Shiga Toxin as a Bacterial Defense against a Eukaryotic Predator, *Tetrahymena thermophila*†

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Bacterially derived exotoxins kill eukaryotic cells by inactivating factors and/or pathways that are universally conserved among eukaryotic organisms. The genes that encode these exotoxins are commonly found in bacterial viruses (bacteriophages). In the context of mammals, these toxins cause diseases ranging from cholera to diphtheria to enterohemorrhagic diarrhea. Phage-carried exotoxin genes are widespread in the environment and are found with unexpectedly high frequency in regions lacking the presumed mammalian “targets,” suggesting that mammals are not the primary targets of these exotoxins. We suggest that such exotoxins may have evolved for the purpose of bacterial antipredator defense. We show here that *Tetrahymena thermophila*, a bacterivorous predator, is killed when cocultured with bacteria bearing a Shiga toxin (Stx)-encoding temperate bacteriophage. In cocultures with *Tetrahymena*, the Stx-encoding bacteria display a growth advantage over those that do not produce Stx. *Tetrahymena* is also killed by purified Stx. Disruption of the gene encoding the StxB subunit or addition of an excess of the nontoxic StxB subunit substantially reduced Stx holotoxin toxicity, suggesting that this subunit mediates intake and/or trafficking of Stx by *Tetrahymena*. Bacterially mediated *Tetrahymena* killing was blocked by mutations that prevented the bacterial SOS response (*recA* mutations) or by enzymes that breakdown H$_2$O$_2$ (catalase), suggesting that the production of H$_2$O$_2$ by *Tetrahymena* signals its presence to the bacteria, leading to bacteriophage induction and production of Stx.

Genes encoding bacterial exotoxins are frequently carried in bacteria by bacteriophages (4, 19). Phage-encoded exotoxins such as cholera toxin, diphtheria toxin, botulinum toxin, and Shiga toxin cause disease in mammals (4). Although these toxins do affect humans and other mammals, these phage-carried exotoxin genes are found at high frequencies in free phages and lysogenic bacteria that are isolated from environments where the presumed corresponding targets are not prevalent (6). These exotoxins kill eukaryotic cells by means of receptors and pathways that are generally conserved among eukaryotic organisms. These observations have led to the hypothesis that humans and other susceptible mammals are neither the original nor primary “targets” of these toxins (31).

If not mammals, then what are the true targets of these ubiquitous exotoxins? One clue may come from a consideration of the bacterial ecology and evolutionary biology. A major source of bacterial mortality is consumption by single-cell eukaryotic predators, such as ciliates and other protozoa (13). The coordinated release of exotoxins, at either the pre- or postdigestive state (31), could comprise one of the bacterium’s major antipredator defense strategies. Hence, humans may be innocent bystanders in the evolutionary battle between protozoans and their bacterial prey.

A family of phages strongly related to the well-characterized coliphage λ genes carry genes encoding Shiga-like exotoxin (Stx). Stx-encoding bacteriophages are associated with a broad range of hosts, including several serotypes of *Escherichia coli*, *Enterobacter cloacae*, *Shigella flexneri*, and *Citrobacter freundii*. One well-studied Stx-encoding strain is *Escherichia coli* O157:H7 strain EDL933. This strain was responsible for the first reported multistate U.S. outbreak of Shiga toxin-caused hemorrhagic colitis in 1982 (40). Related strains continue to cause serious food- and waterborne infections worldwide.

Analysis of the genome sequence of the EDL933 bacterial strain and the lambdoid bacteriophages liberated from it revealed that the disease-causing Shiga toxin is encoded by each of two lysogenic lambdoid prophages, 933W and 933J (32, 34, 36, 38). Lambdoid bacteriophages all share a common developmental program. Upon infection of a bacterial cell, the lambdoid phages choose between two developmental fates. The phage can grow lytically, thereby killing the host. Alternatively, in lysogenic growth, the phage chromosome inserts into the host chromosome and is replicated along with it until a signal that induces lytic growth is perceived by the lysogenized phages. Since transcription of the stx genes is under the control of a promoter that is active only during lytic growth (53), Stx is not produced when the toxin-encoding bacteriophages are in the lysogenic state. Inactivation of the respective bacteriophage repressors (53) causes lytic induction of the lysogenic phases, and exotoxin production increases substantially upon phage lysogen induction (27, 32, 34, 54). Stx is apparently not exported through any bacterial secretory machinery. Its release from the bacteria depends on phage genes that cause bacterial cell lysis (52, 53). These genes are also expressed only during phage lytic growth. Therefore, the Shiga toxin gene can reside harmlessly within the bacterial host until phage induction causes production and release of Shiga toxin.

Stx is a compound A-B$_5$ toxin, consisting of a 32-kDa A subunit and a pentameric 9.7-kDa B subunit. The five B subunits form a disulfide-bonded ring, into which is inserted the
C-terminal end of the A subunit (41–43, 45). Stx toxicity in mammalian cells is mediated by the binding of the B subunit of the holotoxin to its receptor, globotriaosylceramide (Gb3), on the surface of mammalian cells. Holotoxin enters mammalian cells by clathrin-dependent, receptor-mediated endocytosis (42). The B subunit directs the retrograde transport of the A subunit from endosomes to the Golgi apparatus and finally to the endoplasmic reticulum (22, 44), where the catalytic A subunit is cleaved from the holotoxin by furin and released into the cytoplasm (56). Once inside cells, the StxA subunit causes cell death by selectively removing the adenine from position 224 on 22S RNA, thereby inhibiting protein synthesis.

A major source of bacterial mortality is consumption by single-cell protozoan predators. Ciliates, including *Tetrahymena thermophila*, control bacterial densities in many ecosystems (3). Protozoan bacterivory has been shown to reduce bacterial numbers (47) and is therefore accepted as a pivotal process in microbial “population control.” Earlier investigations have demonstrated the utility of using *Tetrahymena* species and *E. coli* as a model predator-prey interaction for studying food web dynamics (23). *Tetrahymena* cells can be grown using only *E. coli* as a food source (23). Methods to quantify predation of bacterial cultures by *Tetrahymena* have been described (11, 49).

We propose that the Stx-encoding bacteriophages evolved to reside within the bacteria to function as part of their antipredator arsenal and that the presence of the toxin gene may confer an evolutionary advantage on the growth and survivability of this bacterial population by killing the predator. We tested this hypothesis by exploring how the presence of an exotoxin-encoding bacteriophage resident within bacteria influences the growth and survival of the bacterial population and a model unicellular eukaryotic predator, *Tetrahymena thermophila*.

A recent study compared the relative survival of Stx+ and Stx− bacteria in cocultures with *Tetrahymena pyriformis* (49). Consistent with a role of exotoxins in augmenting the fitness of bacterial populations that carry them, these investigators found that the ratio of Stx+ to Stx− bacteria increased under these conditions. However, the mechanism by which Stx enhances survivability was not clear from this study. Under the conditions used in those experiments, the presence of Stx-encoding phages increased the survivability of bacteria in food vacuoles inside *T. pyriformis*. However, the results also indicated that increased bacterial survival was largely independent of Stx expression. That study did not examine the effect of bacterial Stx production on the growth and survivability of the *Tetrahymena* predator, a factor that could profoundly influence bacterial survival. The focus of the work presented here is the effect of Stx on *Tetrahymena* viability.

We find that when cocultured with a model predator, *Tetrahymena thermophila*. Stx-encoding bacteria kill this predator. Stx-encoding strains are less efficiently predated than are strains that do not encode this exotoxin. We also found that *Tetrahymena* appears to release a factor that signals the bacteria that a predator is present and stimulates the production of toxin by the bacteria. The results are consistent with a role for bacterial exotoxins in the bacterial antipredator arsenal.
study, *T. thermophila*, acclimated to feed on non-phage-bearing W3110 bacteria, was washed and suspended in medium containing *Stx*′ or *Stx*″ bacteria. Under these culture conditions, *T. thermophila* growth requires the presence of bacteria. Consistent with the use of bacteria as food by *T. thermophila*, when a *Stx*″ bacterial strain that is lysogenized with wild-type bacteriophage λ, a phage that does not encode an exotoxin, is grown in the presence of W3110:λ, the number of *T. thermophila* cells approximately doubles within 6 h (Fig. 1A). In contrast, when *T. thermophila* is fed the Shiga toxin-encoding strain EDL933, the number of *T. thermophila* cells decreases to less than half of the input number of cells by 6 h (Fig. 1A). Hence, EDL933 does not support *T. thermophila* growth; instead, this bacterium causes *T. thermophila* death.

The killing of *T. thermophila* seen in the presence of the Stx-encoding bacteria (Fig. 1A) is not due to a reduction in the amount of their bacterial food source. Figure 1B shows that in coculture with *T. thermophila*, the amount of EDL933 increases over 6 h in coculture. In contrast, the number of W3110:λ cells decreases ~2-fold over the same time period. These observations are consistent with the idea that the presence of Stx-encoding bacteriophages is advantageous to a bacterial population, because when subject to *T. thermophila* predation, the Stx-encoding bacteria survive better than those that do not encode Stx.

Compared to W3110:λ, EDL933 contains approximately 1 Mb of additional DNA, much of which carries other pathogenicity-related genes in addition to *stx*. Thus, to more directly test the idea that Shiga toxin encoded by the lysogenized bacteriophage can kill *T. thermophila*, we determined whether an EDL933 strain bearing complete disruption/deletion of both the *stx*₁ and *stx*₂ gene clusters, EDL933Δstx (18), affects the growth of *T. thermophila*. Similar to the results presented in Fig. 1, when *T. thermophila* is fed the Shiga toxin-encoding strain EDL933, the number of *T. thermophila* cells decreases >2-fold over a 6 h incubation (Fig. 2A). In contrast when *T. thermophila* are cocultured with the EDL933Δstx strain, the number of *T. thermophila* cells increases by ~50% in this time period (Fig. 2A). These observations demonstrate that the killing of *T. thermophila* in cocultures with EDL933 is due to Stx encoded by this bacterial strain. Consistent with the findings in Fig. 1, in coculture with the *T. thermophila*, the number of EDL933Δstx bacteria decreases >2-fold over the 6-h incubation period, while the number of EDL933 bacteria increases slightly (Fig. 2B). This finding supports the idea that Shiga toxin can function as part of an antipredator defense strategy.

To directly demonstrate that Stx can kill *T. thermophila*, we determined whether addition of purified Stx2 could kill *T. thermophila* in axenic cultures. We found that addition of increasing concentrations of Stx2 progressively decreases the number of *T. thermophila* cells that survive after a 6-hour incubation with toxin (Fig. 3). Under our conditions, addition of 25 ng/ml of purified Stx kills ~90% of the cells in culture.
Similarly, partially purified Stx-containing extracts (33) obtained from mitomycin C-induced EDL933 cells also kill *T. thermophila* (not shown). These findings confirm that Shiga toxin is responsible for EDL933-mediated killing of *T. thermophila*.

We wished to delineate the factors that regulate the cytotoxic effect of Shiga toxin on *T. thermophila*. Stx entry into mammalian cells requires the binding of the B subunit of the holotoxin to its Gb3 receptor on the surface of mammalian cells. We reasoned that if the B subunit has a role in Stx-dependent *T. thermophila* killing, addition of excess StxB subunit should compete with holotoxin’s cytotoxic effects. Added partially purified B subunit alone does not exhibit any cytotoxic effect on *T. thermophila* cultures (Fig. 4), a finding that is similar to that obtained with mammalian cells (20, 29). Addition of an increasing weight of the partially purified StxB subunit in the presence of intact Stx progressively inhibits killing of *T. thermophila* caused by the added purified Stx. The inhibitory effect of added B subunit in Stx-mediated killing saturates at between 47 and 63 μg/ml. This finding implies that the added B subunit competes with binding of holo-Stx to specific receptor/trafficking sites in *T. thermophila*. The StxB blockade of holotoxin-mediated killing is specific; addition of similar quantities of BSA (Fig. 4) or boiled B subunit (not shown) does not inhibit Stx-mediated *T. thermophila* killing.

Figure 4 indicates that in order to interfere with Stx-mediated bacterial killing, an excess of StxB over the amount of Stx holotoxin must be added to the cultures. We do not yet know the nature or number of Stx holotoxin receptor/trafficking sites within *T. thermophila*. The requirement for “excess” StxB to block killing indicates that there many such sites, although the observation that the StxB inhibitory effect is saturable suggests that the number of sites is not infinite. Consistent with our findings, excess B subunit is also needed to block Stx holotoxin-mediated killing of mammalian cells (20, 30). Addition of substoichiometric ratios of Stx holotoxin to Gb3 receptor is sufficient to kill mammalian cells (35), and thus excess StxB subunit is needed to saturate the receptors to prevent killing. We speculate that a similar situation occurs with *T. thermophila*.

Nonetheless, regardless of the precise mechanism by which StxB blocks Stx-mediated *T. thermophila* killing, the data in Fig. 4 indicate that the B subunit has a role in mediating cytotoxicity by Stx holotoxin.

The results in Fig. 4 indicate that the B subunit mediates the cytotoxicity of added purified Stx. To determine whether the B subunit also plays a role in the cytotoxic effect of bacteria bearing Stx-encoding bacteriophages (Fig. 1 and 2), we cocultured *T. thermophila* with EDL933Δstx bearing either of two plasmids, one encoding Stx2 holotoxin and the other encoding only the StxA subunit, and measured the growth kinetics of *T. thermophila* and bacteria. When cocultured with EDL933Δstx bearing the Stx holotoxin-encoding plasmid (EDL933Δstx/pStxA), the number of *T. thermophila* cells decreases ~3-fold over a 6-h incubation. The efficiency of *T. thermophila* killing is slightly larger than that seen in cocultures with EDL933, presumably due to the increased production of Stx holotoxin from the plasmid. Consistent with the results presented in Fig. 1 and 2 and our proposal that Stx acts as an antipredator agent, the number of bacterial cells bearing pStxA increases in the presence of *T. thermophila*.

In contrast, *T. thermophila* are not killed when cocultured with EDL933Δstx bearing a plasmid that encodes only the StxA subunit (EDL933Δstx/pStxA). Instead, this *stxA* strain supports *T. thermophila* growth to a similar extent as do non-Stx encoding bacteria (Fig. 1). Also, the number of EDL933Δstx/pStxA cells decreases in the presence of *T. thermophila*, showing that these cells are more efficiently predated than are the *stxA* EDL933Δstx/pStxA cells. Control immunoblots indicate that identical amounts of StxA subunit are produced by the EDL933Δstx/pStxA and EDL933Δstx/pStxAB strains. These findings indicate that the B subunit is involved in killing of *T. thermophila* medi-
indicated. Tetrahymena present at start of incubation. remaining at end of the 6-h incubation relative to number of cells

That 933r, an otherwise isogenic host’s SOS response to DNA damage. Fuchs et al. (16) showed repressor autoproteolysis (9, 37, 46) that occurs during the inactivation of the phage repressor (53). The best-studied state. Lytic induction of lysogenic bacteriophages occurs upon the form of RecA that stimulates repressor autocleavage arises as a consequence of DNA damage (15). Such damage can be caused by numerous agents, including natural products (e.g., microbially produced antibiotics) and reactive oxygen species (ROS). We hypothesized that ROS released by the host’s SOS response to DNA damage. Fuchs et al. (16) showed that 933r, an otherwise isogenic recA mutant of EDL933, has severely reduced virulence in mice. Hence, we used 933r to examine whether the bacterial SOS response plays a similar role in mediating T. thermophila killing. In contrast to the results with wild-type EDL933, when T. thermophila is fed 933r, the number of T. thermophila cells remains unchanged. This finding indicates that recA mutation blocks the bacterially mediated killing of T. thermophila.

The form of RecA that stimulates repressor cleavage is important for the induction of the bacterial SOS response to DNA damage (15). Such damage can be caused by numerous agents, including natural products (e.g., microbially produced antibiotics) and reactive oxygen species (ROS). We hypothesized that ROS released by T. thermophila (14, 39) may indicate the presence of this predator in the absence of any superoxide dismutase (SOD) or catalase, as indicated. Tetrahymena cell counts are given as the number of live cells remaining at the end of the 6-h incubation relative to number of cells present at the start of incubation.

FIG. 5. Effect of antioxidant enzymes on the survival of EDL933 and Tetrahymena in cocultures. Cocultures containing 10^5 Tetrahymena cells/ml and 10^6 bacterial cells/ml were grown in the absence or presence of 4 μg/ml of either superoxide dismutase (SOD) or catalase, as indicated. Tetrahymena cell counts are given as the number of live cells remaining at the end of the 6-h incubation relative to number of cells present at start of incubation.

anted by Stx-encoding bacteria and is essential to its potential role as an antipredator defense molecule.

Shiga toxin is not produced by EDL933 as long as the resident toxin-encoding bacteriophages remain in their lysogenic state. Lytic induction of lysogenic bacteriophages occurs upon inactivation of the phage repressor (53). The best-studied mechanism of repressor inactivation involves RecA-stimulated repressor autoproteolysis (9, 37, 46) that occurs during the host’s SOS response to DNA damage. Fuchs et al. (16) showed that 933r, an otherwise isogenic recA mutant of EDL933, has severely reduced virulence in mice. Hence, we used 933r to examine whether the bacterial SOS response plays a similar role in mediating T. thermophila killing. In contrast to the results with wild-type EDL933, when T. thermophila is fed 933r, the number of T. thermophila cells remains unchanged. This finding indicates that recA mutation blocks the bacterially mediated killing of T. thermophila.

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The data presented here clearly show that Shiga toxin, either released from an induced lysogenic bacteriophage or added exogenously, is capable of killing the single-cell eukaryote Tetrahymena thermophila. To our knowledge, this is the first direct demonstration of Shiga toxin lethality in any protozoan or other bacterial predator. Our results also show that the presence of a Shiga toxin-encoding gene within bacteria decreases T. thermophila predation efficiency. Our findings are consistent with the results of a previous investigation showing that in the presence of Tetrahymena species, bacterial exotoxins augment the fitness of bacterial populations that carry them (49). Together with these previous findings (49), our results strongly suggest that Stx can function as an antipredator defense and support the hypothesis that this toxin may have originated in bacteria as an antipredator adaptation.

There is a growing body of evidence that predation by bacteriophore protozoa can play a large role in shaping the composition of bacterial populations (24, 25, 48, 55). The cytotoxic effect of a bacterial exotoxin on a protozoan predator indicates that these molecules can have important ecological functions within natural microbial communities. Therefore, future studies with T. thermophila can provide information about the evolution and function of bacterioexotoxins in ciliate predators.

Two of our findings suggest that various aspects of the interaction between Shiga toxin-encoding bacteria and mammalian cells have an ancient evolutionary origin in the predator-prey interaction between these bacteria and protozoans. First, the finding that EDL933 bacteria appear to sense the presence of the T. thermophila by detecting the presence of ROS and respond by inducing the synthesis and release of Stx may represent a foreshadowing of the situation found in the mammalian response to bacterial infection. Consistent with the findings of others (14, 39), we have observed that T. thermophila releases H₂O₂ into the medium (data not shown). Similarly, ROS such as H₂O₂ or superoxide, generated and released by leukocytes and neutrophils, activate the SOS response in Shiga toxin-encoding E. coli, leading to toxin release (51) and subsequent death of the “attacking” eukaryotic cell. Since H₂O₂ is present in internal vesicles of Tetrahymena species (14), it is also possible that bacteriophage induction occurs inside the cells after the bacteria are eaten.

Second, similar to its role in mammalian cell killing, we found that the B subunit plays an essential role in Stx-mediated T. thermophila killing. In mammalian systems, Shiga toxin is produced outside of the cell as a consequence of lytic growth of Stx-encoding bacteriophage. Stx entry into mammalian cells occurs by receptor-mediated endocytosis, which requires the binding of the holotoxin’s B subunit to the neutral glycolipid Gb3 receptor. Mammalian cells lacking this glycolipid are immune to the toxin (7). Tetrahymena has all of the machinery for
clathrin-mediated endocytosis (12, 21). Therefore, *Tetrahymena* could use a clathrin-dependent receptor-mediated endocytosis mechanism to import Shiga toxin. However, exhaustive studies performed in our labs and in the laboratories of Clifford Lingwood at the University of Toronto (personal communication) failed to identify any Stx binding lipids in the glycolipid-containing fraction. Moreover, inspection of the *T. thermophila* genome sequence (8, 10) failed to identify any close homologues of the gene that encodes the mammalian Gb3 synthase enzyme (26). These observations are inconsistent with Stx entry into *Tetrahymena* occurring by “standard” Gb3 receptor-mediated endocytosis. Nonetheless, our findings showing that excess B subunit blocks the killing of *T. thermophila* by purified Stx2 holotoxin (Fig. 4) and that expression of the B subunit is required for *T. thermophila* intoxication suggest that alternative Stx receptors could be present in these cells. *Tetrahymena* cells can ingest particulate and soluble nutrients through the oral apparatus and funnel them into their food vacuoles. Hence, in the absence of a Shiga toxin receptor, uptake of either released Stx or Stx-encoding bacteria via *Tetrahymena*’s oral cavity might provide an alternative route or the sole route for toxin entry into these cells. This ability to capture bacteria through their oral apparatus could provide a novel route for toxin entry into *Tetrahymena* cells. If this idea is correct, it suggests that the B subunit affects Shiga toxin cytotoxicity in *Tetrahymena* by influencing intracellular trafficking.

Considering that the lysogenic bacterial host must die to release the Shiga toxin, the fitness benefits of this antipredator defense mechanism cannot accrue to the individual organism but would be to the overall bacterial population, a population that would include cells that are not lysogenic for the toxin-encoding bacteriophage. At first glance this strategy may seem overly “altruistic.” However, the genes encoding this exotoxin are found on mobile, temperate bacteriophages. Due to the high propensity to grow lysically (28), the phage has limited ability to lysogenize naive hosts. The propensity of the phage to lytically infect, and thereby kill, the naive hosts would restrict the benefit of Shiga toxin antipredator activities for the fitness of this segment of the bacterial population. It should be noted that lytic growth in the naive hosts would increase the amount of Stx produced, amplifying the killing capacity of the original sacrificed cell. The ability of nonlysogens to enhance Stx production by a bacterial population has already been demonstrated (17).

Hence, the presence of toxins genes, in this case Shiga toxin genes, in the temperate phages and linkage of toxin expression to lytic infection may provide substantial advantages to bacterial lysogens that “choose” to harbor these phages. Utilization of an exotoxin encoded on an inducible phage is then a cost-effective defensive strategy for the bacterial population. These lysogenic populations sacrifice a few cells to produce a toxin that kills its major predator and produce infectious phage that have the potential to eliminate bacterial competitors (53).

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