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Original article

Identification of a new oxidative stress transcriptional regulator in *Enterococcus faecalis*

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Abstract – *Enterococcus faecalis*, sometimes used as lactic ferment, is able to resist many kinds of environmental stresses and especially the oxidative challenge. In order to identify regulators involved in the oxidative stress response of this commensal bacteria, several mutants affected in known or putative transcriptional regulators annotated in the *E. faecalis* genome (available at the T.I.G.R.) were constructed. A part of these genes encoded proteins belonging to a two-component regulatory system, whereas others were retained on the basis of homology with known oxidative stress transcriptional regulators such as *oxyR* or *soxS* from *Escherichia coli* or *perR* from *Bacillus subtilis*. Comparative proteomic analysis revealed that one mutant, affected in the *ef2958* locus, encoding the most homologous regulator to OxyR, showed some modifications in the protein pattern only after a sublethal H_2O_2 treatment in comparison with the wild-type strain. Indeed, using two-dimensional electrophoresis, five spots significantly less expressed in the *ef2958* mutant cultivated with 2 mmol·L^{-1} H_2O_2 were detected. Taken together, these results allowed us to qualify Ef2958 as a new oxidative stress regulator in *E. faecalis*.

***Enterococcus faecalis* / *ef2958* / transcriptional regulator / oxidative stress**

Résumé – **Identification d'un nouveau régulateur transcriptionnel impliqué dans le stress oxydatif chez *Enterococcus faecalis*.** *Enterococcus faecalis*, parfois utilisé comme ferment lactique, est capable de résister à de multiples stress environnementaux et plus particulièrement au stress oxydatif. Dans le but d'identifier des régulateurs de la réponse au stress oxydatif chez cette bactérie commensale, plusieurs mutants de régulateurs transcriptionnels annotés sur le génome d'*E. faecalis* (disponible sur le site du T.I.G.R.) ont été construits. Certains de ces gènes codent des protéines appartenant à des systèmes de régulation à deux composants, les autres ayant été définis par homologie de séquence avec des gènes codant des régulateurs transcriptionnels de stress oxydatif connus, tels que *oxyR* ou *soxS* chez *Escherichia coli* ou encore *perR* de *Bacillus subtilis*. Des analyses de protéomique comparative ont révélé que le mutant affecté sur le locus *ef2958*, qui montre la meilleure homologie avec le régulateur OxyR, présente des variations significatives de son profil protéique suite à un traitement subléthal en présence d'H_2O_2 comparativement avec celui de la souche sauvage. En effet, dans ces conditions, cinq spots voient leur synthèse diminuée chez le mutant *ef2958*. L'ensemble de ces résultats nous permettent de qualifier la protéine Ef2958 de nouveau régulateur de stress oxydatif chez *E. faecalis*.

***Enterococcus faecalis* / *ef2958* / régulateur transcriptionnel / stress oxydatif**

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1. INTRODUCTION

Enterococcus faecalis is an ubiquitous lactic acid bacteria and is a natural commensal microorganism of the animal and human gut. Although enterococci have long been considered as indicators of poor hygiene during production, this germ is widely used in industrial processes and sometimes constitutes a component of the microflora of fermented foods such as cheeses, sausages, and green olives. The growth of *E. faecalis* in certain cheese varieties contributes to the ripening and development of flavor and can prevent contamination with food pathogens (especially *Listeria*) by producing bacteriocin [19]. Moreover, several enterococcal cultures (mainly *E. faecalis* and *E. faecium*) have been used for their probiotic characteristics [17]. However, in the last decade, the safety of food containing *E. faecalis* has been questioned [4]. Indeed, this bacteria is one of the main germs responsible for nosocomial infections, especially in the USA, and this trend is exacerbated by an increasing multiple-antibiotic resistance [11]. As safety and food quality is a major preoccupation, it is essential that *E. faecalis* strains used in food fermentation or in probiotic preparations should be safe and free of health risks. More information is thus required in order to establish the differences between potentially pathogenic and safe, food-grade *E. faecalis* strains.

An important point is the large capacity of *E. faecalis* to colonize many ecological niches and to cope with several hostile conditions in food and non-food environments. Indeed, *E. faecalis* is known to resist many kinds of stresses such as heat, acid or starvation [7, 13]. One of the major stresses encountered by *E. faecalis* is the oxidative challenge, especially during the infection process as the germ has to cope with the immune system of the host. Investigations conducted in our laboratory showed that *E. faecalis* is strongly resistant to a hydrogen peroxide treatment and that 23 proteins were overexpressed [5]. A comparative genomic approach revealed that this bacteria possesses several genes encoding antioxidant enzymes such as *ahpCF* (alkyl hydroperoxide reductase), *npr* (NADH peroxidase), *sodA* (superoxide dismutase) and catalase. So, identifying which specific regulator gene(s) was(were) expressed in such stress conditions was necessary. Using the genome sequence of *E. faecalis* V583 available at the Institute for Genomic Research (T.I.G.R.), known or putative transcriptional regulators potentially involved in oxidative stress response were identified and mutated.

2. MATERIALS AND METHODS

2.1. Bacterial strains, plasmids, growth and stress conditions

The *E. faecalis* strain used in this study was JH2-2 [26]. *E. coli* EC101 [15] was used as a recipient strain for internal fragment cloning and pUCB300 [6] or pORI19 were used as cloning and integrational vectors. Cultures of *E. faecalis* were grown at 37 °C without shaking in 20-mL glass tubes containing 10 mL of GM17 medium or semi-synthetic medium (Bacto Folic AOAC Medium, Difco, Detroit, MI, USA) supplemented with glucose. Preliminary growth yield studies using different concentrations of glucose led to the choice of 0.2% (wt/vol) glucose to ensure that exhaustion of glucose triggered the transition to the stationary phase [7]. When necessary, erythromycin (Em) and chloramphenicol (Cm) were added at a final concentration of 150 $\mu g \cdot mL^{-1}$ and 20 $\mu g \cdot mL^{-1}$, respectively. *E. coli* strains were cultivated under vigorous agitation at 37 °C in BL medium with Em (300 $\mu g \cdot mL^{-1}$) or Cm (20 $mg \cdot mL^{-1}$) when required. Growth analysis of the mutants affected in a two-component system regulator was monitored at 600 nm (OD_{600}) in moderate stress conditions (25 or 48 °C; or 37 °C in GM17 medium containing 5, 6 or 7% ethanol; 0.05, 0.06 or 0.07% bile salts; 5.5, 6.5 or 7.5% NaCl; or in GM17 medium adjusted to pH 5.6, 5.8 or 6.0 with lactic acid or to pH 9.6, 9.8 or 10 with NaOH) using an

$ELX808_{IU}$ Ultra Microplates Reader driven by the KC4 kineticalc program (BIO-TEK instruments, Winooski, VT, USA). Growth in oxidative stress conditions (1.7, 1.9 or 2.1 mmol·L^{-1} H_2O_2) or at 50 °C of *E. faecalis* strains was monitored in GM17 using a Biophotometer (Eppendorf, Hamburg, Germany).

2.2. Insertional mutagenesis strategy

After PCR amplification, appropriate internal fragments (average size of 300 pb) of genes encoding OmpR-homolog response regulators were purified from the agarose gel, treated with the endonucleases *Eco*RI and *Sph*I, and cloned into the *rep*A^- pORI19 plasmid, using the $RepA^+$ *E. coli* EC101 as recipient strain. The different recombinant plasmids were then individually used to transform *E. faecalis* JH2-2 cells containing the pWV01-derivative *rep*A^{ts} pG^+host3 plasmid (the thermosensitive $RepA^{ts}$ protein is active at 30 °C and allowed replication of pG^+host3 and pORI19 recombinant plasmids) [18]. Insertional mutagenesis was then performed by shifting the culture temperature to 42 °C. Em^R and Cm^S mutant cells were verified by PCR and Southern blot. To construct insertional mutants with a disruption in the *E. faecalis ef1585*, *ef2063* and *lysR* family regulator genes, fragments of about 300 pb from each of these genes were cloned into the insertional vector pUCB300 digested with *Sma*I. The resulting plasmids obtained after transformation into *E. coli* EC 101 were used to transform competent cells of *E. faecalis* JH2-2. Erythromycin-resistant colonies were selected and integrations were checked by PCR and Southern blot analysis. DNA and amino acid sequences were analyzed using the Mac Vector™ (Kodak, Scientific Imaging Systems, Rochester, NY, USA) program and database searches were performed with the BLAST program available on the T.I.G.R. website (http://www.tigr.org). Other standard techniques were carried out as described by Sambrook et al. [20].

2.3. Two-dimensional protein gel electrophoresis

Protein preparation was performed as described by Giard et al. [8] from *E. faecalis* wild-type strains and mutants cultivated in semi-synthetic medium with or without 2 mmol·L^{-1} H_2O_2. The first dimensional electrophoresis was carried out using 17-cm ReadyStrip™ IPG Strips (Bio-Rad Laboratories, Hercules, CA, USA) (pH 4–7) and PROTEAN®IEF Cell apparatus (Bio-Rad) as recommended by the manufacturer. Second dimensions were performed in 14% polyacrylamide gels without stacking gel using the Millipore Investigator™ 2-D electrophoresis system (Millipore, Bedford, MA, USA). 2-D gels were silver-stained and analyzed as described by Giard et al. [8].

3. RESULTS AND DISCUSSION

3.1. Phenotypical analysis of mutants affected in regulators of two-component systems

Using the *E. faecalis* V583 genome sequence provided by the T.I.G.R., 18 loci encoding two-component system elements have been recently identified [12]. Ten of these systems are members of the OmpR family known to be involved in stress response. In a previous study, we constructed eight mutants of regulators included in this family [16]. Each of them was submitted to several moderate stresses but none showed phenotypical modifications compared with the wild-type strain JH2-2 after a sublethal oxidative stress. Nevertheless, following this phenotypical analysis, four regulators were proved to be involved in the stress response of *E. faecalis*. Indeed, the three mutants *err04*, *err08* and *err18* were affected in growth at high temperature (48 °C) compared with the wild-type strain. Furthermore, the *err10* mutant was more tolerant to treatments with bile salts (0.07%) and with heat (48 °C) but less tolerant when grown in an

Table I. Phenotypical analysis of mutants affected in a regulator included in a two-component system.

Locus name[a] (TIGR gene numbers)	Oxidative stress sensitivity	Other stress phenotypes
err04-ehk04 (*ef1703-ef1704*)	no	Heat sensitivity
err08-ehk08 (*ef1864-ef1863*)	no	Heat sensitivity
err10-ehk10 (*ef1050-ef1051*)	no	Heat and bile-salts resistance Acid and NaCl sensitivity
err18[b] (*ef3329*)	no	Heat sensitivity

[a] *err* for enterococcal response regulator, *ehk* for enterococcal histidine kinase. Numbers of the different two-component systems are adapted from the *E. faecalis* V583 description recently done by Hancock and Perego [12]. [b] orphan response regulator.

acid media (pH 3.4) or in the presence of 6.5% NaCl (Tab. I). Moreover, this latter system has recently been described as implicated in the virulence of *E. faecalis* [23].

3.2. Analysis of mutants affected in *perR*, *soxS* and *oxyR* homologous genes

In silico analyses using the *E. faecalis* V583 genome sequence were carried out in order to find genes encoding polypeptides homologous to regulators known to be involved in the oxidative stress response. PerR has been characterized as the major regulator of the inducible peroxide stress response in *B. subtilis* and appeared to be involved in the expression control of antioxidant enzymes such as the alkyl hydroperoxide reductase (*ahpCF*) or catalase (*katA*) [1, 2, 14]. The best BLAST hit result with PerR from *B. subtilis* was the Ef1585 (member of the Fur family) with 65% of identity. After several attempts, no mutant of the corresponding gene was obtained, suggesting a potentially essential role of this locus in *E. faecalis*.

Therefore, the best homolog (35% of identity) to the SoxS regulator from *E. coli* corresponds to the Ef2063 protein, a member of the AraC family. The *soxRS* regulon has been characterized as a defense against oxidative stress in response to $O_2\bullet^-$-generating compounds and contains genes such as superoxide dismutase (*sodA*) [9, 24]. The *ef2063* gene was thus mutated, but no sensitivity toward several oxidant agents of this mutant was detected in our conditions.

Finally, our homology research revealed that mainly five transcriptional regulators, members of the LysR family, were slightly homologous to the *E. coli* OxyR (17 to 23.1% of identity). The LysR family transcriptional regulators are able to regulate diverse genes in many prokaryotes and all those studied control their own expression [21]. OxyR, representative member of this family, was first characterized in *E. coli* and qualified as the main activator in response to H_2O_2 treatment. The *oxyR* regulon, including enzymes such as the alkyl hydroperoxide reductase (*ahpCF*) and catalase (*katG*), was also well described in *E. coli* [3]. So, our homology search led us to construct five *lysR* mutants of *E. faecalis*. Using two-dimensional (2D) electrophoresis, one mutant, affected in the *ef2958* locus encoding the most closely-related regulator to *E. coli* OxyR, appeared to be involved in the oxidative stress response of *E. faecalis*. Proteins were extracted from wild-type and mutant cells in the exponential growth phase

(control) and after a sublethal H_2O_2 treatment (2 mmol·L^{-1}) which has been proved to be the optimal dose for adaptation [5]. No modifications in protein patterns was observed on control gels. On the other hand, as shown in Figure 1, at least five proteins were significantly repressed in the *ef2958* mutant under H_2O_2 condition. This suggests that Ef2958 may act as a transcriptional activator in the presence of H_2O_2. Further investigations are in progress to sequence and identify these first members of the Ef2958 regulon. Experiments are now being carried out to define whether this mutant is affected in its sensitivity against oxidative agents. Since the oxidative stress response is crucial to survival during the infection process, evaluation of the virulence of the *ef2958* mutant cells should also be interesting.

3.3. Sequence analysis of the *ef2958* locus

Analysis of the nucleotide sequence of *ef2958* revealed an open reading frame (ORF) of 882 pb with an ATG codon preceded by a potential ribosome binding site (RBS) sequence, GGAGG, which was complementary to the 3' end of the 16S rRNA of *E. faecalis* CACCUCCAAA [10]. This RBS is placed seven nucleotides upstream of the start codon, which corresponds to the optimal spacing (seven to nine nucleotides) determined by Vellanoweth and Rabinowitz [25]. This locus encoded a protein of 294 amino acids with a calculated molecular mass of 32.6 kg·mol^{-1} and a pI of 5.97. Furthermore, the amino-terminal sequence of Ef2958 presented a highly-conserved region containing a helix-turn-helix DNA-binding motif characteristic of the LysR regulators [21]. In spite of a weak homology, this protein appeared to be the most closely-related polypeptide to OxyR from *E. coli* (23% of identity). Nevertheless, a previous study showed that two highly-conserved cysteine residues at positions 199 and 208 in the sequence of OxyR were necessary to confer the oxidized active form to the regulator [22, 27]. Surprisingly, no cysteine was found in the amino acid sequence of Ef2958. Moreover, this consensus of two cysteines was also absent in the sequence of the other LysR family paralogs of Ef2958. Nevertheless, our combined results indicate that Ef2958 corresponds to a new oxidative stress response regulator.

4. CONCLUSION

Whereas the regulation of the oxidative stress response is well known in *E. coli* or *B. subtilis*, little information is available on non-spore-forming Gram-positive bacteria such as *E. faecalis*. So, the main challenge of this work was to characterize in *E. faecalis* transcriptional regulators specifically involved in such response. In silico analysis was assayed to identify from among the approximately two hundred proteins annotated as potential regulators in *E. faecalis* candidates likely implicated in the oxidative stress response. Several mutants of genes encoding different regulators were obtained. We focused our study on one mutant, affected in the *ef2958* locus encoding the best homolog to OxyR from *E. coli*. Indeed, using a proteomic approach, we showed that the synthesis of at least five proteins was significantly reduced in this mutant after a H_2O_2-treatment compared with the wild-type strain. Furthermore, in spite of a weak similarity with OxyR, homology searches did not reveal any significant identity with known regulatory proteins. So, Ef2958 appears to be the first specific transcriptional regulator involved in the oxidative stress response of *E. faecalis*. Because such a response is important to survive and proliferate inside the host, work is in progress to identify the genes included in the Ef2958 regulon and to evaluate the implication of this regulator in the pathogenesis of *E. faecalis*.

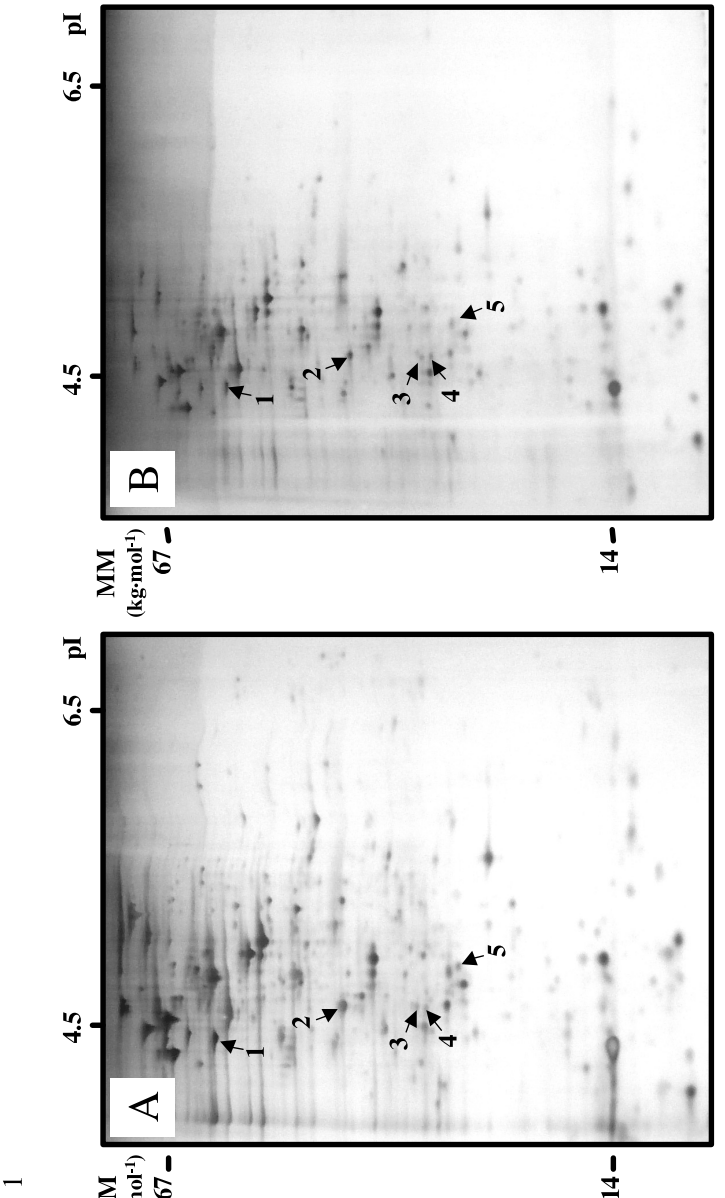

Figure 1. Two-dimensional gel electrophoresis of proteins from *E. faecalis* JH2-2 (A) and *ef2958* mutant (B) cells cultivated with 2 mmol·L^{-1} H_2O_2 for 30 min. Arrows indicate the positions of polypeptides that are present in lower amounts in the *ef2958* mutant than in the wild-type cells.

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