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The mannitol utilization system of the marine bacterium *Zobellia galactanivorans*

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Running title: Mannitol degradation by *Zobellia galactanivorans*

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ABSTRACT

Mannitol is a polyol which occurs in a wide range of living organisms where it fulfills different physiological roles. Particularly, mannitol can account up to 20-30% of the dry weight of brown algae, and is likely to be an important source of carbon for marine heterotrophic bacteria. *Zobellia galactanivorans* (Flavobacteria) is a model to study pathways involved in degradation of seaweed carbohydrates. Annotation of its genome revealed the presence of genes potentially involved in mannitol catabolism, and we describe here the biochemical characterization of a recombinant mannitol-2-dehydrogenase (M2DH) and of a fructokinase (FK). Among the observations, the M2DH of *Z. galactanivorans* was active as a monomer, did not require metal ions for catalysis, and features narrow substrate specificity. The characterized FK was active on fructose and mannose in presence of a monocation, preferentially K^+ . Furthermore, genes coding for both proteins were adjacent in the genome, and located directly downstream three loci likely to encode an ATP binding cassette (ABC) transporter complex, suggesting organization into an operon. Gene expression analysis supported this hypothesis, and showed the induction of these five genes after culturing *Z. galactanivorans* in presence of mannitol as sole source of carbon. This operon for mannitol catabolism was identified in only six genomes of *Flavobacteriaceae* among the 76 publicly available at the time of the analysis. It is not conserved in all *Bacteroidetes* because some species contained a predicted mannitol permease instead of a putative ABC transporter complex upstream M2DH and FK ortholog genes.

INTRODUCTION

Brown algae (Phaeophyceae) are the dominant macroalgae in temperate and polar regions and thus play a crucial role in the primary production of coastal ecosystems (1). They contain large amounts of different structural and storage carbohydrates. For instance, their extracellular matrix is formed by the accumulation of cellulose, fucanes, and alginates (2-4), while they stored carbon by accumulating laminarin and mannitol (5). The potential of this biomass resource for the production of liquid biofuels (6), including ethanol from alginate and mannitol (7, 8), and for the implementation of biorefineries (9), has been recently highlighted.

Mannitol can, depending of species, represent up to 20–30% of the dry weight of brown seaweed (10). In the genomic and genetic model of brown alga *Ectocarpus* sp., formerly included in *E. siliculosus* (11), it has been observed that the content of this polyol varies according to the diurnal cycle (12), and that this compound is likely to act as an osmoprotectant or local compatible osmolyte (13). Mannitol is localized in the cytosol, and is also present at the reducing end of vacuolar laminarin molecules of the M series (in contrast to the G series which contain only glucose residues) (14). Mannitol in brown algae is produced directly from the photoassimilate fructose-6-phosphate (F6P) by two steps: F6P is first reduced by mannitol-1-phosphate dehydrogenase (M1PDH) into mannitol-1-phosphate, which is then converted into mannitol by mannitol-1-phosphatase (M1Pase) (5, 15, 16). Mannitol is thought to be recycled by the successive action of a mannitol-2 dehydrogenase (M2DH) and of a hexokinase (HK) (5, 17), but little is known on the functioning of these enzymes in brown algae (18).

In contrast, mannitol metabolism has been intensively studied in *Escherichia coli* (19) and other bacteria such as *Bacillus subtilis* (20, 21), *Bacillus stearothermophilus* (22), *Clostridium acetobutylicum* (23), and *Streptococcus mutans* (24). In these terrestrial bacteria, mannitol is taken up by a mannitol-specific phosphoenolpyruvate/carbohydrate

phosphotransferase system (PTS), phosphorylated into mannitol-1-phosphate (M1P) during its transport, and M1P is further oxidized to F6P by a M1P-specific dehydrogenase (25) before entering glycolysis. In the soil bacterium *Acinetobacter baylyi* (26), the M1P dehydrogenase is fused to a haloacid dehalogenase (HAD)-like phosphatase domain at the N-terminus that was shown to catalyze M1P phosphatase activity (27). In *C. acetobutylicum* (23) and *B. stearothermophilus* (22), the mannitol catabolic operon is regulated by two mechanisms: a glucose-mediated catabolite repression, and a transcriptional activation mechanism controlled by MtlR using mannitol as an inducer. Other bacteria such as *Pseudomonas fluorescens* are known to contain a mannitol-2-dehydrogenase, an enzyme that oxidizes mannitol into fructose, with mannitol being transported by ATP binding cassette transporters (28). In this organism, fructose is believed to be phosphorylated into fructose-6-phosphate by a kinase coded for by *mtlZ*, a gene of the mannitol catabolic operon (28, 29). In the same way, *Phaeobacter inhibens* DSM17395 imports mannitol via a specific ABC transporter whose corresponding genes are located next to the *mtlK* gene coding for the mannitol-2-dehydrogenase; furthermore, the *frk* gene, encoding a fructokinase, colocalizes with genes corresponding to another ABC transporter (30).

Among the bacteria known to interact with algae, Flavobacteria, and notably several species of *Zobellia*, were found in association with macroalgae (31-33), and isolated from phytoplanktons (34) and from seawater (35). The interactions between *Zobellia* strains and marine algae vary from symbiosis, with some strains inducing the normal differentiation of green macroalgae (36), to algicidal behavior toward dinoflagellate bloom (34). The type species of the *Zobellia* genus, *Zobellia galactanivorans* (formerly known as “*Cytophaga drobachiensis*”), was initially isolated in Roscoff from the red seaweed *Delesseria sanguinea* for its capacity to degrade carrageenans (31). This flavobacterium has been pivotal for the discovery and characterization of enzymes involved in the catabolism of red algal

polysaccharides: kappa-carrageenase (37), iota-carrageenases (38), beta-agarases (39), beta-porphyrases (40), and 3,6-anhydro-L-galactosidases (41, 42). Based on these results, *Zobellia galactanivorans* is gaining interest as a model to study the bioconversion of macroalgal polysaccharides. Furthermore, although this bacterium has been isolated on a red alga, it is also able to metabolize cell wall and storage polysaccharides from brown algae. Indeed it possesses two alginolytic operons induced by the presence of alginate (43), and the two first alginate lyases of this complex system (AlyA1 and AlyA5) have been recently characterized at the biochemical and structural level (44). This bacterium can also grow with brown algal laminarin as the sole carbon source and, among the five putative laminarinases identified in its genome (accession number: FP476056), two GH16 laminarinases, ZgLamA and ZgIamC, have been recently biochemically and structurally analyzed (45). In addition to alginate and laminarin, mannitol is one of the most abundant carbohydrates in brown algae, and the annotation of the *Z. galactanivorans* genome has suggested the presence of proteins potentially involved in the use of this storage compound. Here, we confirm this hypothesis and describe the biochemical characterization of the recombinant mannitol-2-dehydrogenase (ZgM2DH) and fructokinase (ZgFK1) involved in the catabolism of mannitol in *Z. galactanivorans*. This was completed by gene expression analysis indicating that the two corresponding genes are induced by mannitol and organized as an operon.

MATERIALS AND METHODS

Bacterial strain and culture conditions. The type strain Dsij^T of *Z. galactanivorans* (31) was grown in Zobell medium 2216E (tryptone at 5 g l⁻¹ and yeast extract at 1 g l⁻¹ solubilized in seawater) at 20°C. Cells were transferred in 1 ml of Marine Mineral Medium supplemented with 4 ml of glucose or mannitol at 5 g l⁻¹. Briefly, one liter of Marine Mineral Medium is composed of 24.7 g NaCl, 6.3 g MgSO₄·7H₂O, 4.6 g MgCl₂·H₂O, 2 g NH₄Cl, 0.7 g KCl, 0.6 g

CaCl₂, 200 mg NaHCO₃, 100 mg K₂HPO₄, 50 mg yeast extract, 20 mg FeSO₄·7H₂O, in Tris-HCl 50 mM, pH 8.0. Triplicate cultures were made for each substrate condition. Bacteria were grown at 20°C under agitation (180 rpm) until the exponential phase.

Nucleic acid extraction. Genomic DNA was extracted as previously described (46). For RNA extraction, 2 ml of the growth media of cultures at the end of the exponential phase were added to 4 ml of RNA protect reagent. RNAs were then isolated by using the RNA mini kit (Qiagen) following the manufacturer's instructions. Two steps of DNA digestion were performed using the DNase I of Qiagen. RNAs were cleaned-up on mini-column and eluted in 30 µl of RNase-free water. Total elimination of genomic DNA was checked by PCR. For each sample, approximately 250 ng of RNAs were examined by 0.8% agarose gel electrophoresis to check their integrity. The concentrations of RNA samples were determined using a Nanodrop ND-1000 spectrophotometer. The ratio OD₂₆₀/OD₂₈₀ and OD₂₆₀/OD₂₃₀ were calculated to assess the purity of RNA extracts.

RT-PCR and quantitative PCR. Complementary DNA were synthesized on 250 ng of total RNA using the Phusion RT-PCR kit (Finnzymes) with random hexamer primers according to the supplier's instructions, and cDNA samples were diluted to 1 ng µl⁻¹ and stored at -80°C.

For the amplicons named Zg1489-1490, Zg1490-1491, Zg4259-4260, Zg4259-4261, Zg4260-4263 and Zg4262-4264, the PCR reactions were performed using the Advantage 2 PCR kit (Clontech) with 0.2 µM of each primer (Table 1) and 20 ng of cDNA. The PCR program was 94°C for 5 min, 35 cycles (94°C for 30sec, 50°C or 55°C for 30 sec, 72°C for 30 sec or 3 min) followed by 5 min at 72°C. The reaction products were analyzed by 1% agarose gel electrophoresis.

For quantitative PCR, the primers corresponding to the eight candidate genes were designed using the Perl Primer open-sources software (47). Their lengths were between 17

and 22 nucleotides, and T_m between 58 and 60°C. A difference of 1°C was accepted between the forward and the reverse primer. The G/C content of the primers was between 47 and 53%. The dG was greater than -10 kcal mol⁻¹ to avoid the pairing of the primer pairs. The PCR products ranged from 60 to 120 bp and had a G/C content over 60%. The nucleotidic sequences of all primers were compared to the genomic sequence of *Z. galactanivorans* (Barbeyron *et al.*, unpublished data; accession number: FP476056) to check gene-specificity. A PCR on the genomic DNA of *Z. galactanivorans* using the different primer pairs was made to confirm the specific amplification. Quantitative PCR reactions were performed in 96-well plates (ThermoScientific) on a LightCycler 480 (Roche). The composition of each reaction was as follows: 2.5 ng of cDNA, 250 nM of each primer (Table 1), 5 µl of SYBR Green 2X and water for a final volume of 10 µl. The reactions for each gene were technically triplicated. The program was: 5 min at 95°C, followed by 45 cycles of 95°C (10 sec), 51°C (15 sec) and 72°C (15 sec) with a single acquisition mode. Genomic DNA serial dilutions ranging from 10 to 10⁵ copies were amplified by qPCR, in the same run than cDNA samples. The LightCycler480 software was used to obtain Cp values and the PCR efficiencies. The determination of the relative expression was performed with the REST 2009 software using glyA and icdA as reference gene (48). This software determines the ratio corresponding to the relative expression by the equation of Pfaffl: $\text{ratio} = E_{\text{target}}^{\Delta C_t \text{ target}} (\text{check-sample}) / E_{\text{reference}}^{\Delta C_t \text{ reference}} (\text{check_sample})$ where E refers to the efficiency (49). Influence of mannitol on expression of genes of interest was determined by comparing the level of their expression in presence of glucose.

Construction of plasmids for overexpression in Escherichia coli. The coding regions *Zg1491*, *Zg4263* and *Zg4264* were cloned in the expression vector pFO4 as previously described (50). Briefly, the genes were amplified by PCR (used primers listed in Table 1) from *Z. galactanivorans* genomic DNA, PCR fragments were digested by *Bam*HI/*Mfe*I for

Zg1491 and *Bam*HI/*Eco*RI for *Zg4263* and *Zg4264*, ligated in pFO4 vector and recombinant plasmids named pZg1491, pZg4263, and pZg4264 respectively, were transformed in *E. coli* BL21 (DE3). Integrity of their sequences was verified by sequencing.

Nucleotidic and protein sequence analyses. The mannitol degradation related genes of *Z. galactanivorans* were identified in its complete genome (Barbeyron *et al.*, unpublished data; accession number: FP476056) using the program GenDB 2.4 (51). Signal peptide and transmembrane helices were predicted using SignalPv2.0 (52) and TMHMM (53) respectively. The presence of orthologous genes in all available prokaryotic genomes was screened using the genomic BlastP (54) at NCBI (<http://www.ncbi.nlm.nih.gov/genome>, access the 14th of January 2014). BlastP analysis was also done against the UniProtKB/Swiss-Prot database to gain insights on the enzymatic characteristics of the *Zobellia* proteins. The *Z. galactanivorans* target proteins were aligned with their orthologs using MAFFT, applying the iterative refinement method and the scoring matrix Blosum62 (55). To identify putative promoters, intergenic regions were searched for the motif -33/-7 (TTG/TAnnTTTG) with a spacer length ranging from 10 to 30 bp. This motif has been identified as the consensus promoter in *Flavobacterium* species, and is conserved in *Bacteroides fragilis* (56, 57). The TransTermHP (58) software was used to predict putative Rho-independent transcriptional terminators.

Expression and purification of recombinant proteins. *E. coli* strain BL21(DE3) (NovagenR) transformed with plasmids pZg1491, pZg4263, and pZg4264 was grown in ZYP 5052 medium (59) at 20°C for 72 h induced with 0.2% lactose supplemented with 200 µg/ml of ampicillin (final concentration) at 20°C and 200 rpm on an orbital shaker. The purification of recombinant proteins was performed using the system ÄKTA Avant (GE HealthCare) equipped with a HisPrep FF 16/10 column (GE Healthcare) equilibrated in 20 mM Tris-HCl (pH 7.5) buffer containing 200 mM NaCl and 15 mM imidazole. Elution of proteins was

performed using a linear increasing gradient of in 20 mM Tris-HCl pH 7.5 containing 200 mM NaCl and 500 mM imidazole from 0% to 100% within 10 column volumes. Fractions were collected in a 96-well deep plate with 1 ml per well. Aliquots of fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% Criterion precast Bis-Tris gels (Bio-Rad). Protein concentration was measured at 280 nm using a Nanodrop 2000 Spectrophotometer (Thermofisher). A molar extinction coefficient of $58.705 \text{ M}^{-1}.\text{cm}^{-1}$ and a molecular mass of 55.61 kDaltons (kDa) were used for the Zg4263 protein and a molar extinction coefficient of $26.025 \text{ M}^{-1}.\text{cm}^{-1}$ and a molecular mass of 32.69 kDa were considered for Zg4264 to calculate the concentration of pure proteins (Expasy, ProtParam tool, 60). An estimation of the molecular mass of the native recombinant proteins was performed by size exclusion chromatography. Eluted fractions from the Ni^{2+} affinity chromatography were loaded at a flow rate of 1 ml min^{-1} on a calibrated sephacryl HiLoadTM Superdex 200 column (GE Healthcare) equilibrated with buffer containing 20 mM Tris-HCl pH 7.5 and 150 mM NaCl. Fractions were then eluted with the same buffer at a flow rate of 1 ml min^{-1} .

Determination of mannitol-2-dehydrogenase activity. If not noted otherwise, all the assays were carried out in triplicate at 25°C in $100 \mu\text{l}$ reaction volumes. All compounds used were ordered from Sigma-Aldrich. M2DH activity was assayed in both directions, i.e. fructose reduction which produces mannitol and mannitol oxidation which produces fructose, by following changes in absorbance at 340 nm in a microplate Safire2 UV spectrophotometer reader (Tecan). The standard fructose reduction reaction mixture contained 0.1 M Tris-HCl buffer (pH 7), 1 mM fructose, 0.2 mM NADH and 1 to $10 \mu\text{g}$ of purified enzyme. The standard mannitol oxidation reaction contained 0.1 M Tris-HCl (pH 8.5), 1 mM mannitol, 0.5 mM NAD^{+} and 1 to $10 \mu\text{g}$ of recombinant enzyme. Both reactions were initiated by adding substrate.

The dependence of enzyme activities on pH and temperature were determined over a pH range of 5.5 to 9.5 and a temperature range of 10°C to 50°C in steps of 10°C. Buffers used were 0.1 M MES buffer (pH 5.5 to 6.5), 0.1 M Bis-Tris Propane (pH 6.5 to pH 9.5) and 0.1 M Tris-HCl (pH 7 to pH 9). Effects of various metal ions and chemical reagents were examined at 1 and 10 mM final concentrations. For enzymatic characterization, NAD(H) and NADP(H) were tested as potential cofactors and different sugars and polyols (D-fructose, D-glucose, D-mannose, D-galactose, D-xylose, D-mannitol, D-sorbitol, D-arabitol) as putative substrates. Influence of NaCl on M2DH activity was assessed by testing final concentrations ranging from 0 to 2 M. Kinetic parameters K_m and V_m were determined at 25°C for the reduction (at pH 7) and oxidation (at pH 8.5) reactions by varying the concentrations of D-fructose and NADH, and of D-mannitol and NAD^+ respectively. For both activities, one unit corresponded to one μmole of NAD(H) reduced or oxidized per minute and per milligram of protein.

Determination of hexokinase/fructokinase activity. HK/FK activity was determined by an enzyme-coupled assay. The reaction mixture (total volume 100 μl) contained the Zg4264 enzyme in solution in 50 mM Tris-HCl (pH 7.6), and in presence of 1 mM ATP, 100 mM KCl, 1.5 mM MgCl_2 , 1 mM phosphoenolpyruvate, 0.5 mM freshly prepared NADH and 0.2 μl of a mix composed of lactate dehydrogenase (LDH) (900-1400 units/mL) and pyruvate kinase (PK) (600-1000 units/mL) (Sigma). The reaction was initiated with 1 mM fructose. Enzyme activity was measured by following changes in absorbance at 340 nm in a microplate Safire2 UV spectrophotometer reader (Tecan). The temperature and pH optimum were determined in the same conditions that for M2DH activity. D-fructose, D-glucose, D-mannose, D-sorbitol, D-mannitol, mannitol-1-phosphate, glucose-1-phosphate, fructose-1-phosphate, mannose-6-phosphate, glucose-6-phosphate and fructose-6-phosphate were tested as putative substrates, and EDTA, mannose-6-phosphate and fructose-6-phosphate as potential inhibitors. The influence of NaCl on this enzyme was assessed by testing final

concentrations ranging from 0 to 2 M. Kinetic parameters were determined at 25°C and pH 7.5 by varying the concentrations of D-fructose, D-mannose and ATP. One unit corresponded to one μ mole of NADH oxidized per minute and per milligram of protein.

Thermostability analysis. Thermostability of Zg4263 and Zg4264 was studied by dynamic light scattering (DLS). A solution of 50 μ l of each enzyme at 10 mg/ml was filtered on a 0.2 μ m membrane. Using a Zetasizer Nano instrument (Malvern), the protein solution was heated from 10°C to 70°C in steps of 1°C during a total period of 12 h and the hydrodynamic gyration radius (Rg) was measured at each degree. The denaturation temperature was determined as the point of sharp change in gyration radius.

RESULTS

Identification of genes coding for enzymes potentially involved in mannitol degradation.

In the genome of *Z. galactanivorans*, the gene *Zg4263* (1494 bp) is annotated as a putative cytosolic D-mannonate oxidoreductase. The corresponding protein of 498 aa, Zg4263, is 46% identical to the mannitol-2-dehydrogenase (M2DH) of *Pseudomonas fluorescens* DSM 50106 (61) and 42% identical to the M2DH of *Corynebacterium glutamicum* ATCC 13032 (62). These characterized M2DHs belong to the long-chain dehydrogenase/reductase family (LDR). Furthermore, the gene *Zg4264* (885 bp; corresponding to a protein of 295 aa), which is located immediately upstream of *Zg4263*, encodes a putative cytosolic fructokinase (FK) of the pfkB family carbohydrate kinase. BlastP analyses on the *Z. galactanivorans* genome with *Zg4263* and *Zg4264* sequences showed the absence of other genes coding for a putative M2DH, but the presence of another putative cytosolic FK of 315 amino acids (also potentially belonging to the pfkB family carbohydrate kinase) encoded by the gene *Zg1491* (945 bp). There is 26% of identity between the putative *Zg4263* and *Zg1491* FKs. Based on these observations, it was suggested that *Zg4263*, *Zg4264* and *Zg1491* could be implicated in the

utilization of mannitol by *Z. galactanivorans*. To test this hypothesis, these three genes were cloned in *E. coli*. While both *Zg4263* and *Zg4264* genes were over-expressed in the tested conditions, no over-expression was observed for *Zg1491* despite several attempts to improve the conditions of induction. Therefore, we focus our attention on both *Zg4263* and *Zg4264* proteins.

Expression and purification of recombinant *Zg4263* and *Zg4264* proteins.

Recombinant *Zg4263* and *Zg4264* proteins were successfully expressed in high quantity in the *E. coli* BL21 (DE3) expression strain as soluble forms and purified to electrophoretic homogeneity (Fig. S1A and S1B). The final yield after one step of purification by chromatography of affinity was about 30 mg for 200 ml of ZYP medium for *Zg4263* and about 25 mg for *Zg4264* under the same conditions. Proteins eluted from the final size exclusion chromatography indicated an apparent molecular mass of 56 kDa and 32 kDa for the proteins *Zg4263* and *Zg4264* respectively. Comparison of these results with the theoretical mass of 55.6 kDa and 32.7 kDa indicated that both proteins are under monomeric form in solution. Specific activity of the two recombinant enzymes were compared after Ni²⁺-affinity chromatography with and without subsequent gel filtration, and no significant difference was observed. This indicated that the Superdex 200 column did not improve the level of purity of recombinant *Zg4263* and *Zg4264*. These results were confirmed by Dynamic Light Scattering (DLS) experiments. DLS was also used to study the protein thermostability; above 40°C, a sharp increase of the hydrodynamic radius of gyration (R_g) was observed, indicating the beginning of the denaturation of *Zg4263* and *Zg4264*.

Biochemical characterization of *Zg4263*. The specificity of this putative dehydrogenase/oxido-reductase was tested in presence of different sugars and polyols, and at several concentrations. Mannitol, arabitol and sorbitol (at 5, 10 or 50 mM), with NAD⁺ or NADP⁺ as a co-substrate, and fructose, mannose, glucose, galactose, xylose (at 1, 10 or 50

mM), with NADH or NADPH as a co-substrate, were examined as alternative substrates for Zg4263. Purified Zg4263 had a specific activity of 5.64 U/mg for fructose reduction with NADH at pH 6.5. For mannitol oxidation at pH 8.5, the specific activity was 7.18 U/mg. No activity on these substrates were observed when NAD(H) was replaced by NADP(H). This protein is thus a true M2DH and will be named thereafter ZgM2DH. In the direction of oxidation of polyol, only sorbitol at 50 mM, showed an activity representing 14% to the value measured in presence of mannitol with NAD⁺. In the reverse direction, no activity was observed in presence of mannose, glucose, galactose and xylose. These results show that the enzyme features narrow substrate specificity in the both directions, and may only catalyze the production of fructose or mannitol under physiological conditions.

The optimum pH for the mannitol oxidation catalyzed by ZgM2DH was 8.5, with 52% and 84% of the maximum activity at pH 8.0 and pH 9.0 in Tris-HCl buffer respectively. The optimum pH for the fructose reduction was 6.5, with 70% (Tris-HCl buffer) and 76% (Mes buffer) of the maximum activity at pH 6.0 and 7.0 respectively (Fig. 1A and 1B). These values of pH optimum were in accordance with those measured for similar enzymes isolated from diverse bacteria (Table 2). The optimal temperature for mannitol oxydation was 40°C in Tris-HCl buffer pH 8.5 (Fig. 1C), with this value higher than optimum temperature determined in other organisms, except *Thermotoga maritima* (Table 2).

Both M2DH oxidation and reduction activities were measured in presence of different chemicals (Table 3). Results showed that the enzyme was only slightly sensitive to metal chelators such as EDTA (at 1 and 10 mM final concentration), and to mercaptoethanol. Conversely, Ca²⁺, Mg²⁺, K⁺, Li⁺ and NH⁴⁺ inhibited M2DH activity in both directions by 20 to 30%, except NH⁴⁺ which decreased fructose reduction activity by 54%. NaCl at 1M slightly activated the oxidation activity, but inhibited significantly (by 45 %) the reduction activity. Similar effects were observed in presence of 1.5 and 2 M of NaCl (data not shown).

Moreover, both activities of ZgM2DH were inhibited by their reaction products in a similar manner (about 50% at 50 mM). Interestingly, the mannitol production was strongly activated by addition of fructose-6-phosphate (20 mM) which was the product of a FK activity and conversely, this reaction was inhibited (by 25%) with addition of mannitol-1-phosphate (20 mM).

Initial velocities were determined in the standard assay mixture at pH 8.5 for mannitol oxidation and at pH 6.5 for fructose reduction. Substrates and co-factors had hyperbolic saturation curves and the corresponding double-reciprocal plots were linear (Fig. S2). The concentration of mannitol and fructose substrates varied from 0.1 to 50 mM, of NAD^+ from 0.01 to 10 mM, and of NADH from 0.01 to 1 mM since it was the highest nucleotide concentration compatible with the spectrophotometric assay. The results showed that the ZgM2DH enzyme had equivalent K_m and k_{cat} for mannitol and fructose (Table 2). Indeed, specific activities and K_m values were of the same order of magnitude, i.e. 7.18 U/mg protein and 1.12 mM, 5.64 U/mg protein and 2.30 mM for mannitol and fructose respectively.

Biochemical characterization of Zg4264. The recombinant Zg4264 protein catalyzed preferentially the ATP-dependent phosphorylation of fructose into fructose-6-phosphate (specific activity of 2.55 U/mg at 5 mM fructose), but also used mannose as substrate. Indeed, specific activity corresponded to 5% or 16% of the one measured in presence of fructose when mannose was added at final concentration of 10 and 50 mM in the reaction medium respectively. The enzyme was not active on D-glucose, D-galactose, D-xylose, D-sorbitol, D-mannitol, mannitol-1-phosphate, glucose-1-phosphate, fructose-1-phosphate, mannose-6-phosphate, glucose-6-phosphate and fructose-6-phosphate as phosphoryl acceptors. This protein represents a new FK and will be named thereafter ZgFK1.

This fructokinase was active in the pH range of 5.5 to 9, with an optimum at 7.5 (Fig. 2A; specific activity of 3.95 U/mg at 10 mM of fructose), and sharply decreased at more

alkaline pH values (only 30% of activity at pH 8.5), in contrast to what was observed for acidic pH values (73% activity at pH 6). The highest enzyme activity was observed at 40°C (Fig. 2B). For these assays, the reaction mixture contained KCl at a final concentration of 100 mM.

Indeed, Zg4264 was dependent of monovalent cation since no activity was detectable in absence of K^+ . Specific activity was highest in presence of K^+ at 100 mM final concentration (Fig. 2C). Na^+ could partially replace K^+ (about 20% of the activity at Na^+ final concentration of 0.05-1 M), while no activity was observed in presence of $MgCl_2$. This result contrasted with Mg^{2+} requirement featured by several previously characterized bacterial FKs (Table 4). Influence of mannitol-6-phosphate and fructose-6-phosphate on hexokinase activity was determined with fructose or mannose as substrate (final concentration of 10 and 50 mM in the reaction mixture, respectively). The addition of 20 mM final of mannitol-6-phosphate inhibited fructose phosphorylation by 85% and mannose phosphorylation by 80%, while the addition of 20 mM final of fructose-6-phosphate inhibited fructose phosphorylation by 45% and mannose phosphorylation by 70%.

The purified enzyme Zg4264 exhibited typical Michaelis-Menten kinetics when assayed with increasing concentrations of the investigated substrates (D-fructose, D-mannose and ATP) (Fig. S3). The apparent K_m for D-fructose, D-mannose and ATP are 10, 26.6 and 0.31 mM, respectively. Although the K_m for fructose and mannose were in the same range, the k_{cat}/K_m for fructose ($0.136 \text{ mM}^{-1} \text{ s}^{-1}$) was 60 times stronger than the k_{cat}/K_m for mannose ($0.0023 \text{ mM}^{-1} \text{ s}^{-1}$). Similarly, the k_{cat}/K_m for ATP in presence of fructose was $23 \text{ mM}^{-1} \text{ s}^{-1}$ against $1 \text{ mM}^{-1} \text{ s}^{-1}$ in presence of mannose (data not shown).

Organization of the gene clusters related to mannitol degradation in *Z. galactanivorans*. In the genome, two gene clusters were predicted to be involved in the uptake/catabolism of the mannitol (Fig. 3A). The first one, about 6,200 bp long, contained

three genes, *Zg1489*, *Zg1490* and *Zg1491*, annotated as putative SusC-like TBDR, SusD-like and fructokinase *scrk1* proteins respectively. The second cluster, about 6,900 bp long, featured six genes named from *Zg4259* to *Zg4264*. Both clusters were separated by 3,210 kbp in the bacterial chromosome. In this study, we have characterized the proteins *Zg4263* and *Zg4264*. The gene *Zg4259* codes for a conserved unknown protein. The three genes *Zg4260*, *Zg4261* and *Zg4262* corresponded to a putative transmembrane protein complex involved in the uptake of monosaccharides. The predicted aa sequences encoded by these ORFs revealed a high similarity to different components of the ribose transport system in *E. coli* (63, 64). The first gene, *Zg4260*, of 1,062 nt, coded for a peptide of 354 aa with a calculated molecular mass of 32 kDa. The deduced protein showed an overall aa sequence identity of 31% with the ribose binding periplasmic protein *rbsB* of *E. coli*. A putative peptide signal of 29 aa was located at the N-terminal. The second gene, *Zg4261*, of 1,527 nt, encoded a peptide of 509 aa with a molecular mass of 56 kDa. The deduced protein exhibited an overall aa sequence identity of 41% with the ribose ATP-binding protein *rbsA* of *E. coli*. Finally, the third gene, *Zg4262*, of 969 nt, coded for a peptide of 323 aa with a calculated molecular mass of 34 kDa. The deduced protein showed an overall aa sequence identity of 43% with the ribose permease protein *rbsC* of *E. coli*, was predicted to be very hydrophobic, and contains nine transmembrane domains.

In order to test the hypothesis that some of these genes may be expressed on polycistronic messengers, semi-quantitative RT-PCR experiments were carried out on cDNA prepared from RNA extracted from cultures for which the main source of carbon was glucose or mannitol. For the first gene cluster, results showed that the TonB-dependent receptor and the SusD-like genes are expressed in the same polycistronic mRNAs, while it was not the case for the fructokinase *scrk1* and the SusD-like genes (Fig. 3B, left picture). For the second gene cluster, three amplicons (Fig. 3B, middle picture) indicated that *Zg4259* is expressed on

polycistronic mRNAs containing *Zg4260* and *Zg4261*. Then, two long overlapping PCR products were obtained: one showed that the three genes of the putative transport system (rbsB, rbsA, rbsC) were expressed on the same RNA messengers with the gene coding for the *ZgM2DH*, and the second corresponded to the three genes encoding rbsC, *ZgM2DH*, and *ZgFK1* (Fig. 3B, right picture). The occurrence of these both overlapping transcripts suggests that all the genes of this cluster are likely to be expressed on a same polycistronic RNA, even if no PCR product (expected length of 5,820 bp) was observed when attempting to amplify a fragment covering the region from *Zg4260* to *Zg4264*. Similar patterns of amplification were obtained after growing *Z. galactanivorans* in presence of glucose or mannitol as the main source of carbon.

To complete this gene expression analysis, qPCR experiments were carried out for individual genes to test the influence of mannitol on their transcription when compared to culture in presence of glucose. The level of expression was nearly similar under culture in presence of glucose or mannitol for the first cluster (ratio almost equal to 1). (Fig. 3C). In contrast, for the second cluster, genes coding for sub-units of the ABC transporter and for proteins catalyzing the utilization of mannitol were induced simultaneously after culture in presence of mannitol, supporting their organization as a true operon (Fig. 3C).

Taxonomic distribution and organization of genes orthologous to *ZgM2DH* and *ZgFK1* in bacteria. *Z. galactanivorans* genome was analyzed to identify regions of sequence mediating transcription, such as promoters and Rho independent terminators. No promoter sequence matching the consensus identified in other *Bacteroidetes* species (56, 57) was found between the genes *Zg4259* and *Zg4260*. However, directly upstream the start codon of *Zg4259*, this gene encoding a conserved unknown protein, a putative promoter sequence presenting only one mismatch with the consensus sequence (TTG/TANNTTTG) was identified. The length of the spacer separating both the -7 (TANNTTTG) and -33 (TTG)

motifs was 19 bp. Furthermore, this region was relatively poor in GC (27% of GC) compared with the mean value for the whole genome (43% of GC). These parameters were consistent with studies in *Flavobacterium* species, showing that the promoter strength was influenced by the spacer length (19 bp being the optimal value), and was enriched with A and T nucleotides (56). Sequence analyses also revealed the presence of a single putative terminator in this clusters of genes, downstream the locus *Zg4264*.

Orthologs of *Zg4263* and *Zg4264* genes were searched for in genomes of Archaea (338 genomes) and bacteria (19,904 genomes) available in the NCBI genomic blast server at the 14th of January 2014. The genomic vicinity of each identified orthologs was then screened to determine the presence of genes coding for an ABC (ATP Binding Cassette) transporter complex in these microbes. Such cluster of genes involved in mannitol degradation, or mannitol utilization clusters, was absent in Archaea genomes, present in the *Bacteroidetes* phylum, but only in six of the 76 complete genomes available for the *Flavobacteriaceae* family: *Z. galactanivorans* Dsij^T (31), *Gillisia* sp. CAL575 (65), *Lacinutrix* sp. 5H-3-7-4 (66), *Cellulophaga lytica* DSM 7489 (67), *Cellulophaga algicola* DSM 14237 (68) and *Winogradskyella psychrotolerans* RS-3T (69). When considering the entire operon containing genes coding for the conserved unknown protein, rbsB, rbsA, rbsC, M2DH, and FK1, analysis of nucleotide sequences of *Flavobacteriaceae* showed a very high percentage of identity, between 64% (minimum value obtained between *Z. galactanivorans* and *Winogradskyella psychrotolerans* RS-3T) and 75% (maximum value obtained between *Lacinutrix* sp. 5H-3-7-4 and *Winogradskyella psychrotolerans* RS-3T). In the same vein, analysis of aa sequences of the different proteins coded by the mannitol utilization gene cluster showed a highest degree of conservation within the six *Flavobacteriaceae* species considered (Fig. 4) when compared to orthologous genes identified in other bacteria. As an illustration, the identity percentage of putative ABC transporter proteins is between 69% and 89% within these six species, and

decreased to a maximum of 49% with ABC transporter proteins of other bacteria. Interestingly, the organization of the operon observed in the species mentioned above was not conserved in *Bacteroidetes*. Indeed, in the genomes of *Formosa agariphila* KMM 3901T (*Flavobacteriaceae* family) (70), *Marinilabilia salmonicolor* JCM 21150 (*Marinilabiliaceae* family) (71), and *Cytophaga fermentans* JCM 21142T (*Cytophagaceae* family) (72), a gene coding for a predicted mannitol permease was identified upstream orthologs of M2DH and FK1, while no gene for an ABC transporter complex was found.

DISCUSSION

Different metabolic pathways have been described for the use of mannitol by bacteria: the phosphoenolpyruvate-dependent phosphotransferase system (PTS), and a M2DH-based catabolic pathway, this latter involving different types of transporters for mannitol. The PTS system consists of a phospho-carrier or histidine protein (HPr), of a protein kinase enzyme I (EI) and of a substrate-specific enzyme II (EII) (73). This system has been extensively studied in *E. coli* (19), *Bacillus subtilis* (20, 21), *Bacillus stearothermophilus* (22), *Clostridium acetobutylicum* (23), *Streptococcus mutans* (24) and in *Vibrio cholerae* (74). In contrast, in the *P. fluorescens* mannitol operon, this polyol is transported by an ATP binding cassette transporter (MtlEFGK), and oxidized by a M2DH (MtlD) to produce fructose, which is then converted in fructose-6-phosphate by a kinase (MtlZ) (28). Lastly, the *C. glutamicum* mannitol operon contains two structural genes (*mtlT* and *mtlD*), encoding a major facilitator superfamily (MFS) transporter and a M2DH respectively, and a regulator gene (*mtlR*) corresponding to a repressor (75).

In this report, we describe the identification of a cluster of genes involved in mannitol degradation based on the analysis of the genome of the Flavobacteria *Z. galactanivorans*. This cluster is approximately 6.9 kb in size and comprises six genes in the following order: a

conserved unknown protein (*Zg4259*), a putative mannitol ABC transporter complex (*Zg4260*, *Zg4261* and *Zg4262*), a mannitol-2-dehydrogenase (*Zg4263*) and a fructokinase (*Zg4264*). The proteins encoded by the last two enzymes have been purified and successfully characterized: *Zg4263* is a NAD-dependent M2DH (EC 1.1.1.67) which is capable of both mannitol synthesis and degradation, and *Zg4264* is a fructokinase (EC 2.7.1.4), member of the PfkB family of carbohydrate kinases, and active on fructose and mannose in presence of a monocation, preferentially K^+ . This latter observation is in line with the fact that bacterial fructokinases are usually mannofructokinases (77-78).

Analysis of the primary sequence of *ZgM2DH* indicated that this protein is a member of the long-chain MDHs family (79) and more specifically of the polyol-specific long-chain dehydrogenase/reductases subfamily (PSLDR) (80). Most members have been identified by similarity at the level of aa sequence and only a few have been biochemically characterized (61, 81, 82). *ZgM2DH* was active as a monomer, did not require metal ions for catalysis, and features narrow substrate specificity. These observations are in contrast to the results obtained for polyol dehydrogenases characterized in *Saccharomyces cerevisiae* (83), *Rhodobacter sphaeroides* (81), *P. fluorescens* (61) or *Thermotoga maritima* (84) because these M2DHs feature a broader substrate specificity. In addition, most of other characterized M2DHs from bacteria have significantly higher K_m and activity in direction of fructose reduction to produce mannitol than for the oxidation of this polyol, while the M2DH of *Z. galactanivorans* seems to have no preference between fructose and mannitol (Table 2). Moreover, none of the six cysteines present in the *ZgM2DH* protein are likely to be important for catalytic reactions because mercaptoethanol has no effect on both activities of the enzyme. In contrast, oxidation and reduction are inhibited by their reaction products in a similar manner (about 50% at 50mM). Such effect has been already observed for fructose reduction in *T. maritima* TM0298 (84) and for both directions in *S. cerevisiae* (83).

Genes encoding ZgM2DH and ZgFK1 were adjacent in the genome, and located directly downstream four loci coding for a conserved unknown protein and constituents of an ABC transporter complex, thus forming a cluster of six genes. This prompted us to investigate whether the six loci could be part of the same operon. Transcriptional analysis strongly suggests that these genes constitute a genuine operon in *Z. galactanivorans*. Analysis of publicly available prokaryotic genomes showed this operon for mannitol catabolism to be conserved only in five other species of *Flavobacteriaceae*. In addition, M2DH and FK1 genes were retrieved in three species of *Bacteroidetes* (*F. agarophila*, *M. salmonicolor*, and *C. fermentans*) harboring a gene predicted to encode a putative mannitol permease gene instead of genes corresponding to the different sub-units of an ABC transporter.

The occurrence of two types of operons for the degradation of mannitol in a few members of the *Bacteroidetes* is intriguing and questions the evolution of this metabolic pathway in this phylum. It is tempting to speculate that the M2DH and FK genes have been acquired recently by the last common ancestor of the nine strains of *Bacteroidetes* in which both genes have been retrieved, and this assumption is partly supported by the high sequence identity between these genes in these different species. This common ancestor might have been a bacterium associated to brown algae which are known to produce and exude mannitol. Furthermore, the occurrence of such an operon may have conferred an adaptative advantage to this bacterium because mannitol can be used as a carbon and energy source. The observation that extant flavobacterial species containing the mannitol utilization system are not strictly associated to macroalgae suggests that they have probably conserved this operon to degrade mannitol from other marine resources.

The occurrence of two types of mechanisms for the transport of mannitol (primary for the ABC transporter, and secondary for the permease) through the inner membrane in *Bacteroidetes* also suggests that the physiological role(s) and/or the regulation of the mannitol

catabolism may be different among this phylum. Meanwhile, it is difficult to relate these observations with any ecophysiological traits of the nine strains under consideration because there is no clear relationship between their type of mannitol utilization operon and their origin and metabolism. Another interesting aspect to consider for further analysis is the identification of the mechanism(s) used by these bacteria to sense and transport mannitol through the outer membrane.

To go further in the understanding of mannitol catabolism and its physiological important in *Z. galactanivorans*, it will be interesting to study its regulation. In general, changes in expression of genes involved in carbohydrates catabolism occurs via two mechanisms, i.e., catabolic repression (suggesting the existence of a preferential carbon source) and/or specific induction by substrate(s). Usually, transcription of catabolic genes in prokaryotes is regulated by either a repressor or an activator protein. In most of the cases, genes encoding this type of regulators are located nearby the targeted genes, likely in the same cluster/operon although there are exceptions. For instance, in *P. fluorescens* DSM 50156, the gene coding for an activator protein (MtlR) belonging to the Xyl/AraC family and involved in the regulation of the mannitol catabolic pathway is not localized in the region containing all genes involved in this catabolism (29). The genome of *Z. galactanivorans* contains several putative AraC-type transcriptional regulators, but none of them are closely localized to the mannitol utilization cluster. Reverse genetic approach will be very valuable to assess the physiological role(s) of these potential regulators when such methodology will be available for *Z. galactanivorans*.

Reconstruction of metabolic networks from genomic resources, including carbohydrate catabolic pathways, may help to better understand microbial ecophysiology and interactions between microorganisms and their hosts, in particular for algae associated bacteria (85, 86). In this context, our results shed light on evolution of mannitol catabolism in

important environmental bacteria. They pave the way also to better understand the recycling of algal biomass on the shore and for the exploitation of algae as a renewable resource.

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FIGURE LEGENDS

FIG 1 Enzymatic properties of ZgM2DH. (A) Effect of various buffer pH values on the mannitol oxidation activity of ZgM2DH. (B) Influence of various buffer pH values on the fructose reduction activity of ZgM2DH. \square indicated 0.1 M Bis-Tris Propane buffer, \diamond 0.1 M MES buffer, and \triangle 0.1 M Tris-HCl buffer. (C) Effect of various temperatures on the mannitol oxidation activity of ZgM2DH. The enzyme activities observed at pH 8.5 (in A), and at 6.5 (in B), at 40°C, were considered 100%, and all other values are indicated as relative percentages of these activities. Each value represents means \pm SD calculated from three reaction assays for one round of purification.

FIG 2 Enzymatic properties of ZgFK1. (A) Effect of various Tris-HCl buffer pH values on the activity of ZgFK1. (B) Influence of various temperatures on the activity of ZgFK1. (C) Effect of various concentrations of KCl on the activity of ZgFK1. The enzyme activity observed at pH 7.5, 40°C and in presence of 100 mM KCl was considered 100%, and all other values are indicated as relative percentages of this activity. Each value represents means \pm SD calculated from three reaction assays for one round of purification.

FIG 3 Cluster organization and gene expression analysis of genes involved in the catabolism of mannitol in *Z. galactanivorans*. (A) Genetic organization of two putative mannitol utilization clusters. The numbers at the extremities of each operon indicate positions within the bacterial genome. (B) Analysis of the transcription patterns of these two clusters. RT-PCR experiments were performed on RNA extracted from culture with glucose (G) or mannitol (M), and agarose gels showed PCR products indicated in (A) by numbers 1 to 4. MW, molecular mass standard. (C) Differential expression for genes of interest observed between cultures in presence of mannitol and cultures in presence of glucose.

913

914 **FIG 4** Organization of genes related to mannitol catabolism in selected bacteria. Each arrow

915 represents a gene and its orientation. They code for a conserved unknown protein, a ribose

916 binding periplasmic protein (rbsB), a ribose ATP-binding protein (rbsA), a ribose permease

917 protein (rbsC), a mannitol-2-dehydrogenase (M2DH), and a hexokinase (FK1). Numbers

918 correspond to the percentage of identity between genes of *Z. galactanivorans* and orthologous

919 genes in other strains. P indicated a putative promoter, and T a putative terminator. *Z.*

920 *galactanivorans*, *Zobellia galactanivorans* Dsj^T; *G. sp.* CAL575, *Gillisia sp.* CAL575; *L. sp.*

921 5H-3-7-4, *Lacinutrix sp.* 5H-3-7-4; *C. lytica*, *Cellulophaga lytica* DSM 7489; *C. algicola*,

922 *Cellulophaga algicola* DSM 14237; *W. psychrotolerans*, *Winogradskyella psychrotolerans*

923 RS-3T; *F. agariphila*, *Formosa agariphila* KMM 3901T; *M. salmonicolor*, *Marinilabilia*

924 *salmonicolor* JCM 21150; *C. fermentans*, *Cytophaga fermentans* JCM 21142T.

925

926 **TABLES**927 **TABLE 1** Primers used in this study. Restriction sites are indicated in italics.

Primers	Nucleotide sequence (5'-3')	Product length (bp)	T _m (°C)
qPCR primers			
Zg1489-F	ACAAATTCGTCCCTACCCA	111	59
Zg1489-R	TCCTCCCTATTATAGCCATCC		58
Zg1490-F	GAAGACACCATCTATACCCGT	95	59
Zg1490-R	GTAGTATCCTGAATCGCCCA		59
Zg1491-F	CACACCTCATCTATCCGA	79	58
Zg1491-R	GGTAGTGGTAATGGCTCCT		59
Zg4260-F	CTTATGCTGGAGGACTAAAGG	91	58
Zg4260-R	GAGGACACAAACATCGGAC		59
Zg4261-F	CATAGATGAGCCTACCCGA	112	58
Zg4261-R	GCATATCCGATGAGATGACC		58
Zg4262-F	CGTGACCTTTGCCATTCTC	108	59
Zg4262-R	GTAAGCCACCAAATAGCCC		59
Zg4263-F	CTTGCGACAACATTCAGGG	66	60
Zg4263-R	GCGGTGGAAACATAAGTGAG		59
Zg4264-F	TAGTACAGGTAAGCTTGTCCC	75	59
Zg4264-R	TTTATGTTGTCCCAAGCCC		58
cDNA PCR primers			
Zg1489-1490-F	ACAAATTCGTCCCTACCCA	3,289	59
Zg1489-1490-R	GTAGTATCCTGAATCGCCCA		59
Zg1490-1491-F	GAAGACACCATCTATACCCGT	1,669	59
Zg1490-1491-R	GGTAGTGGTAATGGCTCCT		59
Zg4259-4260-F1	GCCATGTGGAATCCAGAAAAGGA	240	68
Zg4259-4260-R-	AAGGCGTTAAGTAAAATGGTAGATAC		72
Zg4259-4260-F2	TCAAATGCGTACAAAATTCCTCATAAC	1,240	72
Zg4259-4260-R	AAGGCGTTAAGTAAAATGGTAGATAC		72
Zg4259-4261-F1	GCCATGTGGAATCCAGAAAAGGA	1,260	68
Zg4259-4261-R	GCTTTTGTATTTTGCATATCGTCCAT		72
Zg4260-4263-F	CTTATGCTGGAGGACTAAAGG	3,543	58
Zg4260-4263-R	GCGGTGGAAACATAAGTGAG		59
Zg4262-4264-F	CGTGACCTTTGCCATTCTC	2,530	59
Zg4262-4264-R	TTTATGTTGTCCCAAGCCC		58
Zg4260-4264-F	TACTTAACGCCTTAGGGTGTGCCGA	5,820	68
Zg4260-4264-R	CCTTCCCTACCGGCTACCATAGCTC		68
Cloning Primers			
Zg4263pFO4-F	GGGGGGGGA7CCAAAAATTATAAATTAAACAGCACTAATCTT	1,491	70 ^a
Zg4263pFO4-R	CCCCCGAA77CTTAATTGTTGTTCTCCTGGTTACGGTTT		70 ^a
Zg4264pFO4-F	GGGGGGGGA7CCAAAAATATAGTCTGTTTGGGGAAGTT	882	70 ^a
Zg4264pFO4-R	CCCCCGAA77CTTATTGGCGACTTTTGATAAAGGCTTGG		72 ^a
Zg1491pFO4-F	GGGGGGGGA7CCAAACCGTATACTGCATTGGAGAATT	942	70 ^a
Zg1491pFO4-R	CCCCCA7GCA7TTATTCCCCGAAAACCATGCTGTTGTC		72 ^a

928 ^a Calculation of T_m does not include the sequences corresponding to the restriction sites,
 929 neither nor the sequence upstream.

930

TABLE 2 Comparison of the biochemical properties of M2DHs from different organisms. Kinetic parameters of ZgM2DH were obtained at 25°C in 0.1 M Tris-HCl and at pH 6.5 for the D-fructose reduction), and pH 8.5 in 0.1 M Bis-Tris Propane buffer and at 25°C for the D-mannitol oxidation. Each value represents the mean of triplicate measurements and the standard errors for parameters are less than 10%.

	Z.	A.	Ps.	Lb.	R.	Le.	T.
Parameters	<i>galactanivorans</i>	<i>fumigatus</i>	<i>fluorescens</i>	<i>sanfranciscensis</i>	<i>sphaeroides</i>	<i>pseudomesenteroides</i>	<i>maritima</i>
Sp. act. ^a (U/mg protein)							
Fructose	5.64	94	55	15.48	60	450	54
Mannitol	7.18	220	31		30	106	
pH optimum							
Fructose	6.50	7.10	7.30	5.80	6.50	5.40	5.50
Mannitol	8.50	10	10	8	9	8.60	8.30
Temp. ^b optimum							
Fructose	40°C	25°C	25°C	35°C			95°C
Mannitol	40°C		25°C				
K_m (mM)							
Fructose	2.30	60	25	24	16.30	44	51
NADH	0.02	0.02	0.15		0.02	0.09	0.05
Mannitol	1.12	13	1.20	78	0.29	12	5.51
NAD ⁺	0.51	0.15	0.15		0.18	0.20	0.14
k_{cat}/K_m (mM ⁻¹ sec ⁻¹)							
Fructose	2.30	1.40	2.20				
NADH	263	4700	436				
Mannitol	6	17	18				
NAD ⁺	13	1400	159				
k_{cat} (sec ⁻¹)							
Fructose	5.27	86	54				
Mannitol	6.70	212	20				
kDa (number of subunits)	56 (1)	58 (1)	54 (1)	53 (1)	52 (1)	36 (4)	34 (4)
Classification	Long-chain dehydrogenase/reductase family (LDR)	Long-chain dehydrogenase/reductase family (LDR)	Long-chain dehydrogenase/reductase family (LDR)		Long-chain dehydrogenase/reductase family (LDR)	Zn2+-containing medium-chain dehydrogenase/reductase family (MDR)	Zn2+-containing medium-chain dehydrogenase/reductase family (MDR)
Reference	This work	(82)	(61, 87)	(88)	(81, 89)	(90)	(84)

^a Sp. act., specific activity; ^b Temp., temperature.

TABLE 3 Effects of metal ions and chemical reagents on the activity of ZgM2DH. The purified enzyme was assayed under standard assay conditions with ions in chloride forms and several chemicals. Values represents percentage of remaining activity under specific condition tested compared to 100% observed in absence of any of these compounds. Each value represents the mean of triplicate measurements and the standard errors for activities are less than 10%.

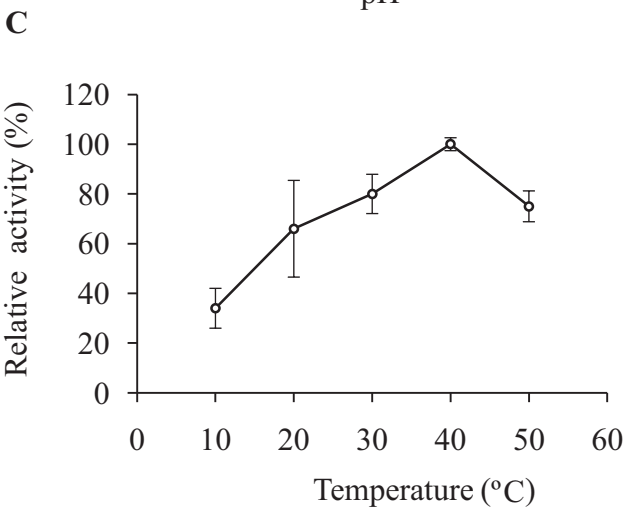
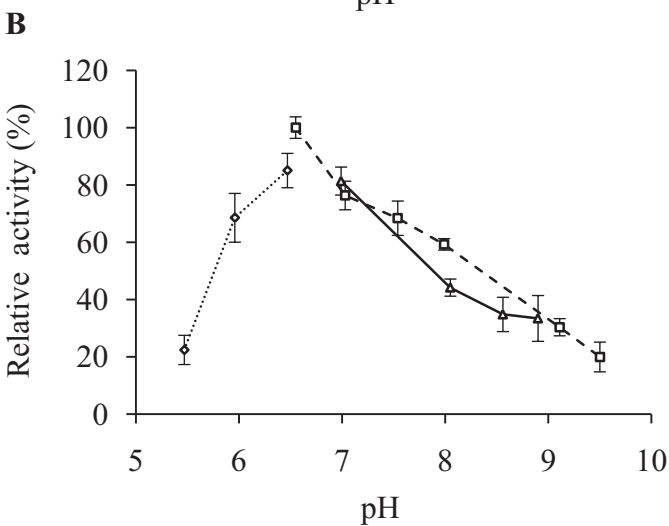
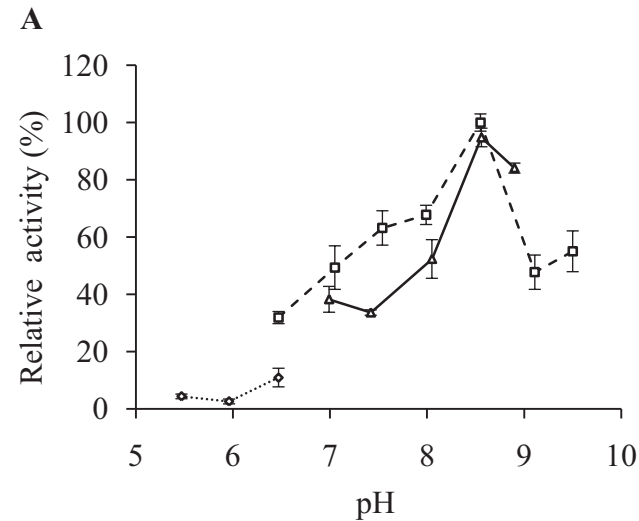
Compounds	Concentration (mM)	Mannitol oxidation Relative activity (%)	Fructose reduction Relative activity (%)
Ca ²⁺	10	73	83
K ⁺	10	79	80
Li ⁺	10	83	86
NH ₄ ⁺	10	77	46
Mg ²⁺	10	103	70
Mercaptoethanol	10	96	90
EDTA	10	115	96
Fructose	20	70	nd ^a
Fructose	50	47	nd
Mannitol	20	nd ^a	73
Mannitol	50	nd	46
Mannitol-1-phosphate	20	95	77
Fructose-6-phosphate	20	93	157
NaCl	100	100	100
NaCl	1000	120	55

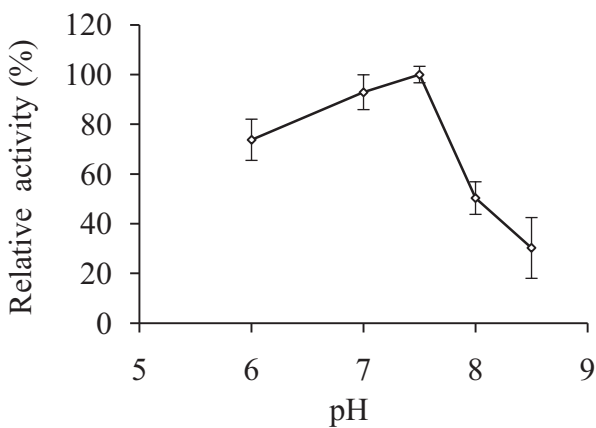
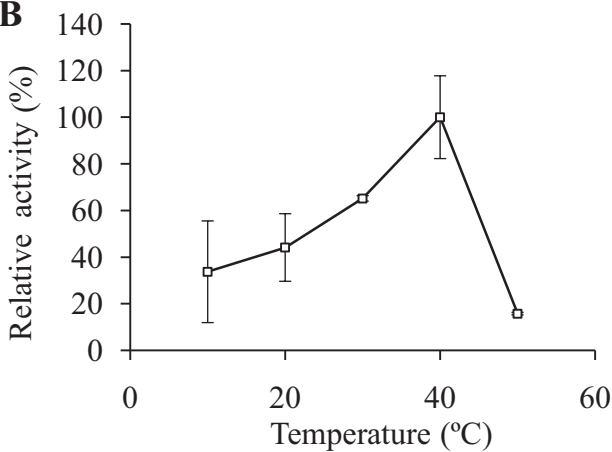
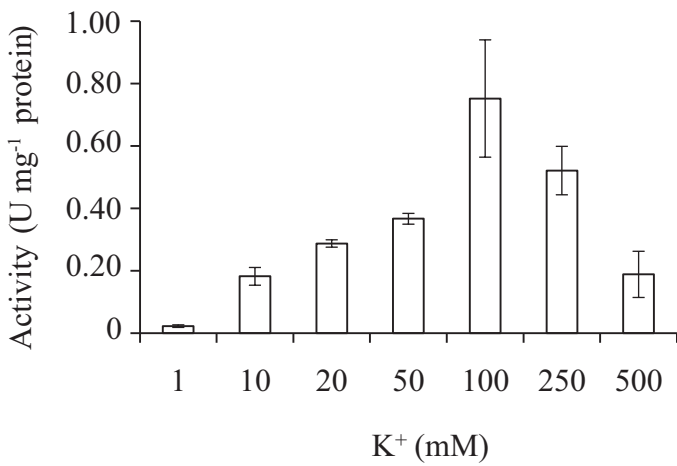
^a nd, not determined.

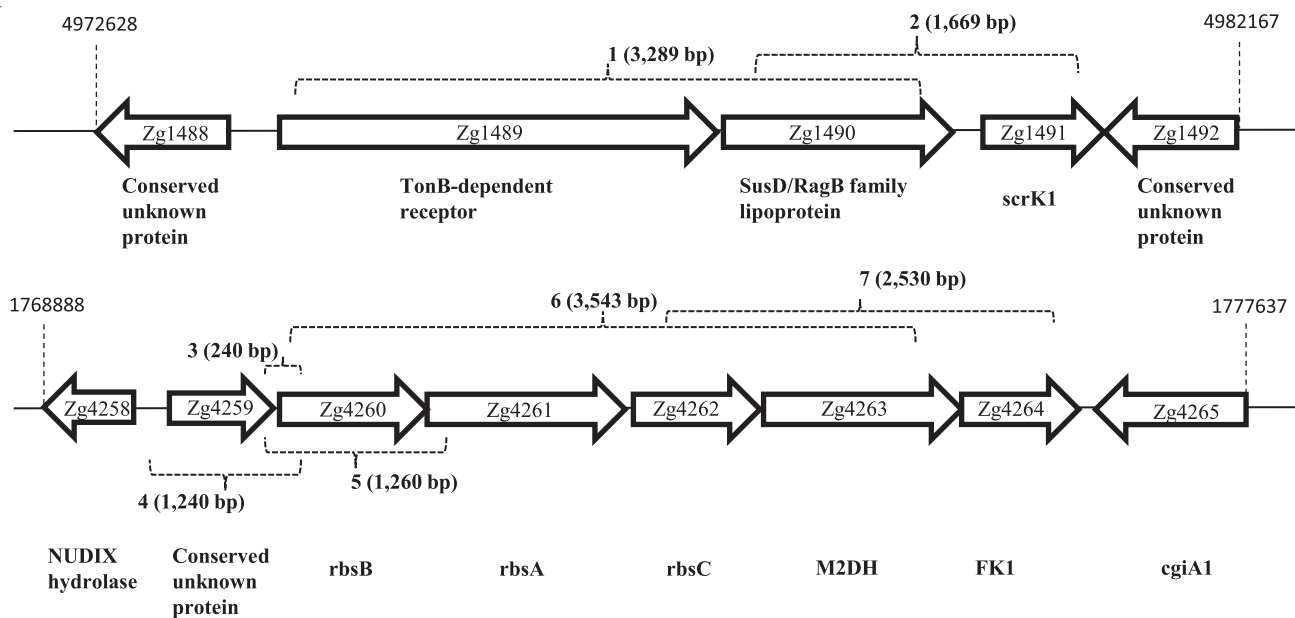
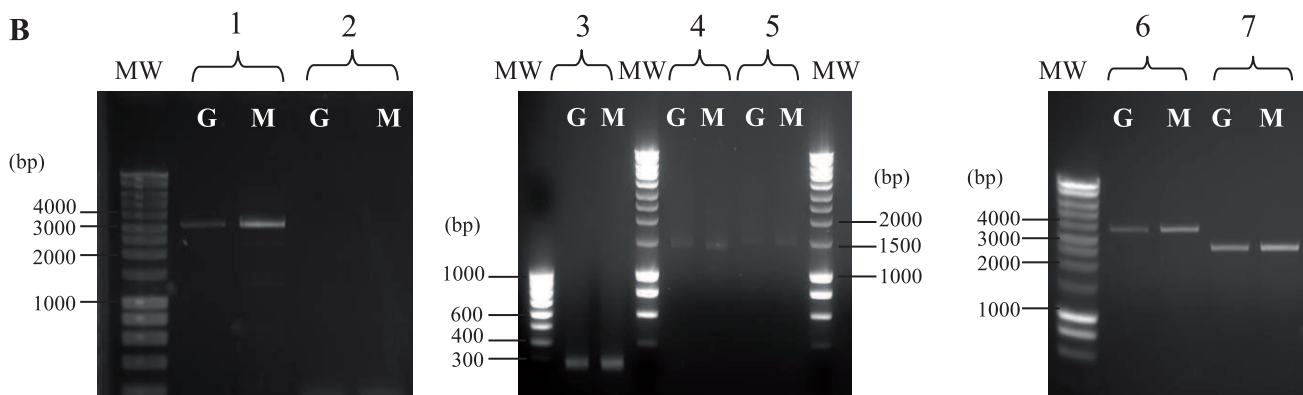
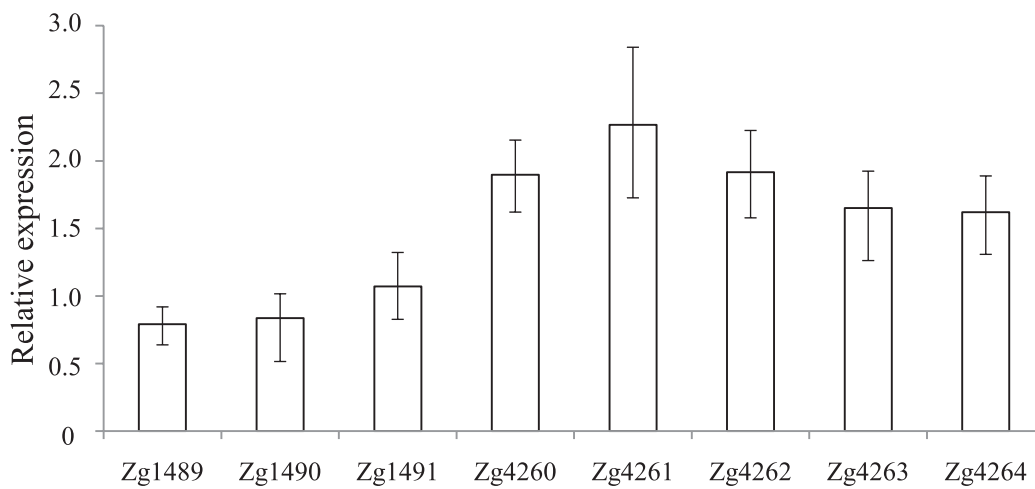
TABLE 4 Comparison of the biochemical properties of fructokinases from different organisms. Kinetic parameters of ZgFK1 were obtained at 25°C and pH 7. Each value represents the mean of triplicate measurements and the standard errors for parameters are less than 10%.

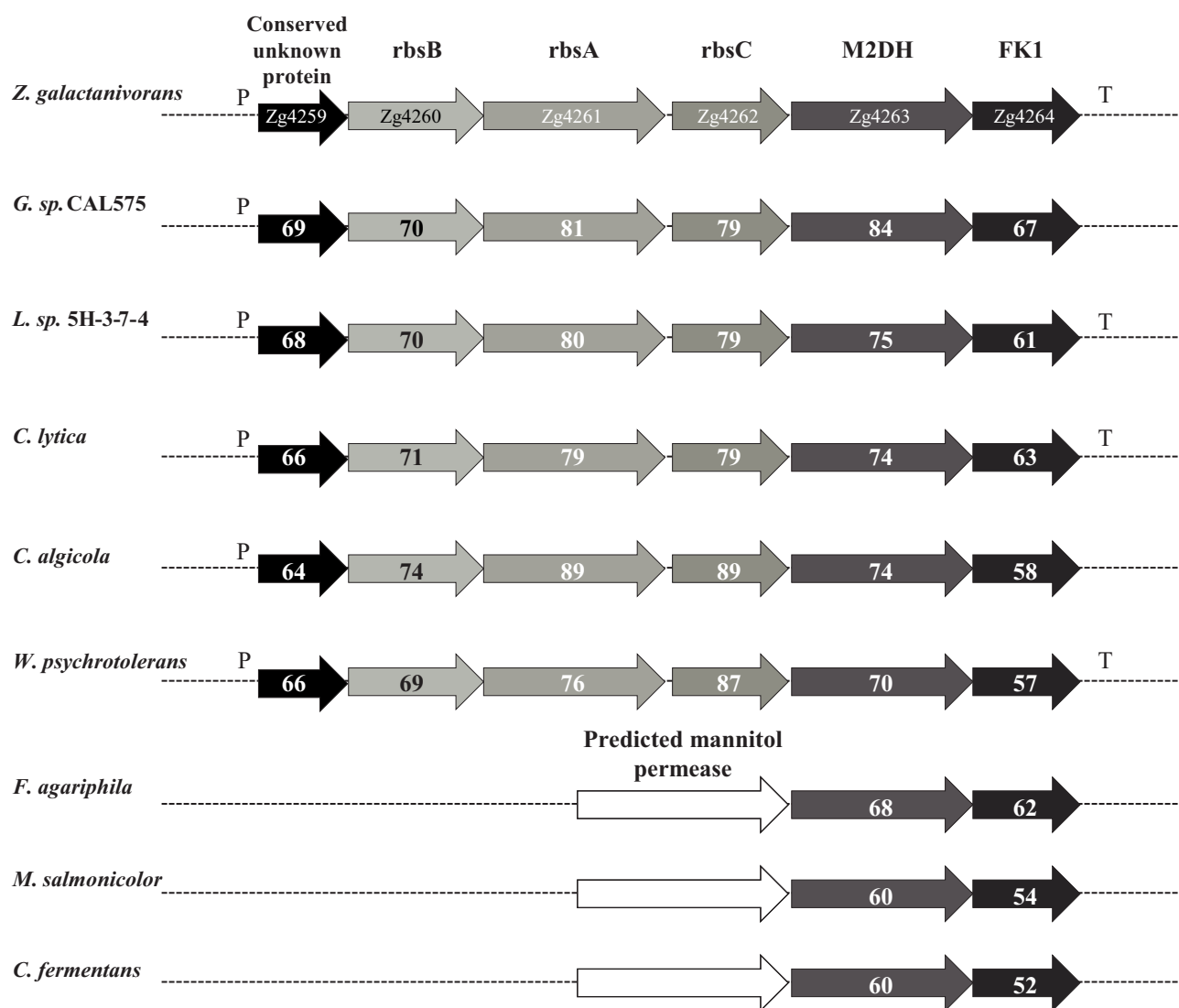
	<i>Z.</i>	<i>B.</i>	<i>Lc.</i>	<i>M.</i>	<i>R.</i>	<i>T.</i>	<i>Z.</i>
<i>Parameters</i>	<i>galactanivorans</i>	<i>longum</i>	<i>lactis</i>	<i>alcaliphilum</i>	<i>leguminosarum</i>	<i>litoralis</i>	<i>mobilis</i>
Sp Act (U/mg protein)							
Fructose	2.55	0.84	112	141	10.80	730	250
Substrate specificity	fructose mannose	fructose	fructose mannose	fructose	fructose	fructose	fructose mannose
pH optimum	7.50	6	7	9	8	7.50	7.40
Temp. ^a optimum	40	50		60		80	
<i>K_m</i> (mM)							
Fructose	10	0.74	0.31	0.26	0.31	2.30	0.7
ATP	0.06	0.76	0.59	1.30		0.81	0.45
Metal requirement	K ⁺		Mg ²⁺	Mg ²⁺	Mg ²⁺	Mg ²⁺	
kDa (number of subunits)	32 (1)	35 (?)	32 (2)	35 (1)	36 (1)	35 (2)	28 (2)
Reference	This work	(91)	(77)	(92)	(93)	(94)	(95)

^aTemp., temperature.



A**B****C**

A**B****C**



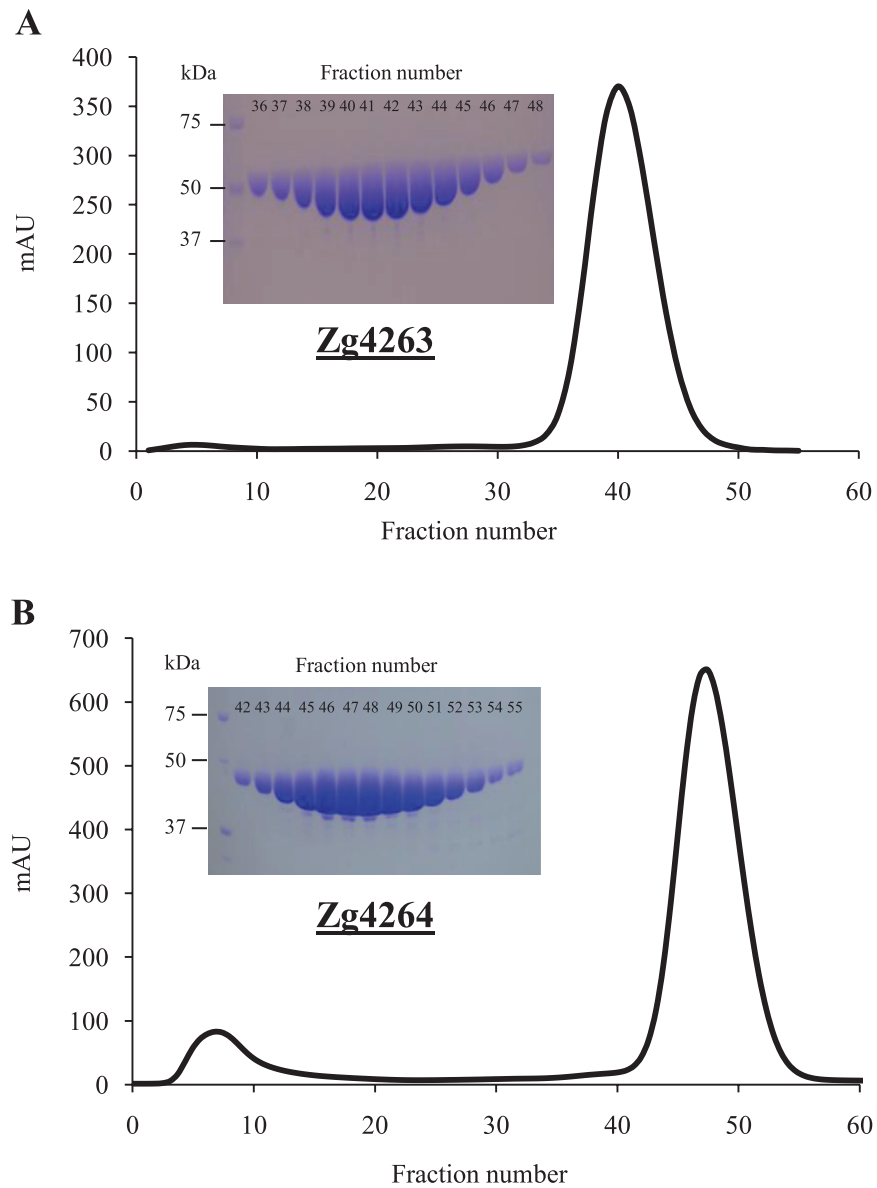


Fig. S1 Purification of Zg4263 (A) and of Zg4264 (B) by affinity chromatography. Proteins were purified by Ni²⁺-affinity chromatography, and eluted fractions were analyzed by SDS-PAGE (inset).

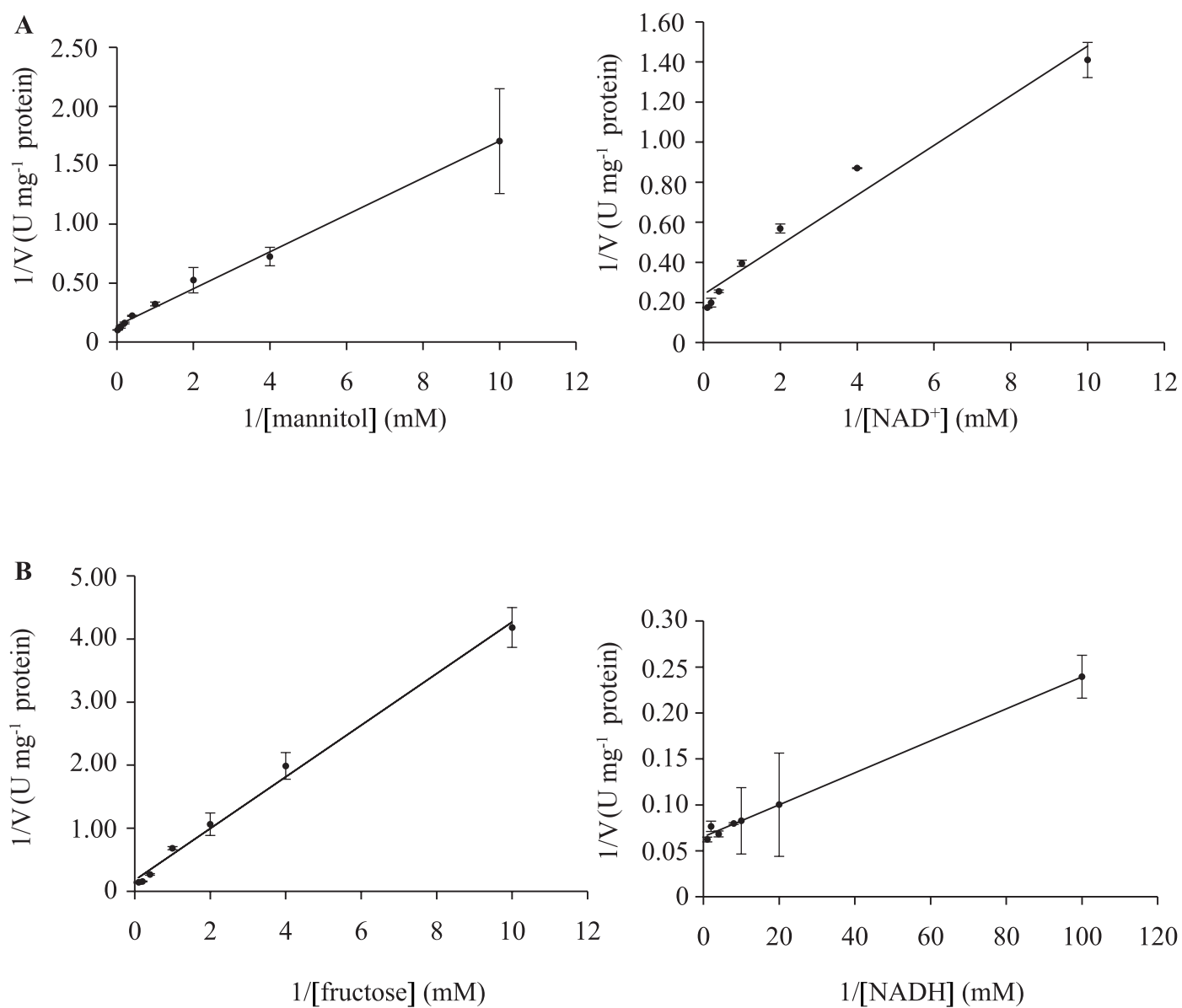


Fig. S2 Determination of kinetics parameters for ZgM2DH.

The Lineweaver-Burk plots for V_m and K_m calculations were obtained for mannitol and NAD⁺ (A) and for fructose and NADH (B). For each substrate, values represent means \pm SD calculated from three reaction assays for one round of purification.

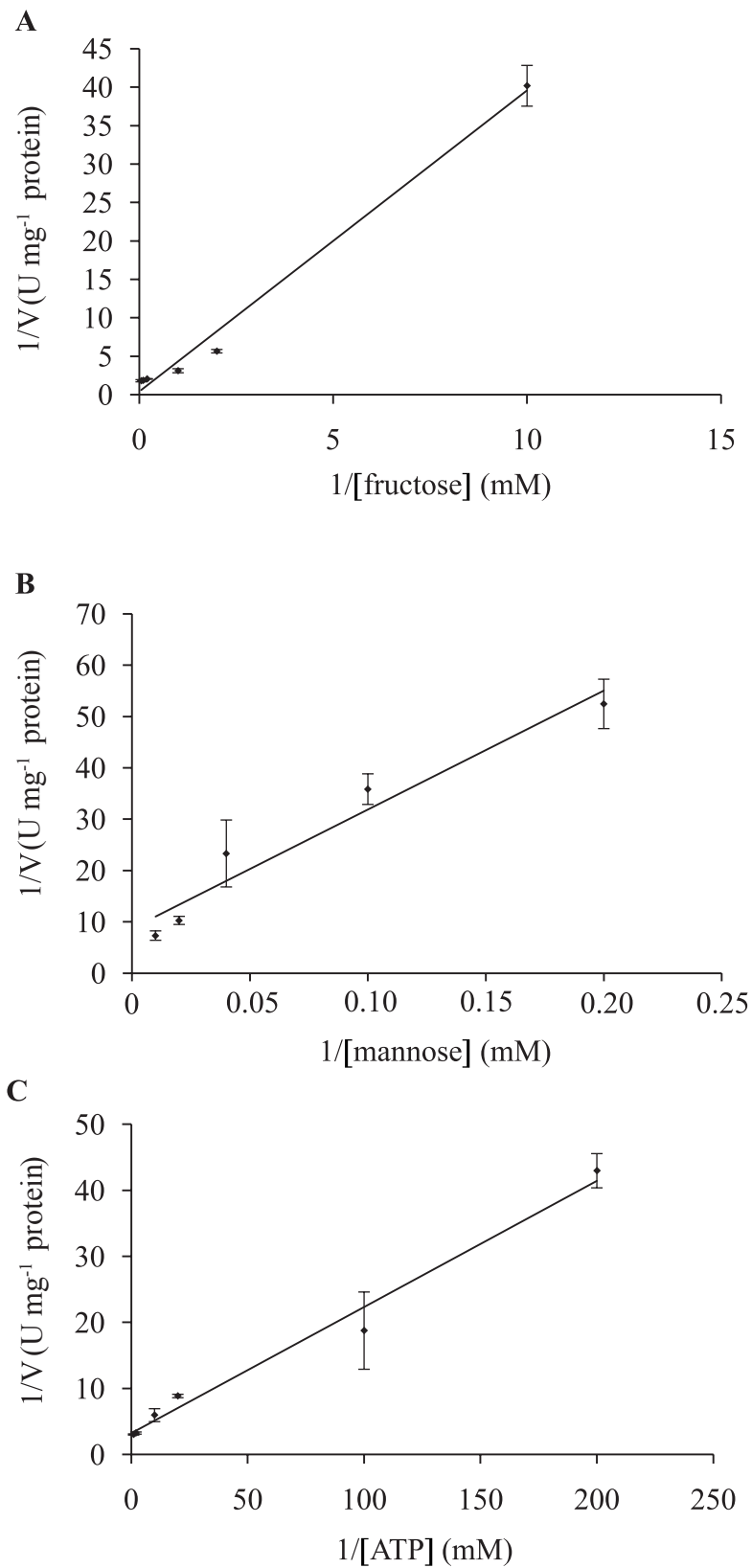


Fig. S3 Determination of kinetics parameters for ZgFK1.

The Lineweaver-Burk plots for K_m and V_m calculations were obtained for fructose (A), mannose (B) and ATP (C). For each substrate, values represent means \pm SD calculated from three reaction assays for one round of purification.