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## ***Enterococcus faecalis* utilizes maltose by connecting two incompatible metabolic routes via a novel maltose-6'-phosphate phosphatase (MapP)**

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### **Summary**

Similar to *Bacillus subtilis*, *Enterococcus faecalis* transports and phosphorylates maltose via a phosphoenolpyruvate (PEP):maltose phosphotransferase system (PTS). The maltose-specific PTS permease is encoded by the *malT* gene. However, *E. faecalis* lacks a *malA* gene encoding a 6-phospho- $\alpha$ -glucosidase which in *B. subtilis* hydrolyses maltose-6'-P into glucose and glucose-6-P. Instead, an operon encoding a maltose phosphorylase (MalP), a phosphoglucomutase and a mutarotase starts upstream from *malT*. MalP was suggested to split maltose-6-P into glucose-1-P and glucose-6-P. However, purified MalP phosphorolyses maltose but not maltose-6'-P. We discovered that the gene downstream from *malT* encodes a novel enzyme (MapP) that dephosphorylates maltose-6'-P formed by the PTS. The resulting intracellular maltose is cleaved

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by MalP into glucose and glucose-1-P. Slow uptake of maltose probably via a maltodextrin ABC transporter allows poor growth for the *mapP* but not the *malP* mutant. Synthesis of MapP in a *B. subtilis* mutant accumulating maltose-6'-P restored growth on maltose. MapP catalyzes the dephosphorylation of intracellular maltose-6'-P, and the resulting maltose is converted by the *B. subtilis* maltose phosphorylase into glucose and glucose-1-P. MapP therefore connects PTS-mediated maltose uptake to maltose phosphorylase-catalyzed metabolism. Dephosphorylation assays with a wide variety of phospho-substrates revealed that MapP preferably dephosphorylates disaccharides containing an O- $\alpha$ -glycosyl linkage.

## Keywords

*Enterococcus faecalis*; phosphotransferase system; maltose transport; maltose-6'-P phosphatase; maltose phosphorylase

## Introduction

The breakdown of the two starch-forming polysaccharides amylose and amylopectin, which contain glucose molecules connected by  $\alpha(1\rightarrow4)$  glycosidic bonds, by the enzyme  $\alpha$ -amylase leads to the formation of the disaccharide maltose (4-O- $\alpha$ -D-glucopyranosyl-D-glucopyranose). Starch is produced by all green plants as an energy store and is therefore an abundant carbon source for bacteria. Plants synthesize  $\alpha$ -amylases mainly during maturation of their fruits and germination of their seeds.  $\alpha$ -Amylase activity is also found in the saliva of humans and animals. In addition, many bacteria possess an  $\alpha$ -amylase able to degrade starch. *Escherichia coli* K12 and many other enterobacteria have the capacity to utilize maltose and maltodextrin (Dippel and Boos, 2005), which results from incomplete hydrolysis of starch from various plant origins. Depending upon the degree of hydrolysis maltodextrin contains variable amounts of glucose, maltose, maltotriose and higher maltose oligosaccharides. Most enterobacteria take up maltose and maltodextrins via an ABC transport system composed of the maltose binding protein MalE, two transmembrane proteins MalF and MalG and the ATP binding protein MalK, which provides the energy for maltose uptake by hydrolyzing ATP. The two membrane proteins and the nucleotide binding protein form a MalF/MalG/(MalK)<sub>2</sub> complex, the structure of which has been solved for the *E. coli* proteins (Daus *et al.*, 2009; Gould and Shilton, 2010). Only maltose carried by the maltose binding protein MalE is recognized by the integral membrane proteins and transported into the cell.

Firmicutes can transport maltose by at least four different systems. Firstly, several lactobacilli, such as *Lactobacillus salivarius*, *Lactobacillus fermentum*, *Lactobacillus buchneri* and *Lactobacillus reuteri* use the MalY protein for maltose uptake. This LacY type ion co-transporting maltose permease was first described in the  $\alpha$ -proteobacterium *Caulobacter crescentus* (Lohmiller *et al.*, 2008). Homologous transporters are also present in the  $\beta$ -proteobacterium *Neisseria meningitidis* and in several verrumicrobiae, such as *Opitutus terrae* or *Pedospaera parvula*. In these organisms the permease gene is located in an operon together with the genes encoding homologues of maltose phosphorylase, phosphoglucomutase and sometimes also aldose 1-epimerase (A. Mazé and J. Deutscher, unpublished observation). It is therefore likely that maltose taken up by MalY is phosphorylated by MalP to glucose and glucose-1-P, with the latter being converted into glucose-6-P by the enzyme phosphoglucomutase.

Secondly, similar to enterobacteria, some firmicutes use a single ABC transporter for the efficient uptake of maltose and maltodextrins. This has been reported for *Listeria monocytogenes* (Gopal *et al.*, 2010), *Lactobacillus casei* (Monedero *et al.*, 2008) and

*Lactococcus lactis* (Andersson and Rådström, 2002). The genes encoding the extracellular enzymes required for the degradation of starch are either included in the *malEFG* operon or sometimes located upstream from it and oriented in the opposite direction, as is the case for *L. lactis* (Andersson and Rådström, 2002). Maltose taken up by the ABC transporter is cleaved by maltose phosphorylase (MalP or Map) into glucose-1-P and glucose, which are both converted into glucose-6-P by the enzymes phosphoglucomutase and glucokinase, respectively. In addition to *malP* a gene encoding the enzyme phosphoglucomutase is therefore frequently associated with the *malEFGK* operon (Gopal *et al.*, 2010; Monedero *et al.*, 2008).

Several other firmicutes also contain an ABC transporter, which, however, preferably transports dextrans. Maltose is efficiently transported by these ABC transporters only when present at high concentrations. Such systems have been described for *Bacillus subtilis* (Schönert *et al.*, 2006) (Fig. 1), *Enterococcus faecalis* (Le Breton *et al.*, 2005) and *Streptococcus pyogenes* (Shelburne III *et al.*, 2008). These bacteria use an inducible phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS) as high affinity transporter for maltose.

A functional PTS is usually composed of five proteins or domains, four of which form a phosphorylation cascade. Phosphorylation of the PTS components occurs at His or Cys residues. Enzyme I autophosphorylates at the expense of PEP, and transfers the phosphoryl group to His-15 in HPr. EI and HPr are the common PTS components usually functioning with all sugar-specific PTS of an organism. P~His-HPr phosphorylates one of several sugar-specific EIAs present in a bacterial cell. An EIIB with the same sugar specificity receives the phosphoryl group from EIIA and transfers it to the carbohydrate bound to EIIC, the integral membrane component of the PTS. The phosphorylated carbohydrate is subsequently released into the cytoplasm. Transport and phosphorylation by PTS components are therefore tightly coupled.

Regarding the transport of maltose, two types of PTS can be distinguished. One uses an EIICB<sup>Mal</sup> (called MalP or GlvC), which in *E. coli* functions together with the glucose-specific EIIA (Pikis *et al.*, 2006), whereas in *B. subtilis* the EIIA<sup>Glc</sup> domain of PtsG was not required for maltose uptake (Schönert *et al.*, 2006). In addition to the gene encoding the PTS permease the operon contains a gene coding for a maltose-6'-P hydrolase (MalA, also called GlvA), an NAD<sup>+</sup>/Mn<sup>2+</sup>-dependent 6-P- $\alpha$ -glucosidase (EC 3.2.1.122) (Rajan *et al.*, 2004; Thompson *et al.*, 1998; Yip *et al.*, 2007) that is assigned to the unique Family 4 of the glycoside hydrolase superfamily (Cantarel *et al.*, 2009; Hall *et al.*, 2009) (Fig. 1). Maltose-6'-P formed during PTS-catalyzed transport is hydrolyzed to glucose-6-phosphate and glucose by maltose-6'-P hydrolase. [The 6'-P notation indicates phosphorylation of the C6 hydroxyl group of the non-reducing glucose moiety of the disaccharide]. Such systems have been reported for *Fusobacterium mortiferum* (Robrish *et al.*, 1994; Thompson *et al.*, 1995), *Clostridium acetobutylicum* (Thompson *et al.*, 2004) and *B. subtilis* (Schönert *et al.*, 2006). A BLAST search revealed that identical operons are present in many other bacilli, geobacilli, clostridia, fusobacteria, leptotrichiae, etc. In addition, putative maltose PTS operons composed of genes coding for an EIICB and a maltose-6'-P hydrolase are found in many proteobacteria, such as serratae, pectobacteria, cronobacter and a few *E. coli* strains. Some of these organisms also contain a gene in the operon encoding a distinct EIIA component.

In contrast to the above bacteria, *E. faecalis* JH2-2 (Le Breton *et al.*, 2005), *Streptococcus mutans* (Webb *et al.*, 2007) *S. pyogenes* (Shelburne III *et al.*, 2008) and *Streptococcus pneumoniae* (Bidossi *et al.*, 2012) transport maltose via an EIICBA protein of the glucose subfamily of PTS transporters (MalT). In most enterococci and streptococci *malT*

(EFT41760 in *E. faecalis* JH2-2) is followed by a gene encoding a protein of unknown function (Fig. 1). In contrast with *B. subtilis*, cells of *E. faecalis* and streptococci lack maltose-6'-P hydrolase, which raises questions pertaining to the route for dissimilation of maltose-6'-P in these organisms. Trehalose-6'-P formed during PTS-catalyzed transport by *L. lactis* is hydrolyzed by the trehalose-6'-P phosphorylase (which exhibits significant similarity to maltose phosphorylase) into glucose-1-P and glucose-6-P (Andersson *et al.*, 2001). It was therefore proposed that in *E. faecalis* JH2-2 maltose-6'-P might be similarly hydrolyzed by maltose phosphorylase (MalP, sometimes also called Map) (Le Breton *et al.*, 2005), the gene of which is divergently oriented next to the transporter gene *malT* (Fig. 1). Indeed, growth of *E. faecalis* on maltose leads to the induction of the *mal* operon encoding maltose phosphorylase, phosphoglucomutase and mutarotase. However, trehalose is a symmetric molecule composed of two non-reducing glucose moieties connected by an  $\alpha(1\rightarrow1)$  glycosidic bond, and phosphorolysis of the phosphorylated disaccharide yields glucose-1-P and glucose-6-P (Andersson *et al.*, 2001). In contrast, maltose contains an  $\alpha(1\rightarrow4)$  glycosyl linkage, and phosphorolysis of maltose-6'-P should predictably yield glucose-1,6-bisphosphate and glucose. Alternatively, phosphorolytic attachment of the phosphoryl group to the reducing glucose moiety would generate glucose-6-P and glucose-4-P. However, glucose-4-P is not a known metabolite, and glucose-1,6-bisphosphate serves only as a requisite cofactor in phosphoglucomutase catalysis. Finally, it was conceivable that maltose might not be phosphorylated during PTS transport similar to the PTS-catalyzed uptake of fucosyl- $\alpha(1\rightarrow3)$ -N-acetylglucosamine by *L. casei* (Rodríguez-Díaz *et al.*, 2012), or less likely, that during translocation the disaccharide was phosphorylated at C6 of the reducing glucose moiety. In light of these uncertainties we have conducted an in-depth study, utilizing genetic and biochemical methods, to elucidate the pathway for the metabolism of this disaccharide in *E. faecalis*.

## Results

### MalP exhibits maltose phosphorylase but not maltose-6'-P phosphorylase activity

Strain JH2-2 used in this study is identical to strain TX4000 (Nallapareddy *et al.*, 2002), the genome of which has recently been determined (see NCBI WebSite: [http://www.ncbi.nlm.nih.gov/genome/808?project\\_id=181499](http://www.ncbi.nlm.nih.gov/genome/808?project_id=181499)). In order to facilitate tracking of the genes described in this study we also mention the gene designations of TX4000 (*EFT*....). The operon located upstream from the previously identified *E. faecalis* JH2-2 PTS permease gene *malT* (*EFT41760*) (Le Breton *et al.*, 2005) is oriented in the opposite direction (Fig. 1) and contains four genes. The first gene (*EFT41759*) encodes a protein exhibiting more than 55% sequence identity to maltose phosphorylase from *Lactobacillus casei* (Monedero *et al.*, 2008) and other firmicutes. This operon seems to play an important role in maltose metabolism in *E. faecalis* because biofilm formation by maltose-grown cells was strongly affected when it was deleted (Creti *et al.*, 2006). In order to test whether *EFT41759* was indeed capable of cleaving maltose-6'-P formed during PTS-catalyzed maltose transport, as suggested in a previous study (Le Breton *et al.*, 2005), we cloned the gene into a His-tag expression vector, purified the protein as described in Experimental procedures and carried out spectrophotometric activity assays. We first tested whether the enzyme was able to phosphorolyse maltose, which would lead to the formation of glucose-1-P and glucose. The formation of glucose was detected by using a coupled NADP-requiring spectrophotometric assay as described in Experimental procedures. The formation of NADPH, which in our assay system is directly related to maltose phosphorylase activity, was followed by measuring the change in absorption at 340 nm. A rapid increase in absorption at 340 nm was observed when maltose was used as substrate (data not shown). However, increase in  $A_{340\text{ nm}}$  was negligible when maltose was replaced with maltose-6'-P. Nevertheless, the possible products of the two presumed modes of the maltose-6'-P phosphorolysis reaction

should have been detected with our assay: Transferring the phosphoryl group to the first glucose moiety of maltose-6'-P would have provided glucose-1,6-bisphosphate and glucose and formation of the latter product would have been measured as described above for the reaction with maltose. Attachment of the phosphoryl group to the reducing glucose moiety would have generated glucose-4-P and glucose-6-P. Formation of the latter compound would have been directly detected by the NADP-dependent glucose-6-P dehydrogenase reaction (see Experimental procedures). The *malP* gene therefore appeared to encode a protein with maltose (but not maltose-6'-P) phosphorylase activity (Fig. 1). Maltose metabolism by *E. faecalis* cannot therefore, follow the pathway previously proposed by (Le Breton *et al.*, 2005).

### Inactivation of the maltose phosphorylase-encoding *malP* gene prevents maltose metabolism

To resolve whether maltose phosphorylase participates in the metabolism of maltose taken up via the PTS transporter MalT, we first disrupted the *malP* gene (Fig. 1) and studied the effect of this mutation on maltose uptake and metabolism. First, the wild-type strain JH2-2 and the *malP* mutant, were grown in carbon-depleted M17 medium (Bizzini *et al.*, 2010) supplemented with either maltose or glucose. While the wild-type strain grew equally well on glucose and maltose, the *malP* mutant failed to grow on maltose (Fig. 2A and B). Strain JH2-2 has previously been shown to efficiently transport 50  $\mu$ M [ $^{14}$ C]-maltose via the PTS protein MalT. A *malT* mutant had almost completely lost maltose uptake. The slow [ $^{14}$ C]-maltose uptake observed for the *malT* mutant was suggested to be catalyzed by the presumed maltodextrin ABC transport system MdxEFG (EFT41965-41967) (Fig. 1) (Le Breton *et al.*, 2005). When carrying out identical transport studies with strain JH2-2 we observed that a significant part of the radioactivity associated with maltose was secreted into the medium in form of a negatively charged compound (Table 1). This was determined by passing an aliquot of the transport assay solution after centrifugation over an ion exchange column, which allowed separation of negatively charged compounds from uncharged or positively charged molecules (see Experimental procedures). The majority of the charged radioactive compound(s) is most likely lactic acid produced during maltose fermentation and subsequently secreted into the medium. [ $^{14}$ C]-Maltose transport studies with the *malP* mutant revealed that although it grew very poorly on maltose-containing medium, it accumulated about three times as much radioactivity as the wild-type strain (Fig. 3). Furthermore, in contrast to the wild-type strain the *malP* mutant secreted only a very small amount of charged radioactive compounds into the medium (Table 1). In summary, these results established that MalP plays an important role in the metabolism of maltose taken up via the PTS. While in the wild-type strain about half of the accumulated radioactive compounds (in the cell pellet after centrifugation of the transport assay mixture) were negatively charged, the radioactive compounds accumulated in the *malP* mutant were mainly (98%) uncharged. It was likely that this uncharged intracellular radioactive compound was [ $^{14}$ C]-maltose, because all glycolytic intermediates carry a negative charge. The question therefore arose whether maltose is *not* phosphorylated during its transport by the PTS or whether it is phosphorylated during the transport, but subsequently *dephosphorylated* inside the cells.

### The gene downstream from *malT* encodes a maltose-6'-P phosphatase

We first tested the hypothesis that maltose might be phosphorylated during its transport by the *E. faecalis* PTS and subsequently dephosphorylated inside the cell in order to serve as substrate for MalP. A clue for such a dephosphorylating activity came from transcription assays of the maltose utilization gene clusters in *S. pyogenes*. The homologues of *malT* and its downstream gene *M5005\_spy1691* were found to be cotranscribed in maltose-grown cells (Shelburne III *et al.*, 2008), suggesting that the protein encoded by *M5005\_spy1691*

also plays a role in maltose metabolism. In *E. faecalis* JH2-2 the corresponding gene *EFT41761* (Fig. 1A) encodes a protein annotated as a member of the endonuclease/exonuclease/phosphatase family. We purified this protein after attaching a His-tag to its N-terminus in order to test whether it plays indeed a role in maltose metabolism. By its annotation as an enzyme that putatively may hydrolyse phosphate esters we suspected that this protein might catalyze the dephosphorylation of intracellular maltose-6'-P to maltose. To test this hypothesis we used the same assay system employed for measuring a potential activity of maltose phosphorylase with maltose-6'-P. While no formation of NADPH occurred in the absence of EFT41761, a rapid increase in NADPH was observed when this protein was added to the assay mixture (Fig. 4). NADPH was also not formed when EFT41761 was present but maltose phosphorylase MalP was lacking in the assay mixture (data not shown). This result strongly suggested that EFT41761 dephosphorylates maltose-6'-P to maltose, which is subsequently cleaved by maltose phosphorylase into glucose-1-P and glucose. To unequivocally confirm the phosphatase activity we incubated maltose-6'-P in the presence and absence of EFT41761 and determined the resulting products by mass spectrometry (MS). In the absence of the enzyme we observed two peaks; one corresponding to maltose-6'-P with one Na<sup>+</sup> ion (MW = 445.08), the other corresponding to maltose-6'-P with two Na<sup>+</sup> ions (MW = 467.07). In the sample incubated with EFT41761 these two peaks were no longer detectable and a new peak at 365.07 corresponding to unphosphorylated maltose with one bound Na<sup>+</sup> ion (MW = 343 + 22) had appeared (Fig. 5A). This result unequivocally established that the gene downstream from *malT*, *EFT41761*, encodes an enzyme with maltose-6'-P phosphatase activity. The question mark in Fig. 1A can therefore be replaced with *mapP*.

To measure the kinetic parameters of the enzyme MapP we used a slightly modified coupled spectrophotometric assay with glucose dehydrogenase (see Experimental procedures). The  $K_M$  of MapP was determined to be 4.1 mM and the  $V_{max}$  was found to be 25  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  of protein providing a  $k_{cat}$  of 128  $\text{sec}^{-1}$ .

### MapP preferentially attacks disaccharide phosphates containing an $\alpha$ -glycosidic bond

In order to determine the specificity of the *E. faecalis* MapP enzyme we used commercially available glucose-6-P and fructose-6-P as potential substrates and in addition synthesized a variety of phosphorylated sugars and sugar derivatives (Table 2). The correct structure of these compounds was confirmed by thin-layer chromatography, mass spectrometry and NMR spectroscopy (Thompson, 2001). The phospho-sugars were incubated with MapP and the products of the reaction were analyzed by mass spectrometry. Neither glucose-6-P, fructose-6-P nor  $\beta$ -methylglucoside-6-P, which contain no or only a small aglycon, were dephosphorylated. Remarkably, MapP preferentially dephosphorylates glucopyranosyl-derived disaccharides and heterosides containing an O- $\alpha$ -glycosyl linkage. For example, the five  $\alpha$ -linked-isomers of sucrose (maltulose-6'-P, leucrose-6'-P, trehalulose-6'-P, turanose-6'-P and palatinose-6'-P) were all entirely or significantly dephosphorylated by MapP (Table 2). Surprisingly, sucrose-6-P itself (which contains an  $\alpha(1\leftrightarrow2)\beta$  glycosidic linkage) was not dephosphorylated. Phosphorylated compounds containing an O- $\beta$ -glycosidic bond (cellobiose-6'-P, gentiobiose-6'-P, arbutin-6-P, etc.) were also not dephosphorylated by MapP. An exception was phosphorylated esculin, which was significantly dephosphorylated by MapP. In esculin, glucose is linked via a  $\beta$ -glycosidic linkage to the hydroxyl group in position 6 of the 6,7-dihydroxychromen-2-on (esculetol) molecule.

A question of considerable interest, was whether MapP would distinguish between phosphate groups bound to the first (non-reducing) and second (reducing) glucose moieties of maltose. To this end, doubly phosphorylated maltose was chemically synthesized, such that both glucose moieties carried a phosphoryl group at the C6-position (maltose-6',6-P<sub>2</sub>,

for its formula, see Table 2). The structure and sites of phosphorylation of maltose-6',6-P<sub>2</sub> were confirmed by thin-layer chromatography, mass spectrometry and NMR spectroscopy. Interestingly, only one of the phosphoryl groups was removed by MapP (compare Fig. 5C and D). This must be the phosphate group attached to the 6'-position because if dephosphorylation had occurred at the 6-position of maltose-6',6-P<sub>2</sub>, maltose-6'-P would have been formed. As described above, maltose-6'-P is efficiently dephosphorylated by MapP leading to the formation of maltose, which, however, was not detected as a reaction product (Fig. 5D). In addition, compared to maltose-6'-P, the rate of dephosphorylation of doubly phosphorylated maltose was significantly slower. These results establish that MapP discriminates between phosphoryl groups bound to the 6- and 6'-position of maltose, and that phosphorylation at C6 of the reducing hexose impedes access of substrates to the active site. MapP therefore seems to interact not only with the non-reducing but also with the reducing glucose moiety of maltose.

Because the mass spectrometry experiments were carried out at saturating substrate concentrations (200 mM) we used the malachite green assay (see Experimental procedures) (Baykov *et al.*, 1988) which allows a more quantitative determination of the amount of phosphate liberated from the various phosphorylated substrates by the action of MapP. We used substrate concentrations of 2 mM, which is in the K<sub>M</sub> range for maltose-6'-P. Under these conditions we observed that only turanose-6'-P was similarly efficiently dephosphorylated as maltose-6'-P (85% of the activity of maltose-6'-P) (Table 2). The other four  $\alpha$ -linked-isomers of sucrose were dephosphorylated with an efficiency ranging from 0.9% for palatinose-6'-P to 18.8% for leucrose-6'-P. Dephosphorylation of maltose-6',6-P<sub>2</sub> was about 15-fold slower than dephosphorylation of maltose-6'-P, thus confirming the inhibitory effect of modifications at the reducing sugar moiety of maltose. Finally, esculin-6-P, the only  $\beta$ -glycoside dephosphorylated by MapP, exhibited 0.6% of the activity obtained with maltose-6'-P, indicating that MapP has only weak affinity for this compound but according to the mass spectrometry data can efficiently dephosphorylate it at high concentrations (Table 2). We also tested MapP-catalyzed dephosphorylation with glucose-6-P, fructose-6-P and sucrose-6-P, but in agreement with the mass spectrometry data no activity was observed.

### The *mapP* mutant accumulates maltose-6'-P

To confirm the role of MapP in maltose fermentation, we constructed a *mapP* mutant and tested its ability to utilize glucose or maltose as carbon source. Surprisingly, in glucose-containing carbon-depleted M17 medium the *mapP* mutant grew significantly and reproducibly faster than the wild-type strain (Fig. 2). We have no explanation for this observation. In contrast to the *malP* strain, the *mapP* mutant was able to grow in maltose-containing medium, although at significantly slower rate than the wild-type strain. The *mapP* mutant probably slowly takes up maltose via the maltodextrin ABC transport system, which exhibits low affinity for maltose (Le Breton *et al.*, 2005). Maltose taken up by the ABC transporter can be directly metabolized via MalP (see Fig. 6). MalP is therefore necessary for the metabolism of maltose taken up by *both*, the PTS and the ABC transport system, which explains why the *malP* mutant had lost the capacity to grow on maltose.

We subsequently studied the effect of the *mapP* mutation on maltose transport and metabolism. As expected from the growth experiments, the *mapP* mutant was able to transport [<sup>14</sup>C]-maltose. However, the amount of radioactivity accumulated inside the cells after 5 min incubation with 55  $\mu$ M [<sup>14</sup>C]-maltose was about 10-times higher than in the wild-type strain and 3.5-times higher than in the *malP* mutant (Fig. 3). It should be noted that these results do not reflect the true transport activity, because, as mentioned above, in the wild-type strain a major part of maltose is secreted as charged compounds into the medium. In contrast to the wild-type strain, the *mapP* mutant secreted only a very small



amount of the radioactivity into the medium, which is probably lactate formed from maltose taken up by the maltodextrin ABC transport system and metabolized via MalP. When we used 250  $\mu\text{M}$  [ $^{14}\text{C}$ ]-maltose the uptake via the ABC transport system increased significantly as deduced from the elevated amount of secreted, negatively charged radioactive compounds (data not shown). Most of the radioactivity accumulated by the *mapP* mutant (in the cell pellet after centrifugation of the transport assay mixture) was present in negatively charged form, presumably as maltose-6'-P (Table 1). In order to confirm this assumption we incubated an aliquot of the intracellularly accumulated radioactive compounds with purified MapP. We observed that in the presence of MapP more than 90% of the intracellularly accumulated negatively charged radioactive compounds were converted into uncharged compounds (Table 2), suggesting that the *mapP* mutant accumulates primarily maltose-6'-P when exposed to a maltose-containing growth medium. The lower amount of accumulated radioactive maltose observed for the *malP* mutant compared to *mapP* (Fig. 3) is probably due to the fact that the latter continues to produce energy by taking up maltose via the ABC transporter and metabolizing it via MalP (Fig. 6).

### Heterologous complementation of a *B. subtilis* *malA mdxG* double mutant with *E. faecalis* *mapP*

In order to demonstrate that *E. faecalis* MapP can dephosphorylate maltose-6'-P *in vivo* also in other bacteria we constructed a *B. subtilis* *malA* mutant, which was expected to accumulate maltose-6'-P owing to the loss of the 6-P- $\alpha$ -glucosidase activity (Thompson *et al.*, 1998). We also inactivated the *mdxG* (former *yvdI*) gene, which encodes a membrane component of the maltodextrin-specific ABC transporter (Schönert *et al.*, 2006) in order to prevent low affinity maltose transport via this uptake system. For this purpose we inserted the pMUTIN plasmid (Vagner *et al.*, 1998) into this gene, which caused not only *mdxG* inactivation but also allowed the IPTG-inducible expression of the downstream genes, including *yvdK*, which codes for a protein with high similarity to maltose phosphorylases (Schönert *et al.*, 2006) (Fig. 1). The resulting double mutant had lost the capacity to grow on minimal medium containing maltose as the sole carbon source. We expected that heterologous complementation of this strain with the *E. faecalis* *mapP* gene cloned into the integrative plasmid pAC7 (Weinrauch *et al.*, 1991) would lead to dephosphorylation of the accumulated maltose-6'-P and therefore restore growth on maltose. In order to test this assumption the *E. faecalis* *mapP* gene was fused to the constitutive promoter and the Shine Dalgarno box of the *B. subtilis* *ptsH* gene and inserted into the *amyE* locus of the *malA mdxG* double mutant. When the empty pAC7 plasmid was inserted into this mutant no growth on IPTG-containing maltose minimal medium was observed. Similarly, when the double mutant was complemented with *mapP* and incubated in maltose minimal medium without IPTG no growth occurred after 36 h. Only when IPTG was included, which leads to the expression of the genes downstream from *mdxG*, the *mapP*-complemented *malA mdxG* double mutant was able to grow on maltose-containing minimal medium at about half the growth rate of the wild-type strain (data not shown). These results suggest that MapP converts maltose-6'-P accumulated by the *B. subtilis* *malA* mutant into maltose, which is subsequently converted into glucose-1-P and glucose by the maltose phosphorylase YvdK. In agreement with this concept, the absence of MapP or the absence of induction of the *yvdK* gene prevented growth of the *malA mdxG* double mutant on maltose minimal medium.

## Discussion

We established in this study that the uptake of maltose by a PTS and its subsequent metabolism in *E. faecalis* follow a quite unusual mechanism. As outlined in Fig. 6, maltose transported by the PTS enters the cells as maltose-6'-P. It is subsequently dephosphorylated

to maltose inside the cells by the maltose-6'-P phosphatase MapP. By using inorganic phosphate, the maltose phosphorylase MalP phosphorylates the resulting intracellular maltose into glucose and glucose-1-P, which are both converted into the glycolytic metabolite glucose-6-P. MapP is a member of the endonuclease/exonuclease/phosphatase family, which includes enzymes with quite different activities, such as DNase I or synaptojanin, a phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase (Woscholski *et al.*, 1997). The kinetic parameters determined for MapP ( $K_M = 4.1$  mM,  $V_{max} = 25$   $\mu\text{mol min}^{-1}$   $\text{mg}^{-1}$  protein;  $k_{cat} = 128$   $\text{sec}^{-1}$ ) are within the usual range of catabolic enzymes.

The presence of the two genes coding for the PTS permease MalT and the maltose-6'-P phosphatase MapP and their identical arrangement in most enterococci and streptococci, such as *S. mutans* (Webb *et al.*, 2007), *S. pyogenes* (Shelburne III *et al.*, 2008) and *S. pneumoniae* (Bidossi *et al.*, 2012) suggest that this kind of maltose utilization is a general phenomenon in these bacterial species. In some streptococci, such as *Streptococcus gallolyticus* and *Streptococcus gordonii*, MapP was called RgfB (regulator of fibrinogen binding). In *S. agalactiae*, *rgfB* is also located downstream from *malT*. Nevertheless, *rgfB* was reported to be co-transcribed with genes encoding a two component sensor kinase and response regulator (Spellerberg *et al.*, 2002). Inactivation of the histidine kinase RgfC affected fibrinogen binding, but it is not known whether alterations in *rgfB* (*mapP*) expression are responsible for this phenotype. Several bacillales (*Macrococcus caseolyticus*, *Paenibacillus polymyxa*, *Bacillus cereus*, etc.) and clostridiales (*Catonella morbi*, *Clostridium botulinum*, *Clostridium perfringens*, etc.) also possess homologues of MalT and MapP. In these organisms, a PTS permease of the glucose subfamily was probably adapted to transport maltose. Indeed, MalT resembles more the glucose-specific PTS transporter PtsG of *B. subtilis* than the maltose-specific MalP. The PTS-mediated uptake of maltose leads to the formation of intracellular maltose-6'-P, which in other bacteria is hydrolyzed into glucose and glucose-6-P by the  $\text{NAD}^+/\text{Mn}^{2+}$ -dependent 6-P- $\alpha$ -glucosidase. Enterococci and streptococci generally lack the maltose-6'-P hydrolase enzyme. Instead enterococci contain upstream from the *malT* gene, which encodes the maltose-specific PTS transporter, an operon encoding three enzymes for the catabolism of maltose taken up without phosphorylation by ABC transporters or ion-symporting permeases (Le Breton *et al.*, 2005). In fact, in several organisms containing a LacY-like maltose permease MalY (Lohmiller *et al.*, 2008) the transporter gene *malY* is followed by the three genes *malP-pgcM-malM* encoding maltose phosphorylase, phosphoglucomutase and mutarotase (A. Mazé and J. Deutscher, unpublished observation). It is therefore tempting to assume that enterococci and streptococci acquired these genes from one of the organisms using MalY for maltose transport. These organisms therefore seem to synthesize a protein for maltose transport (MalT, produces maltose-6'-P), which at the first glance does not seem to be connected to a catabolic route (MalP uses only maltose as substrate). In order to endow enterococci and all other bacteria possessing a *malT* homologue with the capacity to catabolize maltose-6'-P formed during PTS-catalyzed maltose transport, these organisms needed an additional enzyme connecting the PTS uptake route to the maltose phosphorylase-mediated catabolic pathway. For that purpose these bacteria probably acquired the gene located downstream from *malT*, which encodes an enzyme belonging to the large endonuclease/exonuclease/phosphatase family. During the course of evolution it was probably optimized for the dephosphorylation of phosphorylated  $\alpha$ -glucosides, such as maltose-6'-P.

Acquiring a maltose-6'-P phosphatase provided probably an optimal connection between PTS-mediated maltose uptake, and maltose phosphorylase-catalyzed metabolism. Importantly, the number of ATP molecules formed from maltose taken up via an ABC transport system and phosphorylated by maltose phosphorylase, or transported via a PTS and hydrolyzed by a 6-P- $\alpha$ -glucosidase, is principally identical to that obtained by the MapP-catalyzed metabolism of maltose-6'-P formed during MalT-catalyzed transport.

Although MapP dephosphorylates intracellular maltose-6'-P in the first catabolic step by producing Pi and maltose, MalP subsequently uses Pi for the phosphorolysis of the resulting maltose in order to form glucose-1-P and glucose. The enzyme phosphoglucomutase encoded by the gene EFT41758 (Fig. 1A) converts glucose-1-P into glucose-6-P. Glucose and glucose-6-P are also formed when *B. subtilis* MalA hydrolyzes maltose-6'-P taken up via the PTS permease MalP (Fig. 1B). The number of ATP and PEP molecules used for maltose transport and the catabolic intermediates are therefore identical for all three different modes of maltose transport and metabolism.

## Experimental procedures

### Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 4. *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 medium (LB; Difco, New Jersey, USA) containing 0.5% w/v glucose. Growth curves were obtained with carbon-depleted M17MOPS medium, which was prepared as previously described (Bizzini *et al.*, 2010). It was supplemented with either 0.5% w/v glucose or 0.5% w/v maltose. Erythromycin and tetracycline were added when appropriate at concentrations of 150 µg ml<sup>-1</sup> and 5 µg ml<sup>-1</sup>, respectively.

*B. subtilis* strains were grown aerobically at 37°C in LB medium or in minimal salts medium C containing 0.005% w/v tryptophan (Darbon *et al.*, 2002), and supplemented with 0.5% w/v glucose or 0.5% w/v maltose, as indicated. Antibiotics were used at the following concentrations: 5 µg ml<sup>-1</sup> kanamycin and 0.3 µg ml<sup>-1</sup> erythromycin. Conventional transformation of *B. subtilis* was performed by following a published procedure (Anagnostopoulos and Spizizen, 1961). *E. coli* strains were grown aerobically in LB medium at 37 °C and transformed by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories). Aerobic growth of *B. subtilis* and *E. coli* strains was achieved by gyratory shaking at 250 rpm, and was monitored at A<sub>600nm</sub> in a Novaspec II spectrophotometer.

### Purification of His-tagged *E. faecalis* maltose phosphorylase and maltose-6-P phosphatase

In order to purify maltose phosphorylase and maltose-6-P phosphatase the corresponding genes *malP* and *mapP* were amplified by PCR using chromosomal *E. faecalis* JH2-2 DNA as template and the two primer pairs EfMalP\_Up/EfMalP\_Lo and Ef0960BamF/Ef0960SalR, respectively (Table 5). The amplicons were cut with the appropriate restriction enzymes (BamHI/KpnI or BamHI/SalI) and cloned into the His-tag expression vector pQE30 cut with the same enzymes. The correct sequence of the two genes was confirmed by DNA sequencing and purification of the His-tagged proteins was carried out as previously described (Martin-Verstraete *et al.*, 1999).

### Spectrophotometric assays of maltose phosphorylase and maltose-6'-P phosphatase activities

Maltose phosphorylase activity was determined by using a coupled spectrophotometric assay, in which glucose formed from maltose by maltose phosphorylase was converted with ATP and hexokinase into glucose-6-P, which was subsequently oxidized to gluconate-6-P in an NADP-requiring reaction catalyzed by glucose-6-P dehydrogenase. In order to determine the amount of glucose we used the glucose assay reagent of Sigma-Aldrich (30 mg ml<sup>-1</sup>) and followed the formation of NADPH by measuring the A<sub>340nm</sub> with a Kontron Bio-Tek spectrophotometer using the autorate program. The assay mixture contained 500 µl glucose assay reagent, 500 µl 50 mM phosphate buffer, pH 7.0, 10 mM MgCl<sub>2</sub>, 1 mM maltose and 20 µl maltose phosphorylase (1.2 mg ml<sup>-1</sup>). When maltose was replaced with maltose-6'-P

in the assay mixture no increase of the absorption at 340 nm was observed. However, when 40  $\mu$ l maltose-6'-P phosphatase (MapP, 0.8 mg ml<sup>-1</sup>) was added to the assay mixture containing maltose-6'-P an increase of the absorption at 340 nm similar to that detected with maltose and maltose phosphorylase was observed.

To determine the  $k_{cat}$  and  $K_M$  values of Map we slightly modified the assay conditions by replacing the glucose assay kit (enzymes hexokinase and glucose-6-P dehydrogenase) with 5  $\mu$ g of NADH-dependent glucose dehydrogenase (Sigma). The assay mixture therefore contained 30 mM NADH in a total volume of 450  $\mu$ l. The concentration of maltose-6'-P varied from 0.22 to 30 mM. All other conditions were as described above. This assay system reacted more quickly to the formation of glucose and therefore allowed a more accurate determination of the kinetic parameters of MapP.

### Construction of an *E. faecalis malP* defective strain

A mutant defective in maltose phosphorylase was constructed by interrupting the *malP* gene by single recombination using the thermosensitive vector pGhost8 (Maguin *et al.*, 1996). An internal fragment of the *malP* gene was amplified by PCR using chromosomal DNA of *E. faecalis* JH2-2 as template and the primer pair EfMalPmut\_U/EfMalPmut\_L. The amplicon was digested with appropriate restriction enzymes (HindIII and EcoRI) and ligated into pGhost8 cut with the same enzymes. The resulting plasmid was named pGh-malP and used to transform *E. coli* EC101 (Table 4). Purified pGh-malP was electroporated into *E. faecalis* JH2-2 by following a previously described method (Friesenegger *et al.*, 1991). Plasmid integration was induced as previously described (Maguin *et al.*, 1996). Briefly, transformants are grown overnight at the permissive temperature (30°C) in LB medium containing glucose and 5  $\mu$ g ml<sup>-1</sup> tetracycline for plasmid maintenance before an aliquot of the culture was used to inoculate fresh medium (1:500-fold dilution) which was incubated at the restrictive temperature (37°C) thus preventing plasmid replication. When the culture reached an OD<sub>600</sub> of 0.5, serial dilutions were plated on solid LB medium containing glucose and the antibiotic and incubated at 37°C. A single clone was isolated and the interruption of *malP* was confirmed by its maltose-negative phenotype and by PCR. The *malP*-deficient strain was called JH-malP (Table 4).

### Construction of an *E. faecalis mapP* mutant

A mutant deficient in maltose-6'-P phosphatase was constructed by interrupting the *E. faecalis mapP* gene by single recombination using the suicide vector pUCB30bis (Benachour *et al.*, 2007) (Table 4). An internal fragment of *mapP* was amplified by using chromosomal DNA of *E. faecalis* JH2-2 as template and the primer pair MapPBamF/MapPEcoR (Table 5). The amplicon was digested with the restriction enzymes BamHI and EcoRI and ligated into pUCB30bis cut with the same enzymes. The purified plasmid was used to transform *E. faecalis* JH2-2 by electroporation (Friesenegger *et al.*, 1991) and an erythromycin-resistant clone was isolated and called AB01 (Table 4). The correct integration of the plasmid was verified by PCR by using oligo EfmapPconf in combination with a vector-based primer (Table 5).

### Maltose transport studies with [<sup>14</sup>C]-maltose

Transport studies with [<sup>14</sup>C]-maltose were carried out as previously described (Viana *et al.*, 2000). Cells of the *E. faecalis* wild-type strain JH2-2 and the *malP* and *mapP* mutants derived from it were grown in 25 ml LB medium containing 0.5% maltose to an OD<sub>600</sub> of 0.5. Cells were centrifuged and washed twice with 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM MgCl<sub>2</sub>. The cells were resuspended in such a volume of 50 mM Tris-maleate buffer, pH 7.2, containing 5 mM MgCl<sub>2</sub> that the cell suspension exhibited an OD<sub>600</sub> of 10. For maltose transport assays 50  $\mu$ l of the cell suspension was diluted with 0.5 ml

transport buffer (resuspension buffer containing 1% peptone) and preincubated 1 min at 37°C. The transport reaction was started by adding 60 µl of a [<sup>14</sup>C]-maltose solution composed of 54 µl 0.5 mM maltose plus 6 µl [<sup>14</sup>C]-maltose (0.1 mCi ml<sup>-1</sup>) (corresponds to 1.33 × 10<sup>6</sup> cpm). The final maltose concentration in the assay mixture was calculated to be 55 µM. Aliquots of 100 µl were withdrawn after 0.25, 0.75, 1.5, 2.5 and 5 min and mixed with 5 ml of cold resuspension buffer before they were filtered through 0.45 µm pore-size filters (Schleicher und Schuell, Dassel, Germany) and washed twice with 5 ml resuspension buffer. The filters were dried and the radioactivity retained was determined by liquid scintillation counting.

### Isolation and characterization of negatively charged radioactive compounds accumulated during [<sup>14</sup>C]-maltose uptake

*E. faecalis* wild-type and mutant cells were grown as described above and a 275 µl assay mixture for [<sup>14</sup>C]-maltose uptake was prepared for each strain as described above. The cells were incubated for 5 min in the presence of [<sup>14</sup>C]-maltose before a 100 µl aliquot was withdrawn and filtered through a 0.45 µm pore-size filter. Another 60 µl aliquot was rapidly centrifuged and supernatant and cell pellet were separated. The cell pellet was resuspended in 100 µl boiling water to obtain a cell extract. The supernatants and the cell extracts were loaded on 1 ml Dowex AG1-X8 anion exchange columns which were washed three times with 2 ml of water in order to remove uncharged or positively charged compounds. The flow through and the wash fractions were collected, combined and the radioactivity was determined by liquid scintillation counting. Negatively charged compounds were subsequently eluted with three times 1.5 ml 1 M LiCl and the radioactivity was determined by liquid scintillation counting.

In order to test whether treating the negatively charged radioactive compounds accumulated by the *mapP* mutant with maltose-6'-P phosphatase would convert part of them into uncharged compounds strain *mapP* was allowed to take up [<sup>14</sup>C]-maltose before a cell extract was prepared as described above. A 180 µl aliquot of the transport assay was withdrawn, centrifuged and the cells were desintegrated in 300 µl of boiling water. To one 100 µl aliquot of cell extract 50 µl Tris/HCl, pH 7.4 and 20 µl 100 mM MgCl<sub>2</sub> were added, whereas to the second 100 µl aliquot 50 µl of purified MapP dissolved in 50 µl Tris/HCl, pH 7.4 (0.8 mg ml<sup>-1</sup>) was added. The samples were incubated for 1 h at 37°C before charged and uncharged radioactive compounds were determined as described above.

### Synthesis of phosphorylated sugars and sugar derivatives

Phosphorylated O- $\alpha$ -linked disaccharides, including maltose-6'-phosphate, were prepared enzymatically via the  $\alpha$ -glucoside specific PTS present in palatinose-grown cells of *Klebsiella pneumoniae* (Thompson, 2001). Phosphorylation of the primary hydroxyl (C6') group of the non-reducing glucose moiety in O- $\beta$ -linked disaccharides was as previously described (Thompson *et al.*, 2002). In brief, phosphorylation was effected by incubation of the disaccharides with ATP-dependent  $\beta$ -glucoside kinase (BglK, EC 2.7.1.85) from *K. pneumoniae*. Phosphorylated derivatives of both  $\alpha$ - and  $\beta$ -linked disaccharides were isolated by ethanol and Ba<sup>2+</sup> precipitation, and further purified by ion-exchange and paper chromatography. Structures and product purity were confirmed by thin-layer chromatography, mass spectrometry and NMR spectroscopy. The chromogenic analog, p-nitrophenyl- $\alpha$ -D-glucopyranoside 6-phosphate (pNP $\alpha$ G6P) was prepared by phosphorylation of the C6 hydroxyl moiety of pNP- $\alpha$ -D-glucopyranoside with phosphorus oxychloride in trimethyl phosphate containing a small amount of water (Thompson *et al.*, 1995). Treatment of maltose under the latter conditions, causes non-selective phosphorylation at both C6 and C6' primary hydroxyl groups of the disaccharide, producing a mixture of maltose-6'6-diphosphate (maltose-6',6-P<sub>2</sub>), maltose-6'P and maltose-6P.

Maltose-6',6-P<sub>2</sub> was separated from the two mono-phosphate derivatives by gradient elution (0 – 0.5 M NH<sub>4</sub>HCO<sub>3</sub>) from a column of AG1-X8 (formate-form) ion exchange resin. Bicarbonate was removed by lyophilization, and maltose-6',6-P<sub>2</sub> was purified as described above. Its correct structure was confirmed by thin-layer chromatography, mass spectrometry and NMR spectroscopy.

### Mass spectrometric analysis of phosphorylated compounds treated with MapP

Solutions containing the various phosphorylated sugars and sugar derivatives listed in Table 2 at a concentration of 2 M were prepared. 5 µl aliquots of these solutions were added to either 40 µl of purified MapP (0.8 mg ml<sup>-1</sup>, dialyzed against 20 mM ammonium bicarbonate) or to 40 µl of 20 mM ammonium bicarbonate and incubated for 2 h at 37°C. The samples were subsequently lyophilized and rehydrated with 10 µl of water. Aliquots of 1 µl were mixed with 9 µl of a sugar matrix solution and 1 µl was spotted onto the MALDI steel plate. The sugar matrix was freshly prepared and contained 100 µg/ml 2,5-dihydroxybenzoic acid dissolved in a mixture of H<sub>2</sub>O/acetonitrile/N,N-Dimethylaniline (1:1:0.02 vol/vol). The samples were analyzed by MALDI-TOF MS (Voyager DE super STR, AB SCIEX) by irradiating them with a nitrogen laser (337 nm, 10 Hz) integrated in this instrument and recording mass spectra in the reflectron mode using a delay extraction time of 120 ns and a m/z mass range between 200 and 600 Da.

### Detection of MapP-catalyzed formation of phosphate with the malachite green assay

To follow MapP-catalyzed dephosphorylation of the different phospho-compounds over various time periods we used the malachite green assay (Baykov *et al.*, 1988). Dephosphorylation experiments were carried out in 650 µl assay mixtures containing 50 mM Tris/HCl, pH 7.4, 5 mM MgCl<sub>2</sub> and 2 mM of the phosphorylated compounds. The reaction was started by adding MapP and the samples were incubated for various time periods at 37°C. For each phospho-compound the amount of MapP (ranging from 40 µg to 4 mg MapP) was adjusted in such a way that it led to detectable formation of phosphate within 6 min incubation. Aliquots of 100 µl were withdrawn after 0, 1.5, 3, 6, 10 and 20 min incubation and immediately mixed with 25 µl of the malachite green reagent (Baykov *et al.*, 1988). The samples were kept for 20 min at ambient temperature before 375 µl of water was added and the OD<sub>630</sub> determined. In Table 2, the activity measured with the various phospho-compounds is expressed relative to the activity measured with maltose-6'-P, which was set to 100%.

### Construction of a *B. subtilis* *malA* deletion mutant

In order to construct a *malA* mutant we amplified by PCR a 2150 bp fragment covering the entire *malA* gene and upstream and downstream regions by using *B. subtilis* DNA as template and the primer pair malAForBam and malARevKpn (Table 5). The amplicon was cut with BamHI and KpnI and cloned into pUC18 cut with the same enzymes. The resulting plasmid was digested with the restriction enzyme ClaI, which led to the deletion of a 328 bp fragment in the middle of *malA* and also introduced a frame shift. The shortened insert of pUC18 was cut out with BamHI and KpnI and cloned into the vector pMAD (Arnaud *et al.*, 2004) digested with the same enzymes. The resulting plasmid was used to transform *B. subtilis* wild-type strain 168. The plasmid pMAD contains a temperature-sensitive pE194 origin of replication. In order to obtain a *malA* mutant by double cross over we followed a previously described protocol (Aké *et al.*, 2011). The correct deletion and the insertion of a frame shift mutation in the *malA* gene in one of the isolated strains were confirmed by carrying out a PCR amplification with the primer pair VerifMalAFor and VerifMalARev (Table 5), which led to the amplification of a 589 bp fragment instead of the 1012 bp fragment obtained for the wild-type strain 168. This strain was called AM01.

### Construction of a *B. subtilis mala mdxG* double mutant

In order to inactivate one of the membrane components of the *B. subtilis* maltodextrin transporter, which also catalyzes slow uptake of maltose (Schönert *et al.*, 2006), an internal DNA fragment of the *mdxG* gene was amplified by PCR with primers mdxGForHind and mdxGRevBam. The amplicon was digested with HindIII and BamHI and cloned into pMUTIN4 (Vagner *et al.*, 1998) cut with the same enzymes thus providing pMUTIN-mdxG. *B. subtilis* AM01 was transformed with this plasmid to obtain strain AM02 (Table 4), in which transcription of the genes downstream from the disrupted *mdxG* is under control of the IPTG-inducible *Pspac* promoter. Proper integration of the plasmid was confirmed by PCR.

### Complementation of the *B. subtilis mala mdxG* mutant with *E. faecalis mapP*

The *B. subtilis ptsH* promoter and Shine Dalgarno box were amplified with oligos ptsHPromForEco and ptsHPromRevBam and cloned into the EcoRI/BamHI sites of pAC7 providing pAC7-PptsH. Subsequently, the *E. faecalis mapP* gene was amplified using primers mapPForBam and mapPRevSac and cloned into the BamHI/SacI sites of pAC7-PptsH. In the resulting plasmid pAC7-PptsH-mapP (Table 4) the *mapP* gene is expressed under control of the constitutive *ptsH* promoter. It was inserted into the *amyE* locus of the *B. subtilis* 168 chromosome. Kanamycin-resistant integrants resulting from homologous recombination were selected. Integration into the *amyE* locus was confirmed by an amylase-negative phenotype of cells plated on LB agar containing soluble starch (Harwood and Cutting, 1980).

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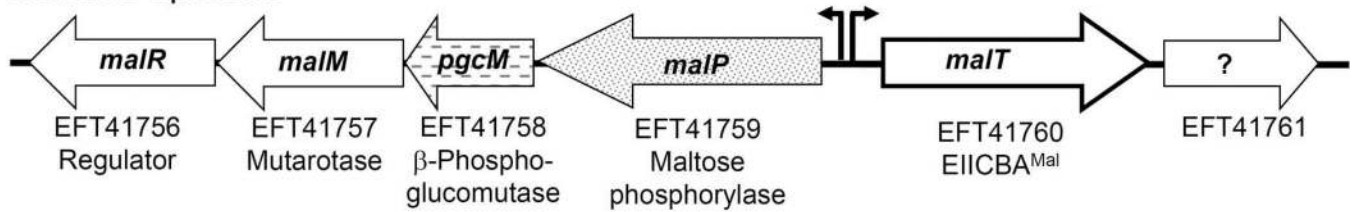


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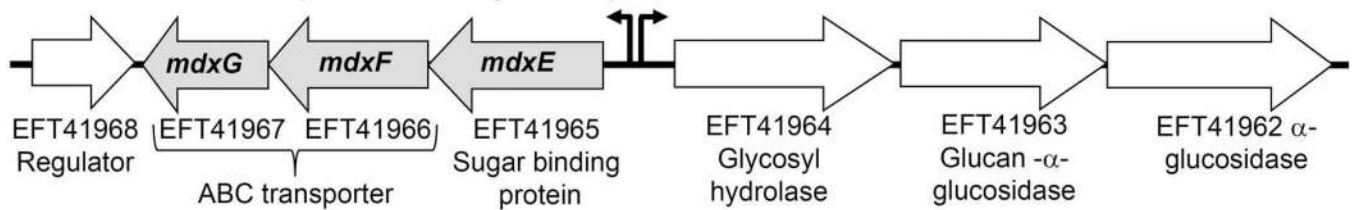
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## *Enterococcus faecalis*

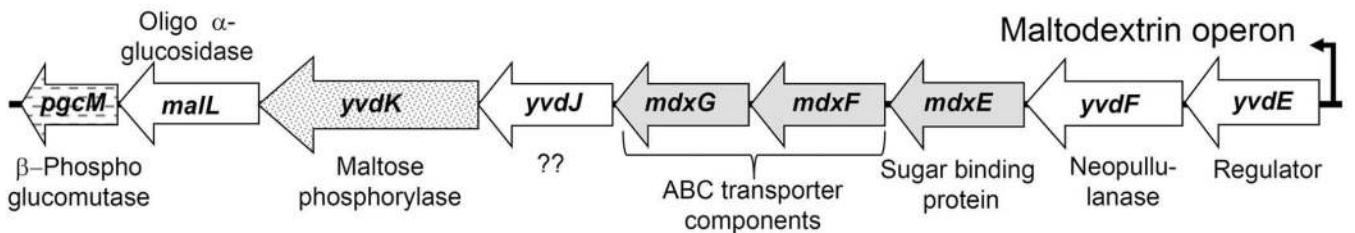
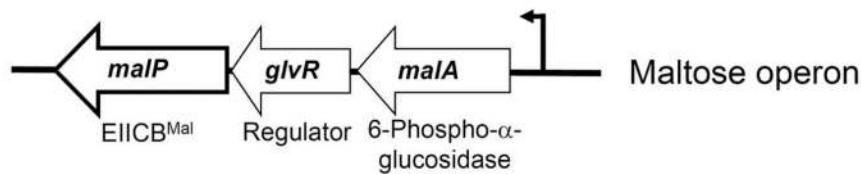
### Maltose operons



### Maltodextrin transport and amylase operons



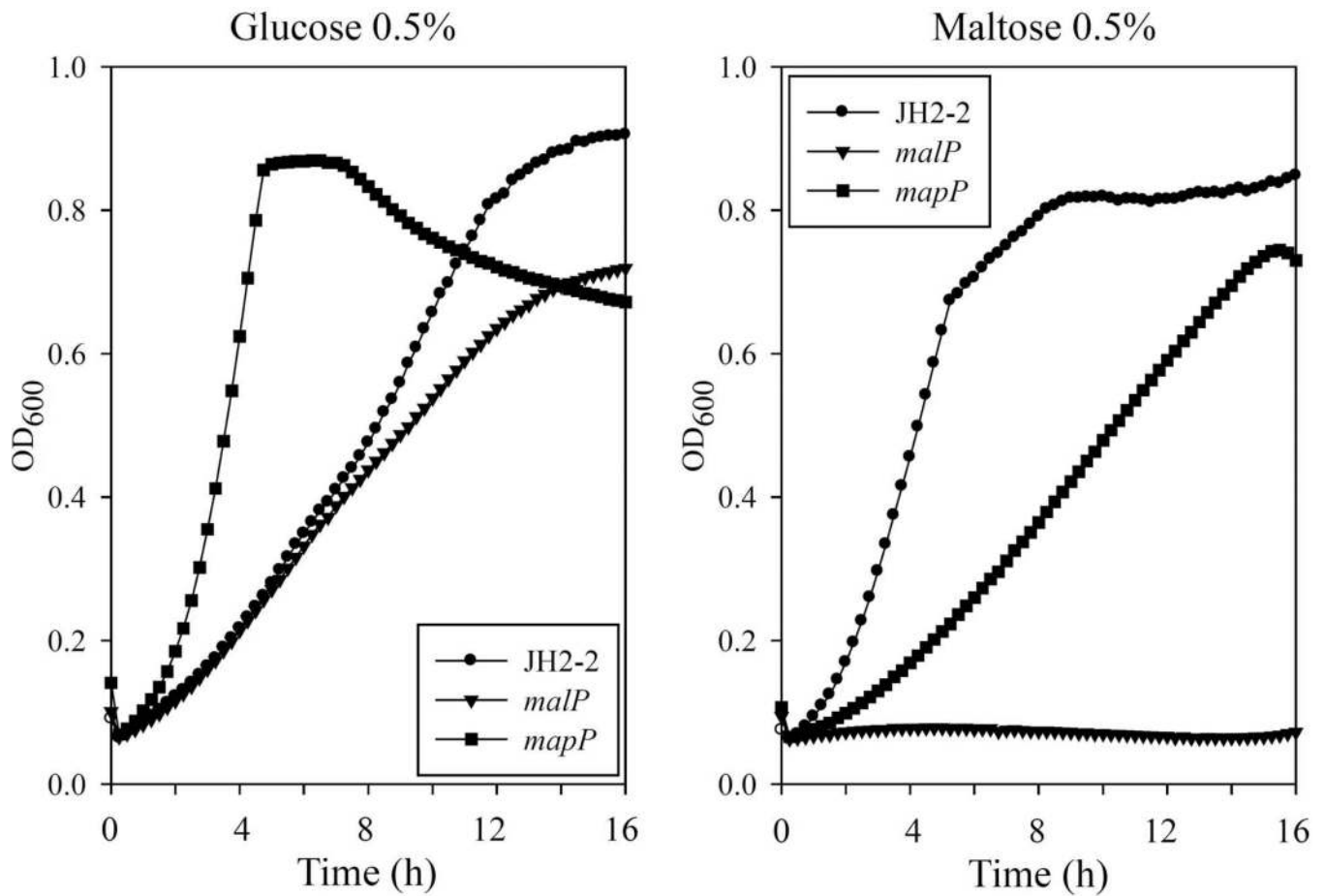
## *Bacillus subtilis*



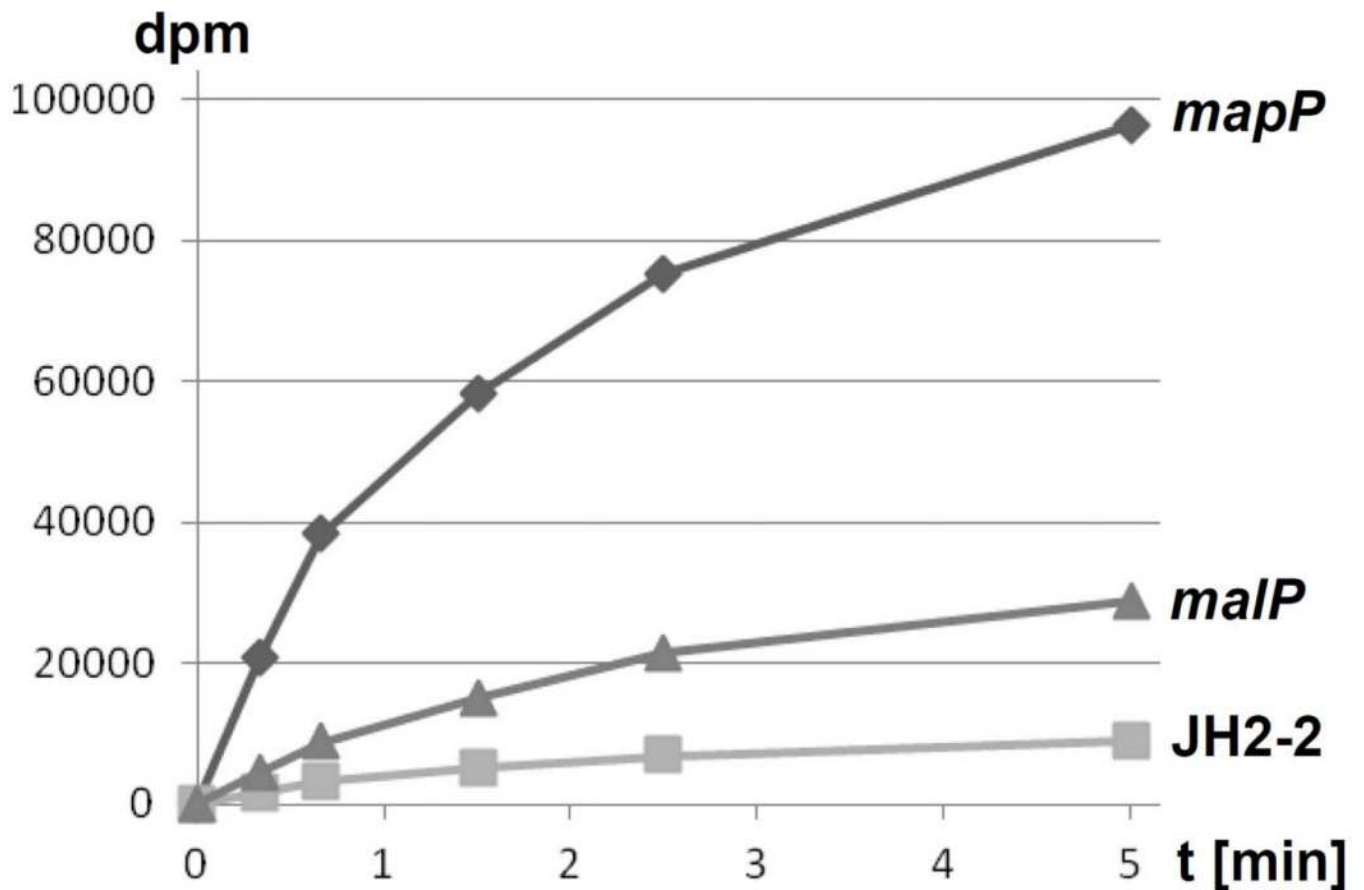
**Fig. 1.**

Comparison of the genes encoding the PTS and ABC transporter involved in maltose uptake in *E. faecalis* JH2-2 and *B. subtilis* 168. (A) In *E. faecalis*, the *malP-pgcM-malMR* operon contains the genes encoding a previously suggested maltose-6'-P phosphorylase (*malP*) (Le Breton *et al.*, 2005), which we showed to function as maltose phosphorylase, a  $\beta$ -phosphoglucomutase (*pgcM*), an aldose-1-epimerase (*malM*) and a transcriptional repressor (*malR*). Oriented in the other direction are *malT*, which codes for a maltose-specific EIICBA of the PTS (Le Breton *et al.*, 2005), and a gene encoding a protein of the endonuclease/exonuclease/phosphatase family of unknown function, which we identified as maltose-6'-P phosphatase (MapP). The operon encoding the ABC transporter for maltodextrins is located in a different place of the genome. Located upstream from the *mdxEFG* operon (grey arrows) and oriented in the opposite direction are three genes encoding enzymes for the degradation of  $\alpha$ -glucose polymers. (B) In *B. subtilis*, the *malP* gene encoding the EIICB<sup>Mal</sup> is associated with the *malA* gene, that encodes an NAD<sup>+</sup>/Mn<sup>2+</sup>-

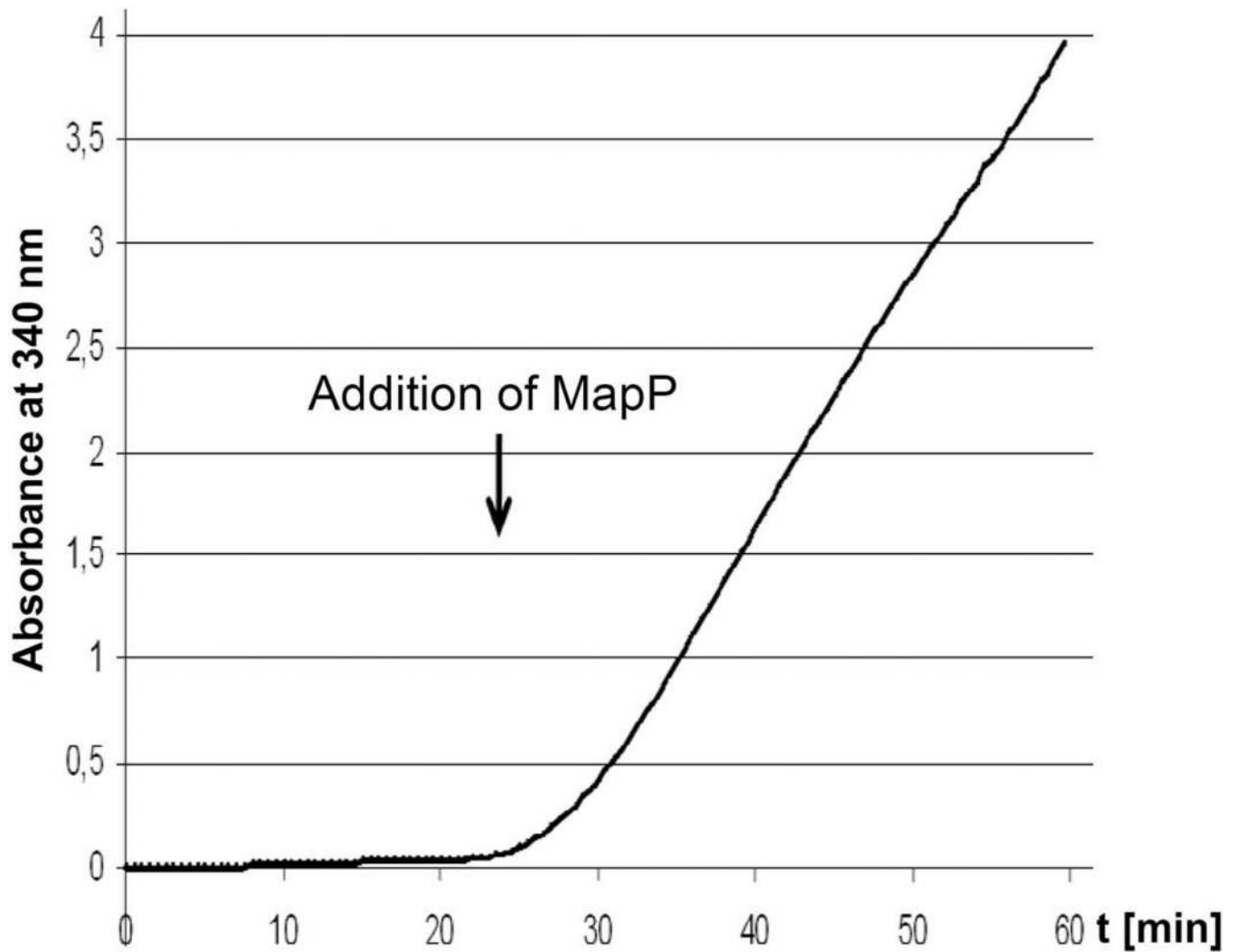
dependent P- $\alpha$ -glucosidase. The operon also includes the gene for a transcription regulator (Yamamoto *et al.*, 2001). Similar to *E. faecalis*, the operon encoding the ABC transporter for maltodextrins (MdxEFG) is located in a different place of the genome. The *mdxEFG* genes are preceded by two genes encoding a LacI-like repressor (*yvdE*) and a maltogenic amylase (*yvdF*) and followed by four genes encoding a protein of unknown function (*yvdJ*), a maltose phosphorylase (*yvdK*), an oligo- $\alpha$ -glucosidase hydrolyzing longer maltodextrins (*malL*), and a phosphoglucomutase (*pgcM*).



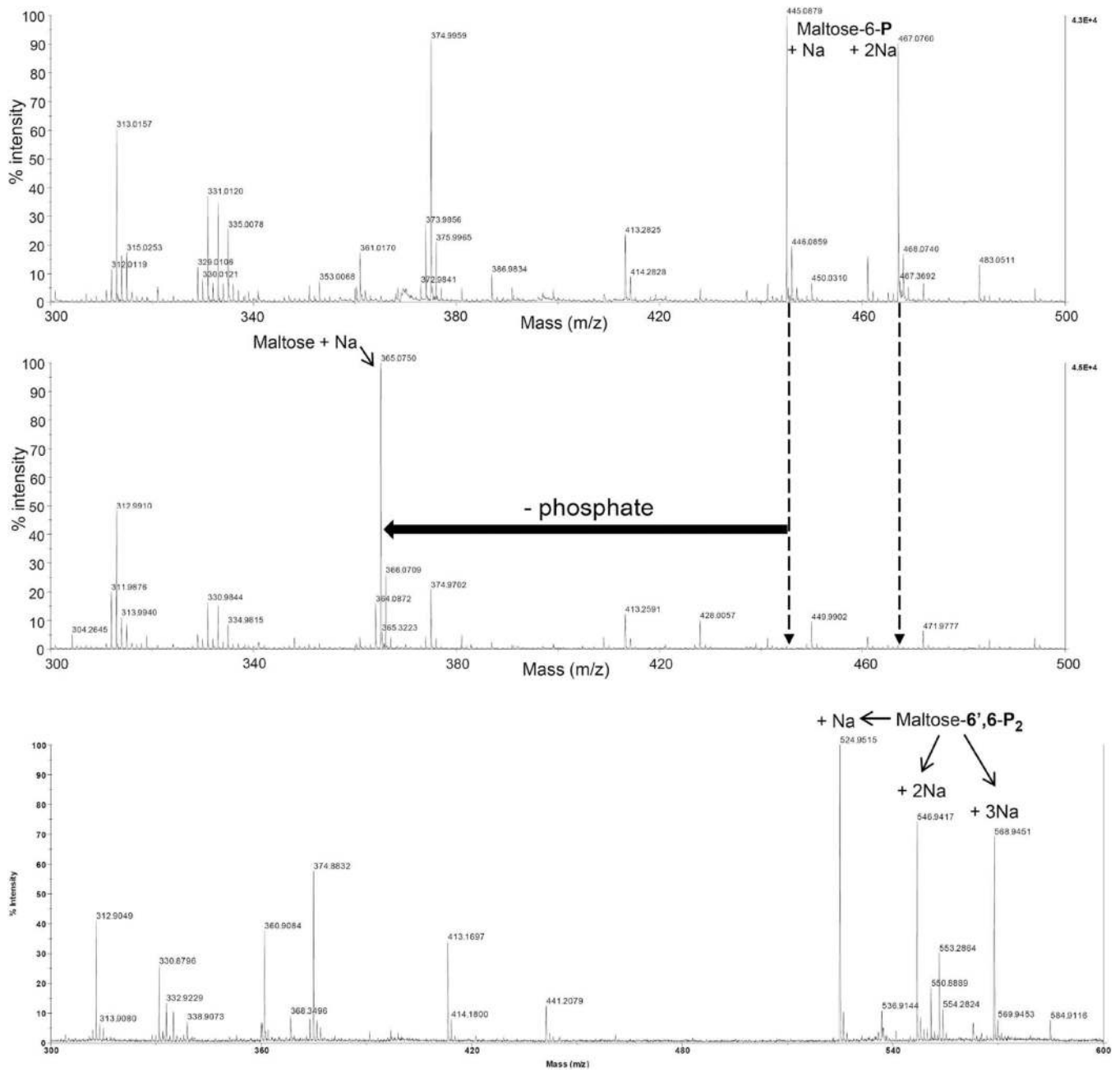
**Fig. 2.** Growth behaviour of the *E. faecalis* wild-type strain JH2-2 and the *malP* and *mapP* mutants derived from it in carbon-depleted M17 medium complemented with (A) 0.5% glucose or (B) 0.5% maltose. Surprisingly, the *mapP* mutant grew faster on glucose-containing medium than the wild-type strain or the *malP* mutant. The latter had lost its capacity to grow on maltose-containing medium, whereas the *mapP* mutant exhibited only significantly reduced growth on maltose. The experiment was carried out three times and results identical to those presented here were always obtained.



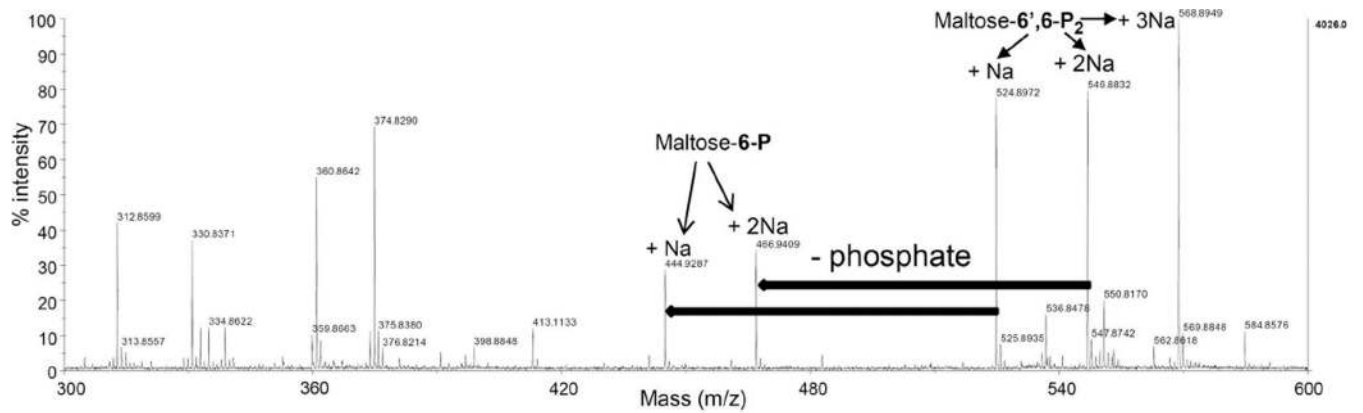
**Fig. 3.** [<sup>14</sup>C]-Maltose uptake studies with the *E. faecalis* wild-type strain JH2-2 (squares) and the *malP* (triangles) and *mapP* (diamonds) mutants derived from it. The experiments were carried out with 55 μM [<sup>14</sup>C]-maltose as described in Experimental procedures. Aliquots of the assay mixture were withdrawn at 0.33, 0.66, 1.5, 2.5 and 5 min and filtered through 0.45 μm pore-size filters. The results presented in this figure are the mean values obtained from three independent experiments. The standard deviations did not exceed 15%.



**Fig. 4.** Coupled spectrophotometric assay of maltose-6'-P phosphatase activity. The reaction mixture contained 500  $\mu$ l glucose assay reagent, 500  $\mu$ l 50 mM phosphate buffer, pH 7, 10 mM  $MgCl_2$ , 1 mM maltose-6'-P and 20  $\mu$ l maltose phosphorylase (1.2 mg  $ml^{-1}$ ). No formation of NADPH (increase of the absorbance at 340 nm) was detected under these conditions. Only when the maltose-6'-P phosphatase (MapP) was added to the reaction mixture (arrow), was glucose produced from maltose-6'-P, and subsequently phosphorylated and oxidized to gluconate-6-P under NADPH formation.

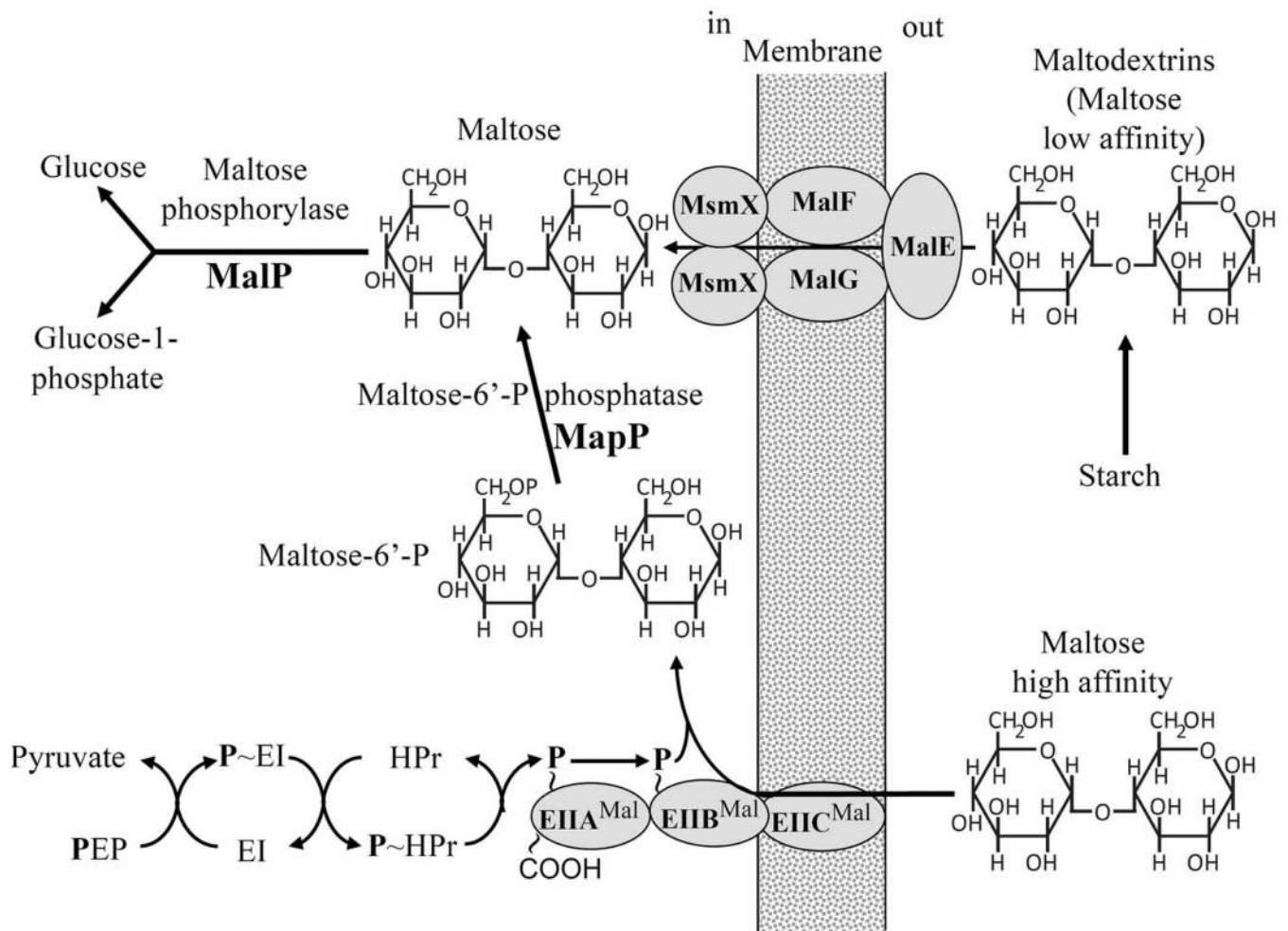






**Fig. 5.**

MapP-catalyzed dephosphorylation of (A and B) maltose-6'-P and (C and D) maltose-6',6-P<sub>2</sub> as evidenced by mass spectrometry. Dephosphorylation assays of maltose-6'-P or maltose-6',6-P<sub>2</sub> with MapP and subsequent analysis by mass spectrometry were carried out as described in Experimental procedures. (A) Untreated maltose-6'-P: The two observed peaks at 445.09 and 467.07 correspond to maltose-6'-P with one [(M+H)+Na<sup>+</sup>] or two [(M+H)+2Na<sup>+</sup>] Na<sup>+</sup> ions, respectively. (B) MapP-treated maltose-6'-P: These two peaks completely disappeared after treatment with MapP and a new single peak at 365.07 appeared, which corresponds to maltose carrying one Na<sup>+</sup> adduct. (C) Untreated maltose-6',6-P<sub>2</sub>: Owing probably to the additional phosphate group, maltose-6',6-P<sub>2</sub> was able to bind up to three Na<sup>+</sup> ions and consequently three peaks at 524.95 [(M+H)+Na<sup>+</sup>], 546.94 [(M+H)+2Na<sup>+</sup>] and 568.94 [(M+H)+3Na<sup>+</sup>] were observed. (D) MapP-treated maltose-6',6-P<sub>2</sub>: These three peaks were still detected after treatment with MapP. Nevertheless, two small additional peaks appeared at 444.93 and 466.94, which correspond to maltose-6-P carrying one [(M+H)+Na<sup>+</sup>] or two [(M+H)+2Na<sup>+</sup>] Na<sup>+</sup> adducts, respectively. These results demonstrate that maltose-6',6-P<sub>2</sub> is a poor substrate for MapP and that only one of the two phosphate groups is removed by this enzyme. No peaks corresponding to maltose carrying one Na<sup>+</sup> adduct (365.07) appeared, which suggests that the enzyme specifically removes the phosphoryl group bound to the non-reducing sugar moiety in maltose-6',6-P<sub>2</sub>. Otherwise maltose-6'-P would have been formed, and (under the reaction conditions employed) should have been efficiently dephosphorylated to maltose (*see*, Fig. 5B). This was not the case.



**Fig. 6.**

Proposed model for maltose uptake and metabolism in enterococci and other MalT- and MapP-containing bacteria. Maltose taken up by the low affinity ABC transport system is metabolized via the usual maltose phosphorylase-mediated pathway. Maltose taken up via the PTS enters the cells as maltose-6'-P. Enterococci and streptococci lack a 6-P- $\alpha$ -glucosidase (MalA) which in other bacteria hydrolyzes maltose-6'-P into glucose and glucose-6-P. In contrast, enterococci and streptococci possess a phosphatase (MapP), which dephosphorylates intracellular maltose-6'-P to maltose, thereby connecting PTS-mediated maltose uptake to maltose phosphorylase-catalyzed metabolism.

**Table 1**

Distribution of charged and uncharged radioactive compounds following the uptake of [ $^{14}\text{C}$ ]-maltose by the *E. faecalis* wild-type strain JH2-2 and the *malP* and *mapP* mutants derived from it.

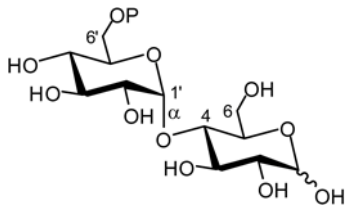
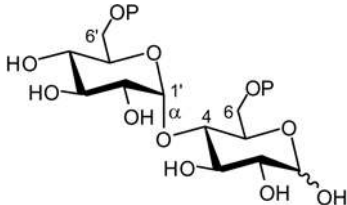
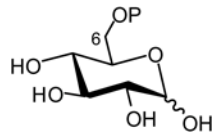
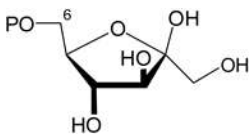
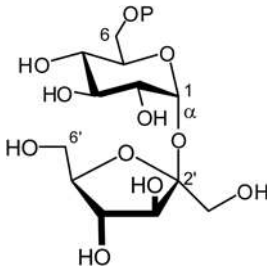
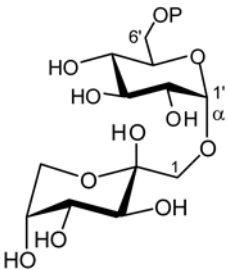
Fraction	JH2-2 (wild-type)		JH01 ( <i>malP</i> )		AB01 ( <i>mapP</i> )	
	Supernatant	Cells	Supernatant	Cells	Supernatant	Cells
Flowthrough <sup>a</sup>	76109 <sup>b</sup>	2484	93678	17441	76532	1133
Elution (Negatively charged)	31857	2812	1901	281	1672	40965

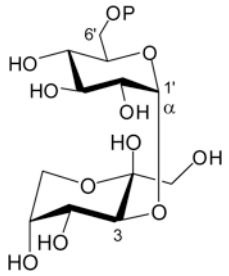
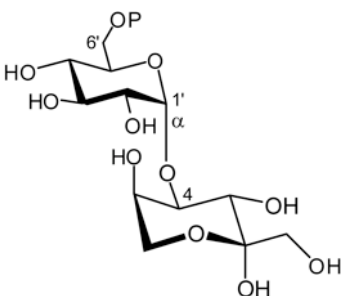
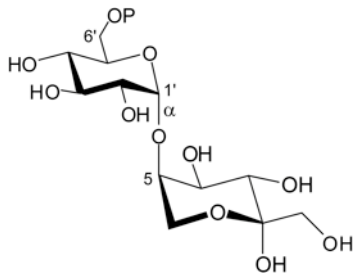
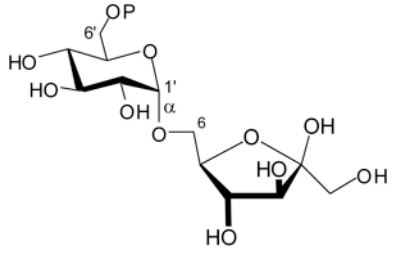
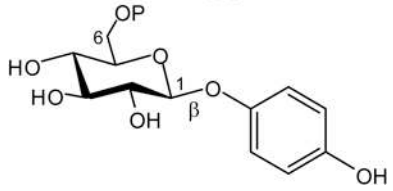
<sup>a</sup>The flowthrough contains uncharged and positively charged compounds.

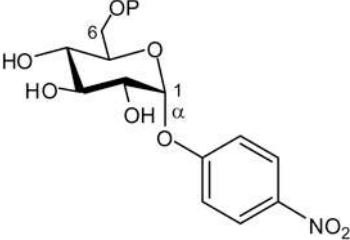
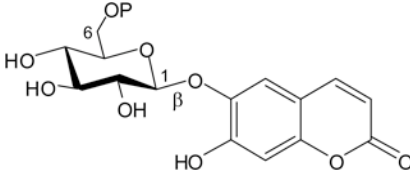
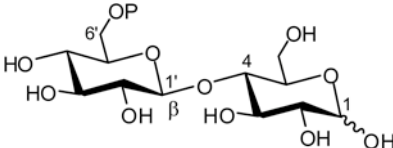
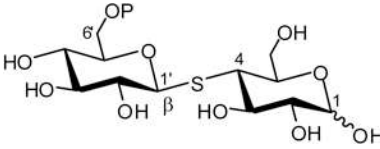
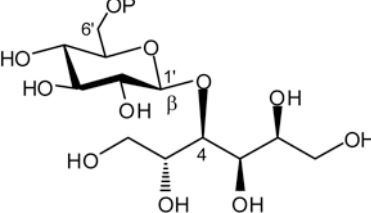
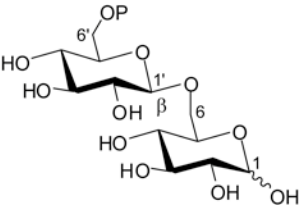
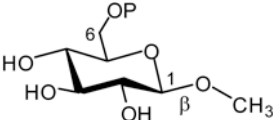
<sup>b</sup>The numbers represent dpm.

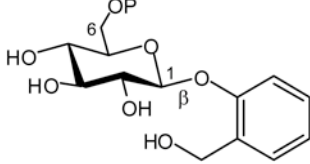
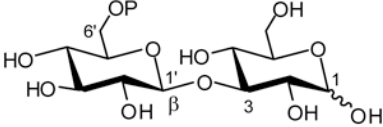
**Table 2**

Dephosphorylation of phosphorylated sugars and sugar derivatives by the enzyme MapP. The formulae of the phosphorylated compounds are also presented.

Name	Glycosidic Linkage	MapP activity <sup>a</sup>	MapP activity <sup>b</sup>	Formulae of phosphorylated sugars
Maltose-6'-P	$\alpha(1\rightarrow4)$	(++) <sup>a</sup>	100	
Maltose-6',6-P <sub>2</sub>	$\alpha(1\rightarrow4)$	(±)	6.0	
Glucose-6-P	$\alpha/\beta$ equilibrium	(-)	(-) <sup>a</sup>	
Fructose-6-P	$\alpha/\beta$ equilibrium	(-)	(-)	
Sucrose-6-P	$\alpha(1\leftrightarrow2)\beta$	(-)	(-)	
Trehalulose-6'-P <sup>c</sup>	$\alpha(1\rightarrow1)$	(±)	2.1	

Name	Glycosidic Linkage	MapP activity <sup>a</sup>	MapP activity <sup>b</sup>	Formulae of phosphorylated sugars
Turanose-6'-P	$\alpha(1\rightarrow3)$	(++)	85.7	
Maltulose-6'-P	$\alpha(1\rightarrow4)$	(±)	8.1	
Leucrose-6'-P	$\alpha(1\rightarrow5)$	(++)	18.8	
Palatinose-6'-P	$\alpha(1\rightarrow6)$	(+)	0.9	
Arbutin-6-P	$\beta$	(-)	nd <sup>d</sup>	

Name	Glycosidic Linkage	MapP activity <sup>a</sup>	MapP activity <sup>b</sup>	Formulae of phosphorylated sugars
p-Nitrophenyl- $\alpha$ -D-glucopyranoside-6-P	$\alpha$	(-)	nd	
Esculin-6-P	$\beta$	(+)	0.6	
Cellobiose-6'-P	$\beta(1\rightarrow4)$	(-)	nd	
Thiocolobiose-6'-P	$\beta(1\rightarrow4)$	(-)	nd	
Cellobiitol-6'-P	$\beta(1\rightarrow4)$	(-)	nd	
Gentiobiose-6'-P	$\beta(1\rightarrow6)$	(-)	nd	
$\beta$ -methylglucoside-6-P	$\beta$	(-)	nd	

Name	Glycosidic Linkage	MapP activity <sup>a</sup>	MapP activity <sup>b</sup>	Formulae of phosphorylated sugars
Salicin-6-P	$\beta$	(-)	nd	
Laminaribiose-6'-P	$\beta(1\rightarrow3)$	(-)	nd	

<sup>a</sup>MapP activity determined by mass spectrometry. (++) = total dephosphorylation; (+) = more than half dephosphorylated; ( $\pm$ ) = less than half dephosphorylated; (-) = no detectable dephosphorylation.

<sup>b</sup>MapP activity determined with the malachite green phosphate assay. The activities determined with the different substrates are expressed relative to the activity measured with maltose-6'-P, which was set to 100%.

<sup>c</sup>The five  $\alpha$ -linked isomers of sucrose-6-P are written in bold letters.

<sup>d</sup>nd stands for not determined.

**Table 3**

Effect of MapP treatment on the negatively charged radioactive compounds accumulated by the *E. faecalis* *mapP* mutant AB01. The experiment was carried out three times and in all cases comparable results were obtained.

	Untreated	MapP-treated
Uncharged Flowthrough <sup>a</sup>	891 <sup>b</sup>	34167
Elution	35334	2619

<sup>a</sup>The flowthrough contains uncharged and positively charged radioactive compounds.

<sup>b</sup>The numbers represent dpm, and are mean values from three independent experiments.



Table 4

## Strains and plasmids used in this study

Name of Stain	Relevant characteristics	Reference or source
<i>E. faecalis</i>		
JH2-2 (TX4000)	Fus <sup>r</sup> Rif <sup>r</sup> ; plasmid-free wild-type strain	(Jacob and Hobbs, 1974)
JH-malP	JH2-2 with pGhost8:: <i>malP</i> insertion	This study
AB01	JH2-2 with pUCB30bis:: <i>mapP</i> insertion	This study
<i>B. subtilis</i>		
168	<i>trpC2</i>	Laboratory stock
AM01	168 <i>malA</i> deletion mutant	This study
AM02	AM01 <i>mdxG</i> ::pMUTIN-I	This study
<i>E. coli</i>		
NM522	<i>supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM) (r<sub>K</sub>- m<sub>K</sub>-) [F<sup>+</sup> <i>proAB lacFZ ΔGM15</i>]; expression of His-tagged proteins</i>	(Gough and Murray, 1983)
EC101	Kan <sup>r</sup> <i>supE thi (lacproAB) (F<sup>+</sup> traD36 proAB lacF ZΔM15) repA</i> , used as host for pGhost8 constructs	(Law <i>et al.</i> , 1995)
<b>Plasmids</b>		
pGhost8	Thermosensitive replication origin, (Tet <sup>R</sup> )	(Maguin <i>et al.</i> , 1996)
pGh-malP	pGhost8 carrying an internal 594 bp fragment of <i>malP</i>	This study
pUCB30bis	<i>E. faecalis</i> suicide vector	(Benachour <i>et al.</i> , 2007)
pUCB30bis-mapP	pUCB30bis carrying an internal 450 bp fragment of <i>mapP</i>	This study
pUC18	Cloning vector	
pAC7	<i>B. subtilis</i> integration vector at the <i>amyE</i> site	(Weinrauch <i>et al.</i> , 1991)
pAC7-PptsH	pAC7 carrying the <i>B. subtilis ptsH</i> promoter and SD box	This study
pAC7-PptsH-mapP	<i>mapP</i> gene expressed from the <i>B. subtilis ptsH</i> promoter	This study
pMUTIN4	<i>B. subtilis</i> gene inactivation vector carrying <i>lacI</i> and <i>Pspac</i> for inducible transcription of downstream genes	(Vagner <i>et al.</i> , 1998)
pMUTIN-mdxG	pMUTIN4 carrying an internal 494 bp fragment of <i>B. subtilis mdxG</i>	This study
pMAD	Thermosensitive replication origin, (Erm <sup>R</sup> )	(Arnaud <i>et al.</i> , 2004)
pMAD-malA	pMAD carrying <i>B. subtilis malA</i> with an internal deletion	This study
pQE30	His-tag expression vector	Qiagen
pQE30-malP	His-tagged maltose phosphorylase	This study
pQE30-mapP	His-tagged maltose-6'-P phosphatase	This study

**Table 5**

## Oligonucleotides used in this study

Name	Sequence	Restriction site
EfMalP_Up	AAAGGATCCATGAAACAAATCAAAC	BamHI
EfMalP_Lo	AGGGGTACCTTAAACATGAACCTTCTTC	KpnI
Ef0960BamF	GGGGGATCCATGAATCTTTTAAACAATCAATAC	BamHI
Ef0960SalR	GCGCGTCGACTTATTTTAGTTGAATGAGTAAGCC	SalI
mapPForBam	GGGGGATCCATGAATCTTTTAAACAATCAATACTC	BamHI
mapPRevSac	TGAGAGCTCAGCGCTTTTCTTATTTTAGTTGAATGAG	SacI
EfMalPmut_U	CAT <u>AA</u> GCTTTCGCACCATTCTGTG	HindIII
EfMalPmut_L	GGGAATTCTGTTGTGCCTACATATACAC	EcoRI
VerifMalP	CCCCATCTAAATCAAACAAAACG	-
MapPBamF	AGAGGATCCATTAAAAAATTAGAGGAG	BamHI
MapPEcoR	TAGGAATTCATAGGCAAAACCTGTACAG	EcoRI
EFmapPconf	CCGATAGTCAGTGATCATTTTGGC	-
PU	TGTA AACGACGGCCAGT	-
PR	CAGGAAACAGCTATGACC	-
malA1ForBam	AGTGGATCCAGTGACTACATCATTGCAAGC	BamHI
malA1RevKpn	CTGAGGTACCAGTTCTTCGAGCTGCATATCAGATC	KpnI
VerifMalAFor	GGGATCGAAGACCGGATGGCGC	-
VerifMalARev	GGCCAGCGCTCGATTCCAAGG	-
mdxGForHind	CATCAAGCTTCCGTGTGGGAATTCGGCG	HindIII
mdxGRevBam	ATAAGGATCCAAAGCGCTGTACCGCGAGC	BamHI
ptsHPromForEco	GAGGAATTC AAGATATTGTGAAGATTGAAA	EcoRI
ptsHPromRevBam	CATGGATCCCATTCTCCTTTTAACTTAAAG	BamHI