

Role of mannitol dehydrogenases in osmoprotection of *Gluconobacter oxydans*

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Abstract *Gluconobacter (G.) oxydans* is able to incompletely oxidize various sugars and polyols for the production of biotechnologically important compound. Recently, we have shown that the organism produces and accumulates mannitol as compatible solute under osmotic stress conditions. The present study describes the role of two cytoplasmic mannitol dehydrogenases for osmotolerance of *G. oxydans*. It was shown that Gox1432 is a NADP⁺-dependent mannitol dehydrogenase (EC 1.1.1.138), while Gox0849 uses NAD⁺ as co-factor (EC 1.1.1.67). The corresponding genes were deleted and the mutants were analyzed for growth under osmotic stress and non-stress conditions. A severe growth defect was detected for Δ *gox1432* when grown in high osmotic media, while the deletion of *gox0849* had no effect when cells were exposed to 450 mM sucrose in the medium. Furthermore, the intracellular mannitol content was reduced in the mutant lacking the NADP⁺-dependent enzyme Gox1432 in comparison to the parental strain and the Δ *gox0849* mutant under stress conditions. In addition, transcriptional analysis revealed that Gox1432 is more important for mannitol production in *G. oxydans* than Gox0849 as the transcript abundance of gene *gox1432* was 30-fold higher than of *gox0849*. In accordance, the activity of the NADH-dependent enzyme Gox0849 in the cell cytoplasm was 10-fold lower in comparison to the NADPH-dependent mannitol dehydrogenase Gox1432. Overexpression of *gox1432* in the corresponding deletion

mutant restored growth of the cells under osmotic stress, further strengthening the importance of the NADP⁺-dependent mannitol dehydrogenase for osmotolerance in *G. oxydans*. These findings provide detailed insights into the molecular mechanism of mannitol-mediated osmoprotection in *G. oxydans* and are helpful engineering strains with improved osmotolerance for biotechnological applications.

Keywords Osmotic stress · Compatible solute · Fructose reduction · Biotransformation · Vitamin C

Introduction

Microorganisms are often exposed to fluctuations of the external osmolarity and have to cope with osmotically induced water fluxes across the cytoplasmic membrane. As a consequence, they must be able to make physiological adjustments to ensure adequate levels of cellular hydration for the protection of cell function and turgor. In prokaryotes, synthesis and accumulation of compatible solutes are among some common strategies to cope with reduced water activity. Small organic compounds such as glycine, betaine, trehalose, ectoine, glutamate, glutamine, mannitol and proline are well known to be compatible with cellular metabolic functions and stabilize the positive turgor pressure inside the cell (da Costa et al. 1998). Mannitol is the most abundant polyol in nature and is used as an osmoprotectant mostly in eukaryotes such as plants and fungi (Empadinhas and da Costa 2008). In a previous study, it was shown that the industrially important microorganism, *Gluconobacter oxydans*, copes with osmotic stress by synthesizing mannitol inside the cell (Zahid et al. 2015). The organism accumulates mannitol as a compatible solute in media with high concentrations of sucrose, glucose, or fructose (Table S1). Additionally, mannitol improved the growth of

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the bacterium when present in low concentrations in high osmotic glucose media. However, until now, the enzymes involved in stress-mediated mannitol production in *G. oxydans* were unknown. There are two potential pathways present in bacteria for the synthesis of mannitol. One includes a mannitol-1-phosphate dehydrogenase and a mannitol-1-phosphatase which convert fructose-6-phosphate to mannitol. There are also reports on a bifunctional enzyme for mannitol biosynthesis in *Acinetobacter baylyi* that combines mannitol-1-phosphate dehydrogenase and phosphatase activities in a single polypeptide chain (Sand et al. 2015). The second pathway is based on a mannitol-2-dehydrogenase reducing fructose directly to mannitol and is frequently found in lactic acid bacteria (Saha 2004). Both, mannitol-1-phosphate dehydrogenase and mannitol-2-dehydrogenase belong to the class of oxidoreductase and require NAD(P)⁺ or NAD(P)H as cofactors.

In the present study, we identified two genes *gox1432* and *gox0849* that potentially encode mannitol dehydrogenases in *G. oxydans* 621H. Both mannitol dehydrogenase-encoding genes were deleted and the effect on osmoprotection in knock-out mutants was analyzed. In addition, both enzymes were overproduced and their catalytic efficiency was investigated. We found that the NADP⁺-dependent mannitol dehydrogenase (Gox1432) was crucial for cell survival under osmotic stress. These findings provide first insight into the mechanism of osmoprotection in *G. oxydans*.

Materials and methods

Chemicals

All chemicals, antibiotics, and media components were purchased from Carl Roth GmbH (Karlsruhe, Germany) and Sigma-Aldrich (Munich-Germany). NADPH, NADH, and NAD⁺ were purchased from Carl Roth GmbH (Karlsruhe, Germany), while NADP⁺ was from Merck (Darmstadt, Germany). Restriction endonucleases, T4 DNA ligase, Taq DNA polymerase, and other PCR reagents were obtained from Thermo Fisher Scientific Biosciences GmbH (Schwäbisch-Gmünd, Germany). Phusion DNA Polymerase was bought from Biozym Scientific GmbH (Oldendorf, Germany). pASK-IBA3 and pASK-IBA5 plasmids were obtained from IBA GmbH (Goettingen, Germany). Oligonucleotides were synthesized by Eurofins MWG Operon (Ebersberg, Germany).

Microorganisms and culture conditions

High-efficiency competent *Escherichia coli* (NEB 5- α) cells were obtained from NEB New England Biolabs Inc. (Frankfurt am Main, Germany) and grown in Lysogeny broth

at 37 °C (Miller 1972). For the overproduction of Gox1432 and Gox0849, *E. coli* transformants were grown in maximal induction medium (Mott et al. 1985) containing ampicillin (100 $\mu\text{g ml}^{-1}$). *G. oxydans* 621H ΔhsdR (DSM 2343; Table 1) was obtained from S. Bringer-Meyer (Research Center Juelich GmbH, Germany) and cultured in yeast extract (0.6 % w/v) mannitol (2 % w/v) medium with cefoxitin (50 $\mu\text{g ml}^{-1}$) in baffled flasks at 30 °C. Strains containing pBBR.p264.ST derivatives were grown in the presence of kanamycin (50 $\mu\text{g ml}^{-1}$). To study the effect of osmotic stress, cultures were grown in YGP medium containing yeast extract (0.6 % w/v), glucose (0.9 % w/v), and 100 mM sterile phosphate buffer (pH 6.8–7.0). The osmolarity of the medium was increased by the addition of sucrose (450 mM). Optical density (OD) was measured photometrically at 600 nm. All experiments were performed in triplicates using two biological replicates.

Standard molecular biology techniques and cloning

All routine molecular biology techniques were performed according to Sambrook et al. (1989). Genomic DNA of *G. oxydans* was isolated using Gen Jet Genomic DNA purification kit from Thermo Fisher Scientific (Schwäbisch-Gmünd, Germany) and was used as template for gene amplification. For cloning of *gox1432* and *gox0849*, primers containing *Eco*11 restriction sites (Table 1) were designed, respectively, using Primer D'signer 1.1 software (www.iba-lifesciences.com). The PCR products and the expression vectors pASK-IBA 3 and pASK-IBA 5 were digested with *Eco*11, purified, and ligated to generate the plasmids pASK.3_ *gox1432*.ST and pASK.5_ *gox0849*.ST, respectively. Both constructs contained an ampicillin resistance cassette, plasmid-encoded ribosomal binding sites, and a C-terminal Strep-tag II (pASK.3_ *gox1432*.ST) or a N-terminal Strep-tag II (pASK.5_ *gox0849*.ST). The plasmids were transferred into *E. coli* (NEB 5- α) according to manufacturer's instructions. Positive clones were selected by colony PCR and the correct insertion of the PCR products was checked by sequencing (StarSEQ GmbH, Mainz, Germany). For the generation of *G. oxydans* strains carrying in-frame deletion mutations for *gox1432* and/or *gox0849*, the codAB markerless in-frame deletion system was used (Kostner et al. 2013). For the generation of the $\Delta\text{gox1432}$ mutant 1 kb of the up- and downstream regions were amplified using primers up_ *gox1432*_Fw/up_ *gox1432*_Rev and do_ *gox1432*_Fw/do_ *gox1432*_Rev (Table 1). The amplified fragments were used as template for a fusion PCR reaction using primers up_ *gox1432*_Fw and do_ *gox1432*_Rev. After digestion using *Kpn*1 and *Xba*1 and ligation in the corresponding sites of pKOS6b, the resulting vector pKos6b Δ 1432 was transferred into competent *E. coli* (NEB 5- α). Finally,

Table 1 Strains, plasmids, and primers

Strains		
<i>E. coli</i> (NEB 5-alpha)	Derivative of DH α <i>fhuA2</i> Δ (<i>argF-lacZ</i>) <i>U169 phoA glnV44 ϕ80Δ(lacZ)</i> <i>M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	New England Biolabs
<i>G. oxydans</i> 621H Δ <i>hsdR</i>	Δ <i>gox2567</i> derivative of <i>G. oxydans</i> 621H (DSM 2343) referred to as wild type throughout the text	S. Bringer-Meyer, research center Juelich GmbH, Germany
Plasmids		
pASK-IBA.3	C-terminal Strep-tag II sequence, <i>tetA</i> promoter/repressor system, Amp ^R	IBA GmbH
pASK-IBA.5	N-terminal Strep-tag II, <i>tetA</i> promoter/repressor system, Amp ^R	IBA GmbH
pBBR.p264.ST	Derivative of pBBR1p264 with C-terminal Strep-tag II sequence from pASK-IBA3 vector	Kallnik et al. (2010)
Primers		
	Primer sequences ^a	Restriction site
RT_Gox1709_Fw	AAACGCAGATGGGTCTGAAG	
RT_Gox1709_rev	CTGACCGGTGCTCATCTTCT	
RT_Gox1432_Fw	CATTGCGTGACTACCTGCTC	
RT_Gox1432_rev	CCTTTACTGCCGATTCTCC	
RT_Gox849_Fw	GACCGAACATCCTGCCATTG	
RT_Gox0849_rev	ATTGACCTCGATCCACTCCG	
pAK.3_gox1432_Fw	ATGGTAGGTCTCA <u>AA</u> TGATTACGCGCGAAACCCTCAAGTC	<i>Eco</i> 31I
pAK.3_gox1432_rev	ATGGTAGGTCTCA <u>G</u> CGCTGGCCGGAATAGCGGCCTTCAC	<i>Eco</i> 31I
pAK.5_gox0849_Fw	ATGGTAGGTCTCA <u>G</u> CGCCATGATCCTGACTTCCCAGACCC	<i>Eco</i> 31I
pAK.5_gox0849_rev	ATGGTAGGTCTCATATCATACTTTGTGACCAGATCCTTCAG	<i>Eco</i> 31I
pBBR.p264.gox1432_Fw	GCGGATATCGGCCCGAAACCACGGAGAATTCCATG	<i>Eco</i> RV
pBBR.p264-gox1432_rev	ATAGGCGCGCCGGCCGGAATAGCGGCCTTC	<i>Asc</i> I
Up-gox1432_Fw	ATAGGTACCTGACGACGGCGGGACCAG	<i>Kpn</i> I
Up_gox1432_rev	GAAGAAGAGGAGGAATTCTCCGTGGTTTCGGGCCGGTCCG	
Do_gox1432_Fw	GGAGAATTCTCCTCTTCTTCAGGGCACAAAAACC	
Do_gox1432_rev	GCGTCTAGATGGAGCGAGCGTTTTCCCGCG	<i>Xba</i> I
Up_gox1432_int_Fw	AGATGCGCTTTGTGATGTCC	
Do_gox1432_int_rev	ACCGGCAGCTTTGATCTTTC	
Up_gox0849_Fw	ATAGGTACCGGCCCGGATGTTTCGAGCTGTC	<i>Kpn</i> I
Up_gox0849_rev	CCAAAACTTGACAGGATCTTTCAGGCGAAAGGACCGGC	
Do_gox0849_Fw	GATCCTGTCCAAGTTTTTGGTGAAGCTTTTTCCAAAAAGC	
Do_gox0849_rev	GCGTCTAGAAGGGGCTTGAGACGCTGGGCGC	<i>Xba</i> I
Up_gox0849_int_Fw	AATCATGTCCGTCTCGGTCT	
Do_gox0849_int_rev	CGAGACGATCTTCGAGAACC	

^a Restriction sites are underlined

pKos6b Δ 1432 was transferred from *E. coli* NEB 5- α into *G. oxydans* with the help of *E. coli* ECG 18 by triparental mating (Condon et al. 1991). The markerless deletion of *gox1432* was generated by plating *G. oxydans* pKos6b Δ 1432 on 5-fluorocytosine containing agar plates as described by Kostner et al. (2013). The construction of the Δ *gox0849* mutant and the double mutant (Δ *gox1432* Δ *gox0849*) was performed analogously. For the complementation of *gox1432* deletion mutants, the vector pBBR.p264.ST was used (Kallnik et al. 2010). Gene *gox1432* including its native ribosomal binding site was amplified using the pBBR.p264.gox1432_Fw and pBBR.p264.gox1432_rev primer pair. The resulting PCR product was digested with *Eco*RV/*Asc*I and cloned into the

*Eco*RV/*Asc*I sites of the pBBR.p264.ST vector. The resulting plasmid, pBBR.p264.gox1432.ST, was transformed into *E. coli* (NEB 5- α) and used for conjugal transfer of the plasmid into the *G. oxydans* Δ *gox1432* strain. All clones and mutants were verified by sequencing (StarSeq, Mainz, Germany).

Reverse transcription quantitative PCR

Reverse transcription quantitative PCR (RT-qPCR) was performed to quantify the relative abundance of transcripts from *gox1432* and *gox0849* in *G. oxydans* using *gox1709* as a reference gene (Rauch et al. 2010). Total RNA from osmotically stressed and non-stressed cultures

(OD₆₀₀ of 0.4–0.6) was isolated by the TRI reagent extraction method (Chomczynski 1993). One hundred milliliter cultures were harvested by centrifugation at 10,000 rpm and 4 °C. The cell pellet was dissolved in 5 ml TRI reagent and lysed by freeze-thaw treatment at –80 °C for 15 min. Total RNA was extracted according to manufacturer's instructions (Sigma-Aldrich, Munich, Germany) and purified using the SurePrep RNA Cleanup and Concentration Kit (Thermo Fisher Scientific, Schwerte, Germany), including a DNaseI treatment. RNA concentration and purity was analyzed spectrophotometrically by the 260/280 nm ratio as well as by denaturing agarose gel electrophoresis. Primers for RT-qPCR were generated using primer3 software (<http://primer3.ut.ee/>) (Table 1). Each PCR reaction contained 250–300 ng RNA and the transcript abundance was quantified using a SYBR Green RT-PCR Kit (Qiagen, Hilden, Germany) with a master cycler gradient from Eppendorf (Hamburg, Germany). A relative value (ΔCt) for the target cDNA concentration in each reaction was determined by subtracting Ct values of the genes of interest from the reference gene and the fold change was expressed as $2^{(\Delta Ct)}$. The efficiency of the primer pairs RT_Gox1432_Fw/RT_Gox1432_rev and RT_Gox849_Fw/RT_Gox849_rev was 96 and 100 %, respectively, and was checked by qPCR using serial dilutions of the template DNA (Stahlberg et al. 2003).

Overproduction and purification of proteins

For the overproduction of Gox1432 and Gox849, *E. coli* DH5 α cells containing plasmid pASK.3_gox 1432.ST or pASK.5_gox0849.ST were grown in 100 ml maximal induction medium (Mott et al. 1985) with ampicillin (100 $\mu\text{g ml}^{-1}$) at 37 °C. The cultures were induced with 200 ng ml⁻¹ anhydrotetracyclin at an optical density of 0.5 and were incubated overnight at 16 °C. Cells were harvested by centrifugation (10,000 rpm, 4 °C, 15 min) and resuspended in 15 ml buffer W (100 mM Tris-Cl, 100 mM NaCl, pH 8.0). After addition of lysozyme, DNaseI and protease inhibitor cocktail (Sigma-Aldrich, Munich, Germany) cells were lysed by sonication (Branson Sonifier Cell Disruptor with Branson Ultrasonics converter, Danbury USA). The proteins were purified by Strep-tactin Superflow® affinity chromatography as described by the manufacturer (IBA GmbH, Göttingen, Germany) and quantified by Bradford method (Bradford 1976). SDS-PAGE and western blots were performed as described by Meyer et al. (2015) according to the methods of Laemmli (1970) and Towbin et al. (1979), respectively. Native PAGE was done on 4–20 % gradient gels (BioRad, Munich, Germany) as described by Rauch et al. (2010).

Enzyme assays

The activities of purified enzymes were measured photometrically by recording the rate of change in absorption of NAD(P)H/NAD(P)⁺ at 340 nm ($\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$). For the analysis of substrate reduction (D-fructose, L-sorbose, or 5-keto D-gluconate), the 1 ml reaction mixtures contained 50 μmol potassium phosphate, pH 7.0, 0.125 μmol NADH or NADPH, and 100 μmol of substrates. For the quantification of polyol oxidation, 50 mM Tris-Cl, pH 9.0 (for Gox0849) or 50 mM sodium carbonate (for Gox1432), pH 10 were used that were supplemented with 0.125 μmol NAD⁺ or NADP⁺ and 100 μmol of substrates. The reactions were started by the addition of substrates. One unit corresponded to the conversion of 1.0 μmol of pyridine dinucleotide per minute. For the determination of activities of mannitol dehydrogenases in the cytoplasm, 100–250 ml stressed or non-stressed cultures were harvested in the mid log phase by centrifugation at 10,000 rpm for 15 min and 4 °C. Cells were resuspended in 10 ml cell suspension buffer containing 100 mM potassium phosphate buffer, pH 7.0, 5 mM MgSO₄·7H₂O and 0.75 mM dithiothreitol. Cells were lysed by sonication and the cell-free extract was centrifuged at 15,000 rpm for 15 min followed by ultracentrifugation at 45,000 rpm for 1 h. The clear supernatant (cytoplasmic fraction) was used to measure the activities of NADP⁺ and NAD⁺-dependent mannitol dehydrogenases at pH 7.0 as described above. Protein concentration was measured according to Bradford (1976).

Determination of glucose consumption and gluconate production

The rate of glucose consumption and gluconate formation was analyzed for the double deletion mutant ($\Delta\text{gox1432} \Delta\text{gox0849}$) in comparison to the wild type. Both cultures were grown in 70-ml YGP media containing 1000 mM glucose in 250-ml baffled flasks. The flasks were inoculated by the addition of 5 ml of an overnight culture and were incubated at 30 °C and a stirring rate of 180 rpm. Samples (0.7 ml) were taken at regular intervals and were centrifuged at 12,000 rpm for 10 min. The supernatant was analyzed on a Aminex-HPX87H column (BioRad, Munich, Germany, 300 mm × 7.8 mm) using 5 mM H₂SO₄ as eluent at 65 °C and a flow rate of 0.3 mL min⁻¹. Glucose and gluconate concentrations were quantified according to standard calibration curves. Analysis of intracellular mannitol content was performed as described by Zahid et al. (2015).

Results

Identification and characterization of mannitol dehydrogenases in *G. oxydans*

Two mannitol dehydrogenases are present in *G. oxydans* that vary with respect to their cofactor specificity (Adachi et al. 1999). One enzyme was described as a NADP⁺-dependent mannitol dehydrogenase (EC 1.1.1.138) and the second mannitol dehydrogenase showed a preference for NAD⁺ (EC 1.1.1.67). The amino acid sequences of these enzymes are not available, and the respective gene loci could not be identified. A search using the NCBI non-redundant BLAST database revealed that *gox1432* and *gox0849* encode putative mannitol dehydrogenases in *G. oxydans*. Both proteins were homologous to several putative mannitol dehydrogenases from acetic acid bacteria. However, these enzymes have not been characterized with respect to their catalytic activities. A BLAST analysis using the SwissProt database indicated several biochemically analyzed homologues of the mannitol dehydrogenase family with identities of 32–40 %, similarities of 50–57 %, and coverage of 84–95 %. Among them was a mannitol-2-dehydrogenase from *Rhodobacter sphaeroides* (Schneider et al. 1993) and *Pseudomonas fluorescens* (Kavanagh et al. 2002) and a D-arabinitol-4 dehydrogenase from *Klebsiella pneumoniae* (Heuel et al. 1998). Gox1432 and Gox0849 contained Rossmann fold NAD⁺/NAD(P)⁺ binding domains (Rossmann et al. 1974) and mannitol dehydrogenase signature sequence motifs (PDOC00751). Gox1432 and Gox0849 were annotated as NADP-D-sorbitol dehydrogenase and NADPH-dependent L-sorbose reductase, respectively (Prust et al. 2005). Because of the unknown substrate spectrum of the predicted mannitol dehydrogenases, the corresponding genes were cloned into pASK-IBA vectors and heterologously overexpressed in *E. coli*. The proteins were purified from crude extract to apparent homogeneity using Strep-tactin affinity chromatography obtaining 1–2 mg of protein from 1 l *E. coli* cultures.

SDS-PAGE and western blot analysis revealed molecular masses of 54.9 kDa for Gox1432 and 55.7 kDa for Gox0849, which are in accordance with the expected mass of 54.6 and 55.0 kDa for the recombinant tagged proteins, respectively (Fig. 1). Similarly, Adachi et al. (1999) reported that the NADP⁺-dependent mannitol dehydrogenase from *Gluconobacter suboxydans* IFO 12528 was a monomeric protein with an apparent molecular mass of 50 kDa. Gel filtration chromatography showed that the NAD⁺-dependent mannitol dehydrogenase had a native molecular mass of 130 kDa and was composed of two identical subunits (Adachi et al. 1999). In agreement, native PAGE revealed that Gox1432 formed monomers, while Gox0849 had a homodimeric structure (data not

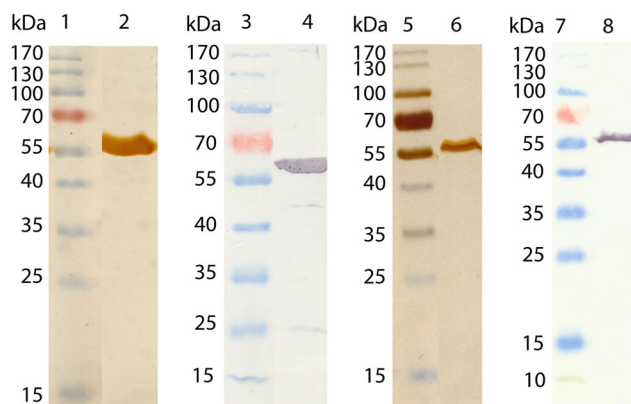


Fig. 1 SDS-PAGE and western blot of purified proteins. Lanes 1, 3, 5, 7: SDS-PAGE (12 % gel) of molecular mass marker (page ruler Prestained Protein Ladder, Thermo Fisher Scientific, Schwerte, Germany). Lane 2: SDS-PAGE of purified Gox1432 (silver staining). Lane 4: western blot of purified Gox1432. Lane 6: SDS-PAGE of purified Gox0849 (silver staining). Lane 8: western blot of purified Gox0849. Molecular mass of the purified proteins was determined by plotting the mobility versus molecular mass of the standard marker proteins

shown). In addition, very faint protein bands were detected for Gox1432 at 100 kDa and for Gox0849 at 160 kDa representing low amounts of homodimers and homotrimers, respectively.

For further characterization, the activity of Gox1432 from *G. oxydans* 621H was determined using different sugars and polyols in the presence of either NAD(P)⁺ or NAD(P)H. It was found that Gox1432 oxidized the polyols D-mannitol, D-sorbitol, D-arabitol, and D-glycerol and was only active with NADP⁺ as an electron acceptor. Furthermore, the enzyme was able to reduce D-fructose, L-sorbose, and 5-keto D-gluconate in the presence of NADPH as an electron donor. The optimum pH for substrate reduction and oxidation was 7.0 and 10, respectively. Gox0849 also showed cofactor preference, but could only use NADH for fructose reduction. No other tested sugars were reduced. Furthermore, only D-mannitol was oxidized by Gox0849 using NAD⁺ as an electron acceptor. The optimum pH for reduction of D-fructose and oxidation of D-mannitol was 7.0 and 9.0, respectively. The kinetic parameters of both proteins were measured for different substrates and pH values (Table 2). At a physiological pH of 7, Gox1432 and Gox0849 reduced most rapidly D-fructose to D-mannitol with catalytic efficiencies of $2.8 \times 10^3 \text{ s}^{-1} \text{ M}^{-1}$ and $2.9 \times 10^3 \text{ s}^{-1} \text{ M}^{-1}$, respectively. In contrast, the rate of mannitol oxidation at pH 7 was 4-fold lower for Gox1432 and 3-fold lower for Gox0849. These data suggest that although the enzymes are able to oxidize mannitol, their primarily function in vivo is the reduction of fructose. High oxidative activities of the enzymes were obtained only at non-physiological pH values of 9–10 with $K_{\text{cat}}/K_{\text{m}}$ of $7.8 \times 10^3 \text{ s}^{-1} \text{ M}^{-1}$ and 6.4×10^3 for Gox1432 and Gox0849, respectively.

Table 2 Kinetic parameters of Gox1432 and Gox0849

Enzyme	Substrate	pH	Cofactor	K_{cat}/K_m ($s^{-1} M^{-1}$) ^e
Gox1432	D-Fructose	7	NADPH ^a	$2.8 \pm 0.35 \times 10^3$
	L-Sorbose	7	NADPH ^a	$3.8 \pm 0.02 \times 10^2$
	D-Mannitol	7	NADP ^{+b}	$6.6 \pm 2.4 \times 10^2$
	D-Mannitol	10	NADP ^{+b}	$7.8 \pm 1.5 \times 10^3$
	D-Sorbitol	10	NADP ^{+b}	$8.9 \pm 0.2 \times 10^2$
Gox0849	D-Fructose	7	NADH ^c	$2.9 \pm 0.03 \times 10^3$
	D-Mannitol	7	NAD ^{+d}	$1.1 \pm 0.06 \times 10^3$
	D-Mannitol	9	NAD ^{+d}	$6.4 \pm 1.05 \times 10^3$

^a K_m NADPH at pH 7.0 for D-fructose reduction was $19.5 \pm 1.0 \mu M$

^b K_m NADP⁺ at pH 10.0 for D-mannitol oxidation was $25.6 \pm 7.2 \mu M$

^c K_m NADH at pH 7.0 for D-fructose reduction was $22.4 \pm 6.0 \mu M$

^d K_m NAD⁺ at pH 9.0 for D-mannitol oxidation was $55.3 \pm 20 \mu M$

^e Substrate concentrations of 10 to 1000 mM were used to calculate K_m and V_{max} using nonlinear regression of the Michaelis–Menten data (GraphPad Prism 6)

Transcript abundance of genes encoding mannitol dehydrogenases in *G. oxydans*

The transcript abundance of *gox1432* and *gox0849* was determined by RT-qPCR in cells grown under unstressed (YGP) and osmotic stress conditions (YGP with 450 mM sucrose). Gene *gox1709* coding for the gluconokinase was used as an internal reference as it is constitutively expressed (Rauch et al. 2010). As shown in Fig. 2, the expression of both genes was not regulated as their quantification cycle (Ct) values did not differ significantly under control and osmotic stress

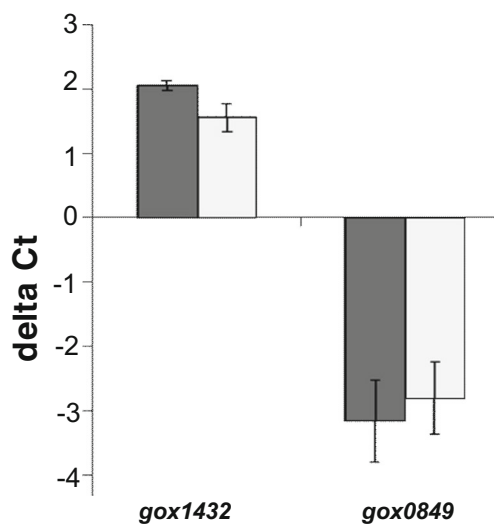


Fig. 2 Transcript abundance of the genes encoding mannitol dehydrogenases (*gox1432* and *gox0849*) in comparison to the house keeping gene *gox1709*. Dark gray bars indicate transcript abundance of indicated genes under non-stress conditions; light gray bars indicate transcript abundance of the corresponding genes under osmotic stress conditions. ΔCt values were determined by subtracting average Ct values of the genes of interest from the reference gene *gox1709*

conditions. However, the transcript abundance of *gox1432* was 3.5-fold higher in comparison to the reference gene indicating its importance for cellular metabolism. In contrast, the amount of transcripts from *gox0849* was about 8-fold lower than the reference gene.

Characterization of mannitol dehydrogenase deletion mutants

To elucidate the function of the two mannitol dehydrogenases in osmotolerance of *G. oxydans*, the deletion mutants $\Delta gox1432$ and $\Delta gox0849$ and the corresponding double mutant were generated. Growth, enzymatic activities, and intracellular mannitol content of all strains were monitored in YGP medium, and 450 mM sucrose was used as an osmoticum as it is not metabolized by *G. oxydans* and does not interfere with glucose metabolism. Growth parameters of all mutants were similar to the wild type under non-osmotic stress conditions with doubling times of 2 h and final optical densities of 2.0 (not shown). Hence, the experiments indicated that *gox1432* and *gox0849* were not essential for growth under standard conditions with glucose as substrate. In YGP medium supplemented with 450 mM sucrose, growth of the wild type and the $\Delta gox0849$ mutant was almost identical with doubling times of 3.2 and 3.3 h, respectively (Fig. 3a). Also the final OD_{600} was similar with 0.91 for $\Delta gox0849$ versus 0.96 for the wild type, indicating the minor importance of mannitol dehydrogenase Gox0849 for cells growth under osmotic stress conditions. In contrast, growth of *G. oxydans* $\Delta gox1432$ was impaired in highly osmotic media. The doubling time was extended to 6 h and the final optical density was severely reduced by 45 % in comparison to the parental strain (Fig. 3a). To examine whether a plasmid-borne allele could complement the genomic deletion of *gox1432*, the mutant was transformed with a broad host range plasmid pBBR1-MCS with the strong promoter p264 in front of gene *gox1432*. The complementation led to slightly improved growth parameters ($t_d = 3.1$ h, final $OD_{600} = 1.4$) in comparison to the wild type (Fig. 3b). These results confirmed the importance of *gox1432* for cell survival and growth under osmotic stress.

The growth defect of cells under osmotic stress was even more pronounced when both mannitol dehydrogenase-encoding genes (*gox1432*, *gox0849*) were deleted. The double mutant ($\Delta gox1432 \Delta gox0849$) showed an extensive lag phase of 8–10 h when grown on YGP + 450 mM sucrose medium followed by poor growth with doubling times of 6 h and a final optical density of 0.4 (Fig. 3a). However, when the double mutant was cultured in YGP + 450 mM sucrose medium in the presence of 5 mM mannitol, growth improved significantly with doubling times of 2.1 h and a final OD_{600} of 2.0. These values were in the same range as for the wild type

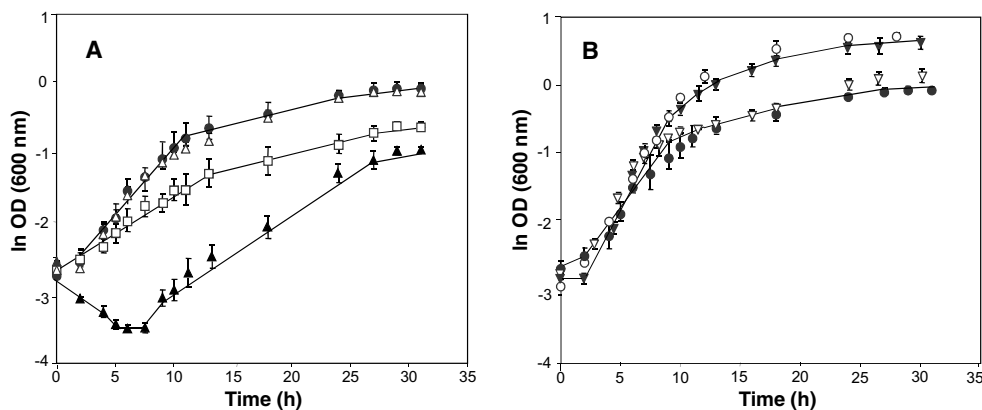


Fig. 3 Growth of wild-type and mutant strains of *G. oxydans* on YPG supplemented with 450 mM sucrose. **a** Growth profile of the wild-type (filled circle), mutant strain $\Delta gox0849$ (open triangle), mutant strain $\Delta gox1432$ (open square), double mutant $\Delta gox1432 \Delta gox0849$ (filled

triangle). **b** Wild-type (filled circle), $\Delta gox1432$ complemented strain (inverted open triangle), double mutant $\Delta gox1432 \Delta gox0849$ with 5 mM mannitol (inverted filled triangle), growth of non-stressed wild type grown on YGP (open circle)

without osmotic stress ($t_d = 2$ h, final $OD_{600} = 2.0$). Hence, low concentrations of mannitol could alleviate osmotic stress from the cells imposed by sucrose (Zahid et al. 2015). The results indicate that the growth retardation observed for the $\Delta gox1432$ mutant and the double mutant under osmotic stress was due to defects in mannitol synthesis. Furthermore, it became evident that *gox1432* encoding the $NADP^+$ -dependent mannitol dehydrogenase was the major player for osmoprotection of *G. oxydans*. However, *gox0849*, encoding the NAD^+ -dependent mannitol dehydrogenase, is important for survival of the cell under osmotic stress when *gox1432* is absent. The slow growth of the double mutant after a long lag phase points towards a third unknown enzyme that is able to produce low amounts of mannitol in the presence of 450 mM sucrose as osmoticum.

Intracellular mannitol formation and activity of cytoplasmic mannitol dehydrogenase

The single and double mannitol dehydrogenase deletion mutants were also analyzed for the presence of intracellular mannitol under osmotic stress induced by 450 mM sucrose (Fig. 4). It was found that the deletion of *gox0849* did not significantly alter the intracellular mannitol accumulation ($1.7 \pm 0.1 \mu\text{mol mg protein}^{-1}$) in comparison to the wild type ($2.0 \pm 0.1 \mu\text{mol mg protein}^{-1}$). In contrast, the $\Delta gox1432$ mutant and the double mutant revealed a 60 to 70 % reduction of intracellular mannitol. These values correlated with the findings from the growth experiments and revealed that the deletion of mannitol dehydrogenases *Gox1432* retarded cell growth under osmotic stress due to a decrease in intracellular mannitol production. Again, it was found that the double deletion mutant had some residual potential for mannitol synthesis ($0.4 \pm 0.1 \mu\text{mol mannitol mg protein}^{-1}$). This finding indicated the presence of a third intracellular mannitol dehydrogenase with limited activity.

To confirm these results, the cytoplasmic fractions of the wild type, the single mutant, and the double mutant strains were prepared from stressed and non-stressed cells. The intracellular pH of acetic acid bacteria is in the range of 7.0 (Sugisawa et al. 1991). Hence, it is tempting to speculate that *Gox1432* and *Gox0849* function as fructose reductases in the cytoplasm of *G. oxydans* as shown for the purified enzymes at pH 7. Therefore, all cytoplasmic fractions were assayed for D-fructose reduction activity (Fig. 5). The maximum reduction rate for D-fructose was $3.0 \pm 0.4 \mu\text{mol min}^{-1} \text{mg}$ of cytoplasmic protein $^{-1}$ as catalyzed by the $NADP^+$ -dependent mannitol dehydrogenase *Gox1432* in the wild-type strain under osmotic stress. In contrast, the activity was only $1.25 \pm 0.2 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$ in the cytoplasm of wild-type cultures grown in the absence of sucrose. Similar rates for NADPH-dependent fructose reduction were found in the cytoplasm of the $\Delta gox0849$ mutant. Under stress and non-stress conditions the NADH-specific reduction rate of D-fructose was only $0.3 \pm 0.03 \mu\text{mol min}^{-1} \text{mg}$ of cytoplasmic protein $^{-1}$ in the

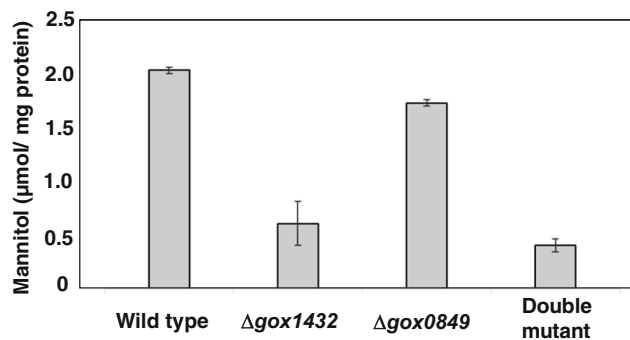
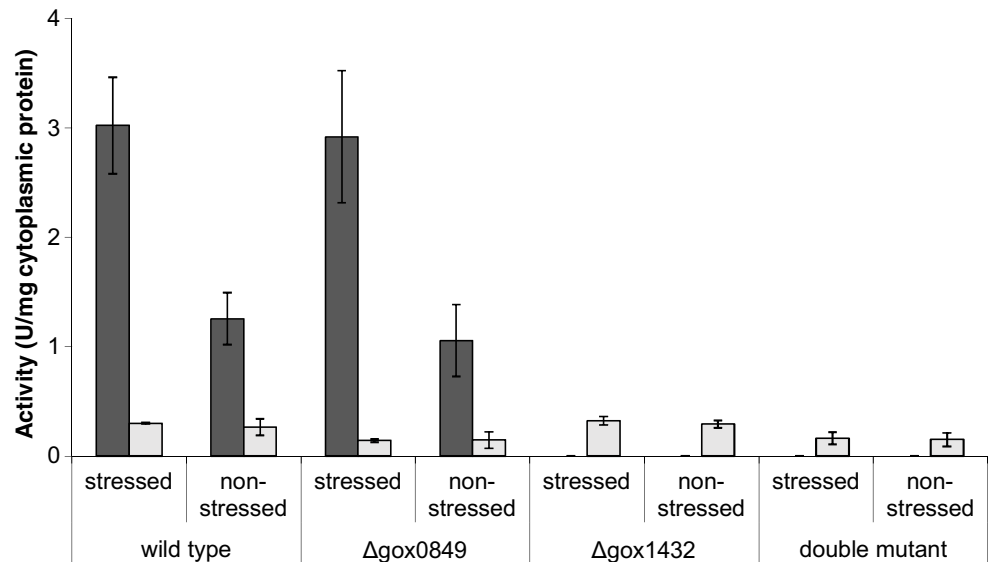


Fig. 4 Effect of deletion of mannitol dehydrogenases on intracellular mannitol formation. The wild-type and the mannitol dehydrogenase deletion mutants were cultivated in YGP medium containing 450 mM sucrose. Analysis of intracellular mannitol content was performed as described by Zahid et al. (2015)

Fig. 5 Activities of mannitol dehydrogenases in the cytoplasmic fractions of the wild-type and mutant strains of *G. oxydans* grown on YGP medium (non-stressed) or YGP medium + 450 mM sucrose (stressed). Dark and light gray bars represent the activity of the NAD⁺ and the NAD⁺-dependent mannitol dehydrogenase, respectively



wild type. NADH-dependent reduction of fructose in *G. oxydans* Δgox0849 was 2-fold lower compared to the wild type. In the Δgox1432 mutant, only NAD⁺-specific mannitol dehydrogenase activity was detected with an activity of $0.3 \pm 0.04 \mu\text{mol min}^{-1} \text{mg}$ of cytoplasmic protein⁻¹. In the double mutant NADPH-dependent D-fructose reduction was completely absent, but the cytoplasm was still reactive with NADH to reduce D-fructose with a specific activity of $0.16 \pm 0.05 \text{ U mg protein}^{-1}$ (Fig. 5). This indicated the presence of another intracellular NAD⁺-specific mannitol dehydrogenase. In addition, the cytoplasmic fractions of all strains were tested for D-mannitol oxidation at pH 7. It was found that the ratio of NAD⁺ and NADP⁺-dependent mannitol oxidation in stressed and unstressed cultures were similar to the rates of fructose reduction but the specific activities were 10-fold lower (data not shown).

One important biotechnological application of *G. oxydans* is the synthesis of gluconate that is used in pharmaceutical and food industries (Roblin et al. 2006). Therefore, the wild-type and the double mutants were tested for their efficiency with respect to gluconate production in YP media containing 1 M glucose as substrate and as osmoticum. It is known, that only low amounts of glucose are transported into the cells for biomass production. Most glucose is oxidized in the periplasm by the membrane-bound glucose dehydrogenase to form δ-gluconolactone, which is then hydrolyzed to gluconate. We observed a severe effect on growth, gluconate production, and pH values of the cultures during glucose-induced osmotic stress (Fig. 6a, b). After a long lag phase, the double deletion mutant reached a final OD₆₀₀ of 0.2 and had a doubling time of approximately 10 h. Consequently, the gluconate production rate of the double mutant strain was almost 4-fold lower compared to the wild-type strain in the exponential growth phase. At the end of the experiment ($t = 30 \text{ h}$), the osmotically stressed wild-type cells produced about 350 mM gluconate in

contrast to the double mutant that formed only 100 mM gluconate. Accordingly, the drop of the pH values during growth of the cultures was different. The pH of the double mutant decreased from 6.5 to 3.5 within 30 h. In the wild type, the same decrease was observed after 14 h. Thus, the deletion of mannitol dehydrogenase-encoding genes had a direct influence on cell division and cellular catalytic efficiencies under osmotic stress. Furthermore, it was found that the deletion mutant accumulated only $0.4 \pm 0.2 \mu\text{mol mannitol mg cell protein}^{-1}$ while the mannitol concentration was $2.0 \pm 0.4 \mu\text{mol mg protein}^{-1}$ in the wild type with glucose as osmoticum (Table S1). In summary, the results demonstrate the necessity of mannitol dehydrogenases for *G. oxydans* to tolerate and survive high osmotic conditions for effective product formation.

Discussion

Microorganisms thrive in different ecological niches depending on local biotic and abiotic factors. Water activity is one of the most important abiotic parameters that directly influence growth of microbial species. Therefore, microorganisms adapt to cope with reduced water activity either by the uptake of extracellular ions (K⁺) or by synthesis and accumulation of small organic molecules referred to as osmolytes or compatible solutes (da Costa et al. 1998; Empadinhas and da Costa 2009; Wood 2015). Numerous low weight organic molecules are known to be compatible solutes such as amino acids (glutamate, proline, glutamine, ectoine), sugars (trehalose, sucrose), phosphodiester (di-myoinositol phosphate), glyceric acid derivatives (mannosylglycerate, glucosylglycerate), and polyols (sorbitol, mannitol) (Wood 2015; Empadinhas and da Costa 2009). These molecules maintain a positive intracellular turgor pressure and secure the hydration of the cell cytoplasm

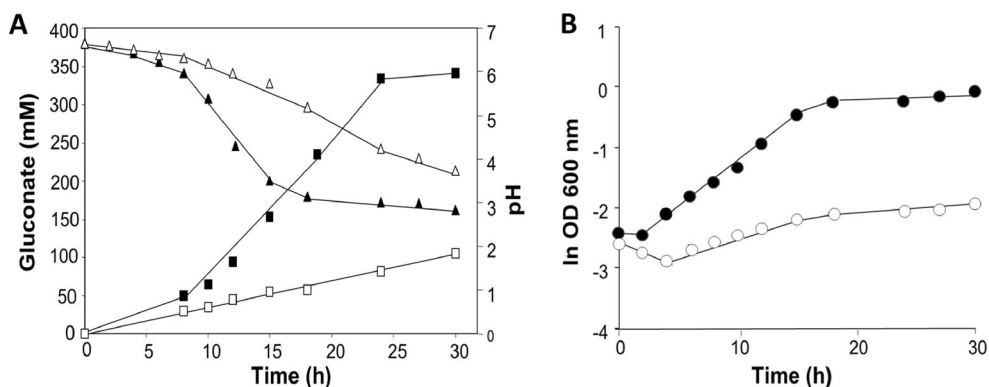


Fig. 6 Effect of deletion of mannitol dehydrogenases on growth and gluconate formation. **a** Correlation between gluconate formation (filled and open squares) and decrease in pH (filled and open triangles) of the wild-type *G. oxydans* (filled symbols) and $\Delta gox1432 \Delta gox0849$ double

mutant (open symbols) on yeast-glucose (1000 mM) media with 100 mM K-phosphate buffer (pH 6.8). **b** Growth profile of the wild-type (filled circle) and the $\Delta gox1432 \Delta gox0849$ double mutant (open circle)

to attenuate external water fluctuations (Cayley et al. 1992; Cayley and Record 2004).

Recently, we have reported the role of D-mannitol as an osmoprotectant in *G. oxydans* 621H (Zahid et al. 2015). Mannitol functions as an osmolyte in plants and fungi (Jennings 1984; Grant 2004; Empadinhas and da Costa 2009) as well as in different bacteria like *Pseudomonas fluorescens* (Kets et al. 1996) and *A. baylyi* (Sand et al. 2013) and in hetero-fermentative lactic acid bacteria (Wisselink et al. 2002). Other short-chain polyols such as glycerol, arabitol, and sorbitol are also known to act as compatible solutes in different microorganisms under osmotic as well as cold and oxidative stress conditions (Sootsuwan et al. 2013; de Barros and Celligoi, 2006; Efiuwewewere et al. 1999; Loos et al. 1994; Hocking 1988). In *G. oxydans*, mannitol was accumulated inside the cells during growth under osmotic stress conditions. Moreover, external mannitol had a positive effect on growth and physiology of cells when added in small amounts to osmotically stressed cultures (Zahid et al. 2015). Hence, it seems obvious that the osmotolerance of *G. oxydans* can be improved by increasing the intracellular mannitol production. Therefore, it was of great interest to unravel the pathway of mannitol synthesis in this organism.

Here, we show that mannitol dehydrogenases are responsible for osmotolerance of *G. oxydans* 621H. This bacterium naturally lives in sugar-rich environments such as fruits, flowers, nectars, wine, and honey syrups (De Muynek et al. 2007). Due to the abundance of nutrients in its environment, *G. oxydans* performs an overflow type of metabolism where most of the sugars, polyols, and alcohols are incompletely oxidized to keto-sugars and acids (De Ley et al. 1984; Deppenmeier et al. 2002; Deppenmeier and Ehrenreich 2009). Due to high product and low growth yield, *G. oxydans* is used for several biotechnological processes in textile, food, pharmaceutical, and fodder industries to produce valuable substances such as D-gluconate, dihydroxyacetone,

L-sorbose (vitamin C production), and 6-amino-6-deoxy-L-sorbose (Miglitol production) (Hommel and Ahnert 1999; Hancock 2009; Macauley et al. 2001; Claret et al. 1994; De Muynek et al., 2007). *G. oxydans* can thrive in concentrated sugar solutions, for example, up to 30 % (w/v) glucose, but its growth and productivity declines by increased osmolarity of medium (Luchterhand et al. 2015; Zahid et al. 2015). Therefore, there is a need to explore the physiology of *G. oxydans* under osmotic stress to understand and improve its bio-catalytic efficiency.

Since mannitol cannot be synthesized by the primary metabolism of *G. oxydans* (Zahid et al. 2015), it can be argued that this compatible solute is produced as a secondary metabolite inside the cell. Fructose is probably the direct precursor which could be formed from glucose by the catalytic activities of a glucokinase, a glucose-6-phosphate isomerase and a phosphofructose phosphatase (Richhardt et al. 2012; Rauch et al. 2010). In addition, the production of fructose 6-phosphate is also possible by using the oxidative pentose phosphate pathway with subsequent dephosphorylation. Finally, fructose would be reduced to mannitol by a mannitol dehydrogenase reaction (Fig. 7). A second pathway for mannitol synthesis is found in fungi which includes the direct production of fructose from glucose by the catalytic activity of a glucose isomerase. However, such an enzyme is not encoded on the chromosome of *G. oxydans* 621H.

Four types of polyol dehydrogenases are known to exist in *Gluconobacter* species which use mannitol (oxidative direction) or fructose (reductive direction) as substrates. These enzymes can be distinguished by their molecular mass, cofactor specificity, and cellular localization: Type 1 comprise membrane-bound and PQQ-dependent polyol dehydrogenases with broad substrate spectrum which are involved in incomplete oxidation of a range of substrates. It has been shown that the enzyme from *G. oxydans* 621H oxidizes mannitol, glycerol and gluconate forming fructose, dihydroxyacetone, and 5-

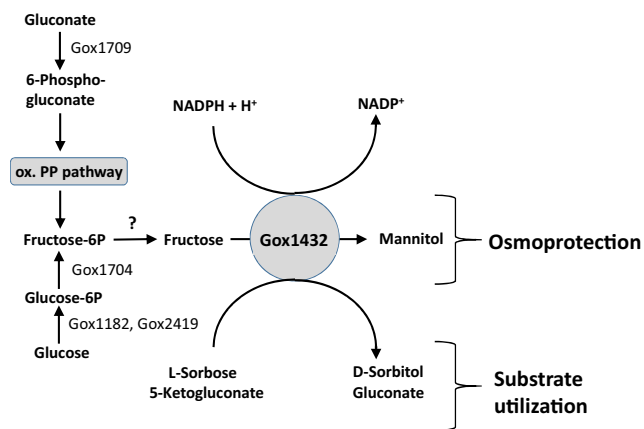


Fig. 7 Function of Gox1432 and putative pathways of mannitol production. *Question mark* indicates an unknown phosphatase. *ox PP pathway* oxidative pentose phosphate pathway

ketogluconate, respectively. The enzyme is also responsible for the oxidation of D-sorbitol to produce L-sorbose, an important intermediate in vitamin C production (Matsushita et al. 2003; Hoshino et al. 2003). Type 2 proteins are NADP⁺-dependent mannitol dehydrogenases with limited substrate spectrum and molecular masses of about 50 kDa per single subunit that convert only mannitol or fructose (Adachi et al. 1999). Type 3 NADP⁺ mannitol dehydrogenases catalyze the reversible oxidation of different polyols such as D-sorbitol, mannitol, and arabitol (Klasen 1994; Shibata et al. 2000; Shinjoh et al. 2002). Blast analysis and multiple alignments indicated that Gox1432 belongs to this group of mannitol dehydrogenases (data not shown). Class 4 comprise NAD⁺ dependent mannitol dehydrogenases which were found in cell extracts from *G. oxydans* LMG 1489 (Parmentier et al. 2005) and *G. suboxydans* IFO12528 (Adachi et al. 1999). Here, we describe the first purified enzyme of this class that is encoded by gene *gox0849*.

Gox1432 and Gox0849 reduced D-fructose to D-mannitol with comparable catalytic efficiencies at pH 7.0. However, neither was able to reduce fructose-6-phosphate to mannitol-1-phosphate. Hence, it is tempting to speculate that there is a phosphatase that is able to dephosphorylate fructose-6-phosphate in the course of mannitol synthesis. Efficient conversion of D-mannitol to D-fructose by Gox1432 and Gox0849 was only observed at non-physiological alkaline conditions. These data indicate that the enzymes work as reductases *in vivo* and can be classified as D-fructose reductases rather than D-mannitol dehydrogenases. Strong evidence was presented that Gox1432 is more important for mannitol production in *G. oxydans* than Gox0849, since its transcript abundance was much higher than the expression level of *gox0849*. This was in agreement with the activity of the NADH-dependent enzyme in the cell cytoplasm being 10-fold lower in comparison to the NADPH-dependent D-fructose reductase Gox1432. The importance of Gox1432 on cell physiology and

compatible solute accumulation was further highlighted by analyzing the corresponding deletion strain. Under osmotic stress condition the growth rate and the final optical density of the $\Delta gox1432$ strain was reduced by 37 and 45 %, respectively, compared to the wild type. Furthermore, deletion of *gox1432* resulted in a complete loss of NADPH-dependent D-fructose reduction in extracts of cells grown under stress and non-stress conditions. As expected, NADH-dependent D-fructose reduction was unaltered. These results indicated that Gox1432 is solely responsible for intracellular NADPH mediated D-fructose reduction. Similar effects were observed in *Zymomonas mobilis*, when the *gfo* gene (encoding the glucose-fructose oxidoreductase) was deleted which is responsible for the synthesis of the osmolyte D-sorbitol (Sootsuwan et al. 2013). The cells became osmosensitive and the growth rates were reduced from 0.18 to 0.08 h⁻¹ in sugar-rich media (Loos et al. 1994; de Barros and Celligoi 2006). The growth defect of *Z. mobilis* Δgfo was completely relieved either by complementation of the *gfo* gene or by the addition of the osmolyte under osmotic stress conditions (Sootsuwan et al. 2013). In almost the same manner, the addition of low amounts of mannitol or the complementation with *gox1432* led to a recovery of normal growth parameters of the $\Delta gox1432$ mutant.

In contrast to the $\Delta gox1432$ strain, growth of the *gox0849* deletion mutant was comparable to wild-type cells in high osmotic media. Interestingly, the deletion of *gox0849* did not result in a complete loss of NADH-dependent D-fructose reduction, indicating the presence of another yet unknown NADH-specific D-fructose reductase with low activity. Evidence in favor of this assumption came from the analysis of the double deletion mutant strain ($\Delta gox1432 \Delta gox0849$) that revealed the same residual activity of NADH-dependent D-fructose reduction under stress and non-stress conditions. However, the unknown mannitol dehydrogenase could not completely restore normal growth of the double deletion mutant since the cultures revealed a long lag phase and reached only 50 % of the final optical density compared to the parental strain.

Sugisawa et al. (1991) deleted the orthologous gene of *gox1432* in *Gluconobacter melanogenus* N44-1 and observed that the mutant strain was unable to grow in media containing 500 mM L-sorbose. Likewise, it was shown that in *G. suboxydans* IFO 3291, the ortholog of Gox1432 is mainly responsible for L-sorbose assimilation (Shinjoh et al. 2002). Furthermore, the enzyme also catalyzes 5-ketogluconate reduction as shown for *G. oxydans* DSM 3503 (Klasen 1994). Analogous to these results, we found that *G. oxydans* 621H $\Delta gox1432$ was not able to grow with L-sorbose and 5-ketogluconate as carbon and energy source, respectively (data not shown). Therefore, Gox1432 is the major factor for intracellular reduction of partially oxidized products that are formed by the membrane-bound polyol dehydrogenase

(Gox0854–0855) (Fig. 7). When the major substrates (D-sorbitol or gluconate) are consumed, incompletely oxidized products (L-sorbose or 5-ketogluconate) are taken up by the cells and the reduced forms are channeled in the central metabolism. L-Sorbose is reduced to D-sorbitol that is converted into D-fructose and finally phosphorylated to fructose-6-phosphate (Richhardt et al. 2012). 5-Ketogluconate is reduced to gluconate and phosphorylated to 6-phosphogluconate by Gox1709. Both intermediates are then channeled into the oxidative pentose phosphate pathway. As shown here, Gox1432 has another important function because it plays a central role in the synthesis of mannitol as osmoprotectant in osmotically stressed *G. oxydans* (Fig. 7). Thus, we suggest that Gox1432 is the key player for survival in the sugar-rich natural habitats of the organism. This information is especially useful to optimize the strategies of engineering *G. oxydans* strains with improved osmotolerance for biotechnological applications.

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Compliance with ethical standards

Ethical statement The authors affirm that the said publication complies with obligations and norms of the ethical standards of research.

Conflict of interest The authors declare that they have no conflict of interest.

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