

Acanthamoeba castellanii Promotes the Survival of *Vibrio parahaemolyticus*[▽]

Michelle A. Laskowski-Arce and Kim Orth*

Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, Texas

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***Vibrio parahaemolyticus* is a food-borne pathogen that naturally inhabits both marine and estuarine environments. Free-living protozoa exist in similar aquatic environments and function to control bacterial numbers by grazing on free-living bacteria. Protozoa also play an important role in the survival and spread of some pathogenic species of bacteria. We investigated the interaction between the protozoan *Acanthamoeba castellanii* and the bacterium *Vibrio parahaemolyticus*. We found that *Acanthamoeba castellanii* does not prey on *Vibrio parahaemolyticus* but instead secretes a factor that promotes the survival of *Vibrio parahaemolyticus* in coculture. These studies suggest that protozoa may provide a survival advantage to an extracellular pathogen in the environment.**

Vibrio parahaemolyticus is a gram-negative, incidental pathogen that causes food-borne gastroenteritis, septicemia, and wound infections. The most common source of infection is contact with undercooked or raw seafood, particularly oysters (9). The pathogenicity of *V. parahaemolyticus* has been attributed to multiple virulence factors, of which the most well studied are the thermostable direct hemolysin (TDH) and the similar TDH-related hemolysin (TRH) (42). These hemolysins are found in most clinical strains and have been associated with cytotoxicity, enterotoxicity, cardiotoxicity, and hemolytic activity (39, 42, 44). *V. parahaemolyticus* also possesses two distinct type III secretion systems (T3SS), which allow for the direct translocation of bacterial proteins into host cells (26). Bacteria use these translocated proteins, known as effectors, to manipulate the host to promote bacterial survival and/or virulence (13). Although the precise role that each T3SS plays in *V. parahaemolyticus* pathogenesis is not understood, it has been shown that the T3SS encoded on the large chromosome (T3SS1) contributes to cytotoxicity toward eukaryotic cells and that the T3SS encoded on the smaller chromosome (T3SS2) is responsible for enterotoxicity (7, 35, 36).

V. parahaemolyticus is commonly isolated from many environmental sources, including marine, estuarine, and coastal waters (20, 21). The prevalence of *V. parahaemolyticus* in the environment and incidence of infection have been linked to rising water temperatures caused by global warming (32). *V. parahaemolyticus* has been found to be associated with various plankton, copepods, and crustaceans; however, sampling from various coastal sites reveals that *V. parahaemolyticus* is present in sediment, as well as free-living in water (5, 8, 19–21). In fact, the number of *V. parahaemolyticus* organisms detected in water samples is greater than or equal to the number associated with plankton, supporting the proposal that free-living bacteria contain a large population of *V. parahaemolyticus* in the environment (5, 20).

In the environment, free-living bacteria coexist with protozoa. The interaction between these organisms is complex and can be parasitic, commensal, or mutualistic in nature. *Acanthamoeba castellanii* is a unicellular free-living protozoan that is found ubiquitously in the environment. This species of protozoa has been isolated from various sources, including estuaries, freshwater lakes, rivers, saltwater lakes, beaches, and sediment (22). Although pathogenic in its own right, many studies have highlighted the role of *Acanthamoeba* spp. as reservoirs and/or vectors of pathogenic bacteria (4, 14). The most well characterized example is that of the intracellular pathogen *Legionella pneumophila*, which has been shown to survive and replicate within both *A. castellanii* and *A. polyphaga* after phagocytosis (12, 23). Moreover, *L. pneumophila* utilizes the same mechanism to survive within both *Acanthamoeba* spp. and macrophages from higher eukaryotes (14). This suggests that the association of bacteria with protozoa has significant consequences for the evolution of bacteria (27). Recently, it was shown that *Vibrio cholerae* is able to survive in the presence of *A. castellanii* and can be detected within amoebae, suggesting that protozoa may serve as hosts for, and promote the spread of, waterborne pathogens in the environment (1, 2). In this study, we examined the interaction between *A. castellanii* and *V. parahaemolyticus*. As these two species occupy similar ecological niches, their interaction likely has significant biological and ecological consequences. In a laboratory coculture microcosm, *A. castellanii* is able to promote the survival of *V. parahaemolyticus*. Surprisingly, this interaction does not involve the intracellular survival of *V. parahaemolyticus* but rather is mediated by a factor secreted by *A. castellanii*. These studies provide the first insight into how the bacterial pathogen *V. parahaemolyticus* may evade phagocytosis and utilize a protozoan factor to promote its own survival in the environment.

MATERIALS AND METHODS

Microorganisms and culture conditions. The *Vibrio parahaemolyticus* strains used in this study are listed in Table 1. To determine the presence/absence of the TDH, genomic DNA was isolated using the Qiagen DNeasy tissue kit according to the manufacturer's protocols. The presence or absence of *tdh* was detected by PCR as described previously (6). The strains POR1, POR2, POR3, LM5312, and LM5674 were maintained on minimal marine medium agar plates (5 mM K₂SO₄, 77 mM K₂HPO₄, 35 mM KH₂PO₄, 20 mM NH₄Cl, 5 mM MgSO₄ · 7H₂O, 2%

* Corresponding author. Mailing address: University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9148. Phone: (214) 648-1685. Fax: (214) 648-1488. E-mail: kim.orth@utsouthwestern.edu.

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TABLE 1. *V. parahaemolyticus* strains used in this study

Strain	Relevant genotype	Relevant phenotype	Source	Reference(s)
RIMD2210633	Wild type	Pandemic isolate, Kanagawa phenomenon positive, TDH ⁺ , competent for secretion/translocation via T3SS1 and T3SS2	Clinical isolate; T. Honda	26
POR1	<i>ΔtdhA ΔtdhS</i>	Lacks TDH, derived from RIMD2210633	Clinical isolate; T. Honda	36
POR2	<i>ΔtdhA ΔtdhS ΔvcrD1</i>	Deficient for secretion/translocation via the T3SS1, derived from POR1	Clinical isolate; T. Honda	34, 36
POR3	<i>ΔtdhA ΔtdhS ΔvcrD2</i>	Deficient for secretion/translocation via T3SS2, derived from POR1	Clinical isolate; T. Honda	34, 36
LM5312	BB22, wild type	Wild-type opaque colony morphology, Kanagawa phenomenon positive, TDH ⁺ , competent for secretion/translocation via T3SS1 and T3SS2	Clinical isolate; L. L. McCarter	17, 31
LM5674	<i>ΔopaR</i>	Lacks quorum sensing regulator OpaR, translucent colony morphology, increased production of lateral flagella, derived from LM5312	Clinical isolate; L. L. McCarter	17, 31
KO2201	Unknown	Environmental isolate, Kanagawa phenomenon positive, TDH ⁺	Blue crab hemolymph; <i>V. parahaemolyticus</i> ATCC 27979	
KO2202	Unknown	Environmental isolate, Kanagawa phenomenon positive, TDH ⁺	Steamed crab; <i>V. parahaemolyticus</i> ATCC 35117	

NaCl, 0.4% galactose, and 1.5% Bacto agar per liter) supplemented with 0.1% sodium pyruvate at 30°C. The strains KO2201 and KO2202 were maintained on marine Luria broth (MLB; 1% tryptone, 0.5% yeast extract, and 3% NaCl per liter) supplemented with 0.1% sodium pyruvate at 30°C. To establish cocultures, all *V. parahaemolyticus* strains were cultured in MLB at 30°C for 16 to 18 h with aeration. *Escherichia coli* K-12 was kindly provided by V. Sperandio (University of Texas Southwestern Medical Center) and was maintained in Luria broth (LB; 1% tryptone, 0.5% yeast extract, and 1% NaCl per liter) at 37°C. To establish cocultures, *E. coli* was cultured in LB at 37°C for 16 to 18 h with aeration.

Anoxic cultures of *A. castellanii* ATCC 30234 were obtained from the American Type Culture Collection and were maintained as monolayers in proteose peptone-yeast extract-glucose medium [PYG; ATCC medium 712; 2% proteose peptone, 0.1% yeast extract, 4 mM MgSO₄ · 7H₂O, 0.4 mM CaCl₂, 0.05 mM Fe(NH₄)₂(SO₄)₂ · 6H₂O, 2.5 mM Na₂HPO₄ · 7H₂O, 2.5 mM KH₂PO₄, 0.1% sodium citrate dihydrate, and 0.1 M glucose, pH 6.5] in 75-cm² tissue culture flasks, incubated at 25°C without aeration. Amoebae were routinely subcultured every 7 days.

Establishment of cocultures. Logarithmic *A. castellanii* cultures were washed and the medium was changed 2 days prior to the start of the coculture. One day prior to coculture, the monolayer was suspended and amoebae were enumerated using a hemocytometer (Reichert). Amoebae were diluted and seeded at a density of 1×10^5 amoebae/ml in PYG in a 24-well tissue culture dish and incubated at 25°C for 24 h. The day the coculture was established (day 0), the medium was removed and replaced with either fresh PYG (cultures with amoebae alone) or PYG containing suspended bacteria at a multiplicity of infection of 10 (approximately 1×10^6 bacteria/ml) (cocultures). For each experiment, one well of amoebae was disrupted by vigorous pipetting and organisms were enumerated; the number of bacteria to be added to the coculture was adjusted accordingly. Cultures of bacteria alone at the same concentration in PYG were also established. Plates were centrifuged at $200 \times g$ for 5 min to facilitate contact between amoebae and bacteria, followed by incubation at 25°C for up to 35 days.

Parachamber cocultures were established as described above with the following modifications. Logarithmic *A. castellanii* cultures were washed and the medium was changed 1 day prior to the start of the coculture. The day the coculture was established (day 0), the monolayer was suspended by tapping the flask, and the number of amoebae was determined. A transwell membrane with a 0.2-μm pore size (Nunc) was inserted into each well of the 24-well tissue culture dish. Amoebae were diluted in PYG to a density of approximately 2.5×10^5 amoebae/ml, and 400 μl of this suspension was added to the top chamber (1×10^5 total amoebae). *V. parahaemolyticus* POR1 was added to the bottom chamber at a density of 2.5×10^6 bacteria/ml in PYG, and 400 μl of this suspension was added to the bottom chamber (1×10^6 total bacteria). As controls, cultures were set up

containing *A. castellanii* alone (top), *V. parahaemolyticus* alone (bottom), and *A. castellanii* and *V. parahaemolyticus* together in the bottom chamber.

Preconditioned *A. castellanii* medium was generated by collecting medium from cultures of *A. castellanii* alone, grown for 1, 4, and 7 days. The medium was centrifuged at $800 \times g$ for 10 min, filtered with a 0.22-μm filter, and used to establish a coculture with POR1 as described above.

Analysis of coculture. Cocultures were monitored visually every day; at various time points, the total number of amoebae was determined by first disrupting each well by vigorous pipetting and counting aliquots with a hemocytometer. The ratio of live to dead amoebae was determined by staining aliquots of the coculture with trypan blue for 10 min (18). At each time point, the number of extracellular bacteria was enumerated by plating serial dilutions on minimal marine medium or MLB supplemented with 0.1% sodium pyruvate (*V. parahaemolyticus*) or LB (*E. coli*) as appropriate. To detect the presence of intracellular bacteria, cocultured *A. castellanii* organisms were gently washed and treated with 200 μg/ml gentamicin in phosphate-buffered saline for 2 hours at room temperature to kill all extracellular bacteria. The cells were gently washed again and lysed in 0.1% Triton X-100 in phosphate-buffered saline for 15 min at room temperature to release intracellular bacteria. Serial dilutions of this suspension were plated as described above. We established that the gentamicin treatment resulted in efficient killing of bacteria without affecting the amoebae and that the Triton X-100 treatment resulted in efficient lysis of *A. castellanii* without affecting the bacteria (data not shown). Parachamber cocultures were visually monitored for up to 21 days and analyzed as described above. Preconditioned-medium cultures were visually monitored for up to 10 days and analyzed as described above.

RESULTS

Survival of *V. parahaemolyticus* in the presence of *A. castellanii*. We initially chose to carry out our experiments with POR1, a strain of *V. parahaemolyticus* that lacks the TDH toxin but is competent for secretion by both T3SSs (34). When this strain was cultured in the *A. castellanii*-specific medium, PYG, the number of CFU/ml decreased sharply over the first 3 days and viable bacteria became undetectable by day 4 (Fig. 1A). We did observe variation between experiments and found that viable bacteria become undetectable between days 4 and 8 when cultured in PYG (data not shown). This is in contrast to what was found when we cultured *E. coli* K-12 in the same

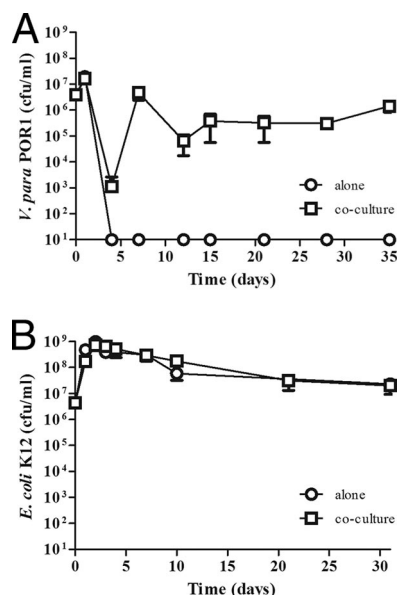


FIG. 1. *A. castellanii* promotes the survival of *V. parahaemolyticus*. *V. parahaemolyticus* (*V. para*) POR1 (A) and *E. coli* K-12 (B) were cultured in the absence (open circles) or presence (open squares) of *A. castellanii* for 35 (A) and 31 (B) days. The number of bacteria was determined by plating serial dilutions on media selective for *V. parahaemolyticus* or *E. coli* as described in Materials and Methods. Results are reported as the number of bacteria recovered per ml; the limit of detection is 10 CFU. Data points represent the means \pm standard deviations (SD) from triplicate cultures and are representative of at least three independent experiments.

medium; viable bacteria can be detected for up to 31 days at approximately 1×10^8 CFU/ml in PYG (Fig. 1B).

When *V. parahaemolyticus* POR1 was cultured in the presence of *A. castellanii*, the number of viable bacteria also initially declined during the first 3 days (Fig. 1A). However, after this decline, POR1 replicated and was able to survive and persist for up to 35 days (Fig. 1A and Table 2). *E. coli* K-12 also survives for up to 31 days in the presence of *A. castellanii* (Fig. 1B). However, this is likely due to the ability of *E. coli* K-12 to survive in the culture medium alone, as the numbers of recovered bacteria are similar in the absence ($2.30 \times 10^7 \pm 0.20 \times 10^7$ CFU/ml) and presence ($2.10 \times 10^7 \pm 1.10 \times 10^7$ CFU/ml) of amoebae at day 31 (Fig. 1B).

The ability of some bacteria to survive in the presence of protozoa such as *A. castellanii* has been attributed to the ability of the bacteria to survive within amoebae (14). To examine whether the survival of POR1 was due to the ability of the bacteria to gain entry into and survive within amoebae, the number of intracellular POR1 organisms was examined by gentamicin protection assays. Unlike what has been described for *V. cholerae*, *L. pneumophila*, and other bacteria, *V. parahaemolyticus* does not reside within *A. castellanii*, as we were unable to recover any intracellular bacteria (1, 2, 12, 23) (Table 2). It should be noted that *V. parahaemolyticus* has been described to enter into a viable but nonculturable (VBNC) state under some long-term-stress conditions (40, 41). As the addition of sodium pyruvate to culture media has been shown to enhance the recovery of bacteria that have entered the VBNC state, the medium was supplemented with 0.1% sodium pyru-

vate (33). Therefore, our inability to recover intracellular bacteria is not due to their entry into a VBNC state. Rather, *V. parahaemolyticus* is able to resist phagocytosis by *A. castellanii* and employ an extracellular strategy of survival.

In an attempt to identify the factors that contribute to this phenotype, we obtained and tested multiple *V. parahaemolyticus* strains in our coculture; the strains, their origins, and their relative genotypes and phenotypes are listed in Table 1. The *V. parahaemolyticus* strain POR1 lacks the TDH; thus, we predict that this virulence factor does not contribute to the survival of the bacteria in the context of our coculture. However, to rule out any involvement of the TDH, we tested the POR1 parent strain, RIMD2210633, which encodes the TDH. To examine the role of the two T3SSs, we tested strains of *V. parahaemolyticus*, POR2 and POR3, containing an in-frame deletion of *vcrD1* and *vcrD2*, respectively, which renders them incompetent for secretion by T3SS1 and T3SS2, respectively (Table 1). We also examined an additional clinical isolate, LM5312, and two environmental isolates, KO2201 and KO2202, all of which encode the TDH (Table 1). KO2201 and KO2202, obtained from the ATCC, were isolated from blue crab hemolymph and steamed crab, respectively. Another strain, LM5674, is derived from LM5312 but lacks the *opaR* gene, which encodes a transcriptional regulator of the LuxR quorum-sensing regulator family (31). Strains lacking *opaR* form translucent colonies and lack the production of the capsule polysaccharide (11). LM5674 also exhibits altered biofilm formation and a hyper-swarming phenotype, which correlates with the overexpression of genes encoding the lateral flagellar system of *V. parahaemolyticus* (10, 17). Similar to what was found for POR1, all of the strains were unable to survive in PYG and none were found to reside within *A. castellanii* (Table 2). Moreover, all of the strains tested were able to survive in the presence of *A. castellanii* for at least 10 days (Table 2). This is consistent with the hypothesis that *A. castellanii* promotes the survival of *V. parahaemolyticus* irrespective of the presence of the TDH, the T3SSs, the quorum-sensing regulator OpaR, or the source of isolation.

Bacterial survival is mediated by a factor secreted by *A. castellanii*. The survival of *V. parahaemolyticus* in the presence of *A. castellanii* does not require the bacteria to gain entrance

TABLE 2. Coculture of various *V. parahaemolyticus* strains with *A. castellanii*

Strain	No. of bacteria recovered (CFU/ml) 10 days after coculture ^a			Mean no. of viable <i>A. castellanii</i> organisms (amoebae/ml) (10 ⁶) \pm SD
	PYG alone	Cocultured with <i>A. castellanii</i>		
		Extracellular	Intracellular	
None	NA	NA	NA	3.95 \pm 0.90
RIMD2210633	ND	1.89 (± 0.74) $\times 10^6$	ND	3.93 \pm 0.34
POR1	ND	2.19 (± 1.44) $\times 10^6$	ND	2.59 \pm 0.30
POR2	ND	1.58 (± 0.35) $\times 10^6$	ND	3.69 \pm 0.59
POR3	ND	1.44 (± 0.08) $\times 10^6$	ND	3.88 \pm 0.28
LM5312	ND	1.91 (± 1.26) $\times 10^5$	ND	4.25 \pm 0.22
LM5674	ND	3.96 (± 1.88) $\times 10^4$	ND	3.83 \pm 0.54
KO2201	ND	2.84 (± 1.01) $\times 10^7$	ND	4.87 \pm 1.40
KO2202	ND	9.97 (± 1.40) $\times 10^6$	ND	3.40 \pm 0.11

^a NA, not applicable; ND, none detected.

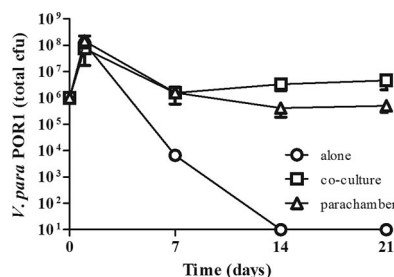


FIG. 2. Survival of *V. parahaemolyticus* is mediated by a factor secreted by *A. castellanii*. *V. parahaemolyticus* (*V. para*) POR1 was cultured in the presence of *A. castellanii* for 21 days but separated from amoebae by a 0.2- μ m-pore-size membrane (triangles). As a control, bacteria were cultured in PYG alone (circles) and in the presence of *A. castellanii* (squares) as in the legend to Fig. 1. The number of bacteria was determined as described for Fig. 1. Results are reported as numbers of total bacteria; the limit of detection is 10 CFU. Data points represent the means \pm SD from triplicate cultures and are representative of at least three independent experiments.

into *A. castellanii*. To determine whether cell contact is necessary for survival, we examined the ability of POR1 to survive in a parachamber with *A. castellanii*. In these experiments, a transwell membrane with a 0.2- μ m pore size is used to physically separate the bacteria and *A. castellanii*. In the parachamber, the amoebae and bacteria are cultured in the top and bottom chambers, respectively. In control cultures, both amoebae and bacteria are added to the bottom chamber. As shown in Fig. 2, POR1 survives equally well when physically separated from *A. castellanii* or when grown in a coculture. At day 1, $1.46 \times 10^8 \pm 0.64 \times 10^8$ CFU were recovered from the parachamber, whereas $0.76 \times 10^8 \pm 0.26 \times 10^8$ CFU were recovered from the coculture. As observed previously, the number of recovered bacteria decreases slightly by day 7 in both the parachamber and the coculture ($1.60 \times 10^6 \pm 1.01 \times 10^6$ CFU and $1.58 \times 10^6 \pm 0.29 \times 10^6$ CFU, respectively) and remains relatively constant over the next 14 days ($0.41 \times 10^6 \pm 0.23 \times 10^6$ CFU and $3.34 \times 10^6 \pm 1.46 \times 10^6$ CFU at day 14 and $0.51 \times 10^6 \pm 0.22 \times 10^6$ CFU and $4.70 \times 10^6 \pm 2.65 \times 10^6$ CFU at day 21 in the parachamber and coculture, respectively). In contrast, bacteria grown in the absence of *A. castellanii* are unable to survive and the bacterial population dies between days 7 and 14 (Fig. 2). Thus, *V. parahaemolyticus* is able to utilize a factor secreted by *A. castellanii* to promote its survival independently of direct contact with the amoebae. In addition, the numbers of bacteria recovered are similar in both the parachamber and the coculture, further supporting the model that *V. parahaemolyticus* resists phagocytosis.

The results from the parachamber experiment indicated that a factor secreted by the amoebae was responsible for the survival of the bacteria. We then tested whether media from a culture of *A. castellanii* alone could recapitulate the same survival effect. Media from 1-, 4-, and 7-day-old cultures of *A. castellanii* alone were used to generate preconditioned media and establish cocultures. However, as shown in Fig. 3, we found that media preconditioned from a culture of *A. castellanii* alone does not support the growth of *V. parahaemolyticus*. This suggests that the amoebae may require the presence of the bacteria to elicit production of the factor. Alternatively, the

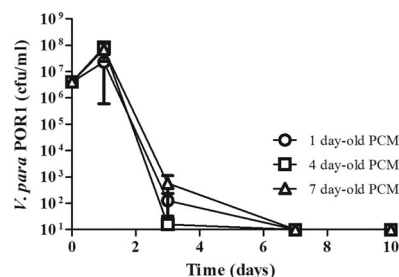


FIG. 3. Medium conditioned from *A. castellanii* alone does not promote the survival of *V. parahaemolyticus*. *V. parahaemolyticus* (*V. para*) POR1 was cultured for 10 days in the presence of media conditioned from 1 (circles)-, 4 (squares)-, and 7 (triangles)-day-old cultures of *A. castellanii* alone. PCM, preconditioned medium. The number of bacteria was determined as described in the legend to Fig. 1. Results are reported as numbers of bacteria recovered per ml; the limit of detection is 10 CFU. Data points represent the means \pm SD from triplicate cultures and are representative of at least three independent experiments.

survival factor produced by the amoebae is either rapidly metabolized or requires continuous production.

***V. parahaemolyticus* is not cytotoxic toward *A. castellanii*.** *V. parahaemolyticus* has been shown to be cytotoxic toward many cell types, and this toxicity has been attributed to the presence of the T3SS1 (7, 25, 34). As cytotoxicity may be a mechanism by which *V. parahaemolyticus* resists phagocytosis and promotes its own survival, we determined the number of viable *A. castellanii* organisms in the coculture. Over 10 days, the number of viable amoebae in PYG alone increases to $3.95 \times 10^6 \pm 0.90 \times 10^6$ amoebae/ml (Table 2). When cocultured with RIMD2210633, POR1, POR3, LM5312, or LM5674, the number of viable amoebae after 10 days is similar to the number of amoebae in PYG alone (Table 2). Furthermore, we do not see an increase in the number of viable amoebae when they are cocultured with POR2, a strain lacking the T3SS1 ($3.69 \times 10^6 \pm 0.59 \times 10^6$ amoebae/ml) (34, 35). Therefore, *V. parahaemolyticus* is not cytotoxic toward *A. castellanii*. The morphology of *A. castellanii* in the presence and absence of bacteria was examined by light and confocal microscopy. No significant alterations in cell morphology were observed (data not shown), suggesting that *V. parahaemolyticus* resists predation by *A. castellanii* through a mechanism other than direct killing of the amoebae.

DISCUSSION

The ability of *V. parahaemolyticus* to survive as a free-living bacterium in the environment requires that it coexist with other organisms, such as protozoa. In this study, we utilized a coculture microcosm to investigate the interaction between *V. parahaemolyticus* and the model protozoan *A. castellanii*. We observe that *V. parahaemolyticus*, unlike some species of bacteria, is able to avoid predation and survive in the presence of *A. castellanii*. Furthermore, the mechanism used by *V. parahaemolyticus* involves extracellular survival and is dependent on a factor produced and secreted by the protozoan. These studies provide the first insight into how these two species may coexist in the environment.

The ability of *A. castellanii* to promote the survival of *V.*

parahaemolyticus does not require direct contact between amoebae and the bacteria. We found that *V. parahaemolyticus* POR1 survived equally well when directly cocultured with *A. castellanii* or when physically separated by a membrane. Thus, survival is mediated by a diffusible factor produced by the amoebae. However, media preconditioned from cultures of *A. castellanii* alone were unable to support the survival of the bacteria. This indicates that the bacteria may require continuous production of the factor or that the factor may be rapidly metabolized. If the factor is metabolized, this would support the idea that *A. castellanii* provides a nutritional source for *V. parahaemolyticus* that satisfies a requirement not fulfilled by the media. This alternative food source may come in the form of dying amoeba or metabolic end products, produced by the amoebae as they grow. This type of cross-feeding between species is known as syntrophy and is common among organisms in a symbiotic relationship (24, 37). Furthermore, it has been suggested that *Mycobacterium avium* survives in the presence of *A. polyphaga* by feeding off by-products secreted by the amoebae (43). However, we found that amoeba lysate could not promote the survival of *V. parahaemolyticus* POR1 (data not shown). Again, this may be due to the rapid metabolism of the factor or the requirement for continuous production. Alternatively, the ability of *V. parahaemolyticus* to survive in the presence of *A. castellanii* may be more complex and involve an active molecular conversation between the two organisms. In fact, the inability of preconditioned media to support survival suggests that production of the survival factor by the amoebae may require the presence of the bacteria. It is becoming increasingly clear that bacteria are capable of interspecies and interkingdom communication and that these processes are important for the evolution and survival of the bacteria (16). As such, the ability to sense and respond to protozoa in the environment would benefit the bacteria, and it may contribute to the persistence of *V. parahaemolyticus* as a free-living organism.

Interestingly, *V. parahaemolyticus* appears to exist in a non-proliferative state in the presence of the amoebae, as the number of viable bacteria does not increase over more than 30 days in coculture. This nonproliferative/nondividing state is in some ways reminiscent of the VBNC state described for various *Vibrio* species, including *V. parahaemolyticus* (40, 41). In both cases, the bacteria are held in a static, nonreplicative state in which they appear to be dormant. This state may be a consequence of limited nutrients late in the coculture. However, if this was the case, we would expect to observe a decrease in the number of bacteria as nutrients become more and more limiting. The fact that we do not see this trend suggests that this state may have more-significant biological relevance. Furthermore, if this state is representative of free-living bacteria in the environment, entry into this state may further facilitate the persistence of bacteria in the environment.

The ability to survive in the presence of amoebae has been reported for other bacteria, such as *V. cholerae*; Abd and colleagues reported that the *V. cholerae* O1 and El Tor strains can survive in the presence, but not the absence, of *A. castellanii* for up to 14 days (1). However, the persistence of *V. cholerae* in the presence of *A. castellanii* is likely due to the ability of *V. cholerae* to survive within this protozoan (1, 2). In contrast, we were unable to recover intracellular *V. parahaemolyticus* under

any conditions. Furthermore, the number of bacteria recovered from the coculture did not change when *A. castellanii* was physically separated from *V. parahaemolyticus*. If *A. castellanii* was able to internalize bacteria, even at a low level, we would expect that the number of viable bacteria recovered would be much higher in the parachamber than in the coculture. Thus, it appears that *V. parahaemolyticus* not only survives in the presence of *A. castellanii* but actively resists predation by the protozoa.

Many species of bacteria have evolved clever mechanisms to resist predation by protozoa (29). *Comamonas acidovorans*, *Flectobacillus* spp., and other bacteria resist engulfment through the formation of long filaments, greater than 10 μm in length (15). Other bacteria, such as *Pseudomonas aeruginosa*, *Janthinobacterium lividum*, and *Chromobacterium violaceum*, target the protozoa with toxins that cause rapid cell lysis (3, 29, 30, 38). Still other species increase their swimming speed to avoid grazing or form biofilms that are resistant to predation (28, 29). We did not observe elongation of the bacteria during coculture or lysis of the amoebae by the bacteria. Furthermore, we observe that the ability to avoid phagocytosis is common for all *V. parahaemolyticus* strains studied. Thus, the mechanism utilized by *V. parahaemolyticus* is independent of the TDH, T3SS machinery, and quorum sensing and is unrelated to the source of isolation. Interestingly, we observed that the strain lacking OpaR, LM5674, was recovered in lower numbers after 10 days of coculture with *A. castellanii* than all other *V. parahaemolyticus* strains tested (Table 2). Thus, while survival and resistance to phagocytosis are not absolutely dependent on OpaR, optimum survival may require OpaR or an OpaR-regulated pathway. Future work will focus on understanding the molecular mechanism used by *V. parahaemolyticus* to resist phagocytosis; this will provide further insight into the survival of this pathogen in the environment.

Overall, these observations have an impact on our understanding of the ability of *V. parahaemolyticus* to persist and spread in the environment. While *V. parahaemolyticus* is a major health and economic issue in Southeast Asia, problems associated with *V. parahaemolyticus* infections in the United States are believed to be largely underdiagnosed and may represent a major health risk (9). Therefore, understanding the mechanisms used by *V. parahaemolyticus* for survival and persistence is essential to control and prevent infection. The ability of amoebae to promote the survival of *V. parahaemolyticus* in coculture may reveal a mechanism by which this pathogen persists in the environment. Future studies will reveal the nature of the molecular relationship between *V. parahaemolyticus* and *A. castellanii* and will focus on the use of this knowledge for the development of tools to prevent the survival and spread of the bacterium in the environment.

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