Enzyme evolution in *Rhodobacter sphaeroides*: selection of a mutant expressing a new galactitol dehydrogenase and biochemical characterization of the enzyme

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A gain of function mutant of *Rhodobacter sphaeroides* Si4, capable of growing on galactitol, was isolated from a chemostat culture. Continuous cultivation was performed for 54 d with a limiting concentration (1 mM) of the substrate D-glucitol and an excess (20 mM) of the non-metabolizable galactitol. The mutant strain, *R. sphaeroides* D, grew in galactitol minimal medium with a growth rate of 0.11 h⁻¹ ($t_d = 6.3$ h). In crude extracts of *R. sphaeroides* D, a specific galactitol dehydrogenase (GDH) activity of 380 mU mg⁻¹ was found, while the wild-type strain exhibited GDH activities lower than 50 mU mg⁻¹ when grown on different polyols. Unlike mannitol, sorbitol or ribitol dehydrogenase from the wild-type strain, the new GDH was expressed constitutively. To study whether it was a newly evolved enzyme or an improved side activity of one of the pre-existing polyol dehydrogenases, GDH was purified to apparent homogeneity by ammonium sulfate precipitation and chromatography on Phenyl-Sepharose, Q-Sepharose, Matrex Gel Red-A and Mono-Q. The relative molecular mass (M_r) of the native GDH was 110 000. SDS-PAGE resulted in one single band that represented a polypeptide with a M_r of 28 000, indicating that the native protein is a tetramer. The isoelectric point of GDH was determined to be pH 4.2. The enzyme was specific for NAD⁺ but catalysed the oxidation of different sugar alcohols as well as different diols and secondary alcohols. The apparent K_m values were: galactitol, 240 mM; D-threitol, 85 mM; 1,2-hexandiol, 0.2 mM; NAD⁺, 12 μ M; L-erythrulose, 144 mM; acetoin, 62 mM; dihydroxyacetone, 48 mM; and NADH, 4 μ M. GDH activity was strictly dependent on the presence of divalent cations. The properties of GDH are different to any of the three polyol dehydrogenases from *R. sphaeroides* Si4. In addition, comparison of the N-terminal amino acid sequence of the isolated GDH with the N-terminal sequence of the other three polyol dehydrogenases clearly demonstrates that GDH is an additional enzyme, so far unrecognized in the wild-type strain.

Keywords: *Rhodobacter sphaeroides*, polyol metabolism, galactitol dehydrogenase, chemostat, enzyme evolution

INTRODUCTION

The ability of bacteria to adapt to novel nutritional conditions is well known (Lerner *et al.*, 1964; Hegeman &

Abbreviations: GDH, galactitol dehydrogenase; MDH, mannitol dehydrogenase; RDH, ribitol dehydrogenase; SDH, sorbitol dehydrogenase.

Dedicated to Professor Dr Gerhard Gottschalk on the occasion of his 60th birthday.

Rosenberg, 1970; Hartley *et al.*, 1972), and mutants with improved or new metabolic capabilities have been isolated readily from cultures grown under selective pressure in a chemostat (Dykhuizen & Hartl, 1983). New metabolic capabilities may arise by regulatory mutations resulting in the overproduction of an enzyme with a marginal specificity for the new substrate (LeBlanc & Mortlock, 1971; Rigby *et al.*, 1974; Chen *et al.*, 1984) or by mutations in structural genes leading to altered or new substrate

specificities of the respective enzymes (Hall, 1981; Doten & Mortlock, 1984; Buchholz *et al.*, 1988; Srinivasan *et al.*, 1993; Lambert *et al.*, 1994). Finally, cryptic genes may be expressed (Hall *et al.*, 1983) which often results in the constitutive formation of the encoded enzyme (Szymona & Doudoroff, 1960; Rode & Giffhorn, 1982, 1983). Therefore, growth of bacteria in a chemostat in the presence of limiting concentrations of a utilizable substrate and excess of a non-utilizable but chemically related substrate can be used as a promising strategy for obtaining enzymes with new or favourably altered properties. In this respect polyol dehydrogenases have been shown to be model systems for studying experimental enzyme evolution. These enzymes are relatively unspecific and can be produced with elevated levels or altered in their specificities under conditions of selective pressure (Wu *et al.*, 1968; Rigby *et al.*, 1974; Wawzonek Thompson & Krawiec, 1983; Doten & Mortlock, 1984; Kyslik & Dobisova, 1986; Buchholz *et al.*, 1988). In view of developing applications of polyol dehydrogenases in synthetic carbohydrate chemistry (Drueckhammer *et al.*, 1991; Schwartz *et al.*, 1994; Mayers-Küntzer *et al.*, 1994), the exploitation of new polyol dehydrogenases using the method of enzyme evolution seems to be a promising approach.

One objective of our studies is the phototrophic bacterium *Rhodobacter sphaeroides* Si4 which grows on a variety of polyols including D-mannitol, D-glucitol (sorbitol), D-arabitol, ribitol and xylitol but does not grow on galactitol. The polyols are metabolized by NAD-linked dehydrogenation to the corresponding sugars by virtue of inducible mannitol dehydrogenase (MDH; EC 1.1.1.67) (Schneider & Giffhorn, 1989), ribitol dehydrogenase (RDH; EC 1.1.1.56) (Kahle *et al.*, 1992) and sorbitol dehydrogenase (SDH; EC 1.1.1.14) (Schauder *et al.*, 1995). Although both SDH and MDH possess marginal specificities for galactitol, apparently the organism cannot take advantage of these activities for utilizing this carbon source for growth. Selective pressure on *R. sphaeroides* Si4 was established by simultaneous exposure of the organism to limiting amounts of the utilizable substrate D-glucitol in the presence of an excess of the non-metabolizable analogue galactitol. Here we report the selection of a galactitol-metabolizing mutant of *R. sphaeroides* Si4, the characterization of a unique and newly expressed galactitol dehydrogenase (GDH) and its suitability for the synthesis of rare sugars from polyols.

METHODS

Organisms and growth conditions. The purple non-sulfur bacterium *Rhodobacter sphaeroides* strain Si4 (DSM 8371) is an isolate from this laboratory (Rode & Giffhorn, 1983). *R. sphaeroides* D is a mutant of strain Si4 capable of growing on galactitol as sole source of carbon and energy. *R. sphaeroides* was grown heterotrophically at 30 °C. The medium for enzyme evolution contained the following components: D-glucitol, 1 mM; galactitol, 20 mM; KH_2PO_4 , 7.4 mM; NH_4Cl , 9.4 mM; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.6 mM; NaCl, 6.8 mM; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.34 mM; 10 × trace element solution SL4 (Pfennig & Lippert,

1966), 1.0 ml l⁻¹; 10 × vitamin solution (Rode & Giffhorn, 1983), 1.0 ml l⁻¹. The pH was adjusted to 6.8 with NaOH. Batch cultivations of *R. sphaeroides* D and Si4 were performed in the same minimal medium with the appropriate carbon source at a concentration of 10 mM.

Enzyme assays and protein determination. Polyol dehydrogenase activities were determined as described previously (Schneider & Giffhorn, 1989). Protein concentrations were determined by the method of Goa (1953) with crystalline bovine serum albumin as standard.

Purification of GDH. Wet cells (61 g) were suspended in 180 ml Bistris/HCl buffer (20 mM, pH 6.5 + 1 mM MgCl_2) and 10 mg DNase I was added. Cells were disintegrated by two passages through a French press at 11 000 N cm⁻². Subsequently the cell debris was removed by centrifugation at 4 °C and 53 000 g for 30 min.

Unless otherwise specified all following operations were carried out at 4 °C.

Step 1. During stirring on ice, saturated $(\text{NH}_4)_2\text{SO}_4$ solution was added dropwise to the crude extract until 30 % saturation was reached. The precipitated proteins were removed by centrifugation at 30 000 g for 30 min. The supernatant was filtered through a nitrocellulose 0.45 µm pore size filter.

Step 2. The protein solution from step 1 was applied to a C 16/20 Phenyl Sepharose column (Pharmacia), containing 23 ml of the resin, equilibrated with Bistris buffer (20 mM, pH 6.5 + 1 mM MgCl_2) with 25 % $(\text{NH}_4)_2\text{SO}_4$ saturation. The proteins were eluted with a linear $(\text{NH}_4)_2\text{SO}_4$ gradient (25–0 % saturation) in 250 ml Bistris buffer (20 mM, pH 6.5 + 1 mM MgCl_2) and 7 ml fractions were collected. Fractions containing high GDH activities were pooled and dialysed against Bistris/HCl buffer (20 mM, pH 6.5 + 1 mM MgCl_2).

Step 3. The dialysed enzyme solution was applied to a XK 50/30 Q-Sepharose column with a bed volume of 80 ml, equilibrated with Bistris/HCl buffer (20 mM, pH 6.5 + 1 mM MgCl_2). The enzyme was eluted with a linear NaCl gradient (0.1–0.4 M) in 480 ml Bistris/HCl buffer and 7 ml fractions were collected. Fractions containing high enzyme activity were pooled and dialysed against Tris/HCl buffer (20 mM, pH 7.5 + 1 mM MgCl_2 + 0.1 M KCl).

Step 4. The dialysed enzyme solution was applied to a C 26/40 Matrex Gel Red A affinity chromatography column with a bed volume of 42 ml, equilibrated with Tris/HCl buffer (20 mM, pH 7.5 + 1 mM MgCl_2 + 0.1 M KCl). The enzyme was eluted with a linear KCl gradient (0.1–0.5 M) in 420 ml Tris/HCl buffer and 5 ml fractions were collected. Fractions containing high GDH activity were pooled and concentrated by ultrafiltration through a YM 10 membrane (Amicon) to a volume of 2 ml and dialysed against Tris/HCl buffer (20 mM, pH 8.0 + 1 mM MgCl_2).

Step 5. The enzyme preparation of step 4 was further purified by FPLC anion exchange chromatography on Mono-Q. The enzyme solution was applied to a Mono Q HR 5/5 column equilibrated with Tris/HCl buffer (20 mM, pH 8.0 + 1 mM MgCl_2 + 0.1 M NaCl). The enzyme was eluted at a flow rate of 1 ml min⁻¹ with a linear NaCl gradient (0.1–0.5 M) in 20 ml Tris/HCl buffer. Fractions of 0.5 ml were collected at a flow rate of 1 ml min⁻¹. Fractions containing GDH activity were pooled.

Gel electrophoresis. Analytical PAGE was carried out in 10 % (w/v) slab gels using the Tris–Glycine buffer system (Davis, 1964). The gels were run at a current of 20 mA. Proteins were

visualized by silver staining (Merril *et al.*, 1981). For activity staining of GDH, the gels were incubated for 5 min in a staining solution containing the following components in a final volume of 10 ml: 0.2 M Tris/HCl (pH 9.0), 5 ml; 90 mM NAD⁺ solution, 100 µl; nitro-blue tetrazolium chloride (10 mg ml⁻¹), 100 µl; 5-methylphenazonium methylsulfate (1 mg ml⁻¹), 100 µl, galactitol solution (0.2 M), 3 ml.

SDS-PAGE was performed in 15% slab gels (Laemmli, 1970). The gels were run at a current of 10 mA per gel. For molecular mass determination of protein subunits, glutamate dehydrogenase (*M_r* 55 600), aldolase (39 200), triose-phosphate isomerase (26 000), trypsin inhibitor (20 100) and lysozyme (14 300) were used as standards.

Linear polyacrylamide gradient gels (4–20%, w/v) were run for 16 h at 150 V and subsequently at 200 V for 6 h (Anderson *et al.*, 1972).

Determination of the isoelectric point. Isoelectric focussing was performed in polyacrylamide gels (5%) containing Servalyt Precotes pH 3–10 (Serva). Focussing was carried out at 400 V for 4 h. The standard test mixture with proteins of known pI included amyloglucosidase (pH 3.5), ferritin (pH 4.4), albumin (pH 4.7), β-lactoglobulin (pH 5.3), conalbumin (pH 5.9), horse myoglobin (pH 7.3), whale myoglobin (pH 8.3), ribonuclease (pH 9.45) and cytochrome c (pH 10.65).

Determination of the reaction products. The sugars produced through oxidation of different polyols by GDH were identified using HPLC. To produce the sugars for product analysis, enzyme reactions were performed in a volume of 500 µl. The reaction mixture contained the following components: 100 mM Tris/HCl (pH 8.5), 9 mg polyol, 10 mg sodium pyruvate, 1.4 µmol NAD, 5 U lactate dehydrogenase, and 0.22 U GDH. After incubation at 28 °C for 24 h, the sugar produced was identified by ligand exchange chromatography with HPLC on a Ca²⁺-loaded cation exchange matrix (Carbohydrate Ca²⁺, 300 × 7.8, BC-100, Benson). The column temperature was 85 °C. Water was used as mobile phase with a flow rate of 0.8 ml min⁻¹. Detection was performed at 35 °C with a refractive index detector (Beckman). The observed retention times were compared with those for authentic sugars, which were 14.6 min for galactitol, 14.8 min for xylitol, 8.5 min for xylose, 8.6 min for galactose, 9.8 min for xylulose and 11.6 min for tagatose.

Chemicals and enzymes. Coenzymes, enzymes and molecular mass standards were purchased from Boehringer Mannheim. Sugars, polyols, diols and alcohols were obtained from Serva or Fluka. Phenyl-Sepharose, Q-Sepharose, Sephacryl S 300 and Mono-Q were from Pharmacia, and Matrex Gel Red A from Amicon.

RESULTS

Selection of a galactitol utilizing mutant of *R. sphaeroides* Si4

R. sphaeroides Si4 is capable of utilizing the five polyols D-glucitol (sorbitol), D-mannitol, D-arabitol, xylitol and ribitol as carbon source, but cannot grow on galactitol (Schneider & Giffhorn, 1989). To select a galactitol-utilizing mutant, an experimental design was chosen that comprises two distinct features: the simultaneous presence of a utilizable and a non-utilizable substrate, and the absence of a mutagen. Selection was performed in a chemostat, with the concentration of the utilizable substrate D-glucitol at 1 mM and the non-utilizable galactitol

Fig. 1. Growth of *R. sphaeroides* Si4 in a chemostat with 1 mM D-glucitol and 20 mM galactitol. Cultivation was performed in a 2 l bioreactor with a constant working volume of 1 l at 30 °C, with aeration (1 l min⁻¹) and agitation (200 r.p.m.). The dilution rate was adjusted to 0.05 h⁻¹. In samples taken from the bioreactor, OD₆₅₀ was monitored.

at 20 mM. The continuous cultivation was carried out in a 2 l bioreactor with a constant working volume of 1 l at a dilution rate of 0.05 h⁻¹. The development of the OD₆₅₀ in the chemostat culture is shown in Fig. 1. After 48 d of growth, a significant increase in OD₆₅₀ appeared, indicating the emergence of a mutant that had acquired the ability to utilize galactitol. When the culture had reached an OD₆₅₀ of 1.2, the cultivation was continued for another 5 d with 1 mM galactitol as sole carbon source. Then cells from the chemostat were plated on galactitol mineral medium (see Methods) and incubated under aerobic conditions. After repeated plating on the same medium, a single colony was isolated and subcultured in the corresponding liquid medium. The galactitol-utilizing mutant thus obtained was designated *R. sphaeroides* strain D. It grew with a rate of 0.11 h⁻¹ on galactitol, much lower than the wild-type strain on D-glucitol (0.24 h⁻¹) (Schneider & Giffhorn, 1989). Like the wild-type strain, the mutant also grew anaerobically in the light and was able to utilize each of the polyols D-glucitol, D-mannitol, D-arabitol, ribitol and xylitol for growth.

Detection of a new GDH activity in cell extracts of *R. sphaeroides* D

Batch cultures of *R. sphaeroides* Si4 and strain D were grown in minimal medium with different polyols and D-malate as carbon source, respectively. From these cultures, cell extracts were prepared and the specific polyol dehydrogenase activities were determined (Table 1). During growth of the wild-type strain Si4 on either D-glucitol, D-mannitol or D-arabitol, both MDH (Schneider & Giffhorn, 1989) and SDH (Schauder *et al.*, 1995) are expressed, resulting in enzyme activities with all three polyols. For growth on xylitol as well as ribitol, the RDH which oxidizes ribitol and xylitol (Kahle *et al.*, 1992) is

Table 1. Polyol dehydrogenase activities in cell extracts of *R. sphaeroides* Si4 (a) and *R. sphaeroides* D (b) after growth in minimal medium with different carbon sources

Enzyme activities given represent mean values of duplicate determinations. The assays were performed in the standard assay system at pH 9.0. The concentrations of the polyols were 100 mM, except galactitol (75 mM).

Carbon source	Specific activity [mU mg ⁻¹] with:					
	Galactitol	D-Glucitol	D-Mannitol	D-Arabitol	Xylitol	Ribitol
(a)						
D-Glucitol	31	590	250	680	42	25
D-Mannitol	34	362	197	463	40	29
D-Arabitol	51	430	279	602	75	18
D-Ribitol	41	590	280	820	350	1120
Xylitol	38	640	295	870	280	920
DL-Malate	9	9	5	17	3	6
(b)						
Galactitol	380	250	50	350	1780	5
DL-Malate	300	30	20	230	1800	0

Table 2. Purification of GDH from *R. sphaeroides* D

Fraction	Total activity (U)	Total protein (mg)	Specific activity (U mg ⁻¹)	Purification (-fold)	Yield (%)
Crude extract	1500	3587	0.4	1.0	100
Ammonium sulfate precipitation	1290	2692	0.5	1.3	86
Chromatography on Phenyl Sepharose	1017	581	1.8	4.5	68
Chromatography on Q-Sepharose	917	297	3.0	7.5	61
Chromatography on Matrex Gel Red-A	653	126	5.2	13	44
Gelfiltration on Superdex 200	540	104	5.2	13	36

also expressed. All three polyol dehydrogenases are suppressed during growth on malate. After growth on any of the polyols, only low GDH activities, caused by a minor activity of SDH (Schauder *et al.*, 1995), can be detected.

In cell extracts of *R. sphaeroides* D grown on galactitol, GDH activity was found which is at least 7.5-fold higher than in extracts of Si4 grown on any polyol (Table 1). In addition, the xylitol dehydrogenase activity was very high, while the specific activities for the other polyols were lower compared to Si4 grown on any of the polyols. Unlike the wild-type, almost the same galactitol and xylitol dehydrogenase activities have been measured in *R. sphaeroides* D after growth on DL-malate, indicating that these activities are expressed constitutively. These results demonstrate that the adaptation to growth on galactitol resulted in an alteration of enzymes. However, from these experiments it could not be deduced whether the properties of one of the three polyol dehydrogenases had changed or an additional polyol dehydrogenase had evolved. Therefore, the new GDH had to be isolated and studied in detail.

Purification of GDH from *R. sphaeroides* D

To obtain sufficient cell material for enzyme purification *R. sphaeroides* D was grown in a 10 l bioreactor on minimal medium with DL-malate as carbon source. The growth rate was 0.29 h⁻¹ ($t_d = 2.4$ h). At the transition to stationary phase, cells were harvested. A yield in dry mass of 1.5 g l⁻¹ was obtained. Enzyme purification was started from 61 g wet cells. Details of the purification procedure are described in Methods and the results are summarized in Table 2. Accordingly, GDH was purified 13-fold to a specific activity of 5.2 U mg⁻¹ with a recovery of 36%. This final enzyme preparation was apparently homogeneous as shown by analytical PAGE and SDS-PAGE, each showing one single protein band (Fig. 2a, b).

Properties of GDH

SDS-PAGE of purified GDH resulted in one single protein band whose mobility corresponded to a M_r of 28000 (Fig. 2b). The molecular mass of the native enzyme was determined by sucrose gradient centrifugation (6–30%, w/v, sucrose) at 140000 g for 5 h with aldolase,

Fig. 2. PAGE of isolated GDH from *R. sphaeroides* D. (a) Analytical PAGE with an enzyme sample of the final purification step; 1, activity stain; 2, silver stain. (b) SDS-PAGE with purified GDH (lane 2) and marker proteins (lane 1). The marker proteins are (from top to bottom): glutamate dehydrogenase ($M_r = 55562$), aldolase (39200), triose-phosphate isomerase (26000), trypsin inhibitor (20100) and lysozyme (14300).

ovalbumin and bovine serum albumin as standards (Schachmann, 1959), as well as by linear gradient PAGE (4–20%) (Anderson *et al.*, 1972). With both methods a M_r of 110000 ± 5000 was determined, demonstrating that GDH is a tetramer of identical subunits. The isoelectric point of GDH was determined to be pH 4.2 by isoelectric focussing.

With respect to the coenzyme, GDH was specific for NAD^+ . Table 3 shows the relative enzyme activities against various polyols, alcohols and sugars. GDH is very unspecific for oxidation of substrates. Galactitol is not the main substrate of GDH, the enzyme activities for the sugar alcohols xylitol and D-threitol being more than fourfold higher. In addition to sugar alcohols, several diols and secondary alcohols are substrates of this enzyme. In the reduction reaction, besides some ketoses, several carbonyl compounds are converted by GDH.

The reaction rates were determined in the standard assay systems at pH 9.0 (oxidation) and pH 6.5 (reduction). The saturation curves for all substrates tested were hyperbolic, and the corresponding double-reciprocal plots were linear. The apparent K_m values determined for galactitol, D-threitol, 1,2-hexandiol and NAD^+ (in the presence of 100 mM 1,2-hexandiol) were 240 mM, 85 mM, 0.2 mM and 12 μ M, respectively. The K_m values for acetoin, L-erythrulose, dihydroxyacetone, and NADH (in the presence of 300 mM acetoin) were 62 mM, 144 mM, 48 mM and 4 μ M, respectively.

The products of galactitol and xylitol oxidation with GDH were identified by HPLC to be tagatose and xylulose, respectively. The configuration of the reaction products (D- or L-sugar) was determined by measuring

their rotation angle in a Perkin-Elmer polarimeter. The product of enzymic galactitol oxidation was identified to be L-tagatose ($[\alpha]_D^{20} = 5.7^\circ$) and of xylitol oxidation to be L-xylulose ($[\alpha]_D^{20} = 32.2^\circ$).

The optimal pH for 1,2-hexanediol oxidation by GDH was pH 10.5 as determined in 100 mM potassium phosphate buffer. For the reduction of acetoin, GDH activity increased with decreasing pH to pH 4.0. At lower pH values, NADH decayed quickly. Because of the limited stability of GDH and NADH at extreme pH values, enzyme assays were routinely performed at pH 9.0 (oxidation) and pH 6.5 (reduction), where GDH exhibited 81 and 63% of the activity measured at pH 10.5 and 4.0, respectively.

GDH activity was clearly dependent on the presence of divalent cations. When GDH solution was dialysed for 24 h against 20 mM potassium phosphate buffer (pH 6.5), only 44% of the initial activity was left. The addition of 1 mM $MgCl_2$, $MnCl_2$, $NiCl_2$ or $CoCl_2$ restored 99, 101, 125 and 121% of the initial GDH activity, respectively. $ZnSO_4$ and $FeSO_4$ were inhibitory to the enzyme. When added at a concentration of 1 mM, the chelating agents EDTA and α, α' -dipyridyl reduced GDH activity to 32 and 87%, respectively, and the inactivation could be completely reversed by the addition of 2 mM $MgCl_2$. The sulfhydryl group modifying reagents iodoacetamide, iodoacetic acid, DTT and arsenic acid had no inhibitory effect on GDH at a concentration of 1 mM, but 1 mM bromosuccinimide inhibited GDH completely.

The stability of GDH was tested in 20 mM Bistris buffer (pH 6.5 + 1 mM $MgCl_2$) in the presence and absence of 30% sucrose. After incubation for 3 months at 4 °C with and without sucrose, 100 and 85% of the initial enzyme activity remained. Accordingly, GDH is stabilized by sucrose. Thermal stability of the enzyme was investigated in the optimal buffer (20 mM Bistris, pH 6.5 + 1 mM $MgCl_2$ + 30% sucrose). The enzyme lost 50% of the initial activity during incubation at 28, 37, 40, 45 and 50 °C after 70 d, 8 d, 5 d, 10 h and 12 min, respectively.

Comparison of GDH with the other polyol dehydrogenases from *R. sphaeroides*

On the basis of its biochemical and kinetic properties, GDH is clearly different to all three polyol dehydrogenases from *R. sphaeroides* Si4 (Table 4). The amino acid sequence of MDH has already been determined (Schneider *et al.*, 1993). RDH (Kahle *et al.*, 1992) and SDH (Schauder *et al.*, 1995) from *R. sphaeroides* Si4 as well as GDH from *R. sphaeroides* D were isolated and subjected to a partial amino acid sequence analysis starting from the N terminus by automated sequencing with a 473A sequencer from Applied Biosystems. The comparative amino acid sequences show (Fig. 3) that although GDH and the other polyol dehydrogenases are not homologous, there is a significant sequence identity between GDH, SDH and MDH. However, there is apparently no identity between any of these enzymes and RDH.

Table 3. Substrate specificity of GDH from *R. sphaeroides* D

Enzyme activities in the direction of oxidation and reduction were measured in the standard assay systems at pH 9.0 and pH 6.5, respectively. The assays each contained 0.05 U (corresponding to 100 % with galactitol or L-tagatose as substrate) of purified GDH and the substrates at saturating conditions. These were 100 mM for substrate oxidation except galactitol (75 mM) and in the reverse direction 200 mM except lactose (50 mM). The activities given are mean values of duplicate determinations. The following compounds were not substrates for GDH: ribitol, L-arabitol, L-threitol, myo-inositol, ethylene glycol, *n*-butanol, ethanol and methanol (substrate oxidation); D-fructose, D-glucose, D-mannose, D-galactose, L-sorbose, D-arabinose, L-arabinose, L-ribulose, D-xylose, D-ribose, lactose, melobiose, maltose, raffinose, furanose, 2-butanone and acetone (substrate reduction)

Substrate oxidation		Substrate reduction	
Substrate	Relative enzyme activity (%)	Substrate	Relative enzyme activity (%)
Galactitol	100	L-Tagatose	100
D-Glucitol	6	D-Tagatose	4
L-Glucitol	83	D-Ribulose	696
D-Mannitol	2	D-Xylulose	42
Xylitol	410	L-Erythrulose	1183
D-Arabitol	64	Acetoin	304
D-Threitol	428	L-Glyceraldehyde	383
<i>m</i> -Erythritol	4	D-Glyceraldehyde	281
Glycerol	51	Dihydroxyacetone	281
1,2-Hexanediol	323		
2-Hexanol	170		
1,2-Pentanediol	269		
1,2-Butanediol	95		
(2S,3S)-Butanediol	74		
(2R,3R)-Butanediol	8		
(2S)-Butanol	46		
(2R)-Butanol	24		
1,2-Propanediol	71		
2-Propanol	42		

Table 4. Properties of polyol dehydrogenases from *R. sphaeroides*

	Molecular mass (kDa)	No. of subunits	Isoelectric point	Requirement for divalent cations	K_m values for the main substrates (mM)	Specific activity (U mg ⁻¹) with:				Reference
						Galactitol	D-Mannitol	D-Glucitol	Ribitol	
GDH	110	4	4.2	+	Galactitol: 240 D-Threitol: 85 1,2-Hexanediol: 0.2	5.2*	0.1	0.3	0	This study
MDH	51.4	1	4.3	—	D-Mannitol: 0.3 D-Arabitol: 1.8 D-Glucitol: 32	3	38	60	0	Schneider & Giffhorn (1989)
SDH	62	2	4.8	—	D-Glucitol: 6.2 Galactitol: 1.5	7	2	42	0	Schauder <i>et al.</i> (1995)
RDH	50	2	6.1	—	Ribitol: 6.3 Xylitol: 77	0	0	6	59	Kahle <i>et al.</i> (1992)

* Due to the low solubility of galactitol in aqueous solution (about 33.3 g l⁻¹) the activity was assayed under non-saturating conditions (see Methods).

GDH:	M	D	Y	<u>R</u>	T	<u>V</u>	F	<u>R</u>		<u>L</u>	<u>D</u>	<u>G</u>	A	C	A	A	V	I		<u>G</u>	<u>A</u>	G		
SDH:		M	<u>R</u>							<u>L</u>	<u>D</u>	<u>G</u>	<u>K</u>	T	<u>A</u>	<u>L</u>	I	I		<u>G</u>	S	<u>A</u>	R	
MDH:			T	<u>R</u>	S	<u>V</u>	T	<u>R</u>	P	S	Y	<u>D</u>	<u>R</u>	<u>K</u>		<u>A</u>	<u>L</u>		I	P	<u>G</u>	I	V	H
<hr/>																								
RDH:	A	E	L	M	Q	G	K	V	A	A														

Fig. 3. N-terminal amino acid sequences of GDH from *R. sphaeroides* D, and SDH, MDH and RDH from *R. sphaeroides* Si4.

DISCUSSION

A spontaneous gain of function mutant of *R. sphaeroides* Si4 capable of growing on the previously non-metabolizable sugar alcohol galactitol was isolated from a chemostat culture grown under selective pressure. Unlike the parental strain, the mutant *R. sphaeroides* D produced elevated levels of a newly expressed GDH. The purified enzyme exhibits a number of properties different to all other polyol dehydrogenases. GDH is inactivated by dialysis or by treatment with EDTA, and its activity can be completely restored by incubation with one of the divalent cations Mg^{2+} , Mn^{2+} , Co^{2+} and Ni^{2+} , but not in the presence of Zn^{2+} which has an inhibitory effect. Unlike GDH of *R. sphaeroides* D, most bacterial polyol dehydrogenases do not require metal ions for catalytic activity (Taylor *et al.*, 1974; Neuberger *et al.*, 1979; Wojtkiewicz *et al.*, 1988; Schneider & Giffhorn, 1989, 1991; Kahle *et al.*, 1992; Schauder *et al.*, 1995). SDH of *Bacillus subtilis* is a remarkable exception. This enzyme requires Zn^{2+} (Ng *et al.*, 1992) similar to mammalian SDHs (Jeffery & Jörnvall, 1988; Jeffery *et al.*, 1984). Another striking characteristic of the *R. sphaeroides* D GDH is its unusual substrate profile. Unlike all other polyol dehydrogenases, GDH oxidizes aliphatic diols and secondary aliphatic alcohols in addition to different sugar alcohols. It is evident that the preferred substrates of this enzyme are aliphatic diols (K_m for 1,2-hexanediol = 0.2 mM) rather than the sugar alcohols (K_m for galactitol = 240 mM). These findings do not only pose the question of how GDH has originated, but also open up interesting perspectives for biotechnical applications of this biocatalyst. The enzymic synthesis of the rare sugar L-tagatose by regioselective oxidation of galactitol at C-5 with GDH has been demonstrated and bioconversions according to previous studies are currently being elaborated (Schwartz *et al.*, 1994). Further, it has been shown that GDH oxidizes the secondary alcohols (2S)-butanol and (2S,3S)-butanediol with relatively high rates, but not primary alcohols. Therefore, it is most likely that the aliphatic diols 1,2-propanediol, 1,2-butanediol, 1,2-pentanediol and 1,2-hexanediol are oxidized at C-2 to yield the corresponding keto-alcohol, indicating a possible application of the enzyme for enantioselective reduction of various keto-alcohols (Keinan *et al.*, 1986).

Apparently, the acquisition of the new metabolic capa-

bility by *R. sphaeroides* Si4 occurred by derepression of a new GDH, and is not caused by the galactitol side activity of overproduced MDH (Schneider & Giffhorn, 1989) or SDH (Schauder *et al.*, 1995) as initially expected. In this respect, galactitol metabolism of *R. sphaeroides* Si4 is different from the acquired xylitol metabolism of *Klebsiella aerogenes* which utilizes xylitol by means of a side activity of an overproduced RDH (Wu *et al.*, 1968; Hartley *et al.*, 1972; Rigby *et al.*, 1974). Rather, galactitol metabolism is similar to acquisitive growth of *R. sphaeroides* on hexoses or D-(−)-tartrate as the result of derepression of a previously unrecognized gluconate-6-phosphate dehydratase (Szymona & Doudoroff, 1960) and D-(−)-tartrate dehydratase (Rode & Giffhorn, 1982, 1983), respectively. Although utilization of some primary aliphatic alcohols by strains of *R. sphaeroides* has been reported (Kondratieva, 1979), substantial studies on the enzymes involved in the oxidation of these substrates are lacking. This study shows that GDH of the *R. sphaeroides* mutant D is different to MDH (Schneider & Giffhorn, 1989), RDH (Kahle *et al.*, 1992) and SDH (Schauder *et al.*, 1995) of the wild-type strain. These differences refer to size of the native enzyme, its subunit composition, metal requirement, and substrate specificity (Table 4). On the other hand, the N-terminal amino acid sequences of GDH, SDH and MDH (Fig. 3) show a significant identity among these enzymes, supporting the idea that they may have evolved from a common ancestor protein. However, it is rather unlikely that GDH has evolved from SDH or MDH during the course of continuous cultivation under selective pressure. Most likely a cryptic gene has been activated. Cryptic genes are phenotypically silent DNA sequences not normally expressed during the life cycle of a bacterial culture (Hall *et al.*, 1983). However, when a culture is exposed to selective pressure during a prolonged time, mutants with new metabolic functions can develop (Hall *et al.*, 1983; Parker *et al.*, 1988). To elucidate the origin of GDH, its true function and its relationship to MDH (Schneider *et al.*, 1993), SDH and other polyol dehydrogenases (Schauder *et al.*, 1995), we will isolate and sequence the structural gene encoding this enzyme.

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