.10
ef0939	methylglyoxal synthase	1627.9	961	1806.1	909.7	-1.84±0.15
ef0630	glyoxalase family protein	1152.1	541.5	945.8	508.4	-2.00±0.14
ef0656	glyoxalase family protein	138.8	112.8	236.1	178.6	-1.28±0.04
ef0666	glyoxalase family protein	2717	1834	2571.2	1811.8	-1.45±0.03
ef0745	glyoxalase family protein	1003.8	974.3	1216.9	1119.1	-1.06±0.03
ef0867	glyoxalase family protein	253.7	114.8	292.7	119.8	-2.33±0.12
ef1669	glyoxylase family protein	260.3	125.4	314.8	161	-2.04±0.04
ef2591	glyoxalase family protein	3065.4	3012.3	3317.9	2821.4	-1.10±0.08
ef3092	glyoxalase family protein	1302.2	1004.4	1238.1	937.8	-1.31±0.01

465 Values are given as means of the fold ratios ± standard deviation (SD).

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468 Table 6. Expression of the genes known to be involved in enterococcal adaptation to oxidative

469 stress.

Locus	**Protein (*Gene*)**	**Expression of genes**		**Ratio**
		GM17	**GM17 +H_2O_2**	**H_2O_2/GM17**
Oxidative stress proteins				
ef0453	Organic hydroperoxide resistance protein (*ohr*)	3712.9	3449.6	-1.07
ef0463	Superoxide dismutase (*sodA*)	4697.8	4342.7	-1.08
ef0606	DNA-binding protein (*dps*)	384.5	370	-1.04
ef1211	NADH peroxidase (*npr*)	4228.5	3577.3	-1.18
ef1338	Thioredoxine reductase (*trx*)	3292.4	2649.6	-1.24
ef1405	Thioredoxine reductase (*trx*)	1787.7	1772.6	-1.01
ef1586	NADH oxidase (*nox*)	5842.3	6058.5	1.04
ef1597	Catalase (*katA*)	2734.8	2806.2	1.03
ef1681	Peptide methionine-S-sulfoxide reductase (*msrA*)	2144	1908.5	1.12
ef2738	Peroxiredoxine reductase (*ahpF*)	3974	3984.6	1.00
ef2739	Alkyl hydroperoxide reductase (*ahpC*)	4536.4	4583	1.01
ef3164	Peptide methionine-S-sulfoxide reductase (*msrB*)	2449	1478.1	-1.65
ef3233	DNA-binding protein (*dps*)	4553.2	4202.9	-1.08
ef3270	Glutathione reductase (*gor*)	2707.7	2879.4	1.06
Oxidative stress related transcriptional regulators				
ef1525	(*fur*)	2925.4	2397.8	-1.22
ef1585	(*perR*)	2741.6	2304.2	-1.19
ef2063	(*soxS*)	1847.9	1969.6	1.07
ef2417	(*zur*)	1017.1	804.2	-1.26
ef2958	(*hypR*)	245.1	256.9	1.05

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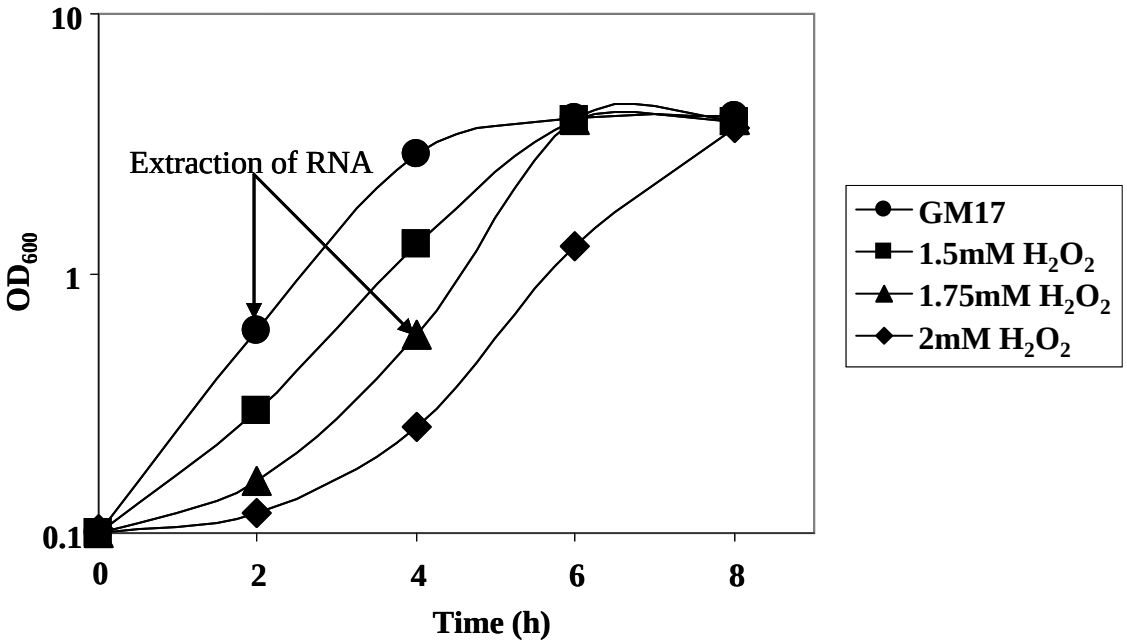
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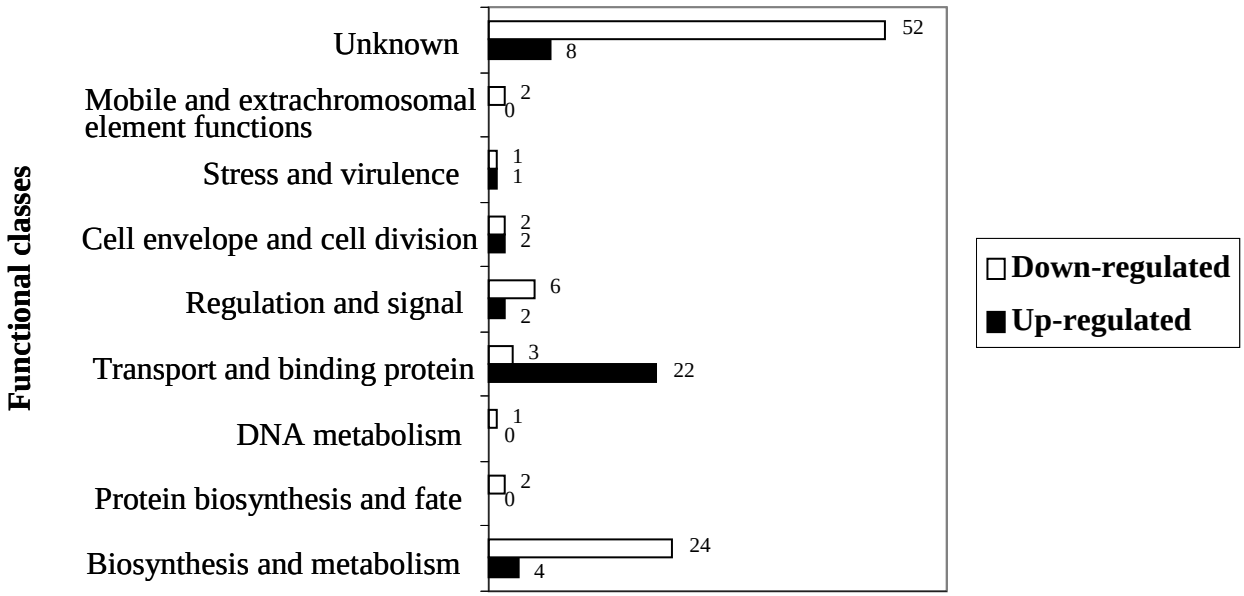
Fig. 1.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Cell envelope and cell division
2
2
Regulation and signal
6
2
Transport and binding protein
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Up-regulated

Fig. 2.

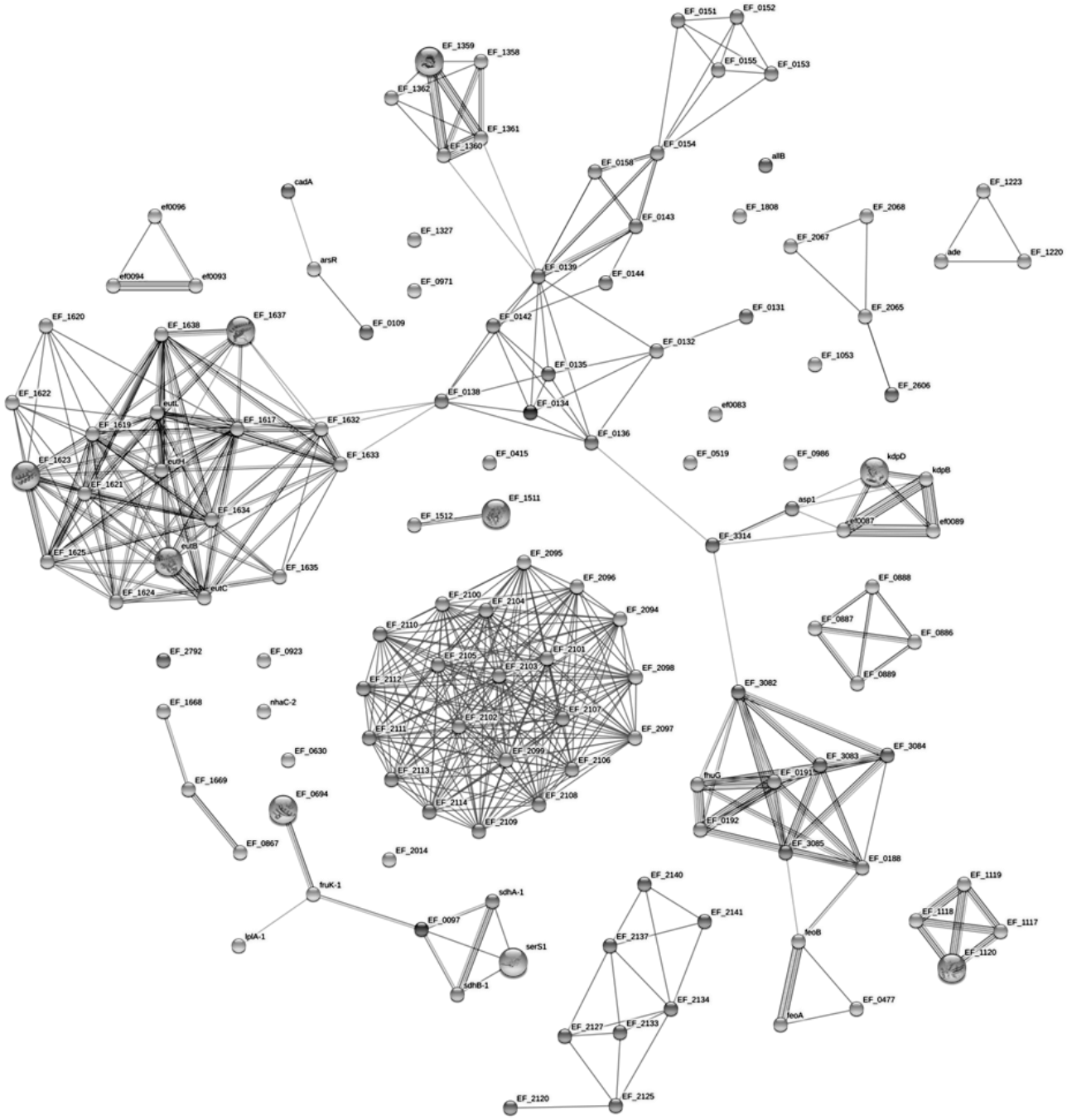

Fig. 3