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Characterization of membrane-bound dehydrogenases of *Gluconobacter oxydans* 621H using a new system for their functional expression

Markus Mientus[1] · **David Kostner**[1] · **Björn Peters**[1] · **Wolfgang Liebl**[1] · **Armin Ehrenreich**[1]

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Abstract Acetic acid bacteria are used in biotechnology due to their ability to incompletely oxidize a great variety of carbohydrates, alcohols, and related compounds in a regio- and stereo-selective manner. These reactions are catalyzed by membrane-bound dehydrogenases (mDHs), often with a broad substrate spectrum. In this study, the promoters of six mDHs of *Gluconobacter oxydans* 621H were characterized. The constitutive promoter of the alcohol dehydrogenase and the glucose-repressed promoter of the inositol dehydrogenase were used to construct a shuttle vector system for the fully functional expression of mDHs in the multi-deletion strain *G. oxydans* BP.9 that lacks its mDHs. This system was used to express each mDH of *G. oxydans* 621H, in order to individually characterize the substrates, they oxidize. From 55 tested compounds, the alcohol dehydrogenase oxidized 30 substrates and the polyol dehydrogenase 25. The substrate spectrum of alcohol dehydrogenase overlapped largely with the aldehyde dehydrogenase and partially with polyol dehydrogenase. Thus, we were able to resolve the overlapping substrate spectra of the main mDHs of *G. oxydans* 621H. The described approach could also be used for the expression and detailed characterization of substrates used by mDHs from other acetic acid bacteria or a metagenome.

Keywords Promoters · Membrane-bound dehydrogenases · Functional expression · Acetic acid bacteria · *Gluconobacter oxydans*

Introduction

The acetic acid bacterium *Gluconobacter oxydans* as well as other acetic acid bacteria are widely used in biotechnology due to their ability of incomplete regio- and stereo-selective oxidation of a great variety of alcohols, polyols, carbohydrates, and related compounds. This incomplete oxidation of substrates is catalyzed by various membrane-bound dehydrogenases (mDHs) (De Muynck et al. 2007; Deppenmeier et al. 2002; Gupta et al. 2001). For the detailed investigation and metabolic engineering of *G. oxydans* strains, an easily applicable markerless deletion system is essential. Such a system was developed by Peters et al. (2013a) for strain *G. oxydans* 621H. It uses 5-fluorouracil together with a plasmid-encoded uracil phosphoribosyltransferase for counter selection. For functional expression of mDHs in *G. oxydans*, the construction of a specialized shuttle expression vector system is necessary. It is preferable to use an acetic acid bacterium as host for the expression of mDHs, because these enzymes need to be integrated in the physiology and electron transport chains of the organism. In recent years, a couple of vector systems, including broad host range vectors and cryptic plasmids for *G. oxydans* and *Acetobacter* were reported (Condon et al. 1991; Fukaya et al. 1985; Merfort et al. 2006a, b; Okumura et al. 1985; Saito et al. 1997; Schleyer et al. 2008; Shinjoh and Hoshino 1995; Tonouchi et al. 2003; Trcek et al. 2000; Zhang et al. 2010), but most of them have limited use or effectiveness (Kallnik et al. 2010; Schleyer et al. 2008). The complete genome sequence of *G. oxydans* 621H revealed more than 75 mostly uncharacterized

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✉ Armin Ehrenreich
aehrenr@tum.de

[1] Lehrstuhl für Mikrobiologie Technische Universität München, Emil-Ramann-Str. 4, 85354 Freising, Germany

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oxidoredutases (Deppenmeier and Ehrenreich 2009; Prust et al. 2005). Among them, eight known and two unknown mDHs were predicted (Prust et al. 2005). Recently, Peters et al. (2013b) undertook a comprehensive in vivo study of the mDHs of *G. oxydans* 621H. This study allowed a precise description of the substrates converted by each of the membrane-bound dehydrogenases of this organism. Many previous studies on mDHs and the substrates they convert were based on single purified activities with little information, which genes code for respective enzymes. Former characterizations of some enzymes indicated that they typically have a broad, often overlapping substrate spectrum (Adachi et al. 2003; Schweiger et al. 2007, 2010, 2013). Peters et al. (2013b) created a set of deletion strains, where all the mDHs of *G. oxydans* 621H were deleted one after the other. In order to characterize the substrate spectrum of the enzymes using these strains, a new miniaturized whole cell 2,6-dichlorophenolindophenol (DCPIP) activity assay was developed. This assay revealed that some of the mDHs have a relatively narrow and broad substrate spectrum (Peters et al. 2013b).

In the present study, we used an expression system for mDHs in *G. oxydans* 621H in order to characterize the expressed enzymes individually. It employed the promoters of the membrane-bound alcohol dehydrogenase (GOX1067–1068) and the membrane-bound inositol dehydrogenase (GOX1857) for functional expression of these enzymes. The backbone of the vector pEXGOX-K (Schleyer et al. 2008) was used to construct the vector pMM4a with the promoter of the pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenase and vector pMM4b with the promoter of the quinoprotein inositol dehydrogenase for the expression of the target mDH (Holscher et al. 2007). The strength of both promoters was determined by *lacZ* reporter constructs. The expression vector pMM4a was then utilized to individually complement the deleted mDHs in the multi-deletion strain *G. oxydans* BP.9 (Peters et al. 2013b). Thus, nine derived strains of *G. oxydans* 621H were constructed, in each of which one membrane-bound dehydrogenase is complemented. These strains were used in the whole DCPIP cell assay to study the compounds oxidized by each enzyme, thereby the resolve overlapping substrate spectra of some enzymes and verify the results of the study of Peters et al. (2013b).

Materials and methods

Bacterial strains and culture conditions

All strains used in this study are listed in Table 1 and all plasmids in Table 2. *Escherichia coli* strains were cultivated in LB medium at 37 °C and 180 rpm on a rotary shaker (Sambrook et al. 1989). For selection of recombinant *E. coli* strains, 50 μg/mL kanamycin was added to the medium. *Gluconobacter oxydans* DSM 2343 (621H) and its derivatives were cultivated in a complex medium containing 5 g/L yeast extract, 3 g/L peptone, and 50 mM of a carbon source (mannitol, sorbitol, fructose, or glucose). The pH was adjusted to pH 6.0 using hydrochloric acid. Additionally, 10 μM thymidine was added to the complex medium for cultivation of Δ*upp* strains (Peters et al. 2013a). *G. oxydans* strains were grown at 30 °C and 180 rpm in test-tubes or 250 mL baffled Erlenmeyer flasks. For selection of recombinant *G. oxydans* strains, 50 μg/mL kanamycin and when required 60 μg/mL cefoxitin were used. For monitoring growth, precultures were grown overnight in fructose medium with kanamycin. The main culture was done in a volume of 25 mL in 250 mL baffle shaking flasks, at 30 °C and 180 rpm.

General molecular biological techniques

Molecular biological techniques were carried out according to standard procedures (Sambrook et al. 1989). *G. oxydans* strains were transformed via electroporation or conjugation as described by Kostner et al. (2013). Enzymes for molecular biology were purchased from Fermentas (Waltham, MA), Finnzyme (Vantaa, Finland), and New England Biolabs (Ipswich, MA). Kits were obtained from Promega (Madison, WI), Axygen (Union City, CA), and Epicenter (Madison, WI). They were used according to the instructions of the manufacturers. Oligonucleotides were designed with CloneManager 9 software (Sci-Ed Softwatre, Cary, NC) and synthesized by Eurofins MWG GmbH (Ebersberg, Germany). Sequencing was also done by Eurofins MWG GmbH.

Determining transcription start points of mDH genes

Total RNA of *G. oxydans* 621H was prepared from 50-mL late exponential culture grown on complex medium with mannitol. The RNeasy Midi kit from Qiagen (Hilden, Germany) was used according to the instructions of the manufacturer with some modifications: cells were resuspended in 200 μL TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5), frozen with liquid nitrogen, and mechanically disrupted in a ball mill; the frozen powder was resuspended in 4 mL RLT buffer (provided) followed by DNase treatment, phenol/chloroform extraction, and precipitation with 0.3 M sodium acetate and 70% (*v*/*v*) ethanol. The isolated RNA was used to determine the transcription start points of mDHs by the RNA ligase-mediated rapid amplification of complementary DNA (cDNA) ends (RLM RACE) in accordance to the manual of the FirstChoice RLM-RACE kit (Ambion). The generated cDNA was then used in a PCR with *Taq* DNA polymerase. The primers used are listed in Supplementary Table S1. The PCR products were purified and directly sequenced without cloning, and the sequence data was analyzed with CloneManager 9 software to identify the transcription starting point. WebLogo alignment (http://weblogo.berkeley.edu) was

Table 1 Strains used in this study

Strain	Properties	Source
Escherichia coli		
E. coli TOP10	*F*−, *mcrA* Δ(mrr-hsdRMS-mcrBC), φ80*lacZ*ΔM15, Δ*lacX*74, *nupG* ,*recA1, ara*D139 Δ(ara-leu)7697, *galE*15, *galK*16, *rpsL*(Str^R), *end*A1 λ$^-$	Invitrogen, CA
E. coli TOP10 pMM3b	Expression of β-galactosidase under control of the promoter P_{adh}	This work
E. coli TOP10 pMM3	Expression of β-galactosidase under control of the promoter P_{idh}	This work
E. coli TOP10 pJV17-*lacZ*	Expression of β-galactosidase under control of the promoter P_{tufB}	This work
E. coli TOP10 pBBR1MCS2-*lacZ*	Expression of β-galactosidase under control of the promoter P_{lac}	This work
E. coli TOP10 pBBR1MCS2	Empty control broad host vector	This work
E. coli HB101	*F*−, *hsdS*20 (r-B, m-B), *supE*44, *ara*-14, *galK*-2, *lacY*1, *proA*2, *rpsL*20, *xyl*-5, *mtl*-1, *recA*13, *Km^R*, *oriColE*1, *RK2-Mob*+, *RK2-Tra*+, and *mH*-1 with plasmid pRK2013	Boyer and Roulland-Dussoix (1969)
Gluconobacter oxydans		
G. oxydans 621H (DSM 2343)	Wild type, Cef^R	DSM2343
G. oxydans DSM 3504	Wild type, Cef^R	DSM3504
G. oxydans 621H Δ*upp*	*upp*-deletion strain, Cef^R and FU^R	Peters et al. (2013a)
G. oxydans 621H Δ*upp*Δ2567–9	*hsd*R-deletion strain, Cef^R and FU^R	Kostner (unpublished data)
G. oxydans BP.9	mDH multi-deletion strain based on *G. oxydans* 621H Δ*upp*, Cef^R and FU^R	Peters et al. (2013b)
G. oxydans BP.9 pMM4a-mADH	Complementation of the membrane-bound alcohol dehydrogenase (GOX1067–8)	This work
G. oxydans BP.9 pMM4a-mSldAB	Complementation of the membrane-bound polyol dehydrogenase (GOX0854–5)	This work
G. oxydans BP.9 pMM4b-mSldAB	Complementation of the membrane-bound polyol dehydrogenase (GOX0854–5)	This work
G. oxydans BP.9 pMM4a-mGDH	Complementation of the membrane-bound glucose dehydrogenase (GOX0265)	This work
G. oxydans BP.9 pMM4a-mPQQ3	Complementation of the membrane-bound PQQ-dependent dehydrogenase 3 (GOX1441)	This work
G. oxydans BP.9 pMM4a-mPQQ4	Complementation of the membrane-bound PQQ-dependent dehydrogenase 4 (GOX0516)	This work
G. oxydans BP.9 pMM4a-mSDH3504	Complementation of the membrane-bound sorbitol dehydrogenase (GOX2094–7) with homologous genes RGLU02119–21 from *G. oxydans* DSM 3504	This work
G. oxydans BP.9 pMM4a-mAcDH	Complementation of the membrane-bound aldehyde dehydrogenase (GOX0585–7)	This work
G. oxydans BP.9 pMM4a-mGlDH	Complementation of the membrane-bound gluconate-2 dehydrogenase (GOX1230–2)	This work
G. oxydans BP.9 pMM4a-mIDH	Complementation of the membrane-bound inositol dehydrogenase (GOX1857)	This work

used to identify putative −10 and −35 regions and the Shine-Dalgarno sequences.

Reporter fusions and β-galactosidase assay

Reporter gene fusions were used to measure the promoter strength in *E. coli* TOP10 and *G. oxydans* 621H Δ2567–9. For their construction, two putative promoter regions of the alcohol dehydrogenase (GOX1067–1068) and the quinoprotein inositol dehydrogenase (GOX1857) were amplified from genomic DNA of *G. oxydans* 621H using primers P3_P_{adh}_LacZ_Fus, P4_P_{adh}_MunI, P3_P_{idh}_LacZ_Fus, and P4_P_{idh}_MunI adding a 5′ *Mun*I restriction site and a 20-bp sequence overhang complementary to the *lacZ* gene. The *lacZ* reporter gene was amplified from genomic DNA of *E. coli* K12 using primers P1_LacZ_HindIII, P2_LacZ_P_{adh}_Fus, and P2_LacZ_P_{idh}_Fus (Table S1) adding a 5′ *Hin*dIII restriction enzyme site and 20 bp sequence overhang complementary to the promoter sequence. The final reporter gene construct was then made by fusion PCR according to the modified long flanking homology (LFH) PCR as described by Wach (1996). The promoter-reporter fusion products were then ligated into a *Mun*I/*Hin*dIII-digested vector pJV17 (unpublished), which is based on pEXGOX-K (Schleyer et al. 2008) in order to obtain the vectors pMM3 (P_{idh}-*lacZ*) and

Table 2 Plasmids used in this study

Plasmid	Description	Source
pJV17	pEXGOX-K derivative expressing *sldAB*; *mob, rep, Km*^{R}, P_{tufB}	Voss (unpublished data) and Schleyer et al. (2008)
pBBR1MCS2	*mob, rep, Km*^{R}, broad host vector	Kovach et al. (1995)
pJV17-*lacZ*	pJV17 derivative containing the promoter P_{tufB}; expressing *lacZ*	This work
pBBR1MCS2-*lacZ*	pBBR1MCS2 derivative expressing *lacZ*	This work
pMM2	pJV17 derivative; *mob, rep, Km*^{R}; empty control plasmid	This work
pMM3	pJV17 derivative containing the promoter P_{idh}; expressing *lacZ*	This work
pMM3b	pJV17 derivative containing the promoter P_{adh}; expressing *lacZ*	This work
pMM4a-mADH	pMM3b derivative expressing membrane-bound alcohol dehydrogenase (GOX1067–8)	This work
pMM4a-mIDH	pMM3b derivative expressing membrane-bound inositol dehydrogenase (GOX1857)	This work
pMM4a-mAcDH	pMM3b derivative expressing membrane-bound aldehyde dehydrogenase (GOX0585–7)	This work
pMM4a-mSDH3504	pMM3b derivative expressing membrane-bound sorbitol dehydrogenase (RGLU02119–21) from *G. oxydans* DSM3504	This work
pMM4a-mGlDH	pMM3b derivative expressing membrane-bound gluconate-2 dehydrogenase (GOX1230–2)	This work
pMM4a-mGDH	pMM3b derivative expressing membrane-bound glucose dehydrogenase (GOX0265)	This work
pMM4a-mPQQ3	pMM3b derivative expressing membrane-bound PQQ-dependent dehydrogenase 3 (GOX1441)	This work
pMM4a-mPQQ4	pMM3b derivative expressing membrane-bound PQQ-containing dehydrogenase 4 (GOX0516)	This work
pMM4a-mSldAB	pMM3b derivative expressing membrane-bound polyol dehydrogenase (GOX0854–5)	This work
pMM4b-mSldAB	pMM3 derivative expressing membrane-bound polyol dehydrogenase (GOX0854–5)	This work

pMM3b (P_{adh}-*lac*Z). Screening for positive *E. coli* clones was done by colony PCR using primers pMM3_check2.for, pMM3b_check1.for, and pMM3_uni2.rev (Table S1). Plasmids pMM3 and pMM3b were verified by sequencing and used to transform *G. oxydans* 621H Δ*upp* Δ2567–9 (Kostner, unpublished data) via electroporation. The β-galactosidase activity assay was performed in accordance to Miller (1972), with the modification that the cells were pelleted and resuspended in the assay buffer. *E. coli* and *G. oxydans* cultures were inoculated to an OD_{600} of 0.1 and incubated in 100 mL baffled shaking flasks (10 mL culture volume) at 37 °C (*E. coli*) or 30 °C (*G. oxydans*). The *E. coli* cells were harvested at an OD_{600} of 0.7 to 0.9 and the *G. oxydans* cells at an OD_{600} of 0.8 to 1.2. They were then washed and resuspended in the assay buffer and lysed by mixing with chloroform and SDS. The reaction was incubated at 28 °C and started by addition of colorless *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG; 4 mg/mL), which is hydrolyzed by β-galactosidase to yellow *ortho*-nitrophenol, monitored photometrically at 420 nm.

Construction of expression vectors

For the construction of the pMM4a-derived expression vectors for the various mDHs, the enzyme-free cloning method according to Tillett and Neilan (1999) was used. The specific primers P_{adh}uniV1.for/rev and P_{adh}uniV2.for/rev (Table S1) were designed to amplify the backbone of pMM4a (Table 2) in two PCRs amplifying the vector-backbone and a complementary smaller product, lacking 16–20 bp upstream and downstream of the respective genes. Corresponding to vector amplification, in two PCRs, the respective membrane-bound dehydrogenase was amplified including an insert with a 16–20-bp upstream and downstream overhang complementary to the vector product. Equimolar amounts of all four PCR products were included in a PCR reaction according to the method described by Tillett and Neilan (1999) in 20 μL hybridization buffer (10 mM Tris-HCl, pH 8.5, 1 mM EDTA, 100 mM NaCl) in 4 cycles. Annealing was done at 65 °C for 2 min and extension at 55 °C for 15 min after denaturation at 95 °C for 3 min. After transformation in *E. coli* TOP10, positive clones were identified by colony PCR and checked by sequencing. Plasmids were transferred to *G. oxydans* BP.9 by conjugation, using tri-parental mating as described by Kostner et al. (2013).

Whole cell DCPIP assay

Activity of mDHs towards many substrates was measured using a high-throughput, whole cell DCPIP assay as described

by Peters et al. (2013b) after incubating the strains for 48 h in 5 mL fructose medium with kanamycin.

Results

Promoters of membrane-bound dehydrogenases

Expression of fully functional membrane-bound enzyme complexes is a delicate undertaking. In order to develop a new vector for the expression of mDHs in *G. oxydans* strains, at first promoter, regions of six mDHs from *G. oxydans* 621H were characterized. The transcription starting points of alcohol dehydrogenase (GOX1067–8), inositol dehydrogenase (GOX1857), polyol dehydrogenase (GOX0854–5), gluconate-2 dehydrogenase (GOX1230–2), D-lactate dehydrogenase (GOX1253), and sorbitol dehydrogenase (GOX2094–7) were identified by 5′ RLM RACE. An overview of the relevant genes with the old and new locus tag at NCBI is given in Table S2. Putative −10 and −35 regions and ribosome-binding sites were determined via WebLogo multiple sequence alignment. All transcription starting points as well as deduced ribosomal binding sites and corresponding −10 and −35 regions are shown in Fig. 1.

Construction of reporter fusions

In order to determine the strength and regulation of native membrane-bound dehydrogenase promoters, the *lacZ* gene was selected as a reporter gene to investigate two mDH promoters, the P_{adh} promoter of the alcohol dehydrogenase and the P_{idh} promoter of the inositol dehydrogenase. The backbone of the vector pJV17 (Fig. 2) was chosen to construct the vectors pMM3 and pMM3b as described in "Materials and methods" for the expression of *lacZ* under control of P_{adh} and P_{idh}, because it has a kanamycin resistance cassette, which can be used as selection marker in *G. oxydans* and *E. coli*.

A map of pMM3b is shown in Fig. 2. Expression of membrane-bound enzymes such as the mDHs is often toxic to the cloning host. Therefore, it is desirable to have a low expression in *E. coli*. To check for this, *E. coli* TOP10 were transformed with the plasmids pMM3 and pMM3b and plasmids pJV17-*lacZ* and pBBR1MCS2-*lacZ* for positive control as well as pBBR1MCS2 as a negative control. The strength of the promoters P_{adh} and P_{idh} were measured in the expression host *G. oxydans* after transformation of the plasmids pMM3 and pMM3b in *G. oxydans* 621H Δupp Δ2567–9 by electroporation.

Measurement of promoter strength

The relative promoter strengths were quantified as β-galactosidase activity in Miller Units (MU). The promoter strength in *E. coli* TOP10 cells of the promoter-reporter fusion P_{idh}-*lacZ* and the promoter-reporter fusion P_{adh}-*lacZ* were very low with 2.93 ± 0.55 and 0.36 ± 0.02 MU, respectively. The plasmid control pBBR1MCS2 (empty plasmid without *lacZ* gene) and promoter P_{tufB} fused to *lacZ* (pJV17-*lacZ*) showed no activity (0.0 MU) when measured in *E. coli*. The positive control plasmid pBBR1MCS2-*lacZ* showed a high activity in *E. coli* with 871.64 ± 31.62 MU (Fig. 3b).

In contrast, the strengths of the promoters P_{adh} and P_{idh} are quite high in *G. oxydans* 621H. The β-galactosidase assays of cells from exponential growth, grown on different carbon sources (mannitol, sorbitol and glucose) for the P_{adh} promoter

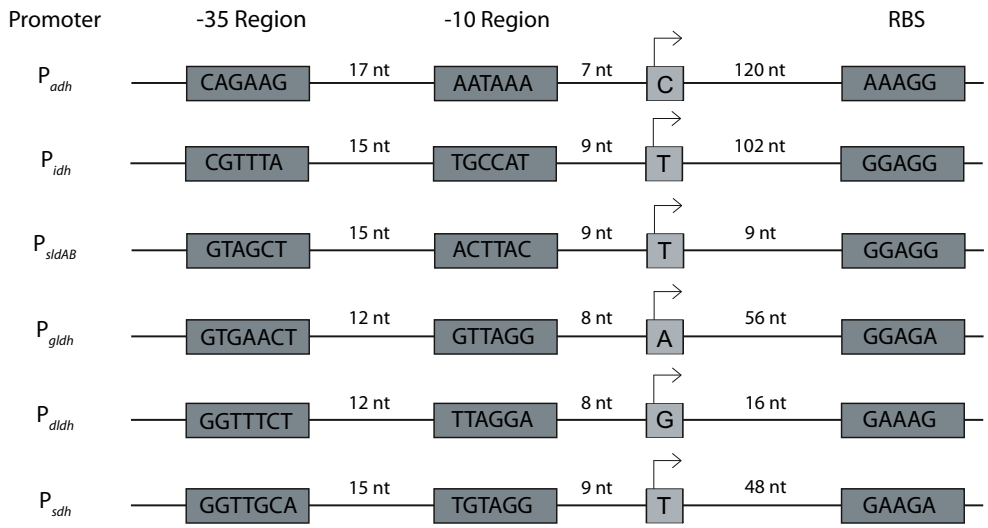

Fig. 1 Promoter regions of membrane-bound dehydrogenases. P_{adh} promoter of alcohol dehydrogenase (GOX1067–8), P_{idh} promoter of inositol dehydrogenase (GOX1857), P_{sldAB} promoter of polyol dehydrogenase (GOX0854–5), P_{gldh} promoter of gluconate-2 dehydrogenase (GOX1230–2), P_{dldh} promoter of D-lactate dehydrogenase (GOX1253), and P_{sdh} promoter of sorbitol dehydrogenase (GOX2094–7). The putative −10 and −35 regions and the putative ribosomal binding site (*RBS*) as predicted by WebLogo alignment are *shaded with gray boxes*. The *arrow* marks the transcription start site. Distances are given in nucleotides (*nt*)

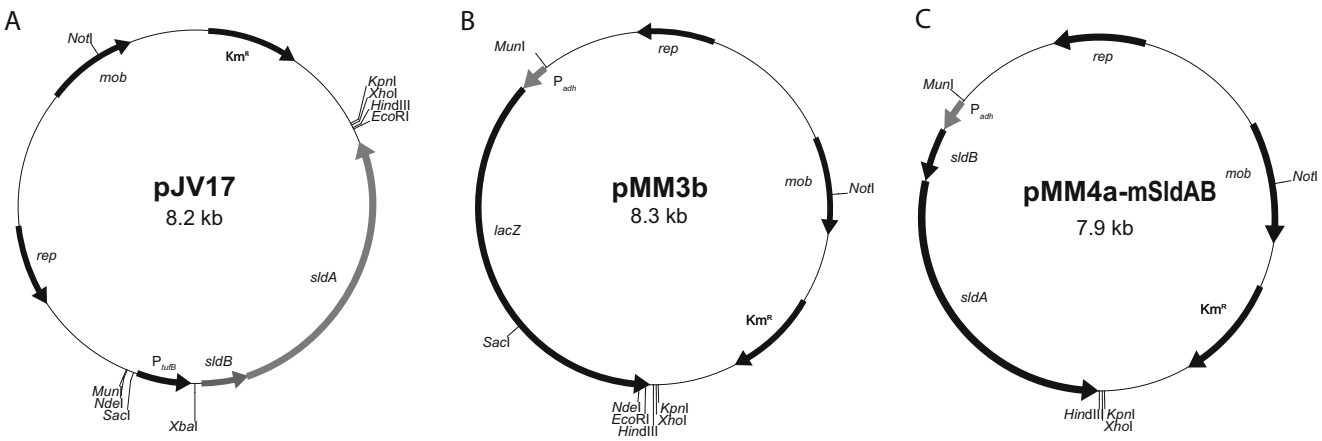

Fig. 2 Vector maps and construction of reporter and expression vectors. **a** Vector map of parental vector pJV17 (pEXGOX-K derivative). **b** Vector map of pMM3b promoter-reporter fusion constructed by inserting the fusion construct of promoter P_{adh} and the *lacZ* gene into the *Mun*I/*Hin*dIII digested pJV17. **c** Vector map of expression vector pMM4a-mSldAB; the promoter P_{adh} and the *sldAB* cluster were combined via fusion PCR and ligated into *Mun*I/*Hin*dIII-digested pJV17. Abbreviations: *rep* origin of replication, Km^R kanamycin resistance determinant, *mob* mobilization protein gene, *lacZ* β-galactosidase gene, P_{tufB} promoter of elongation factor Tu, P_{adh} promoter of the membrane-bound alcohol dehydrogenase (GOX1067–8), *sldA* and *sldB* membrane-bound polyol dehydrogenase (GOX0854–5) genes

in *G. oxydans* 621H Δ*upp* Δ2567–9 (Fig. 3a) were 856.18 ± 131.33 MU with mannitol, 1034.26 ± 148.78 MU with sorbitol and 880.19 ± 1.15 MU with glucose as carbon source. The same assays were performed with the promoter P_{idh}. The results were 35.6 ± 2.05 MU with mannitol and 458.67 ± 5.36 MU with sorbitol but only 4.54 ± 0.11 with glucose (Fig. 3a) as carbon source. Figure S1 gives the activities of pJV17-*lacZ* and pBBR1MCS2-*lacZ* in *G. oxydans* for comparison. The data in Fig. 3a shows that the P_{idh} promoter is repressed in *G. oxydans* by the presence of glucose in contrast to the P_{adh} promoter, which is constitutive under the conditions tested. Both, the promoters are practically not active in *E. coli,* while they showed good expression in *G. oxydans*.

Complementation of the polyol dehydrogenase

The applicability of expression vectors using the promoters P_{adh} and P_{idh} for the expression of functional mDHs was tested by complementation of a *G. oxydans* mutant lacking the polyol dehydrogenase. Such a strain is not able to grow on mannitol or sorbitol due to the missing *sldA* and *sldB* genes, coding for the polyol dehydrogenase (GOX0854–5) (Voss et al. 2010). As a polyol dehydrogenase mutant, the multi-deletion strain *G. oxydans* BP.9 constructed by Peters et al. (2013b), lacking the polyol dehydrogenase among the other mDHs, was used. The complementation plasmids pMM4a-mSldAB and pMM4b-mSldAB were constructed via fusion PCR as described above for the promoter-reporter gene constructs and were ligated into the *Mun*I/*Hin*dIII-cleaved vector pJV17. All plasmids used in this study are listed in Table 2.

Complementation was analyzed in growth experiments in complex medium containing 50 mM mannitol or 50 mM sorbitol as carbon source, supplemented with thymidine but without kanamycin. The kanamycin is not needed because a polyol dehydrogenase is required for growth on mannitol or sorbitol, thereby selecting for the plasmid. *G. oxydans* BP.9 and *G. oxydans* 621H wild type were included as controls. Growth curves of these strains are shown in Fig. 4. The complemented strains *G. oxydans* BP.9 pMM4a-mSldAB and *G. oxydans* BP.9 pMM4b-mSldAB as well as the wild type were able to grow on mannitol (Fig. 4a) and sorbitol (Fig. 4b), while *G. oxydans* BP.9 without any of these plasmids showed no growth. The strain *G. oxydans* BP.9 pMM4a-mSldAB reaching a final OD_{600} of 1.91 grew even better on mannitol (Fig. 4a) than the wild-type strain reaching a final OD_{600} of 1.56 or *G. oxydans* BP.9 pMM4b-mSldAB with a final OD_{600} of 1.22. On sorbitol (Fig. 4b), the growth of both complemented strains and the wild type was nearly equal. After 25 h of growth, the strain *G. oxydans* BP.9 pMM4a-mSldAB reached a final OD_{600} of 1.18. At the same point of time, the strain *G. oxydans* BP.9 pMM4b-mSldAB reached an OD_{600} of 1.19 while the wild-type strain reached an OD_{600} of 1.16. Again, no growth of the multi-deletion strain *G. oxydans* BP.9 on sorbitol was observed.

Characterization of expressed membrane-bound dehydrogenases

Based on the previous results, it became possible to functionally express mDHs. This also opened the possibility to characterize enzymes with overlapping substrate specificities, by expressing them individually in a *G. oxydans* strain lacking all mDHs. The vector pMM4a was used to construct complementation plasmids for all deleted mDHs by expressing them from the P_{adh} promoter. The strain *G. oxydans* BP.9, which has all of its mDHs deleted (Peters et al. 2013b), was used as expression host.

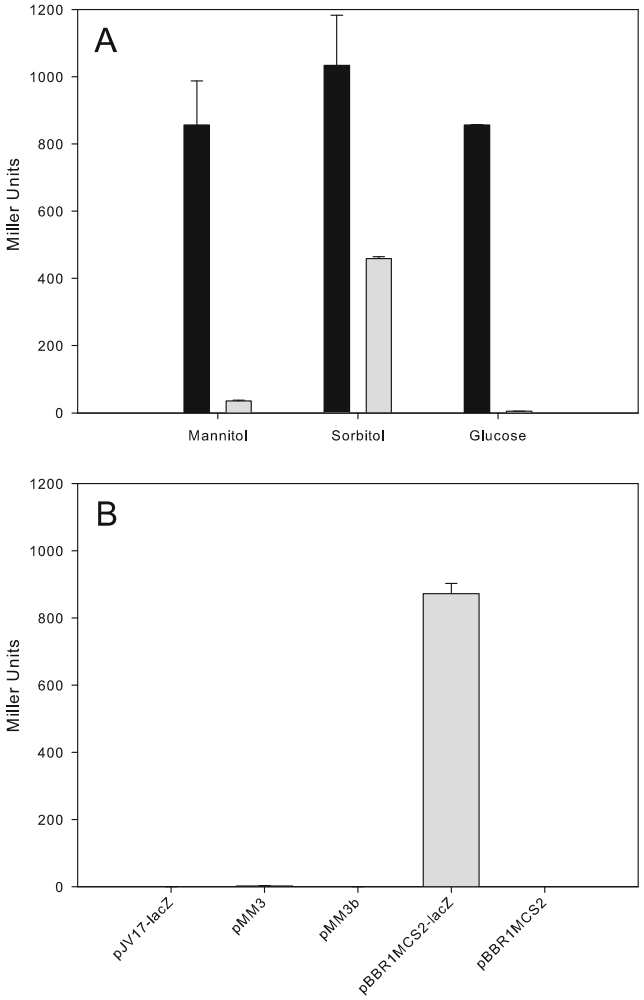

Fig. 3 Promoter activity in *Gluconobacter oxydans* 621H Δ*upp* Δ2567–9 and *Escherichia coli* TOP10. **a** β-Galactosidase activity in Miller units with *lacZ* under control of promoter P_{adh} (*black bars*) and promoter P_{idh} (*gray bars*) in *G. oxydans* 621H Δ*upp* Δ2567–9 grown on mannitol, sorbitol, and glucose. **b** β-galactosidase activity in Miller units with *lacZ* under control of promoter P_{tufB} (pJV17-*lacZ*), promoter P_{idh} (pMM3), promoter P_{adh} (pMM3b), promoter P_{lac} (pBBR1MCS2-*lacZ*), and control vector pBBR1MCS2 (*empty*) in *E. coli* TOP10 grown on LB medium

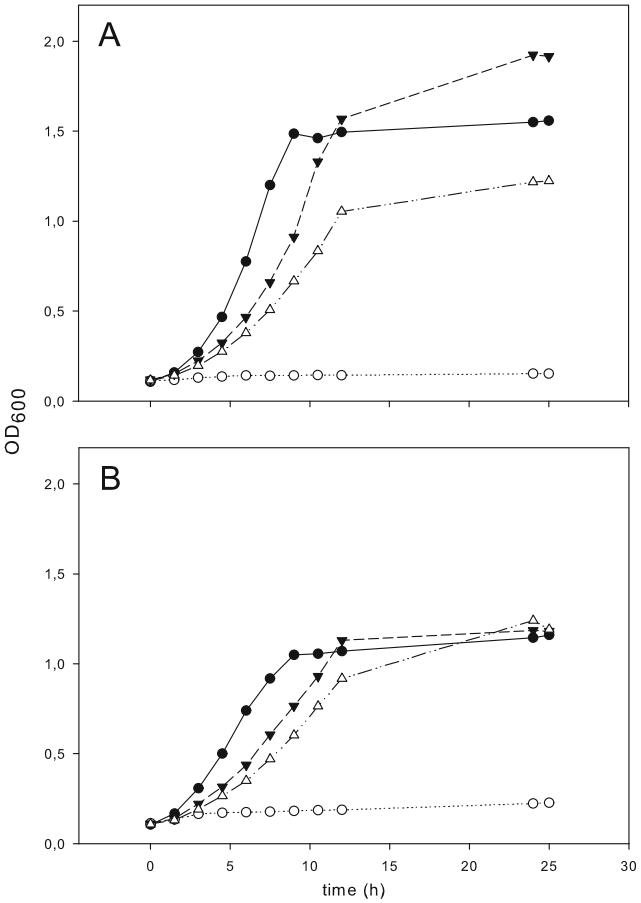

Fig. 4 Comparative growth experiments. *Gluconobacter oxydans* 621H wild type (*filled circles*), *G. oxydans* BP.9 (*empty circles*), *G. oxydans* BP.9 pMM4a-mSldAB (*filled triangles*), and *G. oxydans* BP.9 pMM4b-mSldAB (*empty triangles*) on **a** mannitol and **b** sorbitol

The following complementation plasmids were created for the expression of mDHs under control of the promoter P_{adh}: pMM4a-mSldAB as described above, pMM4a-mADH for the alcohol dehydrogenase (GOX1067–8), pMM4a-mAcDH for the aldehyde dehydrogenase (GOX0585–7), pMM4a-mPQQ3 for the PQQ-dependent dehydrogenase 3 (GOX1441), pMM4a-mPQQ4 for the PQQ-containing dehydrogenase 4 (GOX0516), pMM4a-mGDH for the glucose dehydrogenase (GOX0265), pMM4a-mGlDH for the gluconate-2-dehydrogenase (GOX1230–2), and pMM4a-mIDH for the inositol dehydrogenase (GOX1857). An overview of the relevant genes with the old and new locus tags at NCBI is given in Table S2. For the sorbitol dehydrogenase (GOX2094–7), no substrate oxidation could be expected, because the coding sequence contains an amber stop codon in one subunit (Peters et al. 2013b; Prust et al. 2005), which presumably leads to a non-functional protein. This assumption was confirmed with the respective complementation plasmid pMM4a-mSdh (GOX2094–97): no substrate oxidation was observed (data not shown). For this reason, the homologous membrane-bound sorbitol dehydrogenase from a different strain, *G. oxydans* DSM3504 (RGLU02119–21), was used for complementation, resulting in pMM4a-mSDH3504. The plasmids were transferred into *G. oxydans* BP.9 by conjugation. As vector controls for the complementation experiments, the plasmid pMM2 was transferred into the strains *G. oxydans* 621H Δ*upp* and *G. oxydans* BP.9. The nine constructed complementation plasmids are listed in Table 2 and the nine complemented strains in Table 1. The determination of the substrate spectrum of the expressed mDHs was performed by the whole cell DCPIP assay as described by Peters et al. (2013b). All strains were cultured for 48 h in 50 mM fructose medium containing thymidine and kanamycin, before being assayed. In the assays, 55 different compounds were assayed for each of the nine mDHs, whether they are oxidized by the respective enzyme. The results are

given in Table 3. The inositol dehydrogenase (GOX1857) only oxidized *myo*-inositol and the gluconate-2-dehydrogenase (GOX1230–2) only gluconate. The remaining dehydrogenases showed a broader substrate spectrum. All tested aldehydes (acetaldehyde, butyraldehyde, valeraldehyde, and formaldehyde) were oxidized by the aldehyde dehydrogenase (GOX0585–7). These substrates were also oxidized by the alcohol dehydrogenase (GOX1067–8) together with glyceraldehyde, D-threose, ethanol, isopropanol, 3-hexanol, 1,3-butandiol, 1,2-pentandiol, 1,2-hexandiol, L-fucose, and cellobiose and further 16 substrates. Altogether, the membrane-bound alcohol dehydrogenase oxidized 30 of 55 substrates tested (Table 3). The glucose dehydrogenase (GOX0265) oxidized D-xylose, L-arabinose, D-glucose, D-galactose, D-allose, D-altrose, D-mannose, turanose, and like the alcohol dehydrogenase L-fucose and cellobiose. The second most substrates were oxidized by the polyol dehydrogenase (GOX0854–5). The enzyme exhibited activity for 25 of the 55 substrates tested, e.g., glycerol, *meso*-erythritol, D-arabitol, D-mannitol, D-sorbitol, ribitol, D-ribose, 2,3-butanediol, 2,4-pentandiol, 2-hexanol, and gluconate. The substrates glyceraldehyde, D-threose, D-erythrose, isopropanol, 3-hexanol, 1,3-butandiol, 1,2-pentandiol 1,2-hexandiol, and L-erythrulose were oxidized by the polyol dehydrogenase and the alcohol dehydrogenase. The membrane-bound sorbitol dehydrogenase from *G. oxydans* DSM3504 was successfully expressed in the strain *G. oxydans* BP.9. However, this dehydrogenase has no broad substrate spectrum and oxidizes only mannitol as well as sorbitol. From the substrates tested, no substrate could be identified for the PQQ-dependent dehydrogenase 3 (GOX1441) and the PQQ-containing dehydrogenase 4 (GOX0516).

Discussion

Currently, very little is known about the structure of promoters of mDHs in acetic acid bacteria. Therefore, the regulation and transcription start point of different mDHs of *G. oxydans* 621H was investigated. Transcription start points of six different mDHs (Fig. 1) were determined together with their putative −10 and −35 regions. Up to now, several vector systems for gene expression in acetic acid bacteria and especially in *Gluconobacter* strains were developed and reported. They often use promoters from *E. coli* like the *tufB* and *tac*, the phage lambda PL promoter (Saito et al. 1997), or the well-known *lac* promoter (Tonouchi et al. 2003). Additionally, promoters from *G. oxydans* were used, like the *E. coli* homologous *tufB* promoter (Schleyer et al. 2008; Zhang et al. 2010) or the promoter of the soluble glucose dehydrogenase (*gdh*) (GOX2015) (Merfort et al. 2006a) as well as the putative promoter region of the *adhAB* genes of *G. oxydans* 621H (Kawai et al. 2013). All vectors containing the *tufB* promoter from *G. oxydans* or the *gdh* promoter were reported to be suitable for protein expression in *G. oxydans*. However, both promoters are not well studied for expression of membrane-located enzyme systems. In the study of Kallnik et al. (2010), promoters of ribosomal genes (GOX0264 and GOX0452) were used in expression vectors in *G. oxydans*. In contrast to the other studies, Kallnik et al. (2010) quantified the promoter strength by measurement of β-D-glucuronidase activity in *G. oxydans* and also showed that the vectors pBBR1p264 and pBBR1p452 with ribosomal promoters are suitable for protein production. However, functional expression of membrane-bound enzymes can give rise to special problems, so we suppose that the use of native promoters of membrane-bound proteins is essential for successful and efficient heterologous expression of mDHs in the host strain. This required at first a characterization of these promoters, so six different promoter regions of mDHs, including their transcription starting points and putative −10 and −35 regions were characterized (Fig. 1). The strength and regulation of two of those promoters, the promoter P_{adh} and the promoter P_{idh} were quantified using *lacZ* fusions as a reporter. β-Galactosidase assays revealed that those native promoters of *G. oxydans* were practically silent in *E. coli* (Fig. 3), which means that *E. coli* can be used as an unproblematic cloning host for the construction of vectors used for the subsequent expression of mDHs in *G. oxydans*. Expression of membrane proteins in *E. coli* is reported to be often problematic (Miroux and Walker 1996; Saida 2007), hindering the cloning of the mDHs.

The results of the β-galactosidase activity assays of the P_{adh} promoter in *G. oxydans* 621H Δ*upp* Δ2567–9 (Fig. 3) confirmed prior expression analysis in our laboratory that this is a constitutive promoter. Transcription from the promoter P_{adh} was highly similar during growth on mannitol, sorbitol, and glucose. However, there is also the case of a regulated P_{adh} promoter in *Acetobacter pasteurianus* NCI1380. The activity of the membrane-bound alcohol dehydrogenase was increased up to 10-fold by the addition of ethanol to the medium (Takemura et al. 1993). In our studies, a regulation of P_{adh} in *G. oxydans* was not observed. Also, the results of the assays with the P_{idh} promoter in *G. oxydans* 621H Δ*upp* Δ2567–9 confirmed previous observations (Ehrenreich and Hoffmeister, unpublished data) that the activity of this promoter seems to be regulated by different carbon sources. The highest activity of the P_{idh} promoter was observed on sorbitol, a lower activity on mannitol, while on glucose this promoter is nearly inactive. To prove applicability in expression vectors for mDHs, both promoters were used for the expression of the membrane-bound polyol dehydrogenase. The expression vectors pMM4a-mSldAB and pMM4b-mSldAB (Table 2) were constructed and transferred into the multi-deletion strain *G. oxydans* BP.9 that is devoid of its mDHs, for complementation of the deleted polyol dehydrogenase in this strain. The growth experiments revealed that the complemented strain was able to grow on mannitol (Fig. 4a) and sorbitol (Fig. 4b) irrespective of which the two promoters drove the expression of the polyol dehydrogenase. When compared with each other, the strain *G. oxydans*

Table 3 Oxidation activities of mDHs of *G. oxydans* 621H in complemented multi-deletion strain BP.9 as determined by the whole cell DCPIP assay

Substrate	Enzyme									Peters et al. (2013b)
	mADH	mSldAB	mGDH	mAcDH	mGlDH	mIDH	mPQQ3DH	mPQQ4DH	mSDH3504	
Alcohols (pri.)										
Methanol	+	−	−	−	−	−	−	−	−	mADH
Ethanol	+++	−	−	−	−	−	−	−	−	mADH
1-Hexanol	+++	−	−	−	−	−	−	−	−	mADH
1,3-Propanediol	+++	−	−	−	−	−	−	−	−	mADH
1,4-Butanediol	+++	−	−	−	−	−	−	−	−	mADH
2-Cyclohexylethanol	+++	−	−	−	−	−	−	−	−	mADH
Alcohols (sec.)										
Isopropanol	+++	++	−	−	−	−	−	−	−	mSldAB
2-Hexanol	−	+	−	−	−	−	−	−	−	mSldAB
3-Hexanol	++	+++	−	−	−	−	−	−	−	mSldAB
2,3-Butanediol	−	+++	−	−	−	−	−	−	−	mSldAB
2,4-Pentanediol	−	++	−	−	−	−	−	−	−	mSldAB
2,5-Hexanediol	−	+	−	−	−	−	−	−	−	mSldAB
Alcohols (mix)										
1,3-Butanediol	+++	+	−	−	−	−	−	−	−	mSldAB
1,2-Pentanediol	++	++	−	−	−	−	−	−	−	mSldAB
1,2-Hexanediol	++	+++	−	−	−	−	−	−	−	mSldAB
Alcohols (cyclic)										
1,2-Cyclopentanediol	−	+++	−	−	−	−	−	−	−	mSldAB
1,3-Cyclopentanediol	−	+	−	−	−	−	−	−	−	mSldAB
1,2-Cyclohexanediol	−	+++	−	−	−	−	−	−	−	mSldAB
Aldehydes										
Formaldehyde	+++	−	−	+	−	−	−	−	−	mAcDH
Acetaldehyde	++	−	−	+++	−	−	−	−	−	mAcDH
Butyraldehyde	++	−	−	+++	−	−	−	−	−	mAcDH
Valeraldehyde	++	−	−	+++	−	−	−	−	−	mAcDH
Polyols										
Glycerol	−	++	−	−	−	−	−	−	−	mSldAB
meso-Erythritol	−	++	−	−	−	−	−	−	−	mSldAB
D-Threitol	++	−	−	−	−	−	−	−	−	mADH
L-Threitol	+	−	−	−	−	−	−	−	−	mADH
D-Arabitol	−	+++	−	−	−	−	−	−	−	mSldAB
D-Mannitol	−	++	−	−	−	−	−	−	+++	mSldAB
D-Sorbitol	−	++	−	−	−	−	−	−	++	mSldAB
Ribitol	−	+	−	−	−	−	−	−	−	mSldAB
Aldoses										
Glyceraldehyde	++	++	−	−	−	−	−	−	−	mSldAB
D-Threose	+	++	−	−	−	−	−	−	−	mSldAB
D-Erythrose	+	+++	−	−	−	−	−	−	−	mSldAB
D-Xylose	−	−	+++	−	−	−	−	−	−	mGDH
D-Ribose	−	+	−	−	−	−	−	−	−	mSldAB
D-Arabinose	+	−	−	−	−	−	−	−	−	mADH
L-Arabinose	−	−	+++	−	−	−	−	−	−	mGDH
D-Lyxose	−	0/+	−	−	−	−	−	−	−	mSldAB
D-Glucose	−	−	+++	−	−	−	−	−	−	mGDH
D-Galactose	−	−	+++	−	−	−	−	−	−	mGDH

Table 3 (continued)

Substrate	Enzyme									Peters et al. (2013b)
	mADH	mSldAB	mGDH	mAcDH	mGlDH	mIDH	mPQQ3DH	mPQQ4DH	mSDH3504	
D-Allose	−	−	+++	−	−	−	−	−	−	mGDH
D-Altrose	+	−	+++	−	−	−	−	−	−	mGDH
D-Mannose	0/+	−	++	−	−	−	−	−	−	mGDH
L-Glucose	+	−	−	−	−	−	−	−	−	mADH
Ketoses										
L-Erythrulose	+	++	−	−	−	−	−	−	−	mSldAB
D-Sedoheptulose	+	−	−	−	−	−	−	−	−	mADH
Disaccharides										
Turanose	−	−	+	−	−	−	−	−	−	mGDH
Trehalose	+	−	−	−	−	−	−	−	−	mADH
Cellobiose	+++	−	+	−	−	−	−	−	−	mGDH
Carboxylic acids										
Gluconate	−	+	−	−	+++	−	−	−	−	mSldAB
L-Lactate	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	?
D-Lactate	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	mDLDH
2-Hydroxybutyric acid	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	?
Miscellaneous										
L-Fucose	++	−	0/+	−	−	−	−	−	−	mSldAB
myo-Inositol	0/+	−	−	−	−	+	−	−	−	mIDH

mADH membrane-bound alcohol dehydrogenase (GOX1067–8), *mAcDH* membrane-bound aldehyde dehydrogenase (GOX0585–7), *mSldAB* membrane-bound polyol dehydrogenase (GOX0854–5), *mGDH* membrane-bound glucose dehydrogenase (GOX0265), *mIDH* membrane-bound inositol dehydrogenase (GOX1857), *mGlDH* membrane-bound gluconate-2 dehydrogenase (GOX1230–2), *mPQQ3* membrane-bound PQQ-dependent dehydrogenase 3 (GOX1441), *mPQQ4* membrane-bound PQQ-dependent dehydrogenase 4 (GOX0516), *mSDH3504* membrane-bound sorbitol dehydrogenase (RGLU02119-21), "+++" strong activity >83.25 μM DCPIP have been reduced after 2 h, "++" medium activity ≤83.25 μM DCPIP have been reduced after 2 h, "+" weak activity ≤50.25 μM DCPIP have been reduced after 2 h, "0/+" very low/minimal activity ≤16.92 μM DCPIP have been reduced after 2 h, "−" no activity, *n.t.* not tested, "?" unknown activity

BP.9 pMM4a-mSldAB grew better on mannitol than the strain *G. oxydans* BP.9 pMM4b-mSldAB, but on sorbitol, the growth of both strains was nearly identical. These growth experiments demonstrated that the expression vectors developed in the present study are suitable for the expression of fully active mDHs.

In order to investigate the substrate spectrum of mDHs, in the study of Peters et al. (2013b), the nine mDHs (Prust et al. 2005) were deleted sequentially, leading to strains lacking between one and nine of the mDHs. The last strain of this series of deletion strains, which was missing all important mDHs, was designated *G. oxydans* BP.9. In the present study, this multi-deletion strain was used as the host for the expression of each of the individual dehydrogenase genes to (i) characterize the substrate spectra of the mDHs individually using the whole cell DCPIP assay, (ii) clarify overlapping substrate spectra, and (iii) to verify the results of Peters et al. (2013b). For this purpose, the developed expression vector pMM4a with the promoter P_{adh} was used to express each deleted dehydrogenase and to complement its activities in the multi-deletion strain. This method allowed characterization of several new activities and overlapping substrate spectra of some enzymes. In addition, the enzyme assays in the present study corroborate the findings of Peters et al. (2013b). The membrane-bound alcohol dehydrogenase and the membrane-bound polyol dehydrogenase, both with very broad substrate spectra, seem to be most important enzymes in the membrane of *G. oxydans* 621H, allowing the oxidation of a broad range of substances for energy generation. From 55 substrates, the alcohol dehydrogenase oxidized 30 substrates and the polyol dehydrogenase 25. For nine substrates, an overlapping activity of the alcohol dehydrogenase and the polyol dehydrogenase was detected (Table 3). In the study of Peters et al. (2013b), this overlap was not detectable, because the alcohol dehydrogenase was the first deleted enzyme and the polyol dehydrogenase the last one. In the present study, the multi-deletion strain was complemented with one dehydrogenase at a time, therefore, either the polyol dehydrogenase or the alcohol dehydrogenase are present. This fact made it possible to observe overlapping substrate oxidation. Other overlapping substrate spectra were observed for the same reason. The alcohol dehydrogenase also oxidized all tested aldehydes as well as the aldehyde dehydrogenase. In the case of formaldehyde oxidation, the alcohol dehydrogenase showed an even stronger activity than the aldehyde dehydrogenase. A

similar property of a membrane-bound alcohol dehydrogenase was already observed in *Acetobacter* sp. SKU 14 (Shinagawa et al. 2006). Another improvement was made for the oxidation of L-fucose. In the study of Peters et al. (2013b), the polyol dehydrogenase was designated to be the enzyme, which oxidizes L-fucose. However, in this study, no such activity of the polyol dehydrogenase was observed. Instead, the L-fucose was oxidized by the alcohol dehydrogenase and the glucose dehydrogenase. An additional example for such an overlapping substrate spectrum is the oxidation of cellobiose. This disaccharide is not only oxidized by the glucose dehydrogenase but also by the alcohol dehydrogenase.

In summary, due to the characterization of promoters of mDHs, we were able to develop new expression vectors for the fully functional expression of mDHs in *G. oxydans*. The use of such a vector with the constitutive P_{adh} promoter for the expression of individual dehydrogenases in the multi-deletion strain *G. oxydans* BP.9 has allowed to characterize the substrate spectrum of every single membrane-bound dehydrogenase in vivo, using a whole cell DCPIP assay, extending our knowledge about their substrate spectra and overlapping activities. A functional expression system for known mDHs was introduced that could also be used for the expression of mDHs from other acetic acid bacteria in *G. oxydans* BP.9 and even mDHs from a metagenome.

Compliance with ethical standards

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Conflict of interest Markus Mientus declares that he has no conflict of interest. David Kostner declares that he has no conflict of interest. Björn Peters declares that he has no conflict of interest. Wolfgang Liebl declares that he has no conflict of interest. Armin Ehrenreich declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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