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

KARL-HEINZ SCHNEIDER,1 FRIEDRICH GIFFHORN1 and SAMUEL KAPLAN2*

1Institut für Angewandte Mikrobiologie, Universität des Saarlandes, Im Stadtwald, 6600 Saarbrücken, Germany
2Department of Microbiology and Molecular Genetics, The University of Texas, Health Science Center at Houston, Houston, Texas 77225, USA

(Received 15 January 1993; revised 13 April 1993; accepted 5 May 1993)

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 MDH 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

*Author for correspondence. Tel +1 713 794 1742; fax +1 713 794 1782; e-mail skaplan@utmmg.med.uth.tmc.edu.

Abbreviation: MDH, mannitol dehydrogenase.

The GenBank accession number for the nucleotide sequence data reported in this paper is L13697.
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 (Luxenberg *et al.*, 1978) with either D-mannitol (Sis-mannitol medium) or D-fructose (Sis-fructose medium) or succinate (Sis-succinate medium) as the 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 [α²⁵P]ATP (Amersharm) using polynucleotide kinase from Pharmacia. The labelled oligonucleotides were purified by column chromatography on Nuctrap 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 6x SSC; 1 M-NaCl; 0.15 mg/ml salmon sperm DNA ml⁻¹. 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 6x 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 cefusolidin 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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R. sphaeroides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Si4</td>
<td>Wild-type</td>
<td></td>
</tr>
<tr>
<td>M22</td>
<td>Tn5 mutant of Si4, Km⁺ Man⁻</td>
<td></td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM101</td>
<td>thi-1 (lac proAB) [F traD36, proAB, lacZM15]</td>
<td></td>
</tr>
<tr>
<td>JE85</td>
<td>Phe⁺ mutant of <em>E. coli</em> DH5x</td>
<td></td>
</tr>
<tr>
<td>S17-1</td>
<td>Pro⁺ Res⁺ Mod⁺ recA, integrated plasmid RP4-Tc⁺:Mu-Km⁺::Tn7</td>
<td></td>
</tr>
<tr>
<td>HB101</td>
<td>Res⁺ Mod⁺ recA13 Sm⁺</td>
<td></td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMH1701</td>
<td>Suicide plasmid containing Tn5::B12S, Km’</td>
<td>Hynes <em>et al.</em> (1989)</td>
</tr>
<tr>
<td>pRK415</td>
<td>InclTc lacZ2x mob</td>
<td>Keen <em>et al.</em> (1988)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>ColE1 replicon, Tn⁺ of RK2, Km’</td>
<td>Ditta <em>et al.</em> (1980)</td>
</tr>
<tr>
<td>pBS II KS⁺</td>
<td>Cloning vector, Ap’</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pAK10</td>
<td>Parts of Tn5 with flanking DNA from <em>R. sphaeroides</em> M22 in pUC19</td>
<td>This study</td>
</tr>
<tr>
<td>pAK20</td>
<td>EcoRI/HindIII fragment of pAK10 in pUC19</td>
<td>This study</td>
</tr>
<tr>
<td>pAK30</td>
<td>8 kb DNA fragment from <em>R. sphaeroides</em> Si4 in pRK415 which complements the mutation in <em>R. sphaeroides</em> M22</td>
<td>This study</td>
</tr>
<tr>
<td>pAK36</td>
<td>5.5 kb EcoRI/BglII fragment from pAK30 in pRK415</td>
<td>This study</td>
</tr>
<tr>
<td>pAK60</td>
<td>5.5 kb EcoRI/BglII fragment from pAK30 in pBluescript II KS</td>
<td>This study</td>
</tr>
<tr>
<td>pAK63</td>
<td>As pAK60, but with the EcoRI/BglII fragment in opposite orientation</td>
<td>This study</td>
</tr>
</tbody>
</table>

*University of Texas Health Science Center at Houston.
plated on Sis-succinate plates supplemented with kanamycin and, in order to prevent growth of E. coli, with 10 μg K3TeO3 ml−1 (Moors & Kaplan, 1992). The plates were incubated at 30°C. By replica plating of the grown colonies on Sis-fructose plates and Sis-mannitol plates presumptive mutants that were able to grow on D-fructose but not on D-mannitol were identified.

**Enzyme assays and protein determination.** Polyol dehydrogenase activities were determined as described previously (Schneider & Giffhorn, 1989). Protein concentrations were measured by the method of Gea (1955). Crystalline bovine serum albumin was employed as standard.

**Determination of the N-terminal amino acid sequence of MDH.** The amino acid sequence of the N-terminal region of the isolated MDH was determined by automated Edman degradation using the pulsed liquid phase protein sequencer 477 A with an on-line 120 A PTH analyser (Applied Biosystems). The sequencing was performed by Dr B. Schmidt, Biochemistry II, Universität Göttingen, Germany.

**DNA sequencing.** DNA was sequenced by the dideoxy chain-termination method of Sanger with double-stranded (plasmid) templates using the Bst-Polymerase 7-deaza-dGTP Sequencing Kit from BioRad and dATP (Amersham). A T7-primer was used for the sequencing reactions as well as synthetic oligonucleotides complementary to already sequenced regions on the template.

In addition the same templates and primers were used for automated sequencing with the Applied Biosystems 370A/373A apparatus in the DNA core facility of the Department of Microbiology and Molecular Genetics, University of Texas Medical School, Houston.

**Results**

**Cloning of mtlK from R. sphaeroides Si4**

In order to clone the gene for MDH (mtlK) from *R. sphaeroides* Si4 we obtained partial amino acid sequence information for the enzyme and subsequently deduced the corresponding nucleotide sequences, which could be used to identify mtlK. Purification of MDH from *R. sphaeroides* was performed according to the protocol described previously (Schneider & Giffhorn, 1989). The first 43 amino acids of the enzyme were determined by automated sequencing; the sequence is shown in Fig. 1(a). Four different oligonucleotides were synthesized, the nucleotide sequences of which were deduced from this amino acid sequence. The sequences of the oligonucleotides are shown in Fig. 1(b); all of them have several ‘wobble’ positions. Unfortunately, our attempts to identify mtlK in a cosmid bank of genomic DNA from *R. sphaeroides* Si4 were not successful.

Therefore it was necessary to use a completely different strategy to clone mtlK. Thus, by producing a mutant of *R. sphaeroides* with a transposon inserted in or near mtlK, DNA flanking the transposon could be used as a homologous probe to identify the gene for MDH following suitable enrichment of the mutant organism.

Transposon-induced mutants of *R. sphaeroides* were produced and enriched as described in Methods. Subsequently mutants were selected that did not form colonies on minimal plates with D-mannitol as sole carbon source. These mutants were grown in Sis-succinate medium supplemented with 10% LB medium and 5 g D-mannitol l−1, crude extracts were produced using a French press, and the specific polyol dehydrogenase activities were determined. One of the mutants was able to utilize D-fructose and other sugars as sole carbon source, but neither D-mannitol nor D-arabitol. In the crude extract of this strain no polyol dehydrogenase activity with either D-mannitol or D-arabitol was detectable (Table 2). The mutant strain was designated *R. sphaeroides* M22.

**Table 2. Specific polyol dehydrogenase activities in crude extracts of *R. sphaeroides* strains Si4, M22, and M22 containing pAK30**

<table>
<thead>
<tr>
<th>Polyol in enzyme assay</th>
<th>Specific activity [U (mg protein)−1]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Si4</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>0.09</td>
</tr>
<tr>
<td>D-Arabitol</td>
<td>0.20</td>
</tr>
<tr>
<td>D-Glucitol</td>
<td>0.16</td>
</tr>
<tr>
<td>Ribitol</td>
<td>0.02</td>
</tr>
<tr>
<td>Xylitol</td>
<td>0.01</td>
</tr>
</tbody>
</table>
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 *mltK*. Genomic DNA of *R.
Mannitol dehydrogenase gene of *R. sphaeroides*

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 transconjugant 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...
Fig. 4. Nucleotide sequence of the MDH gene (mtMK) with flanking regions and derived amino acid sequence for the enzyme. The Shine–Dalgarno sequence is underlined. ‘Transposon’ marks the position were TrnS is inserted in R. sphaeroides M22.
Identification of the amino acid sequence of MDH from R. sphaeroides Si4 (Rs mdh) and the mannitol-1-phosphate dehydrogenases from E. coli (Ec mdph) and Enterococcus faecalis (Ef mdph). 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/BglII 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/BglII fragment, the transcription/translation stop cartridge omega spectinomycin'/streptomycin' (Spec'/Strep') cartridge was inserted into the EcoRI site, either end of the fragment. For this purpose the Spec'/Strep' cartridge was cloned in one case into the HindIII 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'/Strep' cartridge was cloned between the EcoRI/BglII fragment and the lac, tet promotors of the vector, there was no complementation of the mutation in M22. 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 mtIK.

For sequencing of mtIK

For sequencing of the mannitol dehydrogenase gene the complementing EcoRI/BglII fragment was cloned in both orientations into pBluescript 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 mtIK.

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 mtIK is preceded by the Shine–Dalgarno-like sequence (Shine & Dalgarno, 1974) GAGGT. A promotor-like region was not found in the sequenced upstream region, which is consistent with the finding that a promotor of the vector was necessary for complementation by the cloned DNA 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 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.
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 SstI site close to the position of insertion of Tn5. In order to sequence the region near the position of insertion of the transposon, the SstI/HindIII 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 & Gifhorn, 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 & Gifhorn, 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 & Gifhorn, 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 & Gifhorn, 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 BamHI 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 BamHI 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 (α-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., 1991b) and the medium-chain family (Jörnvall et al., 1987). In addition a long-chain family (Persson et al., 1991a) 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 (Persson et al., 1991b; 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-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., 1991b). 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., 1991b), 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* S4.

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., 1991b). 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., 1991a). 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.

We thank Dr B. Schmidt, Biochemie II, Universität Göttingen, Germany, for determining the N-terminal amino acid sequence of MDH. Greg Shipley for performing the automated sequencing as well as the synthesis of the oligonucleotides. We gratefully acknowledge Mark Moore for developing the method for enrichment of non-growing mutants of *R. sphaeroides*. We acknowledge the support of numerous colleagues in the laboratory, in particular of Antonius Suwanto and Ellen Neidle.

This work was supported by a grant of the Deutsche Forschungsgemeinschaft to K.-H.S. and by grants GM15590 and GM31667 to S.K.

References


Improved broad host-range plasmids for DNA cloning in Gram-negative bacteria. Gene 70, 191–197.


