

The Rise of Pathogens: Predation as a Factor Driving the Evolution of Human Pathogens in the Environment

Martina Erken · Carla Lutz · Diane McDougald

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Abstract Bacteria in the environment must survive predation from bacteriophage, heterotrophic protists, and predatory bacteria. This selective pressure has resulted in the evolution of a variety of defense mechanisms, which can also function as virulence factors. Here we discuss the potential dual function of some of the mechanisms, which protect against heterotrophic protists, and how predation pressure leads to the evolution of pathogenicity. This is in accordance with the coincidental evolution hypothesis, which suggests that virulence factors arose as a response to other selective pressures, for example, predation rather than for virulence *per se*. In this review we discuss some of those environmental factors that may be associated with the rise of pathogens in the marine environment. In particular, we will discuss the role of heterotrophic protists in the evolution of virulence factors in marine bacteria. Finally, we will discuss the implications for expansion of current pathogens and emergence of new pathogens.

Introduction

The rapidly changing environment is increasingly recognized to have an important role in the emergence of pathogens. In the marine environment, these changes will include increases in seawater temperature, changes of currents, increasing eutrophication, and decreases in salinity [1]. An extensive study spanning 50 years of surface seawater

samples from the North Sea indicate that increasing seawater temperatures favors the spread of *Vibrio* spp. [2], including those pathogenic to humans [3, 4]. It is also becoming clear that infection of humans in many cases is incidental, as virulence factors may have important roles in interactions with predators and non-human hosts in the environment, including bacteriophages, predatory bacteria such as *Bdellovibrio*, protozoans, nematodes, insects, and plants [5]. Thus, the implications are that many of these virulence factors evolve outside of the context of human disease [6]. In the cases presented here, the selection pressure driving the evolution of these virulence factors was likely to be the predation by heterotrophic protists, and thus predation may explain, at least in part, the evolution of pathogens in the marine environment.

Predation as a Driving Force of Evolution

Bacteria and heterotrophic protists have a long history of co-evolution. Predation by heterotrophic protists is considered to be a major mortality factor in the marine environment [7–10] and is therefore a strong driving force for the evolution of defense strategies [11]. These traits that provide grazing defense may also have resulted in enhanced fitness and pathogenicity of these strains in human (or animal/plant) hosts [12, 13]. For example, bacteria can avoid being ingested by altering cell surface components, changing cell morphology (e.g. filamentation), increasing swimming speed, biofilm formation, and quorum sensing-regulated toxin release (for reviews see [11, 14, 15]). Many of these traits may have led to acquired pathogenicity, suggesting that humans are an accidental rather than intended host [12, 16–18]. In contrast, it can be envisioned that predation in the environment could also lead to a decrease in pathogenicity if the traits that are used for anti-predator defense and human infection are different. In this case the cost of predator defenses may have a fitness trade-off resulting in a decrease

M. Erken · C. Lutz · D. McDougald
Centre for Marine Bio-Innovation, School of Biotechnology
and Biomolecular Science, University of New South Wales,
Sydney, New South Wales 2052, Australia

D. McDougald (✉)
Advanced Environmental Biotechnology Centre, Nanyang
Environment and Water Research Institute, School of Biological
Sciences, Nanyang Technological University, Nanyang Avenue,
Singapore 637551, Singapore
e-mail: d.mcdougald@unsw.edu.au

in pathogenicity traits. Indeed, this may in part explain why most of the bacterial strains found in these environments are in fact not pathogens. Thus, we could envision that only a subset of anti-predator defenses would be effective in the human host and, therefore, only strains exhibiting these defenses would be able to colonize human hosts and hence be pathogenic.

The main question then is whether virulence is a result of natural selection for phenotypes such as avoidance of host immune defenses and infectious transmission or whether virulence factors have roles that increase the fitness of the bacteria in another environment or context. The traditional view of host/pathogen co-evolution is that this association would ultimately end in extinction of one or both partner(s), or in pathogen attenuation and ultimately commensalism [19, 20]. Thus, virulence is evidence that the association of the bacterium with the host is recent [21]. However, an alternative view is that virulence could be coincidental, i.e., the expression of virulence determinants gives the bacterium an advantage in an out-of-host context. Levin and Eden [22] suggest in their review on host/pathogen co-evolution that direct selection would result in attenuation of virulence while coincidental selection would result in maintenance of virulence. The authors suggest that the polysaccharide capsule of *Streptococcus pneumoniae* is under direct selection as encapsulated strains are selected for in the host and the unencapsulated strains are avirulent. Conversely, it is likely that the *Escherichia coli* *pap* adhesin that is responsible for urinary tract infection is under coincidental selection as these adhesins cause an inflammatory response in the urinary tract resulting in clearance, and adhering strains have a disadvantage when competing with non-adhering bacteria for colonization of the urinary tract. It can also be envisioned that both direct and coincidental selection occur simultaneously but act on different virulence determinants. For example, the toxin produced by *Vibrio cholerae* that is responsible for the explosive diarrhea in cholera patients is probably under direct selection. A role for the toxin in the environment has not been identified and indeed most *V. cholerae* isolates from the environment are non-toxigenic [23]. Furthermore, the expression of this toxin in the human host is important for the dissemination of the bacterium. In contrast, the toxin-coregulated type IV pilus of *V. cholerae* could be under coincidental selection as this pilus is needed for attachment to the chitinous exoskeleton of copepods which is the preferred niche in the environment [24].

Due to the long history of co-evolution of bacteria and protists, many grazing defense strategies have evolved in different bacterial lineages (Table 1). Some bacteria have the ability to resist digestion after phagocytosis and multiply within the amoeba host without killing it (e.g., *V. cholerae* [25, 26]), while others cause host death (e.g., *Legionella*

pneumophila [27–29]), traits which may contribute to pathogenicity in a human host. In some cases the bacterium can prevent the fusion of the lysosome and phagosome, thereby avoiding digestion [30, 31], while others escape lysosomal digestion by entering the cytoplasm of the predator [26].

In some cases, the genes and/or effector proteins regulating the survival of bacteria in the presence of bacterivorous protists have been identified and often these genes/proteins have a defined role in the pathogenicity of these bacterial pathogens towards the human host. In this review we outline a few of these “virulence factors” and discuss their roles both in the environment and in the human host and present evidence that these factors likely evolved to improve bacterial fitness in the environment rather than for pathogenicity *per se*.

Amoeba as a Selective Force Driving the Evolution of Intracellular Pathogens

Free-living amoeba such as *Dictyostelium discoideum* and *Acanthamoeba castellanii* are useful predator models for the study of how pathogenic bacteria prevent/survive phagocytosis. Both organisms have been extensively studied and share similar characteristics to human macrophages, i.e., both are eukaryotic single cells and phagocytize bacteria. *D. discoideum* shares a range of proteins and metabolic processes with metazoans [32]. In plaque assays Adiba and colleagues demonstrated that *E. coli* strains possessing virulence genes (iron uptake and resistance to serum, bile, and lysozyme/lactoferrin) were resistant to grazing by *D. discoideum* whereas non-virulent strains were eliminated [12]. *D. discoideum* has been used as a model organism for the identification of virulence factors that are important for survival in co-culture with human pathogens such as *Pseudomonas aeruginosa* and *V. cholerae* [33–35].

In some cases co-culture can be advantageous for the prey. In co-culture with *A. castellanii*, *Vibrio parahaemolyticus* was shown to survive in rich peptone-yeast-glucose (PYG) medium, while no viable bacteria could be detected in PYG alone [36]. *V. parahaemolyticus* was able to resist phagocytosis by *A. castellanii*; thus, this growth promotion was not due to intracellular survival of *V. parahaemolyticus* but rather was mediated by a factor secreted by *A. castellanii*. Similar results have been presented with *V. cholerae* where the biofilm biomass was increased in the presence of marine heterotrophic nanoflagellates (*Cafeteria roenbergensis* and *Rhynchomonas nasuta*) compared to non-grazed controls [37, 38]. Supplementation of *V. cholerae* cultures with supernatants from cultures of predators also resulted in an increase in bacterial biomass. While this may be partly due to recycled nutrients made available by predation, there is evidence that the amount of nutrient provided is not

Table 1 Examples of factors involved in pathogenicity of a broad range of bacteria are also involved in anti-predation strategies

System/toxin	Bacterium	Anti-predation	Virulence affecting humans	Reference
T3SS	<i>P. aeruginosa</i>	Kills <i>A. castellanii</i>	Pneumonia	[86]
	<i>L. pneumophila</i>	Parasite inside amoeba and human monocytes	Legionellosis	[31, 101]
	<i>E. coli</i>	Survives inside <i>A. castellanii</i>	Diarrheal disease	[64]
	<i>Chlamydiae</i>	Endosymbiont of <i>Acanthamoeba</i> sp.	Trachoma, genital tract infections, and pneumonia	[102]
	<i>V. parahaemolyticus</i>	Toxic towards a range of protist grazers	Wound infections, septicemia, and acute gastroenteritis	[92]
T6SS	<i>V. cholerae</i>	Cytotoxic against <i>D. discoideum</i>	O1/O139-cholera and non-O1/non-O139 gastroenteritis	[34]
Violacein	<i>C. violaceum</i>	Causes rapid cell death in protists	Opportunistic pathogen	[71, 100]
	<i>J. lividum</i>	Causes rapid cell death in protists	–	–
	<i>P. tunicata</i>	Causes rapid cell death in protists	–	–
Shiga toxin	<i>E. coli</i>	<i>Stx</i> -carrying bacteria kill <i>T. thermophila</i>	Hemorrhagic colitis	[65, 66]

sufficient to fully explain the increase, indicating that a secreted predator cue is involved [39].

Biofilm Formation

The role of biofilms in human infection and disease has been a topic of intense research over more than 40 years. Biofilms are surface-associated communities of microorganisms encased in extracellular polymeric substances (EPS). Biofilms in the human body are the cause of more than 60 % of infections, including dental plaque and periodontitis [40], infections from biomedical implants such as heart valves (endocarditis) and prosthetic joint implants, catheters and stents, infections in the lungs of cystic fibrosis patients often resulting in bronchopneumonia, persistent otitis media, and chronic rhinosinusitis [41]. Biofilms are especially important in chronic infections as bacterial cells in biofilms are up to 1,000-fold more resistant to antimicrobials than their planktonic counterparts [42]. Understanding the nature of this increased resistance is currently a central goal for medical researchers. Because of their recalcitrance to treatment, biofilm infections typically show recurring symptoms after antibiotic treatment and often, must be removed surgically. A large number of medically important bacteria are able to form biofilms including *P. aeruginosa*, *E. coli*, *Staphylococcus* spp., *Salmonella typhimurium*, *L. pneumophila*, *Haemophilus influenzae*, *Vibrio* spp., and *Streptococcus* ssp. [43, 44].

Evidence for the occurrence of biofilms in the environment is present in fossil records, for example, in 3.2 billion-year-old deep-sea hydrothermal rocks from Pilbara Craton, Australia [45]. Biofilms are a characteristic of ancient lineages of Archaea and Bacteria, indicating that biofilm formation is an ancient characteristic of microorganisms [43] and implying that biofilms have a role in increasing the

fitness of microbes in these environments. Biofilms are common in freshwater environments, often forming a slimy covering on rocks and other submerged surfaces. In the marine environment, biofilms form on all submerged surfaces, including the hulls of ships, buoys and pylons, but the major source of marine biofilms is in aggregates of microorganisms and detritus called marine snow [46].

V. cholerae has been observed in the marine environment in biofilms in the form of multicellular aggregated clumps, and it is believed that these biofilms contribute to the persistence of the organism in inter-epidemic periods [47–49]. The formation of biofilms of similar structure has been shown to also occur during human infection [50] and may protect *V. cholerae* from acid pH and/or antibiotics in the human gut. Furthermore, the biofilms of *V. cholerae* that are shed in human stools have increased infectivity compared to planktonic cells and are thus responsible for high rates of reinfection during outbreaks of cholera [50].

Biofilms formed in the environment have been shown to be more resistant to a variety of stresses, including extremes of temperature, pH, and exposure to UV [43]. There is increasing evidence that biofilms are protective against predation. In biofilms formed by the yeast, *Cryptococcus* spp., ciliate grazers showed a strong preference for feeding on the EPS rather than on cells embedding in the biofilm, while in the planktonic phase, high numbers of yeast cells were ingested [51]. Furthermore, the number of metabolically active yeast cells in the biofilm was 1.75 times higher in grazed biofilms when compared to non-grazed controls. Thus, EPS from the biofilm may serve as a preferential nutrient source to some predators, and feeding on EPS as opposed to biofilm cells benefits the biofilm not only from protection against ingestion but also due to the increased nutrients provided to the community through nutrient recycling.

Early biofilms of *V. cholerae* [38] and *P. aeruginosa* [52] formed grazing-resistant microcolonies in the presence of the surface-feeding flagellate, *R. nasuta*, whereas biofilms without the predator remained undifferentiated. Late, more mature biofilms were toxic to *R. nasuta*, while biofilms formed by a quorum sensing mutant supported the growth of the predator. Thus, microcolony formation and the production of toxins are effective anti-predator mechanisms that allow *V. cholerae* and *P. aeruginosa* to persist in the environment. In addition, while *V. cholerae* biofilms were not grazed, planktonic cells were reduced by 97 % in 72 h [38]. In the presence of a predator in planktonic populations, there was a shift from the smooth cell morphotype to the biofilm-enhancing rugose morphotype. Further investigations on the relative contribution of EPS to grazing resistance indicated that while EPS provides protection from grazing, the secretion of an anti-protozoal factor is more important in grazing resistance of late biofilms [39]. In *P. aeruginosa*, it was shown that mature biofilms are resistant to a range of biofilm-feeding predators due to the production of an inhibitor, while microcolony formation was only beneficial in the early stages of biofilm formation [53]. Thus, there are multiple anti-predator mechanisms that are expressed at different stages of the life-cycle of these bacteria.

Evidence that the expression of biofilm-mediated predator defense is a general feature of marine biofilm-forming bacteria has been presented in a study comparing the presence and efficacy of chemical defenses in biofilms and planktonic phases of growth. The bacterial isolates used in the study were obtained from the surface of the marine alga, *Ulva lactuca*, and included representatives of the five main bacterial groups, *Bacteroidetes*, *Planctomycetes*, α -, δ -, and γ -Proteobacteria [54]. When grown planktonically, 90 % of the isolates were edible, resulting in increased predator numbers (180-fold increase on average). In contrast, when the same strains were grown as biofilms, 57 % of the strains caused a significant reduction in predator numbers, indicating that the majority of the strains were grazing resistant only when grown as a biofilm. In semi-natural biofilms formed by microbial populations from the Rhine River, Cologne, Germany, it was shown that the presence of heterotrophic nanoflagellates resulted in an increase in the numbers of microcolonies [55]. Thus, the anti-predator fitness of biofilms is likely to be a conserved grazing resistance mechanism that has evolved because of intense predation pressure in these environments.

Role of Intercellular Communication in Predation Defense and Pathogenicity

Bacteria communicate through the secretion and perception of chemical cues called autoinducers (AI), in a process termed quorum sensing (QS). AIs accumulate in the

environment and when they reach a critical concentration, they bind to and activate intracellular receptors that alter gene expression. QS was first described in *Vibrio fischeri* where it controls luminescence [56]. For many years, it was thought that QS only occurred in a few marine bacteria, but it is now known to occur in many bacteria. In a large number of these, QS is known to regulate virulence (e.g., *Staphylococcus aureus*, *P. aeruginosa*, *E. coli*, *Yersinia* spp., *Aeromonas* spp., and *Vibrio* spp.) [57]. In order to establish an infection, a pathogen must reach a sufficient density so that it can overcome the host defenses before it expresses virulence factors, otherwise the pathogen will be eliminated by the host immune system. Thus, controlling the expression of virulence factors in a cell-density dependent manner gives the pathogen an advantage. The fact that many virulence factors, such as the production and secretion of toxins, proteases, and siderophores, and the expression of pili are QS-regulated has led to the exploration of QS inhibition as a way to treat bacterial infections [58].

Quorum sensing allows a coordinated, synchronized response by the population to the changing environmental conditions, thereby increasing the fitness of these populations and contributing to their persistence in the environment. Evidence for the role of QS in the regulation of anti-predator defenses has been reported for *P. aeruginosa* [52], *V. cholerae* [38], *Chromobacterium violaceum* [59], and *Serratia marcescens* [60] where it was shown that QS mutants have a significantly reduced anti-predator fitness compared to isogenic wild-type strains. In *V. cholerae* [38] and *P. aeruginosa* [52, 61], QS regulates the expression of an unknown toxin that is effective against a range of predators. In the case of *S. marcescens*, the QS-regulation of a filamentous biofilm protected the bacterium from grazing [60]. Indeed, the regulation of biofilm formation and maturation is an important phenotype for environmental persistence.

Shiga Toxin

Phage-encoded exotoxins, such as cholera toxin, diphtheria toxin, and Shiga toxin (Stx), produced by bacterial isolates kill eukaryotic cells by targeting pathways that are generally conserved. The genes encoding the Shiga exotoxins, *stx1* and *stx2*, are carried on a bacteriophage that infects *E. coli*. Shiga toxin-carrying *E. coli* (STEC) can cause severe dysentery-like diarrhea and hemolytic uremic syndrome [62]. Often, these exotoxins are found in environments where there are no mammalian hosts, indicating that these exotoxins may have a role in predation resistance [63].

Stx-producing *E. coli* O157:H7 are able to survive inside the food vacuoles of *Tetrahymena pyriformis* [64]. Stx-bearing *E. coli* was also shown to have a growth advantage

over Stx-negative strains in co-culture with the ciliate *Tetrahymena thermophila* [65], but not when cultured in the absence of the protozoa [64]. When co-cultured with the Stx strain or when pure Shiga toxin was added, the ciliate was killed [65]. The authors suggest that the H₂O₂ released by *T. thermophila* induced production of Stx, thereby preventing predation. In this case the release of a toxin that affects humans was directly controlled by the presence of phagotrophic protists.

Although the principle reservoir of STEC is bovines, reports of isolation of *stx*-carrying *E. coli* [66, 67] and *stx*-encoding phage [63] from the marine coastal environment are increasing. The first report of STEC isolated from marine shellfish originated from the French coastal environment [67]. In a survey by Gourmelon and colleagues [67] covering areas in the English Channel, the Atlantic coast, and Mediterranean Sea, 27.8 % of the samples from oysters, mussels, and cockles were *stx* positive. Due to the methods used in this survey, it was not possible to differentiate between STEC and *stx*-encoding prophage, but the results indicate the potential for infection from ingestion of shellfish from this region. Increased runoff from urban and agricultural areas is probably the cause for the increase in abundance of STEC in the marine environment. The authors suggest that although *stx2*, which is associated with high human virulence, was not detected in any of the samples, the marine environment acts as a reservoir from which new pathogenic STEC strains can arise. In a more recent study off the coast of Morocco, *E. coli* O157:H7 and other non-O157 STEC were found with *stx1*-positive samples comprising 2.1 % of the total shellfish and sediments samples tested. Only shellfish were positive (0.48 %) for *stx2* [66].

In San Diego, California, *stx*-carrying phages were isolated from terrestrial and aquatic environments where no disease had been reported to date [63]. Exotoxin-specific PCR assays showed that 25 % of all samples analyzed were positive for *stx* and 18 % of the water samples (combined marine and freshwater samples) were positive. The authors speculate that these phages may play a role in the evolution of human pathogens due to the ease of virulence transfer to non-pathogenic strains.

Violacein

In 1942 Singh reported that a pigmented extract of *C. violaceum* (along with two other bacteria) prevented ingestion of otherwise edible bacteria by soil amoeba [68]. This purple pigment has been identified as the indole alkaloid, violacein [69, 70]. Violacein was since found to have antimicrobial, antifungal, antiprotist, antiviral, and even antirotifer properties [71, 72]. In particular, the anti-protozoan properties of violacein are well documented. In addition to

C. violaceum, many other bacteria produce violacein, including the freshwater bacterium *Janthinobacterium lividum* and the marine bacteria *Alteromonas luteoviolacea* [71], *Pseudoalteromonas tunicata*, *Pseudoalteromonas ulvae*, *Pseudoalteromonas luteoviolacea*, and *Microbulbifer* sp. [73]. The anti-protozoan activity of *P. tunicata* was determined by bioassay-guided fractionation to be due to violacein as well as the violacein derivative deoxyviolacein. The concentration of violacein in biofilms of different violacein-producing strains was 3 (*P. ulvae*) to 59 (*Microbulbifer* sp.) times higher than in planktonically grown bacteria.

In grazing bioassays Matz and colleagues [59] demonstrated that as little as three violacein-producing bacteria ingested by heterotrophic flagellates resulted in rapid cell death after 20 min and cell lysis within 1 to 2 h. By using a QS mutant strain, they were able to show that toxin production was QS dependent. In experiments with predators exposed to a mixed population of non-toxic and violacein-producing bacteria, flagellate numbers were inhibited, suggesting an important role for violacein in microbial community dynamics. Violacein also displayed activity against a variety of bacterivorous freshwater zooplankton protists and metazoans. For all zooplankton taxa tested, the presence of 9 ng mL⁻¹ violacein, equivalent to the amount present in a mixed population of 10 % *C. violaceum*, resulted in reduced growth rates by 18–100 % [69].

Although *C. violaceum* is not a major pathogen, cases of infections have been increasingly reported. In particular, immune-compromised persons and children are vulnerable to *C. violaceum* infections (e.g. [74–76]). There have also been reports that infections in other mammals have increased [77, 78]. Minor skin trauma appears to be the entrance point for the bacterium into the body, leading to septicemia and liver abscesses, which are often fatal [76]. Violacein induces apoptosis of immune cells such as leukocytes and thus could be important for evasion of the immune system during human infection [79]. As the range of growth temperature of *C. violaceum* is 20–37 °C, rising sea surface temperatures may also increase the prevalence of violacein-producing bacteria.

Type III Secretion System

The type III secretion system (T3SS) consists of more than 20 proteins and delivers bacterial effector proteins into eukaryotic host cells. T3SSs can be found in many Gram-negative bacteria [80–82], as well as in an endosymbiont of *Acanthamoeba*, *Protochlamydia amoebophila*. Examination of the endosymbiont genome shows little evidence of horizontal gene transfer (HGT) after its divergence from *Chlamydiae* approximately 700 million years ago [83]. The fact

that T3SS evolved in this organism long before the appearance of Mammalia ~210 million years ago indicates that the T3SS evolved for a purpose other than virulence as such, thus supporting the “coincidental evolution” hypothesis [84].

The T3SS is essential for the survival of pathogenic *E. coli* inside trophozoites of *A. castellanii* [85] and as a QS-independent anti-predator mechanism in *P. aeruginosa* [86]. The upregulation of T3SS and down-regulation of factors contributing to biofilm formation [86, 87] result in a highly motile phenotype, allowing *P. aeruginosa* to rapidly colonize and kill amoeba cells. In the human host, effectors secreted by the *P. aeruginosa* T3SS have profound effects on the progression of pneumonia, further supporting the hypothesis that the evolution of anti-predator defenses might have increased the success of *P. aeruginosa* as a human pathogen.

V. parahaemolyticus has emerged as the number one cause of seafood-related bacterial illnesses in many countries [88, 89]. The thermostable direct hemolysin (TDH) is an important virulence factor of *V. parahaemolyticus*, and the T3SS is only present in TDH-positive pathogenic strains [90]. *V. parahaemolyticus* has two T3SSs; T3SS-1 is encoded on chromosome 1 and is responsible for cytotoxicity, while T3SS-2 carries 2 *tdh* genes and is encoded on chromosome 2 on a virulence pathogenicity island (VPaI-7) acquired by HGT [90, 91]. Matz and colleagues [92] demonstrated that the T3SS-2 was necessary for resistance to grazing by the flagellate *C. roenbergensis* as the biomass of a T3SS-2 mutant was reduced by 80 % while the wild-type strain reduced the flagellate numbers by 85 %. In microcosm experiments containing a native coastal plankton community, both T3SS-2 positive and negative strains decreased by about 10 % over a five-day-period. In the presence of heterotrophic and photoautotrophic protists, the T3SS-2-negative *V. parahaemolyticus* population declined 100-fold while in T3SS-2-positive microcosms, a stable *V. parahaemolyticus* population was established and increased by 20 % as the numbers of protists decreased by 59 % [92]. Furthermore, with an increase in the temperature from 16 to 30 °C, the maximum cell density of the T3SS-2-positive strain increased while the T3SS-2-negative strain remained suppressed by the ciliate grazer. These data show that the T3SS plays a role in increasing the fitness of *V. parahaemolyticus* in the environment by facilitating invasion of a coastal plankton community and that these strains have an added advantage at higher temperatures which is important in the context of global warming.

Type VI Secretion System

The type VI secretion system (T6SS), first identified by Spaink and co-workers [6], is found in more than 100

sequenced bacterial genomes, a large number of which are Gram-negative bacterial pathogens (e.g., *P. aeruginosa*, *Salmonella enterica*, and *Aeromonas hydrophila*) and many possessing multiple copies [93]. The T6SS has been demonstrated to be involved in pathogenesis in a number of human pathogens [94–96]. This secretion system was demonstrated to be required for cytotoxicity of *V. cholerae* towards *Dictyostelium* and mammalian macrophages by a contact-dependent mechanism [34]. In *A. hydrophila* the expression of T3SS and T6SS, as well as QS and lateral flagella, is positively linked. In fact, the link between regulation of T3SS and T6SSs has been reported for a number of bacteria [97]. However, the majority of bacteria possessing T6SSs are not pathogens, but rather occur in marine and soil environments and there is strong evidence that in many of these cases, the T6SS has a role in interactions with other bacteria [98]. Thus, it is likely that T6SSs have a broad role for increasing bacterial fitness in the environment rather than in pathogen/host interactions *per se*. Supporting this is the fact that T6SS, T3SS, flagellar synthesis, biofilm formation, and quorum sensing are linked in many bacteria and these systems are all known to play roles in environmental fitness. In the case of *V. cholerae*, while there is clear evidence that genes encoded in the T6SS are important for survival in *Dictyostelium* and the deletion of the effector protein, Hcp, had no effect in a mouse model of infection, highlighting that its role in virulence in the human is unclear [34, 99].

Conclusions

Amoeba and macrophages have similar mechanisms of phagocytosis and prey inactivation [100] and therefore, the resistance mechanisms that are important for survival of both ingestion by amoeba and phagocytosis by immune cells seem to be similar, supporting the idea that resistance to amoeba is an important driving force in the evolution of intracellular pathogens [11, 27]. Likewise, pre-ingestional protective mechanisms may also be similar to those needed by pathogenic bacteria to evade eukaryotic immune systems. For example, biofilm production and QS-mediated secretion of virulence factors by *P. aeruginosa* and *V. cholerae* are important for both environmental survival and resistance to predation by heterotrophic nanoflagellates [38, 52, 53, 86, 92]. Thus, the similarities between anti-predator defense mechanisms and defenses against phagocytes suggest a causal link in pathogenicity and environmental persistence. This has profound implications for the evolution of virulent strains in the environment, as many virulence factors that are studied in the context of human disease potentially have functions in natural microbial communities. A better understanding of these roles would allow

us to predict how such pathogens arise and persist in the environment. Therefore, future research should be directed at the better understanding of the role of predation in microbial community function, diversity, and stability.

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