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Cloning and Molecular Analysis of a Mannitol Operon of Phosphoenolpyruvate-dependent Phosphotransferase (PTS) type From *Vibrio cholerae* O395

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Abstract

A putative mannitol operon of the phosphoenolpyruvate phosphotransferase (PTS) type was cloned from *Vibrio cholerae* O395 and its activity studied in *Escherichia coli*. The 3.9 kb operon comprising of three genes is organized as *mtlADR*. Based on the sequence analysis, these were identified as genes encoding a putative mannitol-specific enzyme IICBA (EII^{Mtl}) component (MtlA), a mannitol-1-phosphate dehydrogenase (MtlD) and a mannitol operon repressor (MtlR). The transport of [^{3}H]mannitol by the cloned mannitol operon in *E. coli* was 13.8±1.4 nmol/min/mg protein. The insertional inactivation of EII^{Mtl} abolished mannitol and sorbitol transport in *V. cholerae* O395. Comparison of the mannitol utilization apparatus of *V. cholerae* with those of Gram-negative and Gram positive bacteria suggests highly conserved nature of the system. MtlA and MtlD exhibit 75% similarity with corresponding sequences of *E. coli* mannitol operon genes, while MtlR has 63% similarity with MtlR of *E. coli*. The cloning of *V. cholerae* mannitol utilization system in an *E. coli* background will help in elucidating the functional properties of this operon.

Keywords

Mannitol; *Vibrio cholerae* O395; PTS; II^{Mtl}; MtlD

Introduction

The phosphoenolpyruvate-dependent carbohydrate:phosphotransferase system (PTS) catalyzes concomitant transport and phosphorylation of sugars by a process called group translocation (Hengstenberg et al. 1993; Postma et al. 1993; Lengeler et al. 1994; Barabote and Saier 2005). PTS is a multicomponent system consisting of cytoplasmic and membrane-bound proteins. Two cytoplasmic soluble proteins, HPr (heat-stable protein) and enzyme I, are involved in general phosphorylation of carbohydrates before they are taken up by the membrane bound enzyme II made of A, B and C components, coded contiguously in the genome or assembled functionally after being transcribed from genes located far apart. The general process of carbohydrate transport by the PTS is well known. Enzyme I is phosphorylated first with the utilization of a phosphoenolpyruvate (PEP) resulting in phospho-enzyme I and pyruvate. Phospho-enzyme I in turn phosphorylates an intermediate phosphocarrier protein HPr to form phospho-HPr. The phosphoryl group is finally transferred to the carbohydrate *via* PTS enzyme II (EII) complex (Postma and Lengeler

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1985). The sugar specificity of PTS is attributed to the transmembrane permease component EII.

The 6-carbon sugar alcohol, D-mannitol is abundant in nature, such as in plants, algae, yeasts, fungi and bacteria. Mannitol is a major storage polysaccharide in fungi that also plays important roles in osmoregulation and stress tolerance (Jennings 1984; Stoop and Mooibroek 1998). Many microorganisms have mannitol utilization systems, mainly of PEP-dependent phosphotransferase system (PTS) types. Studies on the mannitol utilization systems from various Gram-negative and -positive bacteria such as *Escherichia coli* (Lee and Saier 1983), *Staphylococcus carnosus* (Fischer and Hengstenberg 1992), *Bacillus subtilis* (Akagawa et al. 1996), *B. stearothermophilus* (Henstra et al. 1996) and *Clostridium acetobutylicum* (Behrens et al. 2001) have suggested functional similarities, albeit organizational differences exist in the structural genes of the mannitol operons in Gram-positive and –negative bacteria.

The importance of PTS-mediated carbohydrate utilization in marine carbon cycling has been emphasized long ago and shown experimentally in vibrios (Meadow et al. 1987). The availability of whole genome sequences of many bacteria has facilitated the cloning and characterization of these, generally multicomponent, PT systems. The whole genome sequence of *V. cholerae* O395 has a putative mannitol operon and we tested the hypothesis that this genetic element plays a role in the utilization of mannitol. This paper describes the cloning of the mannitol operon from *V. choelrae* O395, its functional characterization and comparative analysis of the *Vibrio* mannitol utilization apparatus with other bacterial mannitol utilization systems.

Materials and methods

Cloning of mannitol operon, enzyme I and HPr encoding genes

The bacterial strains and plasmids used in this study are shown in Table 1. Genomic DNA was extracted from *V. cholerae* O395, *V. cholerae* PS-15 and *Escherichia coli* DH5α using the CTAB (cetyl trimethyl ammonium bromide) method (Ausubel et al. 1995). The mannitol operon containing a mannitol-specific enzyme IICBA (*mtlA*), mannitol-1-phosphate dehydrogenase (*mtlD*) and an operon repressor (*mtlR*) genes was amplified using primers ManXh and ManEco containing XhoI and EcoRI restriction sites (Table 2). The resultant PCR product was double digested with XhoI and EcoRI restriction enzymes, and ligated to a similarly digested pHSG396 plasmid (TaKaRa Bio Inc., Japan) to obtain pHSG396/Mtl, and transformed into *E. coli* HB101. The *ptsI* gene encoding enzyme I and the *ptsH* gene encoding HPr were amplified separately using primers PTS1 and PTS2 containing BamHI and XhoI restriction sites, and the resultant PCR product was restriction digested and ligated into the plasmid vector pSP72 (Promega, Madison, WI) to obtain pSP72/PTS. The plasmid construct pSP72/PTS was electro transformed into *E. coli* HB101 harboring pHSG396/Mtl, and the resultant transformant *E. coli* HB/Mtl-PTS was selected on Luria Bertani (LB) agar containing 100 μg/ml ampicillin and 25 μg/ml chloramphenicol.

The *mtlA* of *V. cholerae* O395, *V. cholerae* PS15 and *E. coli* DH5α were amplified using primers listed in Table 2, restriction digested with BamHI and XhoI and ligated into pSP72 to obtain pSP72/*mtlA*^{VC}, pSP72/*mtlA*^{PS15} and pSP72/*mtlA*^{EC}, respectively. The plasmid constructs were electroporated into *E. coli* HB101.

Mannitol fermentation phenotypes on indicator media

The mannitol fermentation phenotype of *E. coli* HB/Mtl-PTS containing the complete mannitol operon, *ptsI* and *ptsH* genes (Table 1) was determined by streaking on MacConkey agar containing 1% mannitol, 100 μg/ml ampicillin and 25 μg/ml chloramphenicol. The

plates were incubated at 37°C for 48 h and the colonies were observed for pink color due to production of acid from mannitol. *E. coli* HB101/pHSG396/pSP72 containing the cloning vectors alone was used as the control strain in all the experiments. The minimum mannitol concentration required to exhibit fermentation phenotype was determined by using MacConkey agar plates containing 0.1, 0.20, 0.3, 0.4, 0.5 or 1% mannitol as the sole source of fermentable carbohydrate. The plates were observed for colonies exhibiting red phenotype after 48 h of incubation at 37°C. Similarly, the mannitol fermentation phenotypes of *E. coli* HB/pSP72/*mtlA*^{VC}, *E. coli* HB/*mtlA*^{PS15} and *E. coli* HB/*mtlA*^{EC} were determined on MacConkey- mannitol-ampicillin agar. *E. coli* HB101/pSP72 was used as the control.

Sugar transport assays

Mannitol transport assay was performed using *E. coli* HB/Mtl-PTS. HB101/pHSG396/pSP72 (harboring plasmid vectors only) served as the control strain to determine the background radioactive sugar uptake. Cells were grown in 10 ml Luria Bertani (LB) broth containing 1 mM mannitol as an inducer of mannitol metabolizing enzymes, 100 μg/ml ampicillin and 25 μg/ml chloramphenicol to an $O.D_{600}$ of 1.0, centrifuged and washed twice with an equal volume of Tris chloride buffer (50 mM, pH 8.0) containing 10 mM $MgCl_2$ and resuspended in the assay buffer consisting of 50 mM Tris chloride (pH 8.0), 10 mM $MgCl_2$, 10 mM phosphoenolpyruvate (PEP), 10 mM potassium fluoride and 0.5 mM dithiothreitol. The cells were equilibrated to room temperature for 10 min and radioactive sugar [^{3}H]mannitol (American Radiolabeled Chemicals Inc., MO) was added to a final concentration of 1.0 mM, and incubated for 10 min at 37°C. Following incubation, the mixture was rapidly vacuum filtered through 0.45 μm cellulose nitrate filters (Sartorius, Hayward, CA), washed immediately with 3 ml of Tris-chloride buffer containing 0.5 mM $HgCl_2$ to quench the sugar uptake reaction. The filters were placed in 4 ml of Sigma-Fluor (Sigma, St. Louis, MO) liquid scintillation fluid. The amount of radioactivity was determined using a LS-6500 liquid scintillation counter (Beckman Coulter, Fullerton, CA).

The kinetics of mannitol transport were studied at various concentrations (0.25, 0.5 and 1 mM) of radiolabeled [^{3}H]mannitol. The cells were prepared as described above for transport studies and incubated with ^{3}[H]-mannitol at room temperature. Aliquots of 200 μl were drawn at intervals of 30 sec and 60 sec and rapidly filtered. The values were plotted on Lineweaver-Burk double reciprocal plot and the apparent V_{max} and apparent K_m values were calculated (Segel 1976).

Inactivation of *V. cholerae* O395 *mtlA*

The *mtlA* gene of *V. cholerae* O395 was inactivated by intron-mediated insertion using the TargeTron method (Sigma-Aldrich St. Louis, MO). Briefly, primers IBS, EBS1d and EBS2 were designed using TargeTron algorithm (http://www.sigma-genosys.com/targetron/), and used to mutagenize a group II intron by PCR, followed by restriction digestion and ligation into plasmid pACD4K to obtain pACD4K/*mtlA*. The ligation mixture was transformed into TOP 10 *E. coli* (Invitrogen, USA). Separately, *V. cholerae* O395 was electroporated with the helper plasmid pAR1219 (Davanloo et al. 1984). Electrocompetent *V. cholerae* O395 harboring pAR1219 was transformed with pACD4K/*mtlA*, and grown in LB containing ampicillin, chloramphenicol and 1% glucose overnight. Following this, 40 μl of the overnight culture was used to inoculate 2 ml of fresh LB broth containing ampicillin, chloramphenicol and 1% glucose to an OD_{600} of 0.2, followed by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 1 mM. The cells were grown for 30 min at 30°C. IPTG induction results in the intron splicing and integration into target DNA, thus inactivating the target gene. The intron splicing simultaneously activates kanamycin resistance and hence, the mutant colonies were selected on LB plates containing

30 μg/ml kanamycin. The insertion of 2 kb plasmid into *mtlA* was confirmed by PCR using primers flanking *mtlA*.

Computer analysis

The deduced amino acid sequences of mannitol PTS proteins of *V. cholerae* O395 were compared to all other known proteins in the NCBI database by BLASTP analysis (Altschul et al. 1997). The two-dimensional structure of EII^{Mtl} was determined by using the TMHMM sever (Transmembrane helix prediction based on hidden Markov models), the results of which were analyzed using the TMRpres2d (Transmembrane Re-presentation in 2-dimensions). Multiple sequence alignments were derived using the CLUSTALW program (Higgins et al. 1994).

Results

Identification and cloning of mannitol operon

The mannitol operon identified in the whole genome sequence of *V. cholerae* O395 is arranged as 3 complete open reading frames (ORFs) over a stretch of 3945 bp corresponding to coordinates 1033774-1037718 of the chromosome II (GenBank accession CP001236). The ORFs are organized 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) (Fig. 1). The 1950 bp *mtlA* encodes a fused IICB and IIA domains with 649 amino acids. The entire mannitol operon, together with the gene encoding enzyme I and the HPr proteins were separately cloned to obtain pHSG396/Mtl and pSP72/PTS constructs. The two plasmid systems were mobilized into *E. coli* HB101 to obtain *E. coli* HB/Mtl-PTS. We used *E. coli* HB101 for mobilizing our mannitol gene constructs, since this strain lacks EII^{Mtl}.

The amino acid sequence of II^{Mtl} from *V. cholerae* shared high degree of homology with corresponding sequences of several *Vibrio* species, being 95% identical and 98% similar with putative II^{Mtl} protein of *V. mimicus*, 83% identical and 90% similar with putative II^{Mtl} protein of *V. harveyi*, *V. alginolyticus*, *V. orientalis*, and *V. furnissii*. II^{Mtl} sequences with more than 80% identity and 90% similarity with *V. cholerae* II^{Mtl} were found in several *Vibrio* spp. including *V. metschnikovii*, *V. coralliilyticus* and *V. vulnificus*. The sequence conservation in EII^{Mtl} across bacterial genera is evidenced by the presence of homologous sequences with high similarity (>75%) in *Tolumonas auensis*, *Providencia rettgeri*, *Enterobacter sakazakii*, *Pectobacterium carotovorum*, *Actinobacillus succinogenes*. With the *E. coli* mannitol specific IICBA component, *V. cholerae* EII^{Mtl} exhibited 67% identity and 80% similarity.

The predicted secondary structure of II^{Mtl} and the putative phosphorylation sites are shown in Fig. 2. The highly conserved nature of the mannitol permease across diverse bacterial groups could be deduced by comparing the amino acid sequence of N-terminal CB domain of II^{Mtl} from *V. cholerae* with sequences of mannitol permeases from different Gram-positive bacteria (Fig. 3). The C-terminal domain of *V. cholerae* II^{Mtl} bearing putative phosphorylation sites has high sequence identity with the MtlF domains of Gram-positive bacteria (Fig. 4).

Mannitol transport

E. coli HB/Mtl-PTS exhibited a mannitol fermentation phenotype (red) on MacConkey agar containing mannitol. The minimum mannitol concentration required to show a fermentation phenotype on MacConkey agar was 0.20% (11 mM). The mannitol transport was measured using [^{3}H]mannitol. At 1 mM final concentration of [^{3}H]mannitol in the assay mixture, the

uptake was determined to be 13.8±1.4 nmol/min/mg protein. The kinetics of uptake was measured over a concentration range of [^{3}H]mannitol and an average K_m value of 2.74±0.2 μM was obtained, while the V_{max} value was 16.6±0.21 nmol/min/mg protein. With the wild type O395 strain, the K_m and V_{max} values were 0.97±0.02 μM and 8.6±0.11 nmol/min/mg protein, while in the case of *V. cholerae* non-O1 strain PS15, the mannitol transport kinetic studies detected a K_m and a V_{max} of 1.18±0.13 μM and 48.1±1.8 nmol/min/mg protein, respectively.

The *mtlA* gene of *V. cholerae* O395 was successfully disrupted by intron-mediated insertion. The mutant strain *V. cholerae* O395 *mtlA*::intron was unable to transport mannitol, as evidenced by the non-fermenting phenotype of the mutant colonies on MacConkey-mannitol agar. In addition, *V. cholerae* O395 *mtlA*::intron failed to ferment sorbitol.

Discussion

Microorganisms evolve to utilize diverse substrates to survive under conditions of low nutrient availability. The polyol sugar mannitol is a carbon source widely utilized by many species of bacteria. Apart from being a carbon source, mannitol is known to contribute to osmoregulation and stress tolerance (Efiuvwevwere et al. 1999). The metabolism of mannitol *via* PTS involves genes organized into an operon in Gram-positive and Gram-negative bacteria (Fischer et al. 1989; Fischer and Hengstenberg 1992; Akagawa et al. 1995; Behrens et al. 2001). The PTS permeases consist of homologous proteins that may have evolved from a common ancestral PTS (Postma and Lengeler 1985). The organization of the mannitol operon in *V. cholerae* consists of an Enzyme II^{Mtl} (*mtlA*) -encoding IICBA complex, followed by *mtlD* and *mtlR*, similar to that reported from many other Gram-negative bacteria (Lee and Saier 1983). In Gram-positive bacteria such as *B. stearothermophilus* (Fig. 1), the enzyme IICB and IIA components are encoded by separate genes, *mtlA* and *mtlF*, respectively (Fischer et al. 1989).

We demonstrated that the II^{Mtl} encoded by *mtlA* is the only mannitol-specific uptake system in *V. cholerae* O395 by insertional inactivation of the gene. The intron insertion into the *mtlA* gene resulted in *V. cholerae* with a mannitol non-fermenting phenotype. The intron insertion into *mtlA* resulted in the disruption of the entire operon as evidenced by the absence of *mtlA*, *mtlD* and *mtlR* RNA transcripts when tested by real-time PCR (data not shown). Further, our results also conclusively prove that a single operon functions to transport and utilize both mannitol and sorbitol in *V. cholerae* O395, since the inactivation of *mtlA* abolished the transport of sorbitol. However, cloning of the 7.2 kb *mtlADR* with promoter sequences did not confer a mannitol fermentation phenotype in *E. coli* HB101, suggesting that *E. coli* enzyme I and HPr components poorly interact with *V. cholerae* EII^{Mtl}. A second plasmid system consisting of *V. cholerae ptsH* and *ptsI* gene sequences was designed, and complementation by this second plasmid system was apparent from red colonies of *E. coli* HB101 on MacConkey-mannitol agar.

Slow fermentation of D-mannitol by toxigenic strains of *V. cholerae* has been used to distinguish them from non-toxigenic strains that ferment mannitol rapidly (Wang et al. 2007). In our study, we used two *V. cholerae* strains for comparison of the functioning of mannitol operon. *V. cholerae* O395 is a toxigenic strain that ferments mannitol very slowly. When streaked on MacConkey-mannitol agar, *V. cholerae* O395 exhibited a white colony phenotype suggestive of non-sorbitol fermentation. When the white colonies were streaked again, light pink colonies appeared, that turned red upon a third streaking on MacConkey-mannitol agar (data not shown). In contrast, *V. cholerae* PS15, a non-O1 serotype, exhibited dark red colonies in the first streak plating, suggesting rapid mannitol utilization. We wanted to determine if this difference could be demonstrated in an *E. coli* background by cloning

EII^{Mtl} (*mtlA*) from *V. cholerae* O395 and *V. cholerae* PS15. Interestingly, HB/$mtlA^{O395}$ and HB/$mtlA^{PS15}$ were both white colonies on MacConkey-mannitol agar. As a positive control, we cloned *mtlA* from *E. coli* DH5α and transformed HB101 (HB/$mtlA^{EC}$) which exhibited dark red colonies on MacConkey-mannitol agar. Since *mtlA* was constitutively expressed from a high copy plasmid, the non-functioning of *V. cholerae mtlA*, from both toxigenic and non-toxigenic strains in *E. coli*, provides indirect evidence to suggest that the differences in rate of fermentation between toxigenic and non-toxigenic strains are not due to differences in the levels of *mtlA* expression. A recent study showed that *mtlR* is not responsible for differences in mannitol fermentation rates between toxigenic and non-toxigenic strains (Wang et al. 2007). These observations together with the results of our study compel us to propose that in *V. cholerae*, the functioning of the mannitol operon is distinct compared to *E. coli*, and the differences in the mannitol utilization between toxigenic and non-toxigenic strains could be due the differences in expression of *ptsH* and *ptsI* genes.

EII^{Mtl} with 649 amino acid residues has a predicted molecular weight of 68.69 kDa. Analysis of the N-terminal amino acid sequence of II^{Mtl} did not reveal the presence of a signal peptide. Using bioinformatic tools, we derived a secondary structure for EII^{Mtl}, which exhibited a hydrophobic transmembrane N-terminal domain and a hydrophilic cytoplasmic domain, akin to the II^{Mtl} of *E. coli* (Lengeler et al. 1994) (Fig. 2). The membrane bound N-terminal portion is 334 amino acids long with 9 predicted transmembrane domains. The cytoplasmic domain corresponds to C-terminal portion of the protein from amino acids 335–649. The larger EII^{Mtl} of *E. coli* has a fused C-terminal domain, which functions independently as EIII (MtlF) in Gram-positive bacteria (Saier et al. 1985). In *E. coli* II^{mtl}, His-554 and Cys-384 have been shown to be the first and second sites of phosphorylation, respectively (Pas and Robillard 1988; Pas et al. 1988). In *V. cholerae* EII^{Mtl}, based on the sequence alignment, the potential phosphorylation sites are predicted to be Cys-389 and His-564 (Fig. 3). We also compared the C-terminal 147 amino acids of *V. cholerae* EII^{Mtl} with corresponding sequences from *E. coli* and *V. parahemolyticus*, and with MtlF from *Staphylococcus carnosus*, *Clostridicum acetobutylicum* and *Bacillus stearothermophilus* (Fig. 4). The first phosphorylation site within a highly conserved PHGT peptide was identified in sequences compared here, except in the MtlF of *C. acetobutylicum* in which tyrosine is replaced by a methionine. However, phosphorylation at these sites in the mannitol permease of *V. cholerae* remains to be experimentally demonstrated. The MtlF domains of *S. carnosus*, *C. acetobutylicum* and *B. stearothermophilus* were 35–44% similar to the corresponding sequence in the C-terminal domain of *V. cholerae* II^{Mtl}.

MtlA simultaneously takes part in phosphorylation and transport of mannitol as mannitol 1-phosphate, that is subsequently oxidized to fructose 6-phosphate by the cytoplasmic enzyme mannitol-1-phosphate dehydrogenase (Wolff and Kaplan 1956; Saier 1977; Lee et al. 1981). Since we cloned the entire mannitol operon containing *mtlD* encoding mannitol phosphate dehydrogenase, the transport of mannitol was essentially “downhill”. The mannitol transport kinetics were determined in *E. coli* HB101 using [^{3}H]mannitol. In the presence of PEP, [^{3}H]mannitol was transported at a rate of 13.8±1.4 nmol/min/mg protein. The apparent K_m value of 2.74±0.2 μM suggests that the mannitol PTS of *V. cholerae* has a very high affinity for its substrate. Since, *E. coli* HB101 has a native MtlD, this will not be a definitive determination of mannitol utilization. Hence, we compared the mannitol transport kinetics of wild type *V. cholerae* O395 and *V. cholerae* PS15, the latter being a non-O1 strain. The K_m of mannitol transport was not significantly different between these two strains, being 0.97±0.02 μM and 1.18±0.13 μM, respectively for *V. cholerae* O395 and PS15 strains, suggesting similar affinities for the substrate. However, the V_{max} value of *V. cholerae* PS15 was 48.1±1.8 nmol/min/mg, much higher than the V_{max} of 16.6±0.21 nmol/min/mg protein obtained with *V. cholerae* O395.

Mannitol fermentation has been used as an important biochemical assay in the identification of of vibrios (West and Colwell 1984). Vibrios, which live in nutrient-limiting oligotrophic waters may vastly benefit physiologically by having an efficient uptake system for mannitol either by biofilm form of association with mannitol-producing algae or the utilization of mannitol released into seawater from decaying seaweeds. The mannitol fermentation phenotype has been correlated with virulence in *V. vulnificus* (Drake et al. 2010). However, the exact mechanism by which mannitol utilization contributes to virulence is not elucidated at the molecular level. While being involved in phosphorylation and uptake of specific sugars, PTS is known to be involved in global regulation of other carbohydrate utilization genes and the Kreb's cycle (Postma and Lengeler 1985). To achieve mannitol transport, all *V. cholerae* PTS components were required in *E. coli*. Transformation of *E. coli* HB101 with *mtlA* (II^{Mtl}) alone did not confer a positive mannitol fermentation phenotype. When *ptsH* and *ptsI* genes were mobilized in a separate plasmid into *E. coli* HB101 with EII^{MtlA}, the host cells utilized mannitol. A similar cloning strategy was previously reported for obtaining a functional PTS system of *Staphylococcus carnosus* (Fischer and Hengstenberg 1992).

This study will be the first step towards understanding the structure-function relationship of the mannitol transporter in *V. cholerae* and its physiological significance in vibrios. We are currently attempting to express and purify EII^{Mtl} and to determine if i) Cys-389 and His-564 are active phosphorylation sites by site directed mutagenesis ii) differences in levels of *ptsH* and *ptsI* gene expression are responsible for differences in mannitol transport rates and utilization between the toxigenic and non-toxigenic strains of *V. cholerae*.

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References

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. Microbiology. 1995; 141:3241–3245. [PubMed: 8574415]

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]

Ausubel, FM.; Brent, R.; Kingsten, RE.; Moore, DD.; Seidman, JG.; Smith, JA.; Struhl, K. Short Protocols in Molecular Biology. 3. John Wiley and Sons; New York: 1995.

Barabote RD, Saier MH Jr. Comparative genomic analyses of the bacterial phosphotransferase system. Microbiol Mol Biol Rev. 2005; 69:608–634. [PubMed: 16339738]

Behrens S, Mitchell W, Bahl H. Molecular analysis of the mannitol operon of *Clostridium acetobutylicum* encoding a phosphotransferase system and a putative PTS-modulated regulator. Microbiology. 2001; 147:75–86. [PubMed: 11160802]

Davanloo P, Rosenberg AH, Dunn JJ, Studier FW. Cloning and expression of the gene for bacteriophage T7 RNA polymerase. Proc Natl Acad Sci USA. 1984; 81:2035–2039. [PubMed: 6371808]

Drake SL, Whitney B, Levine JF, Depaola A, Jaykus LA. Correlation of mannitol fermentation with virulence-associated genotypic characteristics in *Vibrio vulnificus* isolates from oysters and water samples in the Gulf of Mexico. Foodborne Pathog Dis. 2010; 7:97–101. [PubMed: 19785540]

Efiuvwevwere BJO, Gorris LGM, Smid EJ, Kets EPW. Mannitol-enhanced survival of *Lactococcus lactis* subjected to drying. Appl Microbiol Biotechnol. 1999; 51:100–104.

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 IImannitol of *Escherichia coli*. Eur J Biochem. 1992; 204:963–969. [PubMed: 1551396]

Fischer R, Eisermann R, Reiche B, Hengstenberg W. Cloning, sequencing and overexpression of the mannitol-specific enzyme-III-encoding gene of *Staphylococcus carnosus*. Gene. 1989; 82:249–257. [PubMed: 2684783]

Hanahan, D. DNA Cloning. Glover, D., editor. Vol. 1. IRL Press, Ltd; 1985.

Hengstenberg W, Kohlbrecher D, Witt E, Kruse R, Christiansen I, Peters D, Pogge von Strandmann R, Städtler P, Koch B, Kalbitzer HR. Structure and function of proteins of the phosphotransferase system and of 6-phospho-beta-glycosidases in gram-positive bacteria. FEMS Microbiol Rev. 1993; 12:149–163. [PubMed: 8398213]

Henstra SA, Tolner B, ten Hoeve Duurkens RH, Konings WN, Robillard GT. Cloning, expression, and isolation of the mannitol transport protein from the thermophilic bacterium*Bacillus stearothermophilus*. J Bacteriol. 1996; 178:5586–5591. [PubMed: 8824601]

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]

Jennings DH. Polyol metabolism in fungi. Adv Microb Physiol. 1984; 25:149–193. [PubMed: 6099966]

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]

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]

Lengeler JW, Jahreis K, Wehmeier UF. Enzymes II of the phosphoenol pyruvate-dependent phosphotransferase systems: their structure and function in carbohydrate transport. Biochim Biophys Acta. 1994; 1188:1–28. [PubMed: 7947897]

Meadow ND, Revuelta R, Chen VN, Colwell RR, Roseman S. Phosphoenolpyruvate:glycose phosphotransferase system in species of *Vibrio*, a widely distributed marine bacterial genus. J Bacteriol. 1987; 169:4893–4900. [PubMed: 3667518]

Pas HH, Robillard GT. S-phosphocysteine and phosphohistidine are intermediates in the phosphoenolpyruvate-dependent mannitol transport catalyzed by *Escherichia coli* II^{Mtl}. Biochemistry. 1988; 27:5835–5839. [PubMed: 3142516]

Pas HH, ten Hoeve-Duurkens RH, Robillard GT. Bacterial phosphoenolpyruvate-dependent phosphotransferase system: mannitol-specific EII contains two phosphoryl binding sites per monomer and one high-affinity mannitol binding site per dimer. Biochemistry. 1988; 27:5520–5525. [PubMed: 3140890]

Postma PW, Lengeler JW. Phosphoenolpyruvate:carbohydrate phosphotransferase system of bacteria. Microbiol Rev. 1985; 49:232–269. [PubMed: 3900671]

Postma PW, Lengeler JW, Jacobson GR. Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. Microbiol Rev. 1993; 57:543–594. [PubMed: 8246840]

Saier MH Jr, Grenier FC, Lee CA, Waygood EB. Evidence for the evolutionary relatedness of the proteins of the bacterial phosphoenolpyruvate:sugar phosphotransferase system. J Cell Biochem. 1985; 27:43–56. [PubMed: 3884637]

Saier MH Jr. Bacterial phosphoenolpyruvate:sugar phosphotransferase systems: structural, functional, and evolutionary interrelationships. Bacteriol Rev. 1977; 41:856–871. [PubMed: 339892]

Segel, IH. Biochemical calculations: how to solve mathematical problems in general biochemistry. 2. John Wiley & Sons; New York, NY: 1976.

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]

Wang HY, Yan MY, Zhao YW, Kan B. Transcriptional repressor gene--mtlR of mannitol PTS operon in *Vibrio cholerae*. Wei Sheng Wu Xue Bao. 2007; 47:522–525. [PubMed: 17672318]

West, PA.; Colwell, RR. Identification and classification of *Vibrionaceae*-an overview. In: Colwell, RR., editor. Vibrios in the environment. John Wiley & Sons Inc; New York, N.Y: 1984. p. 285-363.

Wolff JB, Kaplan NO. D-Mannitol-l-Phosphate dehydrogenase from *Escherichia coli*. J Biol Chem. 1956; 218:849–869. [PubMed: 13295236]

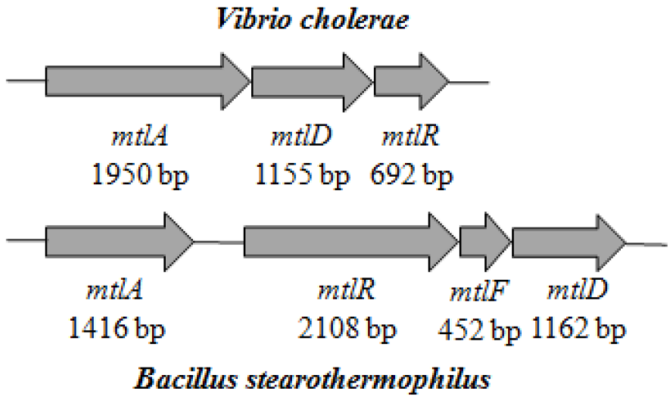

Fig. 1.
Organization of the mannitol operons of Gram-negative (*V. cholerae*) and Gram positive bacteria (*B. stearothermophilus*).

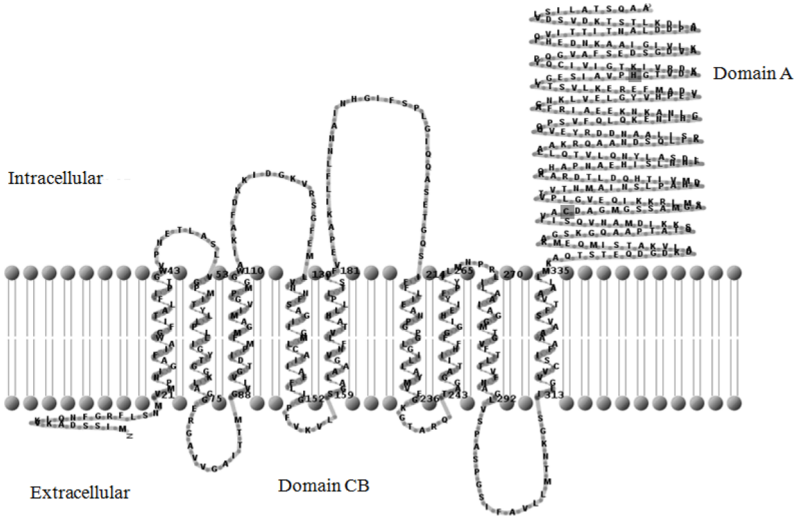

Fig. 2.

Predicted secondary structure of Enzyme II^{Mtl} of *V. cholerae* O395. The two phosphorylation sites Cys 384 and His 554 in *E. coli* EII^{Mtl} correspond to Cys-389 and His-564. These two amino acid residues in domain IIA are shown highlighted.

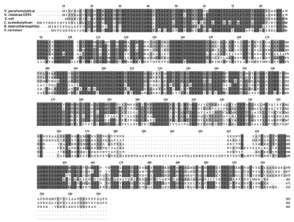

Fig. 3.
Multiple sequence alignment of IICB domain of *V. cholerae* O395 with the corresponding domains of mannitol permeases from different bacteria. The GenBank accession numbers are shown in parentheses against each bacterial species. *E. coli* (V01503), *V. parahemolyticus* (BA000031), *Staphylococcus carnosus* (YP_002634749), *Clostridium acetobutylicum* (AAC12848), *Bacillus stearothermophilus* (P50852).

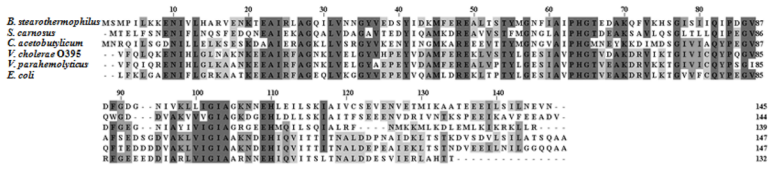

Fig. 4.

Comparison of C-terminal part of EII^{Mtl} from *V. cholerae* O395, *E. coli* (GenBank accession V01503) and *V. parahemolyticus* (GenBank accession BA000031) with MtlF of Gram-positive bacteria *Staphylococcus carnosus* (GenBank accession YP_002634751), *Bacillus stearothermophilus* (GenBank accession Q45420) and *Clostridium acetobutylicum* (GenBank accession AAC12850)

Table 1

Plasmids and bacterial strains used in this study.

Plasmid construct/Strain	Description/genotype	Source/Reference
pSP72/PTS	*ptsH* and *ptsI*; Amp^R	This study
pHSG396/Mtl	*mtlA, mtlD, mtlR*; Cm^R	This study
pSP72/$mtlA^{EC}$	DH5α *mtlA*; Amp^R	This study
pSP72/$mtlA^{O395}$	*V. cholerae* O395 *mtlA*; Amp^R	This study
pSP72/$mtlA^{PS15}$	*V. cholerae* PS15 *mtlA*; Amp^R	This study
pACD4K/*mtlA*	Mutagenic plasmid; Cm^R	This study
pAR1219	Helper plasmid; Amp^R	Davanloo et al. (1984)
E. coli HB101	F^-, *lac*Y1, *gal*K2, (Str^r), *xyl*-5, *mtl*-1.	Hanahan (1985)
E. coli DH5α	F^- *end*A1 *rec*A1 *gal*E15 *gal*K16 *nup*G *rps*L *ΔlacX74 Φ80lacZΔ*M15 *ara*D139 Δ(*ara*,*leu*)7697 *mcrA* Δ(*mrr*$^-$*hsd*RMS- *mcr*BC) λ^-	Invitrogen, Carlsbad, CA.
E. coli HB/Mtl	mannitol operon, *ptsI*, *ptsH*; Amp^R, Cm^R	This study
Vibrio cholerae O395	Wild-type *V. cholerae*	ATCC 39541/Ogawa 395
Vibrio cholerae PS15	Wild-type *V. cholerae* non-O1	This study
V. cholerae O395 *mtlA*:: Intron	*mtlA* mutant, Kan^R	This study

Table 2

Oligonucleotide primers used in this study

Primer	Nucleotide sequences (5′-3′)	Target gene	Product size (bp)
PTS1	gcgggatccactattttcatcgcctatctcct	*ptsI* and *ptsH*	2330
PTS2	gcgctcgaggacggtttacgctcaattgctca		
ManXh	gcgctcgagcggtgtatttttagttgacccgt	Mannitol operon	7256
ManEco	gcggaattcatcatggcgatggatataaccgg		
Mtl-ECF	gcgggatccatgtcatccgatattaagatcaa	*E. coli mtlA*	1914
Mtl-ECR	gcgctcgagttacttacgacctgccagcagttc		
Mtl-VCF	gcgggatccatgatatcatcagacgccaaggt	*V. cholerae mtlA*	1950
Mtl-VCR	gcgctcgagttatgccgcttggctggtggcca		
IBS	aaaaaagcttataattatccttaagagacgaaagcgtgcgcccagatagggtg	Mutagenic primers	-
EBS1d	cagattgtacaaatgtggtgataacagataagtcgaaagcggtaacttacctttctttgt		
EBS2	tgaacgcaagtttctaatttcgatttctcttcgatagaggaaagtgtct		