Proteins involved in difference of sorbitol fermentation rates of the toxigenic and nontoxigenic *Vibrio cholerae* El Tor strains revealed by comparative proteome analysis

Ruibai Wang, Hongzhi Zhang, Haiyan Qiu, Shouyi Gao and Biao Kan*

Address: State Key Laboratory for Infectious Disease Prevention and Control, Department of Diarrheal Diseases, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 100220, PR China

Email: Ruibai Wang - ruibaiwang@yahoo.cn; Hongzhi Zhang - zhanghongzhi@126.com; Haiyan Qiu - qiuhaiyan@126.com; Shouyi Gao - gaoshouyi@icdc.cn; Biao Kan* - kanbiao@icdc.cn

* Corresponding author

**Abstract**

**Background:** The nontoxigenic *V. cholerae* El Tor strains ferment sorbitol faster than the toxigenic strains, hence fast-fermenting and slow-fermenting strains are defined by sorbitol fermentation test. This test has been used for more than 40 years in cholera surveillance and strain analysis in China. Understanding of the mechanisms of sorbitol metabolism of the toxigenic and nontoxigenic strains may help to explore the genome and metabolism divergence in these strains. Here we used comparative proteomic analysis to find the proteins which may be involved in such metabolic difference.

**Results:** We found the production of formate and lactic acid in the sorbitol fermentation medium of the nontoxigenic strain was earlier than of the toxigenic strain. We compared the protein expression profiles of the toxigenic strain N16961 and nontoxigenic strain JS32 cultured in sorbitol fermentation medium, by using fructose fermentation medium as the control. Seventy-three differential protein spots were found and further identified by MALDI-MS. The difference of product of fructose-specific IIA/FPR component gene and mannitol-1-P dehydrogenase, may be involved in the difference of sorbitol transportation and dehydrogenation in the sorbitol fast- and slow-fermenting strains. The difference of the relative transcription levels of pyruvate formate-lyase to pyruvate dehydrogenase between the toxigenic and nontoxigenic strains may be also responsible for the time and ability difference of formate production between these strains.

**Conclusion:** Multiple factors involved in different metabolism steps may affect the sorbitol fermentation in the toxigenic and nontoxigenic strains of *V. cholerae* El Tor.

**Background**

*Vibrio cholerae* is the causative agent of the diarrheal disease cholera. Out of the 200 serogroups of *V. cholerae*, only two biotypes of serogroup O1 (classical and El Tor) and serogroup O139 cause severe diarrhea and epidemic cholera [1], although not all strains in these two sero-

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V. cholerae is capable of metabolizing many types of carbohydrates. Previously, we found that not only is D-sorbitol metabolized by V. cholerae, but it is also fermented at different rates by the toxigenic and nontoxigenic El Tor strains. The toxigenic strains have a low sorbitol fermentation rate and are called slow-fermenting strains, whereas the nontoxigenic strains have a faster sorbitol fermentation rate and are called fast-fermenting strains [6]. The sorbitol fermentation test is included in the Phage-biotyping scheme, which consists of phage typing and biochemical typing and is developed in 1970s in China. This scheme is used to distinguish and type the El Tor strains which are pathogenic and are potential to cause epidemic or not [6]. It is found that the O1 El Tor strains isolated from patients and environmental samples in epidemics are toxigenic and sorbitol slow-fermenting, whereas the strains isolated from environment in non-epidemic periods are nontoxigenic and fast-fermenting.

In some bacteria, D-sorbitol is transported into the cell via the sorbitol specific phosphotransferase system (PTS) or some non-sorbitol specific PTS, and then it is transformed from sorbitol-6-phosphate to fructose-6-phosphate and enters the fructose/mannitol metabolism pathway. All genes involved in the fructose/mannitol metabolism pathway in V. cholerae have been identified and annotated on the genome [7], but the genes involved in sorbitol transportation and transformation are unknown. This approach allowed to identify differences in protein expression associated with sorbitol metabolism difference in the toxigenic and nontoxigenic V. cholerae strains. Differences of formate production, fructose-6-phosphate production and subsequent metabolism were found to be causative mechanisms in the sorbitol fermentation difference in the toxigenic and nontoxigenic V. cholerae strains.

Methods

Bacterial Strains

Two V. cholerae strains of serogroup O1 El Tor (N16961 and JS32) were used to compare protein expression profiles by 2-DE analysis. N16961 is a toxigenic strain whose complete genome was previously published [7], while JS32 is a nontoxigenic strain isolated in 1982 in China. An additional eight toxigenic strains (97005, LN2001-5, GD97-73, ZJ62-10, D118, 93–284, WUJIANG-2 and 63–12) and three nontoxigenic strains (V05-18, 79327 and 60–61) isolated in China were also included in this study (Table 1).

Sorbitol and fructose fermentation tests

Fresh colonies cultured on Luria-Bertani (LB) agar were selected and inoculated statically in 1 ml LB broth at 37°C for 2 hours, to reach the OD₆₀₀ of 0.5 or 1 × 10⁷ CFU/ml equivalently. Then 100 µl cultures were transferred into 3
ml fermentation media (0.01% peptone, 5% NaCl, 2% sorbitol or fructose, and 0.025% phenol red; pH 8–9) and inoculated statically at 37°C. Sugar fermentation was measured as the color change in the medium 4 and 8 hours post-inoculation (yellow, fast fermentation or a positive test; red, slow fermentation or a negative test) [6]. Considering the high concentration of sorbitol in the fermentation medium, fructose at a similar concentration was used as a control sugar in the proteome analysis to eliminate differences in nutrient usage, osmotic pressure and pH in the media with and without sorbitol. pH of the fermentation medium was measured with CPpH 59003-05 (Cole).

**1H-NMR**

One milliliter of the fermentation media cultured with the test strains was collected and centrifuged at 10,000 × g at room temperature for 10 min to clarify the supernatant. The 1H resonance of D2O (10%) was used to lock the field room temperature for 10 min to clarify the supernatant. Consideration of higher concentration of sorbitol in the fermentation medium, fructose at a similar concentration was used as a control sugar in the proteome analysis to eliminate differences in nutrient usage, osmotic pressure and pH in the media with and without sorbitol. pH of the fermentation medium was measured with CPpH 59003-05 (Cole).

**In-gel protein digestion, MALDI-TOF-MS and protein identification**

Protein spots of interest were excised from the gels. After destaining, gel pieces were digested with trypsin (Roche, Germany) for 12 h at 37°C. The extracts were dried and resolubilized in 2 μl of 0.5% TFA. Peptide mass fingerprinting (PMF) measurements were performed on a Bruker Reflex™ III MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) working in reflectron mode with 20 kV of accelerating voltage and 23 kV of reflecting voltage. A saturated solution of α-Cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile and 0.1% trifluoroacetic acid (TFA) was used for the matrix. Mass accuracy for PMF analysis was 0.1–0.2 Da with external calibration; internal calibration was carried out using enzyme autolysis peaks, and the resolution was 12,000. Database searches were performed using the software Mascot v1.7.02 (Matrix Science Ltd.) licensed in-house http://mascot.proteomics.com.cn/search_form_PMF.html against the database of *V. cholerae* N16961 (Version Vib CLEAN 040921, 3814 sequences). Monoisotopic peptide masses were used to search the databases with a mass tolerance of 100 ppm and one partial cleavage. Oxidation of methionine and carbamidomethyl modification of cysteine was considered. Scores greater than 48 were significant (p < 0.05), with more than five peptides matched and sequence coverage greater than 15%.

**Comparative proteome analysis**

*V. cholerae* strains N16961 and JS32 were cultured in 400 ml sorbitol or fructose fermentation media. The *V. cholerae* cell precipitates were washed with precooled low salt PBS (3 mM KCl, 1.5 mM KH2PO4, 68 mM NaCl, 9 mM NaH2PO4) and disrupted and solubilized using lysis solution (7 M Urea, 2 M Thiourea, 4% CHAPS, 50 mM DTT) and sonicated for 2 min on ice using the Sonifier 750 (S&M0202, Branson Ultrasonics Corp., Danbury, CT, USA). After centrifuging at 100,000 × g 15°C for 45 min, supernatant aliquots were stored at -70°C and the protein concentration was determined with the PlusOne 2-D Quant Kit (Amersham Pharmacia, Sweden).

**Sequencing of the gene VCA0518**

The gene VCA0518 (designated in the genome of N16961, GenBank Accession Number NC002506), which corresponds to the fructose-specific IIA/FPR component (PTS system, FIIA), was amplified from all tested strains using primers 5' CCT CTG CAT TTA AGG TGA TGG 3' and 5' TCAG CCT ATA GAT GAG GCA GAG AGG 3' and sequenced. The sequences were searched in the CDD database (v2.16-27036PSSMs, http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) for conserved domain analysis.

**Quantitative real-time PCR (qRT-PCR)**

Total RNA from N16961 and JS32 cultured in sorbitol fermentation media was extracted at the inoculation time points 2, 4, 6 and 8 h with the RNasy Mini Kit (Qiagen). The following primer pairs were used in reverse transcription and amplification of genes: 5' CCG CAG GAA TCG TGT TGA TGG 3' and 5' GCC GTT GTC GGT CTT GAA GGA G 3' for pyruvate dehydrogenase subunit E1 (VC2414); 5' CAG GAC CCT GCC TAC ATC AAC 3' and 5' ACC ATA CGG ATA CCA CCA CCA TTA GGG 3' for pyruvate formate-lyase 1 activating enzyme (PFL) (VC1866); and 5' AAG ATT GGT GTG ATC TTT GGT A 3'.
and 5' CAC TTC TTC GCC TTC TTT GA 3' for the internal standard recA gene. The reaction was performed using the SYBR premix Ex Taq™ (TaKaRa, Dalian, China). The \( \Delta \Delta Ct \) method was used to calculate relative expression of the VC18166 gene to the VC2414 gene in the N16961 and JS32 strains, and normalized with the control gene recA. 

\[ \Delta \Delta Ct = (Ct_{VC1866} - Ct_{VC1866\text{recA}}) - (Ct_{VC2414} - Ct_{VC2414\text{recA}}) \]

where \( Ct \) is the Ct values of VC1866 simultaneously amplified with VC1866 and VC2414, \( Ct_{VC1866\text{recA}} \) and \( Ct_{VC2414\text{recA}} \) indicating the Ct values of recA simultaneously amplified with VC1866 and VC2414, \( Ct_{VC1866} \) and \( Ct_{VC2414} \) indicate the Ct values of VC1866 and VC2414.

**Results**

**Dynamic change of the fermentation medium pH**

We measured the pH of the sorbitol fermentation media of the strains during the fermentation test, by extracting 5 ml of the media serially at each time point, from a volume of 400 ml culture of each strain. The pH-time curves (Fig. 1) demonstrate that the JS32 sorbitol fermentation medium pH dropped gradually over time, while that of N16961 leveled off at pH 6.5 for about 2 hours before dropping again. The change in pH was consistent with the toxigenic and nontoxigenic strains, we examined changes in medium components using \(^1\)H-NMR. The toxigenic and nontoxigenic strains, we examined.

**\(^1\)H-NMR analysis**

In order to understand the differences in pH observed for the toxigenic and nontoxigenic strains, we examined changes in medium components using \(^1\)H-NMR. The majority of the components in the sorbitol fermentation media exhibited similar depletion or formation for JS32 and N16961 (Fig. 2). One exception was the appearance of two volatile compounds (formate and lactic acid). Formate appeared in the JS32 culture earlier than in the N16961 culture, and the different production rates of formate between these two V. cholerae strains were consistent with their pH changes and fermentation rates. At the time of color change in the JS32 fermentation sample, the concentrations of acetic acid and formate in the medium were 30.53 mmol/L and 16.86 mg/L (0.509 mmol/L and 0.367 mmol/L, respectively). In contrast, the acetic acid concentration in N16961 fermentation media was 24.37 mg/L (0.406 mmol/L), and formate was below the level of detection.

**Comparative proteomic analysis**

At the positive time point of the sorbitol fermentation test of JS32 (4 hours), whole cell proteins from four different cultures were prepared and separated by 2-DE. These protein profiles were designated FN, SN, FJ and SJ, indicating samples prepared from N16961 in fructose, N16961 in sorbitol, JS32 in fructose, and JS32 in sorbitol fermentation medium, respectively.

Out of 73 total differential spots identified in the SN/FN and SJ/FJ comparisons, 10 common signified potential proteins of these two comparison groups may be involved in the difference between the sorbitol and fructose metabolism pathway: amino acid ABC transporter, perosamine synthase, malate dehydrogenase, aminotransferase NifS, heat shock protein HtpG, succinyl-CoA synthase, FIIA, glycerol kinase, pyruvate dehydrogenase, and oxygen-insensitive NAD(P)H nitroreductase. Three of these proteins (glycerol kinase, oxygen-insensitive NAD(P)H nitroreductase, and FIIA) were more abundant in sorbitol medium.

Two proteins within the identified 73 spots, the FIIA protein of PTS system and mannitol-1-P dehydrogenase (MtlD), may be involved in the transportation and transformation of sorbitol. FIIA was a common differential protein observed in the comparisons of SN/FN and SJ/FJ, respectively.

![Figure 1](http://www.biomedcentral.com/1471-2180/9/135)

**Figure 1**

pH-time curves of toxigenic strain N16961 and nontoxigenic strain JS32 on sorbitol fermentation media.
Figure 2

$^1$H-NMR spectra of JS32 and N16961 sorbitol fermentation medium. Samples were collected at four time points: the starting time (0 h), the JS32 color change (4 h), the N16961 color change (8 h), and 24 hours. Formate could be seen at 4 h in JS32, while there was no formate peak in N16961.
both of which were more abundant in the sorbitol medium than in the fructose medium (Fig 4A). MtlD was more abundant in sorbitol than in fructose fermentation medium of the sorbitol fast-fermenting strain JS32, but was not found difference in SN/FN comparison of the sorbitol slow-fermenting strain N16961 (Fig. 4B), suggesting its potential role in the sorbitol metabolism difference between these two strains. Two different pl forms of it were also found in SJ sample (Fig. 4B). Two proteins, pyruvate dehydrogenase and PFL, were also found differences in the comparison of sorbitol to fructose fermentation in JS32 and N16961, pyruvate dehydrogenase was more abundant in SJ of SJ/FJ comparison but less abundant in SN of SN/FN comparison, and PFL was more abundant in both FJ and FN (Fig. 4C and 4D). These two enzymes were both involved in pyruvate transformation, and PFL catalyzes pyruvate to produce formate. Their different expression may suggest their roles in formate production in the sorbitol fast- and slow-fermenting strains. In addition, the haemolysin and hcp proteins, which are related to *V. cholerae* pathogenicity, were also abundant spots on the SN gel, showing higher expression levels in N16961.

Figure 3

2-DE gels of whole cell proteins of *V. cholerae* strains JS32 and N16961 cultured in sorbitol and fructose fermentation media. The comparative proteins (comparison between SN/FN and SJ/FJ) were marked and numbered on their more abundant maps.
Sequencing of the VCA0518 gene

Due to the observed differences on the 2-DE gels (the VCA0518 gene product, FIIA component), the VCA0518 gene from all toxigenic and nontoxigenic strains studied were amplified and sequenced (GenBank: EF581766 to EF581778). All of the sequences contained three predicted conserved domains: the fructose specific PTS EIIA component, the EIIA component of PTS, and the HPr protein. The sequences of the nine toxigenic strains were highly similar but differed from the nontoxigenic strains, while three of four nontoxigenic strains had identical sequences. A comparison of amino acid residues of the nontoxigenic and toxigenic strains revealed changes mainly localized at the spacer region between the latter two domains. Nearly all of these residues involved changes in the polarity or acid-alkalinity of the amino acid (Fig. 5). Three of the four nontoxigenic strains (JS32, 79327 and V05-18) lacked a 15 nucleotide (nt) region (AGCTGTGGGAACGAT) from 861 to 875, and the pI's of their proteins changed from 5.88 to 5.75. This data was consistent with the appearance of the FIIA protein spots on the 2-DE gels. The nontoxigenic strain 60–61 did not lack the 15 nt fragment, but amino acid mutations placed it in the farthest phylogenetic cluster from the other strains (data not shown).

qRT-PCR of VC1866 and VC2414

PFL (VC1866) and pyruvate dehydrogenase (VC2414) were identified as spots in the proteomic analysis (Fig. 4) and are involved in the production of fermentation acids. We monitored transcription levels at four time points during the fermentation assay. The transcription levels of both VC1866 and VC2414 of JS32 were higher than those of N16961 in sorbitol fermentation medium at 4 hrs and
reversed at 8 hrs (Fig. 6A). When comparing the relative transcription levels of VC1866 to VC2414 of JS32 and N16961 (Fig. 6B), we found that the relative transcription of VC1866 of JS32 was higher than of N16961 at all time points. JS32 transcription of VC1866 reached a peak five-fold increase at 6 hrs, whereas N16961 transcription was only increased two-fold. No wonder the fast-fermenting strain JS32 showed much higher production of formate than did the slow-fermenting strain N16961.

Discussion

Nontoxigenic V. cholerae strains ferment sorbitol at a faster rate than toxigenic strains, one of phenotyping included in the Phage-biotyping, which has been widely used as a typing scheme in cholera surveillance for many years in China and has been confirmed by thousands of strains [6]. To understand the mechanism of this difference in sorbitol fermentation rate, here we compared the expression of proteins involved in sorbitol fermentation in toxigenic and nontoxigenic strains. The proteome profiles of the cells cultured in sorbitol and fructose medium were very similar with few differential spots, indicating that the status of the cells in these two conditions was similar. Therefore, we could subtract the most commonly expressed constitutive proteins not related to sorbitol fermentation when comparing SN/FN and SJ/FJ. This approach identified two PTS proteins and two proteins involved in formate production.

In general, the specificity of sugar PTSs lies in their EIIA component, while the HPr protein and EI enzyme are encoded by independent genes and are commonly used by different sugar PTS systems. In the conservative domain analysis of the V. cholerae VCA0518 gene, we found that this EIIA component was larruping and it contained three conservative domains, two of which are not sugar-specific. The sequences of the three domains were almost completely identical for all tested strains, further demonstrating their highly conserved nature. We conjectured that the low specificity of the co-expressed HPr and EIIA domains endowed the VCA0518 gene product with a role in sorbitol utilization. Contrary to the conservation of the domains, the entire VCA0518 gene sequences of the 13 tested strains showed obvious differences between the toxigenic and nontoxigenic strains, with the variable amino acid residues located at the spacer region between the domains. These differences may impact the steric conformation and the regulation of this protein, and further impact the efficiency of sorbitol transportation. The regulation of transcription, which maybe also affects the expression of VCA0518 in the sorbitol fast-fermenting and slow-fermenting strains, should also be considered.

MtlD catalyses the transformation of mannitol-1-P to fructose-6-P, the later enters the fructose metabolism pathway. Mannitol and sorbitol are very similar in molecular structure. In Pseudomonas fluorescens, sorbitol is transported by the mannitol PTS system and transformed by polyol dehydrogenase, which has a broad substrate spectrum [14,15]. In a previous study we confirmed the transcriptions of the N16961 VCA1046 gene in sorbitol and mannitol fermentation media [16]. Here, our results indicate that two non-sorbitol specific PTSs are involved in the V. cholerae sorbitol utilization process. This may be similar to the uptake of L-sorbose in Lactobacillus casei where L-sorbose is mainly taken up via EII/Sos and EII/Man plays a secondary role [17]. In Bacillus subtilis, MtlD is required for sorbitol assimilation in addition to the gut operon [18]. Interestingly, both of these PTSs are located on chromosome I of V. cholerae. Several studies indicate that the two chromosomes of V. cholerae are heterologous and that chromosome II may be a megaplasmid captured by an ancestral V. cholerae [7]. The ability to ferment sorbitol used to differentiate V. cholerae strains may provide clues as to both the origins and genetic variation of the toxigenic and nontoxigenic strains.
The traditional sorbitol fermentation test is a phenotypic method using phenol red as the indicator. In our study, we showed that the observed differences in sorbitol fermentation rates were the result of changes in the production rate of formate in the fast-fermenting and slow-fermenting strains. The fact that the ratio of formate to acetic acid was not consistent between the two strains also indicated that, besides the differences early in the metabolic pathway (including the transportation and transformation of sorbitol), pyruvate catabolism could be different in sorbitol fermentation in the toxigenic and nontoxigenic strains. Both pyruvate dehydrogenase and PFL can catalyze the transformation of pyruvate to acetyl-CoA, but they have different electron acceptors and outputs. Their activities affect the relative proportion of the end products [19]. Pyruvate dehydrogenase produces CO2 in addition to acetyl-CoA, while formate is the product of PFL. In the proteomic and qRT-PCR analyses of this study, the respective expression and transcription levels of these two genes were significantly different in the fast-fermenting JS32 and slow-fermenting N16961. Consistent with this fact was that formate was produced earlier in JS32 than in N16961. In a previous study, we had confirmed that the sequences of VC1844 including the promotor region of the toxigenic and nontoxigenic strains could be identical (data not shown). The differential transcription or metabolism of pyruvate was not at VC1844 gene level and there must be a regulation mechanism, which acts at the pyruvate point, differs between the toxigenic and nontoxigenic strains.

The most important difference between the toxigenic and nontoxigenic strains is the presence or absence of the cholera toxin gene ctxAB. When we deleted ctxAB from the toxigenic strains or complemented ctxAB via plasmid into the nontoxigenic strains, we did not observe the reversion of the sorbitol fermentation rate when comparing the mutants with the wild-type strains (data not shown). In the proteomic analysis, we identified two virulence-related proteins. Among them, hemolysin has a predominant role in lethality and confers V. cholerae the ability to prevent clearance and establish prolonged colonization without a requirement for cholera toxin or toxin-coregulated pili [20,21]. V. cholerae Hcp protein is a 28-kDa secreted protein regulated coordinately with hemolysin. The expression of both proteins has been shown to promote expression of virulence determinants in vivo and increase LD50 in the infant mouse cholera model [22,23]. Consistent with their co-regulation relationship, both hemolysin and hcp were more abundant in the N16961 sorbitol culture profiles, suggesting that sorbitol induction and metabolism may have relationship with the regulation of the expression of virulent elements in V. cholerae.

Conclusion
We carried out a comparative analysis of the differences induced by sorbitol between toxigenic (sorbitol slow fermentation) and nontoxigenic (sorbitol fast fermentation) V. cholerae strains. Our results suggest that the differential expression of the FIIA protein and MtlD of mannitol PTS demonstrate changes in the transportation and metabolism of sorbitol, and that pyruvate dehydrogenase and PFL relate to the different production rate of the acid metabolites. The contribution and functional mechanisms of these proteins in the V. cholerae sorbitol fermentation pathway in toxigenic and nontoxigenic strains will require further study.

Authors' contributions
RW carried out the main part of experiments in this study and drafted the manuscript, HZ carried out qRT-PCR and participated in discussion in preparing the manuscript, HQ participated in cultures and sample preparation, SG and BK designed and coordinated the study, and BK revised the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1
The differential protein spots identified by PMF. In this table the protein spots with the differential abundance in the proteome comparisons of SI/FJ and SN/FN in sorbitol and fructose fermentation media respectively, and their PMF identification results, were listed.
Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2180-9-135-S1.doc]

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