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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 nestling in the intestine.

Studying glycerol metabolism is interesting for several reasons. Glycerol is an essential precursor for the synthesis of lipids 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 phosphoesters during transport (see reference 9 for a recent review). Phosphorylation by the general PTS protein P~His_{15}-HPr 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 H_2O_2. Extensive biochemical and structural studies have been conducted with GlpO proteins from *Enterococcus* and *Streptococcus* species (4, 5, 23).

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TABLE 1. Bacterial strains and plasmids used in this study

Strain(s) or plasmid	Description or relevant characteristics	Reference or source
E. faecalis strains		
JH2-2	Fus^r Rif^r; plasmid-free wild-type strain	30
Δ*glpK* mutant	JH2-2 with a 190-bp central deletion in *glpK* introducing a frameshift mutation	This work
Δ*gldA1* mutant	JH2-2 with a 635-kbp deletion in *gldA1* introducing a frameshift mutation	This work
Δ*gldA1* Δ*glpK* mutant	Double mutant with the aforementioned deletions in the *glpK* gene and *gldA1* operon	This work
TX0104	Information and (partial) genome sequence available from the BCM database[a]	B. E. Murray, unpublished data
MMH594	Information and (partial) genome sequence available from the BCM database	27
HH22	Information and (partial) genome sequence available from the BCM database	22
ATCC 19433		American Type Culture Collection
V583	Genome sequence available from TIGR[b]	25
OG1RF	Genome sequence available from the BCM database	10
EF CI isolates 1 to 9	Nine clinical isolates from various backgrounds	J. M. Entenza and J. Huebner, unpublished data
E. coli Top10F′		Invitrogen
Plasmids		
pGEM-T Easy		Promega
pUCB30	oripMB1 *lacZ′* Amp^r Em^r	3
pMAD	oripE194(Ts) Em^r Amp^r *bgaB*	1

[a] BCM data are available at http://www.hgsc.bcm.tmc.edu/projects/microbial/microbial-index.xsp.
[b] 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 phosphoryl donors (11). The ATP-dependent kinases occur mainly in eukaryotes but also in several eubacteria. The phosphoenolpyruvate-dependent enzymes are specific for eubacteria, 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 anoxic 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 depleted of carbohydrates and has the usual β-disodium-glycerophosphate buffer of M17 replaced with 3-*N*-morpholinopropanesulfonic acid (MOPS; Research Organics) 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 aliquots at −20°C. The medium is stable for at least 3 months under these conservation 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. Interexperimental variations were ≤10%. Determinations of H_2O_2 concentrations were done in triplicate. Standard deviations of the means are indicated in the corresponding 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, Germany). 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 Δ*glpK* mutant, the frameshift mutation starts after codon 239 and the first stop is after codon 258. The *glpK* wild-type gene has 501 codons, which means that more than half the encoded protein is deleted in the mutant. In the *gldA1* 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 insertional mutants, an internal fragment obtained by PCR amplification using the *E.*

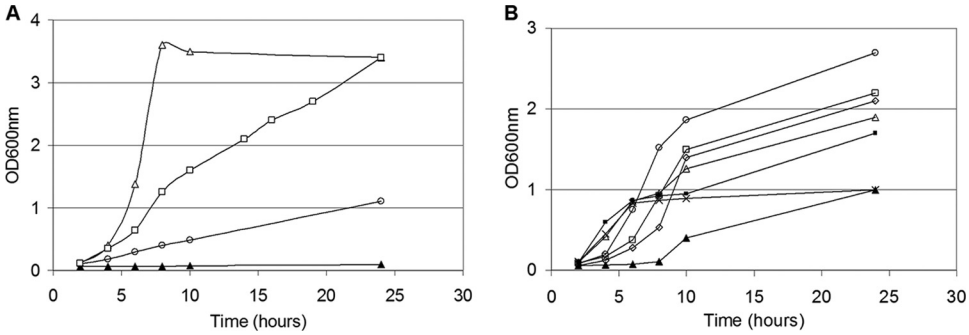

FIG. 1. Aerobic (A) and anaerobic (B) growth of different *E. faecalis* strains on glycerol. For anaerobic growth, 0.26% (wt/vol) fumarate (22.4 mM) was added to the ccM17MOPS growth medium. Symbols: white triangles, TX0104; white squares, JH2-2; white circles, MMH594; black triangles, V583; white diamonds, HH22; black squares, OG1RF; and multiplication signs, ATCC 19433.

faecalis JH2-2 chromosome as a template was ligated into the suicide vector pUCB30 (3). The resulting plasmid obtained after transformation of *E. coli* XL1-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 H_2O_2 concentrations. The production of H_2O_2 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 H_2O_2 concentration, respectively) with 1× reaction buffer so that the H_2O_2 concentration in the assay mixture was between 0.5 and 3 μM. In parallel, H_2O_2 standard curves (0 to 5 μM H_2O_2) were prepared using ccM17MOPS medium supplemented with glycerol and diluted 10- or 100-fold in 1× reaction buffer. Fluorescence was measured using a spectrofluorometer (JY3 D; Jobin Yvon).

RNA isolation and real-time 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 samples of the resulting cDNA synthesis reaction mixtures (diluted 100-fold) were used for subsequent PCR amplification with 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 iCycler 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: $n = 2^{-x}$ for $C_{T\text{mutant}} < C_{T\text{JH2-2}}$, and $n = -2^x$ for $C_{T\text{mutant}} > C_{T\text{JH2-2}}$, where $C_{T\text{mutant}}$ and $C_{T\text{JH2-2}}$ are the C_T values for the mutant and JH2-2 strains and $x = (C_{T\text{mutant}} - C_{T\text{JH2-2}})$.

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 (OD_{600}) 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 OD_{600}s (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 OD_{600} 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 OD_{600} recorded (1 after 24 h of incubation) was for the V583 and ATCC 19433 strains. The strain MMH594 reached the highest final OD_{600} of 2.7. The other strains (JH2-2, HH22, TX0104, and OG1RF) had comparable growth kinetics and final OD_{600} 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 dissimilation 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 [GldA1]), 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

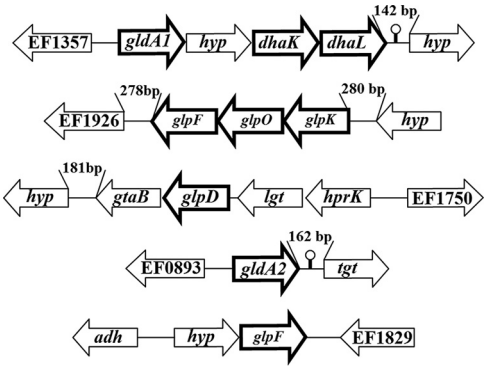

FIG. 2. Operon structures containing genes potentially implicated in glycerol metabolism in *E. faecalis* V583. The genes with assigned putative functions encode the following proteins: *glpF*, glycerol uptake facilitator; *glpO*, glycerol-3-P oxidase; *glpK*, glycerol kinase; *glpD*, glycerol-3-P dehydrogenase; *gtaB*, UTP glucose-1-phosphate uridylyltransferase; *lgt*, prolipoprotein transferase; *hprK*, HPr kinase/phosphatase; *adh*, alcohol dehydrogenase; *gldA*, glycerol dehydrogenase; and *dhaK* and *dhaL*, the two subunits of dihydroxyacetone kinase. Genes related to glycerol metabolism are highlighted in bold in each operon structure. *hyp* indicates genes encoding hypothetical proteins.

band of approximately 3.5 kb, which is consistent with cotranscription of the four genes (data not shown).

Three other genes implicated in glycerol metabolism were grouped into an operon structure coding for glycerol kinase (GlpK), glycerol-3-P oxidase (GlpO), and the glycerol diffusion facilitator protein (GlpF1) (Fig. 2). Cotranscription of the three genes was previously confirmed by Northern blot analysis (20). Furthermore, genes encoding a second putative glycerol dehydrogenase (the EF0895 enzyme [GldA2]) and a second glycerol facilitator (the EF1828 protein [GlpF2]), 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

Comparison	Gene no. (TIGR)	Gene name(s) (this work)	Putative gene product or function	Ratio of mRNA levels
Relative gene expression levels in JH2-2 wild-type strain aerobically grown on glucose or on glycerol	EF0895	*gldA2*	Glycerol dehydrogenase	−3.8
	EF1747	*glpD*	Glycerol-3-P dehydrogenase	−1.4
	EF1358	*gldA1*	Glycerol dehydrogenase	−1.8
	EF1360	*dhaK*	Dihydroxyacetone kinase	−1.7
	EF1927	*glpF*	Glycerol facilitator protein	+2.2
	EF1928	*glpO*	Glycerol-3-P oxidase	+2.9
	EF1929	*glpK*	Glycerol kinase	+3.1
Expression of *dhaK* operon relative to *glpK* operon in JH2-2 wild type aerobically grown:				
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	*gldA2*	Glycerol dehydrogenase	1
	EF1747	*glpD*	Glycerol-3-P dehydrogenase	+2
Expression of *dhaK* operon relative to *glpK* operon in strain MMH594 aerobically grown on glycerol	EF1358, EF1360	*dhaK* operon	Glycerol catabolism	−2
Expression of *dhaK* operon relative to *glpK* operon in strain TX0104 aerobically grown on glycerol	EF1358, EF1360	*dhaK* operon	Glycerol catabolism	+263

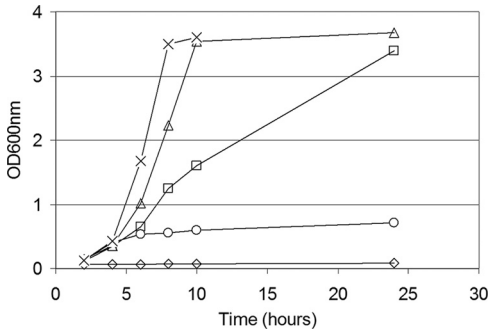

FIG. 3. Aerobic growth of the JH2-2 wild-type strain (squares), the Δ*gldA1* mutant (circles), the Δ*glpK* mutant (triangles), and the Δ*gldA1* Δ*glpK* double mutant (diamonds) on glycerol. When used, catalase was added to growing cultures after 2 h of incubation. Growth in the presence of catalase led to similar results for the JH2-2 wild-type strain, the Δ*gldA1* mutant, and the Δ*glpK* 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.

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 Δ*gldA1* 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 was inactivated by the insertion of a plasmid (data not shown). On the other hand, the strain with a central deletion in the *glpK* gene (the Δ*glpK* mutant) grew much faster than the parental control. Two double mutants were constructed and tested for growth on glycerol. Neither the Δ*gldA1* Δ*glpK* mutant (Fig. 3) nor the Δ*glpK* *dhaK*::*ery* mutant (data not shown) grew under these experimental conditions.

Upon the addition of catalase to the growth medium, the single Δ*gldA1* mutant as well as the wild-type strain grew slightly better than the Δ*glpK* mutant under these conditions (Fig. 3). In contrast, catalase addition had no effect on the Δ*gldA1* Δ*glpK* 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 Δ*dhaK* mutant (data not shown).

Due to the growth-stimulating effect of catalase, we determined extracellular H_2O_2 concentrations for the single mutants and the wild-type strain (Table 3). The results showed that the H_2O_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 Δ*gldA1* mutant. In contrast, H_2O_2 was at the limit of detection (between 7 and 12 μM) in the growth medium of a Δ*glpK* mutant culture (Table 3).

Growth of *E. faecalis* on glycerol under anaerobic conditions requires an electron acceptor, e.g., fumarate (16). Without the addition of fumarate, none of the strains were able to grow in

TABLE 3. Extracellular H_2O_2 concentrations for different strains grown aerobically on glycerol

Time point	Mean extracellular H_2O_2 concn (μM) ± SD for:		
	JH2-2 wild type	Δ*glpK* mutant	Δ*gldA1* mutant
6 h	428 ± 30	7 ± 4	594 ± 103
10 h	341 ± 9	12 ± 6	471 ± 16

the presence or absence of glycerol (data not shown). Growth of the Δ*glpK* mutant was comparable to that of the wild type. In contrast, the Δ*gldA1* mutant (Fig. 4) and the *dhaK*::*ery* mutant (data not shown), as well as the Δ*gldA1* Δ*glpK* double mutant (Fig. 4), did not grow.

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 Δ*glpK* mutant was higher than that of the wild-type strain. Oxidation of glycerol-3-P via glycerol-3-P oxidase generates H_2O_2, and we supposed that the accumulation of this reactive oxygen species to toxic levels may be responsible for the poor growth of the Δ*gldA1* mutant. H_2O_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_2O_2 to water and oxygen. Hence, both results strongly supported our assumption that growth arrest of the Δ*gldA1* mutant was due to the accumulation of toxic levels of H_2O_2. In contrast, the addition of catalase had little influence

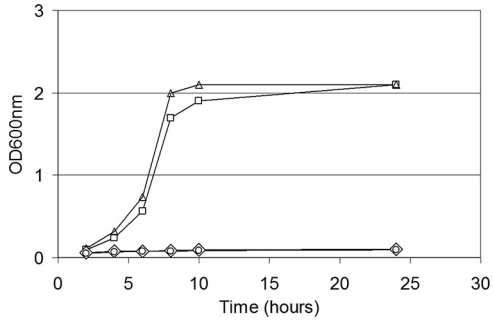

FIG. 4. Anaerobic growth of the JH2-2 wild-type strain (squares), the Δ*gldA1* mutant (circles), the Δ*glpK* mutant (triangles), and the Δ*gldA1* Δ*glpK* double mutant (diamonds) on 0.3% (wt/vol) glycerol in the presence of 0.26% (wt/vol) fumarte.

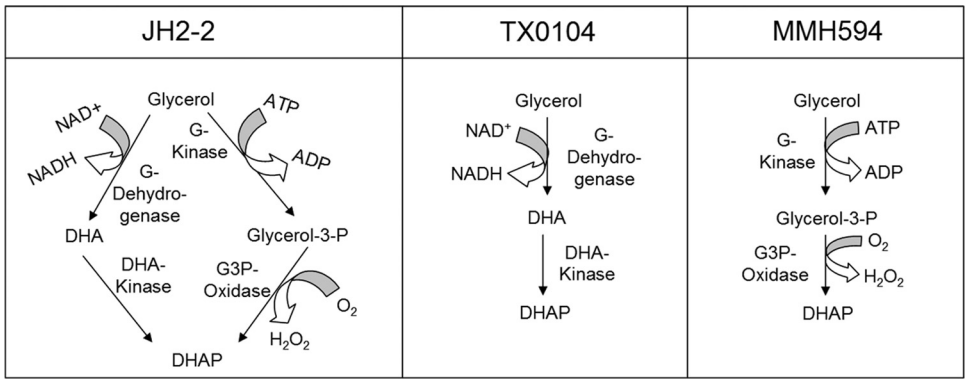

FIG. 5. Proposed model for aerobic glycerol catabolism in strains JH2-2, TX0104, and MMH594. Strains TX0104 and MMH594 utilize preferentially the *dhaK* and *glpK* pathways, respectively, for glycerol dissimilation. In the JH2-2 strain, glycerol flows through both pathways. In the *dhaK* pathway, glycerol is first oxidized to dihydroxyacetone (DHA) by glycerol dehydrogenase (G-dehydrogenase) and then phosphorylated to DHAP by dihydroxyacetone kinase. In the *glpK* pathway, glycerol is first phosphorylated to glycerol-3-P by glycerol kinase (G-kinase) and then oxidized to DHAP by glycerol-3-P oxidase (G3P-oxidase).

on the growth of the mutant with a defective *glpK* gene, and H_2O_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 Δ*gldA1* mutant but not as fast as the Δ*glpK* 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 Δ*glpK* mutant, suggesting that glycerol is dissimilated mainly (if not exclusively) by the *dhaK* pathway in these strains (Fig. 5). This hypothesis is strongly supported by the real-time RT-PCR results, which revealed that the level of expression of the *dhaK* operon in TX0104 is more than 250-fold higher than the level of expression of the *glpK* operon (Table 2). On the other hand, the group of *E. faecalis* isolates showing poor growth on glycerol (typified by strains OG1RF and MMH594) is comparable to the JH2-2 Δ*gldA1* mutant. Real-time RT-PCR experiments showed that the level of expression of the *glpK* operon is twofold higher than the level of expression of the *dhaK* operon in strain MMH594 (Table 2). Measurements of H_2O_2 indicated that this strain, in contrast to strain TX0104, accumulated this peroxide to a high level. The growth arrest could be lifted by the addition of catalase (data not shown), suggesting that in the MMH594 strain, as in the JH2-2 Δ*gldA1* mutant, glycerol is catabolized mainly via the *glpK* pathway (Fig. 5).

Under anaerobic conditions, glycerol can be dissimilated only via the *dhaK* pathway in strain JH2-2. This is consistent with the real-time RT-PCR results, which showed that under these conditions the genes of the *glpK* operon were repressed and those of the *dhaK* operon were highly induced. The combined results confirm the model proposed by Jacobs and VanDemark (16) for glycerol dissimilation under anaerobic conditions. The molecular basis of the strain dependency of growth under these conditions remains unexplained. Moreover, strain V583 seems to have a general, unknown defect in glycerol catabolism since it did not grow at all under aerobic conditions (even when catalase was added to the growth medium) and grew poorly under anaerobic conditions, although it is equipped with the entire set of genes depicted in Fig. 2.

Among the glycerol-metabolizing genes present in operon structures other than the *glpK* and *dhaK* operons, EF0895 seems not to be involved in glycerol catabolism in strain JH2-2. 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 Δ*gldA1* 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 Δ*gldA1* 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_2O_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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