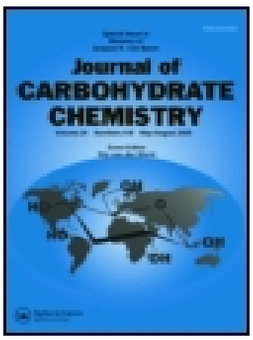


Journal of Carbohydrate Chemistry

ISSN: 0732-8303 (Print) 1532-2327 (Online) Journal homepage: http://www.tandfonline.com/loi/lcar20

# Enzymatic glycosylation of indoxyglycosides catalyzed by a novel maltose phosphorylase from Emticicia oligotrophica

**Faisal Nureldin Awad, Anna Kulinich, Ming Jun Yao, Xu Chu Duan, Zhi Peng Cai, Bin Gu, Li Liu & Josef Voglmeir**

**To cite this article:** Faisal Nureldin Awad, Anna Kulinich, Ming Jun Yao, Xu Chu Duan, Zhi Peng Cai, Bin Gu, Li Liu & Josef Voglmeir (2016): Enzymatic glycosylation of indoxyglycosides catalyzed by a novel maltose phosphorylase from Emticicia oligotrophica, Journal of Carbohydrate Chemistry, DOI: 10.1080/07328303.2016.1238479

**To link to this article:** http://dx.doi.org/10.1080/07328303.2016.1238479

JOURNAL OF CARBOHYDRATE CHEMISTRY
2016, VOL. 0, NO. 0, 1–14
http://dx.doi.org/10.1080/07328303.2016.1238479

Taylor & Francis
Taylor & Francis Group

# Enzymatic glycosylation of indoxyglycosides catalyzed by a novel maltose phosphorylase from *Emticicia oligotrophica*

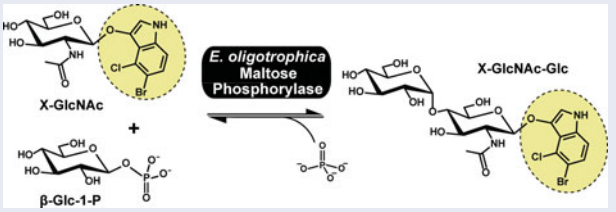
Faisal Nureldin Awad[a,b], Anna Kulinich[a], Ming Jun Yao[a], Xu Chu Duan[a], Zhi Peng Cai[a], Bin Gu[a], Li Liu[a,c], and Josef Voglmeir[a]

[a]Glycomics and Glycan Bioengineering Research Center (GGBRC), College of Food Science and Technology, Nanjing Agricultural University, Nanjing, People's Republic of China; [b]National Food Research Centre, Khartoum North, Sudan; [c]Qlyco Ltd., Nanjing, People's Republic of China

**ABSTRACT**

Maltose phosphorylases (EC 2.4.1.8) catalyze the reversible conversion of maltose to glucose and glucose-1-phosphate in the presence of inorganic phosphate. Herein, we describe for the first time the use of a maltose phosphorylase for the synthesis of various anomerically modified diglycosides. The maltose phosphorylase used was isolated from the bacterium *Emticicia oligotrophica* and showed a high selectivity towards the phosphorolysis of maltose, whereas no phosphorolysis was observed using other glucose-containing disaccharides such as cellobiose, melibiose, sucrose and trehalose. The addition of glucose to various 5-bromo-4-chloro-3-indolyl-glycosides (X-sugars) was used to evaluate the promiscuity of the maltose phosphorylase, and product formation was verified by LC-ESI-MS and MALDI-TOF-MS. The simple expression and purification protocol and the use of maltose as an inexpensive starting material make this maltose phosphorylase from *Emticicia oligotrophica* a valuable novel biocatalyst for the synthesis of glucose-containing glycosides.

**GRAPHICAL ABSTRACT**




**ARTICLE HISTORY**
Received 11 March 2016
Accepted 15 September 2016

**KEYWORDS**
X-glycosides; *Emticicia oligotrophica*; maltose phosphorylase; maltose; phosphorolysis; beta glucose 1 phosphate

**CONTACT** Josef Voglmeir josef.voglmeir@njau.edu.cn and liu.lichen@qlyco.com Qlyco Ltd., Nanjing, People's Republic of China

Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/lcar.

Supplemental data for this article can be accessed on the publisher's website.

**ABBREVIATIONS:** $\alpha$-PGM: $\alpha$-phosphoglucomutase; $\beta$-Glc-1-P: $\beta$-Glucose-1-phosphate; $\beta$-PGM: $\beta$-phosphoglucomutase; DHB: 2,5-dihydroxybenzoic acid; *E. coli*: *Escherichia coli*; EDTA: ethylenediaminetetraacetic acid; ESI-MS: electrospray ionization – mass spectrometry; HPLC: high-performance liquid chromatography; IPTG: isopropyl-$\beta$-D-thiogalactopyranoside; MALDI-TOF: matrix-assisted laser desorption/ionization; PAGE: polyacrylamide gel electrophoresis; PMSF: phenylmethylsulfonyl fluoride; SDS: sodium dodecyl sulphate; SIM: selected ion monitoring; TFA: trifluoroacetic acid; Tris: tris(hydroxymethyl)aminomethane; X-$\beta$-GlcNAc: 5-bromo-4-chloro-3-indolyl-acetyl-beta-D-glucosaminide; X-$\beta$-GlcNAc-Glc: 5-bromo-4-chloro-3-indolyl-acetyl-beta-D-glucosaminide-$\alpha$1,4-Glc

## Introduction

Enzymes involved in bacterial starch and maltose metabolism have been intensively studied for more than 60 years.[1] Maltose degradation can be either catalyzed by the hydrolytic action of $\alpha$-glucosidases, or by maltose phosphorylases in the presence of inorganic phosphate.[2,3] The product of the phosphorolysis reaction, glucose-1-phosphate, can be utilized after subsequent isomerization by phosphoglucomutase for the glycolytic energy pathway in bacteria.[4] Maltose phosphorylase (EC 2.4.1.8) belongs to glycosyl hydrolase family GH 65 and catalyzes the phosphorolysis of maltose with the inversion of anomeric configuration to form $\beta$-glucose-1-phosphate ($\beta$-Glc-1-P) and glucose. Many probiotic as well as pathogenic bacteria have been shown to bear maltose phosphorylase or closely related enzymes in their genome.[5–7] Several maltose phosphorylase homologues have already been cloned and characterized,[8,9] and the crystal structure of the maltose phosphorylase from *Lactobacillus brevis* has been solved.[10] The high specificity of this enzyme towards maltose has permitted applications such as the determination of $\alpha$-amylase activity,[11] or the detection of maltose in oligosaccharide mixtures.[12] Furthermore, maltose phosphorylases have been used in conductometric sensors for detecting phosphate in foodstuffs.[13]

Maltose phosphorylases have also been used for the synthesis of unnatural disaccharides such as glucosylfucose,[14] or for the synthesis of monoglucosides such as salicylglucose.[15] Although a number of novel maltose phosporylases have been reported over the last years, the search for enzymes with broader substrate promiscuity remains challenging.

Here we describe the identification of a previously uncharacterized maltose phosphorylase from *Emticicia oligotrophica*, a bacterium initially isolated from warm spring water in Assam, India.[16] Although the bacterium was discovered almost a decade ago, the biochemical potential of this organism remains unexplored. Using *E. oligotrophica* maltose phosphorylase for the enzymatic synthesis of indoxyldiglycosides is the first step in broadening our knowledge of the biotechnological potential of this microorganism.

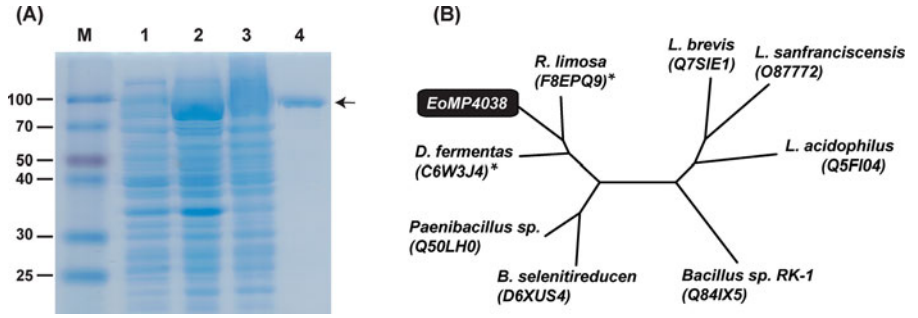



**Figure 1.** SDS-PAGE analysis and phylogenic relationship of EoMP4038. (A) SDS-PAGE at different expression and purification stages: **M** – protein marker; **1** – cell pellet before induction; **2** – cell pellet after induction with IPTG; **3** – supernatant after cell lysis; **4** – purified protein. (B) Phylogenetic relationship of maltose and kojibiose phosphorylases from selected bacterial species to *E. oligotrophica* isoform. UniProt identifiers are shown in parenthesis. Asterisks indicate putative, non-characterized enzymes.

## Results and discussion

### *Cloning, expression, and purification of maltose phosphorylase from E. oligotrophica*

The putative maltose phosphorylase gene was chosen from the annotated genome sequence of *E. oligotrophica* (eo.ggbrc.com) provided by the Pathosystems Resource Integration Center (PATRIC).[17] The open reading frame consisted of 2325 base pairs and was annotated based on its consecutive gene number (EoMP4038) in the genome sequence. The screening of the genome sequence revealed a second open reading frame belonging to glycoside hydrolase family 65, which maltose phosphorylases amongst trehalases and other phosphorylases belong to. However, the low homology of only 18% to any other characterized family member suggests a different functionality of this homologue. The amplified gene product of EoMP4038 was successfully cloned and ligated into a pET30a expression vector construct containing a hexa-histidine fusion tag, to facilitate protein purification using $Ni^{+2}$-nitrilotriacetate agarose affinity chromatography. The concentration of the purified protein was determined to be 1.8 mg/mL. A single protein band with an apparent molecular weight between 70 and 100 kDa was observed using SDS-PAGE (Fig. 1A), which is in good agreement with the theoretical mass of the expression product (89 kDa).

The amino acid sequence of EoMP4038 was compared to the amino acid sequences of various homologues of bacterial origin using a translated nucleotide database search (TBLASTN). The best homology scores were found for kojibiose phosphorylases from *Runella limosa* (UniProt: C6W3J4) and *Dyadobacter fermentas* (UniProt: F8EPQ9), both sharing 68% homology with EoMP4038. Furthermore, EoMP4038 shares close homologies with maltose phosphorylases from *Paenibacillus* sp. (58%, UniProt: Q50LH0), *Bacillus selenitireducens* (56%, UniProt: D6XUS4), *Bacillus* sp. strain RK-1 (47%, UniProt: Q84IX5), *Lactobacillus acidophilus* (47%,

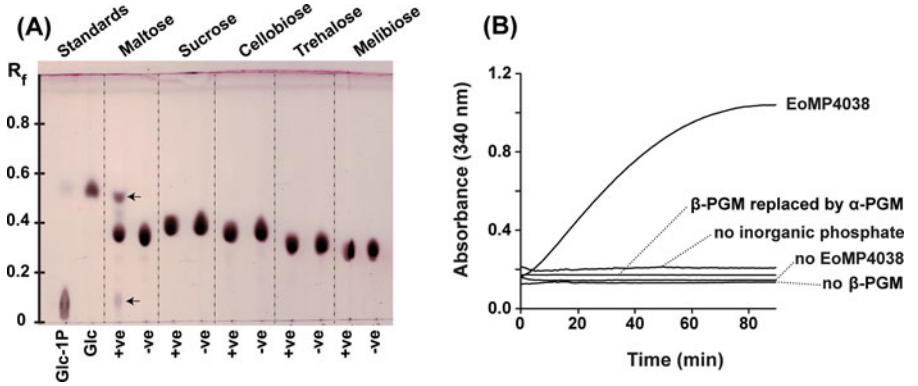



**Figure 2.** Analysis of disaccharide phosphorolysis catalysed by EoMP4038: (A) TLC-based assay; positive (+ve) and negative (−ve) are reaction mixtures with EoMP4038 and without EoMP4038, respectively. Arrows indicate the phosphorolysis products, glucose and glucose-1-phosphate. (B) enzyme-coupled microplate-based assay.

UniProt: Q5FI04), *Lactobacillus brevis* (46%, UniProt: Q7SIE1), and *Lactobacillus sanfranciscensis* (45%, UniProt: O87772) (Fig. 1B, Supplementary Fig. S1).

### *Phosphorolytic specificity of EoMP4038*

The specificity of EoMP4038 phosphorolysis was initially tested by thin layer chromatography (TLC) using a range of disaccharides as substrates. Only maltose was converted to glucose and $\beta$-Glc-1-P, indicating that the enzyme had high selectivity to $\alpha$-1,4-glycosydic linkage. No phosphorolysis was observed for sucrose, cellobiose, trehalose, and melibiose (Fig. 2A). Activity of EoMP4038 towards maltose was further confirmed using a photometric assay by monitoring the formation of NADPH resulting from the coupled enzymatic reactions catalyzed by EoMP4038, $\beta$-phosphoglucomutase and glucose-6-phosphate dehydrogenase (Fig. 2B). The exchange of $\beta$-phosphoglucomutase with $\alpha$-phosphoglucomutase led to no formation of NADPH, which indicates that $\beta$-Glc-1-P, but not $\alpha$-Glc-1-P, was produced from maltose in the phosphorolylis reaction.

The strict specificity of EoMP4038 toward maltose is in agreement with the specificity described for maltose phosphorylases from several other bacteria such as *B. selenitireducens*,[18] *L. acidophilus*[19] and *Paenibacillus* sp.[8] One exception is the maltose phosphorylase from *Bacillus* sp. RK-1, which also showed activity towards the $\alpha$-1,6- and $\beta$-1,6-linkages present in isomaltose and gentiobiose, respectively.[20]

### *Enzymatic properties of EoMP4038*

The pH optimum was determined at pH 7.0, and showed relatively good activities over a pH range between pH 5.0 and 9.0 (Fig. 3A). This pH optimum is comparable with the ones reported for maltose phosphorylases from *Paenibacillus* sp. (pH 6.0 to 7.0)[8] and for the *Bacillus* sp. (pH 7.0 to 7.5).[20] The temperature optimum was 45°C, and although EoMP4038 still performed well up to 65°C, the enzyme's activity

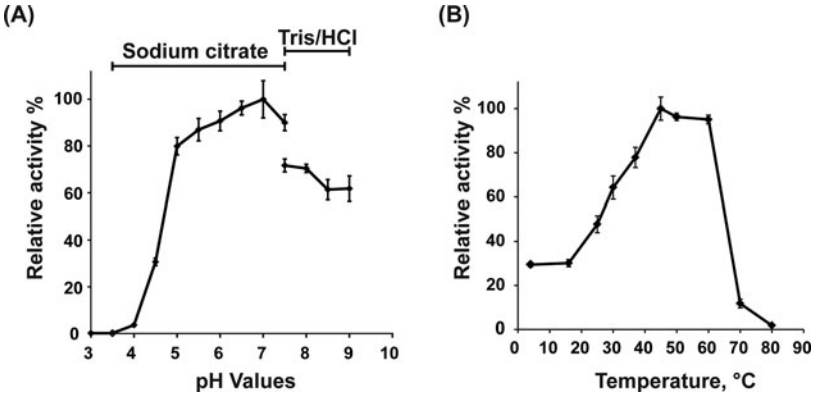



**Figure 3.** Biochemical properties of EoMP4038. (A) Effects of temperature and (B) pH-dependency of on the activity of the enzyme.

declined drastically at temperatures above 70°C (Fig. 3B). This temperature optimum is lower compared to *Paenibacillus* sp. and *Bacillus* sp. maltose phosphorylases (50°C and 65°C, respectively).[8,20]

The kinetic parameters of EoMP4038 phosphorolytic activity are listed in Table 1. The $K_M$ value of 0.59 mM is lower than the data reported for characterized maltose phosphorylases from *Paenibacillus* sp. (4.0 mM)[8] and *Bacillus* sp. (12.3 mM),[20] which might be advantageous when using EoMP4038 in reactions with low maltose concentrations.

## *Synthesis of indoxyldiglycosides by reverse phosphorolysis*

The enzymatic addition of $\alpha$-1,4-linked glucose to various indoxysugars was examined in a coupled reaction. An excess of maltose and inorganic phosphate was used to generate $\beta$-glucose-1-phosphate by phosphorolysis, which was consequently utilized as a glucosyl donor for the glycosylation of the indoxysugars (Fig. 4).

The advantage of this coupled reaction is that inexpensive maltose and inorganic phosphate can be used to generate the glucose donor $\beta$-glucose-1-phosphate. The use of indoxyglycosides as acceptors allowed facile separation by reversed phase HPLC and monitoring of the donor substrates and reaction product due to the presence of the 5-bromo-4-chloro-3-indolyl chromophore.

The conjugation of $\alpha$-1,4-linked glucose was observed when X-$\beta$-GlcNAc, X-$\alpha$-Glc, X-$\beta$-Glc were used as acceptor substrates in a reaction catalyzed by EoMP4038, whereas no product formation was observed for the other acceptor substrates tested (Table 2).

**Table 1.** Kinetic parameters of EoMP4038.

| Enzyme | $K_M$ (mM) | $V_{max}$ (mM/min) | $K_{cat}$ (1/s) |
|---|---|---|---|
| EoMP4038 | 0.59 ± 0.008 | 0.114 ± 0.007 | 0.29 ± 008 |

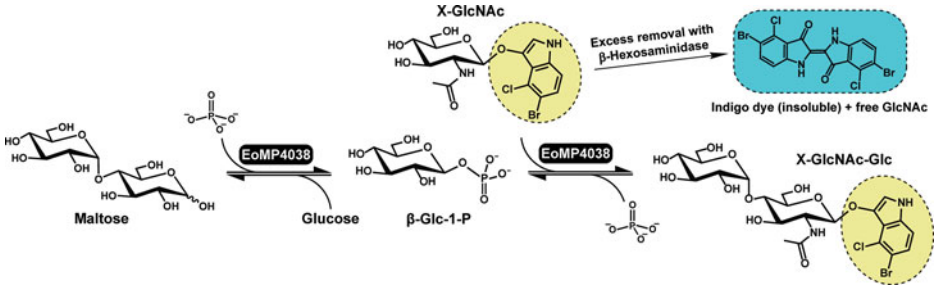



**Figure 4.** Scheme of indoxyldiglycoside synthesis (here exemplified with X-GlcNAc) using maltose phosphorylase in a coupled enzymatic reaction. The aromatic indoxyl groups are highlighted.

The analysis of the enzymatic glucosylation reaction using HPLC-ESI-MS and MALDI-TOF-MS for the acceptor substrate X-$\beta$-acetylglucosamine (X-$\beta$-GlcNAc) is exemplified in Figs. 5 and 6. Although a product yield of only 3.1% could be achieved after 16 h of reaction, the resulting X-$\beta$-acetylglucosamine-$\alpha$1,4-glucose could be cleanly separated using reversed phase HPLC with a retention time of 7.6 min (Fig. 5A). Treatment of the reaction mixture with $\beta$-hexosaminidase led to complete hydrolysis of the starting material X-$\beta$-GlcNAc, resulting in the generation of free GlcNAc and an insoluble, dark blue indigo dye, which can easily be removed by centrifugation. Further sample analysis was performed using HPLC-ESI-MS: signals at m/z values corresponding to both substrate and product (459.00 $[M + H]^+$ and 611.00 $[M + H]^+$, respectively) were detected in reaction mixtures containing EoMP4038, whereas a signal corresponding to the product could not be detected in mixtures which did not contain EoMP4038 (Fig. 5B). In addition, the identity of the synthesized disaccharide was also confirmed by MALDI–TOF mass spectrometry (Fig. 6).

Several maltose phosphorylases have been reported to accept a broader variety of free monosaccharide substrates, such as the maltose phosphorylases from *Lactobacillus acidophilus* and *Propionibacterium freudenreichi* which can also utilize mannose-, fucose-, and xylose[14,19] and achieve product yields of more than 50%. Interestingly, maltose phosphorylase from *Enterococcus hirae* has been used to $\alpha$-glucosylate various alcohols, such as butanol and benzyl alcohol.[15] Furthermore, a maltose phosphorylase from *Bacillus celenitireducens* has been applied to the synthesis of branched glucose containing trisaccharides.[18] However, despite the relatively

**Table 2.** Transglycosylation activity of EoMP4038.

| Acceptor | Expected product | Product yield, % |
|---|---|---|
| X-$\beta$-acetylglucosamine | X-$\beta$-acetylglucosamine-$\alpha$1,4-glucose | 3.1 |
| X-$\alpha$-glucose | X-$\alpha$-glucose-$\alpha$1,4-glucose | 7.8 |
| X-$\beta$-glucose | X-$\beta$-glucose-$\alpha$1,4-glucose | 2.7 |
| X-$\alpha$-galactose | X-$\alpha$-galactose-$\alpha$1,4-glucose | ND |
| X-$\alpha$-fucose | X-$\alpha$-fucose-$\alpha$1,4-glucose | ND |
| X-$\alpha$-mannose | X-$\alpha$-mannose-$\alpha$1,4-glucose | ND |
| X-$\beta$-mannose | X-$\beta$-mannose-$\alpha$1,4-glucose | ND |
| X-$\beta$-xylose | X-$\beta$-xylose-$\alpha$1,4-glucose | ND |

ND – product formation not detected.

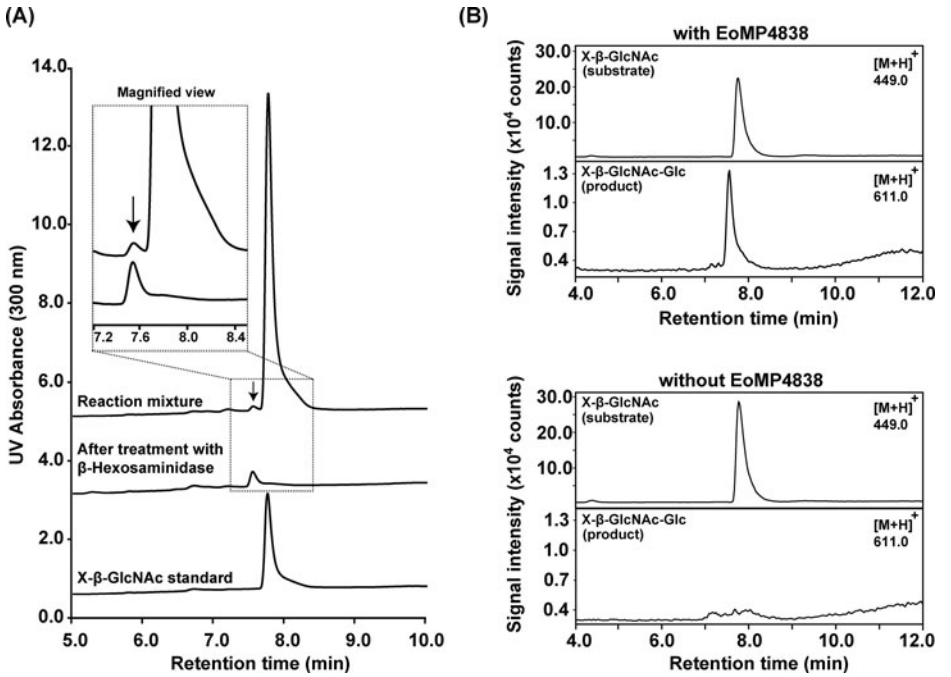



**Figure 5.** HPLC and MS analysis of the enzymatic $\alpha$-glucosylation transfer: (A) HPLC chromatogram of the reaction mixture, purified product (X-$\beta$-GlcNAc-$\alpha$1,4-Glc) and the substrate standard (X-$\beta$-GlcNAc). The arrows indicate the product peak; (B) HPLC-ESI-MS signal response of the corresponding substrate and product masses and substrate masses for reaction mixtures with EoMP4038 (top panel) and without EoMP4038 (bottom panel).

low yield of glucose-modified indoxyglycosides by EoMP4038, this first example of the use of sugar phosphorylases for the synthesis of anomerically modified glycosides shows the potential and promiscuity of these class of enzymes.

## Conclusion

In this report we describe the identification of maltose phosphorylase from *E. oligotrophica*, a maltose-specific enzyme capable of reverse phosphorolysis using various indoxyglycosides as acceptor substrates. The synthesis of several $\alpha$-glucose-terminated indoxyldisaccharides could be demonstrated by analyzing reaction mixtures using LC-MS and MALDI-TOF-MS. The screening of other maltose phosphorylases and the optimization of the reaction conditions to obtain higher product yields are part of our current research effort.

## Experimental

### *Bacterial strains and chemicals*

*Emticicia oligotrophica* strain DSM 17448 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). DNA polymerases were obtained

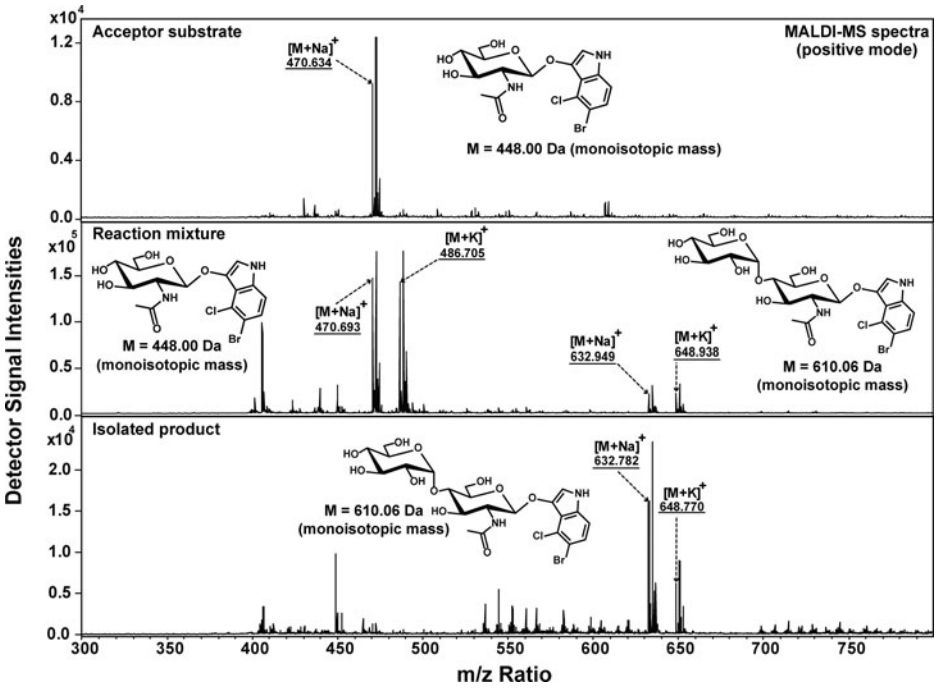



**Figure 6.** MALDI-TOF-MS spectra of the acceptor substrate (X-$\beta$-GlcNAc, top panel), the enzymatic reaction reaction mixture (middle panel), and the isolated product after HPLC purification (X-$\beta$-GlcNAc-$\alpha$1,4-Glc, lower panel).The natural isotope ratio for bromine ($^{79}$Br:$^{81}$Br = 50.7:49.3) and for chlorine ($^{35}$Cl:$^{37}$Cl = 75.8:24.2) substituents of the indoxyl group are the reason for the additional masses (signals with the +2 and +4 Da) observed in the spectra.

from Takara (Japan); restriction endonucleases and T4 ligase were from Thermo Fisher Scientific (Shanghai); DNA Gel Purification Kit and Plasmid Extraction Kit were purchased from Axygen (Beijing); maltose was obtained from Aladdin (Shanghai, China); 5-bromo-4-chloro-3-indolyl-glycoside derivatives of $\alpha$-glucose, $\beta$-glucose, $\beta$-acetylglucosamine, $\alpha$-galactose, $\alpha$-L-fucose, $\alpha$-mannose, $\beta$-mannose and $\beta$-xylose were obtained from Carbosynth (Berkshire, UK). All chemicals used in the work were of the highest grade available.

### *Gene cloning and construction of the expression plasmid*

Genomic DNA from *E. oligotrophica* was isolated as previously described.[21] The putative maltose phosphorylase gene was designated EoMP4038 according to its sequential gene number in the *E. oligotrophica* genome provided by the Pathosystems Resource Integration Center (PATRIC)[17] (eo.ggbrc.com). The gene encoding EoMP4038 was amplified *via* polymerase chain reaction (PCR) with Primestar HS DNA Polymerase, using the genomic DNA of *E. oligotrophica* as a template with the following primer pair: forward, 5′-CGGGATCCATGAAAAATTATATAACACACGATG-3′ and reverse, 5′-CCGCTCGAGTTAGTATTCTACACTGGCCGAAG-3′ containing BamHI and

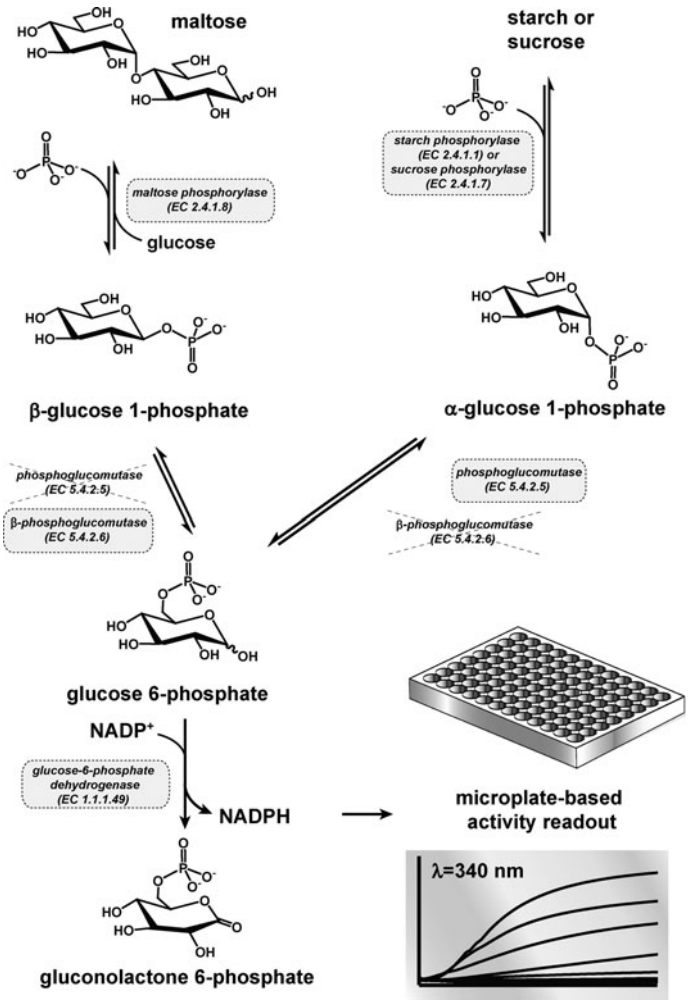



**Figure 7.** Microplate-based spectrophotometric detection of maltose phophorylase activity using a coupled enzymatic assay with $\beta$-phosphoglucomutase and glucose-6-phospate dehydrogenase (left branch). A more commonly applied application of the microplate based assay for determining the activities of starch phosphorylase or sucrose phosphorylase is shown in the right branch of the figure.

XhoI restriction sites (underlined), respectively. In brief, 35 PCR cycles consisting of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 3 min were performed. The resulting PCR product was digested using the corresponding restriction enzymes, purified and ligated into the predigested expression vector pET30a (Novagen). The EoMP4038-containing vector construct was transformed into *E. coli* Mach1 T1 cells (Invitrogen) and plated onto Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl and 1.5% agarose) supplemented with kanamycin (50 $\mu$g/mL). The gene sequence of candidate clones was further verified by Sanger sequencing (Genscript, Nanjing).

### *Expression and purification of recombinant protein*

*E. coli* BL21 (DE3) cells were used as a host for recombinant protein expression which was carried out as described previously.[22] Briefly, cells were cultured in

400 mL of LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) with shaking at 37°C, 250 rpm until the optical density ($OD_{600}$) reached 0.5. Target protein expression was induced by addition of 1 mM isopropyl-$\beta$-D-thiogalactopyranoside (IPTG) with 24 h of further culture shaking at 18°C. Cells were harvested by centrifugation (5000 g, 20 min, 4°C), resuspended in 10 mL of lysis buffer (50 mM Tris/HCl, 1% Triton X-100, 100 mM NaCl and 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 8.0) and disintegrated by ultrasonication (40 on/off cycles with 20 $\mu$m amplitude for 15 s at 4°C) for 20 min in ice water. Cell debris were removed by centrifugation (20000 g, 20 min, 4°C). The resulting supernatant was loaded onto $Ni^{2+}$-nitrilotriacetate agarose affinity column (2 mL bed volume, Qiagen), washed with 15 column volumes of protein binding buffer (50 mM NaCl, and 50 mM Tris/HCl, pH 8.0) and 5 column volumes of washing buffer (50 mM NaCl, 50 mM Tris/HCl, and 10 mM imidazole, pH 8.0) to avoid unspecific protein adsorption. The target protein with a N-terminally fused $His_6$-tag was eluted from the affinity column using elution buffer (50 mM NaCl, 50 mM Tris/HCl, and 500 mM imidazole, pH 8.0). The purity of the recombinant protein was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions as described by Laemmli,[23] and visualized by staining with Coomassie Brilliant Blue G-250. The purest fractions were pooled and protein concentration was performed using a Bradford-based protein quantification assay kit (Sangon Biotech).

### *Chromatographic analysis of phosphorolysis products*

Thin-layer chromatography was employed to detect the products of the phosphorolysis reactions. A 20 µL reaction mixture containing 20 mM $NaH_2PO_4$, 50 mM Tris/HCl buffer (pH 7.5), 0.5 mM $MgCl_2$, 95 mU of EoMP4038, and 50 mM of a disaccharide substrate (maltose, sucrose, cellobiose, trehalose, or melibiose) was incubated overnight at 37°C and spotted on silica-gel TLC plate Merck KG (Type 60 $F_{254}$, Darmstadt, Germany). The plates were developed using a mobile phase mixture and staining solutions as described previously[24] (acetonitrile-acetic acid-$H_2O$, 7:1.5:1.5, v/v/v). Substrate and product spots from the reaction were visualized by soaking the plate in a 5% solution of methanolic $H_2SO_4$ followed by heating at 95°C for 10 min. Samples lacking the enzyme, and sugar standards such as glucose-1-phosphate (Glc-$\alpha$-1P) and glucose were used as controls.

### *Enzymatic assay*

The activity of EoMP4038 was determined using a coupled assay which allows the generation of NADPH to be monitored at a wavelength of 340 nm. Typically, the reaction mixture (25 µL) contained 50 mM maltose, 10 mM $NaH_2PO_4$, 0.2 mM $MgCl_2$, 50 mM Tris/HCl buffer (pH 7.5), 1 mM $NADP^+$, and 0.5 mU, 0.5 mU and 50 mU of EoMP4038, $\beta$-phosphoglucomutase (EC 5.4.2.6 Figs. S2 and 3), and glucose-6-phosphate dehydrogenase (EC 1.1.1.49, Sigma aldrich), respectively. In

addition, an activity assay where $\beta$-phosphoglucomutase was replaced with 0.5 mU of $\alpha$-phosphoglucomutase (EC 5.4.2.2 Figs. S4 and 5) was carried out as a negative control (Fig. 7). Further negative controls included reaction mixtures lacking EoMP4038, inorganic phosphate, or $\beta$-phosphoglucomutase. Absorbance was monitored for 90 min continuously on a 384-well-microplate reader (Thermo Multiskan).

### Determination of the substrate promiscuity of EoMP4038

The synthetic potential of EoMP4038 was evaluated using maltose as a $\beta$-Glc-1-P donor and different 5-bromo-4-chloro-3-indolyl-glycoside derivatives (X-glycosides), including X-$\alpha$-Glc, X-$\beta$-Glc, X-$\beta$-GlcNAc, X-$\alpha$-Gal, X-$\alpha$-Fuc, X-$\beta$-Xyl, X-$\alpha$-Man and X-$\beta$-Man as acceptor substrates. Reaction mixtures (20 µL) contain 50 mM maltose, 50 mM Tris/HCl, pH 7.5, 2.5 mM $NaH_2PO_4$, 0.5 mM $MgCl_2$, 1 mM X-glycoside, and 86 mU of EoMP4038, were incubated at 37°C for 16 h. Reactions were quenched by adding 20 µL of chloroform to the reaction mixture, followed by centrifugation, and the upper phase containing the enzyme-free analyte was collected and subjected to HPLC analysis as previously described,[25] with slight modifications. The analytes were separated using a reversed-phase HPLC column (Phenomenex Hyperclone, 5 µm, C18, 120 Å, 250 × 4.6 mm) at a constant flow rate of 0.8 mL/min and continuous UV-detection at 300 nm. The mobile phases used were aqueous ammonium formate (50 mM, pH 4.5) and acetonitrile (solvents A and B, respectively). After sample injection (10 µL), a linear gradient of 30–60% solvent B was applied from 0 to 5 min, followed by an increase of solvent B to 90% over 1 min, and held at 90% for another 2 min. B was then decreased to 30% over 1 min, and the column was equilibrated under the initial conditions for 6 min. Product and substrate masses were detected by ESI-MS (Nexera MS 2020, Shimadzu Corporation, Kyoto, Japan).

### MALDI –TOF MS analysis of reverse phosphorolysis reaction compounds

The acceptor substrate, reaction mixtures and the $\beta$-hexosaminidase-treated reaction product were lyophilized and resuspended in 10 µL $H_2O$, and then mixed in 1:1 ratio with a solution of 2,5-dihydroxybenzoic acid (DHB) matrix containing 4 mg/mL DHB in 30% acetonitrile, 0.1% TFA, and 1 mM sodium chloride. Mass spectrometry was performed using a Autoflex Speed MALDI-TOF mass spectrometer (Bruker Daltonics, Germany).

### Biochemical analysis

The optimum pH, optimum temperature and kinetic parameters of EoMP4038 were determined by measuring the amount of liberated glucose from maltose during enzymatic phosphorylsis using a glucose oxidase-peroxidase assay kit which

was determined photometrically at 505 nm using 4-aminoantipyrine (Shanghai Ronsheng Biotech Co. Ltd). The incubation time for all assays was 15 min and the reaction was terminated by heating at 90°C for 10 min. For measuring the pH optima, the reaction mixture contained maltose (20 mM), $NaH_2PO_4$ (10 mM), $MgCl_2$ (0.5 mM), and either sodium citrate buffer in the lower pH range (pH 3.5–7.5, 50 mM) or Tris/HCl in the higher pH range (pH 7.5–9.0, 50 mM); Sodium citrate buffer (pH 7.0, 50 mM) was chosen for determining the temperature optimum, by testing the enzyme's activity at various temperatures ranging from 4–80°C. The kinetic parameters of EoMP4038 were also determined photometrically, using maltose concentrations between 0.05 and 5 mM, and by applying the non-linear regression model provided by the Labplot data analysis software (version 2.0.1).

## Funding

This work was supported in part by the Natural Science Foundation of China (grant number 31471703 and A0201300537 to J.V. and L.L.) and the 100 Foreign Talents Plan (grant number JSB2014012 to J.V.).

## Acknowledgments

The authors would like to thank Dr. Louis Conway (GGBRC, Nanjing) for language editing of this manuscript.

## ORCID

Josef Voglmeir http://orcid.org/0000-0002-4096-4926

## References

1. Puyet, A. Expression of bacterial genes involved in maltose metabolism. *World Journal of Microbiology & Biotechnology* **1993**, *9*, 455–460.
2. Andersson, U.; Radstrom, P. Beta-Glucose 1-phosphate-interconverting enzymes in maltose- and trehalose-fermenting lactic acid bacteria. *Environmental Microbiology* **2002**, *4*, 81–88.
3. Le Breton, Y.; Pichereau, V.; Sauvageot, N.; Auffray, Y.; Rince, A. Maltose utilization in Enterococcus faecalis. *Journal of Applied Microbiology* **2005**, *98*, 806–813.
4. Levander, F.; Andersson, U.; Radstrom, P. Physiological role of beta-phosphoglucomutase in Lactococcus lactis. *Applied and Environmental Microbiology* **2001**, *67*, 4546–4553.
5. Huwel, S.; Haalck, L.; Conrath, N.; Spener, F. Maltose phosphorylase from Lactobacillus brevis: purification, characterization, and application in a biosensor for ortho-phosphate. *Enzyme and Microbial Technology* **1997**, *21*, 413–420.
6. Martin, S.A.; Russell, J. B. Transport and phosphorylation of disaccharides by the ruminal bacterium Streptococcus bovis. *Applied and Environmental Microbiology* **1987**, *53*, 2388–2393.

7. Nidetzky, B.; Weinhausel, A.; Griessler, R.; Kulbe, K. D. Enzymatic-Synthesis of Alpha-Glucose-1-Phosphate - a Study Employing a New Alpha-1,4 Glucan Phosphorylase from Corynebacterium-Callunae. *Journal of Carbohydrate Chemistry* **1995**, *14*, 1017–1028.
8. Hidaka, Y.; Hatada, Y.; Akita, M.; Yoshida, M.; Nakamura, N.; Takada, M.; Nakakuki, T.; Ito, S.; Horikoshi, K. Maltose phosphorylase from a deep-sea Paenibacillus sp.: Enzymatic properties and nucleotide and amino-acid sequences. *Enzyme and Microbial Technology* **2005**, *37*, 185–194.
9. Inoue, Y.; Yasutake, N.; Oshima, Y.; Yamamoto, Y.; Tomita, T.; Miyoshi, S.; Yatake, T. Cloning of the maltose phosphorylase gene from Bacillus sp. strain RK-1 and efficient production of the cloned gene and the trehalose phosphorylase gene from Bacillus stearothermophilus SK-1 in Bacillus subtilis. *Bioscience, Biotechnology, and Biochemistry* **2002**, *66*, 2594–2599.
10. Egloff, M. P.; Uppenberg, J.; Haalck, L.; van Tilbeurgh, H. Crystal structure of maltose phosphorylase from Lactobacillus brevis: unexpected evolutionary relationship with glucoamylases. *Structure* **2001**, *9*, 689–697.
11. Poudrier, S. M.; Oyen, M. T. Stable reagent and kinetic assay for alpha-amylase. US patent **1991**, 4990445A 9120.
12. Kamogawa, A.; Yokobayashi, K.; Fukui, T. An enzymatic method for the determination of maltose in the presence of other oligosaccharides. *Analytical Biochemistry* **1974**, *57*, 303–305.
13. Zhang, Z.; Jaffrezic-Renault, N.; Bessueille, F.; Leonard, D.; Xia, S.; Wang, X.; Chen, L.; Zhao, J. Development of a conductometric phosphate biosensor based on tri-layer maltose phosphorylase composite films. *Analytica Chimica Acta* **2008**, *615*, 73–79.
14. Aisaka, K.; Masuda-Kato, T.; Chikamune, T.; Kamitori, K.; Uosaki, Y.; Saito, Y. Enzymatic synthesis of novel disaccharides using disaccharide phosphorylases. *Journal of Bioscience and Bioengineering* **2000**, *90*, 208–213.
15. Kino, K.; Shimizu, Y.; Kuratsu, S.; Kirimura, K. Enzymatic synthesis of alpha-anomer-selective D-glucosides using maltose phosphorylase. *Bioscience, Biotechnology, and Biochemistry* **2007**, *71*, 1598–1600.
16. Saha, P.; Chakrabarti, T. Emticicia oligotrophica gen. nov., sp. nov., a new member of the family 'Flexibacteraceae', phylum Bacteroidetes. *International Journal of Systematic and Evolutionary Microbiology* **2006**, *56*, 991–995.
17. Wattam, A. R.; Abraham, D.; Dalay, O.; Disz, T. L.; Driscoll, T.; Gabbard, J. L.; Gillespie, J. J.; Gough, R.; Hix, D.; Kenyon, R.; Machi, D.; Mao, C.; Nordberg, E. K.; Olson, R.; Overbeek, R.; Pusch, G. D.; Shukla, M.; Schulman, J.; Stevens, R. L.; Sullivan, D. E.; Vonstein, V.; Warren, A.; Will, R.; Wilson, M. J.; Yoo, H. S.; Zhang, C.; Zhang, Y.; Sobral, B. W. PATRIC, the bacterial bioinformatics database and analysis resource. *Nucleic Acids Research* **2014**, *42*, D581–591.
18. Nihira, T.; Saito, Y.; Kitaoka, M.; Otsubo, K.; Nakai, H. Identification of Bacillus selenitireducens MLS10 maltose phosphorylase possessing synthetic ability for branched alpha-D-glucosyl trisaccharides. *Carbohydrate Research* **2012**, *360*, 25–30.
19. Nakai, H.; Dilokpimol, A.; Abou Hachem, M.; Svensson, B. Efficient one-pot enzymatic synthesis of alpha-(1–>4)-glucosidic disaccharides through a coupled reaction catalysed by Lactobacillus acidophilus NCFM maltose phosphorylase. *Carbohydrate Research* **2010**, *345*, 1061–1064.
20. Inoue, Y.; Ishii, K.; Tomita, T.; Fukui, F. Purification and characterization of maltose phosphorylase from Bacillus sp. RK-1. *Bioscience, Biotechnology, and Biochemistry* **2001**, *65*, 2644–2649.
21. Liu, S.; Kulinich, A.; Cai, Z. P.; Ma, H. Y.; Du, Y. M.; Lv, Y. M.; Liu, L.; Voglmeir, J. The Fucosidase-Pool of Emticicia oligotrophica: Biochemical Characterisation and Transfucosylation Potential. *Glycobiology* **2016**, 26, 871–879.

22. Wei, S.; Kulinich, A.; Duan, X. C.; Liu, L.; Voglmeir, J. Discovery and biochemical characterization of UDP-glucose dehydrogenase from Granulibacter bethesdensis. *Protein and Peptide Letters* **2015**, *22*, 628–634.
23. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.
24. Kulinich, A.; Liu, S.; Ma, H. Y.; Lv, Y. M.; Liu, L.; Voglmeir, J. Identification and Characterization of Two Novel Alpha-D-Galactosidases from Pedobacter heparinus. *Protein and Peptide Letters* **2015**, *22*, 1052–1059.
25. Huang, K.; Wang, M. M.; Kulinich, A.; Yao, H. L.; Ma, H. Y.; Martinez, J. E.; Duan, X. C.; Chen, H.; Cai, Z. P.; Flitsch, S. L.; Liu, L.; Voglmeir, J. Biochemical characterisation of the neuraminidase pool of the human gut symbiont Akkermansia muciniphila. *Carbohydrate Research* **2015**, *415*, 60–65.