Deciphering the role of multiple compatible solute transporters from *Vibrio parahaemolyticus*

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Introduction

Vibrio parahaemolyticus is a moderate halophile, capable of causing self-limiting gastroenteritis in humans following the consumption of raw or undercooked shellfish. Severe morbidity and mortality can result if the organism gains entry to an individual through open wounds or upon infection of individuals that are immunocompromised (Yeung and Boor, 2004). *V. parahaemolyticus* is widely distributed in coastal seawaters, areas where the salt concentrations can rapidly fluctuate, and as such, the organism must have the ability to respond to these osmotic shifts if it is to survive. In such conditions, bacterial persistence relies on mechanisms for rapidly balancing the osmotic pressure between the cell and its external environment. A rapid, short term response to osmotic changes is an influx of ions within the cell, utilizing potassium uptake systems (Sleator and Hill, 2001). This functions initially to stabilize the cell, but over time the increased accumulation of these ions can have a deleterious effect on normal cellular functions and enzymatic activity. In order to cope with changes in salinity over an extended period, mechanisms for the synthesis and/or uptake of compatible solutes or their precursors are essential for organisms inhabiting marine environments. Compatible solutes are low molecular weight organic molecules that are capable of accumulating to extremely high intracellular concentrations, subsequently protecting the cell from osmotic stress, but importantly, do not disrupt normal cellular functions (Empadinhas and da Costa, 2008; Bremer and Kramer, 2000). An array of molecules can function as compatible solutes in bacteria, and the uptake of these solutes or their precursor molecules requires specialized transmembrane proteins that have distinct substrate specificities (Bremer and Kramer, 2000). Here we describe our preliminary studies examining a set of recently identified transporters in *V. parahaemolyticus* so as to begin to elucidate their importance in allowing for the organism to survive environmental stressors.

Materials and methods

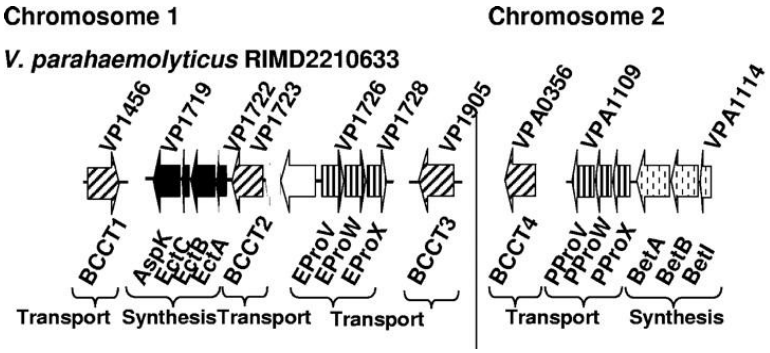
Bacterial growth, RNA isolation, and real-time PCR analysis

Bacterial cultures were grown overnight at 37ºC with agitation (230rpm) in either Luria-Bertani (LB) media or M9 minimal media with 0.4% glucose (Maniatis *et al.*, 1989), each supplemented with NaCl to a 3% final concentration. Fresh cultures (either LB, 3% NaCl or M9, 3% NaCl) were inoculated with a 2% inoculum from overnight cultures and grown at 37ºC with agitation for 4 hours (log phase) or 10 hours (stationary phase). After initial culture, cells were stressed by osmotic upshock by pelleting cells, resuspending in media containing 6% NaCl, and incubating at 37ºC for 30 minutes. Following osmotic upshock, total RNA was isolated from cells using the RNAprotect Bacteria reagent and RNeasy isolation kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. In addition, total RNA was isolated from aliquots of the original log or stationary phase cultures for non-stressed reference samples in expression analysis. Total RNA was DNase treated (Turbo DNase, Invitrogen, Carlsbad, CA), quantitated with a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA), and 500 ng used as template for cDNA synthesis (SuperScript II Reverse Transcriptase, Invitrogen) primed with 200 ng of random hexamers. For real-time PCR, cDNA was diluted 1:125 in sterile water and 2 ul used as template with Fast SYBR Green Master Mix (Invitrogen) on an ABI 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA). Primer sequences are available upon request. Relative expression levels were determined after normalization with 16S rRNA levels and using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001).

Results and Discussion

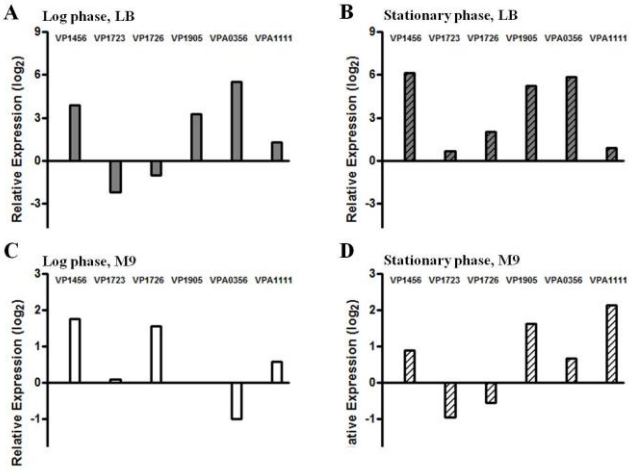
Initial bioinformatic analysis of the genome of *V. parahaemolyticus* led to the identification of several genes potentially involved in the synthesis of compatible solutes, in addition to several encoding for putative betaine/choline/carnitine transporters (BCCTs), which are involved in the uptake of these molecules into the cell (**Figure 1**).

Figure 1: The osmolyte transport and synthesis systems identified in *Vibrio parahaemolyticus* RIMD2210633. Arrows indicate the direction of transcription. BCCT family transporters are represented by diagonal stripes. Solid black arrows represent putative genes involved in ectoine synthesis. Open arrows indicate hypothetical proteins. Vertical stripes represent genes homologous to that which encodes the ProU (ProVWX) transporter. Dashed lines indicate genes involved in glycine betaine synthesis (*betABI*). (Adapted from Naughton *et al.*, 2009)



Interestingly, *V. parahaemolyticus* was found to contain six transport systems, twice the number of transporters as in other *Vibrio* species, possibly allowing this organism to tolerate a wider range of stressors. Indeed, we have observed that *V. parahaemolyticus* is capable of growth at higher concentrations of salt (6 and 9% NaCl) than several other *Vibrio* species tested (Naughton *et al.*, 2009). In an effort to investigate the involvement of these newly identified transporters in the ability of *V. parahaemolyticus* to tolerate high salt, we first measured the expression patterns of the four single-component BCCTs (VP1456, VP1723, VP1905, VPA0356) and the two multi-component (*proVWX*) systems (VP1726 and VPA1111) to determine their basal levels of transcription at optimal growth conditions (3% NaCl), and to evaluate if transcription could be actively induced by osmotic fluctuation.

Figure 2: Quantitative real-time PCR analysis of putative compatible solute transport genes in *Vibrio parahaemolyticus* following growth and osmotic upshock in LB (gray bars) or minimal media (white bars). After initial culture as described in methods, cells were stressed with an osmotic upshock in media containing 6% NaCl for 30 minutes. RNA was isolated and used to generate cDNA for qPCR analysis. Changes in expression levels are relative to the expression observed in similarly cultured cells (log or stationary phase) not subjected to osmotic upshock (relative expression = 0).



Expression of several of the single-component transporters (VP1456, VP1905, and VPA0356) were induced following osmotic upshock when grown in LB, regardless of the phase of growth of the initial culture (**Figure 2, A and B**). LB is a rich media, known to contain both compatible solutes and their precursors (Wood, 2007; Sezonov *et. al.*, 2007), so we therefore also assessed expression levels under conditions in which nutrients were limited during growth and osmotic challenge (**Figure 2, C and D**). Under these conditions, two transporters (VP1456 and VP1726) were slightly increased when log phase cells were given an osmotic shock. A marked difference in expression was observed when stationary phase cells were examined, as the induction of two transporters (VP1905 and VPA0356) was seen, along with a decrease in VP1726 transcript levels. One transporter (VP1723) was not induced under any of the conditions tested thus far.

Conclusions

Bioinformatic analysis of the genome of *Vibrio parahaemolyticus* RIMD2210633 has revealed that this organism possesses a large number of compatible transport and synthesis systems, several more than are found in other *Vibrio* species, which likely contribute to the ability of this organism to grow at high salinities. Expression analysis by real-time PCR revealed that the expression of several of these transporters is rapidly induced following a short osmotic shift, indicating their potential utilization by the organism when under osmotic stress. Future analysis of these transporters by functional complementation using the transporter-deficient *Escherichia coli* strain MKH13 will allow for the determination of the full range of activity and specificity for each individual transporter.

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