Glycerol Is Metabolized in a Complex and Strain-Dependent Manner in Enterococcus faecalis

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Enterococcus faecalis is equipped with two pathways of glycerol dissimilation. Glycerol can either first be phosphorylated by glycerol kinase and then oxidized by glycerol-3-phosphate oxidase (the glpK pathway) or first be oxidized by glycerol dehydrogenase and then phosphorylated by dihydroxyacetone kinase (the dhaK pathway). Both pathways lead to the formation of dihydroxyacetone phosphate, an intermediate of glycolysis. It was assumed that the glpK pathway operates during aerobiosis and that the dhaK pathway operates under anaerobic conditions. Because this had not been analyzed by a genetic study, we constructed mutants of strain JH2-2 affected in both pathways. The growth of these mutants on glycerol under aerobic and anaerobic conditions was monitored. In contrast to the former model, results strongly suggest that glycerol is catabolized simultaneously by both pathways in the E. faecalis JH2-2 strain in the presence of oxygen. In accordance with the former model, glycerol is metabolized by the dhaK pathway under anaerobic conditions. Comparison of different E. faecalis isolates revealed an impressive diversity of growth behaviors on glycerol. Analysis by BLAST searching and real-time reverse transcriptase PCR revealed that this diversity is based not on different gene contents but rather on differences in gene expression. Some strains used preferentially the glpK pathway whereas others probably exclusively the dhaK pathway under aerobic conditions. Our results demonstrate that the species E. faecalis cannot be represented by only one model of aerobic glycerol catabolism.

Enterococcus faecalis is a ubiquitous, low-GC-content, Gram-positive lactic acid bacterium and a natural member of the digestive microflora in humans and many other animals. Although E. faecalis is harmless in healthy individuals, some strains become pathogenic mainly in hospitalized patients undergoing prolonged antibiotic treatments or in patients with severe underlying diseases or an impaired immune system (21). Epidemiological studies have correlated enterococcal infections with anterior colonization of patient gastrointestinal tracts (29), and the patterns of intrinsic as well as acquired antibiotic resistance of these clinical strains (2), combined with the nutritional versatility of enterococci, are important traits for successful nesting in the intestine.

Studying glycerol metabolism is interesting for several reasons. Glycerol is an essential precursor for the synthesis of phospholipids and in many Gram-positive bacteria, including E. faecalis, for the building of (lipo)teichoic acids (6). Glycerol also seems to be an important carbon/energy source for pathogenic bacteria. It has been shown recently that glycerol-catabolizing enzymes in Listeria monocytogenes are important for intracellular growth (17). Furthermore, the importance of glycerol metabolism for pathogenic bacteria can be extrapolated from results of studies of Mycoplasma spp. These bacteria are highly adapted to life within a eukaryotic host, which is reflected by degenerative genome evolution that resulted in extreme genome reduction. However, glycerol is still part of those few carbon sources (glucose and fructose in addition to glycerol for M. pneumoniae) which can be utilized by these pathogens (14). Moreover, glycerol metabolism has a major impact on the pathogenicity of M. mycoides and M. pneumoniae (14, 24).

E. faecalis can also use glycerol as an energy source (15). It is among those bacteria which are equipped with two pathways of glycerol catabolism. One pathway begins with ATP-dependent phosphorylation of glycerol by glycerol kinase (GlpK) to yield glycerol-3-phosphate (glycerol-3-P). GlpK is a member of a large family of carbohydrate kinases including xylulose kinase, gluconate kinase, and others (9). In Gram-positive bacteria such as Bacillus subtilis, E. faecalis, and Enterococcus casseliflavus, GlpK activity is regulated by the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS), a transport system that catalyzes the uptake of numerous carbohydrates and their conversion into their respective phospho-esters during transport (see reference 9 for a recent review). Phosphorylation by the general PTS protein P~His15-HP at a conserved histidine leads to 10- to 15-fold stimulation of GlpK activity (7, 8). The glycerol-3-P formed is then oxidized to dihydroxyacetone phosphate (DHAP), an intermediate of the glycolytic pathway, in a reaction catalyzed by glycerol-3-P oxidase (GlpO) in E. faecalis. This enzyme uses molecular oxygen as the electron sink, which leads to the formation of H2O2. Extensive biochemical and structural studies have been conducted with GlpO proteins from Enterococcus and Streptococcus species (4, 5, 23).

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In the second pathway, glycerol is first oxidized to dihydroxyacetone by a soluble NAD+-dependent glycerol dehydrogenase (11, 16). The dihydroxyacetone formed is then phosphorylated to DHAP by dihydroxyacetone kinase (DhaK). These enzymes can be grouped into two families according to their phosphotransfer cascade leading finally to the formation of 2-phosphoglycerate. The enzymes are specific for eubacteria, mainly in eukaryotes but also in several eubacteria. The phosphoenolpyruvate-dependent enzymes are specific for catabolism, and the phosphotransfer cascade leading finally to the phosphorylation of DhaK has been well studied in Escherichia coli (12). The current model of glycerol metabolism in E. faecalis is based on findings from biochemical studies using cell extracts obtained from cultures grown on glucose (16). The researchers showed that aerobically grown E. faecalis cells possess a glycerol kinase and the ability to oxidize the phosphorylated product. The corresponding activities were absent in cell extracts obtained from cultures grown without agitation, but the authors demonstrated glycerol dehydrogenase activity under these conditions. Therefore, it is generally supposed that glycerol is metabolized via the glpK pathway in the presence of oxygen and by the dhaK pathway under anaerobic conditions. The results of preliminary experiments in our laboratory were inconsistent with this model, and therefore, we decided to look more closely at glycerol metabolism in E. faecalis in a genetic study. The findings showed that fluxes of glycerol through both pathways are complex and strain dependent. Strain dependence could be explained by astonishing expression differences between the glpK and dhaK operons in the different strains.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Strains and plasmids used in this study are listed in Table 1. Overnight cultures of E. faecalis strains were grown on M17 medium (28) supplemented with 0.5% (wt/vol) glucose. Growth experiments on 0.3% (wt/vol) glycerol were realized with cCM17MOPS medium, which is deple- ted of carbohydrates and has the usual β-disodium-glycophosphate buffer of M17 replaced with 3-N-morpholinopropanesulfonic acid (MOPS; Research Orga- nies) added to a final concentration of 42 g liter⁻¹ (19). This medium was prepared as described previously with the exception that after autoclaving and cooling of the medium to room temperature, dithiothreitol was added to a final concentration of 1 mM and the medium was stored frozen in appropriate ali- quots at −20°C. The medium is stable for at least 3 months under these conserva- tion conditions, leading to better reproducibility of results. Experiments under anaerobic conditions were done with 30-ml glass tubes filled with 30 ml of medium, closed with a rubber stopper, and incubated at 37°C without agitation. Subjection of the tubes to a vortex was avoided, but before manipulation, the tubes were gently moved up and down in order to homogenize the tube contents. For anaerobic growth, 0.26% (wt/vol) fumarate (22.4 mM) was added to the cCM17MOPS growth medium. Experiments under aerobic conditions were performed with 150-ml Erlenmeyer flasks containing 15 ml of medium, and the cultures were incubated at 37°C with vigorous agitation (160 rpm) on a rotary shaker. When indicated, catalase from bovine liver (900,000 U ml⁻¹; Sigma) was added to the medium at a final concentration of 500 U ml⁻¹. E. coli strain Top10F' was cultivated at 37°C in Luria-Bertani medium (26) with ampicillin (100 μg ml⁻¹) under vigorous agitation.

Statistical analysis. For growth experiments, data points are mean values calculated from the results of at least three independent experiments. Interex- perimental variations were ±10%. Determinations of H2O2 concentrations were done in triplicate. Standard deviations of the means are indicated in the corre- sponding table.

General molecular methods. PCR amplifications were carried out with Pfu polymerase (Stratagene, Amsterdam, The Netherlands) and triple master mix or master mix (5 Prime, Hamburg, Germany). Plasmids and PCR products were purified using NucleoSpin plasmid and NucleoSpin extract II kits, respectively (Macherey-Nagel, Düren, Germany). Other standard techniques were carried out as described by Sambrook et al. (26).

Construction of mutants. Primers were purchased from Operon (Köln, Ger- many). Sequences and positions of primers used are given in the supplemental material. A detailed protocol for the construction of unmarked deletion mutants using pUCB30 (or pGEM-T Easy; Promega) and pMAD plasmids has been published previously (19). The changes introduced not only a deletion but also a frameshift mutation and stop codons. For the ΔglpK mutant, the frameshift mutation starts after codon 239 and the first stop is after codon 258. The ΔglpK gene, the deletion provokes a frameshift mutation after codon 80 and the first stop is at position 98. Thus, the expression of the mutant gene led to the synthesis of a 97-amino-acid peptide, in contrast to the 379-amino-acid wild-type enzyme. For the construction of inser- tional mutants, an internal fragment obtained by PCR amplification using the E.

### TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain(s) or plasmid</th>
<th>Description or relevant characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. faecalis strains</td>
<td>Fus’ Rif’; plasmid-free wild-type strain</td>
<td>30</td>
</tr>
<tr>
<td>ΔglpK mutant</td>
<td>ΔglpK mutant, the frameshift provokes a frameshift mutation</td>
<td>This work</td>
</tr>
<tr>
<td>ΔglpA1 ΔglpK mutant</td>
<td>Double mutant with the aforementioned deletions in the glpK gene and glpA1</td>
<td>This work</td>
</tr>
<tr>
<td>TX0104</td>
<td>operon Information and (partial) genome sequence available from the BCM database</td>
<td>B. E. Murray, unpublished data</td>
</tr>
<tr>
<td>MMHH594</td>
<td>operon Information and (partial) genome sequence available from the BCM database</td>
<td>27</td>
</tr>
<tr>
<td>HH22</td>
<td>operon Information and (partial) genome sequence available from the BCM database</td>
<td>22</td>
</tr>
<tr>
<td>V583</td>
<td>Genome sequence available from TIGR</td>
<td>25</td>
</tr>
<tr>
<td>OG1RF</td>
<td>Genome sequence available from the BCM database</td>
<td>10</td>
</tr>
<tr>
<td>EF Cl isolates 1 to 9</td>
<td>Nine clinical isolates from various backgrounds</td>
<td>J. M. Entenza and J. Huebner, unpublished data</td>
</tr>
<tr>
<td>E. coli Top10F’</td>
<td></td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM-T Easy</td>
<td></td>
<td>Promega</td>
</tr>
<tr>
<td>pUCB30</td>
<td>oripMBI lacZ’ Amp’ Em’</td>
<td>3</td>
</tr>
<tr>
<td>pMAD</td>
<td>oripE194(Ts) Em’ Amp’ bgaB</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* BCM data are available at http://www.hgsc.bcm.tmc.edu/projects/microbial/microbial-index.xsp.
* TIGR data are available at http://www.tigr.org.

In the second pathway, glycerol is first oxidized to dihydroxyacetone by a soluble NAD+-dependent glycerol dehydrogenase (11, 16). The dihydroxyacetone formed is then phosphorylated to DHAP by dihydroxyacetone kinase (DhaK). These enzymes can be grouped into two families according to their phosphotransfer cascade leading finally to the formation of 2-phosphoglycerate. The enzymes are specific for eubacteria, mainly in eukaryotes but also in several eubacteria. The phosphoenolpyruvate-dependent enzymes are specific for catabolism, and the phosphotransfer cascade leading finally to the phosphorylation of DhaK has been well studied in Escherichia coli (12).
faecalis JH2-2 chromosome as a template was ligated into the suicide vector pUCB30 (3). The resulting plasmid obtained after transformation of E. coli XLI-Blue was used to transform competent cells of E. faecalis JH2-2. Erythromycin-resistant colonies were selected on agar plates containing 150 μg erythromycin ml⁻¹. Integration was verified by PCR analysis.

**Determination of H₂O₂ concentrations.** The production of H₂O₂ was quantified using the Amplex Red hydrogen peroxide/peroxidase assay kit (purchased from Fisher Scientific Bioblock, France) according to the instructions of the manufacturer (Invitrogen). Briefly, at given time points, 1 ml of culture was centrifuged and appropriately diluted (10- or 100-fold for a low or high H₂O₂ concentration, respectively) with 1x reaction buffer so that the H₂O₂ concentration in the assay mixture was between 0.5 and 3 μM. In parallel, H₂O₂ standard curves (0 to 5 μM H₂O₂) were prepared using ccM17MOPS medium supplemented with glycerol and diluted 10- or 100-fold in 1x reaction buffer. Fluorescence was measured using a spectrofluorometer (FJY D; Jobin Yvon).

**RT-PCR**. Total RNAs from E. faecalis were isolated using the RNeasy Midi kit (Qiagen). For real-time reverse transcriptase PCR (RT-PCR), specific primers were designed using the Primer3 software available at http://biotools.umassmed.edu/bioapps/primer3_www.cgi to produce amplicons of equivalent lengths (100 bp). Aliquots of 2 μg of total RNA were reverse transcribed using the Omniscript enzyme (Qiagen) and random hexamer primers according to the manufacturer’s instructions. Five-microliter reactions were amplified using the appropriate forward and reverse primers (1 μM final concentration) and the Quantitect SYBR green PCR mix (Qiagen). Quantification of 23S rRNA levels was used as an internal control because the ratio of 23S rRNA to total RNA is rather constant. Amplification, detection, and RT-PCR analysis were performed in duplicate with three different RNA samples by using the Bio-Rad Cycler IQ detection system. The values used for comparison of gene expression levels in various strains and environments were the numbers of PCR cycles required to reach the threshold cycle (C_T). To relate the C_T value to the abundance of an mRNA species, the C_T was converted into the n-fold difference by comparing the mRNA abundance in the JH2-2 strain to that in the mutant strain. The n-fold difference was calculated by the following formulas: n = 2⁻⁰·⁰ᵀ for C_ᵀ₉₅₂₀ and n = 2⁻² for C_ᵀ₃₅₂₀, where C_ᵀ₉₅₂₀ and C_ᵀ₃₅₂₀ are the C_T values for the mutant and JH2-2 strains and x = (C_ᵀ₉₅₂₀ - C_ᵀ₃₅₂₀).

**RESULTS**

**Growth kinetics on glycerol are strain dependent.** Aerobic growth of 16 E. faecalis isolates on glycerol was tested. The isolates can be divided into four categories (Fig. 1A). One group of strains (3 of 16), represented by strain TX0104, an endocarditis isolate, demonstrated the shortest lag phases and the fastest growth of all strains tested. They entered stationary phase after 8 h of culture. The only strain in the second category, JH2-2, had a lag phase comparable to those of strains of the TX0104 group. However, in contrast to the latter strains, JH2-2 demonstrated biphasic growth. Growth in the first phase was nearly as fast as that of the TX0104 group. Then the strain entered a second phase of slower growth until reaching a final optical density at 600 nm (OD₆₀₀) comparable to those reached by the TX0104 group. The third group comprised most of the strains tested (8 of 16), including strains OG1RF, the most commonly studied research strain, HH22, the first known beta-lactamase-producing strain of enterococci, and MMH594, which caused a nosocomial outbreak in the mid-1980s. All these strains grew slowly on glycerol under aerobic conditions and entered stationary phase at low OD₆₀₀ (between 0.5 and 1, depending on the strain). Finally, the fourth group (4 of 16 strains), typified by the sequenced strain V583 (the first vancomycin-resistant isolate identified in the United States), did not grow at all on glycerol.

The growth of seven strains under anaerobic conditions was tested, and the results are shown in Fig. 1B. The findings indicated that under anaerobic conditions, growth behavior of the different isolates is quite diverse. They differed in the length of the lag phase and the final OD₆₀₀ after 24 h of growth. The longest lag phase observed was that of the sequenced strain V583, for which some growth was visible after 8 h of incubation. The lowest final OD₆₀₀ recorded (1 after 24 h of incubation) was for the V583 and ATCC 19433 strains. The strain MMH594 reached the highest final OD₆₀₀ of 2.7. The other strains (JH2-2, HH22, TX0104, and OG1RF) had comparable growth kinetics and final OD₆₀₀ levels, with intermediate growth curves relative to those of strains MMH594 and ATCC 19433.

**Inventory of genes encoding putative glycerol-metabolizing enzymes.** In silico analysis of the genome sequence of E. faecalis strain V583 using The Institute for Genomic Research (TIGR) database (http://www.tigr.org) revealed that the genes encoding enzymes of glycerol metabolism are clustered mainly in two chromosomal loci but also that other putative glycerol metabolism genes are scattered around the genome. The genes encoding enzymes of the dhaK pathway seem to be organized into a four-gene operon structure coding for glycerol dehydrogenase (the EF1358 protein [GldA]), a small hypothetical protein (the EF1359 protein), and two subunits (the EF1360 product [DhaK] and the EF1361 product [DhaL]) of dihydroxyacetone kinase (Fig. 2). Northern blot analysis using RNA preparations obtained from cultures grown on glycerol and a probe complementary to the dhaK transcript revealed a
Relative gene expression levels in JH2-2
dhaK
Expression of dhaK, dehydrogenase (the EF0895 enzyme [GldA2]), as well as a putative glycerol-3-P dehydrogenase (the EF1747 enzyme [GlpD]), are present in other operon structures (Fig. 2). As judged by BLAST searching using the Baylor College of Medicine (BCM) database (http://www.hgsc.bcm.tmc.edu/projects/microbial/microbial-index.xsp), all these genes are also present in strains OG1RF, HH22, and TX0104.

Analysis of expression of genes with putative functions in glycerol metabolism. Expression of the different genes in the JH2-2 strain was evaluated by real-time RT-PCR using RNA preparations obtained from cultures grown aerobically on glucose and aerobically or anaerobically on glycerol (Table 2). During aerobic growth on glycerol, the expression of gene gldA2 was repressed (3.8-fold) relative to expression in cultures grown on glucose, whereas levels of expression of the glpD gene did not differ significantly under the two conditions. Furthermore, genes of the glpK operon are induced (2- to 3-fold) by glycerol, whereas the dhaK operon is slightly repressed (1.7- to 1.8-fold). However, the glpK and dhaK operons have comparable expression levels in cultures grown on glycerol. This finding suggested that the dhaK operon has a higher expression level than the glpK operon in cultures grown on glucose, which was indeed confirmed by our results showing the level of dhaK expression to be 7.2-fold higher (Table 2). No result was obtained for gene EF1828, encoding a putative second glycerol transporter, by using two different pairs of primers even with chromosomal DNA as the template. This outcome suggests that this gene is absent in strain JH2-2.

Under anaerobic conditions, the glpK operon is strongly repressed (5-fold) and the dhaK operon is highly induced (21-fold) in comparison to expression during aerobic growth on glycerol. The levels of expression of the gldA2 gene under oxic

### TABLE 2. Change in abundances of mRNAs for genes encoding putative glycerol-metabolizing enzymes

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Gene no. (TIGR)</th>
<th>Putative gene product or function</th>
<th>Ratio of mRNA levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative gene expression levels in JH2-2 wild-type strain grown aerobically on glucose or on glycerol</td>
<td>EF0895</td>
<td>gldA2</td>
<td>Glycerol dehydrogenase</td>
</tr>
<tr>
<td></td>
<td>EF1747</td>
<td>glpD</td>
<td>Glycerol-3-P dehydrogenase</td>
</tr>
<tr>
<td></td>
<td>EF1358</td>
<td>gldA1</td>
<td>Glycerol dehydrogenase</td>
</tr>
<tr>
<td></td>
<td>EF1360</td>
<td>dhaK</td>
<td>Dihydroxyacetone kinase</td>
</tr>
<tr>
<td></td>
<td>EF1927</td>
<td>glpF</td>
<td>Glycerol facilitator protein</td>
</tr>
<tr>
<td></td>
<td>EF1928</td>
<td>glpO</td>
<td>Glycerol-3-P oxidase</td>
</tr>
<tr>
<td></td>
<td>EF1929</td>
<td>glpK</td>
<td>Glycerol kinase</td>
</tr>
</tbody>
</table>

Expression of dhaK operon relative to glpK operon in JH2-2 wild type grown aerobically on glucose:

- On glycerol: EF1358, EF1360 | gldA1, dhaK | Glycerol catabolism | 1.0 |
- On glucose: EF1358, EF1360 | gldA1, dhaK | Glycerol catabolism | +7.2 |

Relative gene expression levels in JH2-2 strain grown anaerobically or aerobically on glycerol:

- EF1927, EF1928, and EF1929 | glpK operon | Glycerol catabolism | −5.23 |
- EF1358 and EF1360 | dhaK operon | Glycerol catabolism | +21 |
- EF0895 | glpD | Glycerol-3-P dehydrogenase | +2 |
- EF1747 | glpK operon | Glycerol catabolism | −2 |

Expression of dhaK operon relative to glpK operon in strain MMH594 aerobically grown on glycerol:

- EF1358, EF1360 | dhaK operon | Glycerol catabolism | +263 |
and anoxic conditions are comparable, but the glpD gene is slightly induced (2-fold) in cells grown under anaerobic conditions on glycerol (Table 2).

**Growth of gldA1 and glpK deletion mutants on glycerol.** The JH2-2 strain was chosen for the construction of markerless deletion mutants. Under aerobic conditions, neither the wild-type strain nor the mutants were able to grow without the addition of glycerol, demonstrating that the ccM17MOPS medium used did not contain growth substrates for *E. faecalis* (data not shown). As can be seen in Fig. 3, the ΔgldA1 mutant, with a nonfunctional dhaK pathway, grew more slowly than the wild type. An almost identical result was obtained with a mutant strain in which the dhaK gene (the ΔgldK mutant) grew much faster than the parental control. Two double mutants were constructed and tested for growth on glycerol. Neither the ΔgldA1 ΔglpK mutant (Fig. 3) nor the ΔglpK dhaK::ery mutant (data not shown) grew under these experimental conditions.

Upon the addition of catalase to the growth medium, the single ΔgldA1 mutant as well as the wild-type strain grew slightly better than the ΔglpK mutant under these conditions (Fig. 3). In contrast, catalase had no effect on the ΔgldA1 ΔglpK double mutant (Fig. 3), and the addition of heat-inactivated catalase to the medium had no growth-stimulating effect on the wild type or the ΔdhaK mutant (data not shown).

Due to the growth-stimulating effect of catalase, we determined extracellular H$_2$O$_2$ concentrations for the single mutants and the wild-type strain (Table 3). The results showed that the H$_2$O$_2$ concentration in the first 10 h of culture was between 300 and 400 μM for the JH2-2 wild-type strain and 500 and 600 μM for the ΔgldA1 mutant. In contrast, H$_2$O$_2$ was at the limit of detection (between 7 and 12 μM) in the growth medium of a ΔglpK mutant culture (Table 3).

**FIG. 3.** Aerobic growth of the JH2-2 wild-type strain (squares), the ΔgldA1 mutant (circles), the ΔglpK mutant (triangles), and the ΔgldA1 ΔglpK double mutant (diamonds) on glycerol. When used, catalase was added to growing cultures after 2 h of incubation. Growth in the presence of catalase led to similar results for the JH2-2 wild-type strain, the ΔgldA1 mutant, and the ΔglpK mutant. For clarity, these combined results are represented by one growth curve (multiplication signs). Catalase addition had no effect on the growth of the double mutant.

**TABLE 3.** Extracellular H$_2$O$_2$ concentrations for different strains grown aerobically on glycerol.

<table>
<thead>
<tr>
<th>Time point</th>
<th>JH2-2 (μM)</th>
<th>ΔgldA1 (μM)</th>
<th>ΔglpK (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 h</td>
<td>428 ± 30</td>
<td>7 ± 4</td>
<td>594 ± 103</td>
</tr>
<tr>
<td>10 h</td>
<td>341 ± 9</td>
<td>12 ± 6</td>
<td>471 ± 16</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The previous model of glycerol catabolism (16) proposed distinct functions of the two glycerol dissimilation pathways in *E. faecalis*. From the findings of biochemical studies, it was extrapolated that glycerol is metabolized by the glpK pathway under aerobic growth conditions and by the dhaK pathway under anaerobic conditions. This model is the one currently presented in textbooks (15). However, as we show in this report, based on the characterization of mutants affected in both pathways, aerobic glycerol metabolism is more complex than hitherto thought.

The JH2-2 strain was chosen for a more detailed molecular analysis. A mutant with a defective dhaK pathway grew poorly on glycerol under aerobic conditions, whereas the growth rate of the ΔglpK mutant was higher than that of the wild-type strain. Oxidation of glycerol-3-P via glycerol-3-P oxidase generates H$_2$O$_2$, and we supposed that the accumulation of this reactive oxygen species to toxic levels may be responsible for the poor growth of the ΔgldA1 mutant. H$_2$O$_2$ measurements showed that it accumulated in this mutant to levels higher than those in wild-type cultures. Furthermore, we showed that the mutant grew better than the wild-type after the addition of bovine catalase to the culture. This enzyme catalyzes the dismutation of H$_2$O$_2$ to water and oxygen. Hence, both results strongly supported our assumption that growth arrest of the ΔgldA1 mutant was due to the accumulation of toxic levels of H$_2$O$_2$. In contrast, the addition of catalase had little influence in the presence or absence of glycerol (data not shown). Growth of the ΔglpK mutant was comparable to that of the wild type. In contrast, the ΔgldA1 mutant (Fig. 4) and the dhaK::ery mutant (data not shown), as well as the ΔgldA1 ΔglpK double mutant (Fig. 4), did not grow.

**FIG. 4.** Anaerobic growth of the JH2-2 wild-type strain (squares), the ΔgldA1 mutant (circles), the ΔglpK mutant (triangles), and the ΔgldA1 ΔglpK double mutant (diamonds) on 0.3% (wt/vol) glycerol in the presence of 0.26% (wt/vol) fumarate.
on the growth of the mutant with a defective glpK gene, and H$_2$O$_2$ was at the limit of detection. These combined results are consistent with the suggestion that in the JH2-2 wild-type strain, glycerol is dissimilated by the two pathways under aerobic conditions (Fig. 5), since the parental strain grew better than the ΔgldA1 mutant but not as fast as the ΔglpK strain. This interpretation is supported by real-time RT-PCR results which indicated that levels of expression of the dhaK and glpK operons are comparable in the JH2-2 strain under these conditions.

The results obtained for the JH2-2 strain can be used to propose an explanation for the growth behaviors of the other strains able to grow on glycerol under aerobic conditions. The TX0104 group of strains demonstrated growth kinetics comparable to those of the JH2-2 strain under aerobic conditions (Fig. 5), since the parental strain grew better than the ΔgldA1 mutant but not as fast as the ΔglpK strain. Indeed, expression of this gene, encoding a putative second glycerol dehydrogenase (GldA2), is neither induced by glycerol nor able to compensate for the loss of the GldA1 protein in the ΔgldA1 mutant. Moreover, the physiological role of EF1747, encoding a putative glycerol-3-P dehydrogenase, is to date also not defined since its expression is not significantly influenced by the presence of glycerol and it could not complement the inactive glycerol oxidase in the ΔgldA1 mutant under anoxic conditions.

It would be interesting to compare the results obtained for E. faecalis with results for other bacterial species possessing both pathways of glycerol catabolism. This seems to be the case, for example, for L. monocytogenes, which is characterized by even more complex glycerol metabolism than E. faecalis (13). However, in contrast to E. faecalis, L. monocytogenes loses the ability to grow aerobically on glycerol with the inactivation of the glpK1 gene, which is part of the glpFK operon (18).

In conclusion, the reason why E. faecalis manages two glycerol dissimilation pathways is still a mystery. Our results clearly show that the dhaK pathway is, in terms of lag phase and growth rate, the most appropriate way to metabolize glycerol independently in the presence or absence of oxygen. Nevertheless, under aerobic conditions, most of the E. faecalis strains use the glpK pathway, which is linked to the generation of H$_2$O$_2$ as a by-product since it uses the glycerol-3-P oxidase instead of the glycerol-3-P dehydrogenase to catalyze the reaction. One may speculate that E. faecalis may gain a competitive advantage by releasing this oxidant into its habitat to poison competitors. A second major result of our study is the finding of astonishing diversity in glycerol metabolism patterns among different E. faecalis isolates, demonstrating that the
species could not be represented by only one model of aerobic glycerol catabolism. BLAST searching of the available *E. faecalis* genome sequences and analysis of gene expression by real-time RT-PCR revealed that this diversity is based not on different gene contents but rather on differences in gene expression. To the best of our knowledge, this is the first study demonstrating such impressive expression differences among strains of the same species. An important question raised by our results is whether this phenomenon is limited to glycerol metabolism in particular or if expression differences between strains are a more general characteristic of *E. faecalis*.

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