OSMOTIC TOLERANCE AND VOLUME REGULATION IN *IN VITRO* CULTURES OF THE OYSTER PATHOGEN *PERKINSUS MARINUS*

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ABSTRACT   Growth rate, cell size, osmotic tolerance, and volume regulation were examined in cells of *Perkinsus marinus* cultured in media of osmolalities ranging from 168 to 737 mOsm (6.5–27.0 ppt). Cells cultured at the low osmolalities of 168 and 256 mOsm (6.5 and 9.7 ppt) began log phase growth 4 days postsubculture, whereas cells cultured at the higher osmolalities 341, 433, and 737 mOsm (12.7, 16.0, and 27.0 ppt) began log phase growth 2 days postsubculture. During log phase growth, cells from the higher osmolalities 341, 433, and 737 mOsm had shorter doubling times than cells from the lower osmolalities 168 and 256 mOsm. During both log and stationary phase growth, the mean cell diameter of cells cultured at 168 mOsm was significantly greater than cells cultured at 341 and 737 mOsm; the mean diameters of cells cultured at 341 and 737 mOsm did not differ significantly from each other. *P. marinus* cells cultured in various osmolalities were exposed to artificial seawater treatments of 56–672 mOsm (2.5–24.7 ppt). After the hypoosmotic treatment of 56 mOsm, cells that had been cultured in medium of low osmolality, 168 mOsm, showed only 41% mortality whereas the cells from the 341-, 433-, and 737-mOsm culture groups experienced 100% mortality. During the hyperosmotic shock, all of the groups exhibited mortalities of less than 10%. In *P. marinus* cells cultured in medium of 737 mOsm and then placed in a 50% dilution, cell diameter increased 13%, which was a volume increase of 44.5%, but cells returned to baseline size (size before osmotic shock) within 5 minutes. *P. marinus* cells cultured at low osmolalities can withstand both hypo- and hyperosmotic stress and use volume-regulatory mechanisms during hypoosmotic stress. Results suggest that transferring infected oysters to low salinity will result in strains of *P. marinus* acclimated to low salinity that will be able to withstand periodic events of extremely low salinity.

KEY WORDS: Osmotic tolerance, volume regulation, *Perkinsus marinus*

INTRODUCTION

Perkinsus marinus, a parasite of the eastern oyster, *Crassostrea virginica* (Gmelin), was first reported in the Gulf of Mexico (Mackin et al. 1950) but is now observed in *C. virginica* along the Atlantic west coast from Maine to Florida and in the Gulf of Mexico from Florida to Mexico (Andrews and Hewatt 1957, Mackin 1962, Burreson et al. 1994a). Since the 1950s and especially since 1986, *P. marinus* has been a major cause of mortality in the eastern oyster in the Chesapeake Bay (Burreson and Ragone Calvo 1996).

The eastern oyster, *C. virginica,* is an osmoconformer, but the osmotic tolerances of the parasites *Haplosporidium nelsoni* (MSX) and *P. marinus* living within the oyster are not clearly defined (Ford and Haskin 1988). Salinity is believed to be an important environmental factor that regulates the prevalence and intensity of *H. nelsoni* and *P. marinus*. These two common oyster parasites, however, appear to have differing tolerances to hypoosmotic conditions. Ford (1985) reported a reduced prevalence of *H. nelsoni* in oysters in salinities lower than 15 ppt. Ford and Haskin (1988) showed that some killing of *H. nelsoni* occurred at 15 ppt with maximum elimination at 9 ppt, suggesting that the pathogen is

physiologically unable to tolerate low salinities. *P. marinus* tolerates salinities lower than 12 ppt, but the mechanisms that allow survival in low-salinity environments have not been clearly defined (Ragone and Burreson 1993, Burreson and Ragone Calvo 1996). Studies have shown that low salinity has a retarding effect on *P. marinus* development (Ray 1954, Mackin 1962, Soniat 1985, Burreson and Ragone Calvo 1996). In addition, it has been reported that infection intensity of *P. marinus* is positively correlated with temperature and salinity (Soniat 1985, Soniat and Gauthier 1989, Crosby and Roberts 1990, Burreson and Ragone Calvo 1996). An *in vivo* study of oysters infected with *P. marinus* determined the critical salinity range for pathogenicity to be between 9 and 12 ppt, and that *P. marinus* was less virulent below 9 ppt (Ragone and Burreson 1993). Also, the study reported that lower salinities (6 and 9 ppt) delayed disease development, whereas infections at higher salinities (12 and 20 ppt) increased in intensity and resulted in higher levels of oyster mortality.

Despite these findings, little is known about the osmotic tolerance of *P. marinus* when faced with hypo- and hyperosmotic stress. Studies with both free-living and parasitic protozoa have shown that many protozoa have the ability to adjust their cell volumes when faced with external osmotic changes (Kaneshiro et al. 1969, Da Silva and Roitman 1982, Geoffrion and Larochelle 1984, Ahmad and Hellebust 1986, André et al. 1988, Cronkite and Pierce 1989, Hellebust et al. 1989, Darling and Blum 1990, Darling et al. 1990). Similarly, *P. marinus* may also utilize physiological mechanisms to adjust to its changing osmotic environment. Only one previous study has been conducted on the osmotic tolerance of *P. marinus* in the absence of host influences (Burreson

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et al. 1994b). This study reported that cells cultured at 22 ppt and placed in extreme low-salinity treatments of 0 and 3 ppt had higher than 90% mortality. As a continuation of this work, we investigated the osmotic tolerances and volume-regulatory abilities of *P. marinus* cells, which have been cultured in a range of osmotic conditions (168–737 mOsm or 6.5–27.0 ppt) and exposed to various osmotic treatments (56–672 mOsm or 2.5–24.7 ppt).

MATERIALS AND METHODS

In Vitro Cultures of P. marinus

Cultures of *P. marinus* were maintained in Jeronie La Peyre-Oyster Disease Research Program-1 (JL-ODRD-1) medium (La Peyre et al. 1993) (approximately 737 mOsm or 27.0 ppt) without bovine serum albumin (BSA) in a humid atmosphere at 28 °C in a 5.0% CO_2 incubator. Cells from the BSA-free acclimated cultures were transferred from 737-mOsm culture medium into 168, 256, 341, and 433 mOsm (approximately 6.5, 9.7, 12.7, and 16.0 ppt) media in a gradual procedure in which cells from 737 mOsm were placed into 433, 433 into 341 mOsm, etc., with the stepwise transfer occurring every 3 days. For culture maintenance, subculturing occurred every 2–4 wk. Cultures were seeded at a density of 5×10^6 cells per 5 mL of medium for all experiments, and during these experiments, subculturing occurred every 2 wk. Growth curves for the groups cultured at 168, 256, 341, 433, and 737 mOsm were determined by obtaining cell counts with a hemacytometer (Fisher Scientific) every day for 12 days starting the day after subculture to determine the time period of log phase growth. The growth rate study used cells approximately 20 generations (about 1 y) descending from the original cultures that were first acclimated to the different osmolalities. A generation is defined as one subculture. Cell size experiments used cells that were approximately 25–30 generations descended from the acclimated cultures. The osmotic tolerance experiments used cultures that were 7–10 generations descended from the original groups acclimated to the different osmolalities.

Culture Media

The cell culture medium used for the *P. marinus* cultures was the JL-ODRP-1 (La Peyre et al. 1993) without BSA. Media (100 mL) equivalent to 168, 256, 341, 433, and 737 mOsm were prepared before each subculture for the different culture groups following methods described by La Peyre et al. (1993). In addition to the reported constituents, the culture medium, depending on the desired osmolality (168, 256, 341, 433, and 737 mOsm), also included basal synthetic sea salts (0.3, 0.6, 0.9, 1.2, or 2.2 g), 0.2 g $NaHCO_3$, and KCl (0.0061, 0.0079, 0.0097, 0.0115, or 0.0177 g) dissolved in 91.5 mL tissue culture-grade water.

Cell Sizes of Cultured Cells

Cell diameters of the various *P. marinus* groups in both log and stationary growth phase were measured by using the NIH Image Analysis (Version 1.56) Macintosh computer program for particle size analysis and the MediaGrabber Macintosh program with RasterOps video digitizer board to capture live microscopic images from an inverted Zeiss light microscope (40× objective used in all of the cell size experiments). Cell measurement techniques with image analysis were based on methods described by Weeks and Richards (1993).

Baseline measurements were initially conducted to determine whether the groups cultured in media with osmolalities of 168,

341, and 737 mOsm varied in cell size. For log phase growth size distributions, cells cultured in 168-, 341-, and 737-mOsm media were harvested 6 days after subcultured and transferred to 15-mL microcentrifuge tubes. Each group of cells was declumped by repeatedly withdrawing the cells and passing them through a 3-mL syringe (25G 7/8-inch hypodermic needle). Cells were centrifuged at 800 *g* for 15 minutes, the medium decanted, and the cells resuspended in 10 mL of isotonic seawater. Seawater solutions (173, 365, and 740 mOsm or 6.7, 13.6, and 27.1 ppt) that were isotonic to the culture medium of each group consisted of 97.5 mL tissue culture-grade water, basal synthetic sea salts (0.45, 1.05, or 2.35 g), 0.2 g $NaHCO_3$, KCl (0.0061, 0.0097, or 0.0777 g), and 2.5 mL HEPES buffer (original concentration = 239.02 mg/mL). After resuspending the cell pellets in the isotonic artificial seawater solutions, cell solutions were stirred with a vortex mixer (Fisher Scientific), and a 10-μL sample was withdrawn from each group for cell counts using a hemacycometer. Volumes containing 1×10^5 cells from each group (168, 341, and 737 mOsm) were calculated, and these cell solutions were added to three different cell wells (three wells per group) in a cell well plate. From each well of each of the three groups, three to four images were captured. The number of cells per image ranged from approximately 40 to 70 cells. Clumped cells that could not be easily distinguished were excluded. This cell sizing protocol was also followed to measure cells cultured at 168, 341, and 737 mOsm in stationary phase growth (2 wk after subculture). Mean cell diameters were calculated for the culture groups from both log and stationary growth phase, and the relationship between culture medium osmolality and cell diameter was examined by a one-way analysis of variance. Significant differences between the groups cultured at the three different osmolalities were determined by using the Scheffé *post hoc* multiple comparison test.

Osmotic Tolerance

Buffered artificial seawater (ASW) treatment solutions of 56, 135, 222, 305, 386, and 672 mOsm (approximately 2.5, 5.3, 8.5, 11.4, 14.4, and 24.7 ppt, respectively) were prepared by dissolving synthetic basal salts (Sigma Chemical Co.) (0.0, 0.3, 0.6, 0.9, 1.2, or 2.2 g), 0.1176 g $NaHCO_3$, KCl (0.0014, 0.0044, 0.0061, 0.0078, 0.0097, or 0.0156 g), and 2.5 mL HEPES buffer (original concentration = 239.02 mg/mL) in 97.5 mL of tissue culture-grade water. After adding these constituents, the mixtures were adjusted to a pH of 7.5 and then filter sterilized. All of the treatment solutions, the BSA-free media for the culture groups, and the isotonic seawater solutions (used for cell size experiments) were analyzed on a vapor pressure osmometer (Wescor) to determine osmolalities. Cell density by hemacytometer and cell viability of the *P. marinus* cultures were assessed in each culture group (168, 256, 341, 433, and 737 mOsm). To determine cell viability, a 100-μL subsample was placed in a microcentrifuge tube and 10 μl of 0.05% neutral red stain added. After 10 min, two 10-μl aliquots were placed on the hemacytometer. Both live (stained) and dead (unstained) cells were counted for at least 200 cells. From each group, 2.0×10^6 cells were added to sterile 15-mL centrifuge tubes and the volumes raised to 7 mL with the treatment ASW at the osmolality equivalent to the medium osmolality. Then, 1 mL of each of these cell suspensions was centrifuged at 470 *g* for 5 min. The supernatant was decanted and the pellet resuspended in 1 mL of each of the treatment osmolalities (ASW) in a 24-well tissue culture plate. Thus, *P. marinus* cells cultured in media of 168, 256, 341, 433, and

737 mOsm were placed in ASW treatment osmolalities of 56, 135, 222, 305, 386, and 672 mOsm for 24 hours in 24-well microtiter plates at 28 °C in an incubator without CO_2. After the 24-hour incubation, 100 μL of neutral red was added and gently mixed with a pipette tip. Mortality was assessed by counting live and dead cells in two to three random grid fields with an inverted light microscope (Zeiss) and a 10 × 10-mm ocular micrometer grid. The experiment was repeated three times. Logistic regression analyses with SAS procedure Catmod were utilized to examine the response of the population (culture group) to the treatment osmolality and to calculate predicted mortalities (which describe the response of each population) with 95% confidence intervals for each of the culture groups at each treatment osmolality. A logistic regression model was chosen to represent the binary response of mortality (live *versus* dead). In addition, the actual live and dead cell counts were used for calculating percent mortalities and for an analysis that compares proportions from independent samples (Fleiss 1981).

Cell Size after Hypoosmotic Shock

Cell diameter changes following a hypoosmotic shock were measured with the MediaGrabber and NIH Image Analysis systems. Cells cultured in medium of 737 mOsm were harvested 2–3 wk after subculture, declumped with a 3-mL syringe (25G 7/8-inch hypodermic needle), and centrifuged at 800 g for 15 min. The medium was decanted, and isotonic seawater was added to obtain a volume of 10 mL. Cell density was determined with a hemacy-tometer, and a volume containing 1×10^5 cells was added to a cell well. A volume of 173 mOsm ASW was added to the well to result in a 50% dilution of the original seawater solution. Before adding this calculated volume of the hypoosmotic shock solution, an image was captured to represent time 0. Ten to twenty seconds after the 50% dilution, a second image was captured as time 1. Images were then captured at 1, 3, 5, 7, 10, 12, 15, 20, 30, 45, and 60 min after dilution. The same cells from the same plane were captured as images, and thus, the same population experiencing the shock was represented. These images were analyzed with the NIH Image Analysis system to determine cell diameters at each time interval. The experiment was repeated five times. The first experiment used cells 19 days postsubculture. The second experiment used cells from a different culture 18 days postsubculture and included time points of 0 and 10–20 sec, and 1, 3, 5, 10, 15, 20, and 30 min. The third, fourth, and fifth experiments used cells 20 days postsubculture and were performed consecutively on the same day with cells from the same flask. Experiments 3, 4, and 5 included images captured at 10–20 sec and 1, 3, 5, 10, 15, 20, and 30 min. Cell viability was assessed with the vital stain neutral red before the shock and 30 min after adding the shock solution. The control experiment used cells 21 days postsubculture and followed the protocol described above without adding the shock solution; images were obtained at 0, 1, 3, 5, 10, 15, 20, and 30 min. Cell sizes after hypoosmotic shock were analyzed with the nonparametric Kruskall-Wallis test to first examine the effect of each experiment. To separate out the significant effect of each experiment but still look at the results of all trials together to examine the overall effect of the treatment osmolality on cell size, a mean center standard-ization was used by subtracting the mean cell diameter (total mean diameter for all time points within each experiment) from each data point. A second Kruskall-Wallis test was run on the standard-ized data to examine whether each experiment continued to have a

significant effect on cell diameter. The effect of the experiment was no longer significant, and the experiments were pooled. A third Kruskall-Wallis test was used to determine whether time had a significant effect on cell diameter. Lastly, the Tukey-Kramer multiple comparison *post hoc* analysis was implemented to deter-mine at which time points the mean cell diameters were signifi-cantly different from each other. An unpaired t-test was used to determine whether there was a significant difference between the standardized control diameters and the standardized replicate di-ameters (experiments pooled) both before the shock and 1 min after the shock.

RESULTS

Growth Rate

The results of the growth rate study indicated that log phase growth began approximately 2 days postsubculture for *P. marinus* cells cultured in 341, 433, and 737 mOsm (12.7, 16.0, and 27.0 ppt) media. Cultures from the 168 and 256 mOsm (6.5 and 9.7 ppt) media began log phase growth approximately 4 days postsubcul-ture (Fig. 1). The groups cultured at the higher osmolalities of 341, 433, and 737 mOsm had shorter doubling times compared with the groups cultured at the low osmolalities of 168 and 256 mOsm. For the 168-mOsm cells, 35.2 h were required for one doubling and 35.7 h for the 256-mOsm cells. For the higher osmolality cells from 341, 433, and 737 mOsm, one doubling required 22.8, 25.9, and 24.4 h, respectively.

Cell Sizes of Cultured Cells

During log phase growth, the mean diameters (± standard error) of *P. marinus* cells cultured in media of 168, 341, and 737 mOsm were 11.8 (±0.191), 9.6 (±0.108), and 9.2 (±0.106) μm, respec-tively. The effect of culture medium osmolality on cell diameter
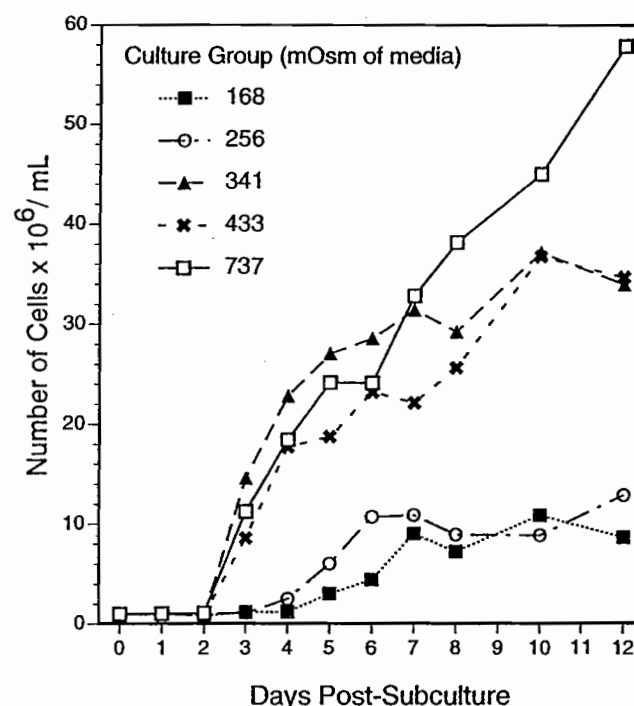

Figure 1. Growth curve of *P. marinus* cells cultured in media of 168, 256, 341, 433, and 737 mOsm (6.5, 9.7, 12.7, 16.0, and 27.0 ppt).

was statistically significant ($P = 0.0001$). Cells cultured at 168 mOsm were significantly larger than cells at either 341 ($P < 0.0001$) or 737 mOsm ($P < 0.0001$). The differences in diameter between cells at 341 and 737 mOsm were not significant ($P = 0.1565$). The mean diameters of stationary-phase *P. marinus* cells cultured at 168, 341, and 737 mOsm were 8.4 (±0.165), 4.7 (±0.070), and 5.1 (±0.093) μm, respectively. As observed with cells from log phase growth, the effect of culture medium osmolality on cell size was statistically significant ($P < 0.0001$). Cells cultured at 168 mOsm were significantly larger than cells at either 341 ($P < 0.0001$) or 737 mOsm ($P < 0.0001$), whereas the difference in cell diameter between the 341- and 737-mOsm groups was only significant at the 5% level of significance ($P = 0.021$).

Osmotic Tolerance

Before osmotic shock, the mean viabilities of the *P. marinus* cells cultured at 168, 256, 341, 433, and 737 mOsm were 88.2%, 96.2%, 99.1%, 99.3%, and 98.8%, respectively. After hypoosmotic treatments, the percent mortality was lower in groups that were cultured in low-osmolality media than in groups from higher osmolalities (Fig. 2). For example, in the extreme hypoosmotic shock of 56 mOsm (2.5 ppt), mortality was 41% in cells cultured at an osmolality of 168 mOsm but was 100% in cells that were cultured at 737 mOsm. Conversely, in the hyperosmotic shock of 672 mOsm (24.7 ppt), groups that had been cultured at low osmolalities as well as high osmolalities all experienced mortalities of less than 10% (Fig. 2). A logistic regression analysis showed that a significant relationship existed between treatment osmolality as a function of mortality ($P < 0.001$). A comparison of proportions from independent samples test showed that the mortality response of the 168-mOsm group was significantly different ($P < 0.001$) from the mortality observed for the 737-mOsm culture group at the 56-mOsm treatment. Predicted mortalities determined from a logistic regression analysis indicated that in low-osmolality treat-

ments, groups cultured at 168 and 256 mOsm have lower mortalities than the groups that had been cultured at 341, 433, and 737 mOsm.

Cell Size after Hypoosmotic Shock

Cell viability was not affected by the 50% dilution; the results of a viability test indicated a 99% viability before the dilution (time 0) and 97% viability 30 min after the dilution. From the first nonparametric analysis, it was difficult to examine the effect of osmolality on cell size because of variability between experiments and variability between experimental conditions. After implementing a mean center standardization, however, nonparametric analysis indicated that each experiment did not have a significant effect on cell diameter ($P = 0.8976$). Therefore, the results from each experiment could then be pooled. The overall response to the 50% hypoosmotic shock was an initial swelling followed by a return to baseline size (Fig. 3). When placed in the 50% dilution treatment, *P. marinus* cells that were cultured at 737 mOsm experienced an initial swelling between 0 and 30 sec after hypoosmotic shock. Cells swelled and returned to baseline size within about 5 min. The mean diameter change during swelling was 0.7 μm. The initial mean cell diameter was 5.7 μm, and thus, the percent diameter increase during initial swelling was approximately 13%, which was a 44.5% change in cell volume. The nonparametric analysis on the pooled, standardized data showed that time had a significant effect on cell diameter, with a tied P-value of <0.0001. The *post hoc* multiple comparison analysis with a $P < 0.05$ level of significance indicated that significant differences existed between the following time points: 0 and <30 sec, <30 sec and 5 min, <30 sec and 15 min, <30 sec and 20 min, and <30 sec and 30 min. The unpaired *t*-test showed no significant difference between the mean diameters of the control group and the experimental groups (all experiments pooled) at time 0 ($P = 0.2931$), but there was a significant difference at a significance level of $P < 0.05$ between the control group and the experimental groups 1 min after the shock ($P = 0.0022$).

DISCUSSION

Continuous cultures of *P. marinus* can be maintained in low-osmolality environments (as low as 168 mOsm or 6.5 ppt). Fur-
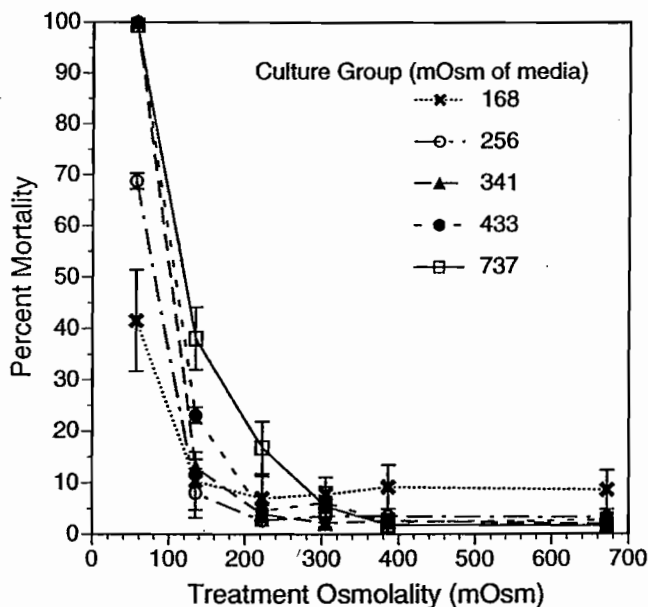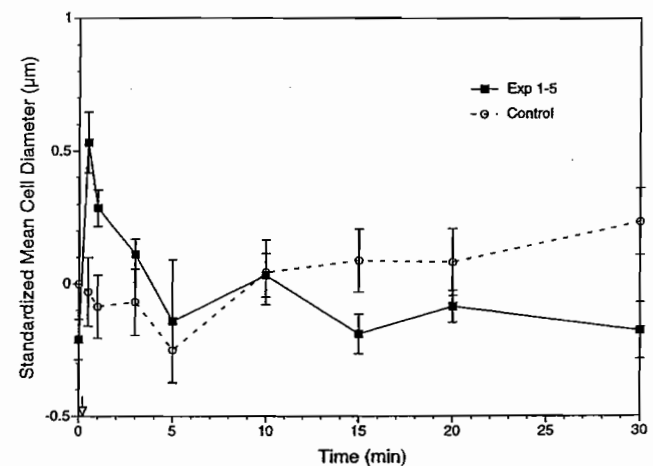

Figure 2. Percent mortality of *P. marinus* cells cultured in media of 168, 256, 341, 433, and 737 mOsm (6.5, 9.7, 12.7, 16.0, and 27.0 ppt) and placed in treatment osmolalities of 56, 135, 222, 305, 386, and 672 mOsm (2.5, 5.3, 8.5, 11.4, 14.4, and 24.7 ppt) for 24 h. Error bars = standard error.

Figure 3. Standardized mean cell diameter (μm) of *P. marinus* cells cultured at 737 mOsm (27.0 ppt) and placed in 50% hypoosmotic shock (arrow) with experiments 1–5 pooled and the control experiment. Arrow indicates actual time of shock; time represents time after shock. Error bars = standard error.

thermore, cells maintained in osmolalities ranging from 168 to 737 mOsm (6.5–27.0 ppt) are tolerant of hypo- and hyperosmotic conditions in the treatment range of 222–672 mOsm (8.5–24.7 ppt). Cells cultured at low osmolalities can also withstand extreme low osmolalities such as 56 mOsm (2.5 ppt) for at least 24 hours. Thus, these experiments have shown that cultured cells of *P. marinus* can survive both hypo- and hyperosmotic stress. During hypoosmotic stress, cells increased in diameter, followed quickly by a return to baseline size (size before osmotic shock), which indicates a volume-regulatory response. This response helps explain why *P. marinus* continues to persist in the Chesapeake Bay despite periods of low salinity that occur during times of high rainfall and runoff into the tributaries.

The growth rate study showed that *P. marinus* cells that were cultured at osmolalities of 341, 433, and 737 mOsm (12.7, 16.0, and 27.0 ppt) reached log phase growth before cells cultured at lower osmolalities of 168 and 256 mOsm (6.5 and 9.7 ppt). In addition, cells from higher osmolalities had greater rates of multiplication (shorter doubling time) during log phase than cells cultured in low osmolalities. These results correspond to a study with trypanosome cultures that showed that media of high osmolality supported greater multiplication rates than low-osmolality media (Da Silva and Roitman 1982).

The osmotic tolerance study indicated that *P. marinus* cells cultured at low osmolalities experienced reduced mortality when placed in extreme hypoosmotic conditions when compared with the groups cultured at higher osmolalities. Because the cells were already acclimated to the stress of a low-osmolality environment, they were able to withstand an extreme low osmolality of 56 mOsm better than cells cultured at much higher osmolalities. Approximately 60% of the 168-mOsm cultured cells survived the extreme low osmolality of 56 mOsm for at least 24 hours. In this study, all of the culture groups had low mortalities (<10%) after hyperosmotic stress. Consequently, *P. marinus* was more tolerant of hyper- than hypoosmotic shock.

This research showed that the stressor did not seem to be the magnitude of the shock, but instead the type of shock (hypo- or hyperosmotic) and the actual osmolality of the challenge treatment. For example, the 737-mOsm cells placed into 222-mOsm treatment (a difference of 515 mOsm) had much higher mortality than cells from 168-mOsm placed into 672-mOsm treatment (a difference of 504 mOsm). Although the magnitude of the shock was about the same, the hypoosmotic rather than the hyperosmotic environment was more stressful, as indicated by higher mortality levels. In addition to the type of stress, the actual osmolality of the stress affected the level of mortality. For instance, cells acclimated to 737 mOsm and placed into 386-mOsm treatment (a difference of 351 mOsm) had much lower mortality (<10%) than cells from 433 mOsm placed into 56-mOsm treatment (a difference of 377 mOsm), which resulted in 100% mortality. Although the magnitude of both of the hypoosmotic shocks was similar, mortality was higher in the treatment with the lowest absolute osmolality, indicating the cells may have a threshold osmolality level needed for survival.

The results from the osmotic tolerance experiment differ from the study by Burreson et al. (1994b), which reported much higher mortality levels in cells acclimated to 737 mOsm and placed in the same hypoosmotic treatments. The study by Burreson et al. (1994b) showed greater than 60% mortality for cells acclimated to 737 mOsm and placed in treatments of 136 mOsm and 213 mOsm, whereas this study reports 15–40% mortality in the same low-

osmotic treatments. One difference is that Burreson et al. (1994b) used *P. marinus* cells cultured in medium with BSA (known as JL-ODRP-1 media), whereas this study used cells cultured in BSA-free medium. However, comparative experiments with cells acclimated to either medium with BSA or BSA-free medium showed no significant difference between the effects of the two types of media on osmotic tolerance. Other factors that may have contributed to the differences in mortality between this experiment and the previous one include reported differences in experimental design such as the age of the cells (numbers of subcultures since isolation and initiation), growth phase of the cells, and type of incubator used (CO_2 or without CO_2). For example, the cells in the previous study were transferred to an incubator without CO_2 for 1 week before use, whereas cells in our experiment were in an environment without CO_2 for only 1 day. The prolonged exposure to an environment without CO_2 may have stressed the cells in the previous study, making them more susceptible to mortality after osmotic shock. Growth rates are reduced in cultures that have been transferred to an incubator without CO_2 when compared with cultures maintained in a 5.0% CO_2 incubator (La Peyre, personal observation).

Cells cultured at the low osmolality of 168 mOsm were significantly larger than cells cultured at the high osmolalities of 341 and 737 mOsm during both log and stationary growth phases. The cells cultured at the high osmolalities of 341 and 737 mOsm, however, were not significantly different from each other in size. The difference in cell size may be due to increased water content required to match the low osmolality of the dilute external medium. A study with red coelomocytes of the euryhaline polychaete *Glycera dibranchiata* showed cells acclimated to a lower osmolality had a higher "body-wall-tissue water content" and greater cellular volume than cells acclimated to a higher osmolality (Costa et al. 1980). An experiment with the amoeba *Acanthamoeba castellanii* indicated that the amount of intracellular water increased when cells were placed in a severe hypoosmotic shock (Geoffrion and Larochelle 1984). Similarly, the gradual acclimation of the *P. marinus* cells from high- to low-osmolality media when developing low-osmolality cultures may have caused an increase in size as water initially diffused into the cells, and the cells cultured in the low osmolality may not have been able to completely return to baseline size during volume regulation because of the stress of the prolonged hypoosmotic environment. Cells must maintain certain levels of metabolites to survive the stress of a low-osmolality environment. These levels of solutes attract water molecules because of simple diffusion, and therefore, an increased intracellular water content results. Studies on the erythrocytes of the bivalve *Noetia ponderosa* (Amende and Pierce 1980, Smith and Pierce 1987) and a report on the euryhaline ciliate *Paramecium calkinsi* (Cronkite and Pierce 1989) indicated that cells may not always completely return to baseline (size before osmotic shock) after volume regulation.

Alternatively, the difference in cell size of the groups cultured at low *versus* high osmolalities may be due to a difference in life stages of the groups that were measured. Cells of *P. marinus* divide by schizogony with a cell increasing in size, acquiring a vacuole, and then releasing several daughter cells (La Peyre and Faisal 1997). This process could have been occurring with some of the cells from the low-osmolality cultures during the cell-size experiment, as both small cells and large cells with smaller cells inside them were observed, whereas the groups measured at higher osmolalities mainly consisted of small cells. Thus, because the

low- and high-osmolality groups had different growth rates, they may not have been at the same growth stage when their cell diameters were measured, which could account for the differences in size between the groups. The cells from higher osmolalities were not observed as a large parent cell dividing into several smaller cells, but instead, one cell often appeared to divide into two (data not shown). *P. marinus* cells with high growth rates appear to divide as one small cell dividing into two cells (La Peyre 1996). The cells at the low osmolality, however, may be larger in size even before schizogony because of an increased internal water content. Further studies examining the relationship between medium osmolality, *P. marinus* growth stage, and cell size would be useful in understanding the role of osmolality in *P. marinus* growth and survival.

During the short-term hypoosmotic stress experiment in this study, *P. marinus* cells followed a typical cell volume response that is observed in other organisms by experiencing an initial swelling and then shrinkage back toward baseline (Costa et al. 1980, Smith and Pierce 1987, Cronkite and Pierce 1989, Darling et al. 1990). The results indicate that *P. marinus* cells do not resist swelling during sudden or extreme external osmolality changes. The size at the maximum swell was significantly different from the initial baseline and the acclimated sizes. The erythrocytes of the clam *N. ponderosa* exhibited a similar pattern when cells acclimated to 935 mOsm were placed in a hypoosmotic shock of 560 mOsm; the cells swelled, thereby increasing their volume by 50% within 5 min followed by a gradual return toward baseline (Smith and Pierce 1987). Because the cells in this study did swell and return to baseline size, the results suggest that *P. marinus* regulates the intracellular osmotic concentration to regulate cell volume during changing external osmolalities. The results reported here along with other studies by our laboratory (data not shown) and by

Paynter et al. (1997) on intracellular osmolytes (i.e., free amino acids) used by *P. marinus* indicate that *P. marinus* cells utilize volume-regulatory mechanisms to compensate for osmotic changes in the external medium. These mechanisms enabled cells in this study to survive a 50% dilution of the external medium. But to better describe the specific volume-regulatory mechanisms used by *P. marinus*, current studies are focusing on measuring the levels of intracellular inorganic ions and organic molecules before, during, and after osmotic shock to determine their role in volume regulation.

The results of these experiments help explain why *P. marinus* continues to persist in the upper portions of the Chesapeake Bay tributaries despite periods of low salinities. Periodic increases in stream flow causing lowered salinities have not greatly affected the abundance of *P. marinus* in Chesapeake Bay tributaries (Burreson and Ragone Calvo 1994, Ragone Calvo and Burreson 1995). The fact that low salinities have not eradicated the pathogen from these areas may be explained by the results in this osmotic tolerance study that indicate *P. marinus* can use volume-regulatory mechanisms to adapt to changing external osmolality and become acclimated to extreme low osmotic conditions. Transferring infected oysters to low salinities may exacerbate the P. marinus problem by allowing acclimation of the parasites to lower salinities, thereby making them more tolerant of extremely low salinities. As a result, strains of *P. marinus* that are tolerant of a wide range of fluctuating salinities, including extremely low-salinity environments, may develop.

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