Cloning, nucleotide sequence and characterization of the mannitol dehydrogenase gene from *Rhodobacter sphaeroides*

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Transposon mutagenesis and antibiotic enrichment were employed to isolate a mutant of *Rhodobacter sphaeroides* Si4 designated strain M22, that had lost the ability to grow on D-mannitol and to produce the enzyme mannitol dehydrogenase (MDH). DNA flanking the transposon in the mutant strain was used as a probe for the identification and cloning of the MDH gene (*mtlK*). A 5.5 kb *EcoRI*/*BglII* fragment from *R. sphaeroides* Si4 was isolated and shown to complement the mutation in *R. sphaeroides* M22. Successful complementation required that a promoter of the vector-plasmid pRK415 be present, suggesting that the *mtlK* gene is part of a larger operon. Using oligonucleotides derived from the N-terminal sequence of MDH as probes *mtlK* was located on the complementing fragment and the gene was sequenced. The *mtlK* open reading frame encodes a protein of 51 404 Da with an N-terminal sequence identical to that obtained from amino acid analysis of the purified MDH. The MDH of *R. sphaeroides* Si4 exhibits distant similarity to the mannitol-1-phosphate dehydrogenases from *Escherichia coli* and *Enterococcus faecalis*, with 28.1% and 26.3% identity, respectively. Mutant strains deficient in MtlK displayed substantial levels of sorbitol dehydrogenase activity, originally thought to be only a minor activity associated with the MDH enzyme. It is likely that we have uncovered an additional polyol dehydrogenase with activity for sorbitol. The *mtlK* gene can be used for overexpression of MDH in *E. coli* in order to obtain sufficient amounts of enzyme for further investigations and applications.

Introduction

Several bacterial polyol dehydrogenases such as sorbitol dehydrogenase (Horwitz & Kaplan, 1964; Schneider & Giffhorn, 1991), mannitol dehydrogenase (Martinez *et al.*, 1963; Schneider & Giffhorn, 1989), ribitol dehydrogenase (Kahle *et al.*, 1992; Taylor *et al.*, 1974), and arabitol dehydrogenase (Neuberger *et al.*, 1979) have been isolated and characterized. Currently the potential use of different microbial polyol dehydrogenases for enzymic polyol determinations is being studied (Berezenko & Sturgeon, 1991; Kiba *et al.*, 1991; Lunn *et al.*, 1989; Schmolke *et al.*, 1990; Schneider & Giffhorn, 1989, 1991). The applicability of these enzymes to polyol analyses is limited for two reasons: most polyol

dehydrogenases are not highly specific and convert more than one polyol (Kiba *et al.*, 1991; Schneider & Giffhorn, 1989, 1991); and the K_m values are often higher than 10 mM, which impedes complete turnover of the substrates (Berezenko & Sturgeon, 1991). Besides screening for new polyol dehydrogenases, investigations pertaining to the fundamentals of substrate specificity and ways to alter specificity of these enzymes offer possibilities of obtaining more suitable enzymes for analytical purposes. For these investigations knowledge about the exact structure of the proteins and the mechanism of the catalysed reactions are necessary. Recently we reported the isolation and characterization of a mannitol dehydrogenase (MDH; EC 1.1.1.67) from *Rhodobacter sphaeroides* Si4 and its application to quantitative D-mannitol determination taking advantage of the unusually low K_m value for D-mannitol (Schneider & Giffhorn, 1989). Because of its monomeric structure this enzyme is particularly suitable for further studies. These studies require that the gene for MDH be available and the primary structure of the enzyme be determined. Until now few investigations concerning the genes for bacterial

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Abbreviation: MDH, mannitol dehydrogenase.

The GenBank accession number for the nucleotide sequence data reported in this paper is L13697.

Table 1. *Bacterial strains and plasmids*

Strain or plasmid	Relevant characteristic(s)	Reference or source
<i>R. sphaeroides</i>		
Si4	Wild-type	Rode & Giffhorn (1983)
M22	Tn5 mutant of Si4, Km ^r Man ⁻	This study
<i>E. coli</i>		
JM101	<i>thi-1</i> (<i>lac proAB</i>) [F <i>traD36</i> , <i>proAB</i> , <i>lacZM15</i>]	Yanisch-Perron <i>et al.</i> (1985)
JE85	Phe ⁻ mutant of <i>E. coli</i> DH5 α	Jesus Eraso*
S17-1	Pro ⁻ Res ⁻ Mod ⁺ <i>recA</i> , integrated plasmid RP4-Tc::Mu-Km::Tn7	Simon <i>et al.</i> (1983)
HB101	Res ⁻ Mod ⁻ <i>recA13</i> Sm ^r	Boyer & Roulland-Dussoix (1969)
Plasmid		
pMH1701	Suicide plasmid containing Tn5-B12S, Km ^r	Hynes <i>et al.</i> (1989)
pRK415	IncP Tc ^r <i>lacZα</i> <i>mob</i>	Keen <i>et al.</i> (1988)
pRK2013	ColE1 replicon, Tra ⁺ of RK2, Km ^r	Ditta <i>et al.</i> (1980)
pUC19	Cloning vector, Ap ^r	Yanisch-Perron <i>et al.</i> (1985)
pBS II KS ⁺	Cloning vector, Ap ^r	Stratagene
pAK10	Parts of Tn5 with flanking DNA from <i>R. sphaeroides</i> M22 in pUC19	This study
pAK20	<i>EcoRI</i> / <i>HindIII</i> fragment of pAK10 in pUC19	This study
pAK30	8 kb DNA fragment from <i>R. sphaeroides</i> Si4 in pRK415 which complements the mutation in <i>R. sphaeroides</i> M22	This study
pAK36	5.5 kb <i>EcoRI</i> / <i>BglII</i> fragment from pAK30 in pRK415	This study
pAK60	5.5 kb <i>EcoRI</i> / <i>BglII</i> fragment from pAK30 in pBluescript II KS	This study
pAK63	As pAK60, but with the <i>EcoRI</i> / <i>BglII</i> fragment in opposite orientation	This study

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polyol dehydrogenases have been performed. Only the ribitol dehydrogenase gene from *Klebsiella pneumoniae* has been sequenced (Loviny *et al.*, 1985). The present communication reports the cloning and sequencing of the MDH gene (*mtlK*) from *Rhodobacter sphaeroides*.

Methods

Bacterial strains and plasmids. These are described in Table 1.

Media. *R. sphaeroides* was grown chemoheterotrophically at 30 °C in LB medium or in 'Sistrom minimal medium A' lacking glutamate and aspartate (Lueking *et al.*, 1978) with either D-mannitol (Sis-mannitol medium) or D-fructose (Sis-fructose medium) or succinate (Sis-succinate medium) as sole carbon source. *Escherichia coli* was grown at 37 °C in LB medium. When appropriate, antibiotics were added to the following concentrations: kanamycin 25 µg ml⁻¹; spectinomycin 50 µg ml⁻¹; streptomycin 50 µg ml⁻¹; ampicillin 75 µg ml⁻¹; tetracycline 1 µg ml⁻¹ for *R. sphaeroides* and 10 µg ml⁻¹ for *E. coli*.

Genetic techniques. Plasmids were mobilized into *R. sphaeroides* by diparental mating with *E. coli* S17-1 or by triparental mating using *E. coli* HB101(pRK2013) as a helper as described by Suwanto & Kaplan (1992). The matings were performed for 8 h at 30 °C on LB plates.

DNA manipulations and Southern hybridizations. Chromosomal DNA from *R. sphaeroides* was isolated by the method of Marmur (1961); plasmid isolation from *E. coli* was performed by the method of Birnboim & Doly (1979). Standard DNA techniques were used as described by Sambrook *et al.* (1989). DNA fragments were isolated from agarose gels using the Gene Clean Kit (Bio 101).

For Southern hybridizations with oligonucleotide probes, the oligonucleotides were labelled with [α -³²P]ATP (Amersham) using polynucleotide kinase from Pharmacia. The labelled oligonucleotides were purified by column chromatography on Nucletrap push columns from Stratagene. Hybridizations were performed for 12 h at 30 °C. The hybridization buffer contained: 50 mM-Tris/HCl, pH 7.5; 1% (w/v) SDS; 1 M-NaCl; 10% (w/v) dextran sulphate; and 0.15 mg salmon sperm DNA ml⁻¹. After hybridization the membranes were washed twice at 40 °C in 6 $\times$ SSC+1% (w/v) SDS and subjected to autoradiography (1 $\times$ SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate). For hybridizations with DNA fragments, the fragments were labelled by biotinylation with the BioNick kit from BRL. After hybridizations had been performed at 60 °C for 12 h in the same buffer as described above the membranes were washed twice at 60 °C in 6 $\times$ SSC+1% SDS. The biotinylated probes were detected using the Photogene system from BRL.

Transposon tagging of *mtlK*. Plasmid pMH1701 containing Tn5 was introduced into *R. sphaeroides* by diparental mating on a sterile filter that was placed on an LB plate (Suwanto & Kaplan, 1992). After mating for 8 h at 30 °C the cells were suspended in 10 ml Sis-mannitol medium and incubated on a rotary shaker at 30 °C for 8 h. Subsequently 50 µg cefsulodin ml⁻¹ and 50 µg methicillin ml⁻¹ were added in order to enrich mutants that were unable to grow with D-mannitol. The combined mixture of these antibiotics effectively kills cells of *R. sphaeroides* growing either chemoheterotrophically or phototrophically, but does not kill cells that are not growing (M. D. Moore & S. Kaplan, unpublished results). Because of the intrinsic resistance of *R. sphaeroides* to most natural and synthetic penicillins, the above procedure has proven extremely useful. The cell suspension was incubated with shaking for 12 h. Cells were harvested, washed twice in Sis-mannitol medium, resuspended in 600 µl Sis-mannitol medium and

Polyol in enzyme assay	Specific activity [U (mg protein) ⁻¹]		
	Si4	M22	M22(pAK30)
D-Mannitol	0.09	0.00	0.19
D-Arabitol	0.20	0.00	0.41
D-Glucitol	0.16	0.19	0.63
Ribitol	0.02	0.01	0.03
Xylitol	0.01	0.01	0.04

Fig. 2. Complementation of the transposon-induced mutation in *R. sphaeroides* M22 by a DNA fragment from *R. sphaeroides* Si4. (a) Restriction map of the 8 kb *EcoRI* fragment from *R. sphaeroides* Si4 that complements the mutation. The fragment has been cloned into the vector pRK415. 'A' marks the position where the transposon is inserted in *R. sphaeroides* M22. This position was identified by comparison with the restriction map of plasmid pAK20, which contains the DNA flanking the transposon in M22. (b) Identification of subfragments of the *EcoRI* fragment that complement the mutation. 'A' marks the position where the transposon is inserted. → indicates that the orientation of the fragment in pRK415 is the same as that of the *tet*, *lac* promoter; ← indicates the opposite orientation. +, Fragment complements the mutation; -, fragment does not complement; ND, not determined.

In order to clone DNA flanking the site of transposon insertion, genomic DNA from *R. sphaeroides* M22 was isolated and digested with *EcoRI*. An *EcoRI* digest produced a fragment that contained the kanamycin resistance gene of the transposon as well as flanking DNA. Using the kanamycin resistance gene of Tn5 as a probe the size of this fragment was determined by Southern hybridization to be approximately 9.5 kb. Because the portion of the fragment contributed by Tn5 is about 6.5 kb in size, the *EcoRI* fragment contained about 3 kb of *Rhodobacter* DNA. *R. sphaeroides* M22

DNA digested with *EcoRI* was separated following electrophoresis in agarose, and DNA of about 9.5 kb in size was isolated from the gel and cloned into pUC19. The desired plasmid (pAK10) containing portions of Tn5 and the flanking DNA of M22 was obtained. In order to remove most of the DNA sequences derived from Tn5 a 3.9 kb *EcoRI*/*HindIII* fragment was subcloned into pUC19. This plasmid was designated pAK20 and contains predominantly *R. sphaeroides* DNA.

The *EcoRI*/*HindIII* fragment of pAK20 was used as a probe for cloning of *mtlK*. Genomic DNA of *R.*

Fig. 3. Identification of those plasmids still containing the N-terminal portion of the *mtlK* gene among plasmids subcloned from pAK60 with deletions of different size which originated from the *EcoRI* site of the *EcoRI/BglII* fragment. (a) Ethidium-bromide-stained agarose gel. Lanes 1–18, plasmids with deletions of different size, digested with *EcoRI/HindIII*; lane 19, size marker (1 kb ladder). (b) Autoradiogram of a Southern hybridization of a blot from the gel shown in (a) with oligonucleotide 1 (Fig. 1b) as probe.

sphaeroides Si4 was digested with *EcoRI* and separated in an agarose gel. Southern hybridization with this probe resulted in a single signal approximately 8 kb in size. *EcoRI*-digested DNA from Si4 of about 8 kb was isolated from an agarose gel, ligated into pRK415, and the resulting construct was transferred into *E. coli* JE85. Plasmids were isolated from the transformants and Southern hybridizations were performed with the *EcoRI/HindIII* fragment of pAK20 as probe. We subsequently identified a plasmid that contained an 8 kb DNA fragment which hybridized with the probe and was designated pAK30. Following transfer of pAK30 to *R. sphaeroides* M22 by triparental mating the trans-conjugant strain was able to grow on D-mannitol. Measurements of polyol dehydrogenase activity in crude extract proved that MDH activity was restored (Table

2), indicating that the cloned *EcoRI* fragment was able to complement the transposon-induced mutation in *R. sphaeroides* M22.

Analysis of the complementing DNA fragment

A restriction map of the 8 kb fragment that complements the mutation in *R. sphaeroides* M22 is shown in Fig. 2(a). Subfragments were cloned into pRK415 and the resulting plasmids were introduced into *R. sphaeroides* M22 by triparental mating. Growth on D-mannitol and MDH activity in crude extracts of the resulting strains was measured. During these investigations it became apparent that only one of two possible orientations of the cloned DNA fragment in plasmid pRK415 was essential for complementation. Most likely a promoter residing

1	GCG AGA GCT ACG ACC GGC TGT TCG CGA TCA ACG TCT CGG GCA CGC TCT TCA TGA TGC AGG CCG TGG CAC GGG CGA TGA TCG CCG GCG GCC GGG GCG	96
97	GCA AGA TCA TCA ACA TGG CAA GCC AGG CCG GCC GCC GCG GCG AGG CCG TGG TGG GCG TCT ATT GCG CGA CCA AGG CCG CCG TCA TCT CCG TCA CCC	192
193	AGA GCG CCG GGC TGA ACC TCA TCC GCC ACG GGA TCA ACG TCA ATG CCA TCG CCC CCG GCG TGG TGG ACG GCG AGC ACT GGG ACG GGG TGG ATG CGA	288
289	AGT TCG CCG ACT ACG AGA ACC TGC CCC GCG GCG AGA AGA AGC GTC AGG TCG GCG CCG CCG TGC CCT TCG GCC GCA TGG GCC GCG CCG AGG ACC TGA	384
385	CCG GCA TGG CGA TCT TCC TCG CCA CGC CCG AGG CCG ACT ACA TCG TGG CCC AGA CCT ACA ACG TGG ACG GCG GCA ACT GGA TGA GCT GAG GCC CAA	480
481	GGC CCG GCC CTC CCC CCG TCG AAC GCG CCC CCT ATC <u>CGA GGT</u> AAT CCC ATG ACC CCG TCC GTC ACC CGT CCC TCC TAT GAC CCG AAG GCG CTC ACT	576
	Met Thr Arg Ser Val Thr Arg Pro Ser Tyr Asp Arg Lys Ala Leu Thr	
577	CCC GGC ATC GTC CAT ATC GGC GTC GGC AAC TTC CAC CCG GCG CAT CAG GCG GTC TAT CTC GAC GAT CTC TTC GCG CTG GGC GAG GGC CAC GAC TGG	672
	Pro Gly Ile Val His Ile Gly Val Gly Asn Phe His Arg Ala His Gln Ala Val Tyr Leu Asp Asp Leu Phe Ala Leu Gly Glu Gly His Asp Trp	
673	GCC ATC CTC GGC GCG GGC GTC CCG CCG ACC GAT GCG CCG ATG CCG GAG GCT CTG GCC GCG CAG GAC AAT CTC TCG ACG GTG ATC GAG CTC GAT CCG	768
	Ala Ile Leu Gly Ala Gly Val Arg Pro Thr Asp Ala Arg Met Arg Glu Ala Leu Ala Ala Gln Asp Asn Leu Ser Thr Val Ile Glu Leu Asp Pro	
	Transposon	
769	GCG GGC CAC CCG GCC CCG CAG GTG GGG GCG ATG GTG GGC TTC CTG CCG GTC GAG GCC GAC AAT GCG GCC CTG ATC GAG GCC ATG TCG GAT CCG CCG	864
	Ala Gly His Arg Ala Arg Gln Val Gly Ala Met Val Gly Phe Leu Pro Val Glu Ala Asp Asn Ala Ala Leu Ile Glu Ala Met Ser Asp Pro Arg	
865	ATC CCG ATC GTC TCG CTG ACC GTG ACC GAG GGC GGC TAT TAT GTC GAT GCC TCG GGC GCC TTC GAT CCG ACG CAT CCC GAT ATC GTG GCC GAT GCG	960
	Ile Arg Ile Val Ser Leu Thr Val Thr Glu Gly Gly Tyr Tyr Val Asp Ala Ser Gly Ala Phe Asp Pro Thr His Pro Asp Ile Val Ala Asp Ala	
961	GCC CAT CCT GCG CCG CCC GCG ACC GCC TTC GGC GCG ATC CTC GCC GCC CTC CCG GCC CCG CCG GAC GCG GGG GTT ACA CCC TTC ACC GTG ATG TCC	1056
	Ala His Pro Ala Arg Pro Ala Thr Ala Phe Gly Ala Ile Leu Ala Ala Leu Arg Ala Arg Arg Asp Ala Gly Val Thr Pro Phe Thr Val Met Ser	
1066	TGC GAC AAC CTC CCC GGC AAC GGC CAT GTC ACC CCG AAC GCC GTG GTG GGC CTG GCC GAG CTC TAC GAC GCC GAG CTT GCG GGC TGG GTG AAG GCG	1152
	Cys Asp Asn Leu Pro Gly Asn Gly His Val Thr Arg Asn Ala Val Val Gly Leu Ala Glu Leu Tyr Asp Ala Glu Leu Ala Gly Trp Val Lys Ala	
1153	CAG GTG GCC TTC CCG AAC GGC ATG GTC GAC CCG ATC ACC CCC GCC ACC GGC CCG CAC GAG CCG GAA CTG CCG CAG GGC TTC GGC CTC GCC GAT CCG	1248
	Gln Val Ala Phe Pro Asn Gly Met Val Asp Arg Ile Thr Pro Ala Thr Gly Pro His Glu Arg Glu Leu Ala Gln Gly Phe Gly Leu Ala Asp Pro	
1249	GTG CCC GTC ACC TGC GAG CCG TTC CCG CAG TGG GTG ATC GAG GAT CAT TTC CCC GCC GGA CCG CCC GCG CTC GAG AAG GTG GGC GTG ACC TTC ACC	1344
	Val Pro Val Thr Cys Glu Pro Phe Arg Gln Trp Val Ile Glu Asp His Phe Pro Ala Gly Arg Pro Ala Leu Glu Lys Val Gly Val Thr Phe Thr	
1345	CCG CAT GTC CAT GCC TAC GAG GCG ATG AAG ATC CCG ATC CTG AAC GGG GGC CAT GCG GTG ATC GCC TAT CCG TCG GCG CTC ATG GAC ATC CAG CTC	1440
	Pro His Val His Ala Tyr Glu Ala Met Lys Ile Arg Ile Leu Asn Gly Gly His Ala Val Ile Ala Tyr Pro Ser Ala Leu Met Asp Ile Gln Leu	
1441	GTG CAC GCG GCC ATG GCC CAT CCG CTG ATC GCG GCC TTC CTG CAC AAG GTC GAG GTC GAG GAG ATC CTG CCC CAT GTC CCG CCC GTG CCC GAC ACC	1536
	Val His Ala Ala Met Ala His Pro Leu Ile Ala Ala Phe Leu His Lys Val Glu Val Glu Glu Ile Leu Pro His Val Pro Pro Val Pro Asp Thr	
1537	AGC ATC CCC GAC TAT CTT ACC CTG ATC GAG AGC CCG TTC TCG AAC CCC GAG ATC GCC GAC ACG ACG CCG AGG CTC TGC CTC GAC GGT TCG AAC CCG	1632
	Ser Ile Pro Asp Tyr Leu Thr Leu Ile Glu Ser Arg Phe Ser Asn Pro Glu Ile Ala Asp Thr Thr Arg Arg Leu Cys Leu Asp Gly Ser Asn Arg	
1633	CAG CCG AAG TTC ATC GTG CCG TCG CTG CCG GAC AAT CTG CCG GCG GGC ACG GTG CCG AAG GGG CTG GTG CTG CTC TCG GCG CTC TGG TGC CCG TAC	1728
	Gln Pro Lys Phe Ile Val Pro Ser Leu Arg Asp Asn Leu Ala Ala Gly Thr Val Pro Lys Gly Leu Val Leu Leu Ser Ala Leu Trp Cys Arg Tyr	
1729	TGC TTC GGC ACG ACG GAC TCG GGC GTT GTG GTC GAG CCG AAC GAT CCG AAC TGG ACG GCG CTG CAG GAC CCG GCG CCG GCG GCG AAG GAG ACG CCG	1824
	Cys Phe Gly Thr Thr Asp Ser Gly Val Val Val Glu Pro Asn Asp Pro Asn Trp Thr Ala Leu Gln Asp Arg Ala Arg Arg Ala Lys Glu Thr Pro	
1825	GCC GAG TGG CTG GCG ATG ACC GAA GTC TAC GGC GAT CTG GCG CAG AAC GAT CTT CTG GCG GCC GAG TTC GCG GCA GCC CTC GAG GCG GTC TGG CCG	1920
	Ala Glu Trp Leu Ala Met Thr Glu Val Tyr Gly Asp Leu Ala Gln Asn Asp Leu Leu Ala Ala Glu Phe Ala Ala Ala Leu Glu Ala Val Trp Arg	
1921	GAC GGG GCC GAG GCG GTG CTG CCG CCG TTC CTC GCG GCC TGA TCC GCA GGG CCC AGC CCG TCG GAG CAC CGA AGC GGA GCC CCT GCC CCT TGC GGC	2016
	Asp Gly Ala Glu Ala Val Leu Arg Arg Phe Leu Ala Ala ---	
2017	GCA CCG TGA GGC GAA ACG ACC GGG CCA CCC CCG GGC CAC CCG CTC GGT AAC ACC ATG GTA TCG CCG AAG AAT GCC GGC GCC TCT GCC GAA CCG GCC	2112
2113	CGG CTG CCG GGC GAG GCG CCG GAC TTG TCA AGG CCG CCG CCC TCG GGT AGA GAG GGC GGG CGT GGC CCC GTT AGC ACA GTG GTA GTG CAG CCG TCT	2208
2209	TGT AAA GCG AAG GTC GTT CGT TCA AAT CCG ACA CCG GGC ATC GCG ATC CTC CCT CCG ACT TCG	2271

Fig. 4. Nucleotide sequence of the MDH gene (*mtlK*) with flanking regions and derived amino acid sequence for the enzyme. The Shine-Dalgarno sequence is underlined. 'Transposon' marks the position where Tn5 is inserted in *R. sphaeroides* M22.

Fig. 5. Alignment of the amino acid sequence of MDH from *R. sphaeroides* Si4 (Rs mdh) and the mannitol-1-phosphate dehydrogenases from *E. coli* (Ec mpdh) and *Enterococcus faecalis* (Ef mpdh). Identical amino acids in all three enzymes are framed.

on the vector was necessary for complementation. Cloned in the correct orientation into pRK415, a 5.5 kb *EcoRI*/*Bgl*III subfragment was the smallest DNA fragment sufficient for complementation. All smaller fragments investigated did not complement the mutation (Fig. 2b).

In order to verify that a promoter present on the vector was required for complementation of the *EcoRI*/*Bgl*III fragment, the transcription/translation stop cartridge omega spectinomycin^r/streptomycin^r (Spec^r/Strep^r cartridge) (Prentki & Krisch, 1984) was inserted at either end of the fragment. For this purpose the *EcoRI*/*Bgl*III fragment was cloned into the *Sma*I site of the multiple-cloning site from pRK415 and the Spec^r/Strep^r cartridge was cloned in one case into the *Hind*III site and in the other case into the *EcoRI* site of this plasmid. The resulting plasmids were introduced into *R. sphaeroides* M22 by triparental mating. When the Spec^r/Strep^r cartridge was inserted into the *EcoRI* site, this plasmid could still complement the mutation in M22. However, when the Spec^r/Strep^r cartridge was cloned into the *Hind*III site, which means that it was located between the *EcoRI*/*Bgl*III fragment and the *lac*, *tet* promoters of the vector, there was no complementation of the mutation in M22. These results proved that a promoter located on the vector was required for complementation by the cloned DNA fragment. In

addition, by having determined the orientation of the gene(s) necessary for complementation we were able to determine the direction of transcription and the orientation of the desired coding sequence.

In order to ascertain that the complementing 5.5 kb *EcoRI*/*Bgl*III fragment contained the structural gene for mannitol dehydrogenase, Southern hybridizations were performed using the four oligonucleotides shown in Fig. 1(b) as probes. Hybridization signals were obtained with all four oligonucleotide probes, indicating that the cloned DNA fragment contained the *mtlK* structural gene.

Sequencing of *mtlK*

For sequencing of the mannitol dehydrogenase gene the complementing *EcoRI*/*Bgl*III fragment was cloned in both orientations into pBluescript II KS. After nested deletions had been produced, Southern hybridizations were performed with the plasmids obtained, which contained inserts of decreasing length, using oligonucleotide 1 (Fig. 1b) as probe (Fig. 3). In this way it was possible to identify the smallest fragment that still hybridized to the probe, indicating that it contained the amino-terminal sequence of *mtlK*. Both strands of the DNA in this region were sequenced, using both manual sequencing methods and automated sequencing with dye primers. To close gaps in the derived sequence, synthetic oligonucleotides were used as primers. The resulting DNA sequence is shown in Fig. 4. The sequence contains an open reading frame starting at nucleotide 529 which encodes the identical amino acid sequence as determined by N-terminal analysis of the isolated MDH (Fig. 1a), with the initiator methionine removed from the mature protein. This proves definitively that this open reading frame encodes the structural gene for MDH. The size of the protein encoded by this gene is 476 amino acids; its molecular mass is 51 404 Da. This is consistent with the molecular mass of isolated MDH, which was determined to be about 50 000 Da (Schneider & Giffhorn, 1989). The ATG start codon of *mtlK* is preceded by the Shine-Dalgarno-like sequence (Shine & Dalgarno, 1974) GAGGT. A promoter-like region was not found in the sequenced upstream region, which is consistent with the finding that a promoter of the vector was necessary for complementation by the cloned fragment.

Screening of the proteins in the data banks GenBank Release 72.0 (6-92), EMBL Release 31.0 (6-92), and SwissProt Release 22.0 (6-92) with the amino acid sequence of MDH, utilizing the University of Wisconsin Genetics Computer Group software program, identified two proteins with a low degree of similarity to MDH: the mannitol-1-phosphate dehydrogenases of *E. coli* (Davis *et al.*, 1988; Jaiang *et al.*, 1991) and *Enterococcus faecalis* (Fischer *et al.*, 1991), with identity values to MDH of

28.1% and 26.3%, respectively. An alignment of the amino acid sequences is shown in Fig. 5.

Identification of the position of insertion of Tn5 in R. sphaeroides M22

The restriction map of plasmid pAK20, which contains a portion of Tn5 and flanking DNA from *R. sphaeroides* M22, showed that the flanking DNA possesses an *Sst*I site close to the position of insertion of Tn5. In order to sequence the region near the position of insertion of the transposon, the *Sst*I/*Hind*III fragment from pAK20 was cloned into M13mp19. After transformation of *E. coli* JM101, single-stranded DNA was isolated and used as template for sequencing. A comparison of this sequence with the sequence of *mtlK* proved that the DNA flanking the transposon is part of the *mtlK* gene. Therefore it was possible to identify the exact position where the transposon was inserted in mutant *R. sphaeroides* M22: after nucleotide 848 of the sequence shown in Fig. 4.

Discussion

For growth on D-mannitol, D-glucitol or D-arabitol *R. sphaeroides* Si4 produces a polyol dehydrogenase which oxidizes all three of the polyols (Schneider & Giffhorn, 1989). The enzyme has been designated mannitol dehydrogenase (MDH) because of its high affinity to D-mannitol. It can be utilized for a quantitative determination of D-mannitol (Schneider & Giffhorn, 1989). Because of its monomeric structure it is particularly suitable for studies aimed at altering the specificity of polyol dehydrogenases. The transposon-induced mutant of *R. sphaeroides* Si4 exhibited no detectable MDH or arabitol dehydrogenase activity. However, the specific sorbitol dehydrogenase activity in crude extracts was about the same in the wild-type and mutant M22 (Table 2). The source of the sorbitol dehydrogenase activity in M22 is unknown. To date only two polyol dehydrogenases of *R. sphaeroides* Si4 are known, MDH and a ribitol dehydrogenase (Kahle *et al.*, 1992; Schneider & Giffhorn, 1989). During growth on D-mannitol, ribitol dehydrogenase is repressed and only a low basal level of activity is detectable. This is also true in the mutant strain M22 (Table 2). Obviously the sorbitol dehydrogenase activity belongs to a third, so far undiscovered, enzyme. This assumption is further supported by the pattern of polyol dehydrogenase activities in strain M22 complemented with pAK30 (Table 2). This strain contains a very high specific sorbitol dehydrogenase activity which is clearly higher than the activity observed with D-arabitol, while the isolated MDH possesses, under saturating conditions, a higher activity with D-arabitol than with D-glucitol (sorbitol) (Schneider &

Giffhorn, 1989). Most likely the sorbitol dehydrogenase activity in M22(pAK30) is the sum of the activities derived from MDH and from the newly discovered sorbitol dehydrogenase.

Using DNA flanking the transposon in M22 as probe, a 5.5 kb DNA fragment from *R. sphaeroides* Si4 has been cloned that is capable of complementing the mutation in M22. For successful complementation a promoter present on the vector was required. The cloned fragment contains the structural gene for MDH as well as 1.9 kb of DNA upstream of *mtlK*. The requirement of a vector promoter for *mtlK* expression indicates that the actual promoter for *mtlK* is not located on the cloned fragment but even further upstream. In addition, DNA fragments smaller than 5.5 kb were not sufficient for complementation, although the *Bam*HI fragment tested (Fig. 2b) contained the complete MDH gene. This result suggests that there is at least one additional gene downstream of *mtlK* that is also required for complementation. Otherwise the *Bam*HI fragment which contained the complete MDH gene should have complemented the mutation in *R. sphaeroides* M22 when cloned in the right orientation into the vector pRK415. Obviously *mtlK* is part of a larger operon.

The *mtlK* structural gene encodes a protein of 476 amino acids (without the initiator-methionine) with a molecular mass of 51400 Da. Screening of the data banks against the amino acid sequence of MDH identified only the mannitol-1-phosphate dehydrogenases of *E. coli* (Davis *et al.*, 1988; Jaiang *et al.*, 1991) and *Enterococcus faecalis* (Fischer *et al.*, 1991) with similarity to MDH. This result was not surprising, because the MDH of *R. sphaeroides* is the first bacterial MDH that has been sequenced. Among bacterial polyol dehydrogenases, only the sequence of the ribitol dehydrogenase from *Klebsiella pneumoniae* has been determined (Loviny *et al.*, 1985). Similarities to this enzyme have not been found. The mannitol-1-phosphate dehydrogenases from *E. coli* and *Ent. faecalis* possess an amino acid identity over the entire protein of 40% (Fischer *et al.*, 1991). The similarity to MDH from *R. sphaeroides*, which does not convert phosphorylated polyols, is more distant, with an identity of 28.1% to the *E. coli* enzyme and 26.3% to the *Ent. faecalis* enzyme. The alignment of the three proteins (Fig. 5) reveals that the homology between them is not equally distributed over the complete sequence. The first 210 amino acids exhibit very few similarities with the exception of a short region near the N-terminus of the proteins which is the putative NAD-binding site (see below). In the region from amino acid 211 to 363, thirty-three amino acids are identical between all three proteins. In addition only a few minor gaps are required to obtain the alignment. The identity between all three proteins in this region is 22%.

This indicates that this portion of the protein may be involved in binding of the substrate (D-mannitol or mannitol 1-phosphate).

As the result of sequence comparisons several different families of dehydrogenases have been found. The best-studied families are the short-chain dehydrogenases (Neidle *et al.*, 1992; Persson *et al.*, 1991*b*) and the medium-chain family (Jörnvall *et al.*, 1987). In addition a long-chain family (Persson *et al.*, 1991*a*) and a family of iron-activated alcohol dehydrogenases (Neale *et al.*, 1983; Scopes, 1983) are known. Common to all NAD-binding dehydrogenases is a short segment, the NAD-binding site. Several studies have demonstrated that the NAD-binding sites contain three strictly conserved glycine residues (Jörnvall *et al.*, 1987; Persson *et al.*, 1991*b*; Wierenga *et al.*, 1985). With respect to the spacing of the glycine residues two different classes exist. The medium-chain dehydrogenases, and many other dehydrogenases (Wierenga *et al.*, 1985), possess a Gly-X-Gly-X-X-Gly-pattern in the NAD-binding domain. Members of the short-chain dehydrogenase family have a different spacing, Gly-X-X-X-Gly-X-Gly (Neidle *et al.*, 1992; Persson *et al.*, 1991*b*). In the MDH from *R. sphaeroides*, starting with amino acid 18 the sequence is Gly-Ile-Val-His-Ile-Gly-Val-Gly, which is similar to the pattern found for members of the short-chain family, although four residues instead of three are located between the first and the second glycine residue. In case of the alcohol dehydrogenase from *Drosophila melanogaster* only two amino acids are located between the two glycine residues (Persson *et al.*, 1991*b*), indicating that small differences in the spacing may be possible. Therefore the segment starting at amino acid 18 is the best candidate for the NAD-binding region of MDH from *R. sphaeroides* Si4.

The MDH of *R. sphaeroides* does not belong to either of the two well-studied dehydrogenase families, short- or medium-chain. The dehydrogenases that belong to the short-chain family are about 250 amino acids in size and possess several conserved residues (Persson *et al.*, 1991*b*). Although the NAD-binding domain of the *R. sphaeroides* MDH is of the same type as in these dehydrogenases, the MDH differs completely with regard to length and all other conserved residues. The enzymes of the medium-chain family, with about 350 residues, are smaller than MDH from *R. sphaeroides* and the arrangement of the domains is different. In the medium-chain dehydrogenases the substrate-binding region of the protein is closer to the N-terminus than the NAD-binding domain (Jörnvall *et al.*, 1987), while the opposite arrangement is found in MDH. In addition, no sequence similarities were found between MDH and medium-chain dehydrogenases. The group of long-chain dehydrogenases comprises enzymes with 450–600 amino acid residues. The

enzymes in this family are more heterogeneous than the members of the other dehydrogenase families. No entire-chain homologies have been detected, only similarities in single segments (Persson *et al.*, 1991*a*). One segment of similarity is the NAD-binding domain. Because MDH from *R. sphaeroides* is also a dehydrogenase with more than 450 amino acid residues, and with no similarities to dehydrogenases of the other families, it can be considered as another member of the heterologous group of long-chain dehydrogenases.

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