Effect of Temperature and Prey Availability on Growth of *Paramoeba invadens* in Monoxenic Culture

JOANNE F. JELLETT* AND ROBERT E. SCHEIBLING

Biology Department, Dalhousie University, Halifax, Nova Scotia B3M 4J1, Canada

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*Paramoeba invadens* Jones 1985 is a pathogenic marine amoeba responsible for mass mortalities of sea urchins (*Strongylocentrotus droebachiensis*) off Nova Scotia between 1980 and 1983. A direct relationship between temperature and sea urchin paramoebiasis has been shown in previous laboratory and field studies. This study examined the effect of prey availability and temperature on the growth of *P. invadens* in monoxenic culture (with the marine bacterium *Pseudomonas nautica*). At 15°C, the specific growth rate of *P. invadens* increased with bacterial prey concentration and was highest at 10^6 bacterial cells ml^-1. Growth rate of *P. invadens* was maximal at 15 to 20°C (which corresponds to annual sea temperature maxima in the natural environment) and the minimum generation time was 19.41 h at 20°C. At 10 and 12°C, generation times were 91.18 and 73.39 h, respectively; at 2 and 5°C, there was no growth. *P. invadens* did not survive in monoxenic culture at 27°C. Growth rates of *P. invadens* in vitro were positively correlated with time to morbidity of infected *S. droebachiensis*

Between 1980 and 1983, sea urchin (*Strongylocentrotus droebachiensis*) populations along the entire Atlantic coast of Nova Scotia were decimated by disease (20, 25–27), resulting in an estimated loss of biomass of >260,000 tons (wt weight) (26). *Paramoeba invadens* Jones, a pathogenic marine amoeba, was identified as the causative agent of this mass mortality (16). These epizootics occurred between August and November, around or shortly after the annual peak of the seawater temperature cycle (25, 27). Outbreaks of disease coincided with unusually high seawater temperatures in these years (25–27), and laboratory studies indicated a direct relationship between temperature and the transmission and progression of sea urchin paramoebiasis, with the lower threshold at ca. 10°C (25, 27).

Although *P. invadens* has been found only in the tissues of diseased *Strongylocentrotus droebachiensis*, it can be cultured polyxenically on mixed marine microorganisms (15–17) and monoxenically on a single bacterial strain (J. F. Jellett and R. E. Scheibling, J. Protozool., in press). Therefore, it is thought to be generally free-living like most other *Paramoeba* spp. (22). This study examined the effect of prey availability and temperature on the growth rate of *P. invadens* in monoxenic culture to gain insight into the ecology of free-living *P. invadens* and the relationship between temperature and paramoebiasis in sea urchins.

**MATERIALS AND METHODS**

**Bacteria.** *Pseudomonas nautica* from a frozen stock (trypsin soy broth with 15% glycerol at −80°C) was cultured at room temperature for 48 h on LIB-X agar (5) made with artificial seawater (ASW; 3.6% [wt/vol] Rila salts mix [Rila Products Ltd., Teaneck, N.J.]). ASW and LIB-X broth initially were filtered (0.45 μm pore size; Millipore) to prevent bacterial flocculation. An isolated colony from the agar plate was used to inoculate 10 ml of LIB-X broth, which was then incubated for 48 h at room temperature. This turbid starter culture was added to 500 ml of filter-sterilized LIB-X broth, incubated at 15°C, and then standardized spectrophotometrically in a Bausch and Lomb Spectronic 20. Cultures were harvested at the end of the log phase (ca. 60 h) by centrifugation at 12,000 × g for 15 min. Cells were then washed three times in ASW and resuspended in 1/100 the original volume.

**Amoebae.** *P. invadens* was isolated from sea urchins (*S. droebachiensis*) infected by exposure to diseased conspecifics by the method of Scheibling and Stephenson (27). Amoebae were isolated from the radial nerve by culture on semisolid, nonnutrient ASW agar (0.6%) along with the associated mixed bacterial population. Squares of the agar containing amoebae and bacteria were removed, and the bacteria were suppressed by using antibiotics. The agar containing the amoebae was then subcultured to solid ASW agar (1.2%) inoculated with a single bacterial strain (*Pseudomonas nautica*) and overlaid with liquid ASW, forming a monoxenic culture. A more detailed description of this method is presented elsewhere (Jellett and Scheibling, in press). Monoxenic cultures of *P. invadens* used in experiments were maintained in stocks by weekly subculturing. Subcultures of the stocks made after 3 weeks were grown for 7 days prior to use in this study.

**Experimental tubes.** All experimental cultures (amoebae and bacteria) and control suspensions (bacteria only) were inoculated into Pyrex glass tubes (16 by 150 mm) containing 1.0 ml of 1.2% ASW agar overlaid with 10.0 ml of filtered ASW and maintained in complete darkness.

**Effect of temperature on *Pseudomonas nautica* in nonnutritive ASW.** In an initial experiment to determine temperature effects on *Pseudomonas nautica*, five groups of tubes were inoculated at 15°C with equal portions of the bacterial suspension (see above) to obtain a final concentration of 2 × 10^8 CFU ml^-1. All tubes were adjusted at a rate of 5°C per day to experimental temperatures of 5, 10, 15, 20, or 27°C and incubated for 17 days.

**Effect of bacterial concentration and temperature on growth of *P. invadens*.** Portions of the bacterial suspension (see above) were transferred aseptically to tubes to obtain various final concentrations. Controls consisted of tubes containing bacteria only. Amoebae from six monoxenic cultures were pooled by gently resuspending and then decanting the overlay liquid from each culture into a sterile flask. Portions

* Corresponding author.
of this suspension were inoculated into the tubes containing bacteria to give approximate final concentrations of either 10 or 100 amoebae ml⁻¹.

To examine the effect of bacterial concentration on the growth rate of *P. invadens*, each of five sets of experimental (amoebae and bacteria) and control (bacteria only) tubes were inoculated with bacterial concentrations of 2.43 × 10³, 5.32 × 10⁵, 2.22 × 10⁶, 1.48 × 10⁷, or 8.17 × 10⁷ CFU ml⁻¹. About 100 amoebae ml⁻¹ were added to the experimental tubes. All tubes were then incubated for 14 days at 15°C.

The effect of temperature on the growth of *P. invadens* was examined in two experiments. In both experiments, experimental and control tubes were inoculated at 15°C with bacteria at 8 × 10⁷ to 4 × 10⁶ CFU ml⁻¹ and adjusted (after the addition of amoebae to the experimental tubes) to different experimental temperatures at a rate of 5°C day⁻¹. In the first experiment, about 10 amoebae ml⁻¹ were used, and each of five sets of experimental and control tubes were incubated at 5, 10, 15, 20, or 27°C for 22 days. In the second experiment, designed to explore the lower temperature limit of *P. invadens*, about 100 amoebae ml⁻¹ were used, and each of two sets of experimental and control tubes were incubated at 2 or 12°C for 29 days.

**Enumeration of amoebae and bacteria.** Immediately following inoculation, and then at 1- to 3-day intervals, three replicate tubes from each experimental and control treatment were randomly selected for enumeration of amoebae or bacteria.

For enumeration of amoebae, 8.0 ml of the resuspended culture fluid was filtered through a type HA filter (0.45 μm; Millipore) at 16.95-kPa reduced pressure. Filters were rinsed with an additional 5.0 ml of ASW, fixed for at least 30 min in 95% ethanol, stained with a modified Papanicolaou technique (M. Feldman, N. N. Ahmed, and K. G. Marshall, MacDonald-Stewart Division of Cytology, Pathology Institute, McGill University; all stains from BD Chemicals), and mounted with Permount containing 1% butylated hydroxytoluene to prevent cellular fading. By this staining method, the nucleus and the parasome, the diagnostic feature of *Paramoeba* spp., were visible. After hardening for at least 2 days at 40°C, filters were counted with a Leitz Laborlux D microscope. A total magnification of 400× proved to be the most effective for counting *P. invadens*. Two hundred fields were counted per filter from center to edge following a schedule of precision at the 90% confidence level (7) and assuming a concentration of at least 1 amoeba per 2 fields. Average counts per field were converted to number of amoebae ml⁻¹.

For enumeration of bacteria, 1 ml of resuspended culture was removed, serial dilutions (10⁻³ to 10⁻¹⁰) were made in 2% NaCl, and duplicate spread plates were made on LBB-X agar. Plates were incubated at room temperature for 48 h. Duplicate counts from plates containing 20 to 200 colonies were averaged for each replicate. A standard plate count method was used because *P. invadens* will not ingest dead bacterial cells (J. F. Jellett, Ph.D. thesis, Dalhousie University, Halifax, Nova Scotia, Canada, 1988), and therefore data on living cells only (as opposed to total cells) were desirable.

At each sampling interval, the mean number of amoebae or bacteria per ml was calculated from three replicates for the experimental and control tubes (six replicates for the initial inoculum). The standard error of the mean number of bacteria or amoebae per ml did not exceed 10% in any case.

Regression analysis was performed on log-transformed amoeba abundance versus time from 2 days to peak concentra-

![FIG. 1. Effect of temperature on the growth of *Pseudomonas nautica* in nonnutrient artificial seawater. Symbols: ⊄, 5°C; ■, 10°C; ▲, 15°C; △, 20°C; □, 27°C. In this and subsequent figures, d = day.](http://aem.asm.org/)

**RESULTS**

**Effect of temperature on the growth of *Pseudomonas nautica* in nonnutrient ASW.** Suspensions of the bacterial prey *Pseudomonas nautica* grown in nonnutrient ASW at an initial concentration of ~10⁶ CFU ml⁻¹ showed little change in viable cell count at 5, 10, 15, and 20°C over 17 days (Fig. 1). At 27°C, however, the viable cell count decreased by two orders of magnitude during this period.

**Effect of bacterial concentration on growth of *P. invadens*.** Growth of *P. invadens* at 15°C occurred at bacterial concentrations of 1.48 × 10⁷ and 8.17 × 10⁷ CFU ml⁻¹ but was minimal at concentrations of 2.43 × 10⁴, 5.32 × 10⁵, and 2.22 × 10⁶ CFU ml⁻¹ (Fig. 2C). A concentration of 8.17 × 10⁷ CFU ml⁻¹ was chosen for use in the temperature experiments because, following an initial drop in population size at 2 days, growth of *P. invadens* generally was sustained at this bacterial concentration over the 14-day experimental period. (At 1.48 × 10⁷ CFU ml⁻¹, the population size of *P. invadens* peaked at around 7 days and then decreased.) Bacterial numbers from experimental tubes with amoebae did not fall below the original inoculum level at any concentration (Fig. 2B). At 2.22 × 10⁶, 1.48 × 10⁷, and 8.17 × 10⁷ CFU ml⁻¹, bacterial numbers in experimental tubes (Fig. 2B) remained relatively constant and similar to those in control tubes with no amoebae (Fig. 2A) throughout the experimental period. However, at concentrations of 2.43 × 10⁶ and 5.32 × 10⁷ CFU ml⁻¹, bacteria in experimental tubes increased to about 2 × 10⁷ CFU ml⁻¹ and remained higher than control suspensions with bacteria only (Fig. 2A and B).

**Effect of temperature on growth of *P. invadens*.** Growth of *P. invadens* varied directly with temperature between 2 and 20°C (Fig. 3C and 4C). In the first experiment, there was no growth at 5°C and the population declined by 61% (from the initial inoculum level) over the 22-day experimental period (Fig. 3C). Growth was slow at 10°C, with a generation time...
FIG. 2. Mean number of amoebae (total organisms) or bacteria (CFU) per ml at 15°C with different bacterial concentrations. Symbols: ×, inoculum (amoebae only); ○, 2.43 × 10⁸ CFU ml⁻¹; ■, 5.32 × 10⁷ CFU ml⁻¹; □, 2.22 × 10⁶ CFU ml⁻¹; ▲, 1.48 × 10⁷ CFU ml⁻¹; △, 8.17 × 10⁷ CFU ml⁻¹. (A) Bacteria from control tubes without amoebae. (B) Bacteria from experimental cultures. (C) Amoebae from experimental cultures.

(g) of ~91 h (Table 1), and rapid at 15 and 20°C, with generation times of 20.33 and 19.41 h, respectively. At 27°C, there was no growth and the population declined to extinction within 13 days. In the second experiment, there was no growth of P. invadens at 2°C and the population declined by 77% over the 29-day experimental period (Fig. 4C). Growth at 12°C (g = 73.39 h) was slightly higher than that at 10°C. At all experimental temperatures, bacterial concentrations in cultures with amoebae were similar to those in control cultures containing no amoebae (Fig. 3A and B and 4A and B).

Linear regressions of log-transformed amoebae abundance versus time were significant (P ≤ 0.05) for each temperature treatment except 5 and 20°C (Table 1). At 5°C, the regression coefficient was not significantly different from 0. At 20°C, the regression was only significant at the P = 0.1
TEMPERATURE AND FOOD EFFECTS ON *P. invadens* GROWTH

![Graph](https://via.placeholder.com/150)

**FIG. 3.** Mean number of amoebae (total organisms) or bacteria (CFU) per ml at different temperatures. Symbols: ×, inoculum; ○, 5°C; ■, 10°C; □, 15°C; ▲, 20°C; △, 27°C. (A) Bacteria from control tubes without amoebae. (B) Bacteria from experimental cultures. (C) Amoebae from experimental cultures.

level, but pooling the sums of squares to increase the degrees of freedom (32) gave a highly significant result (*P* < 0.001). Specific growth rates (μ) calculated for this period of exponential growth are given in Fig. 5 and Table 1. The specific growth rate of *P. invadens* at 12, 15, and 20°C (Table 1) was significantly correlated with median time to morbidity in amoeba-infected sea urchins at the same temperatures (data from reference 25) (*r* = 0.996, *P* < 0.05).

**DISCUSSION**

The relatively rapid growth rates of *P. invadens* under conditions of high temperature and high bacterial concentra-
FIG. 4. Mean number of amoebae (total organisms) or bacteria (CFU) per ml at different temperatures. Symbols: ×, inoculum; ●, 2°C; ○, 12°C. (A) Bacteria from control tubes without amoebae. (B) Bacteria from experimental cultures. (C) Amoebae from experimental cultures.

TABLE 1. Regression analysis of log-transformed amoeba abundance versus timea

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<th>Temp (°C)</th>
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Data were obtained from 2 days to peak concentration or termination of the experiment for all temperature treatments except 27°C from two growth experiments: regression coefficient, degrees of freedom, F value, and corresponding probability. Also given are generation time and specific growth rate (see text for details of calculation).

Growth rates of *P. invadens* in culture were low at 10⁵ to 10⁶ bacteria ml⁻¹ (Fig. 2C), suggesting that these bacterial concentrations may approximate the lower threshold for growth. In feeding studies with other amoebae (*Hartmanella* and *Naegleria* spp.), feeding ceased below 10⁶ to 10⁷ bacterial cells ml⁻¹ (8).

Maximum concentration (3.7 × 10⁴ amoebae ml⁻¹) and density (1,840 amoebae mm⁻²) of *P. invadens* in monoxenic culture (29 days at 12°C) were much higher than those reported for a *Paramoeba* sp. in high-density areas in field
similar in experimental and control tubes, indicating that food was not a limiting factor in the monoxenic cultures.

Phagocytosis of bacteria by \textit{P. invadens} in culture and the presence of membrane vesicles resembling bacteria in various states of degradation in the food vacuoles of amoebae have been documented by light and electron microscopy (Jellett, Ph.D. thesis; Jellett, unpublished observations). \textit{P. invadens} has been observed to ingest bacteria at a rate of one cell every 2 min (Jellett, unpublished observations). At a modest estimate of one cell every 5 min, amoebae in the bacterial concentration experiment, for example, could graze at least $4 \times 10^4$ bacteria ml$^{-1}$ over the course of the 14-day experimental period. Since the numbers of bacteria per ml generally were similar in experimental and control tubes, bacterial growth in the experimental tubes appears to have kept pace with or exceeded (in the case of the lower bacterial concentrations, Fig. 2B) losses due to grazing by amoebae. Bacteria did not grow in control tubes without nutrients. Bacterial growth in the experimental tubes may have been sustained by the release of organic material from dead amoebic cells or by metabolic wastes produced as a result of amoebic growth. Enhancement of bacterial growth by protozoan grazing has been well documented (10, 13, 28, 30, 31, 33, 34; but see reference 6).

Speculation on the origin of \textit{P. invadens} has centered on whether it is an endemic species and triggered to become pathogenic by elevated temperature or an exotic species periodically introduced with warm water masses (16; R. E. Scheibling, Proc. 6th Int. Echinoderm Conf., Victoria, British Columbia, Canada, 1987, in press). Although the amoebae declined in number at the lower temperatures tested, some survived at $2^\circ$C for 29 days and at $5^\circ$C for 18 days. However, it is not clear whether \textit{P. invadens} would survive extended periods of low temperature during winter in North Atlantic coastal areas, and the possibility that it is an exotic species cannot be dismissed. Extinction of \textit{P. invadens} by 13 days at $27^\circ$C indicates that the amoebae off Nova Scotia probably did not originate from tropical water masses. It also indicates that it is unlikely that \textit{P. invadens} caused the recent mass mortality of another sea urchin, \textit{Diadema antillarum}, in the Caribbean (1, 18, 19).

Correlation between growth rate of \textit{P. invadens} in vitro and median time to morbidity of amoeba-infected sea urchins suggests a similar qualitative response of growth rate to temperature in free-living and parasitic \textit{P. invadens}. Paramoebiasis in \textit{S. droebachiensis} is not transmissible below 10 to $12^\circ$C (25, 27). However, in this study, \textit{P. invadens} exhibited slow growth in vitro at 10 and $12^\circ$C. Host resistance may overcome amoebae at these temperatures. At high temperatures, there is evidence of a decrease in the number of coelomocytes (the host defense cells), which may result in decreased host resistance (J. F. Jellett, A. C. Wardlaw, and R. E. Scheibling, Dis. Aquat. Organisms, in press) when amoebic growth rates are maximal.

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\section*{Literature Cited}


