The Role of Aldehyde Dehydrogenase and Hsp70 in Suppression of White Spot Syndrome Virus Replication at High Temperature[▽]

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High temperature (32 to 33°C) has been shown to reduce mortality in white spot syndrome virus (WSSV)-infected shrimps, but the mechanism still remains unclear. Here we show that in WSSV-infected shrimps cultured at 32°C, transcriptional levels of representative immediate-early, early, and late genes were initially higher than those at 25°C. However, neither the IE1 nor VP28 protein was detected at 32°C, suggesting that high temperature might inhibit WSSV protein synthesis. Two-dimensional gel electrophoresis analysis revealed two proteins, NAD-dependent aldehyde dehydrogenase (ALDH) and the proteasome alpha 4 subunit (proteasome $\alpha 4$), that were markedly upregulated in WSSV-infected shrimps at 32°C. Reverse transcription-PCR (RT-PCR) analysis of members of the heat shock protein family also showed that *hsp70* was upregulated at 32°C. When *aldh*, *proteasome $\alpha 4$* , and *hsp70* were knocked down by double-stranded RNA interference and shrimps were challenged with WSSV, the *aldh* and *hsp70* knock-down shrimps became severely infected at 32°C, while the *proteasome $\alpha 4$* knockdown shrimps remained uninfected. Our results therefore suggest that ALDH and Hsp70 both play an important role in the inhibition of WSSV replication at high temperature.

White spot syndrome virus (WSSV) is a notorious pathogen in shrimp aquaculture. It has caused huge economic losses since it was first reported in 1992 in East Asia (6), and the virus is presently endemic in many parts of the world. WSSV is an ovoid to rod-shaped, enveloped, double-stranded DNA virus which belongs to the genus “*Whispovirus*” of the family *Nimaviridae* (35). The destructiveness of WSSV is partly due to its wide host range, which includes shrimp, crayfish, crabs, lobsters, and copepods (2, 9). In shrimps, mortality can reach 100% in 3 to 10 days after WSSV infection (20).

Shrimps are exothermic animals, and unlike the case for endotherms, the body temperature of exotherms is directly susceptible to change depending on environmental conditions. Shrimp culture records show that outbreaks of WSSV are less likely to occur during warm seasons (29, 39), and there is other evidence suggesting that high temperature might protect shrimps from WSSV. Vidal et al. (34) reported that temperatures of 32 to 33°C can significantly reduce mortality in WSSV-infected shrimps. Other research has suggested that the water temperature needs to be maintained at 33°C for only 12 or 18 h per day in order to delay or reduce mortality in WSSV-infected shrimps (26). However, even though the use of warm water for

shrimp cultivation has generally been accepted as beneficial by the shrimp culture industry and is already applied in some commercial shrimp farms, the exact mechanisms that underlie this phenomenon are still unknown. Granja et al. (12) proposed that high temperature might increase cell apoptosis and that this would help to prevent the replication of WSSV. However, apoptosis is no longer thought to be a principal protective factor against WSSV in shrimps (10, 40). Others have suggested that high temperature might directly inhibit WSSV replication (27) or reduce the viral load (13).

In the present study, we attempted to identify host factors that might be related to the inhibition of WSSV replication at high temperature. To identify likely candidates, we first compared the transcriptional patterns of several WSSV genes in WSSV-infected shrimps at 32°C and 25°C and used two-dimensional gel electrophoresis (2-DE) to identify shrimp proteins that were markedly upregulated after WSSV infection at 32°C but that were downregulated or showed a much smaller increase at 25°C. It has also been suggested that heat shock proteins might be involved in the high-temperature repression of WSSV (34), and we therefore used reverse transcription-PCR (RT-PCR) to investigate the mRNA levels of various heat shock family proteins. Lastly, we used gene-specific double-stranded RNA (dsRNA) knockdown to investigate the effects of silencing the candidate genes *in vivo*.

MATERIALS AND METHODS

Virus preparation. The virus used in this experiment was the WSSV Taiwan-1 strain, which was originally taken from a WSSV-infected *Penaeus monodon*

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FIG. 1. RT-PCR time course (0 to 48 hpi) analysis of WSSV gene expression in *P. monodon* at 25°C and 32°C. The expression patterns are for the immediate-early genes *ie1* and *ie2* (A), the early gene *dna pol* (B), and the late structural protein gene *vp28* (C). (D) *P. monodon* *beta-actin* was used as an internal quality control, and primers for an intergenic region (IC) of the WSSV gene were used to check for DNA contamination. Lane headings show hours postinjection. C⁺, positive control from moribund WSSV-infected crayfish; N⁻, negative control (distilled water).

shrimp collected in Taiwan in 1994 (36). Adult specimens of *Litopenaeus vannamei* were infected with this virus, and after 3 days, hemolymph was collected from moribund shrimps, diluted three times with phosphate-buffered saline (PBS), and then stored at -80°C until use.

Shrimps. Adult specimens of *Litopenaeus vannamei* (3 to 4 g) were purchased from the National Taiwan Ocean University, Taiwan, and adult specimens of *Penaeus monodon* (7 to 12 g) were bought from Tung Kang, Taiwan. Shrimps were cultured in 40-liter tanks containing 20 liters of continuously circulated seawater at a salinity of 28 ppt. Before the experiments, all shrimps were acclimated at 25°C for 2 to 3 days, at which time a small part of the pleopod of each shrimp was excised and tested with a commercial WSSV diagnostic kit (IQ2000; IntelliGene) to check whether the shrimp was WSSV-free or not. All shrimps were found to be WSSV-free.

Experimental conditions. In the high-temperature WSSV challenge experiments, the tank water temperature was either maintained at a nominal value of 25°C (in practice, this temperature ranged from 24 to 25°C) or raised 1°C every 12 h to a final temperature of 32°C. When the final temperature was reached, the shrimps (*P. monodon*) were acclimated for a further 72 h and then injected with a WSSV stock that had been filtered through a 0.45-μm membrane. Tissue samples (pleopods, gills, and stomachs) were collected from each group at 0, 2, 4, 6, 8, 12, 24, and 48 h postinjection (hpi).

In the RNA interference (RNAi) experiments, water temperature was adjusted as described above. After 24 h of acclimation, the experimental shrimps (*L. vannamei*) were injected with a specific dsRNA. Forty-eight hours after the dsRNA injection, the shrimps were injected with the WSSV inoculum. The control groups were injected either with PBS or with a nonspecific (*luciferase*) dsRNA 48 h before the WSSV injection. Samples were collected from each group at 24 and 48 hpi. All collection procedures followed the methods of Tsai et al. (33). Samples were stored in liquid nitrogen prior to RNA and protein extraction.

RNA extraction and reverse transcription. To monitor the gene expression pattern of WSSV and to confirm the knockdown effects on specific genes at 25 and 32°C, RNAs were extracted from samples of the experimental shrimps at various times and subjected to reverse transcription. Total RNAs were extracted by use of TRIzol reagent (Invitrogen) according to the manufac-

turer's instructions. The RNA samples were treated with RNase-free DNase I (Invitrogen) at 37°C for 15 min and then reverse transcribed with an oligo(dT) anchor primer (Roche), first-strand reaction buffer, 100 mM dithiothreitol (DTT), a 10 mM concentration of each deoxynucleoside triphosphate (dNTP), RNase Out, and SuperScript III reverse transcriptase (Invitrogen) at 50°C for 1 h. The cDNA products were then used for RT-PCR analysis.

Total protein extraction and Western blot analysis. Stomachs of collected shrimps ($n = 3$) were ground in liquid nitrogen and homogenized with PBS containing a proteinase inhibitor cocktail (Merck). The total protein was quantified by an enzyme-linked immunosorbent assay (ELISA) using Bradford reagents (Bio-Rad). For Western blotting, 20 μg total protein was separated by SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membranes were blocked in 5% skim milk in TBST (0.2 M NaCl, 50 mM Tris-HCl, pH 7.4, 0.5% Tween 20, pH 7.5) overnight at 4°C and then incubated with polyclonal rabbit anti-IE1 (21) and anti-VP28 (32) sera diluted 1:5,000 in TBST for 2 h at room temperature. After being washed in TBST three times, the membranes were incubated with 1:5,000-diluted horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibodies (Sigma). The signals were detected by ECL and Western blotting detection reagents (Amersham Biosciences). Beta-actin protein was used as an internal control.

Two-dimensional gel electrophoresis. For two-dimensional gel electrophoresis, the pooled total protein solution from gills collected from three animals was centrifuged at 4°C for 30 min at 5,000 × g, and the supernatant was mixed with a trichloroacetic acid (TCA)-DTT solution at 4°C. The samples were cooled on ice for 1 h and then centrifuged for 30 min at 10,000 × g at 4°C. The pellet was resuspended in acetone containing 0.1% DTT and then centrifuged again for 30 min. The pellet was dried *in vacuo* and solubilized in rehydration buffer {9.8 M urea, 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 20 mM DTT, 0.5% IPG buffer (pH 3 to 10; Amersham Biosciences)}. Bromophenol blue was added to the solution, and 1-DE was performed.

In the first dimension of 2-DE, isoelectric focusing (IEF) was performed on a 13-cm Immobiline DryStrip gel (Amersham Biosciences) according to the procedures described by Wang et al. (37). After 1-DE, the IPG strips were equilibrated with an SDS equilibration buffer (6 M urea, 2% SDS, 30% glycerol, 50 mM Tris-HCl, pH 8.8, and 1% DTT) for 15 min and then equilibrated with 2.5% iodoacetamide buffer for 15 min. The IPG strips were then placed on a polyacrylamide gel (15% acrylamide, pH 8.8), and the second-dimension electrophoresis was run for 5 to 6 h at 20 mA and 15°C. After electrophoresis, the gels were stained with SyproRuby reagent for 16 h. The protein patterns were scanned using a Typhoon 9400 scanner (Amersham Biosciences) and matched by PD-Quest software (Bio-Rad).

In-gel protein digestion and protein identification. Target protein spots were excised from the 2-DE gels and washed twice with 25 mM ammonium bicarbonate buffer (pH 8.5) in 50% acetonitrile for 15 min. The gels were dehydrated with 100% acetonitrile for 5 min, dried in a vacuum, and then rehydrated for 16 h with 100 ng modified trypsin (Promega) in 25 mM ammonium bicarbonate, pH 8.5, at 37°C. Digested peptides were extracted twice for 15 min each time, using 5% formic acid in 50% acetonitrile. The extracted solutions were dried under vacuum and then dissolved in 0.1% formic acid in 50% acetonitrile. These final solutions were analyzed by liquid chromatography-nano-electrospray ionization-tandem mass spectrometry (LC-nano-ESI-MS/MS). The analysis results were searched by MASCOT, using the NCBI protein and BIO301 expressed sequence tag (EST) databases (<http://bc02.iis.sinica.edu.tw/bio301new/>).

HSP family screening. To understand the expression patterns of the heat shock protein (HSP) family after WSSV infection at 25°C and 32°C, RT-PCR was used for analysis of *hsp10*, *hsp20.6*, *hsp70*, and *hsp90*.

Double-stranded RNA production and injection. The target genes identified by the above screening processes (*aldh*, *proteasome α4*, and *hsp70*) and the *luciferase* control were amplified as sense and antisense DNA strands. The T7 promoter was included at the 5' end of every primer.

Each gene was constructed in a pGEM-T recombinant plasmid and used as a DNA template for PCR. All PCR products were purified using a PCR cleanup system (Geneaid) and then used as templates to synthesize dsRNA. The T7 RiboMAX Express RNAi system (Promega) was used to synthesize dsRNA per the manufacturer's instructions. The dsRNA concentration was determined by a UV spectrophotometer at a wavelength of 260 nm, and quality was confirmed by DNA electrophoresis.

Experimental groups were injected with 0.1 μg/g dsRNA in a total volume of 20 μl, and control groups were injected with 0.1 μg/g nonspecific dsRNA or 20

FIG. 2. Western blotting of WSSV proteins in *P. monodon* at 0 to 48 hpi at 25°C and 32°C. (A) WSSV immediate-early protein IE1. (B) WSSV structural protein VP28. (C) *P. monodon* beta-actin (internal control). C⁺, positive-control sample taken from a WSSV-infected *P. monodon* shrimp at 60 hpi and 25°C.

μl PBS. According to Ongvarrasopone et al. (24), the silencing effect of dsRNA reaches a maximum 48 h after injection of dsRNA, so gills were collected 48 h after dsRNA injection and checked by RT-PCR to confirm both that the target gene had been suppressed successfully and that there was no nonspecific silencing of other genes of interest.

DNA extraction and WSSV detection. For DNA extraction, the pleopods collected from shrimps were homogenized with lysis solution (IntelliGene) and then heated at 95°C for 10 min. The solution was centrifuged at $12,000 \times g$ for 10 min, and the supernatant was mixed with 95% alcohol. This solution was then centrifuged at $12,000 \times g$ for another 5 min, and the pellet was dried and resuspended in distilled water. The DNA samples were analyzed by competitive nested PCR (IQ2000 WSSV detection and prevention system; IntelliGene). The PCR products were analyzed in a 2% agarose gel by electrophoresis. WSSV infection status was then determined according to the kit's instructions, based on the pattern of three bands representing WSSV genes (910, 550, and 296 bp) and one band representing shrimp genomic DNA (848 bp).

Quantitative real-time PCR. SYBR green real-time PCR was used to quantify the viral copy number in WSSV-infected shrimps. The primers used for WSSV detection amplify a highly conserved fragment of the WSSV genome, while internal control primers amplify *Penaeus monodon* 18S DNA (data not shown). The PCR procedure followed an initial cycle of 50°C for 2 min and then 95°C for 10 min, with 40 cycles of 95°C for 15 s and 60°C for 1 min. Real-time PCR was performed in a GeneAmp 9600 thermocycler coupled with a GeneAmp 5700 sequence detection system (PE Applied Biosystems).

RESULTS

Transcriptional and translational patterns of WSSV at high temperature. Figure 1 compares the RT-PCR time course patterns of several WSSV immediate-early, early, and structural protein genes at normal (25°C) versus high (32°C) temperature. Expression of the immediate-early genes (*ie1* and *ie2*) and the early gene (*dna pol*) was first observed at 2 hpi, regardless of temperature. We also noted that transcription levels were initially higher at 32°C than at 25°C. However, while expression levels generally trended upwards at 25°C, they remained more or less constant at 32°C. A fairly similar pattern was also seen for the structural protein gene *vp28*, although expression levels were more variable, and at 25°C, no VP28 expression was seen until 4 hpi. At the protein level, Western blotting failed to detect any IE1 or VP28 at 32°C, even though both proteins were detected at 24 hpi at 25°C (Fig. 2). In sum, these results show that at 32°C, even though the transcripts of these proteins appear at relatively high levels initially, their expression levels fail to increase and no detectable amount of protein is produced.

FIG. 3. 2-DE total protein profiles for WSSV-infected and mock-infected (PBS) *P. monodon* at 25°C versus 32°C. (A to D) Samples were taken 48 h after WSSV or PBS injection. (E) Magnification of the 3 spots of interest (spot 1, calreticulin; spot 2, ALDH; and spot 6, proteasome $\alpha 4$) plus 3 control spots (spot 3, beta-actin; spot 4, arginine kinase; and spot 5, tropomyosin).

Two-dimensional gel electrophoresis of WSSV-infected shrimps at different temperatures. Two-dimensional gel electrophoresis was used to compare the protein profiles of PBS-injected and WSSV-infected shrimps at 25°C and 32°C (Fig. 3). Spots of interest were selected based on increases in expression levels between the 25°C/PBS baseline and the 32°C/WSSV experimental infection conditions. The three spots showing the highest increases were selected, and these differentially expressed proteins were identified by LC-nano-ESI-MS/MS. The results for the spots of interest are summarized in Table 1. The proteasome alpha 4 subunit (spot 6) showed the most upregulation after WSSV infection at 32°C. This protein was also upregulated after WSSV infection at 25°C, although the increase was ~ 3 times smaller. After WSSV infection at 32°C, the expression level of NAD-dependent aldehyde dehydrogenase (ALDH; spot 2) also increased substantially. In contrast, after WSSV challenge at 25°C, the expression of this protein was reduced. Calreticulin (spot 1) also showed increased expression after WSSV infection at 32°C, but the increase was

similar to the increase at 25°C. Since both ALDH and the proteasome alpha 4 subunit showed differently regulated expression results at 32°C versus 25°C, these two proteins were selected for further study.

RT-PCR analysis of heat shock family proteins after WSSV infection at 25°C and 32°C. Transcriptional analysis of heat shock family proteins showed that in WSSV-infected shrimps, *hsp70* was downregulated after WSSV infection at 25°C but was upregulated at 32°C (Fig. 4). No other heat shock family protein exhibited this expression pattern, so only *hsp70* was chosen for further study.

Knockdown of specific host genes by dsRNA. RT-PCR showed that the expression of *aldh*, *proteasome $\alpha 4$* , and *hsp70* mRNAs was almost completely knocked down 48 h after injection of the respective dsRNAs at 25°C and 32°C (Fig. 5). RT-PCR also showed that at 32°C, *aldh* dsRNA had no effect on the expression of *hsp70* (data not shown).

WSSV replication in host gene knockdown shrimps at 25°C and 32°C. In this study, shrimps cultured at 25°C and 32°C

TABLE 1. Protein identification by LC–nano-ESI–MS/MS

Protein rank	Protein name (species, spot)	GenBank accession no.	Sequence coverage (%)	Observed molecular mass (kDa)/pI	Fold change			
					25°C/PBS	25°C/WSSV	32°C/PBS	32°C/WSSV
Experimentally identified spots of interest								
1	Proteasome alpha 4 subunit (<i>Callinectes sapidus</i> ESTs, spot 6)	CV086810	15	24.4/5.7	1	1.40	0.72	4.78
2	NAD-dependent aldehyde dehydrogenase (<i>Litopenaeus vannamei</i> ESTs, spot 2)	CK571503	41	62.4/5.9	1	0.59	0.96	2.71
3	Calreticulin (<i>Litopenaeus vannamei</i> ESTs, spot 1)	CK572545	35	53.7/4.0	1	3.01	0.88	2.56
Control protein spots								
	Beta-actin (<i>Homarus gammarus</i> , spot 3)	CAE46725		43.9/5.5	1	1.01	1.18	1.23
	Arginine kinase (<i>Penaeus monodon</i> , spot 4)	AAO15713		38.8/6.6	1	0.91	0.84	0.96
	Tropomyosin (<i>Anopheles gambiae</i> ESTs, spot 5)	BX607292	19	27.8/4.7	1	0.67	0.64	0.69

were injected with PBS or dsRNA (*aldh*, *proteasome* $\alpha 4$, *hsp70*, or *luciferase* dsRNA) 48 h before being challenged by injection with WSSV. Pleopods were collected from the experimental shrimps at 24 and 48 hpi, and infection levels were diagnosed using an IQ2000 WSSV detection kit (IntelliGene, Taiwan).

At 25°C, WSSV infection was detected in all of the shrimps at 48 hpi, but there was an overall reduction in WSSV severity in the dsRNA groups compared to the PBS controls (Fig. 6). This was presumably due to the nonspecific antiviral effect of dsRNA.

At 32°C and 48 hpi, all of the control group shrimps (PBS and *luciferase* dsRNA groups) and most of the shrimps in the *proteasome* $\alpha 4$ knockdown group were WSSV-free, although a few shrimps had light to moderate infection (Fig. 7). However, all of the shrimps in the *aldh* and *hsp70* knockdown groups had moderate or severe WSSV infection, suggesting that ALDH and Hsp70 might each play a critical role in the suppression of WSSV replication at high temperature.

Viral loads of WSSV-infected shrimps at 25°C and 32°C. To confirm the results of the nested competitive PCR, SYBR

green quantitative real-time PCR was performed to measure the viral copy numbers of WSSV-infected shrimps at 25°C and 32°C. At 25°C, the WSSV copy numbers (Fig. 8) were broadly in agreement with the nested competitive PCR results (Fig. 6), except that the WSSV copy number was unexpectedly low for the *proteasome* $\alpha 4$ knockdown group.

At 32°C, there were no WSSV virions detected in the PBS or *luciferase* knockdown shrimps, and the *proteasome* $\alpha 4$ knockdown group showed fewer than 10 copies/ng (Fig. 8, right panel). However, the viral loads of the *aldh* and *hsp70* knockdown groups were both $\sim 10^5$ copies/ng. Again, these quantitative PCR results are broadly in agreement with the previous nested PCR results.

DISCUSSION

Although our data suggest that WSSV immediate-early, early, and late genes are all transcribed within 2 to 4 hpi, regardless of temperature (Fig. 1), at least two of the corresponding proteins were detected in WSSV-challenged shrimps at 25°C only, not at 32°C (Fig. 2). Thus, the absence of these proteins at 48 hpi in shrimps cultured at 32°C suggests that hyperthermic conditions might activate some host factors that modulate viral gene expression or inhibit protein synthesis.

Three candidate factors were identified by the two-dimensional gel electrophoresis analysis of WSSV-infected versus uninfected shrimps cultured at normal (25°C) or high (32°C) temperature (Fig. 3; Table 1). After infection at 32°C, the upregulation of two of these candidates, ALDH and the proteasome alpha 4 subunit, was in marked contrast to their expression levels after infection at 25°C. Both of these proteins are also involved in pathways related to cell defense and/or cell protection (4, 11, 19), so these two proteins were selected for further study. In contrast, the expression levels of the third candidate, calreticulin, were relatively unaffected by temperature (Table 1), which suggested that calreticulin was unlikely to

FIG. 4. RT-PCR time course (0 to 48 hpi) analysis of *hsp* family proteins in WSSV-infected shrimps at 25°C and 32°C. The internal control is *ef-1α*.

FIG. 5. RT-PCR analysis of gene expression of *aldh*, *proteasome* $\alpha 4$, and *hsp70* at 25°C and 32°C. The internal control is *ef-1 α* . Samples were taken 48 h after injection with the indicated dsRNA.

be involved in the temperature-related suppression of WSSV replication.

In addition to the ALDH and proteasome $\alpha 4$ genes, RT-PCR screening of the heat shock family of proteins showed that *hsp70* was also markedly upregulated at 32°C in WSSV-infected shrimp (Fig. 4). We note that 2-DE is not a good method for monitoring expression of the HSP family because these proteins are very similar in size and pI to the massively expressed shrimp hemocyanin protein.

The next part of our study showed that at 32°C, the specific silencing of *aldh* and *hsp70* by dsRNA interference led to severe WSSV infection in most of the WSSV-challenged shrimps at 48 hpi (Fig. 7). Furthermore, quantitative PCR (Fig. 8) revealed that while specific dsRNA silencing of *proteasome* $\alpha 4$ at 32°C had almost no effect on the high-temperature repression of WSSV, when either *aldh* or *hsp70* was specifically knocked down, WSSV replication was restored at 32°C. It does not necessarily follow from this that ALDH and Hsp70 directly protect the host from the effects of WSSV infection, because it is possible that suppression of these two proteins might in fact make the shrimps more susceptible to stresses in general—including heat stress and the stress of infection. One way to eliminate this possibility would be to demonstrate that upregulation of ALDH and Hsp70 at 25°C results in increased WSSV resistance, but this lies well beyond the scope of the present study. Meanwhile, the data in Fig. 6 to 8 are consistent with the hypothesis that ALDH and Hsp70 are directly involved in high-temperature WSSV resistance, and we consider possible mechanisms below.

If ALDH does in fact mediate the hyperthermic protection of shrimps from WSSV, the underlying mechanism might be an extension of ALDH's basic functions. ALDH oxidizes toxic aldehyde—aldehyde is a strong protein-DNA cross-linker that inhibits DNA replication and macromolecule synthesis—to less harmful carboxylic acid. If high temperatures cause shrimps to suffer from oxidative stress and produce toxins such as aldehyde, then the cells might respond by increasing the levels of ALDH to clear these toxic substances. This detoxification process might then indirectly help the host to resist

WSSV. In the present case, however, this explanation seems unlikely, because no increase in ALDH was seen in the uninfected shrimps at 32°C (Fig. 3; Table 1). We also note, however, that in addition to detoxifying the cell, the upregulation of ALDH during pathogen infections led to suggestions that it might function as a cellular protector (18, 19). Our 2-DE data show that ALDH upregulation occurs in response to WSSV infection at 32°C but not at 25°C (Fig. 3; Table 1). This might explain why ALDH is able to protect the cell against WSSV only under hyperthermic conditions. If so, it raises the question of how WSSV is able to prevent this upregulation at 25°C.

Hsp70 is a powerful indicator of a heat shock response (23), and it is a highly conserved protein that acts as a molecular chaperone and plays a central role in protein folding, regulating the cell cycle, and antiapoptosis (42). Stresses such as hyperthermia, oxidative stress, and bacterial/viral infections can all activate Hsp70 (1). Some viruses, including herpes simplex virus (25) and simian virus 40 (31), recruit Hsp70 to help with viral attachment, replication, and assembly (22). However, other viruses, such as influenza virus (30) and Sendai virus (17), are suppressed by Hsp70, and production of these viruses is inhibited under hyperthermic conditions. Various mechanisms are involved. For instance, influenza virus cannot replicate above 41°C because Hsp70 inhibits nuclear export of the ribonucleoprotein complex (16). Hsp70 also blocks Sendai virus infection at 41°C, by disrupting HN protein assembly (17).

Vidal et al. (34) were the first to suggest that the heat shock protein family might be involved in the high-temperature suppression of WSSV replication, and our present double-stranded RNA interference results for 32°C (Fig. 7 and 8) show that reducing the expression of Hsp70 allows WSSV to replicate in challenged shrimps. (We note, however, that in preliminary studies dsRNA silencing of other heat shock proteins did not have any significant effect on WSSV replication [data not shown].) This suggests that Hsp70 may interact with some vital factors of WSSV to block its replication. One possible candi-

FIG. 6. (A) Nested competitive PCR analysis of WSSV infection levels in shrimps injected with PBS or with *luciferase*, *aldh*, *proteasome* $\alpha 4$, or *hsp70* dsRNA at 25°C. Infection status was determined according to the patterns of bands detected by an IQ2000 kit (see below). The bands at 910, 550, and 296 bp are WSSV DNA, and the band at 848 bp is shrimp DNA. The positive controls (supplied with the kit) show results for 2×10^3 , 2×10^2 , and 2×10^1 copies of a WSSV DNA fragment. The negative control (N) is distilled water. (B) Key to determining WSSV infection status in *Litopenaeus vannamei*. The presence of bands at both 848 bp and 296 bp indicates a very light infection; only one band, at 296 bp, indicates a light infection; bands at both 296 bp and 550 bp indicate a moderate infection; and the appearance of all three bands (910, 550, and 296 bp) indicates a severe infection. -, WSSV-negative sample; +, very light WSSV infection; ++, light WSSV infection; +++, moderate WSSV infection; + + + +, severe WSSV infection; ND, no signal detection.

date is the WSSV structural protein VP28, which has already been reported to interact with heat shock cognate 70 (Hsc70) (41). Hsc70 and Hsp70 share the same open reading frame, and they both belong to the Hsp70 family. It is therefore possible that Hsp70 interacts with VP28 to prevent viral infection; however, this has not yet been investigated experimentally.

It is also possible that Hsp70 and ALDH work in concert to produce high-temperature repression of WSSV. Interactions between these two proteins have not yet been investigated in crustaceans, but it is well known that Hsp70 associates with newly synthesized peptides (5), and in human liver cells, heat shock protein is needed for the correct folding of ALDH2 after its import into the mitochondria (43). Cooperation between

heat shock protein and ALDH has also been proposed to occur during sepsis-induced fever in rats. Chen et al. (3) proposed that an ALDH2 precursor may need to associate with HSPs in order to be transported from the cytoplasm to the mitochondrial matrix. These examples suggest that it might be useful to investigate whether Hsp70 also acts as a molecular chaperone to ALDH in shrimps. However, it is also possible that two separate pathways are involved, and we note that in a preliminary study, when *hsp70* and *aldh* were both silenced simultaneously, the shrimps died very quickly (data not shown).

At 25°C, all of the dsRNA groups showed lower levels of infection than those for the PBS controls (Fig. 7). This is consistent with previous reports showing that dsRNA has a

FIG. 7. WSSV infection levels of shrimps injected with PBS or with *luciferase*, *aldh*, *proteasome* α 4, or *hsp70* dsRNA at 32°C. The positive and negative controls and the infection status key are the same as those for Fig. 6.

nonspecific antiviral effect that reduces mortality after WSSV infection (28).

We also noted that *proteasome* α 4 dsRNA had a very pronounced effect on the WSSV copy number at 25°C (Fig. 8, left panel). As Table 1 shows, *proteasome* α 4 expression was up-regulated after WSSV infection at 25°C. The proteasome is known to combine with ubiquitin to perform nonapoptotic cellular proteolysis (7), and many viruses have evolved to utilize the host proteasome-ubiquitin system for their survival and replication (15). For example, adenovirus cannot replicate its genome if proteasome activity is inhibited (8). It has already

been shown that WSSV encodes ubiquitin ligase (E3) and that this is required for WSSV pathogenesis and replication (14, 38). This suggests that *proteasome* α 4 might also be required for WSSV replication, and if so, then the dsRNA suppression of *proteasome* α 4 at 25°C would account for the observed reduction in WSSV copy number. However, this proposed mechanism would need to be confirmed in future studies.

Taken together, these results suggest that in WSSV-infected shrimps at 32°C, the upregulated host proteins ALDH and Hsp70 may each play a critical role—either

FIG. 8. Effect of temperature on viral loads in dsRNA knockdown shrimps 48 h after challenge with WSSV. (Left) 25°C. (Right) 32°C. Shrimps were injected with PBS or with *luciferase*, *aldh*, *proteasome* α 4, or *hsp70* dsRNA 48 h before challenge.

together or separately—in the suppression of WSSV infection. Clearly, there are still many unknowns, and elucidation of the precise mechanism(s) involved will require further study.

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