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Effects of membrane-bound glucose dehydrogenase overproduction on the respiratory chain of *Gluconobacter oxydans*

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Abstract The acetic acid bacterium *Gluconobacter oxydans* incompletely oxidizes carbon sources as a natural part of its metabolism, and this feature has been exploited for many biotechnological applications. The most important enzymes used to harness the biocatalytic oxidative capacity of *G. oxydans* are the pyrroloquinoline quinone (PQQ)-dependent dehydrogenases. The membrane-bound PQQ-dependent glucose dehydrogenase (mGDH), encoded by *gox0265*, was used as model protein for homologous membrane protein production using the previously described *Gluconobacter* expression vector pBBR1p452. The *mgdh* gene had ninefold higher expression in the overproduction strain compared to the parental strain. Furthermore, membranes from the overexpression strain had a five- and threefold increase of mGDH activity and oxygen consumption rates, respectively. Oxygen consumption rate of the membrane fraction could not be increased by the addition of a substrate combination of glucose and ethanol in the overproduction strain, indicating that the terminal quinol oxidases of the respiratory chain were rate limiting. In contrast, addition of glucose and ethanol to membranes of the control strain increased oxygen consumption rates approaching the observed rates with *G. oxydans* overproducing mGDH. The higher glucose oxidation rates of the mGDH overproduction strain corresponded to a 70 % increase of the gluconate production rate compared to the control strain. The high rate of glucose oxidation may be useful in the industrial production of gluconates and ketogluconates, or as whole-cell biosensors. Furthermore, mGDH was purified to homogeneity by one-step strep-tactin affinity chromatography and characterized. To our knowledge, this is the first report of a membrane integral quinoprotein being purified by affinity chromatography and serves as a proof-of-principle for using *G. oxydans* as a host for membrane protein expression and purification.

Keywords Acetic acid bacteria · Glucose dehydrogenase · Membrane protein · Biotransformation · Overexpression · Protein purification · Gluconate

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Introduction

Acetic acid bacteria oxidize their substrates incompletely under "normal" growth conditions. Among them is the α-proteobacterium *Gluconobacter oxydans*, which is a Gram-negative, obligate aerobic rod-shaped acidophilic organism and belongs to the family Acetobacteriaceae (De Ley et al. 1984). The organism incompletely oxidizes a wide range of carbohydrates and alcohols. The products (aldehydes, ketones, and carboxylic acids) are secreted almost entirely into the medium to near quantitative yields. Furthermore, *G. oxydans* strains are able to grow at low pH values and high sugar concentrations and have low biomass production (Olijve and Kok 1979; Sievers and Swings 2005), which correlates with high oxidation rates and makes the strains suitable for industrial application (Deppenmeier et al. 2002). *G. oxydans* possesses membrane-bound pyrroloquinoline quinone (PQQ)-dependent dehydrogenases that are key enzymes for its overall metabolism. These enzymes are located on the outer leaflet of the cytoplasmic membrane so that the active sites of the dehydrogenases are exposed to the periplasm of the cell and they are linked to the respiratory chain. The

proteins are responsible for the rapid incomplete oxidation of biotechnologically important substrates. Hence, these enzymes are essential for the formation of industrially useful products (Deppenmeier and Ehrenreich 2008). The PQQ-containing membrane-bound glucose dehydrogenase (mGDH) is one of these key enzymes and catalyzes the oxidation of glucose to δ-gluconolactone, which is chemically or enzymatically hydrolyzed to gluconate (Matsushita et al. 1994). Electrons are transferred from the active site of the protein, which is located in the periplasm, to the ubiquinone pool in the cytoplasmic membrane. Reduced ubiquinone is then re-oxidized by the cytochrome bo_3 or CIO-type quinol oxidases (Mogi et al. 2009) that generate the electrochemical proton gradient for ATP synthesis.

An abundant pool of uncharacterized PQQ dehydrogenases in the genomic databases exists. Many of these uncharacterized quinoproteins are predicted to have novel functions that could be useful for biotechnological applications. Here we describe an expression system that is promising for the one-step purification and characterization of PQQ-containing dehydrogenases with unknown function. Using the mGDH as a model enzyme, we show that PQQ-dependent dehydrogenases can be overproduced in *G. oxydans* using the expression vector pBBR1-p452 (Kallnik et al. 2010). The homologously produced mGDH was perfectly incorporated into the overall cellular metabolism, leading to accelerated respiratory rates and improved product formation. Consequently, *G. oxydans* strains overproducing PQQ dehydrogenases are ideally suited as alternate whole-cell oxidative biotransformation factory hosts for the production of compounds with biotechnological applications.

Materials and methods

Materials

All chemicals and reagents were obtained from Carl Roth GmbH (Karlsruhe, Germany) or Sigma-Aldrich (Munich, Germany). Oligonucleotides were synthesized by Eurofins MWG Operon (Ebersberg, Germany). Restriction endonucleases, T4 DNA ligase, Taq DNA polymerase, and PCR reagents were purchased from Fermentas (St. Leon-Rot, Germany). Phusion DNA polymerase was from New England Biolabs (Frankfurt am Main, Germany).

Microorganisms and culture conditions

Escherichia coli DH5α (DSM 6897) was grown in lysogeny broth at 37 °C. *G. oxydans* strains (Table 1) were grown in yeast mannitol (YM) medium, pH 6.5, containing 0.6 % yeast extract, 2 % D-mannitol, and 50 μg/mL cefoxitin at 30 °C in 0.5-L baffled Erlenmeyer flasks (100 mL culture volume) on a Minitron shaker (Infors AG, Bottmingen, Switzerland) with a stirring rate of 180 rpm. For the quantification of the gluconate production and glucose consumption rates, cells were grown on YG medium (YM medium but with the addition of glucose (2 %) instead of mannitol) under the same growth conditions as described above (initial pH 5 after inoculation). The enzymes from *G. oxydans* analyzed in this publication (mGDH, PQQ-dependent sorbitol dehydrogenase (mSldDH) and PQQ-dependent alcohol dehydrogenase (mADH)) are constitutively produced, and the growth substrates have no effect on their activities (Hoffmeister 2006; Hanke et al. 2012). Addition of 50 μg/mL kanamycin or 100 μg/mL ampicillin was used for plasmid maintenance in *G. oxydans* and *E. coli*, respectively. All data presented in this publication are the mean of at least three different biological replicates.

Standard molecular biology techniques and cloning

Routine molecular biological techniques were done according to Sambrook et al. (1989). Genomic DNA from *G. oxydans* 621 H was isolated by cetyl trimethylammonium bromide extraction (Ausubel 2002) and used as a template for the amplification of *gox0265* (*mgdh*). Primers gox0265-p3_f/gox0265-p3_r containing extended 5′ *Bsa*I restriction endonuclease sites were used to amplify *mgdh* including 32 bp of the 5′-UTR and cloned into pASK-IBA3plus as previously described (Schweiger et al. 2010). The resulting construct, pASK3-mgdh, was used as a template to amplify the strep-tag gene fusion product using oligonucleotides pASK3EcoRI_R/pASK3ST_rev. The amplicon was subsequently cloned into the *Eco*RI/*Xho*I sites of the expression vector pBBR1p452 to produce the plasmid pBBR1p452-mgdhST. Constructs were transformed into *E. coli* DH5α and transformants were screened for proper insertion with PCR using sequencing primers pBBR1_f/pBBR1_r (Table 1). Positive transformants were sequenced by the StarSEQ GmbH (Mainz, Germany) and transformed into *G. oxydans* Δ*hsdR* by electroporation using a modified version of Mostafa et al. (2002) as previously described (Kallnik et al. 2010). RT-qPCR was performed as described previously (Kallnik et al. 2010).

Preparation of resting cells

Resting cells of *G. oxydans* Δ*hsdR* were prepared from 100 mL YM cultures. Cells were harvested at an $OD_{600\ nm}$ between 0.8 and 1.2 by centrifugation (20 min, 2,500×*g*) and washed twice with 20 mL 40 mM potassium phosphate buffer (KPB), pH 6. The pellets were then resuspended in 10 mL KPB and used as resting cells.

Table 1 Strains, plasmids, and primers used in this work

Strain or plasmid or primer	Description or primer sequence	Source or added site[a]
Strain		
E. coli DH5α	F$^-$, *sup*E44, *Δlac*U169, *ϕ*80, *lacZ*ΔM15, *end*A1, *hsdR*17 (r_K-m_K+), *thi*-1, λ$^-$, *rec*A1, *gyr*A96, *rel*A1	Hanahan (1983), DSM 6897
G. oxydans 621 H	Cef^R	DSM 2342
G. oxydans Δ*hsdR*	Δ*hsdR* derivative of *G. oxydans* 621 H (deletion of *gox2567*)	S. Bringer-Meyer, Research center Jülich GmbH
Plasmid		
pASK-IBA3plus	C-terminal strep-tag, Amp^R	IBA GmbH
pASK3-mgdh	pASK-IBA3plus derivative with *gox0265* containing a C-terminal strep-tag	This study
pBBR1p452	pBBR1MCS-2 derivative containing the 5′-UTR of *gox0452*, Kan^R	Kallnik et al. (2010)
pBBR1p452-mgdhST	pBBR1p452 derivative expressing *gox0265* containing a C-terminal strep-tag	This study
Primer		
gox0265-p3_f	ATGGTAGGTCTCAAATGTGATGCATCAGAACAACAGAT	*Bsa*I
gox0265-p3_r	ATGGTAGGTCTCAGCGCTTTTCTGATCGGGCAGGGCGTAG	*Bsa*I
pASK3EcoR_f	TGCGAATTCTGATGCATCAGAACAACAGA	*Eco*RI
pASK3ST_r	GCTGCTCGAGAGGTCAAGCTTATTA	*Xho*I
pASK_f	GAGTTATTTTACCACTCCCT	
pASK_r	CGCAGTAGCGGTAAACG	
pBBR1_f	ACTCACTATAGGGCGAATTG	
pBBR1_r	CCCAGGCTTTACACTTTATG	
RTgap_f	TCCGACTTCAACCATGACAA	
RTgap_r	GTTGTCGTACCACGAGCAGA	
RTmgdh_f	AGGCATTCGACGTCTACACC	
RTmgdh_r	ACGACACGATCCAGGAGTTC	

[a] Restriction endonuclease sites underlined

Preparation of membrane fractions and purification of mGDH

To prepare membranes from *G. oxydans* Δ*hsdR*, 400 mL YM cultures with an $OD_{600\ nm}$ between 0.8 and 1.2 were harvested by centrifugation (7,000×*g*, 4 °C, 10 min), resuspended in 10 mL 40 mM KPB, pH 7, and lysed by sonication (15 min at 50 % amplitude with cooling; Branson Sonifier Cell Disruptor with a Branson Ultra Sonics converter, Danbury, USA; cooling: Colora Messtechnik GmbH, Lorch/Württ, Germany). The lysate was cleared by centrifugation (1,300×*g*, 4 °C, 10 min), and the membrane fraction was collected by ultracentrifugation for 1 h at 150,000×*g* and 4 °C. The pellet was washed once with 10 mL of 40 mM KPB, pH 7, and resuspended in 1 mL. For the purification of Gox0265 (mGDH), the membrane fraction was solubilized in 10 mL buffer W (100 mM Tris–HCl, 150 mM NaCl, pH 8.0) containing 1 % (*v*/*v*) Triton X-100. Solubilized membranes were fractionated by ultracentrifugation (150,000×*g*, 4 °C, 1 h), and the supernatant containing the detergent-soluble membrane fraction was applied to a gravity flow strep-tactin affinity column (IBA GmbH, Göttingen, Germany). Affinity chromatography was done according to the manufacturer's instructions except that all buffers contained 0.1 % (*v*/*v*) Triton X-100.

Polyacrylamide gel electrophoresis (PAGE) and western blot

SDS-PAGE was done on a 10 % (*w*/*v*) slab gel as described by Laemmli (1970) with a 5 % (*w*/*v*) polyacrylamide stacking gel. Samples were diluted in sample loading buffer (2 % [*w*/*v*] sodium dodecyl sulfate, 5 % [*v*/*v*] mercaptoethanol, 50 % [*v*/*v*] glycerol, 20 % [*v*/*v*] collecting buffer pH 6.8, 0.001 % [*w*/*v*] bromophenol blue) and boiled for 10 min prior to application. Molecular mass was calculated by comparison to a molecular mass standard (Fermentas, St. Leon-Rot, Germany). Proteins were visualized by silver stain (Blum et al. 1987). Western blot was performed by the method of Towbin et al. (1979), and the strep-tagged protein was detected chromogenically with strep-tactin horseradish peroxidase conjugate according to the manufacturer's protocol (IBA GmbH, Göttingen, Germany).

Protein quantification, enzyme assay, and kinetics

The protein content of resting cells was determined by a modified Biuret assay (Schmidt et al. 1963), the protein content of membrane preparations was quantified by the method of Bradford (1976), and the protein content of purified Gox0265 containing detergent was measured using the DC Protein Assay kit (Bio-Rad, München, Germany). Purified mGDH (0.1–0.5 μg protein) and membrane-bound dehydrogenase activity from membrane preparations (5–9 μg μg protein) or resting cells (turbidity at 600 nm was smaller than 0.2 and was correct by baseline adjustment) was assayed with the electron acceptors phenazine methosulfate (PMS) and dichlorphenolindophenol (DCPIP) at pH 6 or 7 and 25 °C. Toluene was added to 10 % (*v*/*v*) when resting cells were analyzed to allow effective transport of DCPIP and PMS over the outer membrane into the periplasm (Park et al. 1994). The reduction of DCPIP was monitored spectrophotometrically at 600 nm with a Jasco V-600 spectrophotometer (Jasco, Gross-Umstadt, Germany). Temperature was controlled with a Jasco ETC-717 Peltier thermostat, and data were processed with the Spectra Manager III software. Activity was calculated using the extinction coefficient of DCPIP [ε_{DCPIP} = 17.2 mM^{-1} cm^{-1} at pH 6, ε_{DCPIP} = 21 mM^{-1} cm^{-1} at pH 7 (Hölscher and Görisch 2006; Armstrong 1964)]. A 1-mL reaction mixture contained 40 mM KPB pH 6 or 7, 0.67 mM PMS, 0.1 mM DCPIP, and 20–60 mM substrate and enzyme. Reactions were initiated by the addition of substrate or enzyme. One unit was defined as the amount of enzyme activity catalyzing the conversion of 1.0 μmol of substrate per minute. Activity of the purified enzyme was measured at various substrate concentrations, and the kinetic parameters were determined using nonlinear regression of the Michaelis–Menten data (GraphPad Prism).

Determination of oxygen consumption

Oxygen consumption rates of the resting cells and membranes were measured with a Rank oxygen electrode connected to a Rank Brothers electrochemical processor 2000 (Rank Brothers LTD, Cambridge, England, UK). Temperature was controlled with a water bath set to 25 °C. The oxygen electrode was calibrated by adding sodium dithionite to oxygen-saturated buffer (maximal negative output voltage) until complete reduction (minimal negative output voltage). The maximal negative output voltage was set equal to the oxygen content of a saturated aqueous solution at 25 °C (8.11 mg/L), and the minimal negative output voltage was set equal to an oxygen content of zero. The assays consisted of 60 mM substrate, membranes, or resting cells and 40 mM KPB, pH 6, added to 3 mL. Reactions were started by the addition of substrate.

Determination of substrate and product concentrations

To determine the gluconate, 2-keto-D-gluconate, and 5-keto-D-gluconate content in the medium, 1 mL of *G. oxydans* cultures grown on YG was centrifuged for 5 min at 8,000×*g* to remove the cells. The supernatant was filtered through a 0.45-μm membrane and applied to HPLC using an Aminex-HPX87H column (BioRad, 300×7.8 mm) at a flow rate of 0.3 mL min^{-1} with 5 mM H_2SO_4 at 65 °C. Gluconates, 2-keto-D-gluconate, and 5-keto-D-gluconate were detected at 210 nm (Miller et al. 1987), and the concentrations were determined by comparison to corresponding calibration lines (1–150 mM for gluconate, 1–50 mM for 2-keto- D-gluconate and 5-keto-D-gluconate). The glucose content in the supernatant was determined using a D-Glucose/ D-Fructose UV-Test kit according to the manufacturer's instructions (R-Biopharm AG, Darmstadt, Germany).

Results

Two expression vectors were recently constructed for protein overproduction in *Gluconobacter* spp. since it is an important organism for the production of several biotechnologically important products (Kallnik et al. 2010). To date, several recombinant cytoplasmic enzymes have been produced successfully in *G. oxydans* using the expression system described above (unpublished results). However, membrane-bound proteins in acetic acid bacteria are much more important for the process of incomplete oxidation and for the application of these organisms in industrial biotransformation. One example is the PQQ-dependent mGDH from *G. oxydans*, which is of particular importance as it is the key enzyme in the oxidative metabolism of D-glucose and catalyzes the first step in the production of ketogluconates via gluconate. Therefore, gene *gox0265* encoding the mGDH was fused to a strep-tag coding region at the 3′ end and was cloned into the broad-host range expression vector pBBR1p452 (Kallnik et al. 2010). The resulting construct, pBBR1p452-mgdhST, was transformed into *G. oxydans* Δ*hsdR* by electroporation and used for homologous gene expression, as well as protein and strain characterization.

Transcript abundance of the *mgdh* gene

To validate the expression of the plasmid-encoded *mgdh* gene in *G. oxydans* Δ*hsdR* containing pBBR1p452-mgdhST, real-time RT-qPCR was performed and the results were compared with the parental strain containing pBBR1p452.

Table 2 Analysis of transcript abundance of the *mgdh* gene

G. oxydans ΔhsdR harboring plasmid	Cp (*gap*)	Cp (*gdh*)	ΔCp (*gdh–gap*)	Ratio (*gdh/gap*)
pBBR1p452-mgdh-ST	21.5±0.3	17.6±0.1	−3.9	14.9
pBBR1p452	20.8±0.1	20.1±0.1	−0.7	1.6

The quantification cycle (Cp) of the glyceraldehyde-3-phospate dehydrogenase gene (*gap*) was used as a reference to determine the *mgdh* expression ratio of the strains. Transcriptional abundance of the *mgdh* gene was ninefold higher in the strain harboring pBBR1p452-mgdhST compared to the empty vector strain (Table 2). These data indicate that *G. oxydans* is expressing *mgdh* in significantly higher amounts in the expression strain and is a suitable system for the overproduction of mGDH as a model for membrane-bound PQQ-dependent dehydrogenases.

Gluconate production and analysis of whole cells

The gluconate content in growing cultures was monitored to analyze if increased *mgdh* gene expression correlated to higher gluconate production rates. Samples were taken at various points during bacterial growth and gluconate concentration was monitored by HPLC. Total cell counts of the overexpression and control strain were equivalent at corresponding optical densities (about 1×10^9 cells/mL= 1 OD_{600}). Furthermore, no difference in size or cell shape was observed by light microscopy, indicating that optical density was suitable for comparison of growth between the control and overexpression strains. Therefore, gluconate production of the cultures was normalized to optical density since the mGDH overproduction strain had a slightly longer doubling time compared to the parental strain (t_d=2.5± 0.2 vs. 2.2±0.2 h). Steady growth rates in the exponential phase indicated that the number of viable cells was high and constant and that mGDH overproduction did not lead to cell death. The final optical densities of the parental strain and the overexpression strain under the given growth conditions were 0.6±0.1, respectively (Fig. 1b). Also, the pH values during growth of the cultures were the same. The pH decreased from 4.8 after inoculation to 3.2 at the beginning of the stationary phase. The gluconate production rate and the glucose consumption rate of the mGDH overproduction strain were approximately 70 % higher compared to the control strain, respectively. (Fig. 1). At an OD_{600} of 0.6, the mGDH overproduction strain had consumed almost all of the available glucose, whereas there was about 40 mM glucose left in the cultures of the control strain. These results were reflected in the total amount of gluconate production with about 90 mM in the culture supernatant of the mGDH overproduction strain in contrast to about 60 mM in the parental strain. The amount of ketogluconates was low for both strains (<1 mM 5-ketogluconate and up to 4 mM of 2-ketogluconate in the control strain and 1 mM in the overproduction strain, respectively; Fig. 1b).

These data indicated that increased *mgdh* gene expression, observed by RT-qPCR, led to increased mGDH production and subsequent improvements in gluconate production rates. Moreover, gluconate production was proportional to the number of cells per milliliter in the exponential growth phase, demonstrating that mGDH activity was not limited by external parameters, such as pO_2, pH, etc., in either culture.

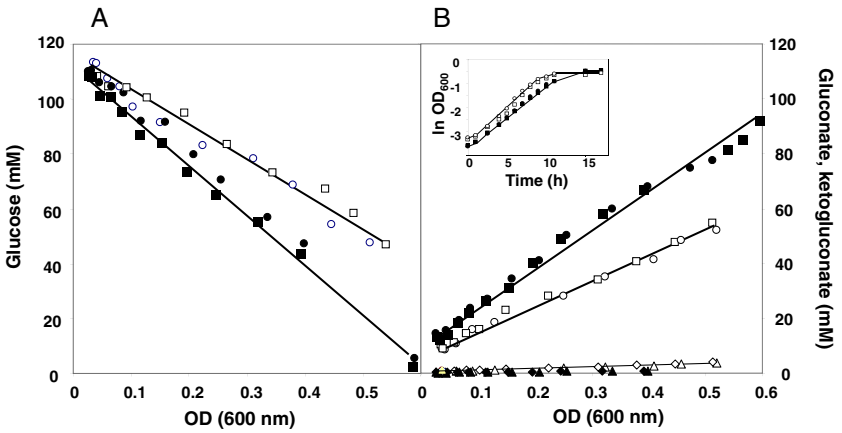

Fig. 1 Effect of mGDH overexpression on gluconate production and glucose consumption in the exponential growth phase in batch culture. **a** Glucose consumption. **b** Gluconate and 2-ketogluconate production. *Open circles* and *squares* represent two independent control strain replicates. *Closed circles* and *squares* represent two independent mGDH overexpression strain replicates. *Triangle* and *diamonds* indicate the 2-ketogluconate concentration (*open symbols*, control strain; *closed symbols*, mGDH overexpression strain). *Inset*: growth curves (*open circles* and *squares* = control strain; *closed circles* and *squares* = mGDH overexpression strain)

Resting cells of *G. oxydans ΔhsdR* harboring the plasmid pBBR1p452-mgdhST and of the control strain containing the empty vector pBBR1p452 were prepared as described in the "Materials and methods." The oxidation of glucose was measured photometrically using the artificial electron acceptors PMS–DCPIP and oxygen consumption was monitored. The specific activity of the *mgdh* overexpressing strain was determined with PMS–DCPIP (3.6±0.2 U/mg) and was 2.2-fold higher in comparison to the control strain (1.6±0.1 U/mg). Similarly, the rate of oxygen consumption (1,440±296 nmol ½ O_2 min^{-1} mg^{-1}) was 1.6-fold higher in comparison to the control strain (923±166 nmol ½ O_2 min^{-1} mg^{-1}), indicating that the increase in gene expression and the observed gluconate production are indeed the result of increased mGDH production and accelerated respiration rates.

Analysis of mGDH membrane activities

Membranes were prepared from the mGDH overexpression strain and from the control strain as described above. Specific activities of the membranes were determined using the electron acceptors PMS and DCPIP at pH 7 using D-glucose or ethanol as substrates. For the mannitol oxidation assay, a buffer with a pH of 5 was used because the oxidizing enzyme (most probably mSldDH) showed no activity at pH 7. The specific activity of the overexpression strain with D-glucose was fivefold higher in comparison to the control, whereas activities with ethanol or D-mannitol were similar for both strains (Fig. 2). These results indicate that mGDH was overproduced in significant amounts and functionally incorporated into the inner membrane. Moreover, it is evident that the production of other major membrane-bound dehydrogenases was not impaired by the overproduction of mGDH.

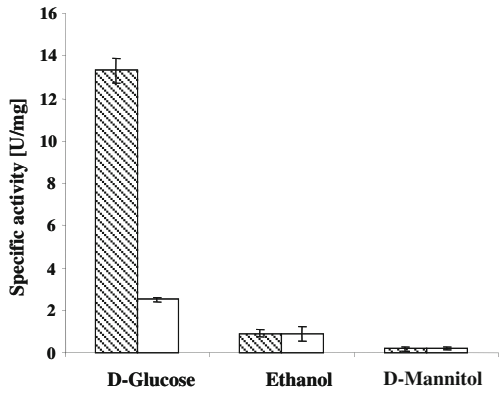

Fig. 2 Comparison of specific membrane activities. Specific activities of membranes (5–9 μg protein) of *G. oxydans ΔhsdR* overexpressing mGDH and the control strain containing the empty vector pBBR1p452 using D-glucose, ethanol, and D-mannitol as substrates. Assays were performed using PMS–DCPIP as electron acceptors at pH 7 (D-glucose, ethanol) or pH 5 (D-mannitol). *Filled bars*, mGDH overexpressing strain; *empty bars*, empty vector control strain. *Error bars*, ± SEM

The oxygen consumption of membranes from the overexpression strain was measured and compared to the control strain to investigate if the respiratory chain was limiting for the oxidation of glucose in the membrane activity assays (Fig. 3). The rate of oxygen consumption was very high for the mGDH overexpression strain (1,075±64 nmol ½ O_2 min^{-1} mg^{-1}) and about threefold faster in comparison to the control strain (Table 3). The oxygen consumption rate of the control strain could be increased about twofold when ethanol was added together with D-glucose as substrates. These data indicate that the wild-type mGDH as well as the mADH channeled electrons into the quinone pool and that the capacity of the end oxidases was still high enough to oxidize the resulting ubiquinol formed from mGDH and mADH. In contrast, the oxidation rate of *G. oxydans* overexpressing mGDH was not increased further by the addition of ethanol (Table 3). This demonstrated that the overexpression of mGDH saturates the electron transport chain with glucose oxidation alone and the rate of oxygen consumption measured represents maximal electron transport capacity.

Purification and characterization of strep-tagged membrane-bound glucose dehydrogenase

Membrane fractions were prepared from 400 mL cultures of *G. oxydans ΔhsdR* harboring pBBR1p452-mgdhST and were solubilized in 1 % (*v*/*v*) Triton X-100 as described in the "Materials and methods." Membrane-bound glucose dehydrogenase was then purified in a single step by streptactin affinity chromatography. Purified mGDH was analyzed by SDS-PAGE and western blot. A single clear band at 89 kDa was observed by SDS-PAGE, matching the predicted size of the recombinant tagged protein of 88.5 kDa. A single band was also detected by western blot, indicating that strep-tagged mGDH was purified to apparent homogeneity

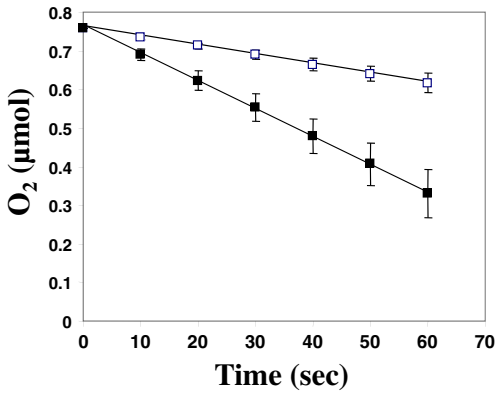

Fig. 3 Oxygen consumption rate of membranes. Rate of oxygen consumption observed with equivalent protein amounts (0.75 mg protein in 3 mL assay). *Closed squares*, mGDH overexpression strain; *open squares*, parental strain. Data are the average of at least three independent biological replicates. *Error bars*, ± SD

Table 3 Oxygen consumption rates of membranes from *G. oxydans* Δ*hsdR* overexpressing mGDH and from the control strain containing empty vector pBBR1p452 with D-glucose or D-glucose/ethanol mixture

	nmol ½ O_2 min^{-1} mg^{-1}	
G. oxydans ΔhsdR harboring plasmid	D-Glucose	D-Glucose and ethanol
pBBR1p452-mgdh-ST	1,075±64	1,110±33
pBBR1p452	368±19	782±85

(Fig. 4). Activities with glucose were determined in the membrane fraction, flow-through, washing fractions, and elution fractions (Table 4). A significant proportion of the mGDH activity remained in the flow-through after purification and only 60 μg of pure protein was obtained from 17 mg of membrane protein. Since the binding capacity of the StrepTactin matrix used in these experiments was in the range of 9 mg protein (calculated for the mGDH with a molecular mass of 88.5 kDa), it is evident that the detergent-solubilized mGDH inefficiently bound the column.

However, the amount of pure enzyme was high enough for protein characterization and the specific activity of purified mGDH was high (150±30 U/mg). Activity of purified mGDH was slightly higher at pH 6 compared to pH 7 and subsequent assays were performed at pH 6. In addition to D-glucose, the activity of purified mGDH was also assayed with several other D- and L-aldoses, aldose derivatives, D-fructose, as well as ethanol. Only oxidation of D-aldoses and aldose derivatives was observed. While D-glucose was the preferred substrate, 2-deoxy-D-glucose, D-allose, D-fucose, and D-xylose were oxidized at greater than 10 % of the glucose rate (Table 5). Kinetic parameters for mGDH D-glucose oxidation were determined from nonlinear regression of the Michaelis–Menten data. The apparent V_{max} and K_M of mGDH D-glucose oxidation were 216±5.4 U/mg and 5.9±0.34 mM, respectively. Glucose was oxidized with good catalytic efficiency, having a k_{cat}/K_M of 5.4×10^4 $s^{-1}M^{-1}$.

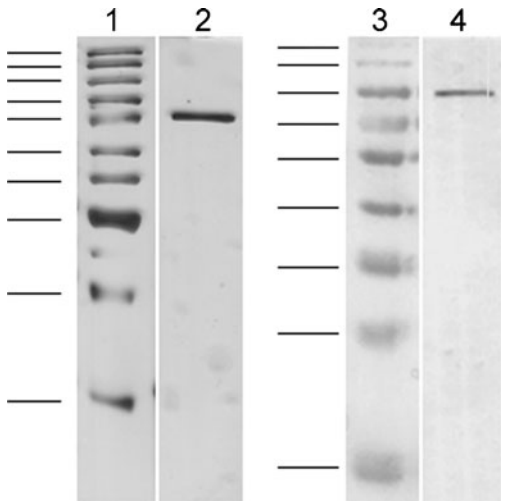

Fig. 4 SDS-PAGE and western blot of Gox0265 purified by StrepTactin affinity chromatography. *1* PageRuler Unstained Protein Ladder (Fermentas, *from the top*: 200, 150, 120, 100, 85, 70, 60, 50, 40, 30 kDa); *2*: purified mGDH (Gox0265); *3*: western blot of PageRuler Prestained Protein Ladder (Fermentas, *from the top*: 170, 95, 72, 55, 43, 34, 26, 17 kDa); *4*: western blot of purified mGDH (Gox0265)

Discussion

G. oxydans produces both a cytoplasmic NADP-dependent GDH and a membrane-bound PQQ-dependent GDH (Buchert and Viikari 1988). Gluconate production in *G. oxydans* is mainly the result of the activity of the PQQ-dependent GDH as the activity of the quinoprotein is 30-fold higher than the activity of the NADP-dependent GDH (Pronk et al. 1989). Bioinformatic analysis and comparison of PQQ dehydrogenases with known 3D structure indicated that mGDH contains five N-terminal transmembrane helices and a large periplasmic domain that contains the active site, including the PQQ and Ca^{2+} binding sites. Homologous mGDHs from *Acinetobacter calcoaceticus*, *Pseudomonas fluorescens*, *E. coli*, and *Gluconobacter suboxydans* have been described and share similar properties. All of these proteins are monomeric with apparent molecular masses between 80 and 88 kDa; however, their kinetic properties vary. The *A. calcoaceticus* mGDH was reported to have a V_{max} between 600 and 720 U/mg and a K_M for glucose of approximately 4 mM at a pH optimum of 8.5 (Matsushita et al. 1989a; Dewanti and Duine 1998). The corresponding enzyme in *P. fluorescens* has similar properties, with a reported V_{max} of 307 U/mg and a K_M for glucose of 6.3 mM at a pH optimum of 8.75 (Matsushita et al. 1980). Interestingly, *E. coli* contains a PQQ-dependent mGDH in spite of the fact that it cannot produce PQQ de novo. Consequently, the apo-mGDH is converted to the holoenzyme by the addition of exogenous PQQ and either Mg^{2+} or Ca^{2+}, which are required for PQQ binding. The *E. coli*-derived mGDH is reported to have a V_{max} from 150 to 400 U/mg and K_M for glucose between 0.9 and 2.1 mM (Yamada et al. 1998; Matsushita et al. 1987; Cozier et al. 1999; Ameyama et al. 1986). Likewise, the reported pH optimum varies between 6 and 8.75. The large differences observed for the *E. coli* mGDH are likely due to the efficiency of incorporation of the exogenous PQQ. To our knowledge, only one mGDH from *Gluconobacter* spp. was purified and characterized to date, a mGDH from *G. suboxydans* IFO12528. This enzyme was solubilized from the membrane fraction with Triton X-100 and purified to homogeneity by conventional column chromatography (Ameyama et al. 1981). The *G. suboxydans* mGDH had a V_{max} of 435 U/mg and the K_M for glucose was 7.7 mM and

Table 4 Purification of mGDH from *G. oxydans* Δ*hsdR*

	Volume [mL]	Volume activity [U/mL]	Protein content [mg/ml]	Specific activity [U/mg]	Total activity [U]	% recovery
Membrane	1	276	17.0	16.2	276	100
Flow-through	10	19.7	5.2	3.8	197	71
Wash fractions	9	3.3	0.7	4.7	29.8	11
Elution fractions	3	2.9	0.02	145	8.7	3.2

had a pH optimum of 6 when PMS–DCPIP were used as electron acceptors. In a separate publication, the *G. suboxydans* IFO12528 mGDH was used for the reconstruction of the respiratory chain by insertion into a phospholipid bilayer containing ubiquinone-10 and the cytochrome *o* terminal oxidase. Under these conditions, the *G. suboxydans* mGDH has a V_{max} with glucose of 381 U/mg (Matsushita et al. 1989b). The results obtained with the mGDH from *G. suboxydans* are in the same range as the mGDH described here (e.g., V_{max} 216 U/mg, K_M 5.9 mM). The mGDH from *G. oxydans* had a broad substrate spectrum. Besides glucose 2-deoxy-D-glucose, allose, fucose, and xylose were oxidized at reasonable rates (i.e., >10 % of the glucose rate). Likewise, mGDHs from *A. calcoaceticus* and *E. coli* have been reported to oxidize 2-deoxyglucose, fucose, L-arabinose, xylose, and galactose with at least half the rate of glucose (Dewanti and Duine 1998; Ameyama et al. 1986; Cozier et al. 1999). In contrast, the *G. suboxydans* and *P. fluorescens* mGDHs are only reported to oxidize glucose (Ameyama et al. 1981; Matsushita et al. 1980, 1989b, Matsushita and Ameyama 1982). The significantly different substrate spectrum among known mGDHs is likely due to varying amino acid composition in the active center. It was observed that a single amino acid substitution of a conserved His787Asn in the *G. oxydans* mGDH changed the substrate specificity from glucose to maltose by creating a more accessible active site (Cleton-Jansen et al. 1991), suggesting that only small changes in the active center are required for drastic effects on substrate recognition.

Table 5 Substrate spectrum of purified mGDH

Substrate	% activity[a]
D-Glucose	100
2-Deoxy-D-glucose	72
D-Allose	43
D-Fucose	33
D-Xylose	11
D-Galactose	7
D-Glucosamine	6
Melibiose	6
D-Maltose	6
L-Arabinose	5
D-Gulose	4

[a] Oxidation was assayed at pH 6 in 40 mM KPB with PMS–DCPIP as electron acceptors. Activities are given as percentage in comparison to D-glucose (150 U/mg). One unit was defined as the amount of enzyme catalyzing the conversion of 1.0 μmol of substrate per min. Oxidation was not observed with assays containing D-mannose, D-ribose, D-arabinose, and D-lyxose, D- or L-altrose, D- or L-talose, and D- or L-idose

The typical expression host for prokaryotic gene expression and protein production is *E. coli.* However, *E. coli* is not always an ideal host, especially for membrane-bound PQQ dehydrogenases since it does not produce PQQ de novo. In contrast, acetic acid bacteria contain the PQQ biosynthetic pathway and have an abundance of naturally occurring PQQ-containing dehydrogenases (Prust et al. 2005). Taking advantage of a recently developed expression vector system for *Gluconobacter* spp. (Kallnik et al. 2010), the *mgdh* gene from *G. oxydans* 621 H was overexpressed and the corresponding membrane-integral mGDH was purified in a single step. In this context, it is important to mention that the overproduction of the mGDH was already attempted in *G. oxydans* 621 H to increase the accumulation of gluconate and to accelerate the complete oxidation process from glucose to 5-ketogluconate (Merfort et al. 2006). In this study, the gene coding for the mGDH was also expressed using the vector pBBR1MCS-2 but without a *G. oxydans*-specific promoter. Hence, the modified strain only displayed an increase in the gluconate production rate of about 11 % compared to 70 % in this publication.

Furthermore, our data showed that the *mgdh* overexpression strain had a much higher mGDH activity compared to the parental strain and other organisms containing the mGDH. For example, *E. coli*, *P. fluorescens*, *A. calcoaceticus*, and *G. suboxydans* membrane fractions had mGDH activities of 241 (Matsushita et al. 1987), 1,400–4,100 (Matsushita et al. 1980), 10,700 (Matsushita et al. 1989a), and 2,860 mU/mg (Matsushita et al. 1989b), respectively, when PMS–DCPIP was used as electron acceptors. Here we showed that the control strain of *G. oxydans* had mGDH membrane activities of 2,500 mU/mg, which is equivalent to wild-type activities found in *G. suboxydans* (Matsushita et al. 1989b), whereas the overexpression strain had a specific activity of 13,300 mU/mg, representing a >5-fold increase in protein production.

Furthermore, oxygen was consumed at a rate of 1,075 nmol ½ O_2 min^{-1} mg^{-1} in the overexpression strain. In contrast, the control strain had only one third of the glucose-dependent oxygen reduction rate of 368 ½ O_2 min^{-1} mg^{-1}. There is only one report that describes an even higher oxygen consumption rate in *G. suboxydans* (Matsushita et al. 1989b). However, it is important to state that the values cannot be directly compared, because different strains (*G. suboxydans* IFO12528, Matsushita et al. 1989b and *G. oxydans* 621 H, this study) were analyzed. Moreover, the growth conditions, membrane preparations, and the protein content determination were different. Nevertheless, it was astonishing that the overproduction of the mGDH in our system led to a threefold increase in the respiratory rate and the question arose whether this rate could even be increased. Therefore, the oxygen consumption in washed membranes of the *G. oxydans* control strain was measured in the presence of two substrates, glucose and ethanol, so that two enzymes, mGDH and mADH, channeled electrons in the respiratory chain. Indeed, after the addition of glucose and ethanol, the overall O_2 consumption rate in the control strain was accelerated by a factor of 2.1. In contrast, the mGDH overproducing strain did not show an enhanced O_2 consumption rate when ethanol was added, indicating that the maximum electron transfer capacity of the respiratory chain was reached in *G. oxydans* overproducing mGDH. Taken together, these data show that electron transfer from glucose to the quinone pool, mediated by mGDH, was limited by the electron consumption rate of the terminal quinol oxidases in the *G. oxydans* overexpression strain. Interestingly, the respiratory rate of the mGDH overproducing *G. oxydans* was similar to that of *Azotobacter vinelandii*, which has the highest known oxygen-dependent respiratory rate found in bacteria and is used to remove O_2 for nitrogenase protection during aerobic growth (Ackrell and Jones 1971).

As mentioned above, the metabolism of *Gluconobacter* spp. is especially designed to perform incomplete oxidation reactions. Some examples are the production of L-sorbose from D-sorbitol (vitamin C synthesis) (Reichstein and Grüssner 1934) and 6-amino-L-sorbose from 1-amino-D-sorbitol for the synthesis of the antidiabetic drug miglitol (Schedel 2000). *Gluconobacter* spp. can also be used industrially to produce D-gluconic acid and 5-keto- and 2-ketogluconic acids from D-glucose (Adachi et al. 2003). These and other carboxylic acids are used industrially in foodstuff, pharmaceuticals, and textiles (Rabenhorst et al. 2001). Industrial applications of gluconate and its derivatives mostly take advantage of their well known ability to chelate di- and trivalent metal ions (Prescott et al. 1953; Dvorkovita and Hawley 1952). Gluconates are also used in the production of fat-soluble dyes, as leavening agent in instant baking powders, and as acid catalysts in the textile industry (Prescott et al. 1953; Sawyer 1964; Mattey 1992). Furthermore, gluconates are particularly desirable in the foodstuff and pharmaceutical industries since they are nontoxic, stable even when sterilized, and very soluble (see Sawyer 1964 for extensive review). Consequently, the production of high quantities of gluconate is of great biotechnological importance, and the wide industrial application of gluconate makes large-scale production attractive. The *G. oxydans* gluconate overproduction strain described here produces gluconate at a rate 70 % higher than the wild type and is potentially useful for the whole-cell production of this important carboxylic acid. Another aspect is that PQQ-dependent mGDHs are very attractive as industrial biosensors and are currently the major enzymes used in self-monitoring of blood glucose sensor systems (D'Costa et al. 1986; Ye et al. 1993; Igarashi et al. 2004). Hence, mGDH overproducing strains as described in this publication might be a suitable source in the future to produce the biosensor and further increase the sensitivity of the glucose-sensing system.

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