The Mannitol Utilization System of the Marine Bacterium *Zobellia galactanivorans*

Agnès Groisillier, Aurore Labourel,* Gurvan Michel, Thierry Tonon

Sorbonne Universités, UPMC Université Paris 06, UMR 8227, Integrative Biology of Marine Models, Station Biologique de Roscoff, Roscoff, France; CNRS, UMR 8227, Integrative Biology of Marine Models, Station Biologique de Roscoff, Roscoff, France

Mannitol is a polyol that occurs in a wide range of living organisms, where it fulfills different physiological roles. In particular, mannitol can account for as much as 20 to 30% of the dry weight of brown algae and is likely to be an important source of carbon for marine heterotrophic bacteria. *Zobellia galactanivorans* (Flavobacteriia) is a model for the study of pathways involved in the 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 a fructokinase (FK). Among the observations, the M2DH of *Z. galactanivorans* was active as a monomer, did not require metal ions for catalysis, and featured a narrow substrate specificity. The FK characterized was active on fructose and mannose in the presence of a monocation, preferentially K^+ . Furthermore, the genes coding for these two proteins were adjacent in the genome and were located directly downstream of 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 culture of *Z. galactanivorans* in the presence of mannitol as the sole source of carbon. This operon for mannitol catabolism was identified in only 6 genomes of Flavobacteriaceae among the 76 publicly available at the time of the analysis. It is not conserved in all Bacteroidetes; some species contain a predicted mannitol permease instead of a putative ABC transporter complex upstream of M2DH and FK ortholog genes.

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 matrices are formed by the accumulation of cellulose, fucanes, and alginates (2–4), while they store 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 highlighted recently.

Depending on the species, mannitol can represent as much as 20 to 30% of the dry weight of brown seaweed (10). In the genomic and genetic model of brown algae *Ectocarpus* sp., formerly included in the species *Ectocarpus siliculosus* (11), it has been observed that the content of this polyol differs according to the diurnal cycle (12) and that it is likely to act as an osmoprotectant or a local compatible osmolyte (13). Mannitol is localized in the cytosol and is also present at the reducing ends 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 (M1P), which is then converted into mannitol by mannitol-1-phosphatase (M1Pase) (5, 15, 16). Mannitol is thought to be recycled by the successive actions of a mannitol-2 dehydrogenase (M2DH) and a hexokinase (HK) (5, 17), but little is known about 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 bac-

teria, mannitol is taken up by a mannitol-specific phosphoenolpyruvate/carbohydrate phosphotransferase system (PTS) and is phosphorylated into 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 M1Pase 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; mannitol is transported by ATP binding cassette (ABC) transporters (28). In this organism, fructose is believed to be phosphory-

Received 27 August 2014 Accepted 21 December 2014

Accepted manuscript posted online 29 December 2014

Citation Groisillier A, Labourel A, Michel G, Tonon T. 2015. The mannitol utilization system of the marine bacterium *Zobellia galactanivorans*. Appl Environ Microbiol 81:1799–1812. doi:10.1128/AEM.02808-14.

Editor: H. Nojiri

Address correspondence to Thierry Tonon, tonon@sb-roscoff.fr.

* Present address: Aurore Labourel, University of Newcastle, Cell and Molecular Biosciences, Medical School, Newcastle upon Tyne, United Kingdom.

A.G. and A.L. contributed equally to this article.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AEM.02808-14>.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AEM.02808-14

lated 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* DSM 17395 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, *Flavobacteriaria*, and notably several species of *Zobellia*, have been found in association with macroalgae (31–33) and have been isolated from phytoplankton (34) and from seawater (35). The interactions between *Zobellia* strains and marine algae range from symbiosis, with some strains inducing the normal differentiation of green macroalgae (36), to algicidal behavior toward dinoflagellate blooms (34). The type species of the *Zobellia* genus, *Zobellia galactanivorans* (formerly known as “*Cytophaga drobachiensis*”), was initially isolated in Roscoff, France, 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-porphyranses (40), and 3,6-anhydro-L-galactosidases (41, 42). Based on these results, *Zobellia galactanivorans* is attracting interest as a model for the study of the bioconversion of macroalgal polysaccharides. Furthermore, although this bacterium has been isolated on a red alga, it can metabolize cell wall and storage polysaccharides from brown algae. Indeed, it possesses two alginolytic operons induced by the presence of alginate (43), and the first two alginate lyases of this complex system (AlyA1 and AlyA5) have recently been characterized at the biochemical and structural levels (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 (GenBank accession number [FP476056](#)), two GH16 laminarinases, *Z. galactanivorans* LamA (ZgLamA) and ZgLamC, have recently been analyzed biochemically and structurally (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 genes encoding these enzymes are induced by mannitol and are organized as an operon.

MATERIALS AND METHODS

Bacterial strain and culture conditions. The type strain of *Z. galactanivorans*, Dsij (31), was grown in Zobell medium 2216E (tryptone at 5 g liter⁻¹ and yeast extract at 1 g liter⁻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, and 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).

Nucleic acid extraction. Genomic DNA was extracted as described previously (46). For RNA extraction, 2 ml of the growth media of cultures at the end of the exponential phase was added to 4 ml of RNeasy Protect

reagent. RNAs were then isolated by using the RNA minikit (Qiagen) according to the manufacturer's instructions. Two steps of DNA digestion were performed using DNase I (Qiagen). RNAs were cleaned up on a minicolumn and were 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 was 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 OD₂₆₀ (optical density at 260 nm)/OD₂₈₀ and OD₂₆₀/OD₂₃₀ ratios were calculated to assess the purity of RNA extracts.

RT-PCR and quantitative PCR (qPCR). cDNA were synthesized from 250 ng of total RNA using the Phusion reverse transcription-PCR (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 were stored at –80°C.

For the amplicons named Zg1489-1490, Zg1490-1491, Zg4259-4260, Zg4259-4261, Zg4260-4263 and Zg4262-4264, the PCRs were performed using the Advantage 2 PCR kit (Clontech) with 0.2 µM each primer (Table 1) and 20 ng of cDNA. The PCR program was as follows: 94°C for 5 min; 35 cycles of 94°C for 30 s, 50°C or 55°C for 30 s, and 72°C for 30 s or 3 min; and finally 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-source software (47). They were 17 to 22 nucleotides (nt) long, with melting temperatures (*T_m*) between 58 and 60°C. A difference of 1°C between the forward and the reverse primer was accepted. The GC content of the primers was between 47 and 53%. The Δ*G* 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 GC content of >60%. The nucleotide sequences of all primers were compared to the genomic sequence of *Z. galactanivorans* (GenBank accession number [FP476056](#)) to check gene specificity. PCR of the genomic DNA of *Z. galactanivorans* using the different primer pairs was carried out to confirm specific amplification. Quantitative PCRs were performed in 96-well plates (Thermo Scientific) on a LightCycler system, model 480 (Roche). The composition of each reaction mixture was as follows: 2.5 ng of cDNA, 250 nM each primer (Table 1), 5 µl of 2× SYBR green, and water for a final volume of 10 µl. The reactions for each gene were carried out in technical triplicate. The program was as follows: 5 min at 95°C, followed by 45 cycles of 95°C (10 s), 51°C (15 s), and 72°C (15 s) with a single acquisition mode. Serial dilutions of genomic DNA ranging from 10 to 10⁵ copies were amplified by qPCR in the same run as the cDNA samples. The LightCycler480 software was used to obtain crossing point (Cp) values and PCR efficiencies. Relative expression was determined with REST 2009 software using *glyA* and *icaA* as reference genes (48). This software determines the ratio corresponding to the relative expression by the equation of Pfaffl: ratio = $E_{\text{target}}^{\Delta C_T} / (E_{\text{reference}}^{\Delta C_T} \times \text{reference (control - sample)})$, where *E* refers to efficiency and *C_T* is the threshold cycle (49). The influence of mannitol on the expression of genes of interest was determined by comparing their expression levels in the presence of glucose.

Construction of plasmids for overexpression in *Escherichia coli*. The Zg1491, Zg4263, and Zg4264 coding regions were cloned into the pFO4 expression vector as described previously (50). Briefly, the genes were amplified by PCR (the primers used are listed in Table 1) from *Z. galactanivorans* genomic DNA; the PCR fragments were digested by BamHI/MfeI for Zg1491 and by BamHI/EcoRI for Zg4263 and Zg4264 and were ligated into the pFO4 vector; and recombinant plasmids named pZg1491, pZg4263, and pZg4264, respectively, were transformed into *E. coli* BL21(DE3). The integrity of their sequences was verified by sequencing.

Nucleotide and protein sequence analyses. The mannitol degradation-related genes of *Z. galactanivorans* were identified in its complete genome (GenBank accession number [FP476056](#)) using the GenDB program, version 2.4 (51). Signal peptide and transmembrane helices were

TABLE 1 Primers used in this study

Primers	Nucleotide sequence (5'–3') ^a	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	CACACCTCATCCTATCCGA	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	GTAAGCCACCAATAGCCC		59
Zg4263-F	CTTGCGACAACATTCAGGG	66	60
Zg4263-R	GCGGTGGAACATAAGTGAG		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-	AAGGCGTTAAGTAAATGGTAGATAC		72
Zg4259-4260-F2	TCAAATGCGTACAAAATTCCTCATAAC	1,240	72
Zg4259-4260-R	AAGGCGTTAAGTAAATGGTAGATAC		72
Zg4259-4261-F1	GCCATGTGGAATCCAGAAAAGGA	1,260	68
Zg4259-4261-R	GCTTTTTGTATTTGCATATCGTCCAT		72
Zg4260-4263-F	CTTATGCTGGAGGACTAAAGG	3,543	58
Zg4260-4263-R	GCGGTGGAACATAAGTGAG		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	GGGGGGGGATCCAAAAATTATAAATTAACAGCACTAATCTT	1,491	70 ^b
Zg4263pFO4-R	CCCCCGAAATTCCTAATTGTTGTTCTCCTGGTTACGGTTT		70 ^b
Zg4264pFO4-F	GGGGGGGGATCCAAAAATATAGTCTGTTTGGGGAAGTT	882	70 ^b
Zg4264pFO4-R	CCCCCGAAATTCCTATTGGCGACTTTTGATAAAGGCTTGG		72 ^b
Zg1491pFO4-F	GGGGGGGGATCCAAAACCGTATACTGCATTGGAGAATT	942	70 ^b
Zg1491pFO4-R	CCCCCATGCAATTATCCCCGAAAACCATGCTGTTGTC		72 ^b

^a Restriction sites are indicated in italics.^b Calculation of T_m does not include the sequences corresponding to the restriction sites or the sequence upstream.

predicted using SignalP version 2.0 (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>; accessed 14 January 2014). BLASTP analysis was also done against the UniProtKB/Swiss-Prot database in order to gain insight into 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 –33 (TTG)/–7 (TANNTTTG) motif with a spacer 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). TransTermHP (58) software was used to predict putative Rho-independent transcriptional terminators.

Expression and purification of recombinant proteins. *E. coli* strain BL21(DE3) (Novagen) transformed with plasmid pZg1491, pZg4263, or

pZg4264 was grown in ZYP 5052 medium (59) at 20°C for 72 h, and protein expression was induced with 0.2% lactose supplemented with 200 µg/ml (final concentration) of ampicillin at 20°C and 200 rpm on an orbital shaker. Recombinant proteins were purified using the ÄKTA avant system (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. Proteins were eluted using a linear increasing gradient (from 0 to 100%) of 20 mM Tris-HCl (pH 7.5) containing 200 mM NaCl and 500 mM imidazole within 10 column volumes. Fractions were collected in a deep 96-well 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 concentrations were measured at 280 nm using a NanoDrop 2000 spectrophotometer (Thermo Fisher). A molar extinction coefficient of 58,705 M^{–1} cm^{–1} and a molecular mass of 55.61 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 used for Zg4264, to calculate the concentration of pure proteins (ExPASy, ProtParam tool [60]). The molecular masses of the native recombinant proteins were estimated by size exclusion chromatography. Eluted fractions from Ni^{2+} affinity chromatography were loaded at a flow rate of 1 ml min^{-1} onto a calibrated Sephacryl HiLoad Superdex 200 column (GE Healthcare) equilibrated with a 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. Unless noted otherwise, all the assays were carried out in triplicate at 25°C in 100- μl 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 Safire2 UV spectrophotometer microplate 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 μg of purified enzyme. The standard mannitol oxidation reaction mixture contained 0.1 M Tris-HCl (pH 8.5), 1 mM mannitol, 0.5 mM NAD^+ , and 1 to 10 μg of recombinant enzyme. Both reactions were initiated by adding the substrate.

The dependences 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 . The buffers used were 0.1 M morpholineethanesulfonic acid (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). The effects of various metal ions and chemical reagents were examined at final concentrations of 1 and 10 mM. 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) were tested as potential substrates. The influence of NaCl on M2DH activity was assessed by testing final concentrations ranging from 0 to 2 M. The kinetic parameters K_m and $V_{\max}$ 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 for the former and those of D-mannitol and NAD^+ for the latter. For both activities, 1 U corresponded to 1 μmol of NAD(H) reduced or oxidized per min per mg of protein.

Determination of HK/FK activity. Hexokinase (HK)/fructokinase (FK) activity was determined by an enzyme-coupled assay. The reaction mixture (total volume of 100 μl) contained the Zg4264 enzyme in solution in 50 mM Tris-HCl (pH 7.6), in the 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 mixture composed of lactate dehydrogenase (LDH) (900 to 1,400 U ml^{-1}) and pyruvate kinase (PK) (600 to 1,000 U/ml) (Sigma). The reaction was initiated with 1 mM fructose. Enzyme activity was measured by following changes in absorbance at 340 nm in a Safire2 UV spectrophotometer microplate reader (Tecan). The temperature and pH optimum were determined under the same conditions as those 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 potential substrates, and EDTA, mannose-6-phosphate, and fructose-6-phosphate were tested 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 1 μmol of NADH oxidized per min per mg of protein.

Thermostability analysis. The thermostabilities of Zg4263 and Zg4264 were studied by dynamic light scattering (DLS). A solution of 50 μl of each enzyme at 10 mg ml^{-1} was filtered on a 0.2- μm membrane. By using a Zetasizer Nano instrument (Malvern), the protein solution was heated from 10°C to 70°C in steps of 1°C for a total period of 12 h, and the radius of gyration (Rg) was measured at each degree. The denaturation

temperature was determined as the point of sharp change in the radius of gyration.

RESULTS

Identification of genes coding for enzymes potentially involved in mannitol degradation. In the genome of *Z. galactanivorans*, the Zg4263 gene (1,494 bp) is annotated as encoding a putative cytosolic D-mannonate oxidoreductase. The corresponding protein of 498 amino acids (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 Zg4264 gene (885 bp; corresponding to a protein of 295 aa), which is located immediately downstream of Zg4263, encodes a putative cytosolic fructokinase (FK) belonging to the PfkB family of carbohydrate kinases. 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 of carbohydrate kinases) encoded by the Zg1491 gene (945 bp). There is 26% 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 into *E. coli*. While both the Zg4263 and Zg4264 genes were overexpressed under the conditions tested, no overexpression was observed for Zg1491, despite several attempts to improve the conditions of induction. Therefore, we focus our attention on the Zg4263 and Zg4264 proteins.

Expression and purification of recombinant Zg4263 and Zg4264 proteins. Recombinant Zg4263 and Zg4264 proteins were successfully expressed in high quantities in the *E. coli* BL21(DE3) expression strain as soluble forms and were purified to electrophoretic homogeneity (see Fig. S1A and B in the supplemental material). The final yield after one step of purification by affinity chromatography was about 30 mg for 200 ml of ZYP medium for Zg4263 and about 25 mg for Zg4264 under the same conditions. The Zg4263 and Zg4264 proteins eluted after the size exclusion chromatography step had apparent molecular masses of 56 kDa and 32 kDa, respectively. Comparison of these results with the theoretical masses of 55.6 kDa and 32.7 kDa indicated that both proteins are in the monomeric form in solution. The specific activities of the two recombinant enzymes were compared after Ni^{2+} 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 levels of purity of recombinant Zg4263 and Zg4264. These results were confirmed by dynamic light scattering (DLS) experiments. DLS was also used to study protein thermostability; above 40°C , a sharp increase in the radius of gyration (Rg) was observed, indicating the beginning of the denaturation of Zg4263 and Zg4264.

Biochemical characterization of Zg4263. The specificity of the putative dehydrogenase/oxidoreductase Zg4263 was tested in the 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 cosubstrate, and fructose, mannose, glucose, galactose, and xylose (at 1, 10, or 50 mM), with NADH or NADPH as a cosubstrate, were examined as alternative substrates

FIG 1 Enzymatic properties of ZgM2DH. (A and B) Effects of various buffer pH values on the mannitol oxidation (A) and fructose reduction (B) activities of ZgM2DH. Symbols: □, 0.1 M Bis-Tris propane buffer; ◇, 0.1 M MES buffer; △, 0.1 M Tris-HCl buffer. (C) Effects of various temperatures on the mannitol oxidation activity of ZgM2DH. The enzyme activity observed at pH 8.5 (in panel A) or pH 6.5 (in panel B) and 40°C was considered 100%, and all other values are expressed as percentages of these activities. Each value represents means $\pm$ standard deviations calculated from three reaction assays for one round of purification.

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 was observed when NAD(H) was replaced by NADP(H). This protein is thus a true M2DH and is referred to below as ZgM2DH. In the direction of polyol oxidation, only sorbitol at 50 mM exhibited activity, representing 14% of the level measured in the presence of mannitol with NAD⁺. In the reverse direction, no activity was observed in the presence of mannose, glucose, galactose, or xylose. These results show that the enzyme features narrow substrate specificity in both directions and may catalyze only 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 maximum activity in Tris-HCl buffer at pH 8.0 and pH 9.0, respectively. The optimum pH for fructose reduction was 6.5, with 70% (Tris-HCl buffer) and 76% (MES buffer) of maximum activity at pH 6.0 and 7.0, respectively (Fig. 1A and B). These pH optimum values were in accordance with those measured for similar enzymes isolated

from diverse bacteria (Table 2). The optimal temperature for mannitol oxidation was 40°C in Tris-HCl buffer at pH 8.5 (Fig. 1C), a value higher than the optimum temperatures determined for other organisms, except *Thermotoga maritima* (Table 2).

M2DH oxidation and reduction activities were measured in the presence of different chemicals (Table 3). The results showed that the enzyme was only slightly sensitive to metal chelators such as EDTA (at final concentrations of 1 and 10 mM) and to mercaptoethanol. In contrast, Ca²⁺, Mg²⁺, K⁺, and Li⁺, inhibited M2DH activity in both directions by 20 to 30%, while NH₄⁺ decreased mannitol oxidation activity by 23% but fructose reduction activity by 54%. NaCl at 1 M slightly increased oxidation activity but significantly inhibited (by 45%) reduction activity. Similar effects were observed in the presence of 1.5 and 2 M 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, mannitol production was strongly activated by the addition of fructose-6-phosphate (20 mM), which was the product of FK activity; conversely, this reaction was inhibited (by 25%) by the 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. The substrates and cofactors had hyperbolic saturation curves, and the corresponding double-reciprocal plots were linear (see Fig. S2 in the supplemental material). The concentrations of mannitol and fructose substrates ranged from 0.1 to 50 mM, that of NAD⁺ ranged from 0.01 to 10 mM, and that of NADH ranged from 0.01 to 1 mM, since that was the highest cofactor concentration compatible with the spectrophotometric assay. The results showed that the ZgM2DH enzyme had equivalent K_m and k_{cat} values for mannitol and fructose (Table 2). Indeed, the specific activities and K_m values were of the same order of magnitude, i.e., 7.18 U/mg protein and 1.12 mM for mannitol and 5.64 U/mg protein and 2.30 mM for fructose.

Biochemical characterization of Zg4264. The recombinant Zg4264 protein preferentially catalyzed the ATP-dependent phosphorylation of fructose into fructose-6-phosphate (specific activity, 2.55 U/mg at 5 mM fructose) but also used mannose as a substrate. Indeed, when mannose was added to the reaction medium at a final concentration of 10 or 50 mM, the specific activity corresponded to 5% or 16%, respectively, of that measured in the presence of fructose. 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 is referred to below as ZgFK1.

This fructokinase was active in the pH range of 5.5 to 9, with an optimum at pH 7.5 (Fig. 2A) (specific activity, 3.95 U/mg at 10 mM fructose), and its activity decreased sharply at more alkaline pH values (only 30% of maximum 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 on a monovalent cation, since no activity was detectable in the absence of K⁺. Specific activity was highest in the presence of K⁺ at a final concentration of 100 mM (Fig. 2C). Na⁺ could partially replace K⁺ (about 20% of the activity at a final concentration of 0.05 to 1 M Na⁺), while

TABLE 2 Comparison of the biochemical properties of M2DHs from different organisms

Parameter ^a	<i>Z. galactanivorans</i> ^b	<i>Aspergillus fumigatus</i>	<i>P. fluorescens</i>	<i>Lactobacillus sanfranciscensis</i>	<i>R. sphaeroides</i>	<i>Leuconostoc pseudomesenteroides</i>	<i>T. maritima</i>
Sp act (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 optimum (°C)							
Fructose	40	25	25	35			95
Mannitol	40		25				
<i>K_m</i> (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
<i>k_{cat}/K_m</i> (mM ⁻¹ s ⁻¹)							
Fructose	2.30	1.40	2.20				
NADH	263	4,700	436				
Mannitol	6	17	18				
NAD ⁺	13	1,400	159				
<i>k_{cat}</i> (s ⁻¹)							
Fructose	5.27	86	54				
Mannitol	6.70	212	20				
Mol mass (kDa) (no. of subunits)	56 (1)	58 (1)	54 (1)	53 (1)	52 (1)	36 (4)	34 (4)
Classification ^c	LDR	LDR	LDR		LDR	MDR	MDR
Reference	This work	81	61, 86	87	80, 88	89	83

^a Each value represents the mean for triplicate measurements, and the standard errors for the parameters are less than 10%.
^b The kinetic parameters of ZgM2DH were obtained at 25°C in 0.1 M Tris-HCl at pH 6.5 for the D-fructose reduction and at 25°C in 0.1 M Bis-Tris propane buffer at pH 8.5 for the D-mannitol oxidation.
^c LDR, long-chain dehydrogenase/reductase family; MDR, Zn²⁺-containing medium-chain dehydrogenase/reductase family.

TABLE 3 Effects of metal ions and chemical reagents on the activity of ZgM2DH^a

Ion or compound	Concn (mM)	Relative activity (%) ^b	
		Mannitol oxidation	Fructose reduction
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
Fructose	50	47	ND
Mannitol	20	ND	73
Mannitol	50	ND	46
Mannitol-1-phosphate	20	95	77
Fructose-6-phosphate	20	93	157
NaCl	100	100	100
NaCl	1,000	120	55

^a The purified enzyme was assayed under standard assay conditions with ions in chloride forms and several chemicals.
^b Percentage of the activity remaining under the specific condition tested relative to the activity observed in the absence of any of these ions or compounds, taken as 100%. Each value represents the mean of triplicate measurements. The standard errors for activities are less than 10%. ND, not determined.

no activity was observed in the presence of MgCl₂. This result contrasted with the Mg²⁺ requirement of several previously characterized bacterial FKs (Table 4). The influences of mannitol-6-phosphate and fructose-6-phosphate on hexokinase activity were determined with fructose or mannose as the substrate (final concentration in the reaction mixture, 10 or 50 mM, respectively). The addition of 20 mM (final concentration) mannitol-6-phosphate inhibited fructose phosphorylation by 85% and mannose phosphorylation by 80%, while the addition of 20 mM (final concentration) 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 substrates investigated (D-fructose, D-mannose, and ATP) (see Fig. S3 in the supplemental material). The apparent *K_m*s for D-fructose, D-mannose, and ATP are 10, 26.6, and 0.31 mM, respectively. Although the *K_m*s for fructose and mannose were in the same range, the *k_{cat}/K_m* value for fructose (0.136 mM⁻¹ s⁻¹) was 60 times higher than that for mannose (0.0023 mM⁻¹ s⁻¹). Similarly, the *k_{cat}/K_m* value for ATP in the presence of fructose was 23 mM⁻¹ s⁻¹, in contrast to 1 mM⁻¹ s⁻¹ in the presence of mannose (data not shown).

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

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 mannitol (Fig. 3A). The first cluster, about 6,200 bp long, contained three genes, *Zg1489*, *Zg1490*, and *Zg1491*, annotated as encoding putative SusC-like TonB-dependent receptor (TBDR), SusD-like, and ScrK1 fructokinase proteins, respectively. The second cluster, about 6,900 bp long, featured six genes named *Zg4259* to *Zg4264*. These two clusters were separated by 3,210 kbp in the bacterial chromosome. In this study, we have characterized the *Zg4263* and *Zg4264* proteins. The *Zg4259* gene codes for an unknown conserved protein. The three genes *Zg4260*, *Zg4261*, and *Zg4262* correspond to a putative transmembrane protein complex involved in the uptake of monosaccharides. The predicted amino acid sequences encoded by these open reading frames (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, codes for a peptide of 354 aa with a calculated molecular mass of 32 kDa. The deduced protein showed an overall amino acid sequence identity of 31% with the *E. coli* ribose binding periplasmic protein RbsB. A putative peptide signal of 29 aa was located at the N terminus. The second gene, *Zg4261*, of 1,527 nt, encodes a peptide of 509 aa with a molecular mass of 56 kDa. The deduced protein exhibited an overall amino acid sequence identity of 41% with the *E. coli* ribose ATP-binding protein RbsA. Finally, the third gene, *Zg4262*, of 969 nt, codes for a peptide of 323 aa with a calculated molecular mass of 34 kDa. The deduced protein showed an overall amino acid sequence identity of 43% with the *E. coli* ribose permease protein RbsC, was predicted to be very hydrophobic, and contained nine transmembrane domains.

In order to test the hypothesis that some of these genes may be expressed on polycistronic messengers, semiquantitative 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, the results showed that the TonB-dependent receptor and *susD*-like genes are expressed in the same polycistronic mRNAs, while this was not the case for the fructokinase *scrK1* and *susD*-like genes (Fig. 3B, left). For the second gene cluster, three amplicons (Fig. 3B, middle)

TABLE 4 Comparison of the biochemical properties of fructokinases from different organisms

Parameters ^a	<i>Z. galactanivorans</i> ^b	<i>Bifidobacterium longum</i>	<i>Lactococcus lactis</i>	<i>Methylobacterium alcaliphilum</i>	<i>Rhizobium leguminosarum</i>	<i>Thermococcus litoralis</i>	<i>Zymomonas mobilis</i>
Sp act (U/mg protein) with 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 optimum	40	50		60		80	
K_m (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⁺	
Mol mass (kDa) (no. of subunits)	32 (1)	35 (?)	32 (2)	35 (1)	36 (1)	35 (2)	28 (2)
Reference	This work	90	76	91	92	93	94

^a Each value represents the mean for triplicate measurements, and the standard errors for parameters are less than 10%.

^b The kinetic parameters of ZgFK1 were obtained at 25°C and pH 7.

FIG 3 Cluster organization and 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 RNAs extracted from cultures with glucose (G) or mannitol (M). Agarose gels show the PCR products indicated in panel A by numbers 1 to 7. MW, molecular mass standard. (C) Differential expression of the genes of interest between cultures in the presence of mannitol and cultures in the presence of glucose. Bars indicate ratios of expression in the presence of mannitol to expression in the presence of glucose.

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). The occurrence of these both overlapping transcripts suggests that all the genes of this cluster are likely to be expressed on the same polycistronic RNA, even if no PCR product (expected length of 5,820 bp) was observed in the attempt to amplify a fragment covering the region from *Zg4260* to *Zg4264*. Similar patterns of amplification were obtained after *Z. galactanivorans* was grown in the 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 relative to transcription in the presence of glucose. The levels of expression were quite similar under culture in the 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 subunits of the ABC transporter and for proteins catalyzing the utilization of mannitol were induced simultaneously after culture in the presence of mannitol, supporting their organization as a true operon (Fig. 3C).

Taxonomic distribution and organization of genes orthologous to those encoding *ZgM2DH* and *ZgFK1* in bacteria. The *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 *Zg4259* and *Zg4260* genes. However, directly upstream of the start codon of *Zg4259*, a gene encoding an unknown conserved protein, a putative promoter sequence presenting only one mismatch with the consensus sequence (TTG/TANNTTTG) was identified. The length of the spacer separating the -7 (TANNTTTG) and -33 (TTG) motifs was 19 bp. Furthermore, this region was relatively poor in GC (27% GC) compared with the mean value for the whole genome (43% GC). These parameters were consistent with studies of *Flavobacterium* species showing that promoter strength was influenced by spacer length (19 bp being the optimal value) and that the sequence of the promoter was enriched with A and T nucleotides (56). Sequence analyses also revealed the presence of a single putative terminator in this cluster of genes, downstream of the *Zg4264* locus.

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 (14 January 2014). The genomic vicinity of each ortholog identified was then screened to determine the presence of genes coding for an ATP binding cassette (ABC) transporter complex in these microbes. Such clusters of genes involved in mannitol degradation, or mannitol utilization clusters, were absent in archaeal genomes and present in the *Bacteroidetes* phylum genomes, but only in 6 of the 76 complete genomes available for the *Flavobacteriaceae* family: *Z. galactanivorans* Dsij^T (31), *Gillisia* sp. strain CAL575 (65), *Lacinutrix* sp. strain 5H-3-7-4 (66), *Cellulophaga lytica* DSM 7489 (67), *Cellulophaga algicola* DSM 14237 (68), and *Winogradskyella psychrotolerans* RS-3^T (69). When the entire operon containing genes coding for the unknown conserved protein, *RbsB*, *RbsA*, *RbsC*, *M2DH*, and *FK1* was considered, analysis of nucleotide sequences

of *Flavobacteriaceae* showed very high percentages of identity, between 64% (minimum value, obtained for *Z. galactanivorans* and *Winogradskyella psychrotolerans* RS-3^T) and 75% (maximum value, obtained for *Lacinutrix* sp. strain 5H-3-7-4 and *Winogradskyella psychrotolerans* RS-3^T). In the same vein, analysis of amino acid sequences of the different proteins encoded by the mannitol utilization gene cluster showed the highest degree of conservation within the six *Flavobacteriaceae* species considered (Fig. 4) compared to orthologous genes identified in other bacteria. As an illustration, the percentages of identity of putative ABC transporter proteins ranged from 69% to 89% within these six species but 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 3901^T (*Flavobacteriaceae* family) (70), *Marinilabilia salmonicolor* JCM 21150 (*Marinilabiliaceae* family) (71), and *Cytophaga fermentans* JCM 21142^T (*Cytophagaceae* family) (72), a gene coding for a predicted mannitol permease was identified upstream of 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, the latter involving different types of transporters for mannitol. The PTS consists of a phosphocarrier or histidine protein (HPr), a protein kinase enzyme I (EI), and 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 *Vibrio cholerae* (74). In contrast, in the *P. fluorescens* mannitol operon, this polyol is transported by an ATP binding cassette transporter (MtlEFGK) and is oxidized by an *M2DH* (MtlD) to produce fructose, which is then converted into 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 an *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 *Flavobacteriia* member *Z. galactanivorans*. This cluster is approximately 6.9 kb long and comprises six genes in the following order: an unknown conserved 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 genes have been purified and successfully characterized: *Zg4263* is a NAD-dependent *M2DH* (EC 1.1.1.67) capable of both mannitol synthesis and degradation, and *Zg4264* is a fructokinase (EC 2.7.1.4), a member of the PfkB family of carbohydrate kinases, and is active on fructose and mannose in the presence of a monocation, preferentially K⁺. The latter observation is in line with the fact that bacterial fructokinases are usually mannofructokinases (76, 77).

Analysis of the primary sequence of *ZgM2DH* indicated that this protein is a member of the long-chain MDH family (78) and more specifically of the polyol-specific long-chain dehydrogenase/reductase subfamily (PSLDR) (79). Most members have

FIG 4 Organization of genes related to mannitol catabolism in selected bacteria. Each arrow represents a gene and its orientation. The genes code for an unknown conserved protein, a ribose binding periplasmic protein (RbsB), a ribose ATP-binding protein (RbsA), a ribose permease protein (RbsC), a mannitol-2-dehydrogenase (M2DH), and a hexokinase (FK1). The percentage of identity of each orthologous gene to the *Z. galactanivorans* gene is given inside the arrow. P, putative promoter; T, putative terminator. *Z. galactanivorans*, *Zobellia galactanivorans* Dsij^T; *G. sp. CAL575*, *Gillisia* sp. strain CAL575; *L. sp. 5H-3-7-4*, *Lacinutrix* sp. strain 5H-3-7-4; *C. lytica*, *Cellulophaga lytica* DSM 7489; *C. algicola*, *Cellulophaga algicola* DSM 14237; *W. psychrotolerans*, *Winogradskyella psychrotolerans* RS-3^T; *F. agariphila*, *Formosa agariphila* KMM 3901^T; *M. salmonicolor*, *Marinilabilia salmonicolor* JCM 21150; *C. fermentans*, *Cytophaga fermentans* JCM 21142^T.

been identified by similarity at the level of amino acid sequence, and only a few have been biochemically characterized (61, 80, 81). ZgM2DH was active as a monomer, did not require metal ions for catalysis, and featured narrow substrate specificity. These observations are in contrast to the results obtained for polyol dehydrogenases characterized in *Saccharomyces cerevisiae* (82), *Rhodobacter sphaeroides* (80), *P. fluorescens* (61), or *Thermotoga maritima* (83), because those M2DHs feature broader substrate specificity. In addition, most of the other bacterial M2DHs characterized have significantly higher K_m s and activity in the direction of fructose reduction to produce mannitol than for the oxidation of this polyol, while the *Z. galactanivorans* M2DH 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 either activity of the enzyme. In contrast, oxidation and reduction are inhibited by their reaction products in a similar manner (about 50% at 50 mM). Such an effect has been already observed for fructose reduction in *T. maritima* TM0298 (83) and for both directions in *S. cerevisiae* (82).

Genes encoding ZgM2DH and ZgFK1 were adjacent in the genome and were located directly downstream of four loci coding for an unknown conserved 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. agariphila*, *M. salmonicolor*, and *C. fermentans*) harboring a gene predicted to encode a putative mannitol permease gene instead of genes corresponding to the different subunits 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 raises questions about the evolution of this metabolic pathway in this phylum. It is tempting to speculate that the M2DH and FK1 genes have been acquired recently by the last common ancestor of the nine strains of *Bacteroidetes* from which both genes have been retrieved, and this hypothesis is partly supported by the high sequence identity between these genes in these different species. This common ancestor might have been a bacterium associated with brown algae, which are known to produce and exude mannitol. Furthermore, the occurrence of such an operon may have conferred an adaptive advantage on this bacterium, because man-

nitro can be used as a carbon and energy source. The observation that extant flavobacterial species containing the mannitol utilization system are not strictly associated with 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 (the ABC transporter is primary and the permease secondary) through the inner membrane in *Bacteroidetes* also suggests that the physiological role(s) and/or the regulation of mannitol catabolism may differ among members of this phylum. Meanwhile, it is difficult to relate these observations to any ecophysiological traits of the nine strains under consideration because there is no clear relationship between the type of mannitol utilization operon and the origin and metabolism of the strain. Another interesting subject for further analysis is the identification of the mechanism(s) used by these bacteria to sense and transport mannitol through the outer membrane.

For further understanding of mannitol catabolism and its physiological importance in *Z. galactanivorans*, it will be interesting to study its regulation. In general, changes in the expression of genes involved in carbohydrate catabolism occurs via one (or both) of two mechanisms: catabolic repression (suggesting the existence of a preferential carbon source) and specific induction by a substrate(s). Usually, transcription of catabolic genes in prokaryotes is regulated by either a repressor or an activator protein. In most cases, genes encoding this type of regulator are located near 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 the genes involved in this catabolism (29). The genome of *Z. galactanivorans* contains several putative AraC-type transcriptional regulators, but none of them are located close to the mannitol utilization cluster. A reverse genetic approach will be very valuable for assessing the physiological role(s) of these potential regulators when this methodology becomes available for *Z. galactanivorans*.

Reconstruction of metabolic networks from genomic resources, including carbohydrate catabolic pathways, may contribute to better understanding of microbial ecophysiology and interactions between microorganisms and their hosts, in particular for alga-associated bacteria (84, 85). In this context, our results shed light on the evolution of mannitol catabolism in important environmental bacteria. They pave the way also to a better understanding of the recycling of algal biomass on the shore and to the exploitation of algae as a renewable resource.

ACKNOWLEDGMENTS

This work was supported by the French National Research Agency via the investment expenditure program IDEALG (ANR-10-BTBR-02). We also acknowledge funding from the Émergence-UPMC-2011 research program. The Ph.D. fellowship of A.L. was funded by the French Ministry of Higher Education and Research.

REFERENCES

- Smith SV. 1981. Marine macrophytes as a global carbon sink. *Science* 211:838–840. <http://dx.doi.org/10.1126/science.211.4484.838>.
- Michel G, Tonon T, Scornet D, Cock JM, Kloareg B. 2010. The cell wall polysaccharide metabolism of the brown alga *Ectocarpus siliculosus*. Insights into the evolution of extracellular matrix polysaccharides in eukaryotes. *New Phytol* 188:82–97. <http://dx.doi.org/10.1111/j.1469-8137.2010.03374.x>.
- Popper ZA, Michel G, Hervé C, Domozych DS, Willats WG, Tuohy MG, Kloareg B, Stengel DB. 2011. Evolution and diversity of plant cell walls: from algae to flowering plants. *Annu Rev Plant Biol* 62:567–590. <http://dx.doi.org/10.1146/annurev-arplant-042110-103809>.
- Deniaud-Bouët E, Kervarec N, Michel G, Tonon T, Kloareg B, Hervé C. 29 May 2014. Chemical and enzymatic fractionation of cell walls from Fucales: insights into the structure of the extracellular matrix of brown algae. *Ann Bot* 114:1203–1216. <http://dx.doi.org/10.1093/aob/mcu096>.
- Michel G, Tonon T, Scornet D, Cock JM, Kloareg B. 2010. Central and storage carbon metabolism of the brown alga *Ectocarpus siliculosus*. Insights into the origin and evolution of storage carbohydrates in Eukaryotes. *New Phytol* 188:67–81. <http://dx.doi.org/10.1111/j.1469-8137.2010.03345.x>.
- Wei N, Quaterman J, Jin YS. 2013. Marine macroalgae: an untapped resource for producing fuels and chemicals. *Trends Biotechnol* 31:70–77. <http://dx.doi.org/10.1016/j.tibtech.2012.10.009>.
- Wargacki AJ, Leonard E, Win MN, Regitsky DD, Santos CN, Kim BP, Cooper SR, Raisner RM, Herman A, Sivitz AB, Lakshmanaswamy A, Kashiwaya Y, Baker D, Yoshikuni Y. 2012. An engineered microbial platform for direct biofuel production from brown macroalgae. *Science* 335:308–313. <http://dx.doi.org/10.1126/science.1214547>.
- Enquist-Newman M, Faust AM, Bravo DD, Santos CN, Raisner RM, Hanel A, Sarvabhowman P, Le C, Regitsky DD, Cooper SR, Peereboom L, Clark A, Martinez Y, Goldsmith J, Cho MY, Donohoue PD, Luo L, Lamberson B, Tamrakar P, Kim EJ, Villari JL, Gill A, Tripathi SA, Karamchedu P, Paredes CJ, Rajgarhia V, Kotlar HK, Bailey RB, Miller DJ, Ohler NL, Swimmer C, Yoshikuni Y. 2014. Efficient ethanol production from brown macroalgae sugars by a synthetic yeast platform. *Nature* 505:239–243. <http://dx.doi.org/10.1038/nature12771>.
- van Hal JW, Huijgen WJ, López-Contreras AM. 2014. Opportunities and challenges for seaweed in the biobased economy. *Trends Biotechnol* 32:231–233. <http://dx.doi.org/10.1016/j.tibtech.2014.02.007>.
- Reed RH, Davison IR, Chudek JA, Foster R. 1985. The osmotic role of mannitol in the Phaeophyta: an appraisal. *Phycologia* 24:35–47. <http://dx.doi.org/10.2216/i0031-8884-24-1-35.1>.
- Cock JM, Sterck L, Rouzé P, Scornet D, Allen AE, Amoutzias G, Anthouard V, Artiguenave F, Aury JM, Badger JH, Beszteri B, Billiau K, Bonnet E, Bothwell JH, Bowler C, Boyen C, Brownlee C, Carrano CJ, Charrier B, Cho GY, Coelho SM, Collén J, Corre E, Da Silva C, Delage L, Delaroque N, Dittami SM, Doubeau S, Elias M, Farnham G, Gachon CM, Gschloessl B, Heesch S, Jabbari K, Jubin C, Kawai H, Kimura K, Kloareg B, Küpper FC, Lang D, Le Bail A, Leblanc C, Lerouge P, Lohr M, Lopez PJ, Martens C, Maumus F, Michel G, Miranda-Saavedra D, Morales J, et al. 2010. The *Ectocarpus* genome and the independent evolution of multicellularity in brown algae. *Nature* 465:617–621. <http://dx.doi.org/10.1038/nature09016>.
- Gravot A, Dittami SM, Rousvoal S, Lugan R, Eggert A, Collén J, Boyen C, Bouchereau A, Tonon T. 2010. Diurnal oscillations of metabolite abundances and gene analysis provide new insights into central metabolic processes of the brown alga *Ectocarpus siliculosus*. *New Phytol* 188:98–110. <http://dx.doi.org/10.1111/j.1469-8137.2010.03400.x>.
- Dittami SM, Gravot A, Renault D, Goulitquer S, Eggert A, Bouchereau A, Boyen C, Tonon T. 2011. Integrative analysis of metabolite and transcript abundance during the short-term response to saline and oxidative stress in the brown alga *Ectocarpus siliculosus*. *Plant Cell Environ* 34:629–642. <http://dx.doi.org/10.1111/j.1365-3040.2010.02268.x>.
- Read SM, Currie G, Bacic A. 1996. Analysis of the structural heterogeneity of laminarin by electrospray-ionisation-mass spectrometry. *Carbohydr Res* 281:187–201. [http://dx.doi.org/10.1016/0008-6215\(95\)00350-9](http://dx.doi.org/10.1016/0008-6215(95)00350-9).
- Rousvoal S, Groisillier A, Dittami SM, Michel G, Boyen C, Tonon T. 2011. Mannitol-1-phosphate dehydrogenase activity in *Ectocarpus siliculosus*, a key role for mannitol synthesis in brown algae. *Planta* 233:261–273. <http://dx.doi.org/10.1007/s00425-010-1295-6>.
- Groisillier A, Shao Z, Michel G, Goulitquer S, Bonin P, Krahulec S, Nidetzky B, Duan D, Boyen C, Tonon T. 2014. Mannitol metabolism in brown algae involves a new phosphatase family. *J Exp Bot* 65:559–570. <http://dx.doi.org/10.1093/jxb/ert405>.
- Iwamoto K, Shiraiwa Y. 2005. Salt-regulated mannitol metabolism in algae. *Mar Biotechnol* 7:407–415. <http://dx.doi.org/10.1007/s10126-005-0029-4>.
- Shao Z, Zhang P, Li Q, Wang X, Duan D. 2014. Characterization of mannitol-2-dehydrogenase in *Saccharina japonica*: evidence for a new

- polyol-specific long-chain dehydrogenase/reductase. PLoS One 9:e97935. <http://dx.doi.org/10.1371/journal.pone.0097935>.
19. Postma PW, Lengeler JW, Jacobson GR. 1993. Phosphoenolpyruvate: carbohydrate phosphotransferase systems of bacteria. Microbiol Rev 57: 543–594.
 20. Akagawa E, Kurita K, Sugawara T, Nakamura K, Kasahara Y, Ogasawara N, Yamane K. 1995. Determination of a 17,484 bp nucleotide sequence around the 39 degrees region of the *Bacillus subtilis* chromosome and similarity analysis of the products of putative ORFs. Microbiol 141:3241–3245. <http://dx.doi.org/10.1099/13500872-141-12-3241>.
 21. Watanabe S, Hamano M, Kakeshita H, Bunai K, Tojo S, Yamaguchi H, Fujita Y, Wong SL, Yamane K. 2003. Mannitol-1-phosphate dehydrogenase (MtlD) is required for mannitol and glucitol assimilation in *Bacillus subtilis*: possible cooperation of *mtl* and *gut* operons. J Bacteriol 185: 4816–4824. <http://dx.doi.org/10.1128/JB.185.16.4816-4824.2003>.
 22. Henstra SA, Duurkens RH, Robillard GT. 2000. Multiple phosphorylation events regulate the activity of the mannitol transcriptional regulator MtlR of the *Bacillus stearothermophilus* phosphoenolpyruvate-dependent mannitol phosphotransferase system. J Biol Chem 275:7037–7044. <http://dx.doi.org/10.1074/jbc.275.10.7037>.
 23. Behrens S, Mitchell W, Bahl H. 2001. Molecular analysis of the mannitol operon of *Clostridium acetobutylicum* encoding a phosphotransferase system and a putative PTS-modulated regulator. Microbiology 147:75–86.
 24. Honeyman AL, Curtiss R, III. 1992. Isolation, characterization, and nucleotide sequence of the *Streptococcus mutans* mannitol-phosphate dehydrogenase gene and the mannitol-specific factor III gene of the phosphoenolpyruvate phosphotransferase system. Infect Immun 60:3369–3375.
 25. Novotny MJ, Reizer J, Esch F, Saier MH, Jr. 1984. Purification and properties of D-mannitol-1-phosphate dehydrogenase and D-glucitol-6-phosphate dehydrogenase from *Escherichia coli*. J Bacteriol 159:986–990.
 26. Sand M, Mingote AI, Santos H, Müller V, Averhoff B. 2013. Mannitol, a compatible solute synthesized by *Acinetobacter baylyi* in a two-step pathway including a salt-induced and salt-dependent mannitol-1-phosphate dehydrogenase. Environ Microbiol 15:2187–2197. <http://dx.doi.org/10.1111/1462-2920.12090>.
 27. Sand M, Rodrigues M, González JM, de Crécy-Lagard V, Santos H, Müller V, Averhoff B. 2014. Mannitol-1-phosphate dehydrogenases/phosphatases: a family of novel bifunctional enzymes for bacterial adaptation to osmotic stress. Environ Microbiol <http://dx.doi.org/10.1111/1462-2920.12503>.
 28. Brünker P, Altenbuchner J, Mattes R. 1998. Structure and function of the genes involved in mannitol, arabinol and glucitol utilization from *Pseudomonas fluorescens* DSM50106. Gene 206:117–126. [http://dx.doi.org/10.1016/S0378-1119\(97\)00574-X](http://dx.doi.org/10.1016/S0378-1119(97)00574-X).
 29. Brünker P, Hils M, Altenbuchner J, Mattes R. 1998. The mannitol utilization genes of *Pseudomonas fluorescens* are regulated by an activator: cloning, nucleotide sequence and expression of the *mtlR* gene. Gene 215: 19–27. [http://dx.doi.org/10.1016/S0378-1119\(98\)00274-1](http://dx.doi.org/10.1016/S0378-1119(98)00274-1).
 30. Wiegmann K, Hensler M, Wöhlbrand L, Ulbrich M, Schomburg D, Rabus R. 2014. Carbohydrate catabolism in *Phaebacter inhibens* DSM 17395, a member of the marine *Roseobacter* clade. Appl Environ Microbiol 80:4725–4737. <http://dx.doi.org/10.1128/AEM.00719-14>.
 31. Barbeyron T, L'Haridon S, Corre E, Kloareg B, Potin P. 2001. *Zobellia galactanovorans* gen. nov., sp. nov., a marine species of Flavobacteriaceae isolated from a red alga, and classification of [*Cytophaga*] *uliginosa* (ZoBell and Upham 1944) Reichenbach 1989 as *Zobellia uliginosa* gen. nov., comb. nov. Int J Syst Evol Microbiol 51:985–997. <http://dx.doi.org/10.1099/00207173-51-3-985>.
 32. Nedashkovskaya OI, Suzuki M, Vancanneyt M, Cleenwerck I, Lysenko AM, Mikhailov VV, Swings J. 2004. *Zobellia amurskyensis* sp. nov., *Zobellia laminariae* sp. nov. and *Zobellia russellii* sp. nov., novel marine bacteria of the family Flavobacteriaceae. Int J Syst Evol Microbiol 54:1643–1648. <http://dx.doi.org/10.1099/ijms.0.63091-0>.
 33. Martin M, Portetelle D, Michel G, Vandenbol M. 2014. Microorganisms living on macroalgae: diversity, interactions, and biotechnological applications. Appl Microbiol Biotechnol 98:2917–2935. <http://dx.doi.org/10.1007/s00253-014-5557-2>.
 34. Skerratt JH, Bowman JP, Hallegraeff GM, James S, Nichols PD. 2002. Algicidal bacteria associated with blooms of a toxic dinoflagellate in a temperate Australian estuary. Mar Ecol Prog Ser 244:1–15. <http://dx.doi.org/10.3354/meps244001>.
 35. Hahnke RL, Harder J. 2013. Phylogenetic diversity of *Flavobacteri* isolated from the North Sea on solid media. Syst Appl Microbiol 36:497–504. <http://dx.doi.org/10.1016/j.syapm.2013.06.006>.
 36. Matsuo Y, Suzuki M, Kasai H, Shizuri Y, Harayama S. 2003. Isolation and phylogenetic characterization of bacteria capable of inducing differentiation in the green alga *Monostroma oxyspermum*. Environ Microbiol 5:25–35. <http://dx.doi.org/10.1046/j.1462-2920.2003.00382.x>.
 37. Barbeyron T, Gerard A, Potin P, Henrissat B, Kloareg B. 1998. The kappa-carrageenase of the marine bacterium *Cytophaga drobachiensis*. Structural and phylogenetic relationships within family-16 glycoside hydrolases. Mol Biol Evol 15:528–537.
 38. Rebuffet E, Barbeyron T, Jeudy A, Jam M, Czjzek M, Michel G. 2010. Identification of catalytic residues and mechanistic analysis of family GH82 iota-carrageenases. Biochemistry 49:7590–7599. <http://dx.doi.org/10.1021/bi1003475>.
 39. Hehemann JH, Correc G, Thomas F, Bernard T, Barbeyron T, Jam M, Helbert W, Michel G, Czjzek M. 2012. Biochemical and structural characterization of the complex agarolytic enzyme system from the marine bacterium *Zobellia galactanivorans*. J Biol Chem 287:30571–30584. <http://dx.doi.org/10.1074/jbc.M112.377184>.
 40. Hehemann JH, Correc G, Barbeyron T, Helbert W, Czjzek M, Michel G. 2010. Transfer of carbohydrate-active enzymes from marine bacteria to Japanese gut microbiota. Nature 464:908–912. <http://dx.doi.org/10.1038/nature08937>.
 41. Rebuffet E, Groisillier A, Thompson A, Jeudy A, Barbeyron T, Czjzek M, Michel G. 2011. Discovery and structural characterization of a novel glycosidase family of marine origin. Environ Microbiol 13:1253–1270. <http://dx.doi.org/10.1111/j.1462-2920.2011.02426.x>.
 42. Ficko-Blean E, Duffieux D, Rebuffet E, Larocque R, Groisillier A, Michel G, Czjzek M. 2015. Biochemical and structural investigation of two paralogous glycoside hydrolases from *Zobellia galactanivorans*: novel insights into the evolution, dimerization plasticity and catalytic mechanism of the GH117 family. Acta Crystallogr D Biol Crystallogr <http://dx.doi.org/10.1107/S1399004714025024>.
 43. Thomas F, Barbeyron T, Tonon T, Génicot S, Czjzek M, Michel G. 2012. Characterization of the first alginolytic operons in a marine bacterium: from their emergence in marine *Flavobacteri* to their independent transfers to marine *Proteobacteria* and human gut *Bacteroides*. Environ Microbiol 14:2379–2394. <http://dx.doi.org/10.1111/j.1462-2920.2012.02751.x>.
 44. Thomas F, Lundqvist LC, Jam M, Jeudy A, Barbeyron T, Sandström C, Michel G, Czjzek M. 2013. Comparative characterization of two marine alginate lyases from *Zobellia galactanivorans* reveals distinct modes of action and exquisite adaptation to their natural substrate. J Biol Chem 288: 23021–23037. <http://dx.doi.org/10.1074/jbc.M113.467217>.
 45. Labourel A, Jam M, Legentil L, Sylla B, Hehemann J-H, Ferrières V, Czjzek M, Michel G. 2015. Structural and biochemical characterization of the laminarinase ZgLamC_{GH16} from *Zobellia galactanivorans* suggests preferred recognition of branched laminarin. Acta Crystallogr D Biol Crystallogr <http://dx.doi.org/10.1107/S139900471402450X>.
 46. Barbeyron T, L'Haridon S, Michel G, Czjzek M. 2008. *Mariniflexile fucanivorans* sp. nov., a marine member of the Flavobacteriaceae that degrades sulphated fucans from brown algae. Int J Syst Evol Microbiol 58: 2107–2113. <http://dx.doi.org/10.1099/ijms.0.65674-0>.
 47. Marshall OJ. 2004. Perl Primer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR. Bioinformatics 20:2471–2472. <http://dx.doi.org/10.1093/bioinformatics/bth254>.
 48. Thomas F, Barbeyron T, Michel G. 2011. Evaluation of reference genes for real-time quantitative PCR in the marine flavobacterium *Zobellia galactanivorans*. J Microbiol Methods 84:61–66. <http://dx.doi.org/10.1016/j.mimet.2010.10.016>.
 49. Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45. <http://dx.doi.org/10.1093/nar/29.9.e45>.
 50. Groisillier A, Hervé C, Jeudy A, Rebuffet E, Pluchon PF, Chevotot Y, Flament D, Geslin C, Morgado IM, Power D, Branno M, Moreau H, Michel G, Boyen C, Czjzek M. 2010. MARINE-EXPRESS: taking advantage of high throughput cloning and expression strategies for the post-genomic analysis of marine organisms. Microb Cell Fact 9:45. <http://dx.doi.org/10.1186/1475-2859-9-45>.
 51. Meyer F, Goesmann A, McHardy AC, Bartels D, Bekel T, Clausen J, Kalinowski J, Linke B, Rupp O, Giegerich R, Pühler A. 2003. GenDB—an open source genome annotation system for prokaryote

- genomes. *Nucleic Acids Res* 31:2187–2195. <http://dx.doi.org/10.1093/nar/gkg312>.
52. Bendtsen JD, Nielsen H, von Heijne G, Brunak S. 2004. Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* 340:783–795. <http://dx.doi.org/10.1016/j.jmb.2004.05.028>.
 53. Krogh A, Larsson B, von Heijne G, Sonnhammer EL. 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol* 305:567–580. <http://dx.doi.org/10.1006/jmbi.2000.4315>.
 54. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402. <http://dx.doi.org/10.1093/nar/25.17.3389>.
 55. Katoh K, Misawa K, Kuma K, Miyata T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res* 30:3059–3066. <http://dx.doi.org/10.1093/nar/gkf436>.
 56. Chen S, Bagdasarian M, Kaufman MG, Bates AK, Walker ED. 2007. Mutational analysis of the *ompA* promoter from *Flavobacterium johnsoniae*. *J Bacteriol* 189:5108–5118. <http://dx.doi.org/10.1128/JB.00401-07>.
 57. Chen S, Bagdasarian M, Kaufman MG, Walker ED. 2007. Characterization of strong promoters from an environmental *Flavobacterium hibernum* strain by using a green fluorescent protein-based reporter system. *Appl Environ Microbiol* 73:1089–1100. <http://dx.doi.org/10.1128/AEM.01577-06>.
 58. Kingsford CL, Ayanbule K, Salzberg SL. 2007. Rapid, accurate, computational discovery of Rho-independent transcription terminators illuminates their relationship to DNA uptake. *Genome Biol* 8:R22. <http://dx.doi.org/10.1186/gb-2007-8-2-r22>.
 59. Studier FW. 2005. Protein production by auto-induction in high-density shaking cultures. *Protein Expr Purif* 41:207–234. <http://dx.doi.org/10.1016/j.pep.2005.01.016>.
 60. Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A. 2005. Protein identification and analysis tools on the ExPASy server, p 571–607. In Walker JM (ed), *The proteomics protocols handbook*. Humana Press, Totowa, NJ.
 61. Brünker P, Altenbuchner J, Kulbe KD, Mattes R. 1997. Cloning, nucleotide sequence and expression of a mannitol dehydrogenase gene from *Pseudomonas fluorescens* DSM 50106 in *Escherichia coli*. *Biochim Biophys Acta* 1351:157–167. [http://dx.doi.org/10.1016/S0167-4781\(96\)00189-3](http://dx.doi.org/10.1016/S0167-4781(96)00189-3).
 62. Kalinowski J, Bathe B, Bartels D, Bischoff N, Bott M, Burkovski A, Dusch N, Eggeling L, Eikmanns BJ, Gaigalat L, Goesmann A, Hartmann M, Huthmacher K, Krämer R, Linke B, McHardy AC, Meyer F, Möckel B, Pfefferle W, Pühler A, Rey DA, Rückert C, Rupp O, Sahm H, Wendisch VF, Wiegäbe I, Tauch A. 2003. The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. *J Biotechnol* 104:5–25. [http://dx.doi.org/10.1016/S0168-1656\(03\)00154-8](http://dx.doi.org/10.1016/S0168-1656(03)00154-8).
 63. Bell AW, Buckel SD, Groarke JM, Hope JN, Kingsley DH, Hermodson MA. 1986. The nucleotide sequences of the *rbsD*, *rbsA*, and *rbsC* genes of *Escherichia coli* K12. *J Biol Chem* 261:7652–7658.
 64. Stewart JB, Hermodson MA. 2003. Topology of RbsC, the membrane component of the *Escherichia coli* ribose transporter. *J Bacteriol* 185:5234–5239. <http://dx.doi.org/10.1128/JB.185.17.5234-5239.2003>.
 65. Maida I, Fondi M, Papaleo MC, Perrin E, Orlandini V, Emiliani G, de Pascale D, Parrilli E, Tutino ML, Michaud L, Lo Giudice A, Romoli R, Bartolucci G, Fani R. 2014. Phenotypic and genomic characterization of the Antarctic bacterium *Gillisia* sp. CAL575, a producer of antimicrobial compounds. *Extremophiles* 1:35–49. <http://dx.doi.org/10.1007/s00792-013-0590-0>.
 66. Klippel B, Lochner A, Bruce DC, Davenport KW, Detter C, Goodwin LA, Han J, Han S, Hauser L, Land ML, Nolan M, Ovchinnikova G, Pennacchio L, Pitluck S, Tapia R, Woyke T, Wiebusch S, Basner A, Abe F, Horikoshi K, Keller M, Antranikian G. 2011. Complete genome sequences of *Krokinobacter* sp. strain 4H-3-7-5 and *Lacinutrix* sp. strain 5H-3-7-4, polysaccharide-degrading members of the family *Flavobacteriaceae*. *J Bacteriol* 193:4545–4546. <http://dx.doi.org/10.1128/JB.00518-11>.
 67. Pati A, Abt B, Teshima H, Nolan M, Lapidus A, Lucas S, Hammon N, Deshpande S, Cheng JF, Tapia R, Han C, Goodwin L, Pitluck S, Liolios K, Pagani I, Mavromatis K, Ovchinnikova G, Chen A, Palaniappan K, Land M, Hauser L, Jeffries CD, Detter JC, Brambilla EM, Kannan KP, Rohde M, Spring S, Göker M, Woyke T, Bristow J, Eisen JA, Markowitz V, Hugenholtz P, Kyrpides NC, Klenk HP, Ivanova N. 2011. Complete genome sequence of *Cellulophaga lytica* type strain (LIM-21). *Stand Genomic Sci* 4:221–232. <http://dx.doi.org/10.4056/sigs.1774329>.
 68. Abt B, Lu M, Misra M, Han C, Nolan M, Lucas S, Hammon N, Deshpande S, Cheng JF, Tapia R, Goodwin L, Pitluck S, Liolios K, Pagani I, Ivanova N, Mavromatis K, Ovchinnikova G, Pati A, Chen A, Palaniappan K, Land M, Hauser L, Chang YJ, Jeffries CD, Detter JC, Brambilla E, Rohde M, Tindall BJ, Göker M, Woyke T, Bristow J, Eisen JA, Markowitz V, Hugenholtz P, Kyrpides NC, Klenk HP, Lapidus A. 2011. Complete genome sequence of *Cellulophaga algicola* type strain (IC166). *Stand Genomic Sci* 4:72–80. <http://dx.doi.org/10.4056/sigs.1543845>.
 69. Kumar Pinnaka A, Ara S, Singh A, Shivaji S. 2013. Draft genome sequence of *Winogradskyella psychrotolerans* RS-3^T, isolated from the marine transect of Kongsfjorden, Ny-Alesund, Svalbard, Arctic Ocean. *Genome Announc* 1:e00630–13. <http://dx.doi.org/10.1128/genomeA.00630-13>.
 70. Mann AJ, Hahnke RL, Huang S, Werner J, Xing P, Barbeyron T, Huettel B, Stüber K, Reinhardt R, Harder J, Glöckner FO, Amann RI, Teeling H. 2013. The genome of the alga-associated marine flavobacterium *Formosa agariphila* KMM 3901^T reveals a broad potential for degradation of algal polysaccharides. *Appl Environ Microbiol* 79:6813–6822. <http://dx.doi.org/10.1128/AEM.01937-13>.
 71. Kumar S, Subramanian S, Raghava GP, Pinnaka AK. 2012. Genome sequence of the marine bacterium *Marinilabilia salmonicolor* JCM 21150^T. *J Bacteriol* 194:3746. <http://dx.doi.org/10.1128/JB.00649-12>.
 72. Starns D, Oshima K, Suda W, Iino T, Yuki M, Inoue J, Kitamura K, Iida T, Darby A, Hattori M, Ohkuma M. 2014. Draft genome sequence of *Cytophaga fermentans* JCM 21142^T, a facultative anaerobe isolated from marine mud. *Genome Announc* 2:e00206–14. <http://dx.doi.org/10.1128/genomeA.00206-14>.
 73. Lengeler JW, Jahreis K. 2009. Bacterial PEP-dependent carbohydrate: phosphotransferase systems couple sensing and global control mechanisms. *Contrib Microbiol* 16:65–87. <http://dx.doi.org/10.1159/000219373>.
 74. Kumar S, Smith KP, Floyd JL, Varela MF. 2011. Cloning and molecular analysis of a mannitol operon of phosphoenolpyruvate-dependent phosphotransferase (PTS) type from *Vibrio cholerae* O395. *Arch Microbiol* 193:201–208. <http://dx.doi.org/10.1007/s00203-010-0663-8>.
 75. Peng X, Okai N, Vertès AA, Inatomi K, Inui M, Yukawa H. 2011. Characterization of the mannitol catabolic operon of *Corynebacterium glutamicum*. *Appl Microbiol Biotechnol* 91:1375–1387. <http://dx.doi.org/10.1007/s00253-011-3352-x>.
 76. Thompson J, Sackett DL, Donkersloot JA. 1991. Purification and properties of fructokinase I from *Lactococcus lactis*. Localization of *scrK* on the sucrose-nisin transposon Tn5306. *J Biol Chem* 266:22626–22633.
 77. Thompson J, Nguyen NY, Robrish SA. 1992. Sucrose fermentation by *Fusobacterium mortiferum* ATCC 25557: transport, catabolism, and products. *J Bacteriol* 174:3227–3235.
 78. Lee JK, Koo BS, Kim SY, Hyun HH. 2003. Purification and characterization of a novel mannitol dehydrogenase from a newly isolated strain of *Candida magnoliae*. *Appl Environ Microbiol* 69:4438–4447. <http://dx.doi.org/10.1128/AEM.69.8.4438-4447.2003>.
 79. Klimacek M, Nidetzky B. 2002. A catalytic consensus motif for D-mannitol 2-dehydrogenase, a member of a polyol-specific long-chain dehydrogenase family, revealed by kinetic characterization of site-directed mutants of the enzyme from *Pseudomonas fluorescens*. *Biochem J* 367:13–18. <http://dx.doi.org/10.1042/BJ20020932>.
 80. Schneider KH, Giffhorn F, Kaplan S. 1993. Cloning, nucleotide sequence and characterization of the mannitol dehydrogenase gene from *Rhodobacter sphaeroides*. *J Gen Microbiol* 139:2475–2484. <http://dx.doi.org/10.1099/00221287-139-10-2475>.
 81. Krahulec S, Armao GC, Bubner P, Klimacek M, Nidetzky B. 2009. Polyol-specific long-chain dehydrogenases/reductases of mannitol metabolism in *Aspergillus fumigatus*: biochemical characterization and pH studies of mannitol 2-dehydrogenase and mannitol-1-phosphate 5-dehydrogenase. *Chem Biol Interact* 178:274–282. <http://dx.doi.org/10.1016/j.cbi.2008.10.001>.
 82. Kulbe KD, Schwab U, Gudernatsch W. 1987. Enzyme-catalyzed production of mannitol and gluconic acid. Product recovery by various procedures. *Ann N Y Acad Sci* 506:552–568.
 83. Song SH, Ahluwalia N, Leduc Y, Delbaere LT, Vieille C. 2008. *Thermotoga maritima* TM0298 is a highly thermostable mannitol dehy-

- drogenase. *Appl Microbiol Biotechnol* 81:485–495. <http://dx.doi.org/10.1007/s00253-008-1633-9>.
84. Dittami SM, Barbeyron T, Boyen C, Cambefort J, Collet G, Delage L, Gobet A, Groisillier A, Leblanc C, Michel G, Scornet D, Siegel A, Tapia JE, Tonon T. 25 July 2014. Genome and metabolic network of “*Candidatus Phaeomarinobacter ectocarpus*” Ec32, a new candidate genus of *Alpha-proteobacteria* frequently associated with brown algae. *Front Genet* <http://dx.doi.org/10.3389/fgene.2014.00241>.
 85. Dittami SM, Eveillard D, Tonon T. 2014. A metabolic approach to study algal-bacterial interactions in changing environments. *Mol Ecol* 23:1656–1660. <http://dx.doi.org/10.1111/mec.12670>.
 86. Slatner M, Nidetzky B, Kulbe KD. 1999. Kinetic study of the catalytic mechanism of mannitol dehydrogenase from *Pseudomonas fluorescens*. *Biochemistry* 38:10489–10498. <http://dx.doi.org/10.1021/bi990327g>.
 87. Korakli M, Vogel RF. 2003. Purification and characterisation of mannitol dehydrogenase from *Lactobacillus sanfranciscensis*. *FEMS Microbiol Lett* 220:281–286. [http://dx.doi.org/10.1016/S0378-1097\(03\)00129-0](http://dx.doi.org/10.1016/S0378-1097(03)00129-0).
 88. Schneider KH, Giffhorn F. 1989. Purification and properties of a polyol dehydrogenase from the phototrophic bacterium *Rhodobacter sphaeroides*. *Eur J Biochem* 184:15–19. <http://dx.doi.org/10.1111/j.1432-1033.1989.tb14984.x>.
 89. Hahn G, Kaup B, Bringer-Meyer S, Sahm H. 2003. A zinc-containing mannitol-2-dehydrogenase from *Leuconostoc pseudomesenteroides* ATCC 12291: purification of the enzyme and cloning of the gene. *Arch Microbiol* 179:101–107. <http://dx.doi.org/10.1007/s00203-002-0507-2>.
 90. Caescu CI, Vidal O, Krzewinski F, Arteni V, Bouquelet S. 2004. *Bifidobacterium longum* requires a fructokinase (Frk; ATP:D-fructose 6-phosphotransferase, EC 2.7.1.4) for fructose catabolism. *J Bacteriol* 186: 6515–6525. <http://dx.doi.org/10.1128/JB.186.19.6515-6525.2004>.
 91. But SY, Rozova ON, Khmelenina VN, Reshetnikov AS, Trotsenko YA. 2012. Properties of recombinant ATP-dependent fructokinase from the halotolerant methanotroph *Methylobacterium alcaliphilum* 20Z. *Biochemistry (Mosc)* 77:372–377. <http://dx.doi.org/10.1134/S006297912040086>.
 92. Fennington GJ, Jr, Hughes TA. 1996. The fructokinase from *Rhizobium leguminosarum* biovar trifolii belongs to group I fructokinase enzymes and is encoded separately from other carbohydrate metabolism enzymes. *Microbiology* 142:321–330. <http://dx.doi.org/10.1099/13500872-142-2-321>.
 93. Qu Q, Lee SJ, Boos W. 2004. Molecular and biochemical characterization of a fructose-6-phosphate-forming and ATP-dependent fructokinase of the hyperthermophilic archaeon *Thermococcus litoralis*. *Extremophiles* 8:301–308. <http://dx.doi.org/10.1007/s00792-004-0392-5>.
 94. Scopes RK, Testolin V, Stoter A, Griffiths-Smith K, Algar EM. 1985. Simultaneous purification and characterization of glucokinase, fructokinase and glucose-6-phosphate dehydrogenase from *Zymomonas mobilis*. *Biochem J* 228:627–634.