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Induced thermotolerance and the heat shock protein–70 family in the Pacific oyster *Crassostrea gigas*

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Abstract

Commercially important oysters are often subject to mass mortality in the field. Rationalizing that heat shock can induce tolerance to stresses other than heat, we are exploring the possibility that this treatment might improve subsequent survival in the field. In characterizing the heat shock response in the laboratory, we found that heat shock of 37°C for 1 h (HS) enabled Pacific oysters (*Crassostrea gigas*) to survive an otherwise lethal heat treatment (43°–44°C, 1 h) (LHT). This induced thermotolerance was retained for at least 2 weeks, the longest period examined. Stress proteins of the 70-kDa family were studied in gills by conventional autoradiography and immunoblotting techniques. Two constitutive isoforms of 77 and 72 kDa were up-regulated, and a 69-kDa protein was induced by HS, its synthesis occurring during and after that treatment. Synthesis of these three proteins was reduced to control levels within 2 days after HS, but their amounts remained higher than in control gills for 2 weeks following HS. Immunolocalization of proteins of the heat stock protein–70 (hsp-70) family in histologic sections of gills was consistent with results from Western blotting.

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Introduction

The ''heat shock response'' has been studied for many years (see Ritossa, 1996; Schlesinger, 1996) and is currently the subject of intense study (see Nover, 1991; Hightower and Hendershot, 1997). Induced thermotolerance is a component of this response (Schlesinger et al., 1982; Lindquist and Craig, 1988; Weber, 1992; Trent et al., 1994) involving the acquisition of tolerance to otherwise lethal temperatures as a result of exposure to a previous ''heat shock'' (a temperature that does not kill, but is well above the physiologic temperature range). It is well known that heat shock can lead to up-regulation of an ensemble of constitutive heat shock proteins, sometimes referred to as hscs, and to the synthesis of other, inducible isoforms (reviewed in Morimoto et al., 1990, 1994; Nover, 1991; Weber, 1992; Parsell and Lindquist, 1993; Feige et al., 1996).

Although the functions of heat shock proteins continue to be elucidated, a well-established role for some of them, including the hsp-70 family, involves their ability to act as molecular chaperones (for history, see Ellis, 1996a). In that capacity they protect other proteins from unfolding, or refold denatured proteins, or target them for degradation, and it is clear that these processes are involved in the induction of thermotolerance (Morimoto et al., 1994; Ellis, 1996b; Feige et al., 1996; Gethig, 1996; Hartl, 1996; Frydman and Höhfeld, 1997).

As part of a broader study on mass mortality of the Pacific oyster *Crassostrea gigas,* we studied the induction of thermotolerance and its relation to the hsp-70 family. The research was motivated by the hypothesis that the induction of thermal tolerance could confer on these animals an enhanced resistance to stress under field conditions. However, to test that hypothesis we first needed to characterize

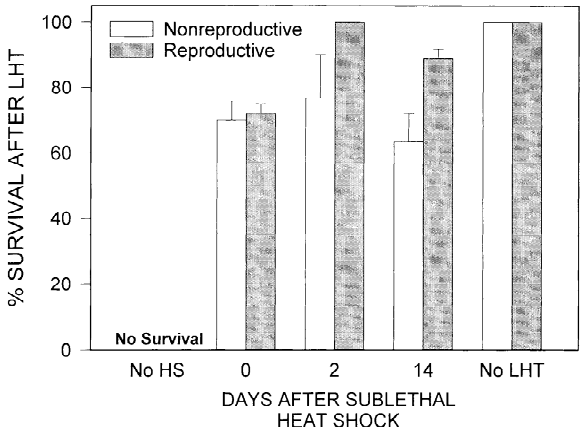

Figure 1. Thermotolerance induced by exposure to 37°C for 1 h (HS). Oysters were given HS conditions and then incubated for 0, 2, and 14 days at the original holding temperature of 11°–12°C before being given a lethal heat treatment (LHT) of 1 h at 44°C. Survival was evaluated after 1 week at 11°–12°C. ''No HS'' refers to oysters given LHT without prior HS; ''No LHT'' refers to animals given HS without subsequent LHT. The bars are means of three groups of 10 reproductive oysters (population 1) or 12 nonreproductive animals (population 3) ± SE.

both the heat shock response and induced thermotolerance in the laboratory, an objective of the present study. To our knowledge nothing has been published on the induction of thermotolerance in intact oysters of any species, and no information is available on any heat shock protein in *C. gigas.*

Results

Heat shock and induced thermotolerance

Our preliminary studies to determine the median lethal temperature (LT_{50}) for *C. gigas* showed that a heat shock of 37°C for 1 h (on animals taken directly from holding temperatures of about 12°C) did not cause any mortality 1 week after the shocked animals were returned to 12°C. These heat shock conditions will be referred to subsequently as ''HS.'' The lowest lethal temperature regimen required to kill all the oysters not experiencing a previous HS was found to vary slightly, but importantly, between different batches of animals: 43°–44°C for 1 h. We refer to this lethal heat treatment as ''LHT'' even though, as we will show, LHT did not kill most oysters previously exposed to HS (i.e., the oysters acquired thermotolerance).

Figure 1 shows representative results on the induction of thermotolerance in these animals. All oysters taken directly from 12°C were killed by LHT (''No HS'' in Figure 1). In contrast, a large proportion of oysters previously given HS, then returned to 12°C, survived exposure to LHT, even when administered immediately after HS (''0'' in Figure 1). As expected, all oysters given HS conditions without subsequent LHT survived (''No LHT'' in Figure 1). Induced thermotolerance was retained for at least 14 days at high survival levels, and no marked differences between reproductively mature and immature animals were noted (Figure 1). Further studies like these revealed similar trends, in some cases resulting in even higher survival rates. No obvious differences in induced thermotolerance related to the sex of the animals were observed.

Table 1 describes the effects of a second LHT given at different times following the first LHT. Unlike a single LHT following HS (Figure 1), a second LHT resulted in high mortality, even if a 2-day recovery period was allowed between the two LHTs.

We found that the lowest temperature required to kill all animals after a 1-h exposure (LHT) varied between different batches of oysters. Thus, in experiments carried out during the spring of 1996, the lethal temperature was determined to be 43.5°C; during the summer of 1996, it was 44.0°C; and during the spring of 1997, it was 43.0°C. Consequently, one is obliged to determine the lethal temperature for each batch of oysters.

Protein synthesis in gills in vitro before and after heat shock

Next we investigated the ensemble of proteins being synthesized, using the same HS protocol as in the studies on induced thermotolerance (Figure 1), except that no LHT was used. Gills were chosen as the tissue for study (see below, Experimental Procedures). Dissected gills from control and heat-shocked oysters were incubated in sterile seawater

Table 1. Survival following sequential lethal heat treatments given after a sublethal heat shock of 37°C for 1 hour.*

Time between LHTs	Survival 1 week after second LHT (%)
None	0
10 min	3
2 h	3
2 d	20

* Thirty animals were used for each treatment: HS, then LHT, then time between first LHT and second LHT (left-hand column).

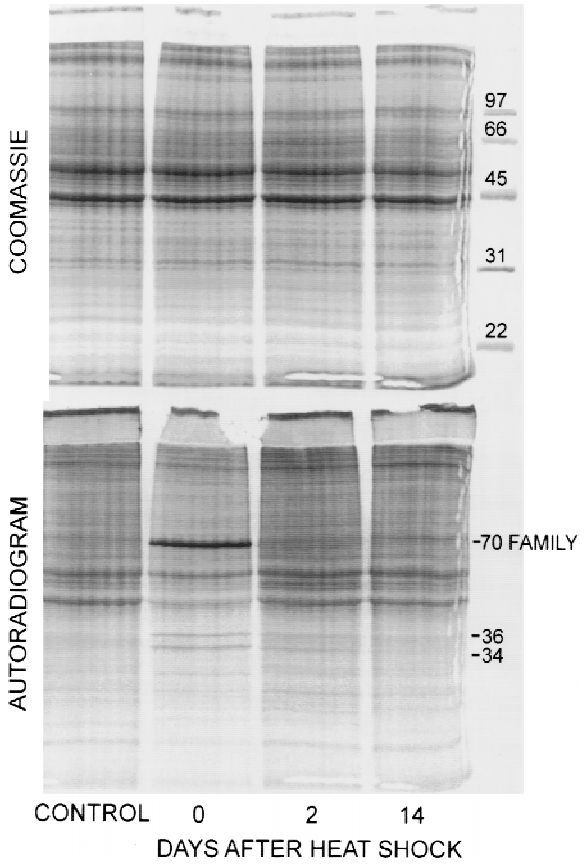

Figure 2. SDS-PAGE (gel above) and autoradiography (gel below) of homogenates of gills from nonreproductive oysters previously incubated with a mixture of ^{14}C-labeled amino acids in vitro (20°–22°C for 2 h). Before gill removal the oysters were exposed to 37°C for 1 h (HS), then held at 12°C for 0, 2, or 14 days. Controls represent gills from oysters not exposed to HS. The lane to the right (top gel) shows protein standards (kDa).

containing ^{14}C-labeled amino acids then prepared for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography as described (Figure 2). The Coomassie-stained protein profiles (upper gel) were quite similar for all treatments, showing no indication of proteolysis. The autoradiogram (below) illustrates the array of proteins being synthesized during the in vitro incubation period. As expected, gills removed immediately following HS (0) exhibited a general but modest depression in the synthesis of most proteins compared with controls. However, an obvious up-regulation of the synthesis of proteins in the 70-kDa range was observed. Later in this article we provide evidence that these proteins belong to the hsp-70 family. Other up-regulated proteins are present, notably two of about 34 and 36 kDa molecular mass, presumably ''small'' heat shock proteins. By 2 days after HS, the pattern of gill protein synthesis in vitro was similar to that in controls, although synthesis of proteins in the vicinity of 70 kDa continued in gills removed from oysters 14 days after HS. Although induced thermotolerance of intact oysters continued for at least 14 days (Figure 1), the synthesis in vitro of gill proteins of the hsp-70 family did not accompany that thermotolerance at an appreciable and corresponding level (Figure 2).

In the studies described in Figure 2, we arbitrarily chose a 2-h incubation of gills with the ^{14}C-labeled amino acid mixture, so shorter incubation periods were examined to determine whether the patterns of synthesis would differ as a function of that variable (Figure 3). We focused on gills from controls and from oysters immediately following HS (0). The results clearly showed an up-regulation of 70-kDa proteins in gills from the latter, regardless of the in vitro incubation period, compared with controls.

Protein synthesis in gills in vitro before and during heat shock

Because thermotolerance seemed to be induced during HS (Figure 1), it was of interest to determine the nature of gill protein synthesis during HS. Figure 4 shows that the profiles of proteins made during the 1-h HS were similar to those made by gills immediately following HS (Figure 3).

Western immunoblotting of the hsp-70 family

We used the same general HS protocol as in the previous studies: controls (C), immediately after HS (0), and 2 and 14 days after HS, those animals being held at 12°C. No LHT was used on any of these oysters. Two apparent constitutive isoforms of about 77 and 72 kDa were observed in gills from controls, and from animals immediately after HS (Figure 5). Hsp-69 was not detected at that time, even though it is being synthesized (Figures 3 and 4), probably owing to the use of very high levels of the radioactive amino acid mixture (25–30 μCi/ml) that detected the synthesis of small amounts of protein not revealed by immunoblotting. Obvious increases in all three proteins took place during the first 2 days after HS, but by 14 days after HS a collective downward trend was evident. Figure 6 quantifies that impression, and also shows that the levels of all three proteins were significantly higher than in the controls, even 14 days after HS. The

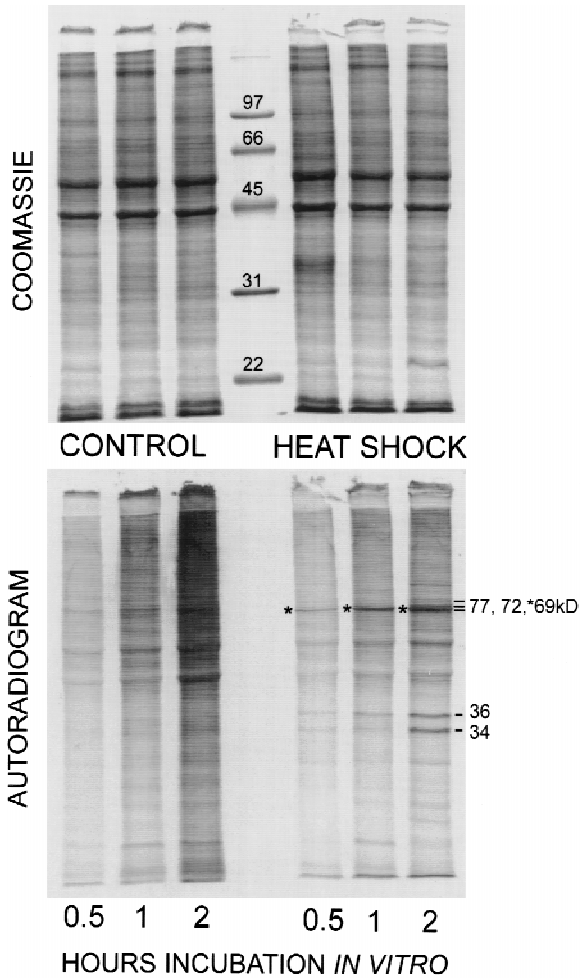

Figure 3. Time course of incorporation of ^{14}C-labeled amino acids into gill proteins in vitro, detected by SDS-PAGE and autoradiography. Conditions are the same as described in the legend to Figure 2, except for the duration of in vitro incubation.

studies described in Figures 5 and 6 used gills from nonreproductive oysters. Comparable studies have been performed on animals with mature gonads, and the results (not shown) were similar to those shown in Figures 5 and 6.

To explore individual variability, and to fill in the gaps in the time between HS and assay by immunoblotting, further studies were performed (Figure 7). These results indicated the appearance of the inducible 69-kDa isoform during the first 4 h after HS, a result that agrees with detection of the synthesis of this protein in gills during HS (Figure 3) and immediately after HS (Figure 2). These results and those presented in Figures 4 and 6 illustrate significant individual variation in the amounts of the three hsp-70 family proteins, but the general trends of their expression are similar.

Hsp-70 localization in gill tissue

Oyster gill sections probed with antibody against the hsp-70 family were labeled to varying degrees in all treatments (Figure 8). Gill tissues from animals 2 days after HS consistently exhibited an increase in both the area of fluorescence labeling (1.9-

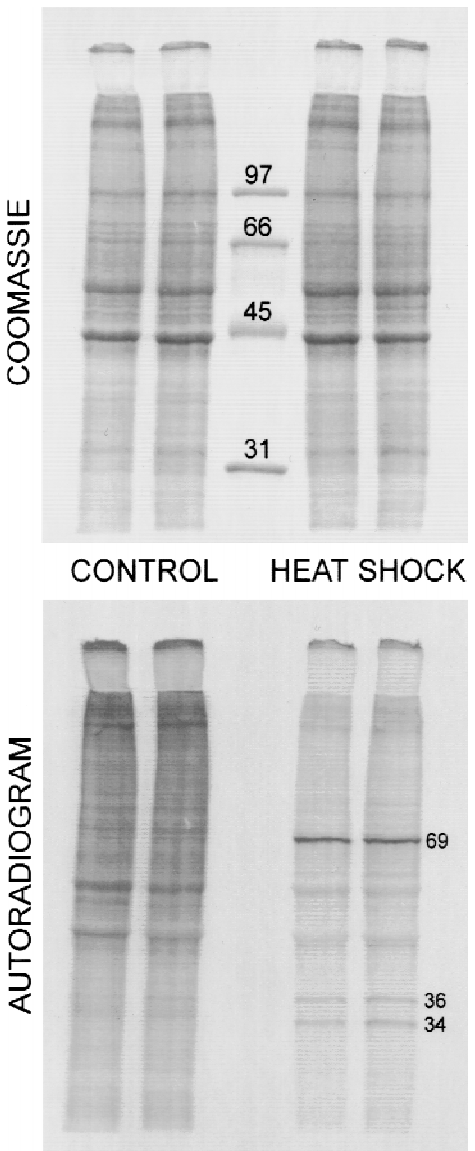

Figure 4. Incorporation of ^{14}C-labeled amino acids into gill proteins during 1-h HS in vitro. Gills from two oysters were studied for control and HS conditions (see legends to Figures 2 and 3).

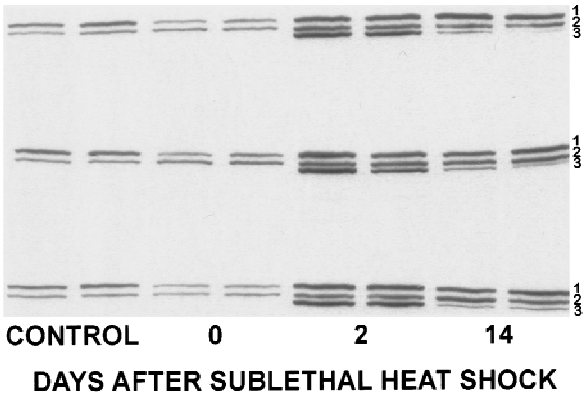

Figure 5. Western immunoblotting to detect proteins of the hsp-70 family in gills from six nonreproductive oysters, displayed in two rows of three above each treatment designation. Note that all these samples were transferred to a single sheet of nitrocellulose (see Experimental Procedures). Treatments were the same as described in the legend to Figure 2 except that the gills were not incubated in vitro after their dissection from the animals. Numbers to the right refer to proteins of approximate molecular mass: 1 = 77 kDa; 2 = 72 kDa; 3 = 69 kDa.

fold increase relative to that in controls) and the intensity. By 14 days after HS, the regions of antibody labeling were low, in some cases below control levels (0.65-fold decrease below control levels).

Discussion

Induced thermotolerance

It is remarkable that these oysters not only tolerate immediate transfer to a temperature that is 25°C above their holding (physiologic) environment, but also acquire from this insult a thermotolerance that remains for at least 2 weeks (Figure 1), a long duration compared with thermotolerance in other animals (see Morimoto et al., 1990, 1994; Nover, 1991; Weber, 1992; Fiege et al., 1996). Although aspects of the heat shock response have been studied in a number of mollusks including mussels (Sanders et al., 1992; Hofmann and Somero, 1995, 1996a, 1996b; Roberts et al., 1997) and limpets (Sanders et al., 1991), we are not aware of any published study of induced thermotolerance in intact oysters.

Induction of thermotolerance in *C. gigas* apparently results immediately from a HS of 1 h at 37°C (Figure 1). However, we have also shown that oysters exposed to HS, followed by one LHT, are all killed by a second LHT immediately thereafter (Table 1). Even a recovery period of 2 days between the two LHTs is not every effective. The fact that some oysters experiencing LHT (with or without preceding HS) take up to a week to die is important to consider when interpreting what the induction of thermotolerance means in these animals. We believe that what takes place during HS involves the initiation of processes that may or may not eventually enable a given oyster to repair the damage caused by HS and LHT during the week following LHT.

That it takes several days to a week for some oysters to die following LHT is not too surprising because similar results have frequently been observed for a variety of cells and tissues. A review by Lepock (1997) provides evidence from a variety of studies indicating that protein denaturation is the most likely candidate for the critical target of hyperthermic killing. Thus, attention to heat shock proteins and molecular chaperones seems highly appropriate when considering the basis of induced thermotolerance, granted that the latter probably also involves other mechanisms.

Involvement of the hsp-70 family

Up-regulation of synthesis of the constitutive 77 and 72-kDa proteins, and induced synthesis of hsp-69 in oyster gills (Figures 2–4) does not continue at the same level during the 2-week period of induced thermotolerance (Figure 1). Nevertheless, the levels of these isoforms remain higher than in controls throughout this same period. It appears that a slow rate of the degradation of these proteins is responsible for this pattern. Although we have not examined here the immediate relation between these

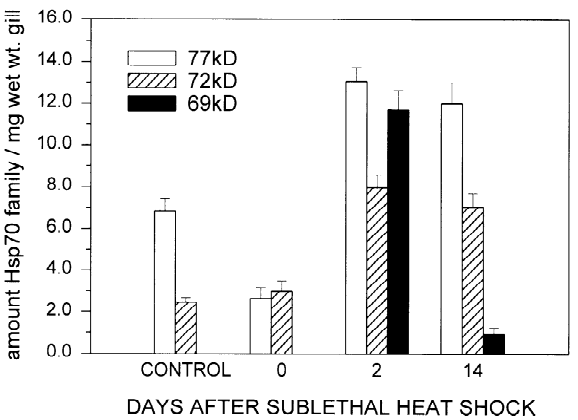

Figure 6. Relative amounts of the hsp-70 family obtained from densitometric scanning of the blots shown in Figure 5. Bars are means ± SE (n = 6).

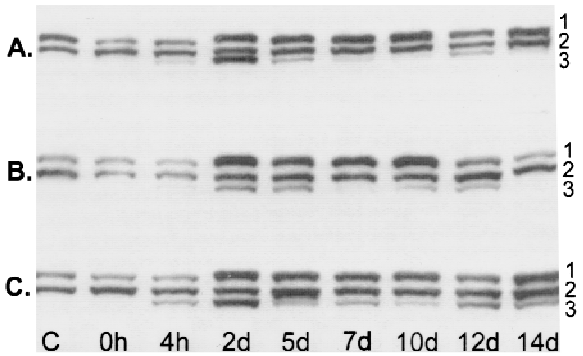

Figure 7. Western immunoblotting to detect proteins of the hsp-70 family in nonreproductive oyster gills from controls and from oysters undergoing HS (1 h, 37°C) then returned to 12°C for the times shown. Results from three different animals are shown (A, B, C). Numbers to the right: 1 = 77 kDa; 2 = 72 kDa; 3 = 69 kDa.

proteins and induced thermotolerance, there is abundant evidence for the general importance of the hsp-70 family in the overall thermotolerance of a variety of cells and intact organisms (Morimoto et al., 1990, 1994; Parsell and Lindquist, 1993; Hofmann and Somero, 1995, 1996a, 1996b; Baler et al., 1996; Dilorio et al., 1996; Feder et al., 1996; Roberts et al., 1997). However, it has also been observed that overexpression of hsp-70 proteins can result in deleterious effects as well as protection against thermal damage (see Feder et al., 1996; Krebs and Feder, 1997).

Studies on the immunolocalization of hsp-70 proteins in sections of gills (Figure 8), using the same antibody employed for Western blotting, showed an overall increase in these proteins 2 days after HS, followed by a significant decrease 14 days after HS. That outcome was consistent with the results from gill homogenates (Figures 5–7). Although overall labeling increased following HS, no apparent change in the spatial distribution of antibody binding was detected in specific regions of the gill filaments. The tissues probed with antibody were deparaffinized 5-µm-thick sections, making it difficult to obtain a complete three-dimensional view of hsp-70 distribution. In future studies we hope to achieve resolution that is adequate to evaluate possible changes in the distribution and expression of hsp-70 proteins in specific cells after heat shock or lethal heat treatment.

Although we have not examined other tissues to the same extent as gills, our current unpublished results using mantle, heart, and adductor muscle indicate their responses to heat shock are similar to those obtained using gill. Tirard et al. (1995) showed that *C. virginica* hemocytes in vitro synthesized and accumulated two proteins of the hsp-70 family for at least 4 days after a heat shock of 1 h at 41° or 46°C (ambient temperature of 20°C). They detected only one isoform in control hemo-

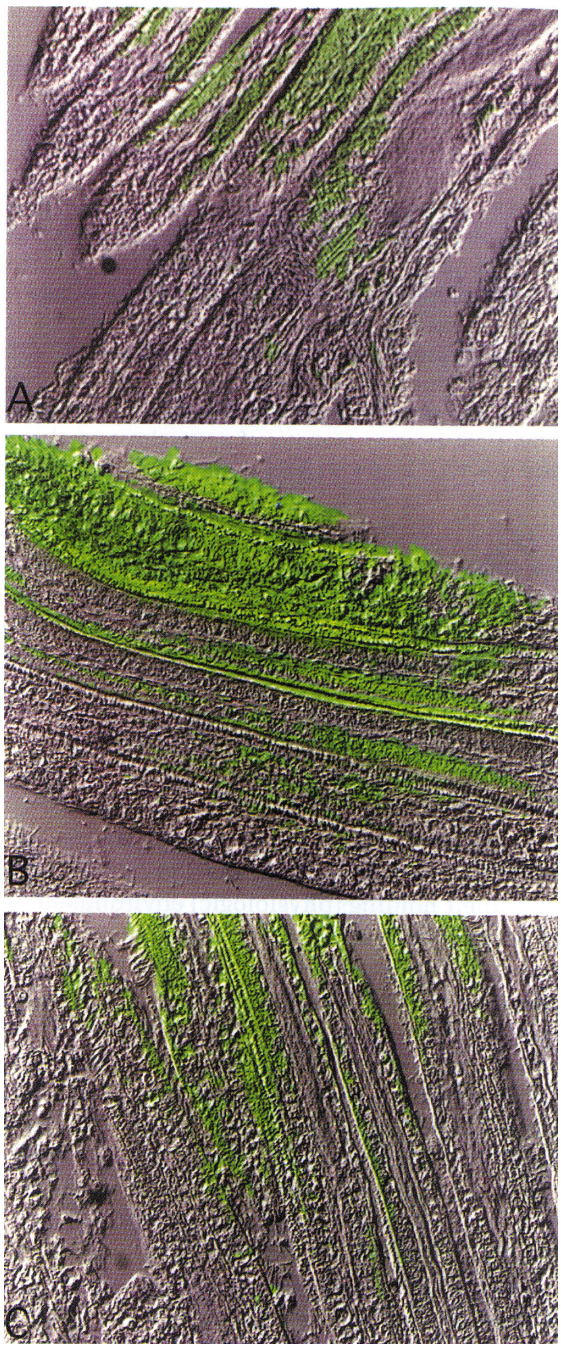

Figure 8. Localization of hsp-70 family in gill tissues by immunofluorescence: (A) control; (B) 2 days after HS; (C) 14 days after HS.

cytes (about 70 kDa) using an antibody from Affinity BioReagents (catalogue number MA3-006). That protein was up-regulated after heat shock, and a thermally induced protein of lower molecular mass (not given) began to accumulate at 2 h after heat shock. Both proteins reached maximum levels 24 h after heat shock and remained higher than control levels for the 4-day period studied. Because we know that the MA3-006 antibody recognizes only hsc-72 and hsp-69 in *C. gigas* (unpublished results), it appears that these results of Tirard et al. (1995) on hemocytes are quite similar to ours on gills, at least for the 69 and 72-kDa proteins.

A recent study by Roberts et al. (1997) on the mussel *Mytilus californianus* is also relevant. They examined the hsp-70 family in gills from mussels in the field and under laboratory conditions, demonstrating two constitutive proteins that may be similar to the 77 and 72-kDa proteins of oyster gills. Interestingly, they also observed a thermally induced protein of slightly lower molecular mass that seems comparable to the 69-kDa isoform of oyster gills. Roberts et al. (1997) also demonstrated the heat-induced synthesis of a small heat shock protein of about 30 kDa, perhaps comparable to the 34 and 36-kDa proteins induced by heat shock in oyster gills. It will be interesting to see how these proteins compare in future work on these animals. More information on the responses of mussels to thermal stress can be found in articles by Hofmann and Somero (1995, 1996a, 1996b) and Roberts et al. (1997).

Finally, we note that hsp-69 may prove to be a useful probe to evaluate whether or not oysters in the field are experiencing stressful conditions. Part of our motivation for examining the heat shock response in *C. gigas* arose from the possibility that this treatment would produce oysters better equipped to resist pathogens or other forms of stress in the field. Some support for this idea comes from Nishimura et al. (1997), who showed that hsp-70 induction protects macrophages infected with *Salmonella choleraesuis* against tumor necrosis factor-α–induced cell death.

Experimental Procedures

Animals

Pacific oysters were spawned at Whiskey Creek Oyster Co. (Tillamook, Ore.) and grown as seed to juveniles in Humboldt Bay, California (Northbay Shellfish Co. or Kuiper Mariculture, Inc.). Animals were collected from Humboldt Bay, California, and shipped overnight to the Bodega Marine Laboratory, where they were placed in 135-L aquaria that received flow-through, sand-filtered, full-strength (32–34 ppt) ambient or chilled seawater (10°–13°C). Oysters were fed daily with an algal slurry of 2×10^9 cells per oyster (Diet C-5, Coast Sea Food, Inc., Quilcene, Wash.) and were starved 24 h prior to use.

Population 1, nonreproductive juveniles, originated from Kuiper Mariculture, Inc., and were maintained for 5 months, as above, before use. Populations 2 (reproductive) and 3 (nonreproductive) were obtained from Northbay Shellfish and were held for about 2 weeks, as above, before use. The size range of animals at the time of use was 4.1–5.8 cm in the maximum dimension.

Heat shock

Oysters were transferred directly from ambient seawater (about 12°C) and heated as a single layer on a perforated disk in a 2-L beaker of preheated, continually aerated seawater in a 12 or 20-L water bath (Neslab RTE211 or Lauda RM20) set to maintain the desired seawater temperature plus or minus 0.1°C. Oysters transferred back to ambient seawater were placed in mesh bags suspended in 20-gal tubs provided with filtered, running seawater, and fed daily.

Conditions used for sublethal HS (1 h, 37°C) and LHT (determined for each batch of oysters, ranging from 43° to 44°C for 1 h) were based on previous unpublished work and confirmed for each population studied. LHT caused 100% mortality within 1 week of return to ambient conditions, while sublethal HS resulted in 100% survival 1 week after return to ambient conditions. Mortality was determined by gaping of the valves and the obvious odor of decay.

Induced thermotolerance and the hsp-70 family

Animals received sublethal HS (37°C, 1 h) and were then maintained at ambient temperature for up to 14 days. At selected times during this period, oysters were given a LHT and assessed for survival 1 week later. To select the best tissue for examination of hsp-70, this protein was initially studied (as described below) in mantle, gill, heart, and adductor muscle. Gills were chosen to illustrate the levels of hsp-70 in experimental animals because of their size, ease of manipulation, and the high resolution of gill proteins by electrophoresis and Western immunoblotting.

Gills were removed from control and heat-shocked oysters, rinsed well in sterile (0.22-μm-filtered) seawater, then briefly in distilled H_2O, blotted, and homogenized on ice at 100 mg wet tissue/ml of buffer K (150 mmol/L sorbitol, 70 mmol/L potassium gluconate, 5 mmol/L $MgCl_2$, 5 mmol/L NaH_2PO_4, 40 mmol/L Hepes, pH 7.4). Aliquots of homogenates were combined with equal volumes of 2× SDS sample buffer (Laemmli, 1970) and heated for 5 min at 100°C. SDS samples (5 μl) were loaded onto 12% polyacrylamide gels, electrophoresed, and then transferred to nitrocellulose membranes (Towbin et al., 1979). Blots were probed with Affinity BioReagents anti-hsp-70, clone 7.10 (catalogue number MA3-001, Golden, Colo.), a monoclonal antibody produced in rats against *Drosophila* hsp-70 (denatured) that is known to recognize heat shock proteins of this family in a wide variety of organisms (Kurtz et al., 1986; Watowich and Morimoto, 1988). Secondary antibody was goat antirat IgG conjugated to horseradish peroxidase (Sigma Chemical Co., St. Louis, Mo.). Labeled proteins were detected with ECL Western blotting reagents (Amersham Corp., Arlington Heights, Ill.) using Kodak BioMax MR single emulsion film (Eastman Kodak Co., Rochester, N.Y.). To quantify relative amounts and estimate molecular weights of detected proteins, films were scanned on a laser-scanning densitometer (Howtek Inc., Hudson, N.H.) and analyzed using ''Quantity One'' software (pdi inc., Huntington Station, N.Y.). Conditions for transfer and detection were optimized at every level, from volume and protein content of samples, to the exposure time of Western blots to x-ray film. Rather than adjust with an internal standard, three 10-well gels were run with two different samples (6 total) from each of the treatments to control for inherent variation between gels or blots. In addition, variation was controlled at the transfer level by sandwiching the 70-kDa slice (visualized with the aid of prestained standards) from each of the gels and transferring them to the same sheet of nitrocellulose, thereby generating only one blot to be incubated, developed, and scanned. Figures 5 and 6 were obtained by this procedure.

Incorporation of ^{14}C-labeled amino acids into gill proteins in vitro

Oysters were given a sublethal HS (1 h, 37°C) and returned to ambient conditions for 0, 2, and 14 days as described above. Gills were then excised, washed thoroughly with sterile seawater, and incubated at room temperature (about 21°C) with a ^{14}C-labeled amino acid mixture (1.85 MBq/mAtom from Amersham Corp.) at 25–30 μCi/ml in sterile seawater. Radiolabeled gills were quickly rinsed, weighed, and homogenized (100-mg wet weight/ml) in buffer K at 0°C. Homogenates were combined with SDS sample buffer, heated, and electrophoresed as described above. The gels were stained with Coomassie blue G, dried onto filter paper, and exposed to Kodak BioMax MR single emulsion film. Molecular weights of synthesized proteins were determined by overlaying the developed film on the Coomassie-stained gel and scanning as described above.

An alternative procedure involved incorporation of ^{14}C-labeled amino acids during HS. Gills were excised and placed directly into seawater plus ^{14}C-labeled amino acids (preheated to 37°C) and incubated for 1 h with swirling, then processed for SDS-PAGE and autoradiography as described above.

Immunofluorescence

A 3–5-mm cross section that included gills, digestive gland, gonad, and mantle tissues was placed in Davidson's invertebrate solution (Shaw and Battle, 1957) and processed for paraffin histology (Luna, 1968). Selected tissue sections (5 μm) were placed on positively charged slides and processed for immunofluorescence according to Dungan and Robertson (1993) with the following modifications. Primary hsp-70 antibody (Affinity BioReagents, MA3-001) was diluted 1:50, and the secondary fluorescent isothiocyanate-labeled rabbit antirat serum (Sigma) was diluted 1:250. Stained tissues were viewed with both interference contrast and epifluorescence optics using an Olympus BH-2 microscope. Images were collected by grabbing frames (Scione Corp.) from the output of an MTI CCD video camera using frame averaging (32 frames/s). Images were imported into NIH Image. Background fluorescence of control tissue was set at a minimal pixel intensity using gain and black level controls on the CCD camera, with identical settings for all samples examined. Pixel intensities were determined for regions exhibiting fluorescence using a region-of-interest (ROI) macro. Fluorescence images were overlaid onto corresponding interference contrast images using Adobe Photoshop 4.0.

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