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NOTE

Identification of the *Vibrio vulnificus htpG* Gene and Its Influence on Cold Shock Recovery

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An *htpG* gene encoding the heat shock protein HtpG was identified and cloned from *Vibrio vulnificus*. The deduced amino acid sequence of HtpG from *V. vulnificus* exhibited 71 and 85% identity to those reported from *Escherichia coli* and *V. cholera*, respectively. Functions of HtpG were assessed by the construction of an isogenic mutant whose *htpG* gene was deleted and by evaluating its phenotype changes during and after cold shock. The results demonstrated that recovery of the wild type from cold shock was significantly faster ($p<0.05$) than that of the *htpG* mutant, and indicated that the chaperone protein HtpG contributes to cold shock recovery, rather than cold shock tolerance, of *V. vulnificus*.

Keywords: *V. vulnificus htpG*, cold shock, heat shock protein, chaperone

Bacteria have evolved elaborate protection systems to allow survival and/or growth during exposure to environmental changes. Change in temperature is one of the most common environmental stresses that bacteria routinely encounter. Unphysiologically high temperatures lead to thermal denaturation of folded proteins, accumulate large aggregates of the thermo-denatured proteins, and thereby endanger bacterial homeostasis (Guisbert *et al.*, 2008). Bacteria respond to increases in external temperature by actively producing a set of specific stress resistance proteins called "heat shock proteins (Hsps)" that play essential roles in restoring cellular homeostasis (Meyer and Baker, 2011). Numerous proteases and molecular chaperones are members of the Hsps and are able to prevent unproductive folding processes leading to unspecific aggregation of proteins, and recognize, refold or eliminate the non-native proteins denatured by the high temperatures (Yura and Nakahigashi, 1999).

On the other hand, drastic decreases in environmental temperature (i.e., cold shock) also have widespread effects on the growth and survival of bacteria. The effects of a sudden temperature downshift on bacteria include a decrease in membrane fluidity, changes in nucleic acid structures, and inefficient protein folding (Phadtare and Severinov, 2010). The cold-shock response is an adaptive mechanism and allows bacteria to grow at low temperature largely by synthesizing several stress resistant proteins, called "cold shock proteins (Csps)" (Weber and Marahiel, 2003). Among the Csps, the main cold shock protein CspA and its homologs act as RNA chaperones in *Escherichia coli* (Goldstein *et al.*, 1990). CspA, which is dramatically and most abundantly induced upon cold shock, plays a critical role in cold adaptation by melting the secondary structures in RNA and thereby facilitating transcription and translation at low temperature (Jiang *et al.*, 1996).

The pathogenic marine bacterium *Vibrio vulnificus* is the causative agent of food-borne diseases such as life-threatening septicemia and possibly gastroenteritis in individuals with underlying predisposing conditions (for a recent review, see Jones and Oliver, 2009). To ensure developing illness, *V. vulnificus* has to cope with ever changing temperatures in environments imposed not only by natural ecosystems but also by current control practices, such as refrigeration to suppress its growth in raw seafood. However, only a few studies have addressed the molecular mechanisms by which *V. vulnificus* can survive fluctuations in temperature (Wood and Arias, 2011). Accordingly, a mutant that was more sensitive to cold shock was screened from a library of *V. vulnificus* mutants generated by random transposon mutagenesis using pRL27 carrying Tn5-RL27(Km^r-*ori*R6 K) (Larsen *et al.*, 2002). A homologue of *E. coli htpG*, encoding a heat shock protein, was identified and cloned by a transposon-tagging method in the present study. The functions of the *htpG* gene were assessed by the construction of an isogenic mutant of *V. vulnificus*, in which the *htpG* gene was inactivated by allelic exchanges, and by evaluating its phenotype changes during and after cold shock.

The bacterial strains and plasmids used in this study are listed in Table 1. Unless noted otherwise, the *V. vulnificus* strains were grown in Luria-Bertani (LB) media supplemented with 2.0% (w/v) NaCl (LBS). When required, M9 (Sambrook and Russell, 2001), in which glucose (0.4%, w/v) was supplemented as a sole carbon source, was used as a minimal medium. Averages and standard errors of the mean

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Table 1. Plasmids and bacterial strains used in this study

Strains or plasmids	Relevant characteristics[a]	Reference or Source
Strains		
V. vulnificus		
MO6-24/O	Clinical isolate; virulent	Laboratory collection
SAC1201	A transposon mutant of MO6-24/O, cold sensitive	This study
SAC1202	MO6-24/O with *DhtpG*	This study
E. coli		
DH5α *λpir*	*supE44 D lacU169 (f80 lacZ DM15) hsdR17 recA1 endA1 gyrA96 thi-1 relAI*; host for p-requiring plasmids	Simon *et al.* (1983)
SM10 *λpir tra*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu l*pir*; Km^r; host for p-requiring plasmids; conjugal donor	Miller *et al.* (1988)
Plasmids		
pDM4	R6K *γ ori*; *sacB;* suicide vector; *oriT* of RP4; Cm^r	Milton *et al.* (1996)
pGEM-T Easy	PCR product cloning vector; Ap^r	Promega
pRL27	Tn5-RL27 (Km^r-*ori*R6 K) delivery vector; Km^r	Larsen *et al.* (2002)
pSC1201	A self-ligated 4.3-kb *Eco*RI fragment of SAC1201 chromosomal DNA with Tn5-RL27 (Km^r-*ori*R6 K); Km^r	This study
pSC1202	pGEM-T Easy with *DhtpG*; Ap^r	This study
pSC1203	pDM4 with Δ*htpG*; Cm^r	This study

[a] Ap^r, ampicillin resistant; Km^r, kanamycin resistant; Cm^r, chloramphenicol resistant

(SEM) were calculated from at least three independent experiments. Data were analyzed by Student's *t* test with the SAS program (SAS software; SAS Institute Inc., USA). Significance of differences between experimental groups was accepted at a *P* value of <0.05.

Identification of *V. vulnificus htpG*

The transposon mutants of *V. vulnificus* in 200 μl LBS cultures contained in 96-well plates (NUNC, USA) were cold shocked by incubating the cultures at 4°C for 24 h. The cultures were then transferred to a 30°C shaking incubator and growth rates of the mutants were compared by measuring A_{600} for 24 h (data not shown). A mutant that showed reproducibly slower growth rate was selected and named *V. vulnificus* SAC1201 (Table 1). The genomic DNA of the mutant SAC1201 was isolated, digested with *Eco*RI and ligated, and the ligation products were used to transform *E. coli* DH5α λ *pir* as described previously (Simon *et al.*, 1983; Larsen *et al.*, 2002). A plasmid, pSC1201 was isolated from the resulting transformant, and the nucleotide sequences of a 150 bp DNA segment flanking the transposon insertion was determined (data not shown). Since a database search for homology to the resulting nucleotide sequences singled out the *htpG* genes of *E. coli* and *V. cholerae* with high levels (72 to 82%) of identity, the open reading frame (ORF) disrupted by the transposon insertion was named *V. vulnificus htpG*.

Fig. 1. Sequence relatedness of HtpG from *V. vulnificus*, *E. coli*, and *V. cholerae*. The amino acid sequences retrieved from GenPept database (NCBI, http://www.ncbi.nlm.nih.gov) (accession numbers YP_004189451.1 for *V. vulnificus*, NP_230631.1 for *V. cholerae*, NP_415006 for *E. coli*) are aligned using the CLUSTALW program (http://www.ch.embnet.org/software/ClustalW.html). Identical sequences (black boxes) and similar sequences (gray boxes) are indicated.

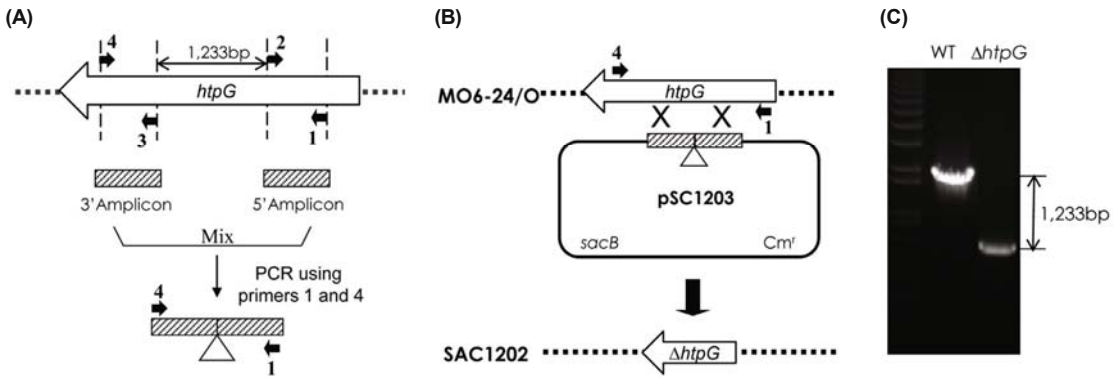

Fig. 2. Allelic exchange procedure and construction of the Δ*htpG* isogenic mutant. (A) Diagram for deletion of 1,233-bp of the ORF of *htpG* by PCR-mediated Linker-Scanning (Murphy *et al*., 2000; Jeong *et al*., 2010). Dashed lines, chromosomal DNA; open arrow, *htpG* ORF; shaded boxes, PCR products for the deletion of the *htpG* ORF; large triangle, deletion of the *htpG* ORF; solid arrows, locations of the oligonucleotide primers used for construction and confirmation of the *ΔhtpG* mutant (1, HTPG5F; 2, HTPG5R; 3, HTPG3F; 4, HTPG3R). (B) Double homologous recombination between strain MO6-24/O and plasmid pSC1203 led to a deletion of the *htpG* gene and resulted in the construction of the mutant SAC1202. Solid line, the plasmid DNA; the large Xes, genetic crossing over. Abbreviations; *sacB*, levansucrase gene; Cm^r, chloramphenicol resistance gene. (C) PCR analysis of MO6-24/O and the isogenic mutant SAC1202 generated by allelic exchanges. Molecular size markers (1 kb plus DNA ladder, Invitrogen, USA) and PCR products are indicated.

Amino acid sequence analysis of *V. vulnificus* HtpG

The amino acid sequence deduced from the *htpG* nucleotide sequence revealed a protein, a heat shock protein HtpG (high temperature protein G), composed of 634 amino acids with a theoretical molecular mass of 72,369 Da and a PI of 4.98. The amino acid composition and molecular weight of this HtpG are similar to those of HtpG from *E. coli* and *V. cholerae*. The amino acid sequences of the *V. vulnificus* HtpG were 71% and 85% identical to those of HtpG from *E. coli* and *V. cholerae*, respectively (Fig. 1). The predicted profile of the hydrophobicity (http//www.expasy.ch) was significantly similar to that of HtpG of *E. coli* and *V. cholera* (data not shown), and is consistent with the fact that the HtpG protein is a cytosolic soluble protein (Bardwell and Craig, 1987). These results suggested that HtpG plays a role in the heat shock response of *V. vulnificus* as previously noted in HtpG of *E. coli* (Bardwell and Craig, 1988; Motojima-Miyazaki *et al*., 2010).

Generation of the *V. vulnificus htpG* deletion mutant

To examine the role of the HtpG in the cold shock response, a *V. vulnificus htpG* isogenic mutant was constructed by allelic exchanges. The *htpG* gene was inactivated *in vitro* by deletion two-thirds (1,233-bp of 1,905-bp) of the *htpG* ORF using the PCR-mediated linker-scanning mutation method as described previously (Murphy *et al*., 2000; Jeong *et al*., 2010). Pairs of primers HTPG5F (5′-AGTTAATGGCCGT TAAGAACTGGC-3′) and HTPG5R (5′-ATGAATTCCAG TCCGTGGTTGAGC-3′) (for amplification of the 5′ amplicon) or HTPG3F (5′-GGAATTCATTTGGCAATGGTG CC-3′) and HTPG3R (5′-AGAAACTCGTGGCTTTCAGT CAGAGG-3′) (for amplification of the 3′ amplicon) were designed using the genomic sequence of *V. vulnificus* MO6-24/O (GenBank accession no. CP002469.1 and CP002470.1, www.ncbi.nlm.nih.gov). The 1,233-bp deleted *htpG* was amplified by PCR using the mixture of both amplicons as the template and HTPG5F and HTPG3R as primers (Fig. 2A). The resulting 672-bp DNA fragment containing the Δ*htpG* was ligated with pGEM-T Easy (Promega, USA) to yield pSC1202. The *ΔhtpG* fragment liberated by digestion of pSC1202 with *SpeI* and *SphI* was isolated and ligated with a suicide vector pDM4 (Milton *et al*., 1996) digested with the same enzymes to generate pSC1203 (Fig. 2B). The *E. coli* SM10 λ *pir, tra* strain (containing pSC1203) (Miller and Mekalanos, 1988) was used as a conjugal donor to *V. vulnificus* MO6-24/O. The conjugation and isolation of the trans-conjugants were conducted using the methods previously described (Kim *et al*., 2011). A double crossover, in which the wild-type *htpG* on the chromosome was replaced with the 1,233-bp deleted *ΔhtpG* allele, was confirmed by PCR as shown in Fig. 2C. PCR analysis of genomic DNA from the wild type MO6-24/O with primers HTPG5F and HTPG3R produced a 1.9-kb DNA fragment, whereas genomic DNA from the *ΔhtpG* mutant SAC1202 resulted in a fragment approximately 0.7-kb in length (Fig. 2C).

Cold shock tolerance of the *htpG* mutant

Cultures of the wild type and *htpG* mutant were grown to exponential phase (A_{600} 0.6) in flasks containing 50 ml of LBS or M9 minimal media at 30°C. Cells were cold shocked by removing the culture and immediately placing it into a 4°C shaking water bath. Samples were then removed at designated time intervals and survivors were determined by enumerating CFU for each cold shock time. The numbers of survivors ($\log_{10}$ CFU/ml) after the cold shock were plotted against time (Figs. 3A and 3B). Upon cold shock, both strains immediately proceeded to an acclimation phase with a slow-rate growth, and then exhibited a continuous decrease of survivors. The acclimation phase length and the death rate of the *htpG* mutant were not significantly different from those of the parental wild type regardless of the media used for the cold shock (Figs. 3A and 3B). This indicated that the HtpG protein does not play an important role in the cold shock tolerance of *V. vulnificus*. However, both strains ex-

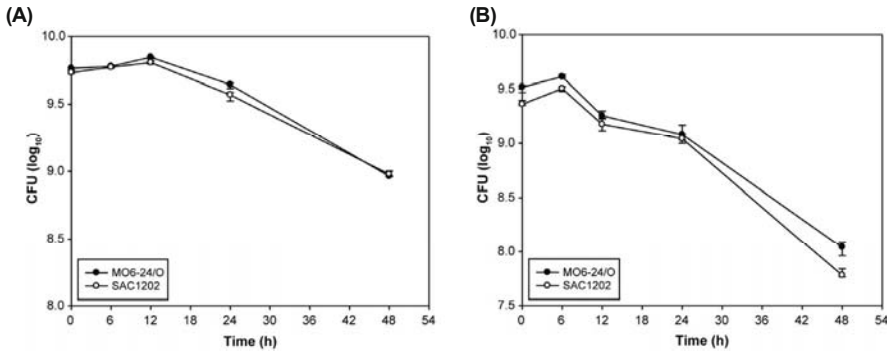

Fig. 3. Survival of the *V. vulnificus* strains during cold shock. Cultures of the wild type (MO6-24/O) and isogenic *ΔhtpG* mutant (SAC1202) were grown to exponential phase (A_{600} 0.6) in LBS (A) and M9 (B) broth as indicated. The cultures were transferred to a 4°C shaking water bath and samples removed at the indicated times were analyzed for survival of the strains by enumerating CFU/ml. Error bars represent the SEM.

hibited a longer acclimation phase and greater numbers of survivors in LBS than M9, indicating that the survival of *V. vulnificus* during cold shock was affected by the type of media.

Cold shock recovery of the htpG mutant

To investigate the role of HtpG in the cold shock recovery, cultures in either LBS or M9 minimal media were inoculated with an initial cell density at an A_{600} of approximately 0.05 and cells were cold shocked by incubating the cultures at 4°C for 24 h. Following the cold shock, the cultures were removed and placed in a 30°C shaking water bath, and then growth rates of the strains were determined by measuring A_{600} at the indicated times (Figs. 4A and 4B). The length of the growth lag period of the *htpG* mutant after the temperature upshift was significantly longer than that of the wild type regardless of the media used. The growth rate of the *htpG* mutant SAC1202 was also significantly lower ($p<0.05$) than that of the parental wild type (Figs. 4A and 4B). As such, absorbance of A_{600} of the *htpG* mutant increased to ca. 0.5, while the wild type strain increased to ca. 0.7 after 4 h incubation at 30°C in LBS (Fig. 4A). In contrast to this difference, unless the wild type and *htpG* mutant were subjected to cold shock at 4°C, the strains did not exhibit any difference in their growth rates nor their growth lag period lengths (Figs. 4A and 4B). The result indicated that the decreased growth rate of the *htpG* mutant at 30°C was largely due to its prior exposure to cold temperature.

The combined results demonstrated that the *htpG* mutant is less effective in growth after cold shock, and indicated that the heat shock protein HtpG plays an important role in the cold shock recovery of *V. vulnificus*. One hypothesis to explain the delayed and lower growth rate of the *htpG* mutant is that the number of the *htpG* mutant cells survived after the cold shock was lower than that of the wild type. However, the numbers of viable cells, counted as CFUs after the cold shock at 4°C for 24 h, were not significantly different between the wild type and *htpG* mutant as mentioned above (Figs. 3A and 3B). Another hypothesis is that the ability of the same numbered cold-survived cells of the *htpG* mutant to adapt to the temperature upshift was deteriorated, delaying regrowth of the mutant at 30°C as observed in Figs. 4A and 4B. This hypothesis is more plausible if HtpG is actively involved in the restoration of the non-native form of proteins or other essential components in cold-exposed cells to biologically active forms during the growth lag period after temperature upshift from 4°C to 30°C.

HtpG is a bacterial heat shock protein that was discovered in *E. coli* through its sequence homology to a eukaryotic Hsp, where it is called Hsp90 (Bardwell and Craig, 1987). Hsp90s constitute one of the most well conserved Hsp families, and were originally identified in the heat shock response (Picard, 2002). Hsp90 is a molecular chaperone wide spread in eukaryotes and promotes the correct folding of client proteins by hydrolyzing ATP and cooperating with a set of co-chaperones (Taipale *et al.*, 2010). Hsp90 is a dimeric protein and undergoes conformational changes that are required for client protein refolding and are modulated by ATP binding and hydrolysis. A large number of client proteins that are dominated by regulatory and signaling proteins depend on Hsp90 to maintain or reach their biologically active states (Picard, 2002; Wandinger *et al.*, 2008; Taipale *et al.*, 2010).

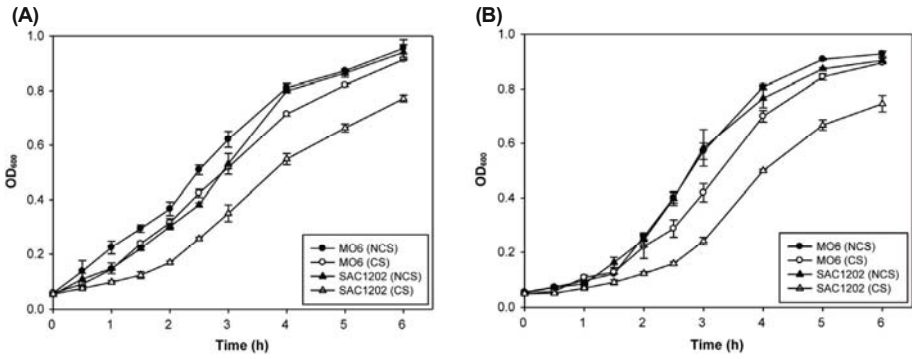

Fig. 4. Growth rates of the *V. vulnificus* strains after cold shock. Growth of the wild type (MO6-24/O) and isogenic *ΔhtpG* mutant (SAC1202), survived after a 24 h cold shock at 4°C (CS), in LBS (A) and M9 (B) was determined by measuring increased absorbance (A_{600}) of the cultures as described in the text. As controls, growth of the strains, not experienced the cold shock (NCS), was also measured as indicated. Error bars represent the SEM.

These Hsp90 dependent cellular functions render that deletion of Hsp90 is lethal in yeast (Borkovich *et al*., 1989).

HtpG and Hsp90 are well conserved in their three dimensional structure and conformational changes of HtpG are also connected to ATP binding and hydrolysis (Shiau *et al*., 2006; Hessling *et al*., 2009). Despite this conservation, to date Hsp70 (the DnaK system) is the only known co-chaperone for HtpG in bacteria (Genest *et al*., 2011). Although deletion of *htpG* in *E. coli* results in a slight increase of protein aggregation in heat-stressed cells (Thomas and Baneyx, 2000) and a slower growth at high temperatures (Bardwell and Craig, 1988), little is known about the physiological roles of HtpG in bacteria. Only ribosomal protein L2 has been identified as a HtpG client protein in *E. coli* (Motojima-Miyazaki *et al*., 2010), and the assembly and stability of phycobilisomes, constituting a large light harvesting complex, require HtpG in cyanobacteria (Sato *et al*., 2010). Interestingly, it appeared in the present study that HtpG, a heat shock protein, is an important player in the cold shock recovery of *V. vulnificus*. However, it is still unclear what proteins denatured by cold shock are indeed restored by HtpG during the temperature upshift from 4°C to 30°C. Undoubtedly, additional work is needed to identify the client proteins of *V. vulnificus* HtpG and to elucidate possible molecular mechanisms by which HtpG may remodel any cold-denatured proteins.

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