Dual Role of Mechanisms Involved in Resistance to Predation by Protozoa and Virulence to Humans

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Most opportunistic pathogens transit in the environment between hosts and the environment plays a significant role in the evolution of protective traits. The coincidental evolution hypothesis suggests that virulence factors arose as a response to other selective pressures rather for virulence *per se*. This idea is strongly supported by the elucidation of bacterial-protozoal interactions. In response to protozoan predation, bacteria have evolved various defensive mechanisms which may also function as virulence factors. In this review, we summarize the dual role of factors involved in both grazing resistance and human pathogenesis, and compare the traits using model intracellular and extracellular pathogens. Intracellular pathogens rely on active invasion, blocking of the phagosome and lysosome fusion and resistance to phagocytic digestion to successfully invade host cells. In contrast, extracellular pathogens utilize toxin secretion and biofilm formation to avoid internalization by phagocytes. The complexity and diversity of bacterial virulence factors whose evolution is driven by protozoan predation, highlights the importance of protozoa in evolution of opportunistic pathogens.

Keywords: pathogenicity, predation, *Vibrio*, evolution, virulence factors, coincidental evolution, protozoa

INTRODUCTION

Many bacterial pathogens are able to survive in non-clinical environments, where environmental conditions play an important role in the persistence and infectivity of those bacteria. In fact, most opportunistic pathogens are not transmitted host to host but rather transit in the environment between hosts for significant amounts of time. Thus, the environment impacts not only their survival but also potential infectivity. For example, global warming has been suggested to be responsible for increased outbreaks of disease caused by *Vibrio* spp., as rising sea surface temperatures promote their growth and virulence in aquatic environments (Vezzulli et al., 2013, 2015). The environmental persistence of bacterial pathogens also depends on their ability to adapt to various stresses. Predation by bacterivorous protists is one of the key mortality factors faced by bacteria in the environment. In response, bacteria have evolved many anti-protozoal mechanisms, such as formation of filaments, increases in swimming speed, surface masking, toxin production and biofilm formation, etc. (Matz and Kjelleberg, 2005). Some mechanisms providing resistance to protozoan grazing may also provide advantages during infection of human and animal hosts. As proposed in the review by Erken et al. (2013), protozoan grazing is "a factor driving the evolution of human pathogens in the environment." This review summarizes the mechanisms of bacterial

pathogens involved in both resistance to predation and human pathogenesis, with an emphasis on advances made in the past 5 years. We compare these mechanisms using examples of intracellular and extracellular pathogens.

PREDATION BY HETEROTROPHIC PROTISTS IMPACTS PATHOGENS IN ENVIRONMENT

Protists, or protozoa, are single-celled eukaryotes that range in size from 2 to 2000 μm. Protozoa are grouped based on morphology and mechanisms of feeding and locomotion into three groups, flagellates, ciliates, and amoebae. Heterotrophic flagellates and ciliates feed by sweeping food particles into a mouth-like cytostome, while amoebae engulf food particles using pseudopodia. After ingestion, food vacuoles (phagosomes) are trafficked through the phagocytic pathway and subsequently fuse with lysosomes (phagolysosome) enabling digestion of food particles (Fenchel, 1987).

Predation by bacterivorous protozoa is a well-known mechanism for top down control of bacterial communities due to their high feeding rates (Sherr and Sherr, 2002) and their ability to predate on surface-associated bacterial biofilms (Parry, 2004). However, there are also studies demonstrating that many bacteria, including pathogenic species, benefit from interactions with protozoa. Protozoa have been called the "Trojan horses of the microbial world" for their ability to promote the survival of pathogenic bacteria in the environment (Barker and Brown, 1994). For example, pathogenic bacteria that have been shown to have increased survival in the environment after interactions with protozoa include *Campylobacter jejuni* (Trigui et al., 2016; Reyes-Batlle et al., 2017), *Francisella tularensis* (Buse et al., 2017), *Listeria monocytogenes* (Fieseler et al., 2014), *Legionella pneumophila* (Cervero-Arago et al., 2014, 2015), *Mycobacterium leprae* (Wheat et al., 2014), *Stenotrophomonas maltophilia* (Cateau et al., 2014), and *Yersinia enterocolitica* (Lambrecht et al., 2013). Although the mechanisms of increased bacterial survival are not fully understood, it has been suggested that bacteria can obtain nutrients from protozoa. Furthermore, protozoa are not only ubiquitous in the environment, but are also members of gut microbiota in many organisms. The interactions between protozoa and other gut microorganisms can be beneficial or harmful to host health (Burgess et al., 2017; Chabe et al., 2017).

In addition, some protozoal species are able to form dormant cysts that are resistant to a variety of stresses, and these cysts provide encased bacteria a protective niche against adverse conditions. For example, *Escherichia coli*, *L. monocytogenes*, *Salmonella enterica,* and *Y. enterocolitica* demonstrated increased resistance to antibiotics and low pH when in *Acanthamoeba castellanii* cysts (Lambrecht et al., 2015). *L. pneumophila* within cysts of *Acanthamoeba polyphaga* was also reported to be resistant to chlorine treatment (Kilvington and Price, 1990). Therefore, protozoa and cysts generated by protozoa can be vectors for pathogenic bacteria and facilitate disease transmission (Denoncourt et al., 2014; Balczun and Scheid, 2017).

In some cases, interactions of bacteria with protozoa induce the expression of virulence traits, and thus, environmental amoebae have been referred to as "training grounds" for bacteria virulence (Molmeret et al., 2005). This is due to the fact that the bactericidal mechanisms used by amoeba and human immune cells, such as macrophage, are conserved. This includes the cell biology of phagocytosis and phagosome maturation. The mechanisms used by macrophage and amoeba to kill engulfed bacterial cells are similar, including H^+–ATPase related acidification of the phagosome, oxidative burst from reactive oxygen and nitrogen species, use of metal transporters for iron and manganese efflux and copper and zinc influx, nutrient deprivation and the battery of antimicrobial proteins and lysosomal hydrolases expressed (Siddiqui and Khan, 2012; German et al., 2013). Many bacteria have evolved anti-digestion mechanisms that allow them to survive inside of protozoa, and some of these mechanisms also contribute to their virulence during infection of human and animal hosts (Gong et al., 2016; Paquet and Charette, 2016). Consequently, intracellular pathogens that inhabit phagosomal compartments interfere with their maturation and/or are resistant to host killing mechanisms. Thus, bacteria that have evolved to escape the bactericidal mechanisms of amoeba will be better protected (or more virulent) when encountering immune cells. Protozoan grazing has been shown to shape phenotypic and genotypic composition of bacteria community (Jurgens and Matz, 2002). Although there is limited experimental data showing that long-term protozoan grazing results in genotypic changes, it has been reported that protozoa can induce the expression of bacterial virulence in the short term, probably due mainly to changes in gene expression. For example, *Mycobacterium ulcerans* when co-cultured with *A. polyphaga* infected the footpads of mice much faster than the *M. ulcerans* only controls (Azumah et al., 2017). *S. enterica* serotype Typhimurium was shown to enter a hyperinvasive state after passage through *A. castellanii* (Carlson et al., 2007), and intracellular growth in *A. castellanii* also induced the invasion and virulence of *L. pneumophila* in mouse infection models (Cirillo et al., 1999).

The micronutrients, Fe, Mn, Zn, and Cu play important roles in the innate immune system and in antimicrobial activity of macrophages in defense against invading microbial pathogens (Hood and Skaar, 2012; Samanovic et al., 2012). Both protozoa and macrophage use the toxicity of Cu and Zn to kill bacteria in mature phagosomes. Some bacteria are able to avoid damage induced by these metals by the use of efflux pumps. Thus, copper resistance increases not only the environmental survival of bacterial pathogens, but also contributes to virulence *in vivo* (German et al., 2013). *E. coli* strains with mutations in Cu(I)-translocating P1B-type ATPase (*copA*), iron uptake transporters (*feoAB* and *entC*) and manganese uptake transporters (*mntH*), as well as *Pseudomonas aeruginosa* mutants in Cu(I)-translocating P1B-type ATPase (*cueA*) showed reduced grazing resistance against *Dictyostelium discoideum* (Hao et al., 2016). Efflux systems are also involved in antibiotic resistance. For example, the *C. jejuni* RND-type efflux pump, CmeABC which is associated with multidrug resistance, may also be involved in virulence as well as survival in *A. polyphaga* (Vieira et al., 2017).

These data highlight the role of these "virulence factors" in persistence and survival in the environment and likely evolved to protect bacteria from predation rather than for protection against antibiotics, given the long history of co-evolution of protozoa and bacteria.

PROTOZOAN GRAZING PROMOTES HORIZONTAL GENE TRANSFER

Horizontal gene transfer (HGT) contributes to bacterial adaptation and evolution. For bacterial pathogens, HGT is involved in resistance to antibiotics and virulence during infection (Juhas, 2015) and protozoan grazing has been shown to play an important role in HGT. Due to the indiscriminate feeding of many protozoa, it is likely that there will be a mix of different bacteria encased in a single food vacuole. This would facilitate the interactions of these bacteria in an enclosed system. For example, *E. coli* strains engulfed by the ciliate, *Tetrahymena pyriformis,* exhibited increased rates of conjugation (Schlimme et al., 1997). After a full digestion cycle, the frequency of conjugation increased 2000- to 4000-fold. *Tetrahymena thermophila* has also been reported to cause an increase in HGT by accumulating phage and susceptible bacteria in the phagocytic vacuoles (Aijaz and Koudelka, 2017). Prophages have been shown to be induced after ingestion due to exposure of the bacterium to oxidative stress in the phagosome, resulting in a switch to the lytic cycle and release of free phage particles. The frequency of lysogen formation was shown to increase sixfold as a consequence of being encased within the phagosome. In both cases, the accumulation of bacteria, phage and DNA in protozoan food vacuoles is proposed to be responsible for the increase in HGT. Protozoan grazing also stimulates biofilm formation by grazing resistant bacteria (Matz et al., 2004, 2005) and it has been proposed that the high cell density in bacterial biofilms is also responsible for increased HGT (Madsen et al., 2012).
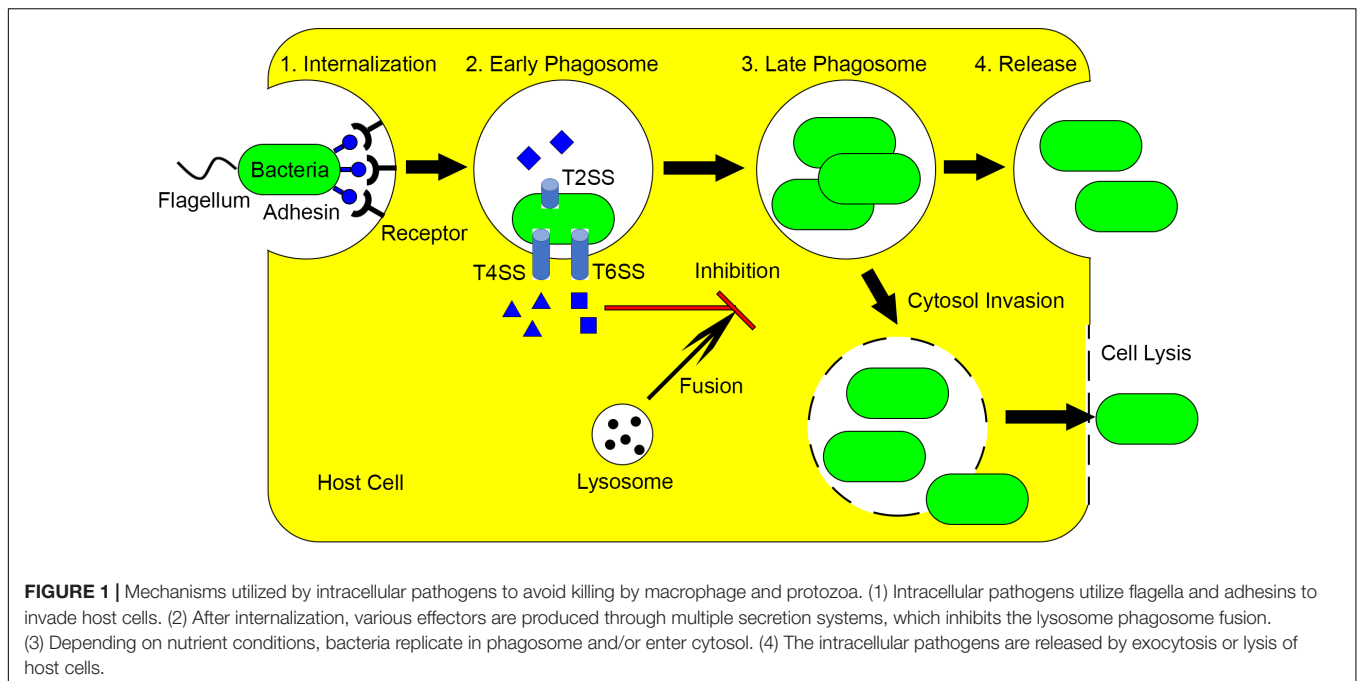
INTRACELLULAR PATHOGENS

L. pneumophila is the causative agent of Legionnaires' disease and is one of the best-studied intracellular pathogens. In the environment, *L. pneumophila* interacts with diverse protozoan hosts, which is critical for its persistence as this organism is not transmitted from person to person (Boamah et al., 2017). This further highlights that environmental protozoa are the true hosts for this pathogen. The *L. pneumophila* life cycle within amoebae and macrophage has been described in detail in numerous reviews (Escoll et al., 2013; Richards et al., 2013; Hoffmann et al., 2014). Briefly, *L. pneumophila* (1) invades the host cell after phagocytosis, forming *Legionella*-containing vacuoles (LCVs), (2) uses the Dot/Icm type IV secretion system (T4SS) to inject approximately 300 effector proteins into the host cell, (3) differentiates into the replicative form and proliferates when nutrients are present, (4) when nutrients become limiting, *L. pneumophila* differentiates into the mature infectious form and enters the cytosol, (5) cells are finally released as free swimming cells after host cell lysis (**Figure 1**). Recently, it has been proposed that the *L. pneumophila* life cycle contains even more developmental stages and cell forms (Robertson et al., 2014).

Host cell invasion by *L. pneumophila* is mainly mediated by surface factors like the flagellum and pilus. *L. pneumophila* has a single monopolar flagellum composed of one major subunit, FlaA. A mutation of FlaA reduces the efficiency of invasion of *A. castellanii* and macrophage due to reduced motility (Dietrich et al., 2001; Schell et al., 2016a). In addition, PilY1 is responsible for the biogenesis of the type IV pili, which is important for twitching motility, surface attachment and host cell invasion (Hoppe et al., 2017). PilY1 is also involved in inhibition of fusion of the lysosome with the phagosome in the social amoebae *D. discoideum* and a mutant of PilY1 demonstrated reduced intracellular growth in macrophage (Shevchuk et al., 2014). The mechanisms facilitating host cell invasion are common in other intracellular pathogens. For example, a pathogenicity island in *Mycobacterium avium* encodes proteins inducing actin polymerization of host cells and mutants of this pathogenicity island are deficient in invasion of both macrophage and *A. castellanii* (Danelishvili et al., 2007).

After internalization, the intracellular survival of *L. pneumophila* largely depends on effectors secreted by the T4SS that have similar functions in amoebae and macrophage, probably due to the high similarity of mechanisms of phagocytic trafficking expressed by these two host cells. Most of the effectors work in a redundant manner to block phagosome-lysosome fusion and promote *L. pneumophila* intracellular survival. This has been summarized in earlier reviews (Finsel and Hilbi, 2015; So et al., 2015). Recently, one of the effectors, a cell membrane-localized iron transporter, IroT, was reported to be involved in ferrous iron acquisition and the mutant is defective in intracellular growth in *A. castellanii* and macrophage (Portier et al., 2015). Iron-dependent virulence is widely distributed among bacterial pathogens (Reinhart and Oglesby-Sherrouse, 2016; Butt and Thomas, 2017; Ramakrishnan, 2017). Although there are limited evidences showing that iron-regulated virulence factors contribute to grazing resistance, it has been reported that *E. coli* strains encoding genes for iron uptake survived better with *D. discoideum* than avirulent strains lacking these genes (Adiba et al., 2010). A recent report demonstrates that *E. coli* strains with mutations in iron uptake genes are attenuated for intracellular survival in *D. discoideum* (Hao et al., 2016). Another *L. pneumophila* T4SS effector, RidL, binds with the retromer (protein complex responsible for recycling transmembrane receptors from endosomes to the *trans*-Golgi network) of host cells and inhibits retrograde trafficking, resulting in better intracellular growth in both *D. discoideum* and macrophage (Finsel et al., 2013). In contrast, the Shiga toxin produced by *Shigella dysenteriae*, the cholera toxin produced by *Vibrio cholerae* and the exotoxin produced by *P. aeruginosa* rely on functional retrograde trafficking to enter the eukaryotic cell cytosol (Johannes and Popoff, 2008), indicating that the intracellular and extracellular pathogens have different strategies for interactions with host cells. Another effector, LegG1, activates the host cell GTPase, Ran on the LCV membrane, causing

FIGURE 1 | Mechanisms utilized by intracellular pathogens to avoid killing by macrophage and protozoa. (1) Intracellular pathogens utilize flagella and adhesins to invade host cells. (2) After internalization, various effectors are produced through multiple secretion systems, which inhibits the lysosome phagosome fusion. (3) Depending on nutrient conditions, bacteria replicate in phagosome and/or enter cytosol. (4) The intracellular pathogens are released by exocytosis or lysis of host cells.

polymerization of microtubules, allowing the LCV to move along the microtubules in host cells. A mutant of LegG1 exhibited compromised intracellular growth (Rothmeier et al., 2013; Hilbi et al., 2014). Interestingly, although the *L. pneumophila* infection generally inhibits chemotaxis of amoebae and macrophage, this inhibition is relieved by LegG1 expression. This may help host cells acquire nutrients from the environment, which then benefits *L. pneumophila* allowing for intracellular multiplication (Simon et al., 2014). Furthermore, many T4SS effectors encoded by the *L. pneumophila* genome are eukaryotic-like proteins which are believed to have been acquired during residence in protozoa, and these eukaryotic-like proteins are used as virulence factors to modulate protist host cell functions (Lurie-Weinberger et al., 2010; Gomez-Valero et al., 2011, 2014). Most of these proteins encode motifs that are involved in protein–protein interactions and mimic host proteins, thereby allowing control of host machinery and intracellular replication. These motifs include F-box, U-box, ankyrin and serine threonine protein kinase motifs.

In addition to the T4SS, the type II secretion system (T2SS) is also important for intracellular survival of *L. pneumophila* in protozoa and macrophage (Rossier et al., 2004) and the more than 25 exoproteins secreted by the T2SS are effective in a host specific manner (Cianciotto, 2009). For example, a T2SS secreted chitinase is responsible for persistence during lung infection in a mouse model of infection, but is not involved in survival in the amoeba, *Hartmannella vermiformis* or human macrophage-like cells (DebRoy et al., 2006). A mutation in a novel T2SS-dependant exoprotein, NttA, reduces intracellular survival in *A. castellanii*, but not other host cells, while the RNase, SrnA, acyltransferase, PlaC and metalloprotease, ProA promote multiplication of *L. pneumophila* in *H. vermiformis* and *Naegleria lovaniensis* only (Tyson et al., 2013).

L. pneumophila has a quorum sensing (QS) system consisting of an autoinducer synthase, LqsA, two sensor kinases, LqsS and LqsT, and a response regulator, LqsR, that controls virulence, motility, filament production, natural competence and the switch between the replicative and infectious forms (Kessler et al., 2013; Schell et al., 2014, 2016b; Personnic et al., 2017). A mutant of LqsR is defective for intracellular growth in *A. castellanii*, *D. discoideum* and macrophage (Tiaden et al., 2007). Interestingly, the autoinducer produced by LqsA, as well as its homologous cholera autoinducer-1 (CAI-1), inhibits the cell migration of *D. discoideum* and macrophage in a T4SS-independent manner, suggesting that these autoinducers play a role in interkingdom signaling (Simon et al., 2015).

Mycobacterium spp., including the causal agent of tuberculosis, *Mycobacterium tuberculosis*, uses similar strategies to infect and replicate in bacteria-containing vacuoles, such as inhibition of phagosome maturation (Awuh and Flo, 2017). It has been reported that *Mycobacterium marinum* can inhibit phagosome-lysosome fusion by inducing host WASH complex dependent actin polymerization in *D. discoideum* and macrophage, although the bacterial effector is still unknown (Kolonko et al., 2014). In *S. enterica* serovar Typhimurium, virulence factors [i.e., *waaL* responsible for ligation of O-antigen to LPS, *invA* and *ssaD* essential structural components of type 3 secretion systems (T3SS), *clpV* chaperone essential for protein secretion by type VI secretion systems (T6SS), and PhoP/PhoQ two-component system] were shown to be involved in intracellular survival in *D. discoideum* (Riquelme et al., 2016). Interestingly, *F. tularensis*, the causal agent of tularemia, exhibits different survival mechanisms in macrophage and amoebae (Ozanic et al., 2015). In macrophages, *F. tularensis* escapes from acidified phagosomes to the cytosol and then proliferates, while in *H. vermiformis*, it replicates in non-acidified phagosomes

(Santic et al., 2011). The Francisella pathogenicity island (FPI) encoding the T6SS is critical for survival in both hosts. One of the FPI proteins, IglC, a homolog of the T6SS Hcp protein (de Bruin et al., 2011), is responsible for escape from phagosome to cytosol (Santic et al., 2005) and invasion of non-phagocytic epithelial cells (Law et al., 2014). Mutants of IglC and the regulator MglA are defective for intracellular survival in both macrophage and *A. castellanii* (Lauriano et al., 2004).

Taken together, the intracellular pathogens possess many dual role factors that mediate their interactions with both protozoa and human phagocytes. Most of the similarities are found between amoebae and macrophage due to the conservative phagocytic process. Intracellular pathogens use surface factors to facilitate invasion and then rely on various effectors delivered by secretion systems to inhibit phagosome maturation, where many effectors are under precise regulation. These dual role mechanisms more likely evolved during the long-term interactions with protozoa in the environment, rather than during infections of human and animals. The temporal and spatial distribution of opportunistic pathogens in the environment between hosts, provides greater chances for them to face grazing pressure by bacterivorous protozoa. As a result, humans and animals become the accidental victims of coincidental evolution and further support the coincidental evolution hypothesis.
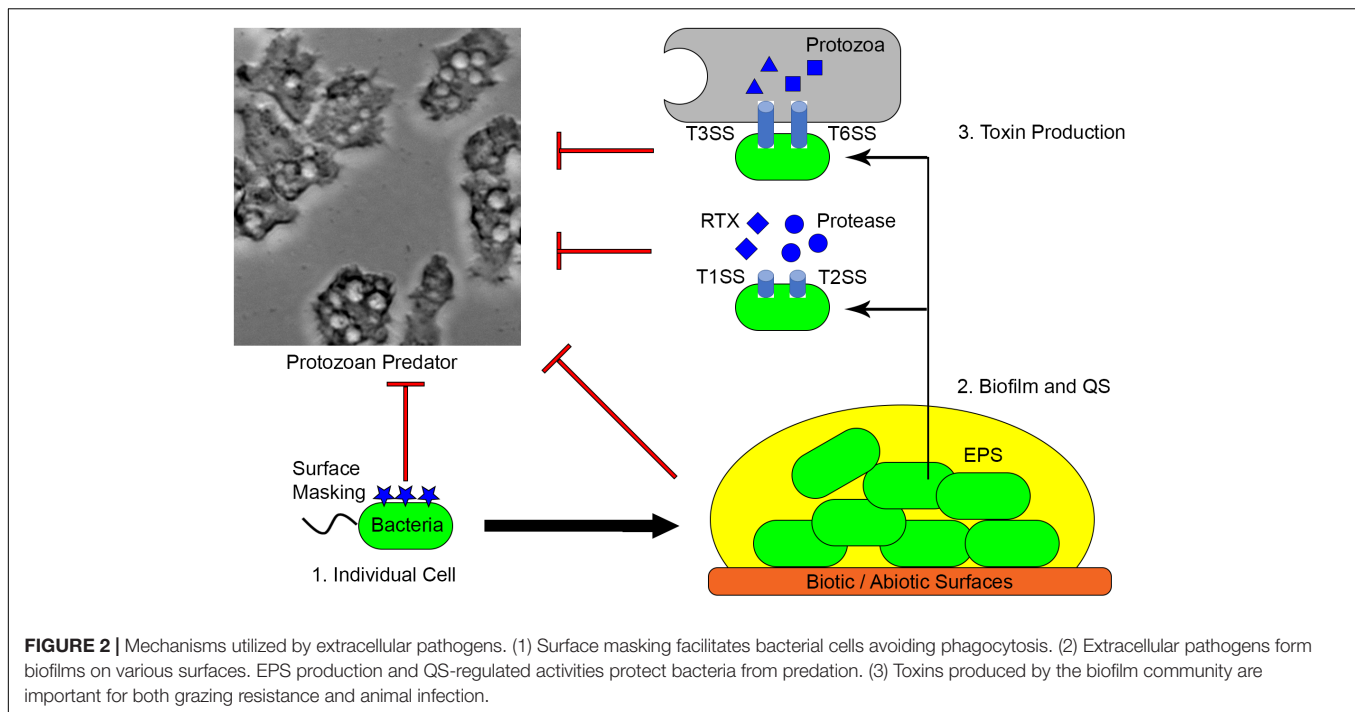
EXTRACELLULAR PATHOGENS

V. cholerae is the causal agent of cholera and is generally recognized as a model for non-invasive diarrheal disease (Sack et al., 2004). *V. cholerae* uses surface masking, toxin secretion and biofilm formation to defend against protozoan grazing and these mechanisms are also important for colonization and infection of humans (**Figure 2**) (Lutz et al., 2013). More than 200 serogroups classified by the lipopolysaccharide (LPS) O-antigen of *V. cholerae* exist, but only O1 and O139 have caused pandemics to date (Chatterjee and Chaudhuri, 2003). The *V. cholerae* LPS, or endotoxin, is an important virulence factor that mediates intestinal attachment and modulates host immunological responses (Chatterjee and Chaudhuri, 2006). The lipid-A of LPS has been proposed to inhibit phagocytosis by *T. pyriformis* (Kovacs et al., 1986). Similarly, production of LPS, capsular polysaccharide and outer membrane proteins protects *Klebsiella pneumoniae* from phagocytosis by *D. discoideum* and macrophage. Mutant strains of *K. pneumoniae* defective in the LPS core, lipid A, palmitoylation, OmpA and OmpK36 are susceptible to phagocytosis (March et al., 2013).

V. cholerae possesses multiple secretion systems that are important for pathogenesis and ecological fitness. The type I secretion system (T1SS) is responsible for delivery of RtxA, a multifunctional autoprocessing repeats-in-toxin (MARTX) toxin, which is involved in the colonization of the small intestine (Olivier et al., 2007) and destruction of host cell actin cytoskeleton (Prochazkova et al., 2009). MARTX is also secreted by the T1SS of *Vibrio vulnificus* and has cytopathogenic activity toward host cells, leading to rounding, cell death and interference

of internalization by host phagocytes (Lee et al., 2007; Kim et al., 2008). *V. vulnificus* RtxA1 can cause damage in a variety of ways, including generation of reactive oxygen species (ROS) (Chung et al., 2010), cytoskeletal rearrangement (Kim et al., 2008), apoptotic cell death (Lee et al., 2008), and interference with the cytosolic Ca^{2+} flux to inhibit phagocytosis (Kuo et al., 2015). *V. vulnificus* produces at least four different types of MARTX (types I–IV), where the plasmid-encoded MARTX type III (RtxA13) is structurally and evolutionarily different to MARTX types I and II (Kwak et al., 2011; Roig et al., 2011). MARTX type III of *V. vulnificus* biotype 2 is involved in the lysis of a wide range of eukaryotic cells, including amoebae, erythrocytes, epithelial cells, and phagocytes. For example, the amoebae *Neoparamoeba pemaquidensis* isolated and purified from turbot (*Scophthalmus maximus*) gills, grew significantly less in the presence of the wild-type strain compared to the $rtxA1_3$ mutant (Lee et al., 2013).

The T2SS of *V. cholerae* is involved in the secretion of cholera toxin (Sandkvist et al., 1997). A recent study has reported that *V. cholerae* protease, PrtV, is transported across the outer membrane by T2SS and then packed into outer membrane vesicles (Rompikuntal et al., 2015). *V. cholerae* PrtV, which causes instant cytotoxic effects during infection in human intestinal cell lines, is also toxic to predators such as the ciliate *T. pyriformis* and the flagellate *Cafeteria roenbergensis* (Vaitkevicius et al., 2006).

Although there are limited studies of T3SS-dependent virulence factors in *V. cholerae*, including VopE (Suzuki et al., 2014), VopF (Tam et al., 2007), and VopX (Alam et al., 2011), and the role of *V. cholerae* T3SS in grazing resistance has not been reported, T3SS has been shown to contribute to anti-protozoal activities in *E. coli* (Siddiqui et al., 2011), *P. aeruginosa* (Matz et al., 2008) and *Vibrio parahaemolyticus* (Matz et al., 2011). Interestingly, pathogenic *E. coli* strains (EHEC or STEC) encode a second T3SS, which may not be a functional secretion system, but is still important for virulence during infection and may be associated with Shiga toxin (Zhou et al., 2014). Shiga toxin is the major virulence factor in EHEC strains, but its role in grazing resistance is under debate. It has been reported that Shiga toxins of *E. coli* O157:H7 provides a survival advantage in *T. pyriformis* food vacuoles (Steinberg and Levin, 2007) and is cytotoxic to *A. castellanii* (Arnold and Koudelka, 2014) and *T. thermophila* (Stolfa and Koudelka, 2012). In contrast, there are also studies showing that Shiga toxin is not important in association of EHEC strains with *A. castellanii* (Chekabab et al., 2012) and may even decrease survival in the presence of *A. castellanii* (Chekabab et al., 2013). Virulence of EHEC strains depends on the induction of Shiga toxin-converting prophage. Bacteriophage-mediated lysis of Shiga toxin-encoding bacteria is necessary for toxicity in protozoa whereas digestion of bacteria by *T. thermophila* is harmless to the cell. Shiga toxin-converting prophage can be induced via treatment with H_2O_2. EHEC strains with mutations in bacterial SOS response genes exhibit increased susceptibility to grazing by *T. thermophila* (Lainhart et al., 2009; Stolfa and Koudelka, 2012). Oxidative stress as a consequence of H_2O_2 excretion by either neutrophils during infection or protist predators could potentially induce the production of Shiga toxin-converting prophage (Licznerska et al., 2016). A phage encoded toxin is also important for the anti-protozoal activity

FIGURE 2 | Mechanisms utilized by extracellular pathogens. (1) Surface masking facilitates bacterial cells avoiding phagocytosis. (2) Extracellular pathogens form biofilms on various surfaces. EPS production and QS-regulated activities protect bacteria from predation. (3) Toxins produced by the biofilm community are important for both grazing resistance and animal infection.

of *Corynebacterium diphtheriae*, as the diphtheria toxin kills *A. castellanii* during co-culturing (Arnold and Koudelka, 2014).

The T6SS of *V. cholerae* injects effector proteins into target cells by the action of a structure analogous to an intracellular membrane-attached contractile phage tail sheath (Basler et al., 2012; Ho et al., 2014). In *V. cholerae* non-O1/non-O139 strains, the T6SS has been shown to have toxic effects toward *D. discoideum* as well as mammalian macrophages (Pukatzki et al., 2006, 2007; Miyata et al., 2011). In addition to the direct role of T6SS in eukaryotic host pathogenesis by the injection of toxins responsible for actin crosslinking, the T6SS also has an indirect role in competition with neighboring bacteria (Dong et al., 2013; Ho et al., 2014).

Biofilm formation is important for both pathogenesis and environmental survival. Colonization of intestinal epithelial cells allows *V. cholerae* to deliver virulence factors effectively and provides resistance against various stresses, such as bile acids and antimicrobial peptides (Silva and Benitez, 2016). Biofilm formation also protects *V. cholerae* against predation while planktonic cells are eliminated. In addition, predation by protozoa promotes *V. cholerae* biofilm formation and induces a smooth to rugose morphotypic shift due to increased extracellular polysaccharide (EPS) production (Matz et al., 2005). EPS as the major component of the biofilm matrix, shields *V. cholerae* from predation by the surface feeding nanoflagellate, *Rhynchomonas nasuta* and *A. castellanii* (Sun et al., 2013). High cell densities within biofilms also allow accumulation of bioactive compounds. For example, production of pyomelanin plays a role in virulence factor expression and colonization by *V. cholerae* (Valeru et al., 2009). Pyomelanin production in *V. cholerae* biofilm also leads to increased ROS levels, which promotes resistance to predation by *A. castellanii* (Noorian et al., 2017).

The high cell density of *V. cholerae* biofilms enables QS, which regulates a number of virulence factors including motility, protease secretion, toxin production via the master regulator ToxR and biofilm formation (Zhu et al., 2002). Both PrtV and the T6SS are QS-regulated. A mutant in the *V. cholerae* QS master response regulator, *hapR*, resulted in reduced grazing resistance in comparison to the wild-type when exposed to *R. nasuta* (Matz et al., 2005). When biofilms of mixed *V. cholerae* wild-type and QS-deficient strains were exposed to predation, the QS mutant was selectively removed, indicating that QS also regulates *V. cholerae* surface components recognized by protozoa (Sun et al., 2013).

Similar to *V. cholerae*, biofilm formation increases *P. aeruginosa* resistance to host defenses and chemotherapy (Mulcahy et al., 2014). In addition, EPS and QS-regulated inhibitors are also responsible for grazing resistance of *P. aeruginosa* biofilms (Matz et al., 2004; Weitere et al., 2005). A newly identified *P. aeruginosa* modulator, GTPase TypA, is responsible for surface attachment and biofilm formation, resistance to antibiotics, and T3SS-dependent virulence. A mutant of TypA showed reduced resistance to *D. discoideum* grazing and increased uptake by macrophage (Neidig et al., 2013).

In contrast to intracellular pathogens, extracellular pathogens use surface factors to avoid internalization and produce various toxins to inhibit protozoan predators. Biofilm formation provides physical protection and accumulates anti-protozoal compounds. These dual role factors also enable extracellular pathogens to colonize epithelial surfaces and deliver toxins to host cells in an effective manner. Notably, the host cell receptors and targets of many dual role toxins are not always conserved between protozoa and human phagocytes, but nonetheless, are effective in both

instances. Thus, toxin production can lead to enhanced virulence during infections.

COINCIDENTAL EVOLUTION HYPOTHESIS

The coincidental evolution hypothesis suggests that virulence factors arose as a response to other selective pressures, such as predation, rather than for virulence *per se* (Levin, 1996; Adiba et al., 2010; Brown et al., 2012; Erken et al., 2013). There are many examples of virulence factors or their homologs that exist in environmental microorganisms that potentially play another functional role in the environment, including but not limited to grazing resistance. In the case of *V. cholerae*, it has been demonstrated that the T6SS of *V. cholerae* is functionally activated under high osmolarity and low temperature conditions, suggesting that the system may be important for the survival of the bacterium in the environment (Ishikawa et al., 2012). Furthermore, the cold shock gene, *cspV*, which controls biofilm formation through modulation of the second messenger cyclic-di-GMP, also regulates T6SS-mediated interspecies killing in a temperature-dependent manner (Townsley et al., 2016). A mutant in *cspV* showed significant defects for attachment and T6SS-mediated killing on the surface of the aquatic crustacean *Daphnia magna* (Townsley et al., 2016). In addition, chitin colonization is important for the long-term environmental persistence of *V. cholerae* (Pruzzo et al., 2008), where *V. cholerae* colonization factor GbpA (Kirn et al., 2005; Wong et al., 2012) and toxin co-regulated pilus (Reguera and Kolter, 2005) are involved in attachment to both chitin and epithelial surfaces.

The evolution of virulence driven by protozoan grazing has also been reported for fungi and viruses. *Cryptococcus neoformans* is a yeast that can cause lung infections in immunocompromised people and invades the brain by using host monocytes as "Trojan horses" (Charlier et al., 2009). Its ability to survive intracellularly has been proposed to have evolved for protection against amoeba predation (Steenbergen et al., 2001; Casadevall, 2012). Although the mechanisms of association are still unknown, *A. castellanii* has been reported to be the carrier or vectors for at least 2 human viruses, adenovirus (Scheid and Schwarzenberger, 2012) and coxsackie B3 virus (Mattana et al., 2006). For coxsackie B3 virus, an enrichment on *A. castellanii* surfaces was observed and the virus was eventually located inside the trophozoite (Mattana et al., 2006).

In some cases, grazing resistance mechanisms benefit the bacterium in association with a broad range of hosts. The methionine sulfoxide reductase can repair ROS-damaged proteins and are important for many bacterial pathogens (Sasindran et al., 2007). Mutation of methionine sulfoxide reductases in *Aeromonas hydrophila* reduces resistance to predation by *T. thermophila* and infection of zebrafish (Pang et al., 2016). Hemagglutinin protease which destroys host cell receptors for several different *V. cholerae* adhesins, is also involved in the degradation of chironomid egg masses (Halpern et al., 2003). Furthermore, QS-regulated production of hemagglutinin protease is important for defense against phages within high cell density bacterial populations (Hoque et al., 2016).

The bacterial–protozoal interaction has been suggested to be one of the oldest interactions between prokaryotic and eukaryotic organisms (Cavalier-Smith, 2002). During their long history of interaction, many virulence factors of bacterial pathogens evolved as adaptations to grazing pressure, rather than for pathogenesis to human and animal hosts. For example, a genome analysis of a *Chlamydia*-related amoebal symbiont indicates that many traits for intracellular survival, including the T3SS, existed in the bacterial genome 700 million years ago, 500 million years earlier than the appearance of Mammalia (Horn et al., 2004).

Our understanding of the pathogenesis of intracellular and extracellular pathogens is distorted due to our tendency to focus on the pathogen in the human host rather than in their natural environmental niche. The functions of virulence genes or their homologs in the environment have for the most part, not been fully explored. Here, we summarized the bacterial mechanisms providing grazing resistance and virulence in model intracellular and extracellular pathogens. For intracellular pathogens, active invasion, blocking of the phagosome lysosome fusion and resistance to phagocytic digestion are critical traits for infecting host cells. Extracellular pathogens, in contrast, rely on toxin secretion and biofilm formation as important mechanisms of protection against predation and pathogenesis. Some bacteria possess traits common in both intracellular and extracellular pathogens. For example, *L. pneumophila* can develop biofilms on environmental surfaces (Abdel-Nour et al., 2013) and *V. cholerae* has been found to proliferate inside of amoebae (Abd et al., 2005; Van der Henst et al., 2015). Since bacterial surface components are involved in the initial contact with host cells, possession of factors mediating active invasion may be used as a key feature to distinguish intracellular and extracellular pathogens. In conclusion, evolution of mechanisms that allow for survival within protozoa may have selected for traits that also allow bacteria to escape that harmful effects of phagocytes.

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