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Characterization of a cryptic, pyrroloquinoline quinone-dependent dehydrogenase of *Gluconobacter* sp. strain CHM43

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ABSTRACT

We characterized the pyrroloquinoline quinone (PQQ)-dependent dehydrogenase 9 (PQQ-DH9) of *Gluconobacter* sp. strain CHM43, which is a homolog of PQQ-dependent glycerol dehydrogenase (GLDH). We used a plasmid construct to express PQQ-DH9. The expression host was a derivative strain of CHM43, which lacked the genes for GLDH and the membrane-bound alcohol dehydrogenase and consequently had minimal ability to oxidize primary and secondary alcohols. The membranes of the transformant exhibited considerable D-arabitol dehydrogenase activity, whereas the reference strain did not, even if it had PQQ-DH9-encoding genes in the chromosome and harbored the empty vector. This suggests that PQQ-DH9 is not expressed in the genome. The activities of the membranes containing PQQ-DH9 and GLDH suggested that similar to GLDH, PQQ-DH9 oxidized a wide variety of secondary alcohols but had higher Michaelis constants than GLDH with regard to linear substrates such as glycerol. Cyclic substrates such as *cis*-1,2-cyclohexanediol were readily oxidized by PQQ-DH9.

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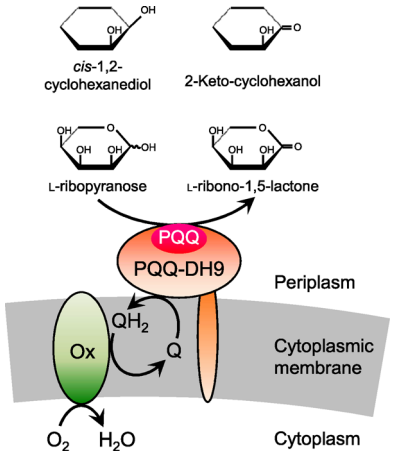
Graphical Abstract



Pyrroloquinoline quinone-dependent dehydrogenase 9 (PQQ-DH9) of *Gluconobacter* sp. strain CHM43 oxidizes cyclic secondary alcohols. Ox, ubiquinol oxidase; Q, ubiquinone.

Keywords: pyrroloquinoline quinone, PQQ-dependent dehydrogenase, acetic acid bacteria, orphan enzyme

Acetic acid bacteria are widely used for industrial purposes because they can oxidize sugars, alcohols, polyols, carbohydrates, and related compounds. Such oxidation reactions are incomplete processes that oxidize substrate only 1 or several times and are conducted by several primary dehydrogenases located on the periplasmic surface of the cytoplasmic membrane (Matsushita *et al.* 1994). For instance, *Gluconobacter* spp. oxidize D-sorbitol by the membrane-bound glycerol dehydrogenase to L-sorbose, which is accumulated in the culture at the nearly stoichiometric levels and is used for the precursor of the industrial vitamin C production (Pappenberger and Hohmann 2014). These dehydrogenases are distinguished by their cofactors: pyrroloquinoline quinone (PQQ; pink in Figure S1), flavin adenine dinucleotide (FAD; yellow in Figure S1), and molybdopterin (green in Figure S1). Based on the genomic data, PQQ-dependent proteins (quinoproteins) can be categorized into 6 groups according to the compositions of their subunits and their primary sequences in acetic acid bacteria (Matsutani and Yakushi 2018); however, a significant number of orphan quinoproteins remain uncharacterized.

According to the draft genome (Matsumoto *et al.* 2018), GenBank accession no. BADZ02000001 to BADZ02000044, we previously reported that the orphan PQQ-dependent dehydrogenase 9 (PQQ-DH9) in *Gluconobacter* sp. strain CHM43 is a homolog of membrane-bound glycerol dehydrogenase (GLDH; also referred to as polyol dehydrogenase or sorbitol dehydrogenase) (Adachi *et al.* 2001; Miyazaki *et al.* 2002). However, the functions of PQQ-DH9 remain unknown (Yakushi *et al.* 2018a). PQQ-DH9 consists of 2 gene products—GLF_2583 and GLF_2584—which are similar to the transmembrane subunit SldB and the catalytic subunit SldA of GLDH, respectively. The GLF_2583 and GLF_2584 proteins have amino acid identities of 97% and 82% to their counterparts SldB (GLF_2777) and SldA (GLF_2776) of the CHM43 strain, respectively.

GLDH has wide substrate specificity; it oxidizes "secondary alcohols" in the D-erythro configuration at the penultimate carbon, according to the Bertrand–Hudson rule (Hann, Tilden and Hudson 1938; Kulhánek 1989; Matsushita *et al.* 2003). Glycerol and D-sorbitol are oxidized by GLDH to yield dihydroxyacetone, which is used as a chemical suntanning agent, and L-sorbose, which is used as a precursor of vitamin C, respectively (Gupta *et al.* 2001; Pappenberger and Hohmann 2014). The oxidation of 1-(2-hydroxyethyl) amino-1-deoxy-D-sorbitol by GLDH produces 6-(2-hydroxyethyl) amino-6-deoxy-L-sorbose, which is a precursor of the antidiabetic drug miglitol (Yang *et al.* 2008). Recently, sugars such as D-fructose and L-ribose have been added to the list of GLDH substrates (Ano *et al.* 2017; Yakushi *et al.* 2018b). Herein, we attempted to characterize the enzymatic properties of PQQ-DH9 by comparing them to those of GLDH. We conducted a membrane-associated enzyme activity assay on a recombinant *Gluconobacter* strain created using a combination of gene deletion and plasmid-based overproduction.

Materials and methods

Chemicals

Yeast extract and Hipolypepton were obtained from Oriental Yeast (Tokyo, Japan) and Nihon Pharmaceutical (Tokyo, Japan), respectively. Restriction endonucleases were obtained from Toyobo (Osaka, Japan). 2,3-Butanediol was obtained from Wako Pure Chemical (Osaka, Japan). All other materials were of analytical grade and were purchased from commercial sources. For the enzyme assays, 2-hexanol, cyclohexanol, and 1-cyclohexylethanol were dissolved in ethanol.

Microorganism culture

Gluconobacter sp. CHM43 (NBRC101659; http://www.nite.go.jp/en/nbrc/index.html) (Moonmangmee *et al.* 2000), its Δ*adhAB* derivative SEI46, and its Δ*adhAB* Δ*sldBA* derivative TORI4 were used in this study (Yakushi *et al.* 2018b). All the *Gluconobacter* strains were cultivated in a sorbitol medium consisting of 50 g of D-sorbitol, 3 g of yeast extract, and 3 g of Hipolypepton per liter. For metabolite analyses, culture media were collected and centrifuged at 10 000 × *g* for 5 min at 4°C to remove the cells. The supernatants

were passed through a filter with 0.4-μm pores (Merck Millipore, Burlington, MA, USA) prior to analysis by high-performance liquid chromatography (HPLC). *Escherichia coli* strain DH5α was used to construct the plasmid (Hanahan 1983), which was cultivated in Luria Bertani medium at 30°C. Tetracycline was used at final concentrations of 10 μg mL^{-1} for *E. coli* and *Gluconobacter*.

Plasmid construction

PCR analysis was performed using the following components: the genomic DNA of *Gluconobacter* sp. CHM43, which had been purified as described in the literature (Marmur 1961); a pair of oligonucleotides [ex-GLF_2583-Xba(+) (5′-*tctaga*acctcaccttctacag-3′; the *Xba*I recognition site is underlined) and ex-GLF_2584-Kpn(−) (5′-*ggtacc*tgcccgcatcaagaaatg-3′; the *Kpn*I recognition site is underlined]; and Herculase DNA Polymerase (Stratagene). Approximately 2.7-kb PCR products were digested with *Xba*I and *Kpn*I prior to insertion into the corresponding sites of pCM62 (Marx and Lidstrom 2001). The resulting p62sGLDH2-11 plasmid was used to transform the TORI4 (Δ*adhAB* Δ*sldBA*) strain via electroporation, as described previously (Yakushi *et al.* 2018b).

Membrane preparation

The TORI4 (Δ*adhAB* Δ*sldBA*) and SEI46 (Δ*adhAB*) strains harboring the pCM62 plasmid and the TORI4 strain harboring the p62SGLDH2-11 plasmid were cultivated in 100 mL of sorbitol medium containing 10 μg mL^{-1} tetracycline; the culture was shaken at 30°C until the late exponential phase of growth was reached. The cells were harvested at 4°C by centrifugation at 10 000 × *g* for 10 min, then washed twice with 10 mM MES-KOH (pH 6.0) containing 2 mM $CaCl_2$. The cell paste was resuspended in 4 volumes of the same buffer (4 mL per 1 g wet weight of the cells). The cell suspensions were passed through a French pressure cell press (1100 kg cm^{-2}) twice. After centrifugation for 10 min at 10 000 × *g* and 4°C to remove cell debris, the supernatants were centrifuged for a further 1 h at 100 000 × *g* and 4°C. The precipitates were resuspended in the same buffer and used as the membrane fraction.

Enzyme assays

Enzyme activity was measured spectrophotometrically at 522 nm by phenazine methosulfate (PMS) reductase activity coupled with 2,6-dichlorophenol indophenols (DCPIP) at 25°C. The reaction mixture contained the enzyme solution, 50 mM Na^+-acetate (pH 5.0), 0.2 mM PMS, 0.11 mM DCPIP, and 100 mM substrate (in the case of 2-hexanol, cyclohexanol and 1-cyclohexylethanol were used at 25 mM and were dissolved in ethanol prior to the enzyme assay). One unit of enzyme activity was defined as the amount of enzyme required to catalyze the conversion of 1 μmol of substrate per min. A millimolar extinction coefficient of 8.6 (cm^{-1} mM^{-1}) was used for DCPIP (Armstrong 1964).

EDTA treatment and holoenzyme formation

The pH of EDTA stock solution was adjusted to 6.0 with NaOH. A membrane suspension comprising 10 mg protein mL^{-1} in 10 mM MES-KOH (pH 6.0) was incubated with 2, 5, 10, and 20 mM EDTA on ice overnight.

The EDTA-treated membranes were sedimented by ultracentrifugation (100 000 × *g*, 1 h, and 4°C) and resuspended in 10 mM MES-KOH (pH 6.0) containing 2 mM $CaCl_2$. This step was repeated once, and the holoenzyme was formed by incubating the resulting membrane suspension at 25°C for 30 min with 5 μM PQQ and 2 mM $CaCl_2$.

Biotransformation

Five milliliters of the reaction mixture consisting of 1.0 mg protein mL^{-1} of the membranes and 100 mM substrates in 50 mM Na^+-acetate (pH 5.0) was incubated in a disposable 50-mL plastic tube with an eight-hole cap (hole diameters of 2 mm), which was shaken at 150 min^{-1} and 30°C for 24 h. An aliquot (500 μL) of the reaction mixture was taken periodically, and the membranes were removed after ultracentrifugation for 1 h at 100 000 × *g* and 4°C. Subsequently, the supernatant was passed through a filter with 0.4-μm pores.

Analytical procedures

The filtered samples were analyzed using an HPLC system (Shimadzu) equipped with a refractive index (RI) detector and a photodiode array (PDA). Distilled and deionized water was used as the mobile phase of a Pb^{2+}-loaded cation-exchange column (SUGAR SP0810; 8.0 mm ID × 300 mm L; Shodex; Showa Denko KK, Kawasaki, Japan) for chromatography at 80°C at a flow rate of 0.5 mL min^{-1}. Chromatographic peak detection and quantification was done using retention time and peak height. Protein concentrations were determined using the modified Lowry method, with bovine serum albumin as the standard (Dulley and Grieve 1975).

Results and discussion

PQQ-DH9 complements L-sorbose production in the GLDH-deficient strain

To characterize PQQ-DH9, we attempted to express the *GLF_2583* and *GLF_2584* genes using the broad-host-range plasmid vector pCM62 in the Δ*adhAB* Δ*sldBA* derivative of CHM43, because membrane-bound alcohol dehydrogenase (ADH) and GLDH are the predominant enzymes of the primary and secondary alcohol dehydrogenases, respectively (Matsushita *et al.* 2003; Yakushi and Matsushita 2010). Therefore, we constructed a Δ*adhAB* Δ*sldBA* strain—which harboring p62sGLDH2-11, a pCM62 derivative carrying the gene for PQQ-DH9 (hereafter, referred to as the *pqq9*$^+$ strain)—and a strain harboring the empty vector pCM62 (hereafter, referred to as the reference strain). For comparison, we constructed a Δ*adhAB* strain harboring the control vector pCM62, which included the *sldBA* gene for GLDH (hereafter referred to as the *sldBA*$^+$ strain).

We examined the function of PQQ-DH9 *in vivo* by cultivating the *Gluconobacter* strains in D-sorbitol, that is, L-sorbose production and the accompanying D-sorbitol consumption were evaluated (Figure 1). Wild-type CHM43 harboring pCM62 was included for comparison. Although the growth of the GLDH-deficient strain was similar to that of the wild-type strain, L-sorbose production by the GLDH-deficient strain (Δ*sldBA* Δ*adhAB*) was abolished (Figure 1 and Figure S2). The *pqq9*$^+$ strain produced L-sorbose at a lower rate than the wild-type. The sorbose production rates were 7.0, 6.7, 2.7, and 0.056 mM h^{-1} for the wild-type, *sldBA*$^+$, *pqq9*$^+$, and reference strains, respectively. D-Sorbitol consumption by the reference strain without L-sorbose accumulation is presumably due to assimilation of D-sorbitol (Soemphol *et al.* 2012). The results indicate that PQQ-DH9 can function as

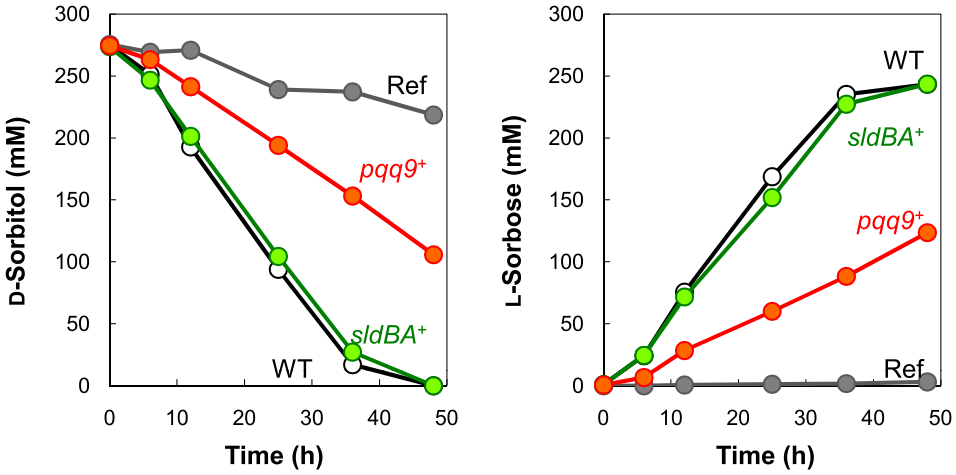


Figure 1. PQQ-DH9 complements deficiency in L-sorbose production by the Δ*adh*AB Δ*sld*BA strain. *Gluconobacter* strains were cultivated on sorbitol medium. The wild-type strain harboring pCM62 (WT, white circles), the Δ*adh*AB Δ*sld*BA strain harboring pCM62 (Ref for the reference strain, gray circles), the Δ*adh*AB strain harboring pCM62 (*sldBA*$^+$, lime green circles), and the Δ*adh*AB Δ*sld*BA strain harboring p62sGLDH2-11 (*pqq9*$^+$, orange circles). D-Sorbitol and L-sorbose levels in the medium were determined by high-performance liquid chromatography.

a primary dehydrogenase in the sorbitol oxidation system of *Gluconobacter*.

Expression of PQQ-DH9

As mentioned above, the reference strain (Δ*adh*AB Δ*sld*BA harboring pCM62), which had PQQ-DH9-encoding genes, produced a marginal amount of L-sorbose. This suggested that the genes were rarely expressed in the chromosome under the cultivation conditions used in the present experiment. The membranes of the reference strain oxidized D-arabitol at a rate below the detection limit, while those of the *pqq9*$^+$ strain had a D-arabitol dehydrogenase activity of 0.20 U (mg of protein)$^{-1}$ (Figure 2). These results suggest that PQQ-DH9 is a cryptic enzyme. It is currently under investigation what culture conditions induce the expression of PQQ-DH9, which is one of the most important issues to be answered. In contrast, the membranes of the *sldBA*$^+$ strain exhibited higher dehydrogenase activity (0.41 U mg of protein)$^{-1}$ owing to the presence of GLDH (Figure 2). The optimum pH for PQQ-DH9 dehydrogenase activity was 5.0-6.0, that is, similar to that for GLDH dehydrogenase activity. These results indicated that PQQ-DH9 was functionally expressed by the constructed plasmid in the *Gluconobacter* strain and supported the suggestion mentioned above that it is expressed in small amounts by the genome.

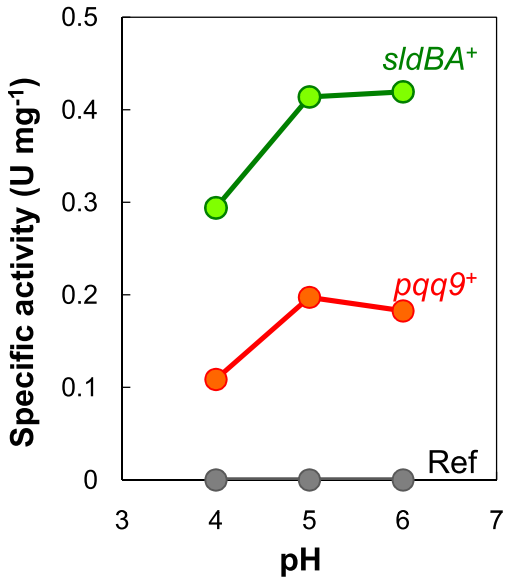


Figure 2. D-Arabitol dehydrogenase assay indicates the functional expression of PQQ-DH9. D-Arabitol dehydrogenase activity in the membranes of the Δ*adh*AB strain harboring pCM62 (*sldBA*$^+$, lime green circles), the Δ*adh*AB Δ*sld*BA strain harboring pCM62 (Ref for the reference strain, gray circles), and the Δ*adh*AB Δ*sld*BA strain harboring p62sGLDH2-11 (*pqq9*$^+$, orange circles) grown on sorbitol medium were determined by PMS/DCPIP assay with 100 mM D-arabitol in 50 mM Na^+-acetate (pH 4.0, 5.0, and 6.0) at 25°C. The membranes of the reference strain (Ref) had activities below the detection limit (0.001 U mg^{-1}).

Substrate specificity of PQQ-DH9

We determined the substrate specificities of the dehydrogenases in membranes at pH 5.0 to evaluate the substrate specificity of PQQ-DH9. The reference strain demonstrated considerable activity on a wide variety of substrates, particularly on sugars, owing to the presence of membrane-bound glucose dehydrogenase (Table 1). Therefore, we evaluated the dehydrogenase activities of PQQ-DH9 and GLDH by subtracting the specific activity of the reference strain from the specific activity of the *pqq9*$^+$ strain or that of the *sldBA*$^+$ strain, and in each case we referred to the result as the Δ activity (Table 1). Similar to the membranes of the *sldBA*$^+$ strain, those of the *pqq9*$^+$ strain oxidized a wide variety of substrates. The *pqq9*$^+$ strain had a higher Δ activity with regard to *cis*-1,2-cyclohexanediol than the *sldBA*$^+$ strain. In addition, L-ribose was oxidized well by both membranes. However, glycerol, ribitol, D-mannitol, D-sorbitol, isopropanol, and 1,2-butanediol were not oxidized well by the *pqq9*$^+$ membranes, that is, the *pqq9*$^+$ membranes had much lower activities with regard to those substrates than the *sldBA*$^+$ membranes.

PQQ-DH9 preferentially oxidized *cis*-1,2-cyclohexanediol over the *trans* isomer (Table 1). The Δ activity values of the *pqq9*$^+$ membranes were 520 and 10 mU mg^{-1} for the *cis* and *trans* forms, respectively. However, the *sldBA*$^+$ strain oxidized the *cis*- and *trans*-forms at 240 and 230 mU mg^{-1},

Table 1. Substrate specificities of pyrroloquinoline quinone-dependent dehydrogenase 9 and glycerol dehydrogenase

	Reference	*pqq9*$^+$		*sldBA*$^+$
	Specific activity (mU mg^{-1})[a]	Specific activity (mU mg^{-1})[a]	Δ activity (mU mg^{-1})[b]	Δ activity (mU mg^{-1})[c]
Glycerol	4.0 ± 5	18 ± 10	14	490
meso-Erythritol	0 (n = 2)	190 (n = 2)	190	330
D,L-Threitol	0 (n = 2)	3.1 (n = 2)	3.1	25
Xylitol	0 (n = 2)	0 (n = 2)	0	3.3
Ribitol	0 (n = 2)	28 (n = 2)	28	260
D-Arabitol	0	170 ± 20	170	400
L-Arabitol	0	0	0	4.6
Galactitol	0 (n = 2)	0 (n = 2)	0	8.5
D-Mannitol	0	27 ± 10	27	210
D-Sorbitol	2.8 ± 3	55 ± 10	52	410
Inositol	31 ± 19	0 ± 0	< 0	< 0
Isopropanol	8.0 ± 8	35 ± 10	27	240
2-Butanol	26 (n = 2)	140 (n = 2)	114	380
2-Hexanol	7.0 (n = 2)	30 (n = 2)	23	78
1,2-Butanediol	2.1 (n = 2)	80 (n = 2)	78	460
1,3-Butanediol	11 (n = 2)	65 (n = 2)	54	140
2,3-Butanediol	26 ± 8	400 ± 50	370	770
2,4-Pentanediol	25 ± 7	85 ± 4	60	240
1,2-Cyclopentandiol	119 (n = 2)	350 (n = 2)	230	630
cis-1,2-Cyclohexanediol	35 ± 13	550 ± 90	520	240
trans-1,2-Cyclohexanediol	0 ± 0	10 ± 0.4	10	230
1-Cyclohexylethanol	6.3 (n = 2)	22 (n = 2)	16	43
Cyclohexanol	5.1 (n = 2)	84 (n = 2)	79	270
Glyceraldehyde	63 (n = 1)	140 (n = 1)	77	120
D-Xylose	220 ± 90	120 ± 50	< 0	23
D-Ribose	14 ± 3	90 ± 30	76	120
L-Ribose	25 ± 2	200 ± 7	180	210
D-Arabinose	9.8 (n = 2)	5.2 (n = 2)	< 0	18
L-Arabinose	157 (n = 2)	120 (n = 2)	< 0	16
D-Lyxose	5.5 ± 3	100 ± 10	95	89
D-Galactose	240 ± 100	130 ± 10	< 0	45
D-Gluconate	140 ± 40	130 ± 10	< 0	82

[a]Specific activity was calculated from 3 independent enzyme assays, shown in mean value ± SD. Otherwise, results of the numbers (n) of enzyme assays indicated in parentheses were used for the calculation of mean value.
[b]Δ activity in the *pqq9*$^+$ membrane was calculated by subtracting the mean specific activity of the reference strain from the corresponding mean specific activity of the *pqq9*$^+$ strain.
[c]Δ activity in the *sldBA*$^+$ membrane was calculated by subtracting the specific activity of the reference strain (n = 1) from the corresponding specific activity of the *sldBA*$^+$ strain (n = 1).

respectively, which was consistent with the previous observation by Moonmangmee *et al.* (2001).

Michaelis constants for the substrates

The K_M values of dehydrogenases in the membrane fractions of the *pqq9*$^+$ and *sldBA*$^+$ strains were determined with regard to glycerol, L-ribose, D-arabitol, 2,3-butanediol, and the *cis*- and *trans*-isoforms of 1,2-cyclohexanediol (Table 2). The K_M values of the *pqq9*$^+$ membranes with regard to glycerol, D-arabitol, and 2,3-butanediol were higher than those of the *sldBA*$^+$ membranes. However, the K_M values of the 2 membranes with regard to L-ribose and *cis*-1,2-cyclohexanediol were comparable. If, as we have suggested (Yakushi *et al.* 2018b), GLDH oxidizes the pyranose form of L-ribose to produce L-ribonolactone, it is plausible that the affinity of PQQ-DH9 for cyclic substrates is comparable to that of GLDH, but its affinity for linear substrates is lower than that of GLDH.

The K_M value of the *pqq9*$^+$ membranes with regard to 1,2-cyclohexanediol was higher for the *trans*-isoform than for the *cis*-isoform, whereas the K_M value of the *sldBA*$^+$ membranes with regard to 1,2-cyclohexanediol was lower for the *trans*-isoform than for the *cis*-isoform (Table 2). The mechanism by which the 2 enzymes discriminate between the *cis*- and *trans*-isoforms is intriguing and requires further biochemical analyses including structural determinations.

Table 2. K_M values (mM) of membrane-associated dehydrogenases with regard to various substrates[a]

	pqq9$^+$	sldBA$^+$
Glycerol	450 ± 40	8.4 ± 0.7
L-Ribose	41 ± 2	61 ± 3
D-Arabitol	88 ± 4	5.5 ± 0.3
2,3-Butanediol	9.4 ± 0.4	1.8 ± 0.2
cis-1,2-Cyclohexanediol	2.8 ± 0.3	4.6 ± 0.6
trans-1,2-Cyclohexanediol	19 ± 3	0.49 ± 0.07

[a]The K_M values were determined by a phenazine methosulfate/2,6-dichlorophenol indophenol assay using membranes from the *pqq9*$^+$ and *sldBA*$^+$ strains grown on sorbitol medium with various concentrations of the substrate in 50 mM Na^+-acetate (pH 5.0) at 25°C, followed by data analysis using KaleidaGraph (ver. 4.5, Synergy Software). See Figure S3.

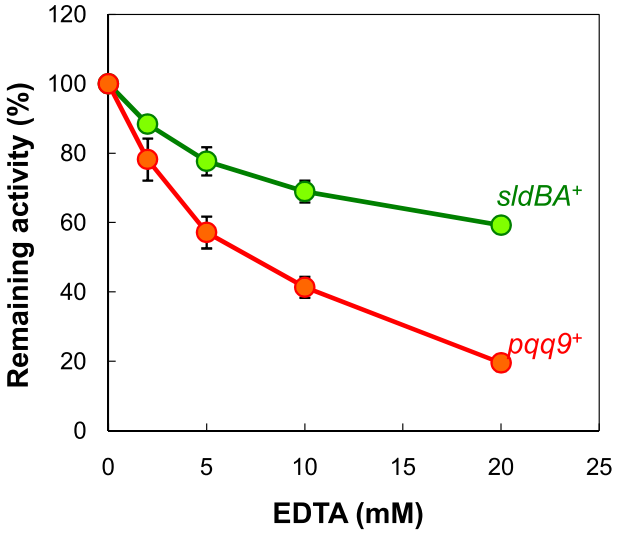


Figure 3. Effects of EDTA treatment on dehydrogenase activity. The membranes of the Δ*adhAB* strain harboring pCM62 (*sldBA*$^+$, lime green circles) and the Δ*adhAB* Δ*sldBA* strain harboring p62sGLDH2-11 (*pqq9*$^+$, orange circles) were treated with 2, 5, 10, and 20 mM EDTA on ice overnight. Then, D-arabitol dehydrogenase activity in the EDTA-treated membrane was determined by PMS/DCPIP assay in 50 mM Na^+-acetate (pH 5.0) at 25°C. The remaining activity relative to that of the control membrane was expressed as a percentage.

Effects of EDTA on dehydrogenase activity

We examined the sensitivity of the dehydrogenases to EDTA. Because PQQ is attached to the enzyme via Ca^{2+} (Ghosh *et al.* 1995), EDTA reversibly inactivates the PQQ-dependent enzyme by chelating Ca^{2+}. Treatment with various concentrations of EDTA of up to 20 mM on ice inactivated D-arabitol dehydrogenase in both the *pqq9*$^+$ and *sldBA*$^+$ membranes (Figure 3). At 20 mM EDTA, the *sldBA*$^+$ membranes retained 60% of the activity of the control membranes in the absence of EDTA, whereas the *pqq9*$^+$ membranes lost 80% of their activity. The results suggest that PQQ-DH9 is less stable than GLDH following treatment with EDTA. We confirmed the recovery of activity by adding 5 μM PQQ and 2 mM Ca^{2+} after removing the EDTA (data not shown). Therefore, the detachment of PQQ and Ca^{2+} from the enzymes accounted for the reduced activities resulting from EDTA treatment, indicating that PQQ-DH9 works in a PQQ-dependent manner. This suggests that PQQ and Ca^{2+} are detached more readily from PQQ-DH9 than from GLDH.

Biotransformation of D-arabitol, 2,3-butanediol, *cis*-1,2-cyclohexanediol, and L-ribose

We examined the oxidation products of D-arabitol, 2,3-butanediol, *cis*-1,2-cyclohexanediol, and L-ribose obtained using the membrane fractions of *sldBA*$^+$ and *pqq9*$^+$, as described in the Materials and Methods section. The reaction products from the 2 membranes were eluted at retention times were close to each other: 20.5, 30.3, 43.2, and 20.6 min for the oxidation products of D-arabitol, 2,3-butanediol, *cis*-1,2-cyclohexanediol, and L-ribose, respectively (Figue S4). Therefore, we concluded that the products resulting from the activity of PQQ-DH9 were the same as those resulting from the activity of GLDH.

We found 2 peaks in the HPLC chromatogram of 2,3-butanediol at retention times of 31.9 and 32.9 min in the product of the 0-h reactions (Figure S4C), both of which were consumed as a function of the duration of the oxidation reaction with the 2 membranes. We refer to the isoforms eluted at 31.9 and 32.9 min as substrates A and B, respectively. Commercially available 2,3-butanediol is a mixture of 3 isoforms: 2*S*, 3*S*-, 2*R*,3*R*-, and the *meso*-form, and substrate B would be one of these isoforms. Substrate B was consumed at a lower rate than substrate A (Figure S5) and oxidized more slowly by the *pqq9*$^+$ membranes than by the *sldBA*$^+$ membranes (Figure S5B). Because the peak intensity of the oxidation product (30.3 min retention time) produced by the *sldBA*$^+$ membranes was higher than that produced by the *pqq9*$^+$ membranes (Figure S5C), the difference in the peak intensities corresponded to the difference in the consumption of substrate B (Figure S5B). Therefore, we suggest that PQQ-DH9 and GLDH oxidize 2,3-butanediol in a stereoisomer-specific manner. The oxidation product of 2,3-butanediol would be acetoin, which is a valuable biochemical used in the dairy, cosmetic, and pharmaceutical industries, as well as in chemical synthesis. *G. oxydans* strain DSM2003 produces (3*S*)-acetoin and (3*R*)-acetoin from (2*S*,3*S*)- and (2*R*,3*R*)-2,3-butanediol, respectively, and it converts *meso*-2,3-butanediol stereoselectively to (3*S*)-acetoin (Wang *et al.* 2013). Since (3*S*)-acetoin and (3*R*)-acetoin run on HPLC as a single peak (Wang *et al.* 2013), the oxidation product (30.3 min retention time) might contain the 3*S*- and 3*R*-isomers of acetoin.

Several attempts have been made to characterize the orphan PQQ-dependent dehydrogenases of *Gluconobacter oxydans* from the metagenome (Mientus *et al.* 2017; Peters *et al.* 2013, 2017). We characterized the GLF_2583-2584 proteins as PQQ-DH9, which was similar to GLDH but had different substrate specificities: PQQ-DH9 oxidized linear substrates—such as glycerol, ribitol, and D-mannitol—at lower rates and with lower affinities than GLDH, whereas it oxidized circular substrates—such as *cis*-1,2-cyclohexanediol and the pyranose form of L-ribose—with affinities and rates comparable to those of GLDH. Since the reaction products of the *pqq9*$^+$ membranes showed similar retention times to those of the *sldBA*$^+$ membranes on HPLC, it is plausibly speculated that 2-keto-cyclohexanol is produced from *cis*-1,2-cyclohexanediol, and L-ribose is converted to L-ribono-1,5-lactone and subsequently to L-ribonic acid, based on analogy with GLDH catalysis (Yakushi *et al.* 2018b). We suggested that PQQ-DH9 is a cryptic enzyme, since the activity of the chromosomal expression level was not detected in our experiment. Culture conditions for the expression of PQQ-DH9 are the important issues to understand the physiological role of this enzyme.

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Supplementary material

Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Author contribution

T.M.N. and T.Y. designed the study. T.M.N. performed the main parts of the experiments. K.N. supported the experiments. M.M. contributed to bioinformatics. T.M.N. wrote the initial draft of the manuscript. N.K. and K.M. supervised. N.K., Y.A., O.A., K.M., and T.Y. edited the manuscript.

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Disclosure statement

No potential conflicts of interest was reported by the authors.

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