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Molecular Cloning and Characterization of Mannitol-1-phosphate dehydrogenase from *Vibrio cholerae*

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Abstract

Vibrio cholerae utilizes mannitol through an operon of the phosphoenolpyruvate-dependent phosphotransferase (PTS) type. A gene *mtlD* encoding mannitol-1-phosphate dehydrogenase was identified within the 3.9 kb mannitol operon of *V. cholerae*. The *mtlD* gene was cloned from *V. cholerae* O395, and the recombinant enzyme was functionally expressed in *E. coli* as a 6×His-tagged protein and purified to homogeneity. The recombinant protein is a monomer with a molecular mass of 42.35 kDa. The purified recombinant MtlD reduced fructose 6-phosphate (F6P) using NADH as a cofactor with a K_m of 1.54 ± 0.1 mM and V_{max} of 320.8 ± 7.81 $\mu\text{mol/min/mg}$ protein. The pH and temperature optima for F6P reduction were determined to be 7.5 and 37°C, respectively. Using quantitative real-time PCR analysis, *mtlD* was found to be constitutively expressed in *V. cholerae*, but the expression was up-regulated when grown in the presence of mannitol. The MtlD expression levels were not significantly different between *V. cholerae* O1 and non-O1 strains.

Keywords

Mannitol; *Vibrio cholerae*; mannitol-1-phosphate dehydrogenase

The polyol, D-mannitol, is abundant in nature and can be found in plants, algae, fungi, and bacteria. Consequently, many microorganisms have evolved physiological capabilities to metabolize mannitol and thereby derive energy. Mannitol is also a major storage polysaccharide in fungi and plays important roles in osmoregulation and stress tolerance [13, 24]. Many microorganisms have mannitol utilization systems, mainly of the phosphoenolpyruvate-dependent phosphotransferase system (PTS) types. The genes participating in mannitol uptake and utilization are organized in operons in both Gram-positive and Gram-negative bacteria [1, 7, 16]. In *E. coli*, mannitol is simultaneously phosphorylated and taken up by PTS proteins to form mannitol 1-phosphate, which is subsequently oxidized to fructose 6-phosphate (Fru6P) by a mannitol phosphate dehydrogenase enzyme (MPD) (EC 1.1.1.17) [17, 29].

Polyol dehydrogenases are broadly classified, based on the sequence analysis, into three groups; long chain dehydrogenases with more than 450 amino acids, medium chain dehydrogenases with up to 350 amino acids, and short chain dehydrogenases with up to 250 amino acids [14, 19, 21]. MtlD has been studied in various Gram-positive and Gram-negative bacteria, and it has been observed that all belong to the medium chain dehydrogenase group [4, 8, 11, 18, 27]. A novel MPD, with broad substrate specificity, has been described from *Pseudomonas fluorescens* [5].

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Genome sequence analysis of *V. cholerae* O395 has revealed that the mannitol operon genome sequence is arranged as 3 complete open reading frames (ORFs) over a stretch of 3945 bp [6]. The ORFs are identified as *mtlA*, encoding a mannitol enzyme IICBA component (EII^{Mtl}), *mtlD*, encoding a mannitol-1-phosphate dehydrogenase, and *mtlR*, encoding a putative operon repressor (MtlR). Recently, we cloned this entire operon and studied the mannitol transport activity in an *E. coli* background [15]. In the present study, we describe the cloning, purification, and functional characterization of a mannitol-1-phosphate dehydrogenase from *V. cholerae* O395.

MATERIALS AND METHODS

Bacterial Strains

Vibrio cholerae O395 is an O1 serotype (ATCC 39541 / Ogawa 395), while *V. cholerae* PS15 is a non-O1 strain. *V. cholerae* O395 EII^{Mtl}::Int is a mutant strain, created during our previous study, in which the mannitol operon is inactivated via the insertion of an intron [15]. *E. coli* SG13009 [9] was used for the expression of a histidine-tagged protein.

Cloning and Expression of the Mannitol-1-Phosphate Dehydrogenase Gene (*mtlD*)

The genomic DNA was extracted from *V. cholerae* O395 using the CTAB (cetyl trimethyl ammonium bromide) method [3]. Primers MPD-F (aaaaagaatgcagttcatttgggt) and MPD-R (ctaatccatctgttgtagagag) were used to amplify the 1155 bp *mtlD* gene from the *V. cholerae* O395 genome for cloning into the expression vector pQE30 (Qiagen, Valencia, CA). The amplification was performed in a 50 µl reaction volume consisting of 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl and 2 mM MgCl₂), 200 µM concentrations of each of the four dNTPs, 25 pico moles of respective primer, 3U of Taq polymerase (MBI Fermentas), and 500 ng of pure genomic DNA. The PCR product was purified using a PCR purification kit (Qiagen, Valencia, CA), cloned into the pQE30 cloning vector to yield pQE30/*mtlD* and transformed into competent *E. coli* SG13009 [pREP4] (Qiagen, Valencia, CA). The 6×His-tagged MtlD fusion protein was purified by using nickel chromatography columns following the manufacturer's protocol (Qiagen, Valencia, CA). Briefly, the recombinant *E. coli* SG13009/pQE30/*mtlD* was grown in LB broth containing 100 µg/ml ampicillin and 25 µg/ml kanamycin to an OD₆₀₀ of 0.5, induced with 1 mM IPTG, and further incubated for 8 h with shaking. The cells were harvested by centrifugation at 3,000 × g for 15 min, and the pellet was resuspended in a lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole) and sonicated 5 times at 50% duty cycle, with a 5 second pause between sonications, in a Branson SLPt sonicator. The mixture was then centrifuged at 11,000 × g for 15 min at room temperature to pellet the cellular debris, and the supernatant was loaded onto an Ni-NTA affinity column. Subsequently, the column was washed twice with a wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0). The bound recombinant protein was then eluted with an elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0) containing increasing concentrations of imidazole (100-500 mM) and dialyzed against a Tris buffer (10 mM Tris Cl pH 8.0, 0.1% Triton X-100) overnight at 4°C. Following this, the recombinant protein was electrophoresed on a 12% sodium dodecyl sulphate (SDS) polyacrylamide gel and visualized by staining with coomassie brilliant blue.

Enzyme Assay

The enzyme activity assay for MtlD was performed in 2.0 ml volumes in glass cuvettes at 30°C as previously described, with slight modifications [20]. The reaction buffer consisted of 50 mM Tris-HCl (pH 7.5), 2.5 mM NADH and 25 µl of the enzyme. The mixture was equilibrated at 30°C for 5 min, and the reaction was initiated by the addition of 2 mM fructose-6-phosphate (F6P). The oxidation of NADH was monitored by recording the decrease in OD₃₄₀ over a period of 10 min. In a separate experiment, the kinetics of MtlD

were determined using various concentrations (0.5 mM, 1 mM, 2 mM and 4 mM) of F6P. The K_m and V_{max} values were calculated from Lineweaver-Burk plots. All enzyme reactions were performed in triplicate.

Effects of pH, Temperature and Metal Ions

The effect of various buffer pH values (6.5, 7.5, 8.5, 9 and 10) on enzyme activity was determined using the F6P reduction assay described above. A Tris-HCl (50 mM) buffer was used for pHs above 7, while a MOPS [3-(N-Morpholino)propanesulfonic acid] buffer was used for assays requiring a pH of less than 7. The effects of temperature variations on the activity of MtlD was determined in the temperature range of 20-55°C. To determine the effects of metal ions, F6P assays were performed with 5 and 10 mM concentrations of $MgSO_4$, $MnSO_4$, $FeCl_3$, $ZnCl_2$, $CoCl_2$, and $LiCl$.

Analysis of mtlD Gene Expression by Real-time Polymerase Chain Reaction (RT-PCR)

V. cholerae O395 was grown in an LB broth to an OD_{625} of 0.5, and 200 μ l of this was used to inoculate fresh LB broths with and without 0.5 % mannitol. The cultures were grown at 37°C, without shaking, to 100 Klett units and the total RNA was extracted from 4 ml of this culture using the RiboPure RNA extraction kit (Ambion, Austin, TX). Contaminant DNA was eliminated by digestion with DNase I. cDNA was synthesized by reverse transcription PCR from 500 ng of the total RNA using an iScript Select cDNA synthesis kit (BioRad, Hercules, CA). Real-time PCR amplifications were performed in 25 μ l volumes containing 10 pico moles of primers MtlD-RT1 (aaggcgtcgcttttagtgaa) and MtlD-RT2 (acatccttggtggaggtgag), 1 μ l of the cDNA template, and 12.5 μ l iQ SYBR Green Super mix (Bio-Rad, Hercules, CA). The experiments were performed in triplicate, on each occasion using RNA from three independent preparations. The house keeping gene *gapA*, encoding glyceraldehyde-3-phosphate dehydrogenase, was used as the internal control. Primers Gap-RT1 (cgtatcggtcgctttcggtt) and Gap-RT2 (caggtcagctgggttacgtt) were used for the amplification of cDNA derived from *gap* RNA transcripts by real-time PCR.

Growth Studies

The growth patterns of *V. cholerae* O395, *V. cholerae* PS 15 (a non-O1 strain) and *V. cholerae* O395 EII^{Mtl}::Int (a mannitol operon-inactivated strain) were studied in both an LB broth and an LB broth containing 0.5% mannitol. In a separate experiment, the growth patterns of WT strains of *V. cholerae* O395 and PS15, both in a minimal medium, were compared. The minimal medium for *V. cholerae* was prepared as has been previously described [12], with 0.5% of mannitol as the sole carbon source. The turbidity was measured in triplicate using a Klett-Summerson's photoelectric colorimeter at 600 nm, and the growth curves were plotted using Sigma Plot (Systat Software Inc., San Jose, CA).

Determination of Protein Concentrations

The protein concentrations in the whole cell lysate and the pure recombinant MPD were determined by the bicinchoninic acid method using the bovine serum albumin standards (Thermo Fisher Scientific, Rockford, IL).

Computer Analysis

The deduced amino acid sequence of MtlD from *V. cholera* O395 was compared to those of other known proteins in the NCBI database by BLASTP analysis [2]. Multiple sequence alignments were derived using the CLUSTALW program [10].

RESULTS AND DISCUSSION

The deduced amino acid sequence of MtlD from *V. cholerae* was seen to be >95% identical with that of MtlD from *V. mimicus* VM603, 80% identical and 89% similar with those of *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus*, and *V. vulnificus*, 72% identical and 84% similar with *Aeromonas hydrophila*, and 63% identical and 77% similar with that of *Yersinia enterocolitica*. High sequence identities (65% identity and 75% similarity) were observed with protein sequences from unrelated bacteria, such as *Reinekea* sp. MED297 (GenBank accession no. ZP_01114874), *Mannheimia haemolytica* (ZP_04978665), *Providencia rettgeri* (ZP_03637680), *Haemophilus somnus*, (YP_001784162), *M. succiniciproducens* (YP_087602), and *Actinobacillus succinogenes* (YP_001343767). MtlD of *V. cholerae* was 64% identical and 77% similar with MtlD of *Enterobacter sakazakii* (YP_001439900), *Yersinia intermedia* (ZP_04636810), *Citrobacter youngae* (ZP_04345304), 62% identical and 76% similar with MtlD of *Klebsiella pneumoniae* (YP_002236036), *Salmonella enterica* subsp. Arizonae (YP_001572890), and 61% identical and 74% similar with *Escherichia coli* 536 (YP_671571). Based on PSI-BLAST (Position-Specific Iterated BLAST) analysis, MtlD was found to show >50% identity and >70% similarity with the putative mannitol-specific IICBA components of *Streptococcus uberis*, *S. mutans*, *Lactobacillus plantarum*, *S. pyogenes*, *Enterococcus faecium*, *Oceanobacillus iheyensis*, *Clostridium difficile*, *Bacillus clausii*, and *B. subtilis*. A comparison of MtlD with other homologous sequences from Gram-negative and Gram-positive bacteria is shown in Fig. 1.

The nucleotide (NAD⁺)- binding consensus sequence GxGxxGxGxxG was identified within the highly conserved N-terminal portion of MtlD corresponding to the amino acid residues 9-19 (Fig. 1). This conserved motif had a sequence GxGxxG, characteristic of medium chain dehydrogenases [28]. The Prosite pattern of mannitol-1-phosphate dehydrogenase (EC 1.1.1.17) [LIVMY]-x-[FS]-x(2)-[STAGCV]-x-V-D-R-[IV]-x-[PS] corresponded to the sequence VGFVDSAVDRIVP between residues 152-164. The highly conserved nucleotide (NAD⁺)-binding the N-terminal domain and the active site of MtlD, in both Gram-negative and Gram-positive, can be seen from the multiple sequence alignment (Fig. 1).

The 1155bp gene, encoding the 384 amino acid long MtlD, was cloned into an expression vector to obtain pQE30/*mtlD*. The 6×His-tagged MtlD protein was expressed and purified from the recombinant construct in *E. coli* SG13009. The purification under native conditions was achieved by elution with a buffer containing 350 mM imidazole (Fig. 2). The total yield of the recombinant protein was approximately 0.865 mg/L of LB broth, with a specific activity of 126.6 U/mg protein. With the crude extract, a specific activity of 3.32 U/mg protein was observed (Table 1). The expression of native protein, using the T7 promoter of the pQE30 vector, yielded active protein that was relatively pure and, despite the high levels of expression, the protein was soluble and could be extracted without the use of harsh reagents.

The 6×His-tagged MtlD protein rapidly reduced fructose-6-phosphate in the presence of NADH. The NADH-dependent activity was very specific, since no activity was found when NADH was replaced with NADPH. The kinetic activities of fructose reduction were determined using various concentrations of F6P. The K_m and V_{max} values, that were determined using the Lineweaver-Burk plot, were 1.51 mM and 322 μmol/min/mg of protein, respectively. None of the metal ions tested in this study had any effect on the activity of MtlD at concentrations of 5 or 10 mM.

The optimal pH for fructose reductase activity was determined to be 7.5 (Fig. 3A). Though *V. cholerae* are known to tolerate, and grow well, in alkaline conditions [22], this

physiology had no bearing on the pH optimum of MtlD, since F6P reduction activity was 84% and 44% at pHs 8.0 and 9.0, respectively. The effect of various temperatures on the activity of the recombinant MtlD was also determined by performing an F6P reduction assay at temperatures ranging from 20-55°C. The relative percent activities of MtlD at different reaction temperatures are shown in Fig. 3B. MtlD seems to be instable at higher temperatures since only 25% of the enzyme activity could be observed at 45°C.

Recently, we reported on the cloning and characterization of the mannitol operon from *V. cholerae* O395 and showed that a single operon functions to transport mannitol in *V. cholerae* by insertional inactivation of the mannitol permease, *mtlA* [15]. The mutant strain EII^{Mtl}::Int has a disrupted mannitol operon, since the intron insertion resulted in the inactivation of downstream gene elements *mtlD* and *mtlR* of the operon, suggesting that *mtlD* and *mtlR* are co-transcribed along with EII^{Mtl}. The inactivation of the whole operon was verified by real-time PCR, which did not detect any RNA transcripts of *mtlA*, *mtlD* or *mtlR* (data not shown). Further, the mutant strain is deficient in both mannitol and glucitol fermentations, but not in fructose utilization, suggesting that in *V. cholerae* a single MtlD protein is involved in both mannitol and sorbitol assimilations. Polyol dehydrogenases are not very specific and utilize multiple polyol substrates [23]. In *B. subtilis*, MtlD is found to interact with the *gut* operon to metabolize glucitol [27]. Some bacteria such as *Enterobacter aerogenes* (previously *Aerobacter aerogenes*) have the distinct enzymes D-mannitol-1-phosphate dehydrogenase and D-sorbitol-6-phosphate dehydrogenase, which oxidize mannitol-1-phosphate and sorbitol-6-phosphate into fructose-6-phosphate [18].

The differential fermentation rates of mannitol are an important biochemical feature used to distinguish between pathogenic and non-pathogenic strains [25]. We studied the growth patterns of wild-type *V. cholerae* O395, an *mtlA* mutant of the same strain and a non-O1 *V. cholerae* strain PS15 in LB broth, with and without 0.5% mannitol. In LB broth without mannitol, the growth rates of the three strains were not significantly different, though the mutant strain of O395 showed slightly enhanced growth (Fig. 4A). In the presence of mannitol, the non-O1 strain grew rapidly while the WTO395 strain showed slightly increased growth (Fig. 4B). These results suggest that the mannitol operon regulates growth not only in the presence of mannitol, but also in the absence of it. The rapid growth of *V. cholerae* PS15 was mannitol-dependent, as evidenced from the higher growth rate in the minimal medium with mannitol as the only carbon source compared to the wild-type *V. cholerae* O395 (Fig. 4C).

The enzyme MtlD has been found to be constitutive in various Gram-positive and Gram-negative bacteria including *E. coli*, and their growth in a mannitol-containing medium enhances the biosynthesis of the enzyme [18]. In our study, we compared the levels of *mtlD* transcripts between *V. cholerae* O395 and *V. cholerae* PS15 grown in the presence and the absence of mannitol (Fig. 5). In both strains, *mtlD* was found to be constitutively expressed even when no mannitol was present in the growth medium, but the expression levels were enhanced when grown in the presence of mannitol. This effect was more pronounced in *V. cholerae* PS15, which is a rapid mannitol fermenter. This difference in the levels of *mtlD* transcripts between O395 and PS 15 were not so significant as to manifest itself in the form of vast differences in the rates of mannitol fermentation. Thus, the differential expression of *mtlD* does not fully explain the differential fermentation rates between the O1 and non-O1 strains used in this study. Further, the *mtlD* RNA does not provide direct evidence for the actual levels of MtlD protein formed in the cell. This is further supported by our observation that higher *mtlD* RNA was observed in O395 growing in LB without mannitol compared to PS15, despite the fact that *V. cholerae* O395 is a slow fermenter of mannitol. However, we could not demonstrate this point due to the non-availability of an anti-MtlD antibody. A recent study, comparing the proteins involved in differential sorbitol fermentation rates

between toxigenic and non-toxigenic strains of *V. cholerae* El Tor, has revealed the possible involvement of fructose-specific EII and mannitol-1-phosphate dehydrogenase [26]. Therefore, a comparison of the levels of MtlD synthesis and activities in *V. cholerae* O1 and non-O1 strains is necessary to elucidate the reasons for differential fermentation rates of mannitol between these serogroups.

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REFERENCES

1. Akagawa E, Kurita K, Sugawara T, Nakamura K, Kasahara Y, Ogasawara N, Yamane K. Determination of a 17,484 bp nucleotide sequence around the 39 degrees region of the *Bacillus subtilis* chromosome and similarity analysis of the products of putative ORFs. *Microbiol.* 1995; 141:3241–3245.
2. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic. Acids. Res.* 1997; 25:3389–3402. [PubMed: 9254694]
3. Ausubel, FM.; Brent, R.; Kingston, RE.; Moore, DD.; Seidman, JG.; Smith, JA.; Struhl, K. *Short Protocols in Molecular Biology*. 3rd edn.. John Wiley and Sons; New York: 1995.
4. Berkowitz D. D-Mannitol utilization in *Salmonella typhimurium*. *J. Bacteriol.* 1971; 105:232–240. [PubMed: 4322346]
5. Brünker P, Altenbuchner J, Kulbe KD, Mattes R. Cloning, nucleotide sequence and expression of a mannitol dehydrogenase gene from *Pseudomonas fluorescens* DSM 50106 in *Escherichia coli*. *Biochim. Biophys. Acta.* 1997; 1351:157–167. [PubMed: 9116029]
6. Feng L, Reeves PR, Lan R, Ren Y, Gao C, Zhou Z, Ren Y, Cheng J, Wang W, Wang J, Qian W, Li D, Wang L. A recalibrated molecular clock and independent origins for the cholera pandemic clones. *PLoS One.* 2008; 3:e4053. [PubMed: 19115014]
7. Fischer R, Hengstenberg W. Mannitol-specific enzyme II of the phosphoenolpyruvate-dependent phosphotransferase system of *Staphylococcus carnosus*. Sequence and expression in *Escherichia coli* and structural comparison with the enzyme II mannitol of *Escherichia coli*. *Eur. J. Biochem.* 1992; 204:963–969. [PubMed: 1551396]
8. Fischer R, Pogge von Strandmann R, Hengstenberg W. Mannitol-specific phosphoenolpyruvate-dependent phosphotransferase system of *Enterococcus faecalis*: molecular cloning and nucleotide sequence of enzyme IIIMtl and the mannitol-1-phosphate dehydrogenase, gene expression in *Escherichia coli* and comparison of the gene product with similar enzymes. *J. Bacteriol.* 1991; 173:3709–3715. [PubMed: 1904856]
9. Gottesman S, Halpern E, Trisler P. Role of *sulA* and *sulB* in filamentation by *Ion* mutants of *Escherichia coli* K-12. *J. Bacteriol.* 1981; 148:265–273. [PubMed: 7026534]
10. Higgins D, Thompson J, Gibson T, Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 1994; 22:4673–4680. [PubMed: 7984417]
11. Honeyman AL, Curtiss R III. Isolation, characterization, and nucleotide sequence of the *Streptococcus mutans* mannitol phosphate dehydrogenase gene and the mannitol-specific factor III gene of the phosphoenolpyruvate phospho-transferase system. *Infect. Immun.* 1992; 60:3369–3375. [PubMed: 1322373]

12. Houot L, Watnick PI. A novel role for enzyme I of the *Vibrio cholerae* phosphoenolpyruvate phosphotransferase system in regulation of growth in a biofilm. *J. Bacteriol.* 2008; 190:311–320. [PubMed: 17981973]
13. Jennings DH. Polyol metabolism in fungi. *Adv. Microb. Physiol.* 1984; 25:149–193. [PubMed: 6099966]
14. Jörnvall H, Persson B, Jeffery J. Characteristics of alcohol/polyol dehydrogenases. The zinc-containing long-chain alcohol dehydrogenases. *Eur. J. Biochem.* 1987; 167:195–201. [PubMed: 3622514]
15. Kumar S, Smith KP, Floyd JL, Varela MF. Cloning and molecular analysis of a mannitol operon of phosphoenolpyruvate-dependent phosphotransferase (PTS) type from *Vibrio cholerae* O395. *Arch. Microbiol.* 2011; 193:201–208. [PubMed: 21184218]
16. Lee CA, Saier MH Jr. Mannitol-specific enzyme II of the bacterial phosphotransferase system. III. The nucleotide sequence of the permease gene. *J. Biol. Chem.* 1983; 258:10761–10767. [PubMed: 6309813]
17. Lee CA, Jacobson GR, Saier MH Jr. Plasmid-directed synthesis of enzymes required for D-mannitol transport and utilization in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA.* 1981; 78:7336–7340. [PubMed: 6801648]
18. Liss M, Horowitz SB, Kaplan NO. D-Mannitol 1-phosphate dehydrogenase and D-sorbitol 6-phosphate dehydrogenase in *Aerobacter aerogenes*. *J. Biol. Chem.* 1962; 237:1342–1350. [PubMed: 14465816]
19. Neidle E, Hartnett C, Ornston LN, Bairoch A, Rekik M, Harayama S. cis-diol dehydrogenases encoded by the TOL pWW0 plasmid xylL gene and the *Acinetobacter calcoaceticus* chromosomal *benD* gene are members of the short-chain alcohol dehydrogenase superfamily. *Eur. J. Biochem.* 1992; 204:113–120. [PubMed: 1740120]
20. Noeldner PKM, Coleman MJ, Faulks R, Oliver RP. Purification and characterization of mannitol dehydrogenase from the fungal tomato pathogen *Cladosporium fulvum* (syn *Fulvia fulva*). *Physiol. Mol. Plant Pathol.* 1994; 45:281–289.
21. Persson B, Krook M, Jörnvall H. Characteristics of short-chain alcohol dehydrogenases and related enzymes. *Eur. J. Biochem.* 1991; 200:537–543. [PubMed: 1889416]
22. Reidl J, Klose KE. *Vibrio cholerae* and cholera: out of the water and into the host. *FEMS Microbiol. Rev.* 2002; 26:125–139. [PubMed: 12069878]
23. Schneider KH, Giffhorn F, Kaplan S. Cloning, nucleotide sequence and characterization of the mannitol dehydrogenase gene from *Rhodobacter sphaeroides*. *J. Gen. Microbiol.* 1993; 139:2475–2484. [PubMed: 8254318]
24. Stoop JM, Mooibroek H. Cloning and characterization of NADP-mannitol dehydrogenase cDNA from the button mushroom, *Agaricus bisporus*, and its expression in response to NaCl stress. *Appl. Environ. Microbiol.* 1998; 64:4689–4696. [PubMed: 9835550]
25. Wang HY, Zheng X, Lou J, Yan MY, Zhao YW, Kan B. Comparison of the transcriptional levels of mannitol PTS operon between epidemic and non-epidemic strains of *Vibrio cholerae*. *Acta Acad. Med. Sin.* 2009; 31:51–54.
26. Wang R, Zhang H, Qiu H, Gao S, Kan B. Proteins involved in difference of sorbitol fermentation rates of the toxigenic and nontoxigenic *Vibrio cholerae* El Tor strains revealed by comparative proteome analysis. *BMC Microbiol.* 2009; 9:135. [PubMed: 19589152]
27. Watanabe S, Hamano M, Kakeshita H, Bunai K, Tojo S, Yamaguchi H, Fujita Y, Wong S, Yamane K. Mannitol-1-Phosphate Dehydrogenase (MtlD) is required for mannitol and glucitol assimilation in *Bacillus subtilis*: Possible cooperation of *mtl* and *gut* operons. *J. Bacteriol.* 2003; 185:4816–4824. [PubMed: 12897001]
28. Wierenga RK, De Maeyer MCH, Hol WGJ. Interaction of pyrophosphate moieties with alpha.-helices in dinucleotide-binding proteins. *Biochemistry.* 1985; 24:1346–1357.
29. Wolff JB, Kaplan NO. D-Mannitol-1-phosphate dehydrogenase from *Escherichia coli*. *J. Bacteriol.* 1955; 112:849–869.

Fig. 1. Multiple sequence alignment. Comparison of *V. cholerae* O395 M1PD with homologous sequences from *V. parahemolyticus* (ZP_05905445), *E. coli* (AP_004192), *Staphylococcus carnosus* (YP_002634752), *Clostridium acetobutylicum* (AAC12851) and *Bacillus stearothermophilus* (AAC44466S). The sequences in boxes with dashed outlines indicate the nucleotide (NAD⁺)-binding site (amino acid residues 5-16) and the protein motif of mannitol-1-phosphate dehydrogenase (amino acid residues 152-164).

Fig. 2. SDS-PAGE analysis of recombinant 6xHis-tagged mannitol-1-phosphate dehydrogenase (MtlD) purified by affinity chromatography. Protein molecular weight marker (lane **M**) (Fermentas, MD), *E. coli* SG13009/pQE30/*mtlD* without IPTG induction (lane **1**), *E. coli* SG13009/pQE30/*mtlD* induced with 1 mM IPTG (lane **2**), and pure recombinant MtlD (lane **3**).

A

B

Fig. 3. pH and Temperature. **A.** Effect of various buffer pH values on the F6P reduction activity of MtlD. **B.** Effect of various temperatures of incubation on the F6P reduction activity of MtlD. The enzyme activities observed at pH 7.5 and 37°C were considered 100%, and all other values are represented relative percentages of the activities at pH 7.0 or at 37°C.

Fig. 4. Growth curves. Cells of *V. cholerae* O395 (wild-type), its insertional mutant $EII^{MutA}::Intron$, and *V. cholerae* PS15 (non-O1) were grown in LB broth (A) and in LB broth with 0.5% mannitol (B). Growth rates of *V. cholerae* O395 and PS15 wild-type strains were compared in minimal medium containing 0.5% mannitol as the only carbon source (C). Overnight cultures were inoculated into fresh LB broth, or the minimal medium, and the growth was monitored by measuring turbidity as Klett units at A_{600} .

Fig. 5. RT-PCR analysis of *mtlD* transcription in *V. cholerae* O395 and *V. cholerae* PS15. Cells were grown in LB broth with or without 0.5% mannitol. The transcript levels were measured in triplicate from three independent RNA preparations and normalized with respect to the expression of a housekeeping gene *gapA*. The error bars represent standard deviations from the mean.

Table 1

The F6P reduction activities of crude and purified 6×His MtlD at pH 7.5.

Protein fraction	Protein (mg/L)	Activity (U/ml)	Specific activity (U/mg protein)
Crude extract	14.56	49.1	3.32
6×His MtlD	0.865	64.6	126.66