Entry and Killing of *Tetrahymena thermophila* by Bacterially Produced Shiga Toxin

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**ABSTRACT** Phage-encoded Shiga toxin (Stx) acts as a bacterial defense against the eukaryotic predator *Tetrahymena thermophila*. It is unknown how Stx enters *Tetrahymena* protozoa or how it kills them. *Tetrahymena* protozoa are phagocytotic; hence, Stx could gain entry to the cytoplasm through the oral apparatus or via endocytosis. We find that Stx2 can kill *T. thermophila* protozoa that lack an oral apparatus, indicating that Stx2 can enter these cells via endocytosis. As opposed to the lack of effect on mammalian phagocytes, Stx2 produced by bacteria encapsulated within phagocytic vesicles is also capable of killing *Tetrahymena*. Addition of an excess of the carbohydrate binding subunits of Stx2 (StxB) and/or ricin (ricin B) blocks Stx2 cytotoxicity. Thus, regardless of whether Stx2 enters the cytoplasm by endocytosis or from the phagocytic vesicle, this transport is mediated by a putative glycoconjugate receptor. Bacteriophage-mediated lysis of Stx-encoding bacteria is necessary for Stx toxicity in *Tetrahymena*; i.e., toxin released as a consequence of digestion of bacteria by *Tetrahymena* is harmless to the cell. This finding provides a rationale as to why the genes encoding Stx are found almost exclusively on bacteriophages; Stx must be released from the bacteria prior to the digestion of the cell, or it will not be able to exert its cytotoxic effect. It also suggests a reason why other bacterial exotoxins are also found only on temperate bacteriophages. Incubation of *Tetrahymena* with purified Stx2 decreases total protein synthesis. This finding indicates that, similar to mammalian cells, Stx2 kills *Tetrahymena* by inactivating its ribosomes.

**IMPORTANCE** *Tetrahymena* is a bacterial predator and a model for mammalian phagocytosis and intracellular vesicular trafficking. Phage-encoded exotoxins apparently have evolved for the purpose of bacterial antipredator defense. These exotoxins kill mammalian cells by inactivating universally conserved factors and/or pathways. *Tetrahymena* and susceptible mammalian cells are killed when exposed to bacteriophage-encoded Shiga toxin (Stx). Stx toxicity in mammalian cells requires Stx binding to the globotriaosyl ceramide (Gb3) receptor, followed by receptor-mediated endocytosis (RME). We show that, similar to mammalian cells, internalized Stx inhibits protein synthesis in *Tetrahymena*. Although *Tetrahymena* lacks Gb3, our results suggest that the cytotoxic effect of Stx on *Tetrahymena* is apparently mediated by a receptor, thereby arguing for the existence of RME in *Tetrahymena*. As opposed to the case with mammalian phagocytes, Stx produced by bacteria inside *Tetrahymena* is cytotoxic, suggesting that these cells may represent a “missing link” between unicellular eukaryotic bacterial predators and phagocytic mammalian cells.

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With approximately 10\(^{31}\) individuals, bacteriophages are likely the most abundant organisms on the planet (1, 2). Bacteriophages often bear genes encoding exotoxins (e.g., Shiga toxin [Stx], diphtheria toxin, cholera toxin, and botulinum toxin) which cause disease in mammals (3). While these phage-encoded toxins harm mammals, these phages can be prevalent where mammals are not found (4). Our earlier work demonstrated that, similar to its effect on mammalian cells, both bacterially produced and purified Stx is capable of killing *Tetrahymena thermophila*. That work also demonstrated that Stx is capable of functioning as part of an antipredator defense strategy, killing *Tetrahymena* and reducing its predation efficiency, allowing the bacteria to survive (5). Hence, susceptible mammals may not be the original or primary “targets” of this exotoxin or others.

Stx is a family of homologous AB\(_5\) exotoxins that share the same enzymatic activity (6, 7). They comprise the enzymatically active (StxA) subunit and a pentamer of receptor binding subunits (StxB), which is necessary for entry and trafficking of the toxin (8). Stx was first identified in *Shigella dysenteriae* serotype 1, but closely related variants are found in *Escherichia coli* and similar bacteria (e.g., Stx1, Stx2, Stx2c to -f) (6, 9–11). Regardless of the source, Stx holotoxin enters mammalian cells by binding a glycosphingolipid, globotriaosyl ceramide (Gb3), present on the cell surface. Gb3 is a glycosphingolipid characterized by Gal(α1-4) Gal(β1-4) Glc(β1-8) ceramide linkages (12). Recognition and binding of the Gal(α1-4) Gal linkage by StxB (6, 9–11). Regardless of the source, Stx holotoxin enters mammalian cells by binding a glycosphingolipid, globotriaosyl ceramide (Gb3), present on the cell surface. Gb3 is a glycosphingolipid characterized by Gal(α1-4) Gal(β1-4) Glc(β1-8) ceramide linkages (12). Recognition and binding of the Gal(α1-4) Gal linkage by StxB (12) and subsequent aggregation of bound Gb3 initiates clathrin-mediated endocytosis (CME) of Gb3-Stx complexes in susceptible mammalian cells (13). Once inside the cell, the Stx-containing endosome undergoes retrograde transport through the Golgi apparatus and into the endoplasmic reticulum (14, 15), where the StxA subunit is cleaved from the holotoxin by furin, leading to release of the StxA...
subunit into the cytosol. The activated Stx subsequently removes a critical adenine residue from the rRNA amino-acyl tRNA-accepting (sarcin-ricin) loop, inactivating the ribosome and eventually killing the cell (16). The position, function, and sequence of this loop are conserved among virtually all organisms, including *Tetrahymena* (17, 18).

Our previous investigations of Stx toxicity to *Tetrahymena* did not determine the mode of entry of Stx or the mechanism by which it kills the organism (5). This information could provide insight into processes related to the evolution of Stx toxicity. *T. thermophila* encodes all of the machinery needed for CME (19). Stx could enter *Tetrahymena* through the plasma membrane by receptor-mediated or non-specific clathrin-mediated endocytosis. Importantly, these cells encode an elaborate set of Rab proteins, suggesting that they are capable of internalizing and appropriately sorting Stx-containing vesicles resulting from CME (20). Protoplasts are also phagocytic and share many features with mammalian phagocytes, especially macrophages. However, macrophages are not susceptible to killing by bacterially produced Stx (21–23), instead responding to Stx intoxication by releasing proinflammatory cytokines.

*T. thermophila* takes up bacteria, nutrients, and other molecules through its oral apparatus, encapsulating these in food vacuoles. Bacteria ingested by *Tetrahymena* remain viable in food vacuoles for a considerable length of time (24, 25). Therefore, it is formally possible that viable Stx-encoding bacteria internalized in food vacuoles by *Tetrahymena* are able to produce toxin that is lethal to this organism. Hence, as opposed to mammalian cells killed by Stx, lethal toxin could enter *T. thermophila* by either or both of two non-mutually exclusive methods, i.e., (i) plasma membrane-mediated endocytosis of cell-free toxin and/or (ii) uptake of Stx-encoding bacteria or cell-free Stx via the oral apparatus.

We showed previously that excess purified StxB specifically prevents *Tetrahymena* killing by purified Stx2 (5). Moreover, preliminary findings indicated that StxB is necessary for the toxicity of bacterially produced Stx to *Tetrahymena* (5). Since StxB is the receptor binding subunit of Stx, these findings suggested that at least part of Stx’s intoxication mechanism is mediated by the binding of Stx to a receptor. Regardless, the nature and location of this putative receptor in *Tetrahymena* are unclear. Moreover, it is unclear whether toxin released from internalized bacteria can also kill these organisms and whether this entry mechanism also involves a receptor.

Once inside mammalian cells, activated Stx inhibits protein synthesis and causes cell death (16). While Stx kills *T. thermophila*, we do not know if it does so in the same way as it does mammalian cells. It has been shown that Stx, though at a very low affinity, inactivates bacterial ribosomes (26), resulting in the death of bacteria that overproduce Stx. *Tetrahymena* ribosomes are more similar to mammalian ribosomes than bacterial ribosomes are and are therefore very likely to be inactivated by Stx.

Here we explore the mechanisms by which Stx enters *Tetrahymena* and how the internalized toxin kills these organisms. Our findings indicate that Stx added to or produced by bacteria in the culture medium enters *Tetrahymena* via a specific receptor. Our data also suggest that this putative receptor is a membrane-bound glycoconjugate. We also show that Stx produced by bacteria encapsulated within food vacuoles is capable of killing *Tetrahymena*. Transport of Stx produced in the food vacuoles to the cytoplasm is also apparently mediated by a glycoconjugate receptor. We find that Stx is a powerful inhibitor of protein synthesis. Although we have not defined the details of how Stx exerts its cytolytic effects, we anticipate that, similar to mammalian cells, this inhibition of protein synthesis leads to cell death.

**RESULTS**

Our previous results (5) showed that purified bacterially encoded Stx kills *Tetrahymena* (strain CU427.4). *A priori*, Stx could enter *Tetrahymena* cells via two alternative, non-mutually exclusive routes. One route entails nonspecific phagocytosis of Stx by *Tetrahymena*; the other involves Stx binding to a cell surface receptor, followed by receptor-mediated endocytosis. Our preliminary results suggested that purified Stx2 enters *T. thermophila* cells via a specific receptor and that this entry is mediated by StxB. We wished to confirm this idea. To do this, we compared the abilities of two different bacterial strains, one of which bears a wild-type 933W prophage that encodes both the A and B subunits of Stx2 (MG1655::933W) and the other of which bears a mutant 933W prophage in which the gene encoding the B subunit has been disrupted (MG1655::933WAB) and hence encodes only the Stx2A subunit. Control immunoblot assays showed that the same amount of the StxA subunit is produced by the two strains (data not shown). When *T. thermophila* is cocultured with MG1655::933W, the number of *T. thermophila* cells is reduced by approximately 60% (Fig. 1A) compared to that of cells that are incubated in the absence of any bacteria. In contrast, the number of *Tetrahymena* cells increases by nearly 2-fold when they are cocultured with MG1655::933WAB. This finding indicates that the StxA subunit alone is insufficient to intoxicate *T. thermophila* and that *Tetrahymena* killing requires both the A and B subunits. This finding is consistent with the suggestions that Stx2 enters *T. thermophila* cells via a specific receptor and that this entry is mediated by StxB.

To further test the idea that Stx intoxication results from toxin binding to a specific receptor in *T. thermophila*, if such a receptor exists, we reasoned that the presence of excess StxB, the receptor binding subunit of Stx, should act as a competitor for Stx holotoxin-mediated killing of *Tetrahymena*, whereas a nongenomic protein would not. To do this, we incubated *T. thermophila* with Stx2 holotoxin in the presence or absence of StxB. Consistent with previous observations, when *Tetrahymena* cells are grown in the presence of purified Stx2 and an ~80-fold molar excess of BSA (bovine serum albumin) the number of viable *Tetrahymena* cells decreases. In contrast, when *Tetrahymena* cells are incubated with purified Stx2 and an ~80-fold molar excess of the StxB subunit, the number of viable *Tetrahymena* cells increases (Fig. 1B), indicating that StxB competes with Stx2 for entry into *Tetrahymena* cells. Our preparation of the StxB subunit contains no proteolytic activity (data not shown). Hence, the ability of StxB, but not BSA, to protect *T. thermophila* from Stx2-induced killing is consistent with the idea that entry of Stx into *Tetrahymena* cells is mediated by a receptor. We hypothesize that each cell bears a number of these receptors, requiring an excess of StxB over Stx2 to block Stx binding and entry into *Tetrahymena* cells.

To further examine the mode of entry of Stx2 into *Tetrahymena* cells, we assessed the ability of Stx2 to kill *Tetrahymena* strain NP1, a temperature-sensitive mutant strain of *T. thermophila*. When grown at the permissive temperature (30°C), NP1 forms a normal oral apparatus; however, at the restrictive temper-
ature (37°C), this strain lacks an oral apparatus (27) and consequently is unable to perform phagocytosis. This leads to the observed smaller size of NP1 at the restrictive temperature (Fig. 2). Consistent with the reported NP1 phenotype (27), at 30°C, this strain efficiently ingests bacteria, as indicated by the presence of mCherry-labeled *E. coli* strain MG1655 in the food vacuoles inside these cells (Fig. 2A). When grown at 37°C, NP1 does not ingest bacteria by phagocytosis, as indicated by the absence of labeled bacteria inside these cells, and does not form food vacuoles (Fig. 2B). Hence, at 37°C, the only route for compounds to enter *Tetrahymena* NP1 cells is via endocytosis. If Stx2 is capable of killing *Tetrahymena* cells at 37°C, this finding would indicate that Stx2 can enter *Tetrahymena* cells by endocytosis. This finding would be consistent with the suggestion that Stx2 enters *Tetrahymena* cells via a specific membrane-bound receptor.

Cells of both wild-type *Tetrahymena* strain CU427.4 and mouthless strain NP1 increase in number when incubated for 6 h in the presence of BSA at 30°C, but NP1 grows to a higher density under these conditions (Fig. 3A). Consistent with the cytotoxic effect of Stx shown previ-
bacteria requires phage-mediated lysis of the cell—it is not se-
stx2 but a receptor or receptor family specific to toxins that bind
putative stx2 receptor in
receptor or family of receptors. This finding also implies that the
cates that stx and ricin share the same putative glycoconjugate
40 ng/ml stx2 when 2

cells after incubation relative to the number of live

data are presented as the number of live tetrahymena cells at the
start of incubation. Error bars represent standard deviations from three or
more independent experiments, with each experiment comprising a mini-
imum of three individual measurements. *, \( P < 0.01 \).

![FIG 4](image)

**FIG 4** Ricin B protects *Tetrahymena* from stx2-mediated killing. *Tetrahymena*
cells were axenically cultured with 40 ng/ml BSA, stx2 holotoxin, or
40 ng/ml ricin B subunit alone or 40 ng/ml stx2 holotoxin in the presence of
2 \( \mu \)g/ml stxB or ricin B as indicated. The number of live *Tetrahymena* cells was
determined after 6 h. Data are presented as the number of live *Tetrahymena*
cells after incubation relative to the number of live *Tetrahymena* cells at the
start of incubation. Error bars represent standard deviations from three or
more independent experiments, with each experiment comprising a mini-
imum of three individual measurements. *, \( P < 0.01 \).

![FIG 5](image)

**FIG 5** Elimination of phagocytosis does not affect killing of *Tetrahymena* by
bacterially produced stx. *Tetrahymena* strain NP1 was cocultured with
EDL933 or EDL933Δstx at 30°C (black bars) or 37°C (white bars). Cultures
were prepared as described in Materials and Methods. Data are expressed as
n-fold changes in cell numbers at the end of a 6-h incubation relative to the
beginning of the incubation. At the start of the incubation, *Tetrahymena* cells
were present at approximately 10^4/ml and bacterial cells were present at 10^8/
ml. Error bars represent standard deviations from three or more independent
experiments, with each experiment comprising a minimum of three individual
measurements. *, \( P < 0.01 \); **, \( P < 0.001 \).

ously (5) and in Fig. 1B, the presence of purified Stx2 inhibits the growth of both of these *Tetrahymena* strains (Fig. 3A) at 30°C. At
37°C, where NP1 lacks the oral apparatus, the number of viable
*Tetrahymena* strain NP1 cells remains static when they are incu-
bated with BSA. Under these conditions, the number of wild-type
*Tetrahymena* cells also remains static. Incubation of NP1 with
purified stx2 at 37°C reduces the number of viable *T. thermophila*
strain NP1 cells by approximately 60% (Fig. 3B). Under identical
conditions, stx2 also similarly decreases the viability of *Tetrahymena*
strain CUL427.4. The ability of Stx2 to kill mouthless *Tetra-
hymena* strain NP1 at the restrictive temperature indicates that Stx
intoxication does not require toxin entry via phagocytosis. This
finding suggests that Stx2 is able to enter *Tetrahymena* cells
through the cell membrane. Stx’s ability to get into *Tetrahymena*
cells through the cell membrane, combined with the finding that
StxB specifically protects against stx2-mediated killing, argues that
*T. thermophila* membranes contain a receptor for Stx.

We wished to gain further insight into the nature of *Tetra-
hymena*’s putative Stx receptor. Since stx2 intoxication of *Tetra-
hymena* requires its B subunit (Fig. 1A) and this subunit binds car-
bohydrates, we postulated that the receptor may be a
glycoconjugate. To test this suggestion, we incubated
*Tetrahymena* cells with the glycolipid binding subunit of ricin, ricin B
(28), and determined whether it could protect *Tetrahymena* cells
from stx2-mediated killing. As shown in Fig. 1B, when incubated
with 40 ng/ml stx2, the number of viable *Tetrahymena* cells de-
creased in the presence of 2 \( \mu \)g/ml BSA but not in the presence of
2 \( \mu \)g/ml stxB. Similar to the findings with StxB, the number of
viable *Tetrahymena* cells was unaffected by the presence of
40 ng/ml stx2 when 2 \( \mu \)g/ml of ricin B was also present (Fig. 4).
Hence, ricin B protects against stx2-mediated killing. The protec-
tion of *T. thermophila* from stx2-mediated killing by ricin B in-
idicates that Stx and ricin share the same putative glycoconjugate
receptor or family of receptors. This finding also implies that the
putative stx2 receptor in *T. thermophila* may be specific not to
Stx2 but a receptor or receptor family specific to toxins that bind
to similar sugar moieties.

Release of Stx holotoxin from EDL933 or other Stx-encoding
bacteria requires phage-mediated lysis of the cell—it is not se-
creted through any bacterial secretory machinery (29). Therefore,
toxin-encoding bacteria in coculture with *T. thermophila* have two
potential routes by which they can deliver toxin into *Tetrahymena*
cells. One route is by bacterial lysis, releasing Stx2 outside the
*Tetrahymena* cell, followed by its entry via endocytosis. Alterna-
tively, bacterially derived toxin may enter *Tetrahymena* cells when
Stx2-encoding bacteria are eaten by *Tetrahymena* cells and these
bacteria, encapsulated within the food vacuoles, lyse, releasing
toxin. We showed previously that *Tetrahymena* releases a factor
that can stimulate SOS-dependent induction of Stx-encoding
prophage (5).

We first determined whether bacterially derived Stx can enter
through the cell membrane via endocytosis. To do this, mouthless
*Tetrahymena* strain NP1 (27) was cocultured with stx1- and
stx2-encoding EDL933 or an stx1 stx2 mutant strain, EDL933Δstx,
which is identical to EDL933 except that its toxin-encoding genes
are inactivated by disruption/deletion (30). The number of viable
*Tetrahymena* NP1 cells in coculture with EDL933 decreased by
approximately 40% at 30°C, the permissive temperature for the
formation of the oral apparatus, and by 60% at the restrictive
temperature (37°C), where NP1 lacks the oral apparatus (Fig. 5).
In contrast, the number of viable NP1 cells in coculture with
EDL933Δstx either increased at 30°C or remained static at 37°C
(Fig. 5). The ability of EDL933 to decrease the number of NP1 cells
at 37°C indicates that entry of Stx through the putative *Tetra-
hymena* cell membrane receptor is a potential route for bacterially
derived Stx.

Although bacterially derived Stx apparently can enter *Tetra-
hymena* cells through the cell membrane (Fig. 5), it is possible that
ingestion of toxin-encoding bacteria and the release of Stx in the
food vacuoles provide an efficient route for Stx entry into *T. ther-
omophila*. If this route is to be effective, some of the bacteria in-
gested by *Tetrahymena* and encapsulated in food vacuoles (Fig. 2)
must remain viable as they pass through the cell. To test this idea,
we followed separately the transit of fluorescently labeled EDL933
and EDL933Δstx bacteria through the cell by microscopy. We found that, consistent with previous results (31), these encapsulated bacteria transited though the cell and were expelled via the cytostome over approximately 90 min (Fig. 6). While there were differences in the survival of EDL933 and EDL933Δstx during their passage through the cells, in both cases, only some of the ingested bacteria were digested and/or lysed during transit. At 60 min, between 20 and 40% of the labeled bacteria remained intact and at 90 min postgestion, 10 to 30% of the labeled bacteria remained intact and within *Tetrahymena* cells.

Since bacteria apparently survive within *Tetrahymena* food vacuoles, it is possible that bacterially derived Stx2 can be released from these organelles and enter the cytoplasm of *Tetrahymena*, causing cell death. To begin to test this idea, we designed an *E. coli* strain that, when transformed with an Stx2-producing plasmid, allows Stx2 to be released only when the bacteria are encapsulated within *Tetrahymena*. This strain, MG1655 (recA)::AcI857 bears a recA mutation and is lysogenized with bacteriophage AcI857. This phage bears a temperature sensitivity mutation in its repressor protein. Consequently, while it is able to form stable lysogens at permissive temperatures (30°C), this phage is unable to maintain lysogeny at temperatures above 33°C. Since any *E. coli* strain that contains a lysogenic prophage can be induced to grow lytically by RecA-mediated bacteriophage repressor autocleavage (32), either stochastically (33) or as a consequence of DNA damage, the presence of the recA mutation ensures that the prophage is not released upon induction. Therefore, the combination of this mutation and bacteriophage AcI857 allows us to induce Stx2 production only from bacteria encapsulated within *Tetrahymena* cells.

We transformed MG1655 (recA)::AcI857, which does not encode Stx, with pStx, a plasmid that directs the constitutive synthesis of low levels of Stx2 holotoxin. Control immunoblot assays indicate that this plasmid produces ~4,500 molecules of intact Stx per cell (i.e., lysis of a saturated overnight culture will contain Stx2 at a concentration of 66 ng/ml). Stx2 is not secreted through any bacterial secretory machinery. Its release from the bacteria depends on phase genes that cause bacterial cell lysis (29). Consequently, at permissive temperatures, the Stx2 constitutively produced within MG1655 (recA)::AcI857 cannot be released from the cell. Alternatively, at the restrictive temperature, MG1655 (recA)::AcI857/psStx lyses readily, releasing the Stx contained within the cell. Control experiments done previously have shown that in MG1655-based AcI857 lysogens are completely lysed within 30 min of incubation at restrictive temperatures (data not shown). We also transformed MG1655 (recA)::AcI857 with the empty vector for use as a control. As another control, we constructed MG1655 (recA)/pStx. Since this strain lacks an inducible prophage, Stx2 would not be released from it unless the cell is killed and lysed by some other means.

These strains, MG1655 (recA)/pStx, MG1655 (recA)::AcI857/pET17b, and MG1655 (recA)::AcI857/psStx, were separately co-cultured with *T. thermophila* strain CU427.4 at the permissive temperature of 30°C for 30 min. Microscopic examination indicates that under these conditions, bacteria fluorescently labeled with mCherry are found in the food vacuoles of approximately 90% of the *Tetrahymena* cells in the culture (data not shown). Subsequently, the free bacteria were washed away by differential centrifugation as described in Materials and Methods. After washing, the cultures were shifted to 42°C and incubated for an additional 4 h.

The number of viable *Tetrahymena* cells co-cultured with MG1655 (recA)::AcI857/pET17b doubled over the incubation period (Fig. 7). In contrast, when MG1655 (recA)::AcI857/psStx cells were co-cultured with *Tetrahymena* cells, the number of viable *Tetrahymena* cells decreased by 40% (Fig. 7). This result is consistent with the idea that bacterially derived Stx is capable of entering the cytosol from the food vacuoles and killing *Tetrahymena* cells.

The number of viable *Tetrahymena* cells incubated with MG1655 (recA)/pStx increased by 50% (Fig. 7). The only difference between this strain and MG1655 (recA)::AcI857/psStx is the presence of the stx prophage.

**FIG 6** Persistence of intact EDL933 and EDL933Δstx in *Tetrahymena*. *Tetrahymena* cells were separately co-cultured with EDL933 (●) or EDL933Δstx (◆), each of which was transformed with a plasmid expressing the red fluorescent protein mCherry. Bacteria not internalized by *Tetrahymena* were removed, and the fluorescence intensity remaining within the *Tetrahymena* cells was quantified at various times as described in Materials and Methods. Fluorescence intensity is expressed as a fraction of that found at t = 0. Each time point represents the average fluorescence intensity of ≥30 cells.

**FIG 7** Stx produced by internalized bacteria is cytotoxic to *Tetrahymena*. *Tetrahymena* cells were co-cultured with MG1655 (recA)::AcI857/psStx, MG1655 (recA)::AcI857/pET17, or MG1655 (recA)/pStx for 30 min at 30°C, uninternalized bacteria were removed by centrifugation, and the bacterium-containing *Tetrahymena* cells were incubated at 42°C as described in Materials and Methods. The *Tetrahymena* cells were enumerated subsequent to washing and again after incubation at 42°C. At the start of the incubation, *Tetrahymena* cells were present at approximately 10^9/ml. The data presented are the number of live *Tetrahymena* cells after incubation relative to the number of live *Tetrahymena* cells at the start of incubation. Error bars represent standard deviations from three or more independent experiments, with each experiment comprising a minimum of three individual measurements. *, P < 0.001.
absence of bacteriophage λcI857 from MG1655 (recA)/pStx. These data indicate that digestion of bacteria by *Tetrahymena* apparently renders the released toxin harmless to the cell and argues that bacteriophage-mediated lysis of Stx-encoding bacteria is necessary for Stx toxicity in *Tetrahymena*.

Taken together, the data in Fig. 5 and 7 show that bacterially produced Stx2 effectively kills *Tetrahymena* regardless of whether the toxin is released into the medium or produced within *Tetrahymena* food vacuoles. Our data suggest that Stx2 transport across the *Tetrahymena* plasma membrane is mediated by a glycoconjugate receptor (Fig. 1, 3, and 4). We wondered whether transport of Stx2 across the food vacuole membrane into the cytosol utilizes the same membrane receptor system. To answer this question, we incubated *Tetrahymena* cells with MG1655 (recA)::λcI857/pStx in the presence of either 2 μg/ml purified StxB or, as a control, 2 μg/ml BSA. The free bacteria were washed away, and the coccultures were moved to 42°C to induce Stx release into *Tetrahymena* food vacuoles. The number of viable *Tetrahymena* cells in cultures incubated with BSA decreased by approximately 40%, while the number of viable *Tetrahymena* cells in cultures incubated with StxB increased slightly (Fig. 8). This finding shows that excess StxB in the medium taken up with bacteria protects *Tetrahymena* cells from bacterially produced Stx and indicates that the B subunit mediates uptake into *Tetrahymena* cells. This observation suggests that bacterially derived Stx2 within *Tetrahymena* food vacuoles enters the cell via a receptor-mediated process that may be identical to Stx2 entry from outside the cell. Hence, these two seemingly disparate modes of entry into *T. thermophila* apparently use the same uptake scheme.

Having gained insight into how Stx enters *T. thermophila* cells, we wished to determine how this toxin kills *Tetrahymena* cells. Stx apparently kills susceptible mammalian cells by inactivating the ribosome and inhibiting protein synthesis (8). To determine whether purified Stx2 inhibits protein synthesis, we incubated *T. thermophila* with purified Stx2 and measured total protein synthesis by pulse-chase using [35S]methionine. In these experiments, we also examined the effect of paromomycin, a potent protein synthesis inhibitor in *Tetrahymena* cells, on [35S]methionine incorporation (34). After a 1-h incubation with Stx, *Tetrahymena* protein synthesis decreases 5-fold compared to that of *Tetrahymena* cells incubated with BSA (Fig. 9). This observation is consistent with the idea that Stx2 inhibits protein synthesis in *T. thermophila*. Interestingly, this decrease in [35S]methionine incorporation is nearly identical to the decrease seen when *Tetrahymena* cells are incubated with paromomycin (Fig. 9). This similarity indicates that Stx2 is also a potent protein synthesis inhibitor. Decreases in protein synthesis caused by both Stx and paromomycin are seen in incubation periods as short as 10 min (data not shown), indicating that both compounds act quickly to inhibit protein synthesis.

**DISCUSSION**

Our earlier work demonstrated that both purified Stx and that produced by bacteria in coculture are capable of killing *T. thermophila* (5). Together with other findings, these observations indicated that Stx can function as part of an antipredator defense strategy, suggesting that susceptible mammals are neither the original nor the primary “targets” of this toxin. Our previous investigations did not discern the mode of entry of Stx into *Tetrahymena* cells and whether the mechanism by which it kills these cells was conserved between single-celled and metazoan eukaryotes. This information could provide insight into the evolution of Stx toxicity.

Stx intoxication of susceptible mammalian cells requires its specific binding to the Gb3 glycolipid receptor located on the plasma membrane. The finding that Stx kills *T. thermophila* under conditions where phagocytosis via the oral apparatus is blocked by mutation indicates that these organisms are able to import Stx through the plasma membrane. *Tetrahymena* contains all of the machinery needed for CME. CME in *Tetrahymena* can occur non-

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**FIG 8** StxB decreases the cytotoxicity of Stx produced by bacteria internalized by *Tetrahymena* cells. *Tetrahymena* cells were cocultured with MG1655 (recA)::λcI857/pStx and either 2 μg/ml BSA or StxB for 30 min at 30°C, unin-ternalized bacteria were removed by centrifugation, and the bacterium-containing *Tetrahymena* cells were incubated at 42°C as described in Materials and Methods. The *Tetrahymena* cells were enumerated subsequent to washing and again after incubation at 42°C. At the start of the incubation, *Tetrahymena* cells were present at approximately 10^9/ml. Data are presented as the number of live *Tetrahymena* cells after incubation relative to the number of live *Tetrahymena* cells at the start of incubation. Error bars represent standard deviations from three or more independent experiments, with each experiment comprising a minimum of three individual measurements.

**FIG 9** Effect of purified Stx2 on *Tetrahymena* protein synthesis. *Tetrahymena* cells (5 × 10^4/ml) were separately incubated with 200 ng/ml Stx2, 200 ng/ml BSA, or 0.5 mM paromomycin for 60 min and subsequently assayed for protein synthesis as described in Materials and Methods. [35S]Methionine incor-poration into protein was determined by scintillation counting and is represented as counts per minute per *Tetrahymena* cell. Error bars represent standard deviations from three or more independent experiments, with each experiment comprising a minimum of three individual measurements. *, P < 0.001.
specifically, via membrane recycling (20, 35–37). However, we find that Stxα-mediated killing of mouthless *Tetrahymena* can be specifically blocked by the addition of an excess of the receptor binding Stxα subunit. It is formally possible that Stxα prevents killing by “inactivating” the Stx holotoxin; however, our Stxα preparation does not contain a protease or other inactivating activities (data not shown). Also, excess Stxα does not appear to inactivate Stx holotoxin Gb3 binding activity (38). Importantly, we also find that Stx holotoxin killing of *Tetrahymena* can be blocked by the carbohydrate binding subunit of ricin, ricin B, but not by a nonspecific protein, BSA. Together, these findings suggest that Stx can be imported through the plasma membrane and this import is mediated by a receptor. Since BSA and StxB pentamers are similar in size and isoelectric point, our results are inconsistent with the idea that StxB enters cells by nonspecific CME.

It is well established that *T. thermophila* behavior responds in a specific and selective way to the addition of effectors (hormones, drugs) that would, at first glance, be expected to affect only cells of multicellular eukaryotes (39–41). It has been conjectured that these behaviors are mediated by ligand–receptor interactions (42). Hence, the suggestion that Stx import into these cells is mediated by a membrane-bound receptor may not be unexpected. However, in no case have ligand–mediated changes in *Tetrahymena* behavior been associated with the binding of these ligands to a specific receptor. While our data do not identify a specific receptor for Stx, the finding that the presence of an excess of the carbohydrate binding subunits of Stx and ricin toxin (StxB and ricin B, respectively) blocks Stx-mediated killing of *Tetrahymena* argues for the presence of a receptor on *Tetrahymena* membranes and that this receptor is a glycoconjugate.

Our analysis of *Tetrahymena* lipid content indicates that the glycolipid Gb3, which serves as the Stx receptor in mammalian cells, is not found in *Tetrahymena* (T. Hennessy, C. Lingwood, and G. Koudelka, unpublished results). Consistent with this finding, bioinformatic analysis shows that *Tetrahymena* does not contain a gene orthologous to that which encodes the mammalian Gb3 synthase (43, 44). Nonetheless, the suggestion that a membrane-bound glycoconjugate may mediate the uptake of bacterial exotoxins is consistent with the observation that pertussis toxin (PTX), a toxin whose import into mammalian cells is also mediated by the binding of a carbohydrate binding subunit to a membrane-bound glycoconjugate receptor, has demonstrable effects on *Tetrahymena* behavior (41). Therefore, while the precise identity of a receptor(s) for both Stxα and PTX in *Tetrahymena* has yet to be determined, we hypothesize that membrane-bound glycoconjugates mediate the import of both toxins.

Both purified Stxα and bacteria can potentially enter *Tetrahymena* cells by phagocytosis via the oral apparatus. The finding that StxBα is necessary for intoxication of *Tetrahymena* by bacterially produced toxin and that excess StxBα, but not BSA, can prevent killing by Stxα produced by bacteria in food vacuoles argues that cytoplasmic import of toxin across the food vacuole membrane may be mediated by a receptor. Since the oral groove is formed by invagination of the plasma membrane, it is not surprising that the internal surface of the resulting food vacuole could contain the putative Stx receptor that is apparently located on the surface of these cells.

*Tetrahymena* cells are bacteriovorous. Live bacteria are captured by the oral groove, encapsulated within phagosomes, and subsequently lysed, presumably as a consequence of acidification of this organelle, which occurs within 10 min postigestion, or fusion of the phagosome with lysosomes, which occurs between 20 and 40 min postigestion (45, 46). In this light, it is surprising that Stxα produced by bacteria in food vacuoles is cytotoxic (Fig. 7). However, similar to the results of others (24, 25), we found that bacteria ingested by *Tetrahymena* cells survive in this phagosome and have a half-life of 20 to 50 min. Moreover, between 10 and 30% of the bacteria survive in the food vacuole for at least 90 min (Fig. 6). Hence, encapsulated bacteria live long enough to deliver cytolethal toxin to *Tetrahymena* cells.

Our previous results indicated that bacterially encoded Stxα can function as part of a bacterial antipredator defense strategy (5). One question that arises from the findings that Stxα produced outside the cell and imported through the cell membrane and Stxα produced inside the phagosome by ingested bacteria are both cytotoxic is: which route of intoxication is most relevant to the antipredator defense mechanism? We found previously that the 50% lethal dose of purified Stxα is ~2.5 ng/ml, which corresponds to approximately 2 × 10^10 molecules/ml. It is unclear how much Stxα is produced by an individual induced bacterium; however, the amount of toxin produced by Stxα-encoding bacteria is proportional to the number of phages produced (47, 48). About 150 lambdoid phages are produced per bacterium. Since Stxα production is linked to the transcription of genes expressed late in the lysis pathway, it is reasonable to assume that a similar (or greater) number of molecules of toxin is released from each induced lysogen. Therefore, approximately 10^8 bacterial cells/ml must lyse to produce enough Stxα to significantly affect the viability of *Tetrahymena* cells. In water, *E. coli* is found at ≤10^7 cells/ml. Thus, if Stxα produced outside the cell mediates its antipredator activity, in order to effectively function as an antipredator defense, virtually all of the cells in the population would need to lyse, a clearly ineffective strategy to facilitate population survival.

Our measurements suggest that at a bacterial concentration of 10^8 cells/ml, each *Tetrahymena* cell ingests ~500 bacterium. *Tetrahy- mena* cells reach a population of size of 10^12/ml in nutrient-poor medium. Hence, after the introduction of bacteria, *Tetrahymena* internalizes ~5 × 10^6 cells/ml “prey” bacteria, leaving at least 9.5 × 10^6 bacterial cells/ml. While we have not precisely measured the number of internalized bacteria that are induced to produce phage or digested by *Tetrahymena*, and even if *Tetrahymena* cell densities are substantially higher than 10^8/ml, it is apparent that intoxication by ingested bacteria is likely the most relevant to the antipredator defense mechanism.

The results in Fig. 7 show that only toxin that is produced by bacteria bearing temperate bacteriophage is able to kill *Tetrahymena* cells. Stxα constitutively produced in phagosomes by bacteria lacking a bacteriophage or bearing a phage that cannot be induced is not cytotoxic (Fig. 7). That is, toxin released from the bacteria as a consequence of digestion by *Tetrahymena* cells is not cytotoxic. These observations suggest that (i) phage induction and consequent toxin release are triggered prior to the toxin-inactivating action of bacterial digestion and (ii) the cytotoxic activity of Stxα produced by bacteria in phagosomes apparently requires that production of toxin be coupled with phage-mediated lysis of the bacterial cell. Since the digestive processes that lead to bacterial lysis apparently inactivate Stxα, these findings provide a rationale for why the genes encoding Stxα are found almost exclusively on bacteriophages; toxin must be released from the bacteria prior to the digestion of the cell, or it will not be able to exert its cytotoxic
effect. We suggest that similar reasons underlie the finding that several other bacterial exotoxins are found only on temperate bacteriophages.

Inspection of the data in Fig. 6 shows that, in otherwise isogenic strains, the half-life of bacteria bearing genes encoding Stx survive 2.5 times longer inside Tetrahymena cells than do bacteria that do not harbor these genes. This difference causes the amount of viable Stx-encoding bacteria to be 3-fold higher at 90 min than the number of Stx− bacteria. Similar findings were reported in reference 25. Hence, Stx appears to partially protect phagocytosed bacteria from digestion. Although the precise cause is likely different, this observation is similar to that made with Legionella pneumophila; i.e., strains of L. pneumophila that are pathogenic to mammals are less susceptible to digestion than are nonpathogenic strains. Apparently, pathogenic L. pneumophila prevents the food vacuoles from fusing with lysosomes (49).

With this in mind, we envision two potentially overlapping mechanisms by which Stx enhances the survival of ingested bacteria in Tetrahymena cells. In both cases, some, but not all, of the bacteria containing a prophage and toxin may be induced to produce phage and consequently toxin. According to one idea, transport of this toxin into the cytoplasm inhibits protein synthesis and blocks phage and consequently toxin. According to one idea, transport of this toxin into the cytoplasm inhibits protein synthesis and blocks phage and toxin. Alternatively, Stx released into the cytoplasm or contained within the food vacuole alters the trafficking of the Stx-containing bacteria so that they are less efficiently targeted to lysosomes.

Similar to our observation in Tetrahymena cells, bacteria bearing genes that encode Stx2 also survive better in human macrophages than do otherwise isogenic bacterial strains bearing mutations in these genes (50). Nonetheless, despite the Stx-mediated increase in intracellular bacterial survival, human monocytes have been found to be resistant (21–23) or, at best, weakly susceptible (50) to this toxin. Apparently, the failure of this bacterially expressed Stx to efficiently kill macrophages stems from the inability of this toxin to be exported into the cytoplasm and the subsequent delivery of the vesicle containing the toxin and bacteria to lysosomes (51, 52). Hence, although the structures and mechanism of phagocytosis and vesicular transport by these two cell types appear to be highly similar (19, 53), in Tetrahymena, the toxin produced by internalized bacteria can clearly be trafficked to the cytoplasm to exert its cytolethal effect. In sensitive mammalian cells, vesicles containing Stx bypass lysosomes and the toxin is brought to the cytoplasm via retrograde transport (54–56). It remains to be determined whether Stx produced by ingested Stx-encoding bacteria uses this same or a different pathway to reach the Tetrahymena cytoplasm.

**MATERIALS AND METHODS**

**Strains and chemicals.** EDL933 was obtained from the ATCC. EDL933Δstx, an EDL933 variant containing deletions of both the stx1 and stx2 genes (30), was a gift from Christine Miller, Institut National de la Recherche Agronomique. E. coli strain YYC7 was obtained from the CGSC. Strain MG1655 (recA) was created as described earlier (57). T. thermophila strains CU427.4 and NP1, a mouthless strain (27), were obtained from the Tetrahymena Stock Center (Cornell University).

Purified Stx2 toxin and anti-Stx antibodies were obtained from Toxin Technologies (Sarasota, FL) and BEI Resources (Manassas, VA). Bacterial strains EDL933:mCherry and EDL933Δstx2:mCherry were made by transforming either strain with the pmCherry vector from Clontech (Mountain View, CA).

**Purification of StxB.** StxB was purified from MG1655 transformed with pSU108, a plasmid that directs the overproduction of StxB, and was a gift from Ludger Johannes. The protein was purified as described previously (56). The purified protein was stored at −80°C in phosphate-buffered saline and supplemented with 10% glycerol.

**Construction of MG1655;393W and MG1655;393WΔrec.** The MG1655;393W lysogen was constructed as previously described (58), using wild-type 393W bacteriophage isolated by spontaneous induction of bacterial strain EDL933. A portion of the stxB gene from this strain was subcloned into plasmid pET17b. A DNA fragment containing the gene for chloramphenicol resistance (cat) was amplified from pACYC184 and inserted into the PflMI site of the stxB gene, and the recombinant plasmid, pStxBΔc, was transformed into MG1655. The MG1655;393WΔrec strain was created by P1 transduction (59) of strain MG1655;393W using a lysate from MG1655/pStxBΔc and selecting for chloramphenicol resistance. Disruption of the stxB gene was confirmed by PCR analysis and sequencing of PCR products derived from the relevant region of the MG1655;393WΔrec chromosome.

**Construction of MG1655 (recA);αcl857 and Stx variant strains.** Bacteriophage αcl857, which has a temperature sensitivity mutation in the cI protein that makes it unable to maintain lysogeny at temperatures above 33°C (60), was purified from lysogenized strain YYC7. YYC7 was grown overnight in Luria broth (LB) at 30°C. The culture was diluted 1:50 in LB and grown to an optical density of 0.6. The mid-log-phase culture of YYC7 was incubated at 42°C, inducing the phage and lysing the cells. A few drops of chloroform were added to the culture, the lyse was vortexed, and the debris was removed by centrifugation at 18,000 × g. The supernatant was spotted onto a lawn of MG1655 (recA) in top agar. Plaques were scraped and streaked onto LB agar plates. Individual colonies were tested for the ability to produce phage at 42°C. The resulting strain, MG1655 (recA);αcl857, was transformed with either pStxB (5) or pET17b (EMB Biosciences).

**Protection from Stx2 killing by ricin B.** Stationary-phase cultures of T. thermophila (CU427.4) were diluted 5-fold in proteose peptone plus FeCl3 and grown for 3 days at 30°C. The cells were washed twice in 10 mM Tris–HCl (pH 7.4) and suspended in the same medium. Washed cells were added to M9 plus 0.08% Na-citrate at a density of 10⁶/ml. These cells were incubated for 6 h at 30°C with 40 ng/ml Stx2 and 2 μg/ml BSA, StxB, or ricin B. As a control, Tetrahymena cells were also separately incubated with 40 ng/ml BSA. Tetrahymena cell density was determined (5) at the beginning and end of the incubation period. Tetrahymena cells killed by Stx are not visible by Lugol staining because they are lysed. Each measurement was performed in triplicate, and the data were averaged. The data shown represent the average of nine or more replicates.

**Effect of Stx2 on Tetrahymena strain NP1.** Saturated cultures of T. thermophila strains NP1 and CU427.4 were diluted 5-fold in EPP medium (27) and grown for 3 days at 37°C. The cells were washed twice in 10 mM Tris–HCl (pH 7.4) and suspended in the same medium. Washed cells were added to M9 plus 0.08% Na-citrate at a density of 10⁶/ml. These cells were incubated for 6 h at 37°C in the presence of 40 ng/ml BSA or Stx2. As a control for growth at 37°C, the experiment was replicated at 30°C. Tetrahymena cells were enumerated as described above. Each measurement was performed in triplicate, and the data were averaged. The data represent the average of nine or more replicate experiments.

**Coculture of EDL933 with Tetrahymena strains NP1 and CU427.4.** T. thermophila strains NP1 and CU427.4 were prepared as described above, except that the washed, resuspended cells were added to medium containing bacterial cells that had been previously grown to saturation at 37°C in M9 plus 0.08% glucose, washed twice in M9 plus 0.08% Na-citrate, and suspended in the same medium. The cocultures containing 10⁴ Tetrahymena cells/ml and 10⁵ EDL933 bacterial cells/ml were incubated for 6 h at 37°C. As a control for growth at 37°C, the experiment was replicated at 30°C. Tetrahymena cells were enumerated as stated above. Each measurement was performed in triplicate, and the data represent the average of nine or more replicate experiments.
Bacterial survival within *Tetrahymena* food vacuoles. *T. thermophila* CU427.4 and bacterial strains EDL933:MCherry and EDL933:astx-mCherry were prepared as described above, except that the final *Tetrahymena* cell density was approximately 5 × 10^6/ml and bacterial cultures were supplemented with 100 μg/ml ampicillin. The bacterial cells produce mCherry, a red fluorescent protein with an excitation maximum at 587 nm and an emission maximum at 610 nm (61). To allow uptake of bacteria by *Tetrahymena*, cocultures containing *Tetrahymena* cells at approximately 5 × 10^5/ml and bacterial cells at 10^7/ml were incubated for 30 min at 30°C. After 30 min, the cocultures were washed free of bacteria by 3-fold dilution in 10 mM Tris-HCl (pH 7.4) and pelleted at 1,000 rpm for 5 min. Pelleted *Tetrahymena* cells containing bacteria were washed twice more and resuspended in 1 ml of 50% proteose peptone with FeCl₃. Bacterium-containing cells were incubated at 30°C, and samples were taken for microscopic examination every 30 min for 2 h. Samples were prepared by adding sodium azide to a final concentration of 0.1% to kill the *Tetrahymena* cells. Samples were loaded onto slides and inspected at ×60 magnification using differential interference contrast (DIC) and a rhodamine red filter on a Zeiss Axio Imager Z1 microscope. Images were taken using high-resolution AxioCam MRm digital camera. Average fluorescence was quantified by analyzing fluorescent images using AxioVision version 4.8.2 (manufactured by Zeiss Microimaging GmbH).

Killing and StxB subunit competition from within *Tetrahymena* food vacuoles. *Tetrahymena* strain CU427.4 and bacterial strains MG1655 (recA)::ΔlcrE5/pStx, MG1655 (recA)::ΔlcrE5/pet17, and MG1655 (recA)::ΔlcrE5/pStx were prepared as described above, except that bacteria were grown at 30°C. After incubation at 30°C for 30 min, the *Tetrahymena* cells were washed free of uninternalized bacteria as described above, except that they were resuspended in a final volume of 4 ml of M9 plus 0.08% Na-citrate. The *Tetrahymena* cells containing internalized bacteria were then incubated at 42°C for 6 h. MG1655 (recA)::ΔlcrE5/pet17 acts as the toxin negative control, while MG1655 (recA)::ΔlcrE5/pStx acts as the phage negative control. To determine if StxB affects bacterially mediating killing of *Tetrahymena*, cocultures were supplemented with 2 μg/ml BSA or StxB. *Tetrahymena* cells were enumerated as described above. Each measurement was performed in triplicate, and the data were averaged.

The data represent the average of nine or more replicate experiments.

Effect of StxB on *Tetrahymena* protein synthesis. *Tetrahymena* cells were prepared as described above, except that their final density was approximately 5 × 10^6/ml. The *Tetrahymena* cells were subsequently incubated with 200 ng/ml StxB for 60 min. Subsequently, the *Tetrahymena* cells were washed twice in M9 plus 0.08% glucose and 2 μCi of [35S]methionine was added to 100 μl of *Tetrahymena* cells and incubated for an additional 15 min at 25°C. Next, 10 μl of 50 mM Casamino acids was added to the mixture and the mixture was incubated for an additional 5 min at 25°C. Cells were lysed by adding 50 μl of *Tetrahymena* lysis buffer (62) and incubating the mixture for 30 min on ice. Cellular debris was removed by centrifugation at 18,000 × g for 15 min. Total protein in the supernatant was precipitated by incubation with 12.5% trichloroacetic acid on ice for 10 min. Precipitate was collected by vacuum filtration through a glass fiber filter using a vacuum manifold and washed three times with 1 ml acetone. The total amount of labeled protein synthesized was measured by counting with a scintillation counter.

Statistical methods. Except where indicated, the error bars shown in the figures are standard deviations of multiple (more than nine) replicate experiments. Student two-tailed, two-sample, equal-variance *t* tests were used to determine the significance of differences in the measured data.

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