**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-α-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 phosphorylates 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. MalP was suggested to split maltose 6-P into glucose 1-P and glucose 6-P. However, purified MalP phosphorylates 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 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 catalyses 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-catalysed metabolism. Dephosphorylation assays with a wide variety of phospho-substrates revealed that MapP preferably dephosphorylates disaccharides containing an O-α-glycosyl linkage.

**Introduction**

The breakdown of the two starch-forming polysaccharides amylose and amylopectin, which contain glucose molecules connected by α(1→4) glycosidic bonds, by the enzyme α-amylase leads to the formation of the disaccharide maltose (4-O-α-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 α-amylases mainly during maturation of their fruits and germination of their seeds. α-Amylase activity is also found in the saliva of humans and animals. In addition, many bacteria possess an α-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 hydrolysing ATP. The two membrane proteins and the nucleotide binding protein form a MalF/MalG/(MalK)2 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. First, 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 protein functions together with the sugar-specific PTS of Lactobacillus casei (Andersson and Rådström, 2002). The genes encoding the enzyme phosphoglucomutase is present in many other bacilli, geobacilli, clostridia, fusobacteria and sometimes also aldose 1-epimerase (A. Mazé and J. Deutscher, unpubl. obs.). It is therefore likely that maltose taken up by MalY is phosphorolysed by MalP to glucose and glucose 1-P, with the latter being converted into glucose 6-P by the enzyme phosphoglucomutase. Second, 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 malEFG operon (Monedero et al., 2008; Gopal et al., 2010).

Several other firmicutes also contain an ABC transporter, which, however, preferably transports dextrins. 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 EIIAs 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 EIICBMal (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/Glc domain of PtsG is 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+/Mn2+-dependent 6-P-α-glucosidase (EC 3.2.1.122) (Thompson et al., 1998; Rajan et al., 2004; 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-catalysed transport is hydrolysed 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, fusob-
bacteria, 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 serratiae, 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), Strepptococcus mutans (Webb et al., 2007) S. pyogenes (Shelburne III et al., 2008) and Strepptococcus pneumoniae (Bidossi et al., 2012) transport maltose via an EIICBA protein of the glucose subfamily of PTS transporters (MalT). In most enterococci and streptococci 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-catalysed transport by L. lactis is hydrolysed by the trehalose 6′-P phosphohydrolase (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 hydrolysed 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 α(1→1) glycosidic bond, and phosphorolysis of the phosphorylated disaccharide yields glucose 1-P and glucose 6-P (Andersson et al., 2001). In contrast, maltose

![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.](image)
contains an α(1→4) 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-catalysed uptake of fucosyl-α(1→3)-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). 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 malP (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-catalysed 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 phosphorolysae 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 A340 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 (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 μM [14C]-maltose via the PTS protein MalT. A malT mutant had almost completely lost maltose uptake. The slow [14C]-maltose uptake observed for the malT mutant was suggested to be catalysed 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. [14C]-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 [14C]-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

**Table 1.** Distribution of charged and uncharged radioactive compounds following the uptake of [14C]-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 (malP) | AB01 (mapP) |
|------------------------|------------------|------------|-------------|
|                        | Supernatant      | Cells      | Supernatant | Cells | Supernatant | Cells |
| Flowthrough*            | 76109*           | 2484       | 93678       | 17441 | 76532       | 1133  |
| Elution (negatively charged) | 31857  | 2812       | 1901        | 281   | 1672        | 40965 |

*a. The flowthrough contains uncharged and positively charged compounds.
   b. The numbers represent dpm.
annotation as an enzyme that putatively may hydrolyse phosphate esters we suspected that this protein might catalyse 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+ ion (m/z = 445.09), the other corresponding to maltose 6′-P with two Na+ ions (m/z = 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+ ion (m/z = 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_0 of MapP was determined to be 4.1 mM and the V_max was found to be 25 μmol min⁻¹ mg⁻¹ of protein providing a k_cat of 128 s⁻¹.

**MapP preferentially attacks disaccharide phosphates containing an α-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 et al., 2001). The phospho-sugars were incubated with MapP and the products of the reaction were analysed by mass spectrometry. Neither glucose 6-P, fructose 6-P nor β-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-α-glycosyl linkage. For example, the five α-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 α(1→2)β glycosidic linkage] was not dephosphorylated. Phosphorylated compounds containing an O-β-glycosidic bond (cellobiose 6′-P, maltulose 6′-P, leucrose 6′-P, turanose 6′-P, palatinose 6′-P and trehalulose 6′-P) were all dephosphorylated by MapP (Table 2). The phospho-sugars were incubated with MapP and the products of the reaction were analysed by mass spectrometry.
Fig. 5. MapP-catalysed dephosphorylation of (A and B) maltose 6'-P and (C and D) maltose 6',6-P2, as evidenced by mass spectrometry. Dephosphorylation assays of maltose 6'-P or maltose 6',6-P2 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+) or two [(M + H) + 2Na+] Na+ 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+ adduct.

C. Untreated maltose 6',6-P2: Owing probably to the additional phosphate group, maltose 6',6-P2 was able to bind up to three Na+ ions and consequently three peaks at 524.95 [(M + H + Na+) 546.94 [(M + H) + 2Na+] and 568.94 [(M + H) + 3Na+] were observed.

D. MapP-treated maltose 6',6-P2: 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+) or two [(M + H) + 2Na+] Na+ adducts respectively.

These results demonstrate that maltose 6',6-P2 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+ 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-P2. 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.

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 maltP 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 maltP 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...
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\(^a\) | MapP activity\(^b\) | Formulae of phosphorylated sugars |
|---------------|--------------------|----------------------|----------------------|-----------------------------------|
| Maltose 6'-P  | \(\alpha(1\rightarrow4)\) | \((++)^a\)           | 100                  | ![Formula](image)                |
| Maltose 6',6-P\(_2\) | \(\alpha(1\rightarrow4)\) | \((\sim)^a\)        | 6.0                  | ![Formula](image)                |
| Glucose 6-P   | \(\alpha/\beta\) equilibrium | \((-)^a\)           | \((-)^a\)            | ![Formula](image)                |
| Fructose 6-P  | \(\alpha/\beta\) equilibrium | \((-)^a\)           | \((-)^a\)            | ![Formula](image)                |
| Sucrose 6-P   | \(\alpha(1\rightarrow2)\beta\) | \((-)^a\)           | \((-)^a\)            | ![Formula](image)                |
| Trehalulose 6'-P\(_c\) | \(\alpha(1\rightarrow1)\) | \((\sim)^a\)        | 2.1                  | ![Formula](image)                |
Table 2. cont.

| Name          | Glycosidic linkage | MapP activity¹ | MapP activity² | Formulae of phosphorylated sugars |
|---------------|--------------------|----------------|----------------|-----------------------------------|
| Turanose 6′-P | α(1→3)             | (++)           | 85.7           |                                   |
| Maltulose 6′-P| α(1→4)             | (±)            | 8.1            |                                   |
| Leucrose 6′-P | α(1→5)             | (++)           | 18.8           |                                   |
| Palatinose 6′-P| α(1→6)            | (+)            | 0.9            |                                   |
| Arbutin 6-P   | β                  | (−)            | nd             |                                   |
Table 2. cont.

| Name                                      | Glycosidic linkage | MapP activity | MapP activity | Formulae of phosphorylated sugars |
|-------------------------------------------|--------------------|---------------|---------------|-----------------------------------|
| p-Nitrophenyl-α-D-glucopyranoside 6-P     | α                  | (-)           | nd            | ![Chemical Structure](image1)      |
| Esculin 6-P                                | β                  | (+)           | 0.6           | ![Chemical Structure](image2)      |
| Cellobiose 6′-P                           | β(1→4)             | (-)           | nd            | ![Chemical Structure](image3)      |
| Thiocellobiose 6′-P                       | β(1→4)             | (-)           | nd            | ![Chemical Structure](image4)      |
| Cellobiitol 6′-P                          | β(1→4)             | (-)           | nd            | ![Chemical Structure](image5)      |
| Gentiobiose 6′-P                          | β(1→6)             | (-)           | nd            | ![Chemical Structure](image6)      |
transport [14C]-maltose. However, the amount of radioactivity accumulated inside the cells after 5 min incubation with 55 μM [14C]-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 μM [14C]-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 3), 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 with 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).

Table 2. cont.

| Name                      | Glycosidic linkage | MapP activitya | MapP activityb | Formulae of phosphorylated sugars |
|---------------------------|--------------------|----------------|----------------|-----------------------------------|
| β-methylglucoside 6-P     | β                  | (-)            | nd             | ![Formula](image)                 |
| Salicin 6-P               | β                  | (-)            | nd             | ![Formula](image)                 |
| Laminaribiose 6-P         | β(1→3)             | (-)            | nd             | ![Formula](image)                 |

a. MapP activity determined by mass spectrometry. (++) = total dephosphorylation; (+) = more than half dephosphorylated; (±) = less than half dephosphorylated; (-) = no detectable dephosphorylation.
b. 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%.
c. The five α-linked isomers of sucrose 6-P are written in bold letters.
d, not determined.

Table 3. Effect of MapP treatment on the negatively charged radioactive compounds accumulated by the E. faecalis mapP mutant AB01.

|                      | Untreated | MapP-treated |
|----------------------|-----------|--------------|
| Uncharged flowthrougha | 891b      | 34167        |
| Elution              | 35334     | 2619         |

The experiment was carried out three times and in all cases comparable results were obtained.
a. The flowthrough contains uncharged and positively charged radioactive compounds.
b. The numbers represent dpm, and are mean values from three independent experiments.

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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-α-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-mdxG 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 phosphorolyses 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, \(V_{max} = 25 \mu\text{mol} \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}\); \(k_{cat} = 128 \text{ s}^{-1}\)) 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 hydrolysed into glucose and glucose 6-P by the NAD'/Mn2+-dependent 6-P-α-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 mapP-pgdM-malM encoding maltose phosphorylase, phosphoglucomutase and mutarotase (A. Mazé and J. Deutscher, unpubl. obs.). 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-catalysed 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 α-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-catalysed 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 hydrolysed by a 6-P-α-glucosidase, is principally identical to that obtained by the MapP-catalysed metabolism of maltose 6'-P formed during MalT-catalysed transport. Although MapP dephosphorylates intracellular maltose 6'-P in the first catabolic step by producing Pi and maltose, MalIP 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 hydrolyses 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 grown at 37°C without shaking in 100 ml sealed bottles filled with 20–50 ml of Luria-Bertani medium (LB; Difco, NJ, 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 and 5 μg ml⁻¹ respectively.

Bacillus 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⁻¹ kanamycin and 0.3 μg ml⁻¹ 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 A600 in a Novaspec II spectrophotometer.
**Table 4.** Strains and plasmids used in this study.

| Name of strain | Relevant characteristics | Reference or source |
|----------------|--------------------------|---------------------|
| E. faecalis JH2-2 (TX4000) | Fus’ Rif’; plasmid-free wild-type strain | Jacob and Hobbs (1974) |
| JH-malP | JH2-2 with pGhost8::malP insertion | This study |
| AB01 | JH2-2 with pUCB30bis::mapP insertion | This study |
| B. subtilis 168 | trpC2 | Laboratory stock |
| AM01 | 168 malA deletion mutant | This study |
| AM02 | AM01 mdxG:pMUTIN-I | This study |
| E. coli NM522 | supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM) (r- m-)
[ 프로AB lacPZ ΔGM15]; expression of His-tagged proteins | Gough and Murray (1983) |
| EC101 | Kan’ supE thi (lacrproAB) (F’ traD36 proAB lacf ZΔM15) repA, used as host for pGhost8 constructs | Law et al. (1995) |

**Plasmids**
- pGhost8: Thermosensitive replication origin (Tet<sup>+</sup>)
- pGH-malP: pGhost8 carrying an internal 594 bp fragment of malP
- pUCB30bis: E. faecalis suicide vector
- pUCB30bis-mapP: pUCB30bis carrying an internal 450 bp fragment of mapP
- pUC18: Cloning vector
- pAC7: B. subtilis integration vector at the amyE site
- pAC7-PptSH: pAC7 carrying the B. subtilis ptsH promoter and SD box
- pAC7-Ptsh-mapP: mapP gene expressed from the B. subtilis ptsH promoter
- pMUTIN4: B. subtilis gene inactivation vector carrying lacI and Pspac for inducible transcription of downstream genes
- pMUTIN-mdxG: pMUTIN4 carrying an internal 494 bp fragment of B. subtilis mdxG
- pMAD: Thermosensitive replication origin (Emr<sup>+</sup>)
- pMAD-malA: pMAD carrying B. subtilis malA with an internal deletion
- pQE30: His-tag expression vector
- pQE30-malP: His-tagged maltose phosphorylase
- pQE30-mapP: His-tagged maltose 6-P phosphatase

**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 EMalP_Up/EMalP_Lo and EF0960BamF/EF0960SalR respectively (Table 5). The amplimers 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 catalysed 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>340</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 μl 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 phosphatase was observed.

To determine the k<sub>cat</sub> and K<sub>m</sub> values of MapP we slightly modified the assay conditions by replacing the glucose assay kit (enzymes hexokinase and glucose 6-P dehydrogenase) with 5 μg of NAD-dependent glucose dehydrogenase (Sigma). The assay mixture therefore contained 3 mM NAD in a total volume of 450 μl. 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).
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 amplimer was digested with appropriate restriction enzymes (HindIII and EcoRI) and ligated into pGhGhost8 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) 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 [14C]-maltose

Transport studies with [14C]-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 OD600 of 0.5. Cells were centrifuged and washed twice with 50 mM Tris-HCl buffer, pH 7.2, containing 10 mM MgCl2. The cells were resuspended in such a volume of 50 mM Tris-maleate buffer, pH 7.2, containing 5 mM MgCl2 that the cell suspension exhibited an OD600 of 10. For maltose transport assays 50 μl of the cell suspension was diluted with 0.5 ml of the cell suspension was diluted with 0.5 ml transport buffer (resuspension buffer containing 1% peptone) and pre-incubated 1 min at 37°C. The transport reaction was started by adding 60 μl of a [14C]-maltose solution composed of 54 mCi ml⁻¹ maltose (0.1 mCi ml⁻¹) (corresponds to 1.33 × 10⁷ cpm). The final maltose concentration in the assay mixture was calculated to be 55 mM. 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.

### Table 5. Oligonucleotides used in this study.

| Name       | Sequence                          | Restriction site |
|------------|-----------------------------------|-----------------|
| EfMalP_Up  | AAAGGATCCATGAAACAAATCAAC          | BamHI           |
| EfMalP_Lo  | AAGGGGTACGCTTAAACACTGATCTTCTTCC  | KpnI            |
| Ef0960BamF | GGGGGATCCATGAAACAAATCAAC          | BamHI           |
| Ef0960SacR | GGCGGTGACATTTTTTTTTTTTTTTTTTGAAGC| Salt            |
| mapPForBam | GGAGGATCCATGAAACAAATCAAC          | BamHI           |
| mapPRevSac | TGAAGGTCAGGGCTTCTTCTTCTTTTTAGTT  | SacI            |
| EfMalPmut_U| CATAACTTTTGCACCTATTTCTGTG         | HindIII         |
| EfMalPmut_L| GGGATTTTGTGTGCCTATACAC            | EcoRI           |
| VerifMalP  | CCCCCTCTAAATCACAAACAAAACG         | –               |
| MapP BamF  | AAGGGATCCATGAAACAAATCAAC          | BamHI           |
| MapP EcoR  | GGAAATTCATGAAACAAACAAAACGTATAC   | EcoRI           |
| EfMapPconf | CCGATAGTGATGATGATTTTG              | –               |
| PU         | GTGAAAACGACGCGGCAG                | –               |
| PR         | CAGGAAAAGCTATGACC                 | –               |
| mal1ForBam | AGGGGATCCAGTACATACATTTTTGCAAC    | BamHI           |
| mal1RevKpn | CTCGAAGTACGACGGCGGCGGC           | KpnI            |
| VerifMalAFor| GTGAAAACGACGCGGCAG                | –               |
| VerifMalARev| GGCGGTGACATTTTTTTTTTTTTTTTGAAGC| –               |
| mdxGForHind| CATCAAGGCTTCCGTGTTGGGAATCTGGCG   | HindIII         |
| mdxGForBam | ATAGGCTACGACGGCGGCGGC             | BamHI           |
| ptsHPromForEco | GAGGATTCCCAGATTTTGAAAGTTGAAA     | EcoRI           |
| ptsHPromRevBam | CATGATGTCAGGATTTTGAAAGTTGAAA    | BamHI           |

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 MapP BamF/MapPEcoR (Table 5). The amplimer 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).
Isolation and characterization of negatively charged radioactive compounds accumulated during \([^{14}C]\)-maltose uptake

*E. faecalis* wild-type and mutant cells were grown as described above and a 275 \(\mu l\) assay mixture for \([^{14}C]\)-maltose uptake was prepared for each strain as described above. The cells were incubated for 5 min in the presence of \([^{14}C]\)-maltose before a 100 \(\mu l\) aliquot was withdrawn and filtered through a 0.45 \(\mu m\) pore-size filter. Another 60 \(\mu l\) aliquot was rapidly centrifuged and supernatant and cell pellet were separated. The cell pellet was resuspended in 100 \(\mu l\) 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 \([^{14}C]\)-maltose before a cell extract was prepared as described above. A 180 \(\mu l\) aliquot of the transport assay was withdrawn, centrifuged and the cells were desintegrated in 300 \(\mu l\) of boiling water. To one 100 \(\mu l\) aliquot of cell extract 50 \(\mu l\) Tris/HCl, pH 7.4 and 20 \(\mu l\) 100 mM MgCl\(_2\) were added, whereas to the second 100 \(\mu l\) aliquot 50 \(\mu l\) of purified MapP dissolved in 50 mM Tris/HCl, pH 7.4 (0.8 mg ml\(^{-1}\)) and 20 \(\mu l\) 100 mM MgCl\(_2\) were 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 *et al.*, 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\(^{2+}\) 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 analogue, 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\(_2\)), maltose 6'-P and maltose 6-P. Maltose 6',6-P\(_2\) was separated from the two mono-phosphate derivatives by gradient elution (0–0.5 M NH\(_4\)HCO\(_3\)) from a column of AG1-X8 (formate-form) ion exchange resin. Bicarbonate was removed by lyophilization, and maltose 6',6-P\(_2\) 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 \(\mu l\) aliquots of these solutions were added to either 40 \(\mu l\) of purified MapP (0.8 mg ml\(^{-1}\), dialysed against 20 mM ammonium bicarbonate) or to 40 \(\mu l\) of 20 mM ammonium bicarbonate and incubated for 2 h at 37°C. The samples were subsequently lyophilized and rehydrated with 10 \(\mu l\) of water. Aliquots of 1 \(\mu l\) were mixed with 9 \(\mu l\) of a sugar matrix solution and 1 \(\mu l\) was spotted onto the MALDI steel plate. The sugar matrix was freshly prepared and contained 100 \(\mu g\) ml\(^{-1}\) 2,5-dihydroxybenzoic acid dissolved in a mixture of H\(_2\)O/acetoniitriile/N,N-Dimethylaniline (1:1:0.02 v/v). The samples were analysed 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-catalysed formation of phosphate with the malachite green assay

To follow MapP-catalysed 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 \(\mu l\) assay mixtures containing 50 mM Tris/HCl, pH 7.4, 5 mM MgCl\(_2\) 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 \(\mu g\) 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 \(\mu l\) were withdrawn after 0, 1.5, 3, 6, 10 and 20 min incubation and immediately mixed with 25 \(\mu l\) of the malachite green reagent (Baykov *et al.*, 1988). The samples were kept for 20 min at ambient temperature before 375 \(\mu l\) of water was added and the OD\(_{570}\) 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 restriction enzyme Clal, which led to the deletion of a 328 bp fragment in the middle of *maIA* and also introduced a frameshift. 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 *maIA* mutant by double cross over we followed a previously described protocol (Aké *et al.*, 2011). The correct deletion and the insertion of a frameshift mutation in the *maIA* gene in one of the isolated strains were confirmed by carrying out a PCR amplification with the primer pair VerilMalAFor and VerilMalAREv (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 catalysts 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 pMMUTI4 (Vagner *et al.*, 1998) cut with the same enzymes thus providing pMMUTIN-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-*P*ptsH. Subsequently, the *E. faecalis* mapP gene was amplified using primers *mapPForBam* and mapP*RevSac* and cloned into the BamHI/SacI sites of pAC7-*PtsH*. In the resulting plasmid pAC7-*PtsH-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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