

Characterizing the Hexose-6-Phosphate Transport System of *Vibrio cholerae*, a Utilization System for Carbon and Phosphate Sources

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The facultative human pathogen *Vibrio cholerae* transits between the gastrointestinal tract of its host and aquatic reservoirs. *V. cholerae* adapts to different situations by the timely coordinated expression of genes during its life cycle. We recently identified a subclass of genes that are induced at late stages of infection. Initial characterization demonstrated that some of these genes facilitate the transition of *V. cholerae* from host to environmental conditions. Among these genes are uptake systems lacking detailed characterization or correct annotation. In this study, we comprehensively investigated the function of the VCA0682-to-VCA0687 gene cluster, which was previously identified as *in vivo* induced. The results presented here demonstrate that the operon encompassing open reading frames VCA0685 to VCA0687 encodes an ABC transport system for hexose-6-phosphates with K_m values ranging from 0.275 to 1.273 μM for glucose-6P and fructose-6P, respectively. Expression of the operon is induced by the presence of hexose-6P controlled by the transcriptional activator VCA0682, representing a UhpA homolog. Finally, we provide evidence that the operon is essential for the utilization of hexose-6P as a C and P source. Thereby, a physiological role can be assigned to hexose-6P uptake, which correlates with increased fitness of *V. cholerae* after a transition from the host into phosphate-limiting environments.

The life cycle of the facultative human pathogen *Vibrio cholerae* is marked by repetitive transitions between aquatic environments and the host gastrointestinal tract. Besides many other variable conditions, *V. cholerae* has to adjust to different qualities and quantities of nutrient sources. This variability is emphasized by the fact that utilization of nutrients under laboratory conditions, such as in full broth or a chemically defined minimal medium, represents no growth limitation for clinical *V. cholerae* isolates (1).

In the open sea, bacteria such as *V. cholerae* face C, N, and P limitation and are restricted to limited nutrients on a picomolar or nanomolar scale, whereby substrates become available and accessible only in specific habitats (2). Therefore, marine bacteria are found close to organic particles ranging from micrometer- to millimeter-sized aggregates. These organic particles derive from different sources, such as lysed or dead phytoplankton, zooplankton, or fecal pellets. They deliver organic and inorganic substrates in concentrations that are up to 4 orders of magnitude greater than those found in particle-free open seawater (for a recent review, see reference 2). Therefore, many plants and animals in the ocean serve as microbial niches for *V. cholerae* (3–6). For example, copepods or other crustaceans contain or are covered with chitin, a β ,1-4-linked polymer of 2-acetamido-2-deoxy- β -D-glucopyranoside (GlcNAc)_n and its deacetylated form, chitosan. These substrates are utilized by *Vibrio* sp. as C and N sources (7–9). In addition, biofilm formation on chitinous surfaces plays a crucial role in *V. cholerae* persistence in the aquatic environment (5, 10, 11). Therefore, biofilms play a more vital role, as thought for *V. cholerae*. For example, as recently shown in our laboratory, extracellular DNA represents a component of the *V. cholerae*-produced biofilm matrix that can further be utilized as a P source (12). Whether or not hexose-6-phosphate (hexose-6P) can be found in the environment is not known to us, since we found no study addressing the environmental monitoring of these substrates.

After oral infection of its human host, *V. cholerae* passes the stomach and enters the gastrointestinal tract. *V. cholerae* colonizes primarily the small intestine while penetrating the intestinal mu-

cin close to the epithelial barrier, where nutrient-rich cells are located and bacterial propagation takes place (13). As shown by scanning electron microscopy in the infant mouse model (14), *V. cholerae* cells rapidly penetrate the intervillous space and associate with the mucosal surface close to crypts. Besides penetrating the mucus lining, products of mucin or mucous surfaces can serve as a C source. This was shown recently (15), whereby the uptake system for sialic acids was identified in *V. cholerae* and found to be encoded on the *Vibrio* pathogenicity island. Within the host, major changes in *V. cholerae* gene expression take place, whereby virulence gene regulation plays a central role in controlling several regulatory cycles and numerous genes (16). Examples are the well-characterized ToxRS and TcpPH regulons that represent the virulence gene expression system that results in colonization and toxin production (17). Once *V. cholerae* has colonized its human host, its growth ability is exceptional. It was reported that cell numbers reach up to 10⁸ cells/g stool excreted by cholera patients (18). Thereby, a highly active metabolism is necessary to support *V. cholerae* cells to such high growth rates within the host. *In vivo* expression technology (IVET) and recombination-based IVET (RIVET) were used to identify *in vivo*-induced genes in *V. cholerae* (19–25). Using such techniques, it was possible to identify and characterize several virulence genes under infection conditions, such as genes encoding cholera toxin and toxin-coregulated pili. Additionally, in these studies, many other genes encoding so-far-uncharacterized substrate metabolism and uptake systems were

Received 3 October 2012 Accepted 5 February 2013

Published ahead of print 15 February 2013

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doi:10.1128/JB.01952-12

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference
<i>E. coli</i> strains		
DH5 α pir	F ⁻ Δ (<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1 λ::pir</i>	28
SM10 λ pir	<i>thi recA thr leu tonA lacY supE RP4-2-Tc::Mu λ::pir</i>	29
<i>V. cholerae</i> strains		
E7946	O1 El Tor, Ogawa; Sm ^r (WT)	30
Δ VCA0685-7	Deletion of VCA0685, VCA0686, and VCA0687 in E7946; Sm ^r	This work
Δ uhpA	Deletion of VCA0682 in E7946; Sm ^r	This work
VCA0687:: <i>phoA</i>	Insertion of pGPphoA downstream of VCA0687 in E7946; Sm ^r Ap ^r	This work
Δ uhpA VCA0687:: <i>phoA</i>	Insertion of pGPphoA downstream of VCA0687 in Δ uhpA; Sm ^r Ap ^r	This work
Plasmids		
pGP704	<i>ori6K mobRP4</i> , Ap ^r	29
pCVD442	<i>ori6K mobRP4 sacB</i> , Ap ^r	30
pTRC99A	pBR322 origin, Ap ^r	31
pGPphoA	pGP704 with promoterless <i>phoA</i> of SM10 λ pir, Ap ^r	32
pGPphoA-VCA0687	pGPphoA with VCA0687 gene fragment, Ap ^r	This work
pCVD Δ VCA0685-7	pCVD442 with upstream fragment of VCA0685 and downstream fragment of VCA0687, Ap ^r	This work
pCVD Δ uhpA	pCVD442 with up- and downstream fragments of VCA0682, Ap ^r	This work
pTRCuhpA	pTRC99A with reading frame VCA0682, Ap ^r	This work
pTRC0685-7	pTRC99A with reading frames VCA0685 to VCA0687, Ap ^r	This work

found to be induced *in vivo*. Recently, Schild et al. used RIVET in combination with the infant mouse model to identify late *in vivo*-induced genes (26). They showed that the induction of these genes facilitates the transition fitness of *V. cholerae* cells when they depart from the infection site of the host and enter the aquatic environment (27). For example, genes involved in chitin degradation or synthesis of the second messenger *c*-di-GMP have been characterized as being induced late under infection conditions and enhance survival in pond water after *in vivo* passage (16, 26). Interestingly, a considerable number of late *in vivo*-induced genes encode different metabolic enzymes and substrate uptake systems. For most of them, the exact function and transported substrates have not been comprehensively investigated. One example is the uncharacterized late *in vivo*-induced gene cluster encoded by hypothetical genes VCA0685 to VCA0687, which have been demonstrated to play a role in pond water survival after release from an *in vivo* colonization model, e.g., the infant mouse model (16, 26). In the present study, we have characterized VCA0685 to VCA0687 as an ABC transport system for hexose-6P, including its regulation and utilization of hexose-6P as a carbon and phosphate source. The results indicate that the utilization of hexose-6P as a phosphate source by *V. cholerae* is advantageous for subsequent survival under phosphate-limiting conditions.

MATERIALS AND METHODS

Strain construction and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1, and the oligonucleotides used are listed in Table 2. Unless noted otherwise, strains were grown with aeration in Luria-Bertani (LB) broth at 37°C. *V. cholerae* AC53, a spontaneous streptomycin-resistant mutant of *V. cholerae* O1 El Tor clinical isolate E7946 (34), was used as the wild-type (WT) strain in all experiments. For genetic manipulations, *Escherichia coli* strains DH5 α pir and SM10 λ pir were used (28, 29, 35). Antibiotics and other supplements were used at the following final concentrations: streptomycin (Sm), 100 μ g/ml; ampicillin (Ap), 50 μ g/ml in combination with other antibiotics, 100 μ g/ml otherwise; glucose, 0.2%; glycerol, 0.4%; sucrose, 10%.

DNA manipulation and construction of suicide plasmids, reporter fusions, and mutant strains. Chromosomal DNA was isolated as described by Grimberg et al. (36), whereas PCR products and digested plasmid DNA were purified with the QIAquick gel extraction and QIAquick PCR purification kits (Qiagen). PCRs for sequencing and subcloning were carried out with the Phusion High-Fidelity polymerase (NEB). For all other reactions, *Taq* DNA polymerase (NEB) was used.

Deletion mutants were constructed as described by Donnenberg and Kaper (31). PCR fragments of approximately 500 bp upstream and downstream of the gene of interest were amplified with oligonucleotides x-y-1 and x-y-2 or x-y-3 and x-y-4, where x represents the gene and y represents the restriction site/enzyme used (Table 2). After enzymatic digestion, PCR fragments were ligated into similarly digested pCVD442, resulting in the corresponding knockout plasmids (Table 2).

Derivatives of pGPphoA (33) were constructed to obtain chromosomal transcriptional fusions of *phoA* to respective genes. As the *phoA* gene acts as a useful genetic marker in *V. cholerae*, promoterless *phoA* was used to generate the VCA0687::*phoA* operon fusion. The gene fragment corresponding to VCA0687 containing the translational stop codon was amplified by PCR with oligonucleotides VCA0687-SacI-phoA-5' and

TABLE 2 Oligonucleotides used in this study

Primer	Sequence (5' to 3') ^a
VCA0685-NcoI-2	TTT <u>CCATGGG</u> TTTTGCATTAACAATCATTGGGTT
VCA0685-SacI-1	TTT <u>GAGCTC</u> ATTACGCTAGCGAACGGGGCT
VCA0687-XbaI-4	TTT <u>TCTAGACT</u> CAATTTTGAAGACTATGTGCTC
VCA0687-NcoI-3	TTT <u>CCATGGC</u> AGAAATAGTGATAATACCCGTAG
VCA0687-KpnI-phoA-3'	TTTGGTACCATGATCCGCTTTGCTTTGCAG
VCA0687-SacI-phoA-5'	TAT <u>GAGCTC</u> TTAGATGCCAACTTGCGCCG
UhpA-XbaI-1	AAATCTAGAGCATCTCCACGTTTCAGCCCTAG
UhpA-KpnI-2	AAAGGTACCATGATGTTTATCACCTAGCAAAATC
UhpA-KpnI-3	AAAGGTACCATGATCCGCTTTGCTTTGCAG
UhpA-SacI-4	ATTGAGCTCAACGGATTTCGGAATTTGCTCTT
UhpA-EcoRI-5'	TTTGAATTCGTATTTGCTAGGTGATAAACATCA
UhpA-XbaI-3'	TTT <u>TCTAGAGT</u> TAACTTCCACGAGCAGTTG
VCA0685-EcoRI-5'	AAAGAAATCAAGGACACAAGGGAGCATCAA
VCA0687-XbaI-3'	TTT <u>TCTAGAGG</u> CTTTACTCAGGGTATTATCAC

^a Restriction sites are underlined.

VCA0687-KpnI-phoA-3' (Table 2). The PCR product was digested with SacI and KpnI and ligated into pGPphoA digested with the corresponding enzymes, resulting in plasmid pGPphoA-VCA0687 (Table 1).

In the case of the suicide plasmid derivatives used to generate deletion knockout mutants and transcriptional fusions, generated plasmids (Table 1) were first transformed into *E. coli* Sm10λpir and subsequently conjugated into *V. cholerae*. The resulting conjugants were purified via selection for Sm^r and Ap^r colonies. Insertion of pGPphoA-VCA0687 into the VCA0687 locus on the *V. cholerae* chromosome by homologous recombination resulted in transcriptional fusions to the VCA0687-encoding gene. For generation of chromosomal deletions in the case of pCVD442 derivatives (Table 1), sucrose selection was used to obtain Ap^s colonies (31). Correct chromosomal insertions or deletions were confirmed by PCR (data not shown).

Construction of expression plasmids. All expression and complementing plasmids were constructed in a similar manner. PCR fragments of the respective genes, containing their own ribosomal binding sites, were generated with oligonucleotides x-y-5' and x-y-3', where x represents the gene and y represents the restriction site/enzyme used (Table 2). PCR fragments digested with the respective restriction enzymes were ligated into similarly digested plasmid pTRC99A as indicated (Tables 1 and 2) and shown in Results and Discussion.

Growth kinetics. Growth kinetics were determined in transparent 24-well plates (Greiner) with a 1-ml culture volume, providing good aeration with a well-to-culture volume of 3:1, with M9 minimal medium supplemented with carbon sources such as glucose (11 mM), fructose-6P (10 mM, disodium salt hydrate; Sigma-Aldrich), and glucose-6P (10 mM, disodium salt hydrate; Sigma-Aldrich). The optical density at 600 nm (OD₆₀₀) was monitored every 30 min in a FLUOstar OMEGA plate reader (BMG Labtech) under shaking conditions at 37°C. For presentation of data, at least three independent growth curves were monitored for each strain tested. The mean values were calculated and plotted. Standard deviations are not shown, since only growth and nongrowth phenotypes were evaluated.

Alkaline phosphatase assays. To determine the enzymatic activities for the transcriptional *phoA* fusions, alkaline phosphatase assays were performed as described previously (33, 37). The activities were expressed in Miller units, given by $A_{405} \times 1.000 / (A_{600} \times \text{ml} \times \text{min})$. Note that no intrinsic alkaline phosphatase background activity was measured for *V. cholerae* strain E7946 in the presence of glucose or glucose-6P (data not shown).

¹⁴C-labeled compounds. For substrate uptake and kinetics, labeled substrates were used as follows, [U-¹⁴C]D-fructose-6P and [U-¹⁴C]D-glucose-6P in disodium salt with specific activities of 248 and 245 mCi/mmol (Hartmann Analytic), respectively.

Uptake studies. For uptake studies, the WT and ΔVCA0685-7 mutant strains transformed with the pTRC99A control plasmid and the ΔVCA0685-7 mutant strain transformed with complementing plasmid pTRC0685-7 and induced with 0.05 mM isopropyl-β-D-thiogalactopyranoside (IPTG) were used. To demonstrate the uptake of [¹⁴C]labeled hexose-6P, cells were inoculated to an OD₆₀₀ of 0.1 in M9 supplemented with glycerol (0.4%) and hexose-6P (10 mM) and grown to an OD₆₀₀ of 0.5, representing the mid-log growth phase. M9 medium supplemented with glycerol and hexose-6P allows growth on both substrates and thereby induction of the hexose-6P uptake system. Note that glycerol was added as a C source to allow hexose-6P uptake-deficient mutants to grow. This growth strategy was used to generate energized cells in the mid-log growth phase by allowing induction of VCA0685-to-VCA0687 expression. The cultures were washed twice and resuspended in M9 to an OD₆₀₀ of 0.5. Uptake studies were carried out at room temperature. The reaction was started with the addition of either 2.5 μl of [U-¹⁴C]fructose-6P (0.4 mM) and 7.5 μl of fructose-6P (0.4 mM) or 2.5 μl of [U-¹⁴C]glucose-6P (0.4 mM) and 7.5 μl of glucose-6P (0.4 mM) per ml of culture. After 30, 60, 90, 210, and 330 s, samples of 500 μl were taken, filtered through membranes (0.45-μm pore size; Schleicher & Schuell MicroScience), and washed un-

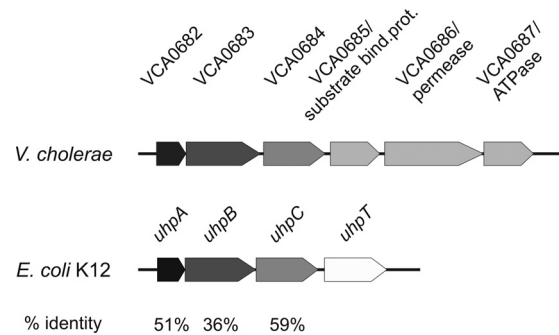


FIG 1 Overview of the genomic organization of hexose-6P uptake systems in *V. cholerae* and *E. coli*. The genomic region of *E. coli* responsible for hexose-6P uptake consists of three regulatory genes, *uhpA*, *uhpB*, and *uhpC*, that control the expression of the corresponding transport protein UhpT (42). In *V. cholerae*, homologous genes are located on the small chromosome and are similarly arranged. They are regulatory genes *uhpA*/VCA0682, *uhpB*/VCA0683, and *uhpC*/VCA0684. While no *uhpT* homologue is found in *V. cholerae*, three additional genes are present: VCA0685 (substrate-binding protein [bind. prot.]), VCA0686 (permease), and VCA0687 (ATPase). According to NCBI BLASTP (57), *V. cholerae* ORFs VCA0682 to VCA0684 show amino acid identities to corresponding *E. coli* K-12 proteins, as indicated at the bottom.

der suction with 10 ml NaCl (100 mM). The membranes were then placed in vials containing 4 ml of scintillation fluid (Ultima Gold; PerkinElmer). To correlate the total added radioactivity, 500 μl of the whole reaction mixture was added directly to separate vials containing 4 ml of scintillation fluid. All samples were counted in a liquid scintillation analyzer (Tri-Carb 2300TR; Packard).

For substrate-dependent uptake kinetics, only the WT strain was used and cultures were prepared as previously described. The uptake reactions were each started with the addition of 25, 18.75, 12.5, 7.5, 5, or 2.5 μl of [U-¹⁴C]D-fructose-6P (0.04 mM) per ml of culture and 37.5, 31.25, 25, 18.75, or 12.5 μl of [U-¹⁴C]D-glucose-6P (0.04 mM) per ml of culture, respectively. After 10, 20, and 30 s, samples of 500 μl were taken, filtered through membrane filters, washed under suction with 100 mM NaCl, and measured as previously described. To detect the entire added radioactivity per volume tested, 500 μl of the whole reaction mixture was added directly to vials containing 4 ml scintillation fluid. Rates of substrate transport were determined from the number of counts obtained after 20 s of incubation with the labeled substrate, as described by Xavier et al. (38). It was verified that a linear correlation of the number of counts versus time was observed for at least 30 s for all of the substrate concentrations tested.

RESULTS AND DISCUSSION

VCA0685 to VCA0687 represent a hexose-6P uptake system.

The gene cluster containing open reading frames (ORFs) VCA0685 to VCA0687 was found to be induced under late infection conditions (26, 39). Furthermore, it has been shown that its function contributes to survival after *in vivo* passage and subsequent transition into pond water (26). VCA0685 to VCA0687, originally annotated as ferric iron transporter system components (39), were recently investigated as a putative ABC iron uptake transport system showing 69, 65, and 60% amino acid identity to the AfuABC system of *Actinobacillus pleuropneumoniae* (40). Functional complementation analysis in iron transport-deficient *Shigella flexneri*, Fur-dependent reporter gene induction in *E. coli*, and mutant analysis in *V. cholerae* showed no evidence of iron uptake activity of VCA0685 to VCA0687 (40). However, in *V. cholerae*, VCA0682 to VCA0687 represent a putative gene cluster (Fig. 1) that corresponds in part to homologous genes of the *uhp* operon in *E. coli* (41, 42). As demonstrated in *E. coli*, UhpT is an

antiporter for hexose-6P uptake (43) and UhpABC represent the sensor kinase-regulating components (44). To characterize cellular growth ability on hexose-6P substrates, two mutant strains were tested. In one mutant strain (Δ VCA0682), the transcriptional activator homologue VCA0682 (*uhpA*) was deleted and a second mutation comprises the deletion of three reading frames, constituting the putative hexose-6P uptake system, i.e., VCA0685, VCA0686, and VCA0687 (39), referred to here as VCA0685-7. The growth kinetics of these deletion mutants and the parental WT were monitored in M9 minimal medium supplemented with different sole C sources such as glucose (Fig. 2A), glucose-6P (Fig. 2B), or fructose-6P (Fig. 2C). All of the strains showed similar growth on M9 supplemented with glucose, except the VCA0685-7 complementation strain, which reached only 70% of the maximum cell density (OD_{600}) of the WT. This effect is probably due to a reduced fitness of these cells bearing the burden of expressing the VCA0685-7 gene products from plasmid pTRC99A, thus reaching only partial maximum growth ability in M9 glucose. Such an effect was not observed in M9 hexose-6P-supplemented medium, where physiologically relevant complementation activity was observed. The deletion mutant strains were unable to grow in hexose-6P-supplemented medium. Complementation of the growth phenotype of both mutant strains was observed by the expression of UhpA or VCA0685-7 from pTRC99A even without the addition of IPTG, because of leaky promoter activity (Fig. 2B and C). Notably, growth in M9 glucose medium started immediately, whereas prolonged lag phases of up to 25 h were observed in the WT strain on hexose-6P substrates. Interestingly, expression of the putative response regulator UhpA from pTRC99A substantially shortened the lag phase (Fig. 2A and B).

Additionally, we observed a decline in cell density during the late exponential growth phase (e.g., Fig. 2A). Most likely, this is an effect of stationary-phase resting cells, which may undergo lysis, change their shape, or form aggregates. It was also noted that the maximum OD_{600} reached by cells grown in M9 glucose was 2 to 3 times as high as that of cells grown in M9 hexose-6P. This represents a substrate concentration effect, since if we increased the glucose-6P concentration to 25 mM, then the OD_{600} increased to 1.4, which is similar to the observed density of cells grown in M9 glucose (data not shown). Finally, by reviewing published and deposited genome information on sequenced *Vibrio* sp. (KEGG, J. Craig Venter Institute, or the BROAD Institute), it seems that the VCA0682-to-VCA0687 gene cluster characterized here is represented inconsistently. Sequenced O1, O139, or non-O1/non-O139 *V. cholerae*, as well as *V. mimicus*, *V. farnesi*, *V. metschnikovii*, and *V. anguillarum*, isolates showed the presence of this gene cluster, whereas no such gene cluster was found in *V. fischeri*, *V. parahaemolyticus*, or *V. vulnificus*. Future bioinformatic analysis will address the genomic view of this gene region. Taken together, these results demonstrate that the components of VCA0685-7 and the putative activator UhpA are essential for the utilization of hexose-6P substrates.

Hexose-6P uptake and kinetics. On the basis of the growth phenotypes shown in Fig. 2, we further characterized the specific transport kinetics of the VCA0685-7 deletion mutant compared to those of WT cells and complemented mutant strains. For example, the uptake of [14 C]fructose-6P and [14 C]glucose-6P was monitored (Fig. 3A and B). Substrate accumulations of 49.5% (fructose-6P) and 55% (glucose-6P) after 6 min of incubation were observed for the WT, and 33% (fructose-6P) and 25% (glu-

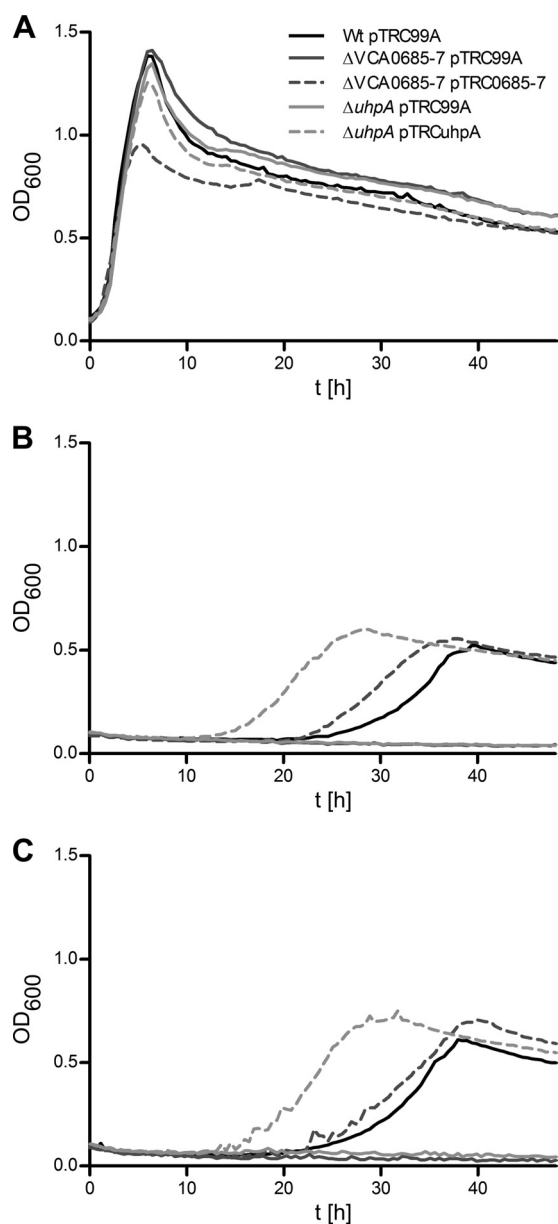


FIG 2 Growth curves of *V. cholerae* WT and mutant strains in M9 minimal medium supplemented with hexoses as carbon sources. Shown are the growth kinetics of the WT, the Δ VCA0685-7 and Δ uhpA deletion mutants, and the Δ VCA0685-7/pTRC0685-7 and Δ uhpA/pTRCuhpA complemented mutants in M9 minimal medium supplemented with glucose (A), fructose-6P (B), or glucose-6P (C). Data represent mean values of at least three independent measurements.

ucose-6P) accumulations were observed for the Δ VCA0685-7 mutant complemented with plasmid pTRC0685-7. In contrast, less than 0.1% of [14 C]fructose-6P or [14 C]glucose-6P accumulated in the Δ VCA0685-7 mutant strain containing control plasmid pTRC99A. For complementation, IPTG induction was necessary to enhance the P_{trc} promoter activity of pTRC99A (32), allowing the expression of VCA0685-7 and monitoring of hexose-6P uptake, whereas no IPTG induction was necessary to complement the growth phenotypes (Fig. 2). As further shown in Fig. 3A and B, uptake of [14 C]glucose-6P was quickly saturated, in <2 min, in-

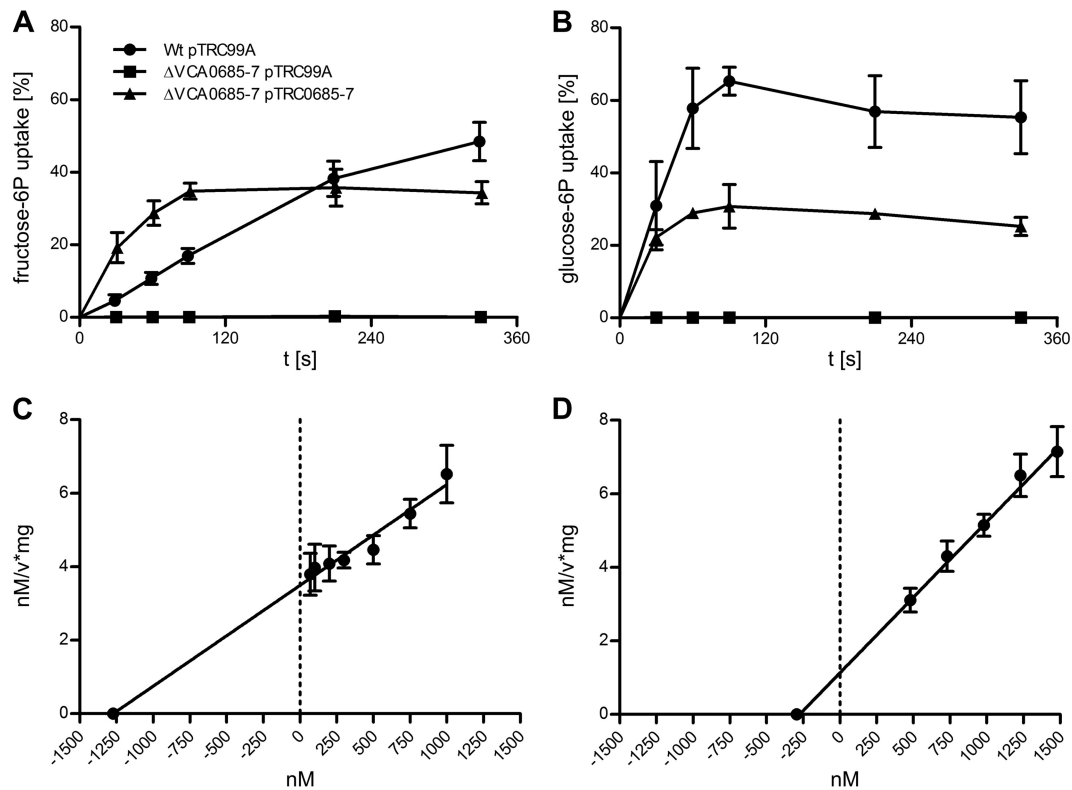


FIG 3 Hexose-6P uptake by *V. cholerae* WT and mutant strains. The time-dependent uptake of [U-¹⁴C]D-fructose-6P (A) and [U-¹⁴C]D-glucose-6P (B) by the WT/pTRC99A strain and the Δ VCA0685-7/pTRC99A and Δ VCA0685-7/pTRC0685-7 mutant strains was determined as a percentage of the total added radioactivity. Shown are mean values of at least four independent measurements. The error bars indicate standard deviations. (C, D) The uptake velocities at specific substrate concentrations were determined by measurements at three consecutive time points. Shown are mean values of at least two independent experiments. The error bars indicate standard deviations. From Hanes-Woolf plots, the calculated apparent K_m was 1.273 μ M with a V_{max} of 364.5 nM/min/mg protein for fructose-6P (C). As shown in plot D, the apparent K_m was 0.275 μ M with a V_{max} of 243.4 nM/min/mg protein for glucose-6P.

indicating that the extracellular [¹⁴C]glucose-6P concentration becomes limited for further uptake after 55% substrate accumulation. In contrast, [¹⁴C]fructose-6P uptake showed a lower velocity and did not reach saturation before the 6-min time point. Differences were also observed for uptake activities of complementing strains, where neither fructose-6P nor glucose-6P uptake reached WT levels, suggesting that expression of VCA0685-7 from pTRC99A is insufficient, resulting in partial complementation. According to Michaelis-Menten kinetics, the V_{max} and K_m values for the [¹⁴C]fructose-6P and [¹⁴C]glucose-6P uptake of the *V. cholerae* WT strain were determined (Fig. 3C and D). At least five different substrate concentrations were analyzed to determine the velocity of the uptake dynamics, according to the analysis method of Hanes (45). For [¹⁴C]glucose-6P and [¹⁴C]fructose-6P, V_{max} values of 243.4 and 364.5 nM/min/mg were determined, and the apparent K_m values deduced were 0.275 and 1.273 μ M, respectively. Interestingly, the two substrates show similar V_{max} values but the K_m values differ by \sim 5-fold. This finding correlates with glucose-6P transport saturation observed already after 2 min (Fig. 3B) and not observed for fructose-6P (Fig. 3A). The most plausible explanation is that for glucose-6P, the V_{max} of the Uhp transporter is maintained, while decreasing substrate concentrations are well above the K_m value in the medium. In contrast, for fructose-6P, the decreasing substrate concentration in the medium is approaching the K_m value and therefore causing lower uptake activity. According to the literature, K_m values for the glucose-6P

uptake system were determined between 20 and 500 μ M (46). It was suggested that these values were influenced by various P_i concentrations in the buffer or medium used, which may act as a competitive inhibitor (46). It is noteworthy that the Uhp transport components characterized here differ significantly from the known system components of *E. coli* or *Salmonella enterica* serovar Typhimurium (Fig. 1). According to bioinformatic analyses, the *V. cholerae* uhp-encoded transport components do not belong to the electrochemical gradient-coupled permease family, also termed secondary active transporters. Instead, the system characterized here constitutes an ATP-binding cassette (ABC) transporter system that resembles a primary active transporter family (47). As shown in *V. cholerae* (Fig. 1), three additional ORFs are present that replace the otherwise occurring single permease-encoding uhpT gene in *E. coli*. As bioinformatic analyses revealed, the three genes observed in *V. cholerae* encode sequences homologous to primary active transporters. These products are a substrate-binding protein, a transmembrane protein, and a nucleotide-binding domain-containing protein resembling an ABC-type transporter (48). The apparent K_m values of a well-characterized prototype ABC transporter, the disaccharide maltose transport system of *E. coli* (49), were determined to be 0.8 to 1.4 μ M (50), depending on the absence or presence of the maltose-binding protein, respectively. Another example is the binding protein-dependent ABC iron transporter FbpABC of *Haemophilus influenzae*. Its Fe³⁺ uptake was determined to have apparent

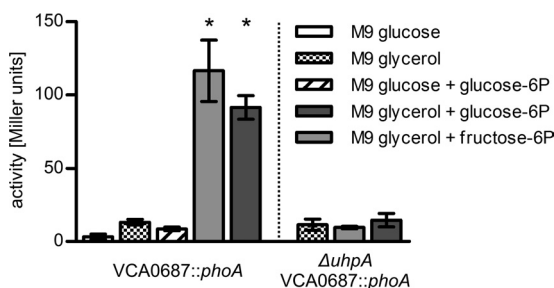


FIG 4 PhoA activities of *V. cholerae* VCA0687::phoA and Δ uhpA VCA0687::phoA strains grown in M9 supplemented with various carbon sources. Strains were grown in M9 medium as indicated. Alkaline phosphatase activities (in Miller Units) of cultures of the VCA0687::phoA and Δ uhpA VCA0687::phoA transcriptional fusion strains grown for 8 h were measured. Shown are the means of at least four independent measurements. The error bars indicate standard deviations. *, significant differences between the respective data sets in comparison to the M9 glycerol control condition (Kruskal-Wallis test followed by posthoc Dunn's multiple comparisons; $P < 0.05$).

K_m of 0.9 to 1.2 μ M, depending on the presence or absence of the iron-binding protein (51). Hence, this identified ABC hexose-6P transport system has a K_m value that is similar to those of other ABC transporters and indicates higher hexose-6P substrate sensitivity, as known for the Uhp system in *E. coli*. This raises an interesting view, whereby *V. cholerae* cells may have an advantage over members of the *Enterobacteriaceae* flora by utilizing such substrates in the human gut. Whether this provides a competitive growth advantage needs to be proven *in vitro* or *in vivo*. The uhp system was found to be induced late *in vivo*, and *in vivo* colonization showed no effect on competition between WT *V. cholerae* and a uhp knockout strain (26). Therefore, the uhp system should play only a minor role in the colonization phase and this indicates that utilization of hexose-6P *per se* is not required for successful colonization. However, WT cells did show higher survival fitness after being released into the environment (26), for reasons addressed in more detail below.

Regulation of the hexose-6P uptake system encoding homologues of uhp genes in *V. cholerae*. To characterize the transcriptional regulation of the VCA0685-7 operon in *V. cholerae*, we generated chromosomal transcriptional fusion strains consisting of a promoterless phoA reporter gene fused to VCA0687 (Fig. 1) (33). The resulting PhoA activity represents the transcriptional activity of the last gene of the putative uhp cluster, VCA0687. To test whether hexose-6P substrates such as fructose-6P or glucose-6P induce VCA0687 gene transcription, *V. cholerae* reporter strains were grown in M9 minimal medium supplemented with glycerol or glucose in the presence or absence of hexose-6P (Fig. 4). *V. cholerae* cultures grown in M9 glycerol reached mid-log phase (OD₆₀₀ of 0.5) after 8 h and late exponential growth phase (OD₆₀₀ of 1.5) after 16 h (data not shown). It was necessary to use glycerol to determine gene induction in a Δ uhpA background, since this strain would not grow on hexose-6P sources. In the presence of hexose-6P, 10- and 12-fold induction of VCA0687::phoA expression was observed with fructose-6P and glucose-6P, respectively (Fig. 4). This is consistent with data from *E. coli* showing 50-fold induction of uhp genes (44). In contrast, cultures grown in M9 glucose and glucose-6P showed no induction, indicating that catabolite repression might control uhp gene expression. However, neither cyclic AMP (cAMP; 3 mM) addition to cultures growing in

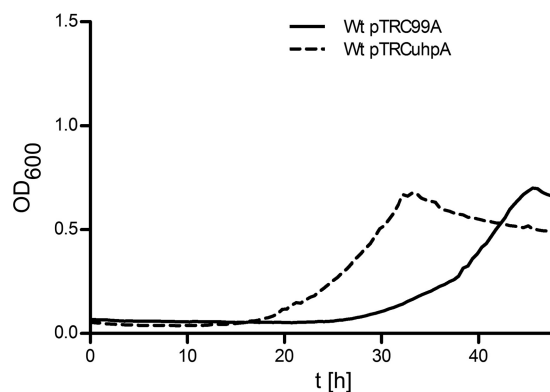


FIG 5 Growth curves of WT *V. cholerae* and in *trans* expression of UhpA. Shown are the growth kinetics in M9 glucose-6P of the WT strain transformed with plasmids pTRC99A (control) and pTRCuhpA. Data represent mean values of at least three independent OD₆₀₀ measurements.

such a medium nor cAMP addition to M9 glucose-6P cultures grown for 8 h showed induction of uhp genes (data not shown). Therefore, we exclude an influence of cAMP-cAMP receptor protein on uhp gene transcription at this point. Further characterization is necessary to elucidate any participation of catabolite repression in uhp gene regulation. So far in *E. coli*, only glucose-6P has been found to bind to a membrane receptor compound (52), which most likely constitutes the sensor kinase complex UhpBC (53), whereas fructose-6P binding could not be demonstrated. This might indicate that fructose-6P has to be converted to glucose-6P before induction can take place (for an overview, see reference 46). Additionally, a VCA0682 deletion mutant missing a uhpA homologue was tested. VCA0682 (39), showing 51% identity to response regulator UhpA (44), may represent the transcriptional activator of the uhp genes in *V. cholerae*. As shown in Fig. 4, in a Δ uhpA mutant strain, VCA0687::phoA expression does not respond to the inducing substrate fructose-6P or glucose-6P. Concordant with this observation, Δ uhpA prevents growth on hexose-6P, while expression of uhpA in *trans* from the complementation plasmid restores growth (Fig. 2). Thus, the results indicate that deletion of the transcriptional activator VCA0682 (UhpA) leads to loss of uhp gene expression. It is important to note that no uhp gene induction was observed when cells were grown in the presence of glycerol, glucose, or glucose and glucose-6P in combination (Fig. 4).

To correlate the observed prolonged lag phase of cell growth (Fig. 2) with uhp gene induction, additional experiments were performed. First we found that UhpA expression from pTRCuhpA shortened the lag phase when it was transformed into the WT strain (Fig. 5). Next, VCA0687::phoA fusion activity was monitored during the entire growth phase at 6, 10, 18, 24, 30, and 48 h (Fig. 6). The results show that at time points prior to 18 h, no significant induction could be observed. However, PhoA activity increased at 24 h, reaching a maximum at 30 h, and stayed high until 48 h. Thus, evidence is provided that uhp gene induction correlates with a prolonged observed lag phase and consequently an inhibition of uhp gene induction is responsible for the prolonged lag phase.

Survival under phosphate-limiting conditions. It was previously shown (26) that the hexose-6P uptake system components characterized here are induced late *in vivo*, suggesting the presence

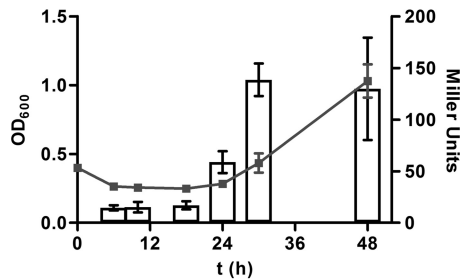


FIG 6 *uhp* gene expression of VCA0687::*phoA* fusion activity along the growth curve. Shown are the mean values and standard deviations of four independent growth curves (OD₆₀₀, left y axis) of VCA0687::*phoA* in M9 glucose-6P measured at 6, 10, 18, 24, 30, and 48 h. At the same time points and from these cultures, PhoA activities (in Miller Units, right y axis) of VCA0687::*phoA* were determined; mean values and standard deviations are shown.

of the inducing substrate hexose-6P in the gut. Furthermore, the Δ VCA0686 mutant strain showed 5-fold less fitness than the WT in pond water survival after *in vivo* passage (26). Interestingly, this defect was compensated when the pond water was supplemented with M9 minimal medium salts. Thus, we speculated that the lack of at least one component of M9 minimal medium salts was the cause for the observed fitness disadvantage. Inorganic phosphate is especially limited in aquatic environments, and *V. cholerae* faces a 3-fold drop in the concentration of phosphate when transiting from cholera patient stool to pond water (54). Interestingly, *E. coli* is not able to grow on glucose-6P as the sole P source (55), since UhpT is an antiporter that imports hexose-6P by exporting cellular P_i (43). In contrast, the *V. cholerae* transporter described here should allow the utilization of hexose-6P as a C and P source. Thus, we investigated whether hexose-6P utilization accounts for increased cell survival under phosphate-limiting conditions. It is known that polyphosphate storage enhances the ability of *V. cholerae* to survive stress in a low-phosphate environment (56). Therefore, our experiments were carried out under such conditions, whereby the WT and Δ VCA0685-7 mutant strains were cultured for 40 h in low-P_i (150 μ M) M9-Tris (pH 7, 100 mM) glycerol medium supplemented with hexose-6P (10 mM). After growth in this medium, the WT and mutant strains were washed and mixed at a ratio of 1:1 to achieve equal cell densities (OD₆₀₀ of 0.5). Subsequently, the cells were incubated in either low-phosphate M9-Tris or standard M9-Tris medium, both lacking any C source. Next, the time-dependent number of CFU/ml and competitive index (CI) were determined. We observed an \sim 5-fold lower CI of cells deficient in hexose-6P utilization after 12 to 24 h of incubation in low-phosphate M9-Tris, whereas in standard M9-Tris medium, no survival defect was observed (Fig. 7). This result is consistent with the previous observation that a Δ VCA0686 mutant exhibits a fitness disadvantage compared to the WT in pond water after *in vivo* passage. Thus, the data indicate that previous exposure to hexose-6P contributes to enhanced cell survival under phosphate-limiting culture conditions. The transition from the host to an aquatic reservoir represents such a scenario, providing an explanation for a physiological role for the hexose-6P uptake system in the life cycle of *V. cholerae*. Since the Uhp system in *V. cholerae* most likely includes an ABC transporter without P_i antiport, acquisition of phosphate should be considered. Therefore, it was tested whether this hexose-6P uptake system is already induced by phosphate limitation. Using the transcriptional *phoA*

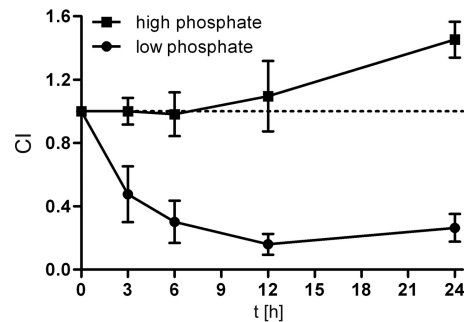


FIG 7 VCA0685-7-dependent survival under phosphate limitation. Shown is the CI, which reflects the survival ratio of the WT and Δ VCA0685-7 mutant strains. The CI is expressed as a ratio of Δ VCA0685-7 CFU to WT CFU at 3 to 24 h normalized for the input ratio at time zero. Closed circles show CI values derived from cultures incubated in low-phosphate M9-Tris, and square symbols indicate CI values of cultures incubated in standard phosphate M9-Tris medium. Prior to M9-Tris incubation, cultures were grown in M9 supplemented with glycerol and glucose-6P (for details, see the text). Shown are the means of at least four independent measurements. Error bars indicate standard deviations.

fusion to VCA0687, we tested whether phosphate limitation alone results in induction of the VCA0685-7 operon. The mean PhoA activities of the VCA0687::*phoA* strain were determined to be 5.5 Miller units in standard M9-Tris medium and 11.9 Miller units in low-phosphate M9-Tris. Thus, phosphate starvation does not result in significant induction of the hexose-6P uptake system. Also, on the basis of the finding that a relatively high phosphate concentration is present in cholera patient stool (54), it seems to be very unlikely that phosphate starvation triggers hexose-6P uptake at late stages of infection.

In summary, this study characterized the hexose-6P substrate uptake system of *V. cholerae*. The reported induction of this system under infection conditions indicates that the corresponding substrate in the host is available to *V. cholerae*. In general, the Uhp regulatory system comprising UhpABC is highly conserved in *E. coli* and *V. cholerae*. However, in this report, distinct differences are revealed. For example, the hexose-6P permease-encoding *uhpT* gene of *E. coli* is absent from *V. cholerae*. Instead, a primary transport system showing lower K_m values for glucose-6P uptake than UhpT in *E. coli* is present. Initially, we got interested in characterizing this late *in vivo*-induced operon because it was reported to facilitate the fitness of *V. cholerae* in the transition phase when the bacteria are released from the host back into the environment. By unraveling the function of the VCA0685-7 components as a hexose-6P uptake system, this work provides a first clue to how this uptake system contributes to enhanced survival fitness. As shown here, glucose-6P utilization serves as a suitable substrate to prepare *V. cholerae* cells for better survival in phosphate-limiting environments. Hence, these data suggest that hexose-6P substrates serve not only as valuable carbon sources but also as substrates from which cells benefit in the transition phase, most likely by building up their endogenous phosphate storage.

ACKNOWLEDGMENTS

This work was supported by Austrian Science Fund (FWF) grants W901 (DK Molecular Enzymology) to M.M., A.S., J.R., and S.S. and P22986 to S.S.

We thank C. Schweinzer for carefully reading the manuscript.

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