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

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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. \textit{msrA} and \textit{msrB} mutants were exposed to an internal oxidative stress by growing them under aerobic conditions on glycerol. Interestingly, the \textit{msr} mutants behave completely differently under these conditions. The \textit{msrA} mutant is inhibited, whereas the \textit{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 \textit{Enterococcus faecalis}. Our results strongly suggest that in the \textit{msrA} mutant, glycerol is catabolized via the GlpK pathway leading to increased synthesis of H\textsubscript{2}O\textsubscript{2}, which accumulates to concentrations inhibitory to growth in comparison with the parent strain. In contrast in the \textit{msrB} mutant, glycerol is metabolized via the DhaK pathway which is not accompanied by the synthesis of H\textsubscript{2}O\textsubscript{2}. The molecular basis for the differences in glycerol flux seems to be due to expression differences of the two glycerol-catabolic operons in the \textit{msr} mutants.

\section*{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-\textit{S}-SO and Met-\textit{R}-SO (Weissbach \textit{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 sulf oxide reductases (Msr) have been described, with MsrA specific to the \textit{S}-epimer and MsrB specific to the \textit{R}-epimer (Boschi-Muller \textit{et al.}, 2008). Whereas the biochemistry of these enzymes has been extensively investigated (Boschi-Muller \textit{et al.}, 2005), the actual \textit{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 \textit{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 \textit{et al.}, 2004).

\textit{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 \textit{msrA} and one \textit{msrB} gene. Deletion mutants have been recently constructed and characterized (Zhao \textit{et al.}, 2010). This showed that Msr enzymes are important for the oxidative stress response and contribute to virulence in \textit{E. faecalis}. \textit{E. faecalis} is also a metabolically versatile organism which could use many different carbohydrates for its growth. \textit{E. faecalis} has a highly developed glycerol metabolism (Bizzini \textit{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 GlpK pathway leads to the formation of \( \text{H}_2\text{O}_2 \) since the glycerol oxidase uses molecular oxygen as the electron sink:

\[
\text{Glycerol-3-phosphate} + \text{O}_2 \rightarrow \text{dihydroxyacetone phosphate} + \text{H}_2\text{O}_2
\]

We showed recently, using peroxidase mutants constructed in strain JH2-2, that the \( \text{H}_2\text{O}_2 \) 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, \( \text{H}_2\text{O}_2 \) temporally accumulated and was at the basis of a reduction in growth rate (La Carbona et al., 2007).

In the present work, we submitted \( \text{msrA} \) and \( \text{msrB} \) mutants to an internal oxidative stress by growing them aerobically on glycerol. This revealed that mutations in \( \text{msrA} \) or \( \text{msrB} \) differently influence the flow through the two glycerol-catabolic pathways and that this was due to differences in expression of the corresponding operons in the \( \text{msrA} \) and \( \text{msrB} \) 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 \textit{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 \textit{E. faecalis} strains were performed on M17 medium (Terrazhi & 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 cM17 MOPS medium which is depleted of carbohydrates and the usual β-disodium-glycerophosphate buffer of M17 is substituted with MOPS (Research Organics) added to a final concentration of 42 g l⁻¹ (Bizzini et al., 2010). Experiments under aerobic conditions were done in 150 ml Erlenmeyer flasks with 15 ml medium, and the cultures were incubated at 37 °C with vigorous agitation (160 r.p.m.) on a rotary shaker. When required, catalase from bovine liver was added to the medium at a final concentration of 500 U ml⁻¹.

#### Determination of \( \text{H}_2\text{O}_2 \) concentration.

The production of \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) concentrations, respectively) with 1× reaction buffer so that the \( \text{H}_2\text{O}_2 \) concentration in the assay was between 0.5 and 3 µM. In parallel, \( \text{H}_2\text{O}_2 \) standard curves (0–5 µM \( \text{H}_2\text{O}_2 \)) were prepared using 1× reaction buffer. The fluorescence was measured using a spectrofluorometer (JY D Jobin Yvon).

#### Construction of \( \text{araC} \) mutant.

An internal fragment of the \( \text{araC} \) gene was obtained by PCR amplification using oligonucleotides \( \text{araC}1 \) (5′-CTATGGATCTTGAAGCCTGGTATTGCG-3′; BamHI restriction site underlined) and \( \text{araC}2 \) (5′-CTATGCGAGCTTCACTTCTTTATTGCG-3′; SalI restriction site underlined) and the \( \text{E. faecalis} \) JH2-2 chromosome as template. The amplicon obtained was ligated into the suicide vector pUCB30 (Benachour et al., 2007). The resulting plasmid obtained after transformation of \textit{Escherichia coli} XL1Blue was used to transform competent cells of \textit{E. faecalis} JH2-2. Erythromycin-resistant colonies were selected on agar plates containing 150 µg erythromycin ml⁻¹. Integration was verified by PCR analysis using plasmid-based primers in combination with oligonucleotides \( \text{araC}3 \) (5′-CTT-TAGATTGAAGGATTTGCC-3′) and \( \text{araC}4 \) (5′-CCAAAGGCACTTAA-AACCG-3′).

#### RNA isolation, RACE-PCR and real-time qPCR.

Total RNA of \textit{E. faecalis} was isolated using the RNasy Midi kit (Qiagen). Mapping of the transcriptional start site and real-time qPCR analysis were performed on RNA extracted from JH2-2 cultures grown aerobically on glycerol. RACE-PCR was performed using a 5′ RACE kit (Roche) according to the manufacturer’s instructions. The oligonucleotides used were: 1358RACE1 (5′-GACACCAATGTCGAAAGGCG-3′); 1358RACE2 (5′-CTTCTCTCGAACCATTGCC-3′); 1358RACE3 (5′-GTCACACGTTTGAGAACGCG-3′); 1929RACE1 (5′-GCCTGTAAGTCC-CAATATGGG-3′); 1929RACE2 (5′-GGTATAATTTCCAAAGAGGCG-3′); 1929RACE3 (5′-GAATCCATATTTCTAGTGCC-3′). For real-time qPCR, specific primers were designed using the Primer3 software available at http://biotools.umassmed.edu/biapps/primer3 www.cgi to produce amplicons of 100 bp. Total RNA (2 µg) of the JH2-2 wild-type strain as well as from \textit{msr} mutants was reverse transcribed using the Omniscript enzyme (Qiagen) and random hexamer primers according to the manufacturer’s instructions. An aliquot (5 µl) of the resulting cDNA synthesis reaction mixture (100-fold diluted) was used for subsequent PCR amplification with the appropriate forward and reverse primers (1 µM final concentration) and the QuantTect SYBR Green PCR mix (Qiagen). Quantification of 23S rRNA levels was used as an internal control. Amplification, detection and real-time qPCR analysis were performed in duplicate with three different RNA samples, using the Bio-Rad iCycler iQ detection system (BioRad). 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). To relate the Ct value to the abundance of an mRNA species, \( C_0 \) was converted to ‘n-fold difference’ by comparing mRNA abundance in the JH2-2 wild-type strain to that obtained with mutant strains. The n-fold difference was calculated by the formula \( n=2^{-\frac{C_{\text{mutant}}-C_{\text{JH2-2}}}{C_{\text{JH2-2}}}} \), when \( C_{\text{mutant}}<C_{\text{JH2-2}} \) and \( (n=-2) \) when \( C_{\text{mutant}}>C_{\text{JH2-2}} \), with \( x=(C_{\text{mutant}}-C_{\text{JH2-2}}) \).

### RESULTS AND DISCUSSION

According to databases at TIGR and the Human Genome Sequencing Center at Baylor College of Medicine, \textit{E. faecalis} harbours one \( \text{msrA} \) and one \( \text{msrB} \) 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 \( \text{H}_2\text{O}_2 \) by the
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 explained 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 Msr 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

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**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>Fus$^b$ Rif$^b$, 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>

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**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.
not be repaired. However, how the presence of either epimer differently influences the glycerol flux is not easy to imagine. There may be two possible explanations: the presence of oxidized methionines may alter (i) activities of enzymes of the two glycerol catabolic pathways or (ii) a protein implicated in the regulation of expression of the two operon structures encoding glycerol metabolic genes. The latter was experimentally analysed by real-time qPCR (Table 3). This showed that in the msrA mutant, expression of the _glpK_ operon is 4.5 times higher than expression of the _dhaK_ operon. In contrast, in the msrB mutant, the _dhaK_ operon is expressed eightfold more than the _glpK_ operon. Compared with expression in the wild-type strain, the _glpK_ operon is expressed four times more in the _msrA_ mutant and expression of the _dhaK_ operon is 13-fold higher in the _msrB_ mutant. The combined results strongly suggest that the differences in glycerol fluxes in the _msr_ mutants can be explained by differences in the expression of the two glycerol catabolic operons.

Regulation of expression of glycerol catabolic operons seems to be complex. From a previous study we learned that both operons are subject to carbon catabolite repression (CCR) in _E. faecalis_ (Leboeuf _et al._, 2000; unpublished results). CCR corresponds to the phenomenon that micro-organisms in the presence of a preferred carbohydrate repress expression of other carbon catabolic operons encoding enzyme activities implicated in the metabolism of less favourable carbon sources. In _E. faecalis_, CCR is mediated via a negative regulatory mechanism involving three components: a _trans_-acting factor called catabolite control protein A (CcpA), _cis_-acting sequences termed catabolite responsive elements (cre-sites) and the HPr protein of the phosphoenolpyruvate-sugar-phosphotransferase system (Deutscher _et al._, 2006). CcpA is a DNA-binding protein and it binds to cre sequences in the presence of its co-repressor HPr phosphorylated on a serine residue at position 46 in this protein (Deutscher _et al._, 2006). Analysis of the sequences upstream of both glycerol-catabolic operons revealed the presence of two potential cre sites in each promoter region (Fig. 3). In order to position these cre sites with respect to the promoters _P_dhaK_ and _P_glpK_, the transcriptional start sites have been mapped by RACE-PCR. _P_dhaK_ displays a −10 (TATACT) and −35 (TTCAAT) regions separated by 21 bp which differ at 1 and 3 positions to the consensus sequences (TATAAT and TTGACA) of bacterial promoters, respectively (Fig. 3). _P_glpK_ displays a −10 (TGTAAT) box with one difference in the consensus sequence and an ideal −35 (TTGACA) region with a distance between the two promoter elements of 20 bp. Relative to the transcriptional start, the first cre site (cre1) overlaps with the −10 box in the promoter region of the _dhaK_ operon, whereas _cre2_ is situated between the +1 and the RBS in the _glpK_ operon. In both cases, the second cre site (cre2) is located upstream of the −35 promoter element at positions −43 and −56 for the _dhaK_ operon and −92 and −105 for the _glpK_ operon (Fig. 3).

As already mentioned, CcpA acts as a negative regulator on carbon catabolic genes in the presence of a preferred carbon source. Nevertheless, due to the presence of two cre sites in each promoter region, regulation of the glycerol-catabolic operons might be more complex. Therefore, we tested if CcpA has an influence on expression of these operons in cultures grown aerobically on glycerol. Total RNA was isolated from a previously characterized _E. faecalis ccpA_ mutant (Leboeuf _et al._, 2000) grown aerobically on glycerol, and expression of both glycerol operons was analysed by real-time qPCR. This showed that expression of _dhaK_ and _glpK_ operons was comparable in the mutant and the parent strain (data not shown).

In addition to global regulation by CcpA, carbon catabolic operons are also subject to specific regulation. For example, the _glp_ regulon of _Escherichia coli_ is under negative regulation of GlpR (Schweizer _et al._, 1985) and the _dhaK_ operon of _Escherichia coli_ is regulated by the transcription activator DhaR (Bächler _et al._, 2005). In both cases, the gene encoding the regulator is located adjacent

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**Table 2.** _H_2_3_0_2_ concentrations of cultures of the JH2-2 wild-type and _msr_ mutants grown aerobically on glycerol

Determinations of _H_2_3_0_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>
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</tbody>
</table>
to 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

Two experiments are shown: (i) modification of dhaK and glpK operon expression at different time points within the same strain and (ii) comparison of expression of dhaK and glpK operons between different strains at comparable growth (assessed by using OD₆₀₀ measurements).

<table>
<thead>
<tr>
<th>Strain and comparative growth time</th>
<th>Factor for dhaK</th>
<th>Factor for glpK</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JH2-2 (15 h/6 h)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>msrA (10 h/6 h)</td>
<td>-2.05</td>
<td>+4.51</td>
</tr>
<tr>
<td>ΔmsrB (7 h/4 h)</td>
<td>+8.07</td>
<td>-2.94</td>
</tr>
<tr>
<td>(ii)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔmsrA (10 h)/JH2-2 (6 h)</td>
<td>1</td>
<td>+3.98</td>
</tr>
<tr>
<td>ΔmsrB (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 electrophoregrams 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′TGWNANCNGNTNWCA; 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.
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