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PHYSIOLOGY

Enzymes Required for Maltodextrin Catabolism in *Enterococcus faecalis* Exhibit Novel Activities

Philippe Joyet,[a] Abdelhamid Mokhtari,[a,b] Eliette Riboulet-Bisson,[c] Víctor S. Blancato,[d] Martin Espariz,[d] Christian Magni,[d] Axel Hartke,[c] Josef Deutscher,[a,e] Nicolas Sauvageot[c]

Micalis Institute, INRA, AgroParisTech, Université Paris-Saclay, Jouy-en-Josas, France[a]; Department of Biology, 8 May 1945 University, Guelma, Algeria[b]; Normandie University, UNICAEN, U2RM Stress/Virulence, Caen, France[c]; Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina[d]; Centre National de la Recherche Scientifique, UMR8261 (affiliated with Université Paris Diderot, Sorbonne Paris Cité), Expression Génétique Microbienne, Institut de Biologie Physico-Chimique, Paris, France[e]

ABSTRACT Maltose and maltodextrins are formed during the degradation of starch or glycogen. Maltodextrins are composed of a mixture of maltooligosaccharides formed by α-1,4- but also some α-1,6-linked glucosyl residues. The α-1,6-linked glucosyl residues are derived from branching points in the polysaccharides. In *Enterococcus faecalis*, maltotriose is mainly transported and phosphorylated by a phosphoenolpyruvate:carbohydrate phosphotransferase system. The formed maltotriose-6″-phosphate is intracellularly dephosphorylated by a specific phosphatase, MapP. In contrast, maltotetraose and longer maltooligosaccharides up to maltoheptaose are taken up without phosphorylation via the ATP binding cassette transporter MdxEFG-MsmX. We show that the maltose-producing maltodextrin hydrolase MmdH (GenBank accession no. EFT41964) in strain JH2-2 catalyzes the first catabolic step of α-1,4-linked maltooligosaccharides. The purified enzyme converts even-numbered α-1,4-linked maltooligosaccharides (maltotetraose, etc.) into maltose and odd-numbered (maltotriose, etc.) into maltose and glucose. Inactivation of *mmdH* therefore prevents the growth of *E. faecalis* on maltooligosaccharides ranging from maltotriose to maltoheptaose. Surprisingly, MmdH also functions as a maltogenic α-1,6-glucosidase, because it converts the maltotriose isomer isopanose into maltose and glucose. In addition, *E. faecalis* contains a glucose-producing α-1,6-specific maltodextrin hydrolase (GenBank accession no. EFT41963, renamed GmdH). This enzyme converts panose, another maltotriose isomer, into glucose and maltose. A *gmdH* mutant had therefore lost the capacity to grow on panose. The genes *mmdH* and *gmdH* are organized in an operon together with GenBank accession no. EFT41962 (renamed *mmgT*). Purified MmgT transfers glucosyl residues from one α-1,4-linked maltooligosaccharide molecule to another. For example, it catalyzes the disproportionation of maltotriose by transferring a glucosyl residue to another maltotriose molecule, thereby forming maltotetraose and maltose together with a small amount of maltopentaose.

IMPORTANCE The utilization of maltodextrins by *Enterococcus faecalis* has been shown to increase the virulence of this nosocomial pathogen. However, little is known about how this organism catabolizes maltodextrins. We identified two enzymes involved in the metabolism of various α-1,4- and α-1,6-linked maltooligosaccharides. We found that one of them functions as a maltose-producing α-glucosidase with relaxed linkage specificity (α-1,4 and α-1,6) and exo- and endoglucosidase activities. A third enzyme, which resembles amylomaltase, exclusively transfers glucosyl residues from one maltooligosaccharide molecule to another. Similar enzymes are present in numerous other *Firmicutes*, such as

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Address correspondence to Axel Hartke, axel.hartke@unicaen.fr, or Josef Deutscher, Josef.Deutscher@grignon.inra.fr.

P.J. and N.S. contributed equally to this research.

streptococci and lactobacilli, suggesting that these organisms follow the same maltose degradation pathway as *E. faecalis*.

KEYWORDS maltodextrin catabolism, α-1,4-glucosidase, α-1,6-glucosidase, glucosyl transferase, *Enterococcus faecalis*

Numerous bacteria are able to utilize maltose and maltodextrins as carbon and energy sources. Depending on the organism, the uptake of these compounds is catalyzed by one of at least three different transport systems. Maltose and maltodextrins, which are taken up by an ATP binding cassette (ABC) transporter (1) or via the LacY-like MalY permease (2, 3), enter bacterial cells without modification. Nevertheless, their catabolism can largely vary from one organism to another. It usually includes α-glucosidases, which liberate either glucose or maltose from the nonreducing end of maltose or maltooligosaccharides, and maltose or maltodextrin phosphorylases, which use inorganic phosphate to phosphorolyze α-1,4 linkages by liberating glucose-1-phosphate (glucose-1-P) from the nonreducing end of maltose or maltodextrins. Maltose or maltooligosaccharides taken up via the phosphoenolpyruvate:carbohydrate phosphotransferase system (PEP:PTS) arrive in phosphorylated form in bacterial cells (4, 5), and their catabolism therefore requires different sets of enzymes. In most organisms, maltose-6′-phosphate produced during PTS-catalyzed transport is hydrolyzed by a 6-phospho-α-glucosidase (6-P-α-glucosidase) (6, 7).

While the transport of maltose and maltooligosaccharides has been studied in detail in several bacteria, including *Enterococcus faecalis* (8, 9), less is known about their subsequent degradation. In addition, there is no common pathway of maltodextrin catabolism, but depending on the organism, different catabolic routes are used. Maltose and maltodextrin catabolism has been extensively studied in *Escherichia coli* (1). This organism takes up maltose and maltooligosaccharides up to at least maltoheptaose via an ABC transport system. Four different enzymes, a maltodextrin phosphorylase (MalP) (1), a glucogenic α-1,4-glycosidase (MalZ) (10), an amylomaltase (MalQ) (11), and a debranching enzyme (GlgX) (12), are required for their subsequent catabolism.

Maltose and maltodextrin catabolism has also been studied in *Bacillus subtilis*, the model organism of *Firmicutes* (13). This bacterium takes up maltose via a PTS and uses an NAD-dependent 6-P-α-glucosidase to hydrolyze maltose-6′-P produced during PTS-catalyzed transport into glucose-6-phosphate (glucose-6-P) and glucose (6). Maltotriose and higher maltooligosaccharides are taken up by an ABC transporter. *B. subtilis* uses two different α-1,4-glucosidases for the degradation of maltodextrin. The glucogenic α-1,4-glucosidase MalL (YvdL) stepwise liberates glucose from maltooligosaccharides up to maltopentaose (4), whereas the maltogenic α-1,4-glucosidase YvdF liberates maltose preferably from longer maltooligosaccharides (13). MalL also hydrolyzes maltose into two glucose molecules, and *B. subtilis* therefore has no need for an amylomaltase. *B. subtilis* also possesses a debranching enzyme (pullulanase), AmyX, which was proposed to hydrolyze α-1,6 linkages (13).

Enterococci and streptococci transport maltose mainly via a PTS permease, MalT (14), but in contrast to *B. subtilis*, they lack a 6-P-α-glucosidase. Instead, they possess a phosphatase (MapP), which transforms maltose-6′-P formed by PTS-catalyzed transport into maltose (8), which is subsequently phosphorolyzed to glucose and glucose-1-P. *E. faecalis* also transports maltotriose preferably via MalT; intracellular maltotriose-6″-phosphate is subsequently converted to maltotriose by the enzyme MapP (9). However, the enzymes catalyzing the further catabolism of maltotriose and higher maltodextrins, which are taken up via an ABC transporter, were not known. Interestingly, maltodextrin utilization by *E. faecalis* affects the colonization of the gastrointestinal tract (15) and of the liver and kidney (9).

We demonstrate here that two enzymes, the genes of which are located upstream from the maltodextrin ABC transporter operon (9) but oriented in opposite direction (Fig. 1), play a major role in maltodextrin catabolism. One functions as a maltose-

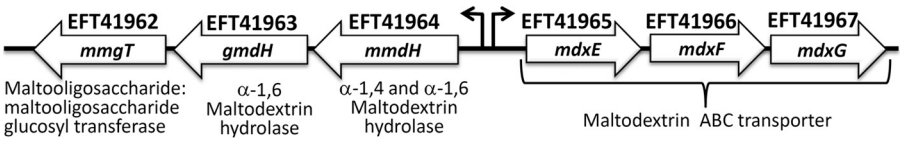

FIG 1 Genetic organization of the *E. faecalis* JH2-2 chromosomal region containing the genes *mmdH*, *gmdH*, and *mmgT*, which encode enzymes involved in maltodextrin catabolism. A second operon located upstream from *mmdH* and transcribed in the opposite direction contains genes which encode three subunits of a maltodextrin-specific ABC transporter. Protein sequences can be searched for with the EFT numbers on the NCBI website (https://www.ncbi.nlm.nih.gov/pubmed).

producing α-1,4- as well as α-1,6-exoglucosidase but also exhibits endoglucosidase activity with cyclodextrins. The other is a glucose-producing α-1,6-glucosidase. A third gene encodes an enzyme, which like *E. coli* MalQ, functions as an amylomaltase but exclusively transfers glucosyl residues; its physiological role remains unknown.

RESULTS

***EFT41964* (*mmdH*) is required for the catabolism of linear α-1,4-linked maltodextrins.** The genes *mmgT* to GenBank accession no. *EFT41964* of *E. faecalis* JH2-2 are located upstream from an operon which encodes an ABC transporter catalyzing the uptake of maltotetraose and longer maltooligosaccharides (Fig. 1) (9). Genes with sequences nearly identical to those of *mmgT* to *EFT41964* are found in all *E. faecalis* strains for which the genome has been sequenced, and they are usually annotated as α-amylase genes. We therefore suspected that they might play a role in maltodextrin metabolism. Compared to growth on glucose and maltose, the wild-type strain grew similarly well on maltotriose but significantly slower on maltotetraose (Fig. 2A) and still slower on longer maltooligosaccharides and maltodextrin (data not shown). We subsequently constructed mutants of each of the three genes and tested the effects of the various mutations on the growth behavior on linear and cyclic α-1,4-linked maltooligosaccharides up to maltoheptaose and on maltodextrin. While the *EFT41964* mutant grew normally on glucose and maltose, it did not grow at all on maltotriose and maltotetraose (Fig. 2B). In contrast to maltotetraose, but similar to maltose (8), maltotriose is taken up and phosphorylated by the PTS permease MalT. Intracellular maltotriose-6″-P is subsequently dephosphorylated to maltotriose by the phosphatase MapP (9), thus making it a substrate for EFT41964. Complementation of the mutant strain with the *EFT41964* wild-type allele expressed from the agmatine-inducible *aguB* promoter restored growth on maltotriose and maltotetraose, confirming that EFT41964 is essential for their catabolism (Fig. 2C). Among the various linear maltooligosaccharides tested, the wild-type strain was not able to utilize the α-1,6-linked isomaltose and isomaltotriose (data not shown).

Purified EFT41964 (MmdH) functions as maltose-producing α-1,4 maltodextrin hydrolase. In order to determine the activity of EFT41964, we purified the His-tagged protein, as described in Materials and Methods, and incubated maltodextrin with the purified enzyme, which resulted in the production of a major compound comigrating with maltose during thin-layer chromatography and a minor product comigrating with glucose (Fig. 3, lane MD). We subsequently incubated the enzyme with maltose and maltooligosaccharides ranging from maltotriose to maltoheptaose. Thin-layer chromatography suggested that EFT41964 transforms even-numbered maltooligosaccharides into only maltose and odd-numbered maltooligosaccharides into maltose and glucose (Fig. 3, lanes G2 to G7). This assumption was confirmed by following the hydrolysis of maltotriose or maltotetraose in a two-step spectrophotometric assay coupled to the NAD-dependent oxidation of glucose, as described in Materials and Methods. When we used maltotriose as the substrate, we first followed the NAD-dependent oxidation of glucose until the reaction reached equilibrium, with almost no further increase of the absorption at 340 nm. We subsequently added *E. faecalis* maltose phosphorylase (8) and observed a second increase, which was due to the oxidation of glucose formed from maltose by the action of maltose phosphorylase (Fig. 4). The glucose dehydro-

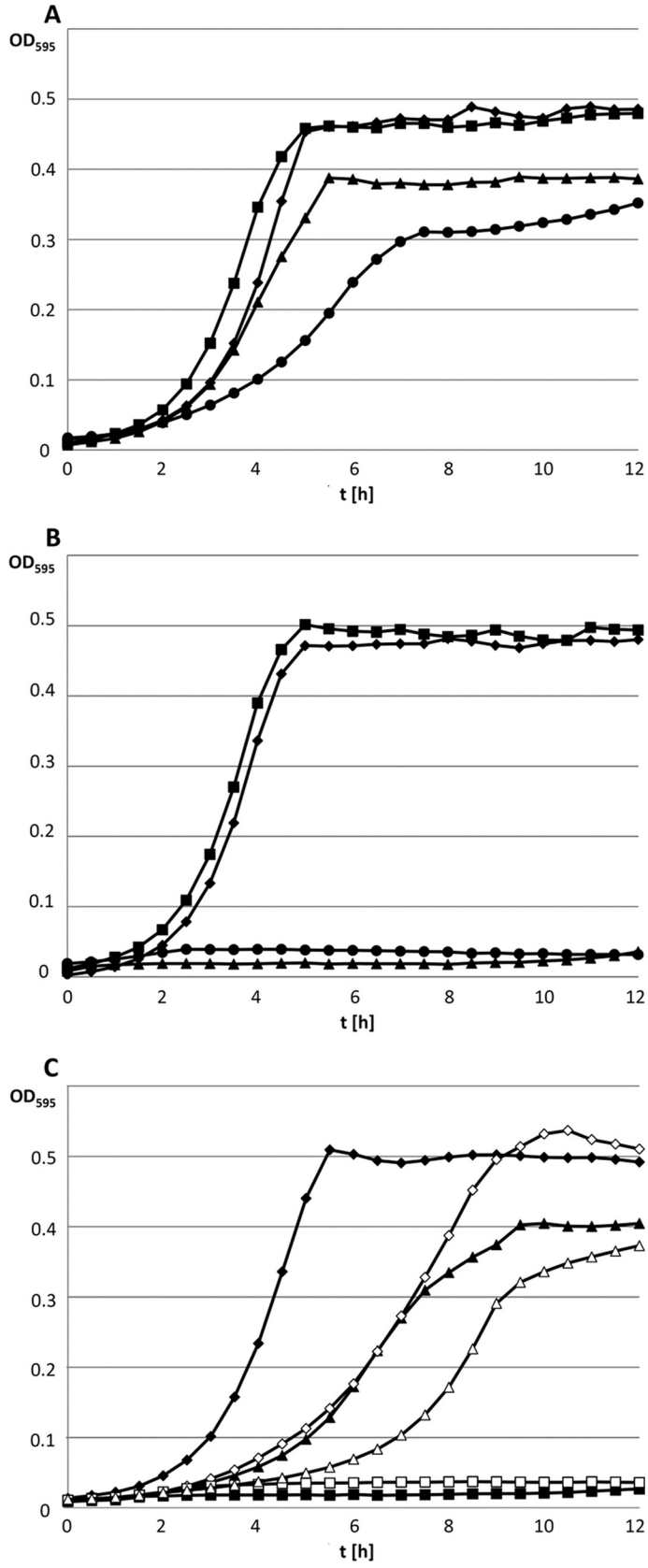

FIG 2 Growth studies with the *E. faecalis* wild-type strain (A), the *EFT41964* (*mmdH*) mutant derived from it (B), and the complemented mutant (C). (A) The *E. faecalis* wild-type strain JH2-2 was grown in M17cc medium supplemented with 0.3% (wt/vol) glucose (◆), maltose (■), maltotriose (▲), or maltotetraose

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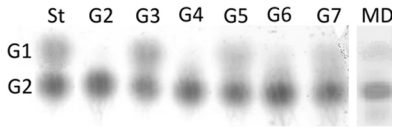

FIG 3 Hydrolysis of maltooligosaccharides by the enzyme EFT41964 (MmdH). Maltose (lane G2) and linear α-1,4-maltooligosaccharides ranging from maltotriose to maltoheptaose (lanes G3 to G7), as well as maltodextrin (MD), were incubated with purified His-tagged EFT41964 at 37°C for 3 h. Aliquots (5 μl) of the reaction mixture were subsequently spotted on silica gel plates, and the reaction products were separated by chromatography, as described in Materials and Methods. After drying, the plate was sprayed with Molisch's reagent (35) in order to visualize the reaction products. Lane St, standards for glucose (G1) and maltose (G2). Lanes G2 to G7 and MD were separated by two different chromatographies.

genase-coupled spectrophotometric assay with maltotriose revealed a K_m of 2.7 mM and a V_{max} of 82 μmol · min^{-1} · mg of protein^{-1}, which corresponds to a k_{cat} (per second) of 91. While a similar K_m value (2.4 mM) was obtained with 4-nitrophenyl-α-maltoside as the substrate, the V_{max} was almost 2-fold higher (176 μmol · min^{-1} · mg of protein^{-1}). In contrast, 4-nitrophenyl-α-glucoside is not a substrate of EFT41964. When the coupled spectrophotometric assay was carried out with maltotetraose, EFT41964 was not able to release glucose from the tetrasaccharide. An increase in the optical density at 340 nm (OD_{340}) was observed only when maltose phosphorylase was added, indicating that EFT41964 forms two molecules of maltose when it hydrolyzes maltotetraose. In addition, only very slight yellow coloration was observed with 4-nitrophenyl-α-maltotrioside, which is probably hydrolyzed to maltose and 4-nitrophenyl-α-glucoside (data not shown). These results unequivocally confirmed that EFT41964 functions as a maltose-producing α-1,4 maltodextrin hydrolase, and we called it MmdH.

MmdH also functions as an endoglucosidase, because it is able to hydrolyze the α-1,4 bonds in α- and β-cyclodextrins. Purified MmdH efficiently produces maltose and a small amount of glucose from α- and β-cyclodextrins (data not shown). The formation of glucose from β-cyclodextrin, which is composed of seven (odd-numbered) glucose residues, was not surprising. However, α-cyclodextrin is composed of six glucose residues and after linearization should have produced only maltose similar to maltohexaose (Fig. 3, lane G6). Why MmdH nevertheless forms glucose is presently unknown. Despite this activity, *E. faecalis* does not grow on β-cyclodextrin and grows only very slowly on α-cyclodextrin. The slow growth on α-cyclodextrin disappeared when *mmdH* was inactivated (data not shown). Interestingly, α- and β-cyclodextrins bind with high affinity to the maltodextrin binding protein of *Lactobacillus casei* and induce the closed active conformation (16). The slow growth of *E. faecalis* on α-cyclodextrin might therefore be due to slow utilization of the cyclic compound, but a slight contamination with linear maltodextrins cannot be excluded.

Interestingly, a *malP* mutant defective in maltose phosphorylase, which in *E. faecalis* is essential for maltose utilization (8, 14), was able to grow on maltotriose, although at a 3-fold-lower rate than the wild-type strain. A likely explanation for this result might be that MmdH hydrolyzes maltotriose to maltose and glucose, and glucose supports the slow growth of the *malP* mutant. The previously reported accumulation of maltose in a *malP* mutant incubated with labeled maltose (8) probably prevents more efficient growth.

FIG 2 Legend (Continued)
(●). Growth was monitored with a BioTek microplate reader by measuring the OD at 595 nm over a time period of 12 h. (B) An identical growth study was carried out with the *EFT41964* (*mmdH*) mutant. (C) The *E. faecalis* wild-type strain JH2-2 transformed with empty plasmid pAGEnt (diamond), the *EFT41964* mutant transformed with the empty pAGEnt (square), and the *EFT41964* mutant transformed with plasmid pAGEnt carrying the *EFT41964* wild-type allele (triangle) were grown in M17cc medium supplemented with 40 mM agmatine and 0.3% (wt/vol) maltotriose (filled symbols) or maltopentaose (open symbols). *t*, time.

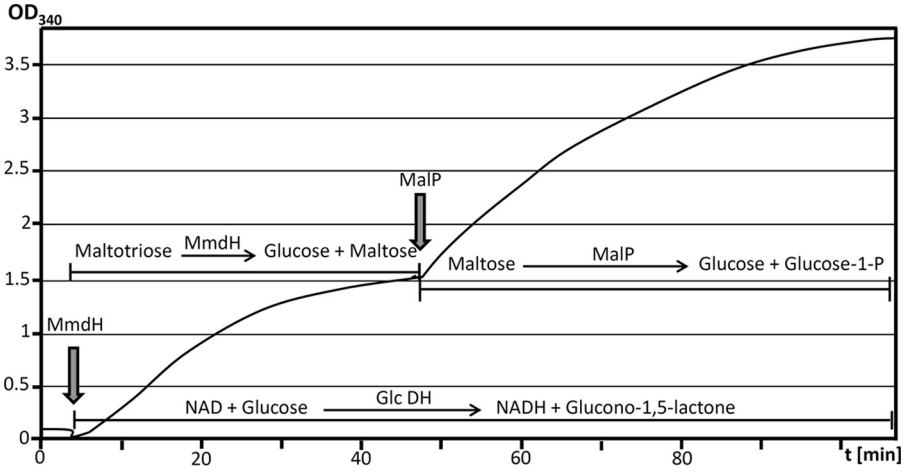

FIG 4 Two-step coupled spectrophotometric assay of maltotriose hydrolysis by the enzyme EFT41964 (MmdH). The assay mixture, which contained maltotriose, NAD^+, and glucose dehydrogenase (Glc DH), was prepared as described in Materials and Methods. After preincubation for about 5 min, the reaction was started by adding EFT41964, and the increase in the OD at 340 nm (NADH formation) was recorded (black curves). In the first step, we followed the NADH-producing oxidation of glucose formed during the EFT41964-catalyzed hydrolysis of maltotriose to maltose and glucose. When the reaction reached equilibrium, which under the employed reaction conditions happened after about 45 min, we added maltose phosphorylase of *E. faecalis*. In the second step, we thus followed the oxidation of glucose formed by the phosphorolysis of maltose to glucose and glucose-1-P. Interestingly, the change in OD_{340} (ΔOD_{340}) of the second step (2.2 absorption units) was significantly larger than the ΔOD_{340} of the first one (1.5 absorption units). The transformation of maltose into glucose and glucose-1-P disturbs the equilibrium reached during the first reaction, and more maltotriose is converted to glucose and maltose, which is probably responsible for the stronger ΔOD_{340} during the second step.

MmdH also functions as maltose-producing α-1,6 maltodextrin hydrolase. Several bacteria were reported to utilize isopanose (17, 18), a linkage isomer of maltotriose, in which the reducing glucose residue is bound via its hydroxyl group at C-6 to the C-1 of the preceding glucose moiety (Fig. 5). In contrast to maltotriose, which is mainly taken up by the PTS permease MalT, isopanose is transported by *E. faecalis* via the MdxEFG-MsmX ABC transporter (9), and the wild-type strain grows on the triose. Interestingly, deletion of *mmdH* prevented growth on isopanose (Fig. 6). However, complementation of the *mmdH* mutant only partially restored growth on maltotriose (Fig. 2C) and did not restore growth at all on isopanose (data not shown). As described below, MmdH hydrolyzes isopanose about 6 times more slowly than maltotriose, which might explain why the complementation allowed slow growth on maltotriose but not on its linkage isomer isopanose.

Depending on whether MmdH hydrolyzes the α-1,4 or α-1,6 linkage in isopanose, glucose and either isomaltose or maltose will be formed, respectively (Fig. 5). Incubation of isopanose in the presence of purified MmdH followed by thin-layer chromatography suggested that similar to maltotriose, isopanose is also hydrolyzed to maltose and glucose and not to glucose and isomaltose (Fig. 7). This result suggested that MmdH hydrolyzes not only α-1,4, but also α-1,6 glucosidic linkages. In order to confirm

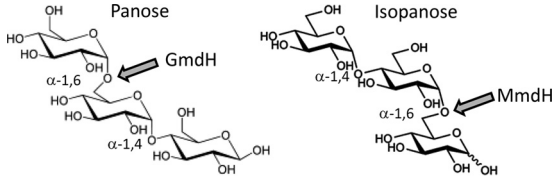

FIG 5 Structure of the maltotriose linkage isomers panose and isopanose. In contrast to maltotriose, which contains only α-1,4 linkages, each of the two isomers contains one α-1,4 and one α-1,6 linkage. The arrows indicate the cleavage sites of panose by GmdH (EFT41963) and of isopanose by MmdH (EFT41964).

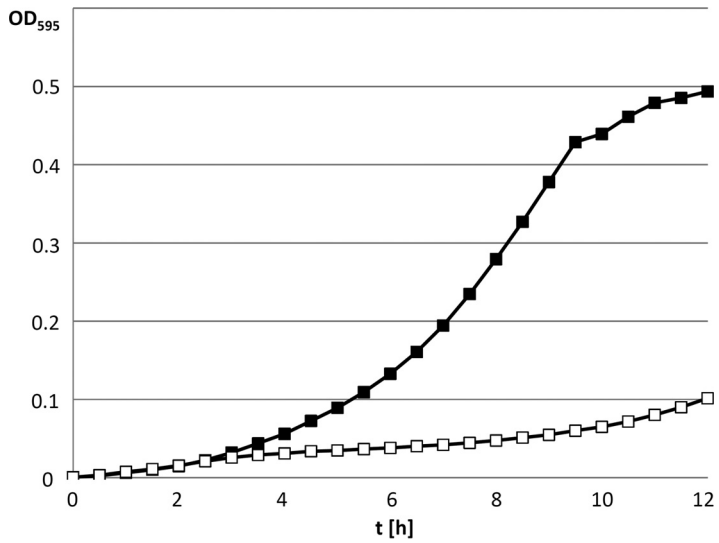

FIG 6 Growth studies with the *E. faecalis* wild-type strain JH2-2 (■) and the *EFT41964* (*mmdH*) mutant derived from it (□) in M17cc medium containing 0.3% panose. The other experimental conditions were the same as those described in the Fig. 2 legend.

this unexpected result, we carried out a coupled two-step spectrophotometric assay. An increase in OD_{340} and therefore glucose oxidation occurred during both steps, similar to that shown in Fig. 4. MmdH must therefore release maltose from the nonreducing end of isopanose and thus function as a maltose-producing α-1,6 maltodextrin hydrolase (Fig. 5). The V_{max} of the hydrolysis of isopanose was about 6-fold slower (13 μmol · min^{-1} · mg of protein^{-1}) and the K_m 2-fold higher (5.9 mM) than the values determined for maltotriose, which is probably partly responsible for the slightly slower growth of *E. faecalis* on isopanose than on maltotriose (compare Fig. 2A and 6).

A spectrophotometric assay revealed that MmdH also hydrolyzes maltotriose-6″-P, the product formed during the PTS-catalyzed transport of the triose, to glucose and probably maltose-6′-P (data not shown). However, the activity was 30-fold slower than with unphosphorylated maltotriose. In contrast, MmdH was not able to hydrolyze isomaltotriose (Fig. 7) or isomaltose (data not shown), which both contain only α-1,6 linkages. As mentioned before, *E. faecalis* is not able to grow on these two oligosaccharides.

EFT41963 (GmdH) functions as glucose-producing α-1,6 maltodextrin hydrolase. Among the five maltotriose linkage isomers (19), *E. faecalis* also utilizes panose (20), in which the first two glucose residues at the nonreducing end are connected

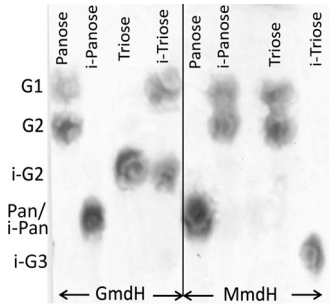

FIG 7 Separation of the hydrolysis products of panose, isopanose (i-panose), maltotriose (triose, G3), and isomaltotriose (i-triose, I-G3) by thin-layer chromatography after incubation with either GmdH or MmdH. The various maltooligosaccharides were incubated with purified His-tagged GmdH or MmdH at 37°C for 3 h. Aliquots (5 μl) of the reaction mixture were subsequently spotted on thin-layer plates (Kieselgel 60; DC-Fertigplatten), and the reaction products were separated by chromatography, as described in Materials and Methods. G1 stands for glucose and G2 for maltose formed during the reactions.

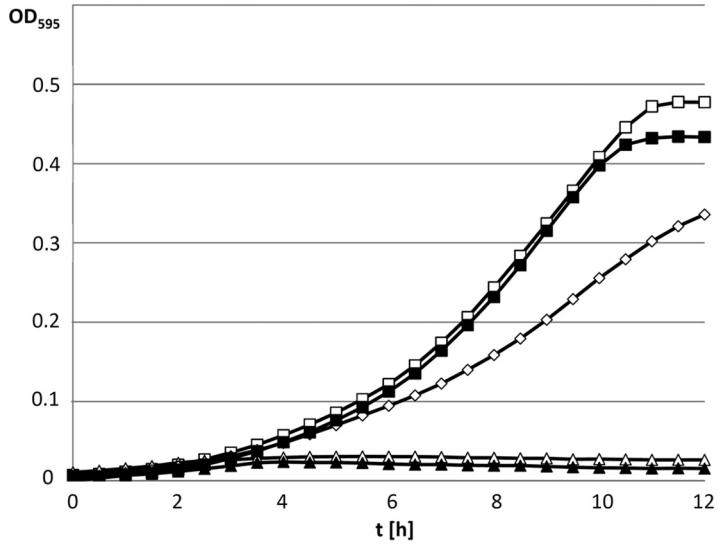

FIG 8 Growth studies with the *E. faecalis* wild-type strain, the *EFT41963* (*gmdH*) mutant derived from it, and the complemented mutant. The *E. faecalis* wild-type strain JH2-2 (■), JH2-2 transformed with empty pAGEnt (□), the *EFT41963* mutant (▲), the *EFT41963* mutant transformed with either empty pAGEnt (△) or with plasmid pAGEnt:gmdH, which contains the *gmdH* allele under the control of the agmatine-inducible *aguB* promoter (◇), were grown in M17cc medium containing 40 mM agmatine and 0.3% panose. The experimental conditions were the same as those described in the Fig. 2 legend.

via an α-1,6 linkage (Fig. 5). Panose is taken up by the maltodextrin ABC transporter (9). In contrast to the wild-type strain, a mutant deleted for GenBank accession no. *EFT41963* was not able to grow on this carbon source (Fig. 8). Complementation of the *EFT41963* mutant with the wild-type gene restored growth on panose, confirming that EFT41963 is involved in the catabolism of the triose.

Depending on whether the α-1,6 or α-1,4 linkage is hydrolyzed, panose will be converted to glucose and either maltose or isomaltose, respectively (Fig. 5). In order to distinguish between the two possibilities, we purified EFT41963, as described in Materials and Methods. Incubation of panose in the presence of purified EFT41963, followed by thin-layer chromatography, suggested that the trisaccharide is hydrolyzed to maltose and glucose, thus indicating that EFT41963 hydrolyzes the α-1,6 linkage (Fig. 7). In order to confirm the presumed α-1,6-glucosidase activity, we carried out a coupled two-step spectrophotometric assay (Fig. 4). As observed for the hydrolysis of isopanose by MmdH, an increase in OD_{340} and therefore glucose oxidation occurred during both steps (data not shown). EFT41963 therefore functions as a glucose-producing α-1,6-maltodextrin hydrolase, and we called it GmdH (for glucogenic maltodextrin hydrolase), because it releases glucose from the nonreducing end of panose (Fig. 5) and probably other maltooligosaccharides beginning with an α-1,6 linkage. The V_{max} of panose hydrolysis by GmdH (12 μmol · min^{-1} · mg of protein^{-1}) and the K_m (6.2 mM) are very similar to the values determined for isopanose hydrolysis by MmdH, which probably explains why, similar to isopanose, panose is also less efficiently utilized by *E. faecalis* than maltotriose (compare Fig. 2A and 6 with Fig. 8).

Thin-layer chromatography indicated that GmdH slowly transforms isomaltose into glucose (data not shown) and isomaltotriose into glucose and isomaltose (Fig. 7), although *E. faecalis* cannot grow on these two compounds. The release of glucose from isomaltose and isomaltotriose by the enzyme GmdH was also confirmed by carrying out the glucose dehydrogenase-coupled spectrophotometric assay (data not shown). The formation of glucose from isomaltotriose was found to be 2.5 times faster than that from isomaltose. In contrast, GmdH was not able to detectably hydrolyze pullulan, a polysaccharide in which maltotriose units are connected via α-1,6 linkages (data not shown).

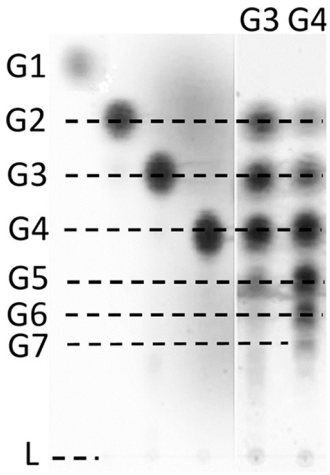

FIG 9 The enzymatic activities of MmgT. The purified His-tagged enzyme MmgT was incubated with either maltotriose (G3) or maltotetraose (G4) at 37°C for 3 h. Aliquots of the reaction mixture were subsequently loaded on silica gel plates, and the reaction products were separated by thin-layer chromatography, as described in Materials and Methods. Reaction products were revealed by spraying the dried thin-layer plate with Molisch's reagent (35). MmgT disproportionated maltotriose into maltose and maltotetraose and some maltopentaose. Similarly, maltotetraose was transformed into maltose and maltotriose, as well as maltopentaose and maltohexaose. Even traces of maltoheptaose were formed under the employed reaction conditions. G1, glucose; G2, maltose; G3 to G7, maltotriose to maltoheptaose, respectively; L, loading line.

EFT41962 (MmgT) functions as maltooligosaccharide:maltooligosaccharide glucosyl transferase. We also inactivated the third gene in the amylase operon but could not attribute a physiological role to the encoded protein, which is also annotated as α-amylase. The resulting mutant grew identically to the wild-type strain on all maltooligosaccharides tested (data not shown). We nevertheless purified MmgT (GenBank accession no. EFT41962), as described in Materials and Methods, and tested whether it might react with any of the maltooligosaccharides utilized by *E. faecalis*. Subsequent thin-layer chromatography revealed that while the enzyme did not react with maltose (data not shown), it disproportionated maltotriose into maltose and maltotetraose together with a small amount of maltopentaose (Fig. 9). The function of the enzyme therefore resembles that of amylomaltases, except that MmgT does not liberate glucose but transfers it to another maltotriose molecule. A similar glucosyl transfer reaction occurred when MmgT was incubated with maltotetraose. Thin-layer chromatography revealed that the tetrasaccharide is converted to a mixture of maltose, maltotriose, maltopentaose, maltohexaose, and even a small amount of maltoheptaose (Fig. 7). Again, no glucose could be detected among the reaction products. In order to confirm the observed combined α-1,4-glucosidase and glucosyltransferase activities of MmgT, we also carried out two-step spectrophotometric assays coupled to glucose dehydrogenase and either maltose phosphorylase or MmdH. When MmgT was incubated with maltotriose, no increase in the OD_{340} occurred, confirming that the enzyme does not liberate glucose from the trisaccharide. However, when maltose phosphorylase was also present in the assay mixture, the OD_{340} increased, confirming that MmgT liberates maltose from maltotriose and transfers the remaining glucose to another maltotriose molecule. When we carried out spectrophotometric assays with maltotetraose, again, MmgT did not release a detectable amount of glucose from the tetrasaccharide (data not shown). By including MmdH in the assay mixture, we could show that MmgT rather liberates maltotriose from maltotetraose probably by hydrolyzing the α-1,4 linkage at the reducing end of the tetrasaccharide (data not shown). The glucosyl residue probably remains bound to the enzyme before it is transferred to another maltotetraose molecule. MmgT did not react with panose and isopanose, the linkage isomers of maltotriose (data not shown), suggesting that the enzyme hydrolyzes only α-1,4-linked linear maltooligosaccharides.

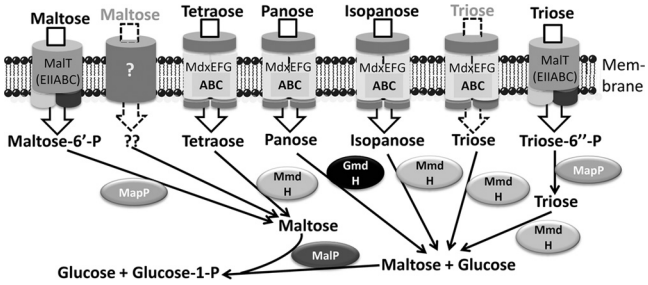

FIG 10 Schematic presentation of the *E. faecalis* uptake systems and main catabolic pathways for maltose, maltotriose (triose), maltotetraose (tetraose), and the maltotriose linkage isomers panose and isopanose. In contrast to maltotetraose and longer maltooligosaccharides, maltose (8) and maltotriose (9) are mainly transported via the PTS permease MalT and therefore arrive in phosphorylated form in the cell. During the first catabolic step, the two phosphocompounds are dephosphorylated by the phosphatase MapP. Maltotriose is also slowly transported by MdxEFG-MsmX and maltose by an unknown transporter (gray letters, dotted arrows, question marks) (9). Unphosphorylated odd-numbered α-1,4-linked linear maltooligosaccharides are hydrolyzed by the enzyme MmdH to glucose and maltose and even-numbered to maltose only. Maltose is subsequently phosphorolyzed to glucose and glucose-1-P by the enzyme maltose phosphorylase. The two linkage isomers of maltotriose, panose and isopanose, which each contain one α-1,6 linkage, are hydrolyzed to glucose and maltose by the glucogenic α-1,6-specific glucosidase GmdH and the maltogenic glucosidase MmdH, respectively.

DISCUSSION

Numerous bacteria utilize maltose and linear α-1,4- and α-1,6-linked maltooligosaccharides. However, their transport and catabolism vary largely from one organism to another. Maltose and maltooligosaccharide uptake can be catalyzed by ABC transporters, PTS permeases, or LacY-like transporters. The use of different systems probably reflects the optimal adaptation of the organisms to the environments in which they are exposed to maltodextrin. The pathogen *Neisseria meningitidis*, which colonizes the nasopharynx in about 15% of the human population, is frequently exposed to high concentrations of maltose and maltooligosaccharides, and uptake by the ion-driven permease MalY might be sufficient to reach high intracellular concentrations (3). Uptake via an ABC transporter (1) and especially via a PTS permease (9) allows the accumulation of maltooligosaccharides and their efficient catabolism even when their extracellular concentration is low, such as in soil. The energetic costs are similar for ion-driven and ABC-catalyzed transport but are lower for PTS-catalyzed uptake, because the substrate arrives already in phosphorylated form in the cell and is primed for further catabolism. The higher energetic transport costs can be compensated in bacteria which use a phosphorylase for the catabolism of maltose and maltodextrins. The same kind of "energy saving" also applies for the special case of enterococci and streptococci, which in the first catabolic step dephosphorylate maltose-6′-P and maltotriose-6″-P produced during PTS-catalyzed uptake (8, 9). Maltose formed directly from maltose-6′-P by MapP or from maltotriose-6″-P by the combined action of MapP and MmdH is subsequently phosphorolyzed to glucose and glucose-1-P (Fig. 10).

Bacterial catabolism of maltose and maltodextrin varies even more strongly than their transport. The most cost-efficient way of maltodextrin catabolism occurs via phosphorolysis, where longer maltodextrins$_{(n)}$ are stepwise phosphorolyzed to maltodextrins$_{(n-1)}$ plus glucose-1-P by using inorganic phosphate as a phosphoryl donor. This mode of catabolism is operative in *E. coli*, where the maltodextrin phosphorylase MalP efficiently phosphorolyzes maltopentaose and longer maltooligosaccharides by liberating glucose-1-P from their nonreducing end. The enzyme is much less efficient with maltotetraose, does not react with maltose and maltotriose, and therefore degrades longer maltooligosaccharides mainly to maltotetraose and a small amount of maltotriose (1). Their further catabolism is achieved by the glucogenic α-1,4-glycosidase MalZ, also called maltodextrin glucosidase, which hydrolyzes maltooligosaccharides by liberating glucose from their reducing end (21), but this enzyme does not recognize maltose (22). In contrast to MalP, the glucose-producing glucosidase is not cost-

efficient, because one ATP has to be hydrolyzed for each glucose molecule liberated by MalZ to transform it to glucose-6-P. The catabolic energy costs will only be low if the ratio of glucose molecules liberated by MalP to MalZ is high.

The combined action of MalP and MalZ leads to the transformation of longer maltodextrins into maltose (10). However, *E. coli* possesses neither a maltose-specific α-1,4-glucosidase nor a phosphorylase able to support growth on maltose. Instead, maltose catabolism in *E. coli* requires the amylomaltase MalQ, which catalyzes the transglycosylation/disproportionation of maltooligosaccharides by removing a glucose residue from the nonreducing end of maltooligosaccharides. While glucose is released and further catabolized, the part of the molecule that remains bound to the enzyme is transferred to a maltose or maltooligosaccharide molecule (11). The possible advantage of *E. coli* using the cumbersome amylomaltase instead of the phosphorylase reaction is not understood.

Streptococcus mutans also uses a glycosyltransferase, MalQ, which, similar to amylomaltase from *E. coli*, removes glucose from maltose and maltotriose and transfers the remaining glucose or maltose residue to another maltooligosaccharide molecule. However, this organism probably spends less energy than *E. coli* for maltooligosaccharide catabolism, because it contains no homologue of the glucogenic α-glucosidase MalZ. It only uses the phosphorylase GlgP, which releases glucose-1-P from maltodextrin and amylodextrin but not from maltose and maltotriose (23).

Although *E. faecalis* contains a maltose phosphorylase, it also possesses an amylomaltase-like enzyme (MmgT) with α-1,4-hydrolase and glucosyl transferase activities. It cuts off glucose residues from one maltooligosaccharide and transfers them to another. In contrast to MalQ of *E. coli* (1, 11) and *S. mutans* (23), MmgT does not react with maltose. In *E. coli* and *S. mutans*, MalQ is essential for the catabolism of maltose because the organisms lack a functional maltose phosphorylase. In contrast, in bacteria which contain a maltose phosphorylase, such as *E. faecalis* and *Clostridium butyricum* (24), MalQ-like proteins react with maltotriose and higher maltooligosaccharides. The physiological function of MmgT is not known, because its deletion had no significant effect on the utilization of the maltooligosaccharides tested in the growth studies.

In organisms which (similar to enterococci and streptococci) use only maltose-producing α-1,4- and α-1,6-glucosidases, the energy costs for the catabolism of maltotetraose and longer maltooligosaccharides are probably slightly lower than in *E. coli*. The combined action of MmdH and MalP leads to the transformation of maltotetraose into two molecules of glucose-1-P and glucose and of maltopentaose into two molecules of glucose-1-P and three glucose molecules. *B. subtilis* probably incurs the highest catabolic energy costs, because the main enzymes of maltodextrin catabolism seem to be a maltogenic α-1,4-glucosidase (YvdF) that liberates maltose from longer maltooligosaccharides (maltopentaose to maltoheptaose) (13), and a glucogenic α-1,4-glucosidase (MalL or YvdL), which stepwise liberates glucose from maltooligosaccharides up to maltopentaose (25). MalL also hydrolyzes maltose into two glucose molecules. Consequently, maltooligosaccharides are entirely converted to glucose molecules, which need to be phosphorylated with ATP. It should be noted that *B. subtilis* contains a presumed maltose phosphorylase, YvdK, of unknown physiological role (4).

E. faecalis contains a debranching enzyme, GmdH, which hydrolyzes α-1,6 linkages by liberating glucose from the nonreducing end, similar to most maltodextrin-utilizing bacteria. Surprisingly, MmdH exhibits both α-1,4- and α-1,6-glucosidase activities. It hydrolyzes α-1,6 linkages by liberating maltose from the nonreducing end. GmdH and MmdH are required for the catabolism of panose and isopanose, respectively, two maltotriose linkage isomers (Fig. 5) taken up by the ABC transporter (Fig. 10). GmdH also liberates glucose from isomaltose and isomaltotriose. Its sequence and substrate specificity strongly resemble those of α-1,6-glucosidases in *B. subtilis* (YcdG), *Bacillus cereus* (26), and *Bacillus coagulans* (27).

In *E. faecalis*, the genes encoding the enzymes required for the transport and catabolism of maltose and maltodextrins are organized in four different operons. The

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Bacterial strains		
Enterococcus faecalis		
JH 2-2	Fus^r Rif^r, plasmid-free maltotetraose-positive wild-type strain	36
mmdH mutant	JH2-2 carrying stop codons at beginning of *mmdH* gene	This study
gmdH mutant	JH2-2 carrying stop codons at beginning of *gmdH* gene	This study
mmgT mutant	JH2-2 carrying stop codons at beginning of *mmgT* gene	This study
JH2-2/pAGEnt	JH2-2 harboring empty pAGEnt	This study
JH2-2*mmdH*/pAGEnt	*mmdH* mutant harboring empty pAGEnt	This study
JH2-2 *mmdH*/pAGEnt:*mmdH*c	*mmdH* mutant carrying pAGEnt containing *mmdH* under the control of the agmatine-inducible *aguB* promoter	This study
JH2-2*gmdH*/pAGEnt:*gmdH*c	*gmdH* mutant carrying pAGEnt containing *gmdH* under the control of the agmatine-inducible *aguB* promoter	This study
Lactococcus lactis		
IL1403	Wild-type strain	37
Escherichia coli		
Top10F′	F′ [*lac*Iq Tn*10* (Tet^r)] *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ*80lacZ*ΔM15Δ *lacX74 recA I araD139* Δ(*ara-leu*) *7697galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
BL21(DE3)	*fhuA2* [*lon*] *ompT gal* (λ *DE3*) [*dcm*] Δ*hsdS* λ *DE3* = λ *sBamHI* Δ*EcoRI-B int*::(*lacI*::*PlacUV5*::*T7 gene1*) *i21* Δ*nin5*	New England BioLabs
Plasmids		
pMAD	pE194^{ts} origin of replication; Erm^r, Amp^r, *bgaB*	31
pAGEnt	5.08 kb; Cm^r *aguR* P*aguB* (promoter inducible with agmatine)	32
pAGEnt:66c	pAGEnt plasmid with *mdxF* gene under control of the agmatine-inducible *aguB* promoter	This study

[a]Fus^r, fusidic acid resistance; Rif^r, rifampin resistance; Tet^r, tetracycline resistance; Str^r, streptomycin resistance; Erm^r, erythromycin resistance; Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance.

regulation of their expression is poorly understood. Only the *malP* operon is required for all substrates. Expression of the *mdx* operon is not needed for maltose and maltotriose utilization, and MmdH from the upstream operon is not required for maltose catabolism. Differential expression of these operons based on multiple regulators and/or inducers is therefore expected.

The genes required for maltose and maltodextrin utilization are usually subjected to catabolite repression. Indeed, the *E. faecalis mdxE* and *mmdH* genes contain potential *cre* sequences, the binding sites of the catabolite control protein A–Ser46-phosphorylated HPr (P-Ser46-HPr) complex (28). Maltooligosaccharide utilization in *Firmicutes* is also subjected to inducer exclusion (29). While in enterobacteria, EIIA^{Glc} interacts with and inhibits MalK, P-Ser46-HPr has recently been reported to interact with MalK1 of *L. casei* (16), and a similar mechanism is probably operative in *E. faecalis*. P-Ser46-HPr-mediated catabolite repression takes about 30 min to become effective, whereas P-Ser46-HPr-requiring inducer exclusion leads to an almost instant stop of maltodextrin uptake (29) and hence formation of the inducer.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. faecalis* strains used in this study are listed in Table 1. Mutants were derived from a maltotetraose-positive clone isolated from our stock of strain JH2-2 (9), which is also called TX4000. *E. faecalis* strains were routinely grown at 37°C without shaking in 100-ml sealed bottles filled with 20 to 50 ml of Luria-Bertani (LB) medium (Difco, NJ, USA) containing 0.5% [wt/vol] glucose. Growth studies were carried out with carbon-depleted M17/MOPS (morpholinepropanesulfonic acid) medium (M17cc), which was prepared as previously described (30). It was supplemented with 0.3% (wt/vol) glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, maltodextrin, panose, or isopanose. Erythromycin and chloramphenicol were added, when appropriate, at concentrations of 150 $\mu g \cdot ml^{-1}$ and 10 $\mu g \cdot ml^{-1}$, respectively.

E. coli strains used for protein purification and cloning experiments were grown aerobically by gyratory shaking at 250 rpm in LB medium at 37°C and transformed by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories). Growth was followed by measuring the absorption at 600 nm in a Novaspec II spectrophotometer.

Construction of *E. faecalis mmgT, gmdH,* and *mmdH* mutants. The *E. faecalis mmgT* (*EFT41962*), *gmdH* (*EFT41963*), and *mmdH* (*EFT41964*) mutants were constructed by introducing two stop codons at the beginning of each open reading frame (ORF) in order to produce small truncated proteins. For amplification of the PCR fragments required for the various genes, we used the primer pairs listed in

TABLE 2 Primers used in this study

Primer	Sequence (5′ to 3′)[a]	Orientation[b]
pMAD_41964for	AAAGTTGGATCCTTTCGGATCTTCGCCATTTTC	+
pMAD_41964_rev	CAAAGGATCCCAGCCATCATTTC	−
mut64stop_for	TCCAACTAGTGCAA**TGA**GATCCTTATC	+
mut64stop_rev	TTGCACTAG**TTA**GACAGAGGCAATGTC	−
pMAD_41963_for	ACGATCCATGGGATTTTTAATACTGGC	+
pMAD_41963_rev	GGCTGGATCCCTTGATAATCCGCAATATC	−
mutEFT41963_for	TTGAACATATGAGC**TAG**GATCAACAACCAGGT	+
mutEFT41963_rev	AGCTCATATG**TTA**AAACTGAAAAATCATAG	−
pMAD_41962_for	GAAGGGATCCCCGCGAATGGGCC	+
pMAD_41962_rev	AAAAGTCGACCAATCGCCTTAGCAGAATGAGAGG	−
mut62stop_for	CGTTGCTAGC**TTA**AAAACTCCGAGGATA	−
mut62stop_rev	TAAAGCTAGC**TAG**GGGGATGGCATTGG	+
RPV_AGEnt_RBS	CATGATGTGTTCCTCCTAAAAG	
FRV_AGEnt_Term	AATTACAGCACGTGTTGCTTT	
pAGEnt_64_for	AAAAACTGCAGATGAACACTGCTGCAATCTAC	+
pAGEnt_64_rev	AAAACTAGTTTATGCTTGATATAAAAGAAAAC	−
pAGEnt_63_for	CTTTTAGGAGGAACACATCATGGAAAAACATTGGTGGC	+
pAGEnt_63_rev	AAAGCAACACGTGCTGTAATTTTATTGACTCACTTTCACAAC	−
D-64-BglII	ACGTAGATCTATGAACACTGCTGCAATCTACC	+
R-64-HindIII	ACGTAAGCTTTTATGCTTGATATAAAAGAAAACC	−
D-63-BamHI	ACGTGGATCCATGGAAAAACATTGGTGGCAAG	+
R-63-HindIII	ACGTAAGCTTTTATTGACTCACTTTCACAAC	−
D-62-BamHI	ACGTGGATCCATGGAACAATGGTGGAAAAATGC	+
R-62-SalI	ACGTGTCGACTTATTGGAAGACGCCTGCCTG	−

[a]Underlined sequences correspond to restriction sites and bold letters indicate stop codons.
[b]+, oriented in the direction of transcription; −, oriented opposite of the direction of transcription.

Table 2. The amplified DNA fragments were cloned into the vector pMAD (31). The resulting plasmids were inserted into the genome of *E. faecalis* strain JH2-2 by double crossing over. For the identification of transformed plasmids, we used the procedure described by Mokhtari et al. (8).

Complementation of the *E. faecalis mmdH* and *gmdH* mutants. For complementation of the *E. faecalis EFT41963* (*gmdH*) and *EFT41964* (*mmdH*) mutants, the corresponding wild-type genes were cloned into the vector pAGEnt (32). This vector contains the gene *aguR*, which encodes the activator of the agmatine operon *aguBDAC*, and the *aguB* promoter. The expression of genes cloned into the multiple-cloning site, which follows the *aguB* promoter in pAGEnt, is therefore induced by agmatine. For complementation of the *EFT41964* (*mmdH*) mutant, the wild-type allele was amplified by PCR, and the resulting product was digested with the restriction enzymes PstI and SpeI and then ligated into the pAGEnt vector cut with the same enzymes. The ligation mixture was used to transform the intermediary host *Lactococcus lactis* strain IL1403. For complementation of the *EFT41963* (*gmdH*) mutant, the required pAGEnt-derived plasmid was constructed with the NEBuilder HiFi DNA assembly cloning kit (New England BioLabs), according to the supplier's recommendation.

Growth studies with wild-type and mutant strains. The media used for the growth studies were inoculated with overnight cultures, which were started from the glycerol stocks kept at −20°C. Each overnight culture was washed once with one volume of physiological water, and the final OD at 600 nm was adjusted to 2.0. A 96-well plate was used, and each well was filled with 200 μl of M17cc medium supplemented with different sugars per well and inoculated with 2 μl of the washed overnight culture. Three drops of paraffin oil were added on top of the medium to avoid desiccation and to create anaerobic conditions. A model 680 microplate reader (Bio-Rad Laboratories) was used, and the temperature was kept at 37°C. The absorption at 595 nm was measured every 30 min over a total period of 12 h. Growth studies under all different conditions tested were carried out at least three times.

Synthesis of maltotriose-6″-P. Maltotriose phosphorylated at the O-6 position of the glucose residue at the nonreducing end was prepared enzymatically using the α-glucoside-specific PTS present in palatinose-grown cells of *Klebsiella pneumoniae* (33).

Purification of His-tagged *E. faecalis* proteins. In order to purify MmdH (EFT41964), GmdH (EFT41963), and MmgT (EFT41962) with an N-terminal His tag, we first amplified the corresponding genes by PCR using *E. faecalis* JH2-2 DNA as the template and the appropriate primer pair (R-62-SalI and D-62-BamHI, R-63-HindIII and D-63-BamHI, or R-64-HindIII and D-64-BglII in Table 2), creating restriction sites at the ends of the amplified DNA. We subsequently cut the amplified DNA fragments with the appropriate restriction enzymes and cloned them into the His tag expression vector pET-18a (Novagen) restricted with the same enzymes. After verification of the correct sequences of the amplified genes, the resulting plasmids were used to transform *E. coli* strain BL21(DE3). A transformant for each amplified gene was subsequently grown in 500 ml of LB medium supplemented with ampicillin until the medium reached an OD_{500} of about 0.5. Expression of the different *E. faecalis* genes was subsequently induced by growing the cells for 3 h at 28°C in the presence of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation, and the preparation of crude extracts and purification of the His-tagged proteins on nickel-nitrilotriacetic acid (Ni-NTA) columns under nondenaturing conditions were carried out as described in reference 34.

Maltose phosphorylase of *E. faecalis* strain JH2-2 is encoded by the gene with GenBank accession no. *EFT41759*. The His-tagged enzyme was overexpressed in *E. coli* from the expression vector pQE30 and purified as previously described (8).

***In vitro* maltooligosaccharide hydrolysis and thin-layer chromatography.** In order to determine the enzymatic activities of the three purified enzymes (MmdH, GmdH, and MmgT), which are all annotated as α-glucosidases, 10 μl of a solution of various maltooligosaccharides (10 μg/ml) in 50 mM Tris-HCl (pH 7.4) was mixed with 10 μl of a solution containing a concentration of 1 μg/ml of one of the enzymes. The assay mixture, which also contained 1 mM $CaCl_2$, was incubated for 3 h at 37°C. Aliquots of 5 μl were spotted on thin-layer plates, and the products formed from the various maltooligosaccharides were subsequently separated by thin-layer chromatography on silica gel glass plates (Kieselgel 60; DC Fertigplatten, Macherey-Nagel, Germany). The mobile phase for the chromatography was composed of 60% propan-1-ol, 10% propan-2-ol, 10% ethanol, and 20% water. After drying, the plates were first sprayed with Molisch's reagent (5% α-naphthol, 95% ethanol) (35) and subsequently with 0.1 N sulfuric acid, which allowed the visualization of mono- and oligosaccharides.

Spectrophotometric enzyme activity assays. In order to test whether the purified *E. faecalis* enzymes form glucose from maltooligosaccharides, we carried out spectrophotometric assays. The detection of glucose was coupled to its NAD^+-dependent oxidation to glucono-1,5-lactone by the enzyme glucose dehydrogenase (Sigma). The assay mixture contained a total volume of 350 μl of 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM NAD^+, maltooligosaccharide at 2.5 mM, and 0.1 mg of glucose dehydrogenase. After preincubation for 5 to 10 min, 2.5 μg of one of the three purified enzymes was added. The formation of NADH was monitored by measuring the OD at 340 nm with a Uvikon 9X3W double-beam spectrophotometer (Kontron Bio-Tek) using the Autorate program.

The formation of maltose from maltooligosaccharides was monitored by coupling the enzymatic reaction to two additional enzymatic steps. First, maltose was phosphorolyzed by purified *E. faecalis* maltose phosphorylase to glucose and glucose-1-P, and glucose was subsequently oxidized by glucose dehydrogenase, as described above. The assay mixture contained a total volume of 350 μl of 100 mM phosphate buffer (pH 7.0), 5 mM $MgCl_2$, 2 mM NAD^+, the maltooligosaccharide at 0.5 mM, 0.1 mg of glucose dehydrogenase, and 20 μg of purified *E. faecalis* maltose phosphorylase (8). After preincubation for 5 to 10 min, the reaction was started with 5 μg of one of the three purified enzymes.

In order to test whether the purified enzymes can transform panose or isopanose into glucose and maltose, we carried out a two-step spectrophotometric assay. In the first step, we followed the formation of glucose, as described above, until the reaction reached equilibrium (after about 45 min under the employed reaction conditions). We subsequently added 25 μg of maltose phosphorylase to the assay mixture. If during the first reaction maltose had been formed simultaneously with glucose, a further increase in the OD at 340 nm should occur, because the phosphorylase will liberate additional glucose from maltose but not from isomaltose.

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We declare no conflicts of interest.

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