Mutations in msrA and msrB, encoding epimer-specific methionine sulfoxide reductases, affect expression of glycerol-catabolic operons in Enterococcus faecalis differently

Chen Zhao,1,2 Alain Bizzini,3 Xiaolin Zhang,2 Nicolas Sauvageot1 and Axel Hartke1

1Université de Caen Basse-Normandie, EA4655 U2RM Stress/Virulence, 14032 CAEN France
2Academy of State Administration of Grain, 11 Baiwanzhuang Avenue, Xicheng District, 100037 Beijing, PR China
3Service of Infectious Diseases, University Hospital Center Lausanne (CHUV), Lausanne, Switzerland

This study aims to define the cellular roles of methionine sulfoxide reductases A and B, evolutionarily highly conserved enzymes able to repair oxidized methionines in proteins. msrA and msrB mutants were exposed to an internal oxidative stress by growing them under aerobic conditions on glycerol. Interestingly, the msr mutants behave completely differently under these conditions. The msrA mutant is inhibited, whereas the msrB mutant is stimulated in its growth in comparison with the parent strain. Glycerol can be catabolized by either the GlpK or DhaK pathways in Enterococcus faecalis. Our results strongly suggest that in the msrA mutant, glycerol is catabolized via the GlpK pathway leading to increased synthesis of H2O2, which accumulates to concentrations inhibitory to growth in comparison with the parent strain. In contrast in the msrB mutant, glycerol is metabolized via the DhaK pathway which is not accompanied by the synthesis of H2O2. The molecular basis for the differences in glycerol flux seems to be due to expression differences of the two glycerol-catabolic operons in the msr mutants.

INTRODUCTION

Oxidative stress is an inevitable consequence of aerobic metabolism. The reactive oxygen species formed can damage biomolecules and may alter their biological functions. Organisms have developed mechanisms to defend themselves against reactive oxygen species either by eliminating them before they could provoke damages or by repairing the injured molecules (Imlay, 2008). Oxidation of methionine leads to the formation of methionine sulfoxide (MetSO) as an equimolar mixture of two epimers at the sulphur atom, i.e. Met-S-SO and Met-R-SO (Weissbach et al., 2002). The presence of MetSO in proteins may impair their biological functions. The existence of evolutionarily highly conserved enzymes able to reduce MetSO back to methionine suggests that these damages present a problem for organisms. Two non-homologous and structurally unrelated methionine sulfoxide reductases (Msr) have been described, with MsrA specific to the S-epimer and MsrB specific to the R-epimer (Boschi-Muller et al., 2008). Whereas the biochemistry of these enzymes has been extensively investigated (Boschi-Muller et al., 2005), the actual in vivo roles and the proteins that depend on MsrA/MsrB repair function are less understood. In order to shed light on the real world functions of these enzymes, protein targeting has recently been studied in Escherichia coli. The authors demonstrated that activity of a key protein (Ffh, a protein of the signal recognition particle) in this process, which is characterized by a methionine-rich domain of crucial importance, is dependent on MsrA/MsrB repair (Ezraty et al., 2004).

Enterococcus faecalis is a Gram-positive lactic acid bacterium and a major commensal of the gastrointestinal tract of humans and animals. This bacterium harbours one msrA and one msrB gene. Deletion mutants have been recently constructed and characterized (Zhao et al., 2010). This showed that Msr enzymes are important for the oxidative stress response and contribute to virulence in E. faecalis. E. faecalis is also a metabolically versatile organism which could use many different carbohydrates for its growth. E. faecalis has a highly developed glycerol metabolism (Bizzini et al., 2010). Indeed, glycerol can be catabolized by two different pathways. Either it is first oxidized by glycerol dehydrogenase and then phosphorylated by dihydroxyacetone kinase (DhaK pathway) or it is first phosphorylated by glycerol kinase and then oxidized by glycerol-3-phosphate oxidase (GlpK pathway). In both cases, this leads to the
formation of the glycolytic intermediate dihydroxyacetone. The corresponding genes of both pathways are clustered in two operon structures. As we showed recently, glycerol can be catabolized by either of the two pathways under aerobic conditions (Bizzini et al., 2010). Analysing different *E. faecalis* isolates showed also that the pathway which is preferentially used under these conditions is strain dependent.

Catabolism of glycerol via the G lpK pathway leads to the formation of H$_2$O$_2$ since the glycerol oxidase uses molecular oxygen as the electron sink:

Glycerol-3-phosphate $+$ O$_2$ $\rightarrow$ dihydroxyacetone phosphate $+$ H$_2$O$_2$

We showed recently, using peroxidase mutants constructed in strain JH2-2, that the H$_2$O$_2$ formed provokes an internal oxidative stress which leads rapidly to growth inhibition of the mutants compared with the wild-type strain (La Carbona et al., 2007). Peroxidases reduce hydroperoxide to water using reducing equivalents of NAD(P)H (Imlay, 2008). However, even in the wild-type strain, H$_2$O$_2$ temporally accumulated and was at the basis of a reduction in growth rate (La Carbona et al., 2007).

In the present work, we submitted *msrA* and *msrB* mutants to an internal oxidative stress by growing them aerobically on glycerol. This revealed that mutations in *msrA* or *msrB* differentially influence the flow through the two glycerol-catabolic pathways and that this was due to differences in expression of the corresponding operons in the *msrA* and *msrB* mutants. Our work demonstrates, for the first time to our knowledge, a link between regulation of gene expression and methionine oxidation, and establishes a new in vivo role of these protein repair enzymes.

**METHODS**

**Bacterial strains and media.** The bacterial strains used in this study are listed in Table 1. Overnight cultures of *E. faecalis* strains were performed on M17 medium (Terzaghi & Sandine, 1975) supplemented with 0.5% (w/v) glucose (GM17) in 30 ml glass tubes without agitation. Growth experiments on 0.3% glycerol (w/v) were realized with 0.5% (w/v) glucose (GM17) in 30 ml glass tubes without agitation. Growth experiments on 0.3% glycerol (w/v) were realized with 0.5% (w/v) glucose (GM17) in 30 ml glass tubes without agitation. Growth experiments on 0.3% glycerol (w/v) were realized with 0.5% (w/v) glucose (GM17) in 30 ml glass tubes without agitation. Growth experiments on 0.3% glycerol (w/v) were realized with 0.5% (w/v) glucose (GM17) in 30 ml glass tubes without agitation.

**Determination of H$_2$O$_2$ concentration.** The production of H$_2$O$_2$ was quantified using the Amplex red hydrogen peroxide/peroxidase assay kit (Invitrogen) according to the manufacturer’s instructions. Briefly, at given time points, 1 ml culture was centrifuged and appropriately diluted (10- or 100-fold for low or higher H$_2$O$_2$ concentrations, respectively) with 1× reaction buffer so that the H$_2$O$_2$ concentration in the assay was between 0.5 and 3 μM. In parallel, H$_2$O$_2$ standard curves (0-5 μM H$_2$O$_2$) were prepared using 10- or 100-fold diluted cCM17 medium supplemented with glycerol (dilution of the medium was done in 1× reaction buffer). The fluorescence was measured using a spectrofluorometer (JY3 D Jobin Yvon).

**RESULTS AND DISCUSSION**

According to databases at TIGR and the Human Genome Sequencing Center at Baylor College of Medicine, *E. faecalis* harbours one *msrA* and one *msrB* gene located at different parts of the chromosome. Markerless single and double deletion mutants as well as complemented strains have been constructed and tested for sensitivity to exogenously added oxidants and for virulence in four different animal models (Zhao et al., 2010).

In a previous study we used aerobic glycerol metabolism to provoke an intracellular generation of H$_2$O$_2$ by the
Table 1. Bacterial strains and plasmids used in the study

<table>
<thead>
<tr>
<th>E. faecalis strain</th>
<th>Relevant characteristic(s)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>JH2-2</td>
<td>FusR, RifR, plasmid-free wild-type strain</td>
<td>Yagi &amp; Clewell (1980)</td>
</tr>
<tr>
<td>ΔmsrA</td>
<td>JH2-2 with a 300 bp central deletion in msrA</td>
<td>Zhao et al. (2010)</td>
</tr>
<tr>
<td>ΔmsrB</td>
<td>JH2-2 with a 324 bp central deletion in msrB</td>
<td>Zhao et al. (2010)</td>
</tr>
<tr>
<td>msrA complemented strain</td>
<td>ΔmsrA strain complemented by allelic exchange</td>
<td>Zhao et al. (2010)</td>
</tr>
<tr>
<td>msrB complemented strain</td>
<td>ΔmsrB strain complemented by allelic exchange</td>
<td>Zhao et al. (2010)</td>
</tr>
<tr>
<td>ccpA::erm</td>
<td>JH2-2 with an integration of plasmid pUCB30 in the ccpA gene</td>
<td>Leboeuf et al. (2000)</td>
</tr>
<tr>
<td>Δers</td>
<td>JH2-2 with a 209 bp central deletion in the ers gene</td>
<td>Riboulet-Bisson et al. (2008)</td>
</tr>
<tr>
<td>araC::erm</td>
<td>JH2-2 with an integration of plasmid pUCB30 in the araC gene</td>
<td>This study</td>
</tr>
</tbody>
</table>

glycerol-3-phosphate oxidase (La Carbona et al., 2007). We showed that this leads to a stressing situation not only in mutants affected in antioxidative activities but also in wild-type cells. In the present work we used this system to study how msr mutants behave when exposed to such a metabolically derived intracellular oxidative stress (Fig. 1). The JH2-2 wild-type strain reached OD_{600} 3.5 after 24 h, whereas the isogenic msrA mutant grew poorly on glycerol and reached OD_{600} 1.5 at the end of the experiment. In contrast, the msrB mutant grew faster than the parental strain and entered stationary phase at OD_{600} 3.8 after 10 h of growth (Fig. 1). These phenotypes could be reversed by complementation (Fig. 1).

Compared with previous results obtained with mutants affected in either of the two glycerol catabolic operons (Bizzini et al., 2010) the msrA mutant shows similar growth characteristics to a strain with an inactive gldA1 gene encoding glycerol dehydrogenase, which is the first gene of the dhak operon. Furthermore, growth of the msrB mutant is comparable with a strain with an inactive glpK gene, the first gene of the glpK operon encoding glycerol kinase. In the gldA1 mutant, glycerol can only be metabolized by the GlpK pathway, which led to the synthesis of H_{2}O_{2} by glycerol-3-phosphate oxidase. The growth defect of this mutant was effectively due to an accumulation of this oxidant and it could be lifted by the addition of catalase to the growth medium (Bizzini et al., 2010). On the other hand, in the glpK mutant, glycerol can only be degraded by the DhaK pathway. No H_{2}O_{2} is formed during the transformation of glycerol to DHAP via this pathway which explains the faster growth of the glpK mutant compared with the gldA1 mutant and the parental strain. The results obtained with the msr mutants suggested therefore that glycerol is mainly catabolized via the GlpK pathway in the msrA mutant and via the DhaK pathway in the msrB mutant (Fig. 2). It is important to note that glycerol is metabolized simultaneously via both pathways in JH2-2 wild-type cells (Bizzini et al., 2010). If our working hypothesis that glycerol is catabolized by either of the two glycerol metabolic pathways in the msr mutants is correct, H_{2}O_{2} should accumulate in the culture supernatant of the msrA mutant, whereas H_{2}O_{2} production of the msrB mutant should be weak. As shown in Table 2, this is indeed the case. H_{2}O_{2} concentration per 10^8 c.f.u. in the first 10 h of culturing was between 300 and 400 μM for the msrA mutant, which is roughly 3–4 times higher than measured for the JH2-2 wild-type cells. The msrB mutant produced 15 and 30 μM H_{2}O_{2} per 10^8 c.f.u. (Table 2). Furthermore, to test if H_{2}O_{2} was causing growth inhibition in the msrA mutant, catalase was added to the culture. The msrA mutant grew well in the presence of this enzyme and even slightly faster than the msrB mutant in the absence of catalase (Fig. 1). Altogether, the combined results supported our hypothesis that glycerol flux through both pathways is diametrically opposed in the msrA and msrB mutants.

While this is an interesting observation, the understanding of this phenomenon on the molecular level is challenging. According to the in vitro activities of Mr enzymes, inactivation of the msrA gene should lead to the accumulation of only the Met-SO epimer in proteins, whereas in the msrB mutant the Met-R-SO epimer should

![Fig. 1. Aerobic growth on glycerol of the wild-type strain JH2-2 (○), the ΔmsrA mutant (□), the ΔmsrB mutant (△) as well as the msrA complemented strain (■) and the msrB complemented strain (▲). In some experiments, catalase was added after 2 h of growth, as indicated by an arrow. Under these conditions, growth of all the strains was comparable and for clarity reasons, the dashed line (+) represents these results.](image-url)
GTAAT box with one difference in the consensus.

The determinations of H$_2$O$_2$ concentrations were done in triplicate; data are means ± SD. Values are in µM per 10$^6$ c.f.u.

<table>
<thead>
<tr>
<th>Sample time (h)</th>
<th>JH2-2</th>
<th>ΔmsrA</th>
<th>ΔmsrB</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>96.1 ± 4.5</td>
<td>324.8 ± 120</td>
<td>33.2 ± 4.5</td>
</tr>
<tr>
<td>10</td>
<td>137.3 ± 22</td>
<td>404.8 ± 103.7</td>
<td>15.9 ± 3.9</td>
</tr>
</tbody>
</table>
Glycerol-catabolic genes, i.e. *glpD* encoding aerobic glycerol-3-phosphate dehydrogenase and *dhaK* encoding a subunit of dihydroxyacetone kinase, respectively. Analysis of the adjacent chromosomal regions of the *glpK* and *dhaK* operons of *E. faecalis* revealed the presence of a gene encoding a regulator belonging to the AraC family in front of the *dhaK* operon. Furthermore, the *E. faecalis* regulator Ers has recently been shown to regulate indirectly the *glpK* operon when anaerobically grown on glycerol (Riboulet-Bisson et al., 2009). Total RNA of an *araC* and *ers* mutant extracted from cultures grown aerobically on glycerol has been analysed by real-time quantitative PCR which showed that expression of neither of the two glycerol-catabolic operons is significantly different to expression in the parent strain (data not shown). From these data, it might be speculated that the two glycerol-catabolic operons are controlled by other unknown regulators. Experiments designed to get a deeper knowledge of regulation of expression of these operons have been initiated.

Table 3. Change in abundance of mRNA of glycerol-catabolic operons in the JH2-2 wild-type and *msr* mutants grown aerobically on glycerol

<table>
<thead>
<tr>
<th>Strain and comparative growth time</th>
<th>Factor for <em>dhaK</em></th>
<th>Factor for <em>glpK</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) JH2-2 (15 h/6 h)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>msrA</em> (10 h/6 h)</td>
<td>−2.05</td>
<td>+4.51</td>
</tr>
<tr>
<td>Δ<em>msrB</em> (7 h/4 h)</td>
<td>+8.07</td>
<td>−2.94</td>
</tr>
<tr>
<td>(ii) Δ<em>msrA</em> (10 h)/JH2-2 (6 h)</td>
<td>1</td>
<td>+3.98</td>
</tr>
<tr>
<td>Δ<em>msrB</em> (7 h)/JH2-2 (15 h)</td>
<td>+13.15</td>
<td>−2.3</td>
</tr>
</tbody>
</table>

Fig. 3. Analysis of the promoter regions of the *dhaK* (a) and *glpK* (b) operons. The ORFs are represented by open arrows and their orientation indicates the transcriptional direction. The putative Rho-independent terminators (TT) are shown. The transcriptional start site (+1), the putative −10 and −35 motifs, ribosome-binding sites (RBS) and the start codons are indicated and underlined. The +1 was determined by 5′RACE-PCR and the electrophoreograms obtained are shown. Two putative cre-sites present in each promoter region are boxed and their positions relative to the start site are indicated. *glpK–cre1* is identical to the cre consensus sequence (5’TGW/NANCNNTWCA; see Deutscher et al., 2006 for more details) whereas the other cre sites display 1 (*dhaK–cre2* and *glpK–cre2*) or 2 (*dhaK–cre1*) mismatches.
ACKNOWLEDGEMENTS

The technical assistance of Annick Blandin was greatly appreciated. We thank J.C. Giard and E. Bisson for the gift of the ers mutant strain.

REFERENCES


Edited by: J. Stülke