Identification of membrane-bound quinoprotein inositol dehydrogenase in *Gluconobacter oxydans* ATCC 621H

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The GOX1857 gene, which encodes a putative membrane-bound pyrroloquinoline quinone (PQQ)-dependent dehydrogenase in *Gluconobacter oxydans* ATCC 621H, was characterized. GOX1857 was disrupted and the oxidizing potential of the resulting mutant strain was compared to that of the wild-type. In contrast to the wild-type, the mutant was unable to grow with *myo*-inositol as the sole energy source and did not show any *myo*-inositol dehydrogenase activity *in vitro*, indicating that GOX1857 encodes an inositol dehydrogenase. The association of inositol dehydrogenase with the membrane and the requirement for the cofactor PQQ were confirmed. Inositol dehydrogenase exhibited optimal activity at pH 8.75. As indicated by cultivation on different substrates, inositol dehydrogenase was repressed by D-glucose.

INTRODUCTION

The acetic acid bacterium *Gluconobacter oxydans* is characterized by its ability to incompletely oxidize various sugars, alcohols and polyols in the periplasm (Matsushita *et al.*, 1994). Substrates are oxidized stereo- and regio-selectively and the resulting aldehydes, ketones and organic acids are excreted into the medium. *G. oxydans* is used in several industrial processes; e.g. in the production of L-sorbose, an intermediate in the synthesis of vitamin C (reviewed by Bremus *et al.*, 2006). The key oxidation reactions in these processes are catalysed by membrane-associated dehydrogenases that are part of the respiratory chain (Matsushita *et al.*, 1994; Goodwin & Anthony, 1998) and often contain pyrroloquinoline quinone (PQQ) as cofactor. Several PQQ-dependent dehydrogenases of *G. oxydans* have been identified and characterized: the quinoprotein glucose dehydrogenase (Ameyama *et al.*, 1981), the quinoprotein glycerol dehydrogenase, which also oxidizes D-gluconate, D-mannitol and other polyols (Matsushita *et al.*, 2003), and the quinohaemoprotein alcohol dehydrogenase (Matsushita *et al.*, 1994). In *G. oxydans* strain IFO3244, a quinoprotein quinate dehydrogenase has been detected (Vangnai *et al.*, 2004). During growth of *G. oxydans* in complex medium supplied with sugars, incomplete oxidation of sugars by the membrane-bound dehydrogenases provides energy for growth, while the carbon for growth can be obtained from both the sugar and the complex components, i.e. yeast extract or tryptone. Channelling of sugars into the biosynthetic pathways is

mediated by cytosolic NAD(P)-dependent dehydrogenases (Matsushita *et al.*, 1994).

Although several oxidation reactions and the enzymes involved have been described, the oxidation potential of *Gluconobacter* strains has not yet been fully elucidated. Recently, the complete genome of *G. oxydans* ATCC 621H (DSM 2343) has been sequenced (Prust *et al.*, 2005), revealing the presence of 75 uncharacterized dehydrogenases, 23 of which are predicted to be membrane-bound. Three of these membrane-bound dehydrogenases share high homology with known quinoproteins, while others belong to flavin-containing enzyme families (Prust *et al.*, 2005). In this study, we investigated the GOX1857 gene, which encodes one of the putative membrane-bound quinoprotein dehydrogenases in *G. oxydans* ATCC 621H. To characterize the catalytic activity of the encoded enzyme, GOX1857 was disrupted and properties of the mutant were compared to those of the wild-type strain.

METHODS

Bacterial strains and culture conditions. The relevant characteristics and references for the strains and plasmids used in this work are listed in Table 1. *Escherichia coli* strains were grown in Luria–Bertani medium (Sambrook *et al.*, 1989). *G. oxydans* ATCC 621H was routinely grown in complex medium containing 0.5% yeast extract and 0.3% tryptone, pH 6.0, supplemented with 250 mM D-mannitol (mannitol medium). Growth was recorded by measurement of the optical density at 620 nm (OD₆₂₀). When appropriate, D-mannitol was replaced by D-sorbitol, D-glucose or *myo*-inositol. When D-glucose was used, potassium succinate (100 mM) was added as a buffering agent. For growth screening, cells pre-grown in mannitol medium and pelleted at 3000 g for 15 min were used. Growth tests were carried out in complex

Abbreviations: DCPIP, 2,6-dichlorophenol indophenol; PMS, phenazine methosulphate; PQQ, pyrroloquinoline quinone.

Table 1. Strains and plasmids

Strain or plasmid	Characteristics	Reference
Strains		
<i>G. oxydans</i> ATCC 621H (DSM 2343)	Wild-type strain	Gillis & de Ley (1980)
<i>G. oxydans</i> DW1	ATCC 621H derivative, Gm ^R inserted in GOX1857 gene	This study
<i>G. oxydans</i> TH1	ATCC 621H derivative, <i>pqqA</i> ::Km ^R	Hölscher & Görisch (2006)
<i>E. coli</i> DH5α	<i>supE44 ΔlacU169 (φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Hanahan (1983)
<i>E. coli</i> HB101	<i>supE44 hsdS20(r_B⁻ m_B⁻) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</i>	Boyer & Roulland-Dussoix (1969)
Plasmids		
pBBR1MCS-2	Broad-host-range cloning vector, Km ^R	Kovach <i>et al.</i> (1995)
pRK2013	Helper plasmid for triparental mating, Km ^R	Figurski & Helinski (1979)
pKmob18GII	Gene replacement vector, <i>gusA</i> , Km ^R	Katzen <i>et al.</i> (1999)
pTB9042	pKmobGII derivative containing an internal fragment of GOX1857, Km ^R	This study
pTB9049	pKmobGII derivative containing an internal fragment of GOX1857 disrupted by the Gm ^R gene, Km ^R Gm ^R	This study
pTB9058	pBBR1MCS-2 derivative containing the complete GOX1857 gene including its putative promoter region, Km ^R	This study

medium containing various substrates at a concentration of 20 mM. Antibiotics were used at the following concentrations: kanamycin, 50 µg ml⁻¹, gentamicin, 10 µg ml⁻¹ (*E. coli*) or 50 µg ml⁻¹ (*G. oxydans*), cefoxitin, 50 µg ml⁻¹.

General genetic techniques. DNA work was performed according to standard protocols (Ausubel *et al.*, 2002; Sambrook *et al.*, 1989). For PCR reactions, genomic DNA isolated from *G. oxydans* ATCC 621H was used as the template. *Pfu* DNA polymerase (Promega Biosciences) was used for PCR-amplification of inserts for cloning, and *Taq* DNA polymerase (Rapidozym) was used for PCR amplification in test reactions (e.g. colony PCR).

Vector construction. To generate a gene replacement vector for inactivation of GOX1857 in *G. oxydans* ATCC 621H, a 1.47 kb internal fragment of GOX1857 was amplified with forward primer 5'-GGAATTCAACCGCGACAATGTCGGCAAG-3' (restriction site underlined) and reverse primer 5'-GCGAAGCTTCGGCATGCTCC-ATTTACACCTTG-3' and cloned between the *Eco*RI and *Hind*III sites of pKmob18GII, resulting in plasmid pTB9042. The GOX1857 fragment within pTB9042 was interrupted by insertion of the gentamicin resistance cassette of vector pBBR1MCS-5 between the *Not*I and *Aat*II sites, resulting in plasmid pTB9049.

For mutant complementation, a 2.65 kb fragment containing the complete GOX1857 gene including its putative promoter region was amplified with forward primer 5'-GGAATTGCAATCTTCTGATC-GCTCCAG-3' and reverse primer 5'-TGCAAGCTTCTGCGCTC-TTATCTTCGGAG-3' and cloned between the *Eco*RI and *Hind*III sites of plasmid pBBR1MCS-2, resulting in plasmid pTB9058.

Conjugational plasmid transfer into *G. oxydans*. Plasmids were transferred into *G. oxydans* by triparental mating using *E. coli* DH5α bearing the respective vector as the donor and *E. coli* HB101 bearing plasmid pRK2013 as the helper strain. The three strains were grown to late exponential phase, pelleted, resuspended in mannitol medium and mixed in a 1:1:1 ratio. The mixture was plated on mannitol medium agar and incubated overnight at 30 °C. The resulting cell patches were scraped from the plates and streaked on

selective mannitol medium agar containing cefoxitin and the appropriate selective antibiotic (kanamycin or gentamicin). Plates were incubated for 2–4 days until kanamycin/gentamicin-resistant colonies appeared.

Preparation of crude extracts and membrane fractions. *G. oxydans* was grown in complex medium containing different carbon sources to late exponential phase (OD₆₂₀ 0.9). Cells were harvested by centrifugation at 5000 g for 10 min, washed once with 10 mM Tris/HCl buffer, pH 7, and resuspended in the same buffer. Cells were broken by three cycles of ultrasonication using a Branson sonifier 250 (intensity 4, 20 % duty cycle, 5 min). The supernatant obtained after centrifugation of broken cells at 10 000 g for 10 min was used as the crude extract. The crude extract was centrifuged at 100 000 g for 60 min and the pellet was washed once with 10 mM Tris/HCl buffer, pH 7, resuspended in the same buffer and used as the membrane fraction.

Dehydrogenase assay. Dehydrogenase activity was determined photometrically at 25 °C in a dye-linked system containing 2,6-dichlorophenol indophenol (DCPIP) and phenazine methosulphate (PMS). The reaction mixture contained enzyme solution, buffer (see below), 33 mM substrate, 0.67 mM PMS, 0.1 mM DCPIP and 4 mM sodium azide. One unit of dehydrogenase activity was defined as the reduction of 1 µmol DCPIP per min, corresponding to the oxidation of 1 µmol substrate per min. Inositol dehydrogenase activity was routinely measured at 600 nm in 167 mM Tris/HCl, pH 8.75 (ε_{DCPIP} = 23 mM⁻¹ cm⁻¹). Dehydrogenase activities with ethanol, D-gluconate, glycerol, D-mannitol and D-sorbitol were measured at 520 nm in McIlvaine buffer at pH 5.0 (ε_{DCPIP} = 10.5 mM⁻¹ cm⁻¹). Glucose dehydrogenase activity was measured at 600 nm in McIlvaine buffer at pH 6.0 (ε_{DCPIP} = 17.2 mM⁻¹ cm⁻¹). For preparation of apoenzymes, samples were incubated with 10 mM EDTA for 60 min at 30 °C. For reconstitution of quinoprotein apoenzymes to the holoenzymes, samples were incubated with 16.5 µM PQQ and 20 mM MgSO₄ or 20 mM CaCl₂ for 10 min at 25 °C, without prior removal of EDTA. The PQQ-deficient *G. oxydans* mutant TH1 only produces quinoprotein apoenzymes

(Hölscher & Görisch, 2006) so EDTA treatment was not necessary. Here, samples were incubated with 16.5 μ M PQQ and 10 mM MgSO_4 to obtain quinoprotein holoenzymes.

Sequence analysis. The sequence of the GOX1857 gene of *G. oxydans* ATCC 621H was obtained from the NCBI web site (<http://www.ncbi.nlm.nih.gov>; accession number YP_192251). The predicted amino acid sequence of GOX1857 was compared to other published sequences using the National Center for Biotechnology Information BLASTP search tool (<http://www.ncbi.nlm.nih.gov/blast/>). Sequences were aligned with the CLUSTALW program located at the European Bioinformatics Institute website (<http://www.ebi.ac.uk/clustalw/>). Analysis of the putative promoter region of GOX1857 was carried out with the Neural Network Promoter Prediction tool (http://www.fruitfly.org/seq_tools/promoter.html), and transmembrane helices in proteins were predicted with the TMHMM tool located at the Center for Biological Sequence Analysis website (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>).

RESULTS

Generation of a mutant with a defective GOX1857 gene

GOX1857 was disrupted in *G. oxydans* ATCC 621H using the gene replacement vector pKmob18GII, which contains the *gusA* marker (Katzen *et al.*, 1999). Since pKmob18GII carries a kanamycin resistance gene, a gentamicin cassette was used for gene inactivation. Double-crossover mutants were identified by blue/white screening as described previously (Katzen *et al.*, 1999; Hölscher & Görisch, 2006); blue colonies could be observed 2 days after plating. A colourless, kanamycin-sensitive colony representing a double-crossover mutant (GOX1857::Gm^R) was isolated and named DW1. The disruption of GOX1857 by the gentamicin cassette and the absence of the vector within the genome of DW1 were confirmed by PCR.

Growth of *G. oxydans* wild-type and mutant DW1 with different substrates

Growth of *G. oxydans* wild-type and mutant DW1 was tested in complex medium containing 20 mM of one of the following substrates: *meso*-erythritol, ethanol, D-fructose, D-fucose, D-gluconate, D-glucose, glycerol, *myo*-inositol, maltose, D-mannitol, ribitol, D-ribose, D-sorbitol, L-sorbose, sucrose and xylitol. Cultures were started with an OD₆₂₀ of 0.05. With all substrates tested except *myo*-inositol, no significant difference in growth was observed between the wild-type strain and mutant DW1 in two independent experiments. Both strains grew to final optical densities varying between 0.2 and 1 with *meso*-erythritol, D-fructose, D-gluconate, D-glucose, glycerol, D-mannitol and D-sorbitol. Poor growth, limited to a maximum OD₆₂₀ of 0.1, was found with ethanol, xylitol, ribitol and L-sorbose. No growth was found with D-fucose, D-ribose, sucrose and maltose or with no added substrate. With *myo*-inositol (1,2,3,5/4,6-cyclohexanehexol), the wild-type grew to a final OD₆₂₀ of 0.15, whereas mutant DW1 did not grow at all. Therefore, it was concluded that DW1 had a defective inositol dehydrogenase. However, growth of *G. oxydans*

wild-type with 20 mM *myo*-inositol in liquid culture was rather poor. Also, cultivation with *myo*-inositol concentrations of 100–500 mM did not result in higher final cell densities. On complex medium agar containing *myo*-inositol (100 mM), the wild-type strain grew slowly; small colonies appeared 2–3 days after plating. No growth was found for mutant DW1 on the same agar.

Dehydrogenase activities in wild-type and mutant DW1 with different substrates

Dye-linked dehydrogenase activities with different substrates were assayed in crude extracts of *G. oxydans* wild-type and mutant DW1 grown in complex medium containing 250 mM D-sorbitol. With ethanol, D-gluconate, D-glucose, glycerol, D-mannitol and D-sorbitol as substrate, both strains exhibited similar dehydrogenase activities (data not shown). No dye-linked dehydrogenase activity was found in either the wild-type or DW1 with quinate or shikimate as substrate. In contrast, with *myo*-inositol, dehydrogenase activity was present in crude extracts of the wild-type but was absent in DW1 extracts (Table 2). The effect of the growth substrate on *myo*-inositol dehydrogenase activity in the wild-type, as shown in Table 2, is discussed below.

Properties of inositol dehydrogenase

Dye-linked *myo*-inositol dehydrogenase in crude extracts of *G. oxydans* exhibited optimal activity at pH 8.75 in Tris/HCl buffer. Activity was nearly absent below pH 5 (McIlvaine buffer) and above pH 10.5 (CAPS buffer). Therefore, all further measurements were carried out at pH 8.75. The apparent K_m value for oxidation of *myo*-inositol in crude extracts using the DCPIP-PMS assay was 5 mM. Maximal activity was already obtained at 33 mM *myo*-inositol; higher *myo*-inositol concentrations even led to a slight decrease in dehydrogenase activity. In crude extracts from cells grown on D-sorbitol, maximal specific activity amounted to about 90 mU per mg protein. Crude extracts of the wild-type, but not of mutant DW1, also oxidized *allo*-inositol (1,2,3,4/5,6-cyclohexanehexol) and *muco*-inositol (1,2,4,5/3,6-cyclohexanehexol). The apparent K_m values for *allo*- and *muco*-inositol (10–15 mM) were slightly higher than that for *myo*-inositol. Maximal specific activities were obtained at 60–70 mM *allo*-inositol and 33 mM *muco*-inositol and corresponded to 160 % and 67 % of the specific activity with *myo*-inositol, respectively. Again, higher inositol concentrations slightly inhibited dehydrogenase activity.

The major portion of dye-linked *myo*-inositol dehydrogenase activity was membrane-associated and amounted to 600 mU per mg membrane protein. In the fraction containing soluble proteins, a specific activity of only 30 mU was found. Activity of PQQ-dependent enzymes requires the presence of divalent metal ions such as Ca^{2+} or Mg^{2+} , which are coordinated to the PQQ in the active site (Goodwin & Anthony, 1998). In membranes of the *G. oxydans* mutant TH1, which is unable to synthesize PQQ (Hölscher & Görisch, 2006), *myo*-inositol dehydrogenase

Table 2. Dye-linked *myo*-inositol dehydrogenase activities in crude extracts of *G. oxydans* wild-type and GOX1857::Gm^R mutant DW1 after growth on different substrates

Growth substrate	Specific <i>myo</i> -inositol dehydrogenase activity [mU (mg protein) ⁻¹]*	
	Wild-type	Mutant DW1
D-Sorbitol	94 ± 16	< 5‡
D-Mannitol	24 ± 2	< 5
D-Glucose	< 5	< 5
D-Sorbitol + <i>myo</i> -inositol	87†	< 5
D-Mannitol + <i>myo</i> -inositol	27†	ND
D-Glucose + <i>myo</i> -inositol	< 5	ND
D-Sorbitol, OD ₆₂₀ 0.15§	39†	ND
<i>myo</i> -Inositol, OD ₆₂₀ 0.15§	214†	NG

ND, Not determined; NG, no growth under these conditions.

*Means ± SD of results of independent triplicate growth tests.

†Means of results of independent duplicate growth tests.

‡*myo*-Inositol dehydrogenase activity found in crude extracts of the complemented mutant DW1-pTB9058 was 75 mU mg⁻¹.

§Strains were harvested at an optical density of 0.15 (see text for details).

activity was absent, but could be restored by addition of PQQ and Mg²⁺. Furthermore, treatment of wild-type crude extracts with 10 mM EDTA completely inactivated inositol dehydrogenase. Without prior removal of EDTA, activity was restored by addition of PQQ and 20 mM Mg²⁺ or Ca²⁺.

Inositol dehydrogenase activity after growth on different substrates

To study the effect of different growth substrates on inositol dehydrogenase activity, *G. oxydans* wild-type was routinely grown to an OD₆₂₀ of 0.9. After growth on D-sorbitol, dye-linked *myo*-inositol dehydrogenase activity in crude extracts amounted to about 94 mU per mg protein (Table 2). A similar specific activity was obtained when cultivation was carried out with a mixture of D-sorbitol (250 mM) and *myo*-inositol (100 mM). After growth on D-mannitol (250 mM) or a mixture of D-mannitol and *myo*-inositol, *myo*-inositol dehydrogenase activity reached only about 27 % of the activity of sorbitol-grown cells. When D-glucose (250 mM) or a mixture of D-glucose and *myo*-inositol were used for cultivation, *myo*-inositol dehydrogenase activity was negligible (Table 2). With *myo*-inositol as sole substrate (100 mM), growth of the wild-type was restricted to a final OD₆₂₀ of 0.15 (see above). Using *myo*-inositol-grown cells, specific *myo*-inositol dehydrogenase activity in crude extracts was about twofold higher than after growth on D-sorbitol to an OD₆₂₀ of 0.9 and about fivefold higher than after growth on D-sorbitol to an OD₆₂₀ of 0.15. Under the cultivation conditions tested, i.e. with D-sorbitol, D-mannitol, D-glucose or a mixture of D-sorbitol and *myo*-inositol, dye-linked *myo*-inositol dehydrogenase activity was not detected in crude extracts of mutant DW1 (Table 2).

Complementation of mutant DW1

Mutant DW1 could be complemented by plasmid pTB9058, which contained the GOX1857 gene including its own putative promoter region. As with the wild-type strain, mutant DW1 bearing pTB9058 grew with *myo*-inositol in liquid culture and on agar. Crude extracts of the complemented mutant oxidized *myo*-, *allo*- and *muco*-inositol.

Gene GOX1857 and the amino acid sequence of quinoprotein inositol dehydrogenase

DNA sequence analysis suggested that GOX1857 is organized in a monocistronic operon. The region upstream of GOX1857 most probably represents a promoter (promoter prediction score of 0.98). The open reading frame consists of 2367 bp encoding a putative 85.4 kDa protein. A BLASTP search and pairwise alignments using CLUSTALW revealed that the quinoprotein inositol dehydrogenase encoded by GOX1857 showed the highest sequence identity to the putative quinoprotein quinate dehydrogenase from *Pseudomonas fluorescens* Pf-5 (46.7 %; accession number YP_262726) and the putative quinoprotein glucose dehydrogenase from *Pseudomonas syringae* (46 %; accession number AAO56073). Maximal sequence identity with biochemically characterized proteins was found with the quinoprotein quinate dehydrogenase from *Acinetobacter* sp. ADP1 (40.7 %; accession number Q59086) and the quinoprotein glucose dehydrogenase from *E. coli* (37.7 %; accession number P15877) (Fig. 1). The predicted topology of quinoprotein inositol dehydrogenase is similar to that of the well-studied *E. coli* quinoprotein glucose dehydrogenase, which is made up of five N-terminal transmembrane helices and a catalytic C-terminal domain facing the periplasm (Yamada *et al.*, 1993). Regions with similarity to the tryptophan docking motifs of the so-called

QIDH GOX	---MNSGPR-ILSMIGILGALMAAFLLIEGLHLIILGCSWFYTLAGIALAASSVYMIR	56
QGDH ECO	MAINNTGSRRLVLTALFAALCGLYLLIGGGWLVAIGCSWYYPITAGLVMLGVANMLWRS	60
	* * * * *	
QIDH GOX	NILSTWIALGLLVATALKSLAEVGTSEFWSESR--LIVFLCVALLIATLMAPLWLSGPGRRY	114
QGDH ECO	KRAALWLYAALLLGTMIWCVVEVGFDFWALTPRSDDLVEFGIWLILPFVWRRLVIP---	116
	* * * * *	
QIDH GOX	FTRPVTGATSGALGAIIVAFLAG--MFRVHPTIAPQDTT---HPQETASTADSDQPGHDW	169
QGDH ECO	-----ASGAVAALVVALLISGGILTWAGENDPQEINGTSLADATPAEAI SPVADQDW	168
	* * * * *	
QIDH GOX	PAYGRTASGTRYASTQINRDNVGLRVAVTYRTGDMALNG---AEFGGTPIKIGDTVY	225
QGDH ECO	PAYGRNQEGQRTSPLKQINADNVHNLKEAWVFRTGDVKQPNDPGEITNEVTPIKVGDTLY	228
	***** * * * * *	
QIDH GOX	ICSPHNIVSALDPDTGTGTEKWKFDPHAQTKVWQR---CRGVGYWH---DSTATDANAFCAS	279
QGDH ECO	LCTAHQRLFALDAASGKEKWHYDPELKINESPQHVTGCRGVSYHEAKAETASPEVMADCPR	288
	* * * * *	
	262	
QIDH GOX	RIVLTITIDARLITIDARTGQACTDFGTNGNVNLLTGLGPTAPGSYYPTAAPLVAGDIVVV	339
QGDH ECO	RIILPVNDGRLIAINAENGLCETPANKGVNLQSNMPTDKPGLYEPTSPPIITDKTIVM	348
	* * * * *	
QIDH GOX	GCRIADNERICEPSGVVRCYDVRTGAQVWAWDATNPHRGTTPLAECEIYPAETPNKWCTA	399
QGDH ECO	AGSVTDNFSIRETSGVIRGFDVNTGELLWAFDPGAKCPNAIPSE-HITFNSPNSWAPA	407
	* * * * *	
	353 354	404
QIDH GOX	SYDPKLNLVFFPLGNQTPDFWGGDRSKASDEYNDAFVAVDAKTGDERWHFRTANHDLDVY	459
QGDH ECO	AYDAKLDLVYLFPMGVTTDPDIWGNRTPEQERYASSILALNATTGKLAWSYQTVHHDLWDM	467
	* * * * *	
	424	466
QIDH GOX	DATAQPILYDIPDGHCCTIRPAIIAMTKRCQIEVLDRRDGTPIVPVEMRKVPQDCAPEHQY	519
QGDH ECO	DLPAQPTLADIT-VNGQKVEVIYAPAKTGNIFVLDRRNGELVVPAPKEKVPQ-GAAKGDY	525
	* * * * *	
QIDH GOX	LAPEQPYSALSIG-TERLKPSDMWGGTIFDQLLCRIQFASYRYEGEFTPVNEKQATIIYP	578
QGDH ECO	VTPTQPFSELSFRPTKDLSGADMWGATMFDQLVCRVMFHQMRYEGIFTTPSE-QGTLVFP	584
	* * * * *	
QIDH GOX	GYGGINWGGGAVDESTGILLVNDIRMAQWGKFMKQE-----EARRSGFKPSSEGEYSEQ	633
QGDH ECO	GNLGMFEWGGISVDPNREVAIANPMALPFVSKLIPRGPNPMEQPKDAKGTGTESGIQPK	644
	* * * * *	
QIDH GOX	KGTPWGVVRSMFFSPAGLPCVKPPYGTNNAIDLRSGKVKWSMPLGTIQDMPVHGMVPGIA	693
QGDH ECO	YGVYPYVTLNPFLLSPFLPCQPAWGYISALDLKTNEVVVKRIGTPQDSMPFPMPVPVP	704
	* * * * *	
QIDH GOX	IPLGMPTMSGPLATHTGLVFFSGTLDNYVRALNTDIGEVVWKARLPVSAQAAPMSYMSDK	753
QGDH ECO	FNMGMPMLGGPISTAGNVLFIAATADNYLRAYNMSNGEKLWQGRLPAGGQATPMTEYVN-	763
	* * * * *	
QIDH GOX	TGKQYLVVTAGGLTRSGVDKNRGDYVIAYALPSEF-	788
QGDH ECO	-GKQYVVISAGHGSGFGT--KMGDYLVAYALPDDVK	796

Fig. 1. Alignment of the amino acid sequences of *G. oxydans* quinoprotein inositol dehydrogenase (QIDH GOX) and *E. coli* quinoprotein glucose dehydrogenase (QGDH ECO). The asterisks denote identical residues in both sequences. Regions of transmembrane helices determined for the glucose dehydrogenase (Yamada *et al.*, 1993) and predicted for the inositol dehydrogenase are underlined. Functionally important residues located in the active site of glucose dehydrogenase are numbered and in bold.

‘propeller fold’, the common structure of PQQ-dependent dehydrogenases (Goodwin & Anthony, 1998; Toyama *et al.*, 2004), were also found in the inositol dehydrogenase (not shown). As revealed by the resolved X-ray structures of several alcohol dehydrogenases, the PQQ molecule is supported by a Trp or Phe residue, which corresponds to Trp-404 in the modelled structure of the *E. coli* glucose dehydrogenase (Cozier & Anthony, 1995). A Trp residue was also found in the respective region of inositol dehydrogenase (Fig. 1). In alcohol dehydrogenases, the PQQ molecule is covered by an unusual bis-cysteine ring made up of two adjacent cysteines, which is replaced by a histidine (His-262) in the model glucose dehydrogenase (Fig. 1). This histidine residue is also required for high affinity to glucose (Cozier *et al.*, 1999). Inositol dehydrogenase contains neither two adjacent cysteines nor a histidine residue at

the corresponding site. However, the enzyme shares the conserved Asp residue present in all studied PQQ-dependent dehydrogenases, which is believed to initiate the catalytic reaction and corresponds to Asp-466 in the *E. coli* glucose dehydrogenase. Furthermore, inositol dehydrogenase shares the conserved Asp, Asn and Thr residues of glucose dehydrogenases (Asp-353, Asn-354, Thr-424; Fig. 1), which most probably interact with the metal ion in the active site (Cozier & Anthony, 1995).

DISCUSSION

To characterize GOX1857, encoding a putative PQQ-dependent dehydrogenase in *G. oxydans*, we generated a mutant with a defective GOX1857 gene. Growth screening and dehydrogenase assays with a selection of sugars and

sugar alcohols revealed that only with *myo*-inositol and other inositols as substrates did the GOX1857 knockout mutant DW1 differ from the wild-type. Whereas the wild-type grew, albeit slowly, on *myo*-inositol and oxidized *myo*-inositol *in vitro*, no growth and no dehydrogenase activity were found for DW1 with this substrate. Therefore, we concluded that GOX1857 encodes an inositol dehydrogenase.

Dye-linked inositol dehydrogenase activity was associated with the membrane of *G. oxydans* wild-type. In a PQQ-deficient *G. oxydans* mutant (Hölscher & Görisch, 2006) or after treatment of wild-type crude extracts with a chelating agent, a procedure that converts quinoproteins into the apoform (Mutzel & Görisch, 1991; Adachi *et al.*, 2001), inositol dehydrogenase activity was absent. These results confirmed that the enzyme is a membrane-bound quinoprotein, as had previously been concluded from sequence analysis of the encoding gene (Prust *et al.*, 2005). In *G. oxydans*, membrane-bound quinoproteins are involved in energy generation by mediating incomplete oxidation (Matsushita *et al.*, 1994). Therefore, we assume that quinoprotein inositol dehydrogenase is required for growth of *G. oxydans* with *myo*-inositol as sole energy source.

Expression of inositol dehydrogenase is regulated. Our results demonstrate that different specific activities of inositol dehydrogenase are obtained in cultures grown on different substrates. Using chip-based genome-wide transcription analysis, GOX1857 had previously been shown to be highly expressed upon growth on D-sorbitol and D-mannitol, but not upon growth on D-glucose (M. Hoffmeister & A. Ehrenreich, personal communication). Consistently, in our study, inositol dehydrogenase activity was found after growth with D-sorbitol and, to a lesser extent, with D-mannitol, but not after growth with D-glucose. When D-sorbitol, D-mannitol or D-glucose were used in combination with *myo*-inositol in the growth medium, the respective inositol dehydrogenase activities were not altered, although the highest inositol dehydrogenase activity was indeed obtained after growth on *myo*-inositol alone. The lack of activity after growth on D-glucose or a mixture of *myo*-inositol and D-glucose suggests that inositol dehydrogenase is subject to catabolite repression by D-glucose. As described above, reduced activities were also obtained after cultivation with mixtures of *myo*-inositol and D-mannitol or *myo*-inositol and D-sorbitol. The reasons for the regulatory effects of D-sorbitol and D-mannitol on expression of inositol dehydrogenase are not understood at present.

To our knowledge, this is the first identification of a gene encoding a membrane-bound inositol dehydrogenase. In early biochemical studies, *myo*-inositol dehydrogenase activity was detected in membranes of *Acetobacter suboxydans* (now *G. oxydans*) KLUYVER-DE LEEUW (Rapin *et al.*, 1967). Oxidation of *myo*-inositol was determined by measuring oxygen consumption, and, under certain conditions, Mg^{2+} ions had a positive effect on activity.

Subsequently, the partial purification of a membrane-bound *myo*-inositol dehydrogenase from *G. oxydans* NCIB 621 (ATCC 621) was described (Criddle *et al.*, 1974, 1977). In these experiments, *myo*-inositol dehydrogenase activity was measured in a dye-linked system, indicating the involvement of a flavin or quinone cofactor. The highest *myo*-inositol dehydrogenase activity in membrane preparations of strain NCIB 621 was obtained in sodium phosphate buffer at pH 6.2, whereas the pH optimum of the quinoprotein inositol dehydrogenase described in our study is in the alkaline range (pH 8.75). However, this difference could be due to the different assay conditions used; the assay of Criddle and co-workers also contained DCPIP as the electron acceptor, but lacked PMS as a mediator. In our experiments, omitting PMS resulted in a general drop of *myo*-inositol dehydrogenase activity; however, similar to the findings of Criddle and co-workers, activity without PMS was higher at pH 6 than at pH 8.75 (data not shown). The K_m value of 60 μ M determined for the *myo*-inositol dehydrogenase of strain NCIB 621 (Criddle *et al.*, 1977) is significantly lower than the K_m value of 5 mM obtained in our study. In strain NCIB 621, solubilization of membrane-bound *myo*-inositol dehydrogenase was achieved with sodium deoxycholate, which was subsequently removed by gel chromatography (Criddle *et al.*, 1974). This information might be helpful for future attempts to purify quinoprotein inositol dehydrogenase from *G. oxydans* ATCC 621H.

Whereas information on membrane-bound inositol dehydrogenases is scarce, soluble NAD-dependent *myo*-inositol dehydrogenases and their genes have been characterized in several species including *Bacillus subtilis* (Ramaley *et al.*, 1979; Fujita *et al.*, 1991), *Klebsiella pneumoniae* (Berman & Magasanik, 1966) and *Sinorhizobium meliloti* (Galbraith *et al.*, 1998). *myo*-Inositol is a common compound in soil and plants that can be used for growth by these bacteria. The genome of *G. oxydans* ATCC 621H encodes proteins with similarity to NAD-dependent *myo*-inositol dehydrogenases and additional enzymes involved in cytoplasmic *myo*-inositol metabolism. Thus, in *G. oxydans*, oxidation of inositol might occur via both a cytoplasmic NAD-dependent and a membrane-bound PQQ-dependent dehydrogenase, as found for several other substrates (Matsushita *et al.*, 1994). However, as shown with mutant DW1 lacking the membrane-bound inositol dehydrogenase, the cytoplasmic inositol dehydrogenase cannot substitute for the function of the membrane-bound enzyme, i.e. provide enough energy for growth. Nonetheless, for unknown reasons, we found that *myo*-inositol is a rather poor growth substrate also for the wild-type under the conditions used.

As indicated by the lack of the respective activities in mutant DW1, quinoprotein inositol dehydrogenase also oxidized *allo*- and *muco*-inositol. The reaction products of quinoprotein inositol dehydrogenase are currently unknown; however, *myo*-inositol is probably oxidized to 2-keto-*myo*-inositol (*myo*-inosose-2) as reported for the soluble

myo-inositol dehydrogenases (e.g. Ramaley *et al.*, 1979) and the membrane-bound *myo*-inositol dehydrogenase described by Criddle *et al.* (1974).

Inositol dehydrogenases have been shown to participate in biotechnologically relevant processes; for example, they are involved in the synthesis of aminoglycoside antibiotics (Walker, 1995) and of the drug candidate D-*chiro*-inositol for treatment of type 2 diabetes and polycystic ovary syndrome (Yoshida *et al.*, 2006). Furthermore, *myo*-inositol dehydrogenases can be used in enzymic assays for diagnosis of diabetes in its early stages (Yamakoshi *et al.*, 2003). Further characterization of the membrane-bound quinoprotein inositol dehydrogenase, e.g. after purification of the protein, will elucidate its potential for future biotechnical applications.

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