**Bacteroides thetaiotaomicron** Outer Membrane Vesicles Modulate Virulence of *Shigella flexneri*

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**ABSTRACT** The role of the gut microbiota in the pathogenesis of *Shigella flexneri* remains largely unknown. To understand the impact of the gut microbiota on *S. flexneri* virulence, we examined the effect of interspecies interactions with *Bacteroides thetaiotaomicron*, a prominent member of the gut microbiota, on *S. flexneri* invasion. When grown in *B. thetaiotaomicron*-conditioned medium, *S. flexneri* showed reduced invasion of human epithelial cells. This decrease in invasiveness of *S. flexneri* resulted from a reduction in the level of the *S. flexneri* master virulence regulator VirF. Reduction of VirF corresponded with a decrease in expression of a secondary virulence regulator, virB, as well as expression of *S. flexneri* virulence genes required for invasion, intracellular motility, and spread. Repression of *S. flexneri* virulence factors by *B. thetaiotaomicron*-conditioned medium was not caused by either a secreted metabolite or secreted protein but rather was due to the presence of *B. thetaiotaomicron* outer membrane vesicles (OMVs) in the conditioned medium. The addition of purified *B. thetaiotaomicron* OMVs to *S. flexneri* growth medium recapitulated the inhibitory effects of *B. thetaiotaomicron*-conditioned medium on invasion, virulence gene expression, and virulence protein levels. Total lipids extracted from either *B. thetaiotaomicron* cells or *B. thetaiotaomicron* OMVs also recapitulated the effects of *B. thetaiotaomicron*-conditioned medium on expression of the *S. flexneri* virulence factor IpaC, indicating that *B. thetaiotaomicron* OMV lipids, rather than a cargo contained in the vesicles, are the active factor responsible for the inhibition of *S. flexneri* virulence.

**IMPORTANCE** *Shigella flexneri* is the causative agent of bacillary dysentery in humans. *Shigella* spp. are one of the leading causes of diarrheal morbidity and mortality, especially among children in low- and middle-income countries. The rise of antimicrobial resistance combined with the lack of an effective vaccine for *Shigella* heightens the importance of studies aimed at better understanding previously uncharacterized aspects of *Shigella* pathogenesis. Here, we show that conditioned growth medium from the commensal bacterium *Bacteroides thetaiotaomicron* represses the invasion of *S. flexneri*. This repression is due to the presence of *B. thetaiotaomicron* outer membrane vesicles. These findings establish a role for interspecies interactions with a prominent member of the gut microbiota in modulating the virulence of *S. flexneri* and identify a novel function of outer membrane vesicles in interbacterial signaling between members of the gut microbiota and an enteric pathogen.

**KEYWORDS** *Shigella flexneri*, *Bacteroides thetaiotaomicron*, microbiota, outer membrane vesicles, *Shigella*, enteric pathogens, gut microbiome, intracellular pathogen, virulence regulation

*S. flexneri* is an enteric pathogen that causes bacillary dysentery in humans (1). After being ingested, *S. flexneri* passes through the stomach and small intestine before entering the colon, where the expression of invasion genes (2) carried on its virulence plasmid (3) enables it to invade the colonic epithelium (4). Coordination of invasion, vacuole lysis, and intercellular spread by *S. flexneri* relies on the timed expression
of a complex array of virulence genes (5). The genes involved in this virulence process are controlled by a master transcriptional regulator, VirF, which directly regulates icsA, a virulence gene encoding a protein necessary for S. flexneri motility within host cells, and virB, a gene encoding a secondary transcriptional regulator required for virulence (6, 7). VirB, in turn, regulates a variety of type three secretion system (T3SS) and invasion genes such as ipaA, -B, -C, and -D (8).

Prior to gaining access to the intestinal epithelium for invasion, S. flexneri encounters a variety of potential obstacles in the colon, including the trillions of established bacteria that make up the human gut microbiota (9). However, the role of the gut microbiota in S. flexneri pathogenesis remains largely unknown. While conventional guinea pigs and mice are resistant to colonization, indicating a possible role for the microbiota in resistance to infection (10). Monoassociating germfree animals with Escherichia coli prior to challenge with S. flexneri restores resistance to S. flexneri colonization, but monoassociating mice with Bacteroides, a prominent member of the human gut microbiota, has no effect on S. flexneri colonization. Interestingly, diassociating mice with both E. coli and Bacteroides has the largest impact on S. flexneri colonization, causing S. flexneri to decrease to undetectable levels in the colon (10–12). Together, these studies suggest that the gut microbiota may function in preventing S. flexneri infection of the colon; however, since E. coli is often only a minor constituent of the human gut microbiota (13), and small-animal models fail to recapitulate many aspects of shigellosis in humans (14), the relevance of these data for S. flexneri infection in humans is unclear.

Another bacterium found in the gut, Lactobacillus, has been of interest to researchers as a probiotic due to its inhibitory effects on enteric pathogens (15). Consistent with the possibility that Lactobacillus is protective against Shigella, a metagenomic study looking at the association between gut microbiota composition, Shigella levels, and diarrheal status in children in low-income countries found that children who were colonized by certain species of Lactobacillus had moderate-to-severe diarrhea less often than expected when they were also colonized by Shigella (16). This possible role for Lactobacillus spp. in preventing symptomatic S. flexneri infection is further supported by experiments using cell culture models that demonstrate that Lactobacillus spp. can inhibit Shigella attachment to and invasion of colonic epithelial cells (17–19). However, similarly to E. coli, Lactobacillus spp. are minor constituents of the gut microbiota (20). Furthermore, only a subset of species of Lactobacillus has been observed to stably colonize the intestinal tract, while others are known to only transiently colonize the gut (21). Our understanding of the interactions between S. flexneri and prominent members of the gut microbiota and what effects these have on S. flexneri virulence remains limited.

The gut microbiota is an incredibly diverse community comprised of hundreds of bacterial species. Most of these bacteria belong to just two phyla, Bacteroidota and Firmicutes (formerly called Bacteroidetes and Firmicutes, respectively) (22). While a number of genera from Bacillota can be found in the human gut microbiota, Bacteroidota are largely represented by just four genera, the most abundant of which are Bacteroides and Prevotella (23). In humans, there appears to be a tradeoff between having a Prevotella dominated microbiota and having a Bacteroides-dominated one (24–26), with studies suggesting that diet may play a fundamental role in enriching for either Prevotella, which is more abundant in people of non-Western societies, or Bacteroides, which is more abundant in people of Western societies (27–30). In people from the United States and Western Europe, Bacteroides is often the most abundant genus in the gut microbiota (24, 31). Because they are major constituents of the human gut microbiota and are prevalent among humans from different populations, Bacteroides species have been proposed as model organisms for studying gut microbes (23).

One of the best-studied Bacteroides species, Bacteroides thetaiotaomicron, has been shown to impact the virulence of a number of enteric pathogens (32). B. thetaiotaomicron releases succinate that increases virulence gene expression (33), proteases that
cleave the T3SS (34), and fucose that represses T3SS gene expression of enterohemorrhagic E. coli (35). Additionally, B. thetaiotaomicron polysaccharides have been shown to inhibit toxin release by Clostridium difficile (36). In this study, we examine the effects of B. thetaiotaomicron on S. flexneri virulence. We show that B. thetaiotaomicron suppresses S. flexneri invasion through the downregulation of S. flexneri T3SS gene expression. This suppression is mediated by B. thetaiotaomicron outer membrane vesicles (OMVs), which fuse to S. flexneri and posttranscriptionally repress its master virulence regulator, VirF. This demonstrates that a prominent member of the human gut microbiota impacts Shigella pathogenesis and that OMVs can modulate the gene expression of enteric pathogens.

RESULTS

Bacteroides thetaiotaomicron-conditioned medium inhibits Shigella flexneri invasion. To determine whether interspecies interactions with members of the human gut microbiota impact S. flexneri virulence, we studied the effect that the common gut microbe B. thetaiotaomicron has on S. flexneri invasion and cell-to-cell spread (plaque formation) in a monolayer of cultured epithelial cells. We simulated S. flexneri encountering an established gut microbiota by growing S. flexneri in cell-free conditioned medium (CM) collected from late-stationary-phase B. thetaiotaomicron cultures. S. flexneri grown in brain heart infusion (BHIS) supplemented with B. thetaiotaomicron CM had an 11-fold reduction in invasion rate relative to S. flexneri grown in BHIS alone (Fig. 1A).

Additionally, S. flexneri grown in the presence of B. thetaiotaomicron CM formed fewer plaques than S. flexneri grown in BHIS alone (Fig. 1B and C), consistent with the lower
rate of invasion. Together, this indicates that *B. thetaiotaomicron* releases a factor that inhibits *S. flexneri* invasion.

Because it is possible that the observed decrease in invasiveness was due to effects of *B. thetaiotaomicron* CM on *S. flexneri* growth rate, we measured the growth of *S. flexneri* in the presence and absence of *B. thetaiotaomicron* CM. The only effect noted on *S. flexneri* growth rate by *B. thetaiotaomicron* CM was during the first 2 h of growth, when the doubling time of *S. flexneri* grown in BHIS supplemented with *B. thetaiotaomicron* CM (37.6 min) was significantly longer than its doubling time in BHIS alone (29.2 min, *P* = 0.022, Fig. 1D). However, during mid- and late log phase, the growth phase used for the invasion assay, the doubling times of *S. flexneri* in the presence and absence of *B. thetaiotaomicron* CM were nearly identical (25.7 min versus 25.9 min, *P* = 0.64, Fig. 1D), indicating that the decrease in *S. flexneri* invasion following growth in *B. thetaiotaomicron* CM was not due to an effect on growth.

*B. thetaiotaomicron* CM reduces the levels of *S. flexneri* virulence factors.

Invasion of eukaryotic cells by *S. flexneri* is type three secretion system (T3SS) dependent. Since growth of *S. flexneri* in the presence of *B. thetaiotaomicron* CM resulted in reduced invasiveness of *S. flexneri* (Fig. 1A), we hypothesized that *B. thetaiotaomicron* CM represses *S. flexneri* T3SS protein expression. To test this, *S. flexneri* was grown in BHIS supplemented with increasing proportions of *B. thetaiotaomicron* CM, and the amount of the *S. flexneri* T3SS protein IpaC was determined by Western blotting. *B. thetaiotaomicron* CM inhibited the expression of IpaC in a dose-dependent manner (Fig. 2A). This repression was not unique to IpaC. Other T3SS proteins (IpaA, IpaB, and IpaD) and a non-T3SS-associated virulence factor (IcsA) were also produced in smaller amounts when *S. flexneri* was grown in the presence of *B. thetaiotaomicron*-conditioned medium (Fig. 2B). In addition to cell-associated virulence factors, secreted virulence factors, including IcsA, IpaA, IpaB, and IpaC, were also decreased in the presence of *B. thetaiotaomicron* CM relative to BHIS (see Fig. S1 in the supplemental material), suggesting that *B. thetaiotaomicron* CM reduces the production of *S. flexneri* virulence factors rather than triggering their secretion.

*B. thetaiotaomicron* CM reduces *S. flexneri* virulence genes at the transcriptional and posttranscriptional level. The inhibitory factor produced by *B. thetaiotaomicron* could be affecting *S. flexneri* virulence gene expression directly or indirectly by affecting expression of upstream regulators. In *S. flexneri*, VirF is the master virulence transcriptional regulator, which activates expression of a second regulatory gene, virB. VirB regulates other virulence genes, including the *ipa* genes. Additionally, *icsA* is directly regulated by VirF but not by VirB (6–8) (Fig. 3A). Since both *icsA* and *ipa*
protein levels were reduced, this suggested that *B. thetaiotaomicron* CM represses *virF*. To test whether *B. thetaiotaomicron* CM affects *S. flexneri* virulence gene expression, the relative virulence gene expression level of *S. flexneri* grown in the presence or absence of *B. thetaiotaomicron* CM was determined by quantitative reverse transcription-PCR (RT-qPCR). *virB* and *icsA* expression was repressed 5.7- and 6.4-fold, respectively, in the presence of *B. thetaiotaomicron* CM. Additionally, *VirB'*s downstream targets *ipaB* and *ipaC* were repressed 8.8- and 9.0-fold (Fig. 3B), consistent with *B. thetaiotaomicron* CM repression of *virB*. Surprisingly, *virF* levels were unchanged. This indicated either that *B. thetaiotaomicron* CM repression of its two targets, *virB* and *icsA*, was independent of VirF or that VirF levels were reduced posttranscriptionally. To determine if *B. thetaiotaomicron* CM regulates VirF posttranscriptionally, an S tag was fused in frame to the 3’ end of *virF*, and the tagged gene was expressed from its native promoter on a plasmid. Western blot analysis of S-tagged VirF showed that VirF protein levels were reduced in *B. thetaiotaomicron* CM relative to BHIS (Fig. 3C). These data, showing that *B. thetaiotaomicron* CM reduces VirF S-tag
levels (Fig. 3C), but not virF gene expression (Fig. 3B), suggest that *B. thetaiotaomicron* CM affects VirF levels posttranscriptionally.

**B. thetaiotaomicron inhibitory factor is not a secreted metabolite or protein.** To characterize the nature of the *B. thetaiotaomicron* secreted inhibitory factor, we looked for evidence that the inhibitory factor was either a secreted metabolite or a secreted protein. Bacteria of the gut microbiota, including *Bacteroides* species, are known to excrete a variety of short-chain fatty acids (SCFAs) (37, 38), and enteric pathogens have been shown to modulate their virulence genes in response to exogenous SCFAs (39–41).

To determine whether *S. flexneri* was responding to a secreted metabolite, such as an SCFA, the small molecules (<10 kDa) were separated from proteins and other large molecules using a protein concentrator (Fig. 4A). The inhibitory activity of fractionated *B. thetaiotaomicron* CM was associated with the retentate; the small molecules in the flowthrough had no effect on the virulence protein levels (Fig. 4B, lanes 5 and 6). We repeated the fractionation of *B. thetaiotaomicron* CM with a 100-kDa-molecular-weight-cutoff filter. Similar to what was observed with the 10-kDa-cutoff filter, the majority of the inhibitory activity remained in the retentate (Fig. 4B, lanes 9 and 10), suggesting that the active factor was not a small molecule. As expected, fractionating BHIS with the protein concentrators had no effect on IpaC expression relative to unfractionated BHIS (Fig. 4B, lanes 3 and 4 and lanes 7 and 8). The lack of effect of the flowthrough on the level of *S. flexneri* virulence proteins indicates that the inhibition is due to the presence of a high-molecular-weight factor and further demonstrates that it is not a result of depletion of a growth factor from the conditioned medium.

To assess whether the inhibitor was a protein, *B. thetaiotaomicron* CM was treated with proteinase K. However, proteinase K treatment of *B. thetaiotaomicron* CM did not eliminate its ability to inhibit *S. flexneri* IpaC production (Fig. 5A), suggesting the active component was not a protein. It was possible that the *B. thetaiotaomicron* secreted factor was a soluble metabolite that nonspecifically stuck to the protein concentrator during fractionation and was recovered in the retentate fraction. To rule this out, we fractionated *B. thetaiotaomicron* CM by ultracentrifugation. This separated the supernatant, containing soluble molecules, from a pellet, containing the lipids and other insoluble components of the CM. These two fractions were then tested individually for their
The effect on *S. flexneri*, where it was observed that *S. flexneri* grown in medium containing the resuspended pellet had reduced IpaC expression (Fig. 5B, lane 6), while *S. flexneri* grown in medium supplemented with the supernatant (Fig. 5B, lane 5) had IpaC levels comparable to those of the BHIS control (lane 1). This indicated that the active component in *B. thetaiotaomicron*-conditioned medium was insoluble in aqueous solution and likely to be lipid associated. To ensure that the inhibitory factor being ultracentrifuged out of *B. thetaiotaomicron* CM was specific to the CM, BHIS was fractionated in the same way. Neither the supernatant nor the pellet isolated from BHIS had any effect on *S. flexneri* IpaC levels (Fig. 5B, lanes 1 to 3).

**B. thetaiotaomicron** lipids and OMVs inhibit *S. flexneri* virulence factors. To directly determine whether the inhibition of *S. flexneri* virulence factor production was due to the presence of *B. thetaiotaomicron* lipids in the culture supernatant, we extracted the total lipids from a pellet of stationary-phase *B. thetaiotaomicron* and grew *S. flexneri* in the presence of these lipids. Growth in the presence of total *B. thetaiotaomicron* lipids repressed IpaC expression (Fig. 5C). This effect was specific to *B. thetaiotaomicron* lipids, since an equivalent amount of lipids from *E. coli* had no effect (Fig. 5C).

Gram-negative bacteria, including *B. thetaiotaomicron*, are known to produce outer membrane vesicles (OMVs). OMVs have been shown to be involved in cell-to-cell communication (42). Because the size and lipid nature of the inhibitory factor were consistent with OMVs, we purified OMVs from the *B. thetaiotaomicron* CM for analysis. Transmission electron microscopy (TEM) confirmed the presence of small spherical structures (Fig. 6A) ranging from 20 nm to 100 nm in diameter, with an average diameter of 51 nm (Fig. 6B). These structures were similar in both size and appearance to OMVs previously isolated from other *Bacteroides* species (43). Additionally, we validated that the vesicles isolated from *B. thetaiotaomicron* CM were OMVs by utilizing *B. thetaiotaomicron* strains expressing tagged proteins known to localize to either the outer membrane (OM) or OMVs extracted from these strains. Consistent with the results of Valguarnera et al. (44), BT_1488 was localized to the isolated extracellular vesicles, while BT_0418 was found in the OM and not in the

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**FIG 5** Active component of *B. thetaiotaomicron* CM is lipid associated and proteinase K resistant. (A) To degrade proteins, BHIS and *B. thetaiotaomicron* CM were treated with 50 µg/mL of proteinase K. Each was then mixed 1:1 with untreated BHIS. Total proteins were collected from *S. flexneri* grown to mid-log phase in either proteinase K-treated BHIS or proteinase K-treated *B. thetaiotaomicron* CM and used for an anti-IpaC Western blot assay. (B) BHIS and *B. thetaiotaomicron* CM were ultracentrifuged to separate the water-soluble supernatant from the insoluble pellet. The supernatant was mixed 1:1 with BHIS, while the pellet was resuspended in BHIS. Total proteins were collected from *S. flexneri* grown to log phase in unfractionated medium (U) or BHIS supplemented with either the supernatant (S) or pellet (P) fractions and used for an anti-IpaC Western blot assay. (C) *S. flexneri* was grown in the presence of *B. thetaiotaomicron* lipids, *E. coli* (Ec) lipids, or an equal volume of saline. The effect of these lipids on *S. flexneri* IpaC levels was determined by Western blotting. For all three panels, anti-SecA was used as a loading control. Each fractionation or isolation and its corresponding Western blot assay were performed at least 3 independent times.
extracellular vesicles (Fig. S3). This indicates that the purified fraction contained *B. thetaiotaomicron* OMVs and was not significantly contaminated with cellular membranes.

To determine whether *B. thetaiotaomicron* OMVs are the factor responsible for repressing *S. flexneri* virulence in *B. thetaiotaomicron* CM, the invasion rate of *S. flexneri* grown in the presence of *B. thetaiotaomicron* OMVs was measured. The amount of OMVs added was normalized to protein levels in the purified vesicles and is consistent with concentrations used in previous studies (45, 46). We observed that when treated with *B. thetaiotaomicron* OMVs, *S. flexneri* was less invasive than *S. flexneri* treated with an extract of uninoculated BHIS (mock extract) (Fig. 6C). Similar to the results with *B. thetaiotaomicron* CM, *B. thetaiotaomicron* OMVs inhibited expression of *S. flexneri* *IpaC* (Fig. 7A) and the virulence factors *IcsA*, *IpaA*, and *IpaB* (Fig. 7B). As determined by RT-qPCR, this reduction in protein levels was associated with reduced expression of the virulence genes *virB*, *icsA*, *ipaB*, and *ipaC*. Relative to growth in BHIS alone, *S. flexneri* grown in the presence of *B. thetaiotaomicron* OMVs had reductions in expression of 3.9-fold for *virB*, 3.5-fold for *icsA*, 5.8-fold for *ipaB*, and 5.7-fold for *ipaC*. Also consistent with the results from *B. thetaiotaomicron* CM treatment, *virF* gene expression was unchanged by growth in the presence of the *B. thetaiotaomicron* OMVs (Fig. 7C), but protein levels of VirF were reduced (Fig. 7D). Thus, the gene and protein expression patterns observed in *B. thetaiotaomicron* CM were recapitulated by *B. thetaiotaomicron* OMVs. The level of repression of the virulence genes by the vesicles is somewhat less than that observed with the conditioned medium. This may reflect differences in the amount of the inhibitory factor between the purified vesicles and the crude conditioned

**FIG 6** *B. thetaiotaomicron* CM contains OMVs that inhibit *S. flexneri* invasion. (A) Extracellular vesicles were concentrated from *B. thetaiotaomicron* CM, purified on a density gradient, and counterstained with 2% uranyl acetate for visualization by transmission electron microscopy. A representative image taken at >60,000 magnification is shown. Bar, 200 nm. (B) The diameters of 400 OMVs were measured using ImageJ (88). (C) A gentamicin protection assay was used to determine the invasion rate of *S. flexneri* grown in the presence of 20 μg/mL of *B. thetaiotaomicron* OMVs or an equal volume of mock extract. P value was determined by a two-tailed, unpaired t test from 4 biological replicates (*, P < 0.05). Error bars indicate standard error of the mean.
medium, or there may be an additive effect of the vesicles and some other component in the conditioned medium.

**B. thetaiotaomicron OMV lipids inhibit IpaC.** Since *B. thetaiotaomicron* lipids and OMVs both repress *S. flexneri* IpaC levels, we wanted to determine whether *B. thetaiotaomicron* OMV lipids alone were sufficient to inhibit *S. flexneri* IpaC expression. Lipids extracted from purified *B. thetaiotaomicron* OMVs repressed *S. flexneri* IpaC levels (Fig. 7E), indicating that the inhibitory effect of the OMVs is due to the OMV lipids themselves and not to the presence of an unknown cargo contained in the *B. thetaiotaomicron* OMVs. Performing the same OMV and lipid extraction starting with BHIS (mock lipids), and growing *S. flexneri* in the presence of this extract had no effect on IpaC levels (Fig. 7E), demonstrating that the extracted lipids that inhibit *S. flexneri* virulence protein levels are specific to *B. thetaiotaomicron* OMVs and not present in the uninoculated BHIS medium.
B. thetaiotaomicron OMVs directly interact with S. flexneri. Because the effect of B. thetaiotaomicron-produced OMVs on S. flexneri invasion is due to the lipids in B. thetaiotaomicron OMVs, we hypothesized that the OMVs were fusing with S. flexneri’s membrane and that the presence of B. thetaiotaomicron lipids in the membrane of S. flexneri could initiate the observed changes in S. flexneri virulence protein levels. To test whether the B. thetaiotaomicron vesicles directly contact S. flexneri, B. thetaiotaomicron OMVs were stained with the lipophilic fluorescent dye FM4-64 FX and washed extensively to remove unbound dye. The stained B. thetaiotaomicron OMVs were then coincubated with S. flexneri, and the amount of fluorescence associated with the S. flexneri cells was determined over time. After 30 min of coincubation, S. flexneri exhibited a fluorescent signal of 24.2 relative fluorescent units (RFU)/optical density at 650 nm (OD$_{650}$), and after 2 h of coincubation, the fluorescence of S. flexneri increased to 36.6 RFU/OD$_{650}$ (Fig. 8). This suggested that the fluorescently labeled OMVs fused with S. flexneri. Since free FM4-64 FX can directly bind to S. flexneri, we controlled for free dye carryover by performing the same staining and washing procedure on saline that did not contain OMVs. After 30 min and 2 h of coincubation with the stained and washed control saline, S. flexneri exhibited fluorescence of only 1.1 and 1.3 RFU/OD$_{650}$ respectively. This ruled out dye carryover from the staining and washing procedures as the cause of S. flexneri fluorescence. To further control for release of dye from the vesicles during coincubation, labeled vesicles were soaked in buffer for the same amount of time used in the transfer experiment. After soaking, the vesicles were removed by ultracentrifugation, and S. flexneri was incubated with either the soaked OMVs or the buffer in which the OMVs had been soaked. While some fluorescence was measured in S. flexneri that was resuspended in the soaking buffer, the amount of fluorescence was over 10 times lower than that of S. flexneri that was incubated with the soaked OMVs. Additionally, the fluorescence of S. flexneri that was coincubated with soaked OMVs was indistinguishable from that of S. flexneri that was coincubated with unsoaked OMVs (Fig. S4). Together, this suggests that passive transfer of dye from the OMVs to S. flexneri via diffusion of dye away from the stained vesicles is only a minor contributor to S. flexneri fluorescence and indicates that fusion or direct contact between the OMVs and S. flexneri is likely occurring.

**DISCUSSION**

Despite the fact that S. flexneri must interact with the gut microbiota before establishing infection in the colon, the impact of these interactions on S. flexneri pathogenesis remains poorly understood. In this study, we show that the common gut microbe B. thetaiotaomicron produces OMVs that repress S. flexneri virulence gene expression and inhibit S. flexneri invasion.
These results have implications in the broader context of *S. flexneri* pathogenesis. First, it is possible that gut microbiotas enriched for *B. thetaiotaomicron* have a protective effect, making the people that harbor these microbiotas more resistant to *S. flexneri* infection. In humans, *Bacteroides* is often the most abundant genus in the gut microbiota. However, it also the most variable, ranging from comprising the majority of gut microbes in some people to comprising only a small minority in others (22, 24). Our data show that the inhibitory effect of *B. thetaiotaomicron* on *S. flexneri* virulence protein levels is dose dependent, with a higher proportion of *B. thetaiotaomicron* CM leading to a stronger reduction in the T3SS protein IpaC (Fig. 2A), and that the presence of OMVs in *B. thetaiotaomicron* CM is responsible for its inhibitory effect on *S. flexneri* invasion (Fig. 6). We speculate that if the abundance of *B. thetaiotaomicron* in the gut microbiota were to correlate with the accumulation of more *B. thetaiotaomicron* OMVs in the lumen of the colon, then it is conceivable that individuals with higher proportions of *B. thetaiotaomicron* could be given some degree of protection from *Shigella* infection. However, existing data suggesting that *B. thetaiotaomicron* plays a protective role in *S. flexneri* infection are scarce. One study examined the composition of the gut microbiota in children in low-income countries in the presence or absence of *Shigella* (16). These data show that the relative abundance of *Bacteroides* is lower in children who are colonized by *Shigella* and have diarrhea than in children who are colonized by *Shigella* but do not have diarrhea. While this correlation between *Bacteroides* proportion and disease state is consistent with the possibility that *Bacteroides* plays a protective role in *Shigella* infection, it is worth noting that a similar decrease in abundance of *Bacteroides* was observed in children who had diarrhea relative to children who did not have diarrhea, even when these children did not have *Shigella*. Thus, it is difficult to parse out whether these effects are due to interactions between *Bacteroides* and *Shigella*, or whether they are simply due to effects of diarrhea on gut microbiota composition. More research is needed to determine whether the abundance of certain species in the gut microbiota can affect susceptibility to *S. flexneri* infection.

Alternatively, it is possible that, rather than repressing *S. flexneri* virulence, *B. thetaiotaomicron* functions to help coordinate *S. flexneri* invasion. The low infectious dose of *Shigella* (47) suggests that its invasion process is both highly coordinated and efficient. Furthermore, a number of cues, including temperature (48), pH (49), oxygen tension (50), bile salts (51), and osmolarity (52), which help coordinate *S. flexneri* invasion have been identified. Perhaps *B. thetaiotaomicron*-produced OMVs are functioning as another cue that prevents *S. flexneri* from prematurely expressing its T3SS in the lumen of the colon, before other cues closer to the colonic epithelium trigger T3SS expression at the appropriate time. This paradigm, where members of the gut microbiota secrete cues that are sensed by enteric pathogens and used to coordinate infection, has been reported in other enteric pathogens and used to coordinate infection (33, 35).

The inhibitory effect of *B. thetaiotaomicron* CM on *S. flexneri* virulence is due to the presence of *B. thetaiotaomicron* OMVs (Fig. 6 and 7). OMVs derived from commensal bacteria are known to modulate the intestinal immune response, deliver molecules that protect mice from colitis, and function in interkingdom signaling between bacteria and intestinal cells, while OMVs from pathogenic bacteria have been shown to deliver virulence factors to intestinal cells (53–58). In the context of interbacterial interaction, OMVs have been observed to affect population and community dynamics by functioning in both intraspecies and interspecies signaling (42, 59, 60). In this study, we add to the variety of ways that OMVs are known to function in interspecies interaction by showing that OMVs secreted by one species of bacteria can modulate the virulence gene expression of another species. Additionally, by showing that gut microbiota-derived OMVs can modulate the invasiveness of an enteric pathogen, we have identified a possible new function for bacterial OMVs in the colon. Whether this inhibitory effect is specific to *B. thetaiotaomicron* OMVs and *S. flexneri* virulence genes or is more generalizable to other combinations of gut microbes and enteric pathogens is unknown.

The mechanism by which *B. thetaiotaomicron* OMVs repress *S. flexneri* virulence gene
expression remains to be determined. The interaction between the bacteria involves direct contact between \textit{B. thetaiotaomicron} OMVs and \textit{S. flexneri}, the majority of which occurs within the first 30 min of coincubation (Fig. 8). This suggests that the vesicles are fusing with the \textit{S. flexneri} cells. Additionally, incubation with \textit{B. thetaiotaomicron} OMVs leads to a decrease in protein levels of the \textit{S. flexneri} master virulence regulator, VirF (Fig. 7D). The link between OMV fusion and repression of VirF remains uncharacterized; however, it is possible that the fusion of lipids contained in \textit{B. thetaiotaomicron} OMVs into the membrane of \textit{S. flexneri} triggers stress responses. Virulence gene expression in \textit{S. flexneri} and other enteric pathogens is known to be closely intertwined with bacterial stress responses (61–66). \textit{B. thetaiotaomicron} OMVs fusing to \textit{S. flexneri} may activate envelope stress response two-component systems, and then these directly or indirectly transduce the signal of \textit{B. thetaiotaomicron} OMV fusion from the outer membrane of \textit{S. flexneri} to VirF. While \textit{B. thetaiotaomicron} OMV-induced repression of VirF protein was observed, no change in virF RNA was detected (Fig. 7C). This suggests posttranscriptional regulation or effects on the stability of VirF. To date, two examples of posttranscriptional regulation of VirF have been described. Specifically, deletion of two genes involved in tRNA modification, tgt and miaA, has been shown to decrease the translation efficiency of virF despite having no effect on its transcription (7, 67, 68). Future studies will be aimed at determining how OMV fusion leads to the observed decrease in VirF.

Our data suggest that the particular component of \textit{B. thetaiotaomicron} OMVs that causes the repression of \textit{S. flexneri} virulence gene expression is a lipid. Total lipid extracts from both \textit{B. thetaiotaomicron} stationary-phase cell pellet and \textit{B. thetaiotaomicron} OMVs repress \textit{S. flexneri} Ipac protein expression, while an equivalent amount of lipids from \textit{E. coli} does not (Fig. 5C). The particular \textit{B. thetaiotaomicron} lipid or lipids that are responsible for this effect remain unknown. Since \textit{E. coli} lipids do not induce the same effect, it does not appear to be a general lipid effect that reduces Ipac levels. Furthermore, this specificity for \textit{B. thetaiotaomicron} lipids indicates that the responsible inhibitory lipid(s) is likely enriched in \textit{B. thetaiotaomicron} relative to \textit{E. coli}, or absent from \textit{E. coli} altogether. \textit{B. thetaiotaomicron} membrane sphingolipids are a possible candidate. Unlike \textit{E. coli} and almost all other bacteria, members of the phylum \textit{Bacteroidota} (including \textit{B. thetaiotaomicron}) contain membrane sphingolipids, which comprise over 50% of the total lipids in \textit{B. thetaiotaomicron} OMVs (69–71). Sphingolipids of both mammalian and bacterial origin can be found in the colon, where \textit{B. thetaiotaomicron} sphingolipids are known to promote intestinal homeostasis (72, 73). In the environment, the bacterium \textit{Algoriphagus machipongonensis} induces multicellular rosette formation of the choanoflagellate \textit{Salpingoeca rosetta} by releasing OMVs that fuse to \textit{S. rosetta}. The active signals contained in the OMVs that induce \textit{S. rosetta} rosette formation are sulfonolipids, a structural analogue of sphingolipids (74, 75). A similar signaling mechanism may be occurring in \textit{S. flexneri}, whereby \textit{B. thetaiotaomicron} sphingolipids, released as part of OMVs, fuse to \textit{S. flexneri} and cause the observed repression of virulence gene expression.

\textbf{MATERIALS AND METHODS}  
\textbf{Bacterial strains and growth conditions.} Bacterial strains and plasmids used in this study can be found in Table S1 in the supplemental material. \textit{S. flexneri} and \textit{E. coli} strains were maintained at −80°C in tryptic soy broth (TSB) containing 20% (vol/vol) glycerol. \textit{S. flexneri} was grown aerobically at 37°C on TSB agar with 0.01% Congo red dye. Overnight cultures in TSB were subcultured 1:100 into the indicated growth medium and grown aerobically (200 rpm, 37°C). \textit{E. coli} strains were grown on Luria-Bertani (LB) agar (1% Tryptone, 0.5% yeast extract, 1% NaCl, and 1% agar) and in LB broth (1% Tryptone, 0.5% yeast extract, 1% NaCl) under the same growth conditions as \textit{S. flexneri}.

\textit{B. thetaiotaomicron} strains were maintained at −80°C, in brain heart infusion (BHI; Bacto) containing 5 mg/L hemin and 20% (vol/vol) glycerol. \textit{B. thetaiotaomicron} was grown on BHI agar supplemented with yeast extract (0.5% [wt/vol]), sodium bicarbonate (0.2% [wt/vol]), 5 mg/L hemin, and l-cysteine (free base, 0.1% [wt/vol]). For liquid culture, \textit{B. thetaiotaomicron} was grown in supplemented brain heart infusion (BHI) liquid brain heart infusion (Bacto), yeast extract (0.5% [wt/vol]), 5 mg/L hemin, l-cysteine (free base, 0.1% [wt/vol]), and 50 mM HEPES sodium salt (pH 7.5). \textit{B. thetaiotaomicron} was cultured in an anaerobic chamber (Coy) using an atmosphere of 85% N\textsubscript{2}, 10% CO\textsubscript{2}, 5% H\textsubscript{2} at 37°C. Antibiotics were used at the indicated concentrations: gentamicin (20 μg/mL) and erythromycin (25 μg/mL).
Construction of plasmids. C-terminally S-tagged (76) VirF (VirF-S-tag) was constructed using the primers indicated in Table S2. The promoter region and coding sequence of virF were amplified with the primers from the virulence plasmid of S. flexneri 2457T along with the S tag, which was included in the sequence of the reverse primer. The region directly downstream of virF on the virulence plasmid was amplified as well. These two pieces were attached using splicing by overlap extension PCR (77) and then ligated into the EcoRl and Sall sites of the low-copy-number vector pWKS30 (78).

Cell culture media and growth conditions. Minimal essential medium (MEM; Gibco) containing heat-inactivated fetal bovine serum (Gibco; 10% [vol/vol]), tryptone phosphate broth (Bacto; 10% [wt/vol]), 1× nonessential amino acids (Gibco), and 2 mM glutamine was used to grow Henle cells (intestine 407; ATCC CCL-6). Henle cells were incubated at 37°C with 5% CO₂.

B. thetaiotaomicron cell-free CM. To generate B. thetaiotaomicron-conditioned medium (CM), B. thetaiotaomicron was grown anaerobically on BHI agar plus gentamicin. BHIS was inoculated with a single colony of B. thetaiotaomicron and grown anaerobically for 40 h to late stationary phase. The stationary-phase culture was centrifuged at 13,000 × g for 10 min. The supernatant was filter sterilized using a 0.22-μm polycarbonate filter (PES), pH adjusted to 6.8 with 1 M NaOH, and then passed through a second 0.22-μm filter.

Virulence assays. S. flexneri invasion was measured by a gentamicin protection assay (79). S. flexneri was grown to late log phase in the indicated growth medium. Approximately 10⁸ S. flexneri CFU (multiplicity of infection of ~100) were added to a confluent monolayer of Henle cells in a 35-mm, 6-well polystyrene plate (Corning) and centrifuged at 1,000 × g for 10 min. The plate was incubated for 30 min at 37°C and 5% CO₂, after which each well was washed 4 times with phosphate-buffered saline (PBS-D) (1.98 g/L KCl, 8 g/L NaCl, 0.02 g/L KH₂PO₄, 1.39 g/L K₂HPO₄), filled with MEM containing 20 μg/mL gentamicin, and incubated for another hour. The wells were then washed twice more with PBS-D and lysed at room temperature for 10 min with 0.1% Triton X-100 to recover intracellular bacteria. Tenfold serial dilutions of both the input and output bacteria were plated, and invasion efficiency for each sample was calculated as percent invasion (output CFU/input CFU). For plaque assays, S. flexneri was grown to log phase in LB, and 5 × 10⁶ bacteria were added to a confluent layer of Henle cells in a 6-well polystyrene plate and centrifuged at 1,000 × g for 10 min. The plate was incubated for 30 min, after which each well was washed 4 times with PBS-D, and the medium was replaced with MEM containing 0.45% glucose and gentamicin for 24 h. After 24 h, the medium was replaced with MEM containing only gentamicin, and the plate was incubated for 48 h more. The wells were washed with PBS-D, fixed with 80% methanol for 5 min, and then stained with 0.5% crystal violet for visualization.

SDS-PAGE and immunoblotting. S. flexneri was grown to late log phase in the indicated growth medium, centrifuged, resuspended in Lammlli SDS sample buffer (5% β-mercaptoethanol, 3% [wt/vol] SDS, 10% glycerol, 0.02% [wt/vol] bromophenol blue, 63 mM Tris-Cl [pH 6.8]) (80) at a concentration of approximately 2 × 10⁸ CFU/mL, and boiled for 5 min. To isolate secreted proteins, Halt protease inhibitor (ThermoFisher) was added to S. flexneri supernatant, which was then concentrated 15-fold using a 10-kDa molecular-weight cutoff filter (Amicon). Concentrated supernatant was normalized to the OD₆₅₀ of the bacterial culture from which it was collected and then resuspended in 4× Laemmlli SDS sample buffer.

RNA isolation. S. flexneri was grown in the indicated growth medium to late log phase. Four milliliters of late-log-phase cell suspension was mixed with 1 mL of an ice-cold solution containing 95% ethanol and 5% phenol (pH 4.5) and then kept on ice until ready for further processing. Once all samples were ready, the cells were pelleted by centrifugation, resuspended in 100 μL of 1-mg/mL lysozyme in Tris-EDTA (TE) buffer, and incubated at room temperature for 5 min. One milliliter of RNA-Be (Tel-Test Inc.) was added to the lysozyme-treated cells, 200 μL of chloroform was added, and then the solution was centrifuged at 4°C and 21,400 × g for 15 min. The aqueous phase was collected, mixed with an equal volume of isopropanol, and stored at −80°C overnight. Samples were centrifuged at 21,400 × g for 20 min to pellet precipitated RNA, which was then washed once with ice-cold 75% ethanol, air dried, resuspended in water, and DNase treated per the manufacturer’s instructions (Turbo DNA-Free; Invitrogen).

cDNA synthesis and qPCR. Two micrograms of RNA was reverse transcribed into cDNA using the Superscript III kit (Thermo Fisher) per the manufacturer’s instructions. Primers for quantitative PCR (qPCR) were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). For real-time qPCR, cDNA was diluted 1:10 and used with Power SYBR green (Thermo Fisher). The qPCR was run on an Applied Biosystems Viia7 instrument as previously described (81). Relative expression of virulence genes was calculated by using the threshold cycle (ΔΔC_T) method and normalized to the mean for two reference genes, secA and gyrA.

Fractionation of B. thetaiotaomicron CM. B. thetaiotaomicron CM was fractionated by size using 10-kDa and 100-kDa-molecular-weight cutoff centrifugal filters (Amicon). Filters were rinsed once with sterile saline. Then, 2.5 mL of B. thetaiotaomicron CM or BHIS was added to the filter and centrifuged at 3,000 × g at 4°C until all but 100 μL of liquid had flowed through the filter. The flowthrough was mixed 1:1 with BHIS, while the top fraction was resuspended in 5 mL of BHIS for S. flexneri growth. For ultracentrifugation, 2.5 mL of BHIS and B. thetaiotaomicron CM was centrifuged at 135,000 × g for 2 h. The supernatant was collected. The resulting pellet was resuspended in 1 mL of sterile saline and centrifuged a
second time. The supernatant was mixed 1:1 with fresh BHIS, while the pellet was resuspended in 5 mL of fresh BHIS for S. flexneri growth.

Proteinase K treatment of B. thetaiotaomicron CM. BHIS and B. thetaiotaomicron CM were treated with 50 μg/mL of proteinase K at 37°C for 3.5 h. Proteinase K-treated BHIS and B. thetaiotaomicron CM were then each mixed 1:1 with fresh BHIS for S. flexneri growth.

Isolation and normalization of total lipids. Stationary-phase B. thetaiotaomicron was pelleted by centrifugation for 10 min at 13,000 × g. Alternatively, large-scale preparations of crude B. thetaiotaomicron OMVs were isolated by centrifuging 75 mL of B. thetaiotaomicron CM for 3.5 h at 38,400 × g. Total B. thetaiotaomicron lipids from either cell pellets or crude OMVs were extracted by the method of Bligh and Dyer (82). For a negative control, the crude OMV isolation and lipid extraction were performed starting with 75 mL of uninoculated BHIS to generate a sample referred to as “mock lipids.” E. coli total lipid extracts were purchased (Avanti Polar Lipids). Extracted or purchased lipids were dried under a stream of nitrogen and then stored at −20°C until use.

For relative quantitation, lipids were resuspended in saline and then normalized by their fluorescence in the presence of the lipophilic fluorescent dye FM4-64 FX by a method adapted from a previously published protocol (83). Briefly, 2-fold serial dilutions of the lipid samples were made, and 50 μL of each dilution was mixed with 150 μL of 6.67 μg/mL FM4-64 FX (final concentration, 5 μg/mL) and incubated for 10 min at room temperature. Fluorescence was measured (excitation [Ex]/emission [Em], 510/585 nm) on a FlexStation 3 plate reader. Serial dilutions falling within the linear range of the assay were used to determine relative concentrations of the lipid preparations. To standardize lipid concentrations, lipid samples were adjusted with saline, such that a 50-μL aliquot gave a signal of 240 relative fluorescent units (RFU) in a 200-μL reaction mixture (5 μg/mL FM4-64 FX). To assess the effect of lipids on S. flexneri IpaC levels, normalized lipid stocks (240 RFU) were diluted 6-fold in S. flexneri growth medium for a final lipid concentration of 40 RFU.

OMV isolation. To isolate outer membrane vesicles (OMVs), 240 mL of B. thetaiotaomicron CM, prepared as described above, was concentrated to 7 mL using either 100-kDa-cutoff centrifugal filters (Amicon) or a tangential flow filtration device with a 100-kDa-cutoff filter (VivaFlow 50R; Sartorius). Concentrated B. thetaiotaomicron CM was then ultracentrifuged at 135,000 × g for 2 h to pellet crude B. thetaiotaomicron OMVs. Depending on the downstream application, the crude OMVs were either washed once with saline or further purified on an OptiPrep (Sigma) density gradient modified from a published protocol (84). Briefly, the crude OMVs were resuspended in 45% (vol/vol) OptiPrep and added to the bottom of an Ultraclear (Beckman) centrifuge tube. Five layers of OptiPrep, decreasing in increments of 5% each layer down to 20% OptiPrep, were layered on top of the OMVs. The OMVs were then ultracentrifuged at 4°C for 20 h at 150,000 × g. The density gradient was collected in 12 fractions, and SDS-PAGE was performed to identify the fractions that contained OMVs. OMV-containing fractions were then pooled and diluted in 10-fold-excess Dulbecco’s phosphate-buffered saline supplemented with salts (DPBS) (0.2 g/L KCl, 0.2 g/L KH2PO4, 11.7 g/L NaCl, 1.15 g/L Na2PO4, 0.1 g/L MgCl2-6H2O, and 0.1 g/L CaCl2) and the OMVs were collected by centrifugation at 38,400 × g for 3.5 h. The entire OMV isolation protocol was also performed on 240 mL of sterile BHIS to generate a sample for negative controls, referred to as mock extract.

IM/OM preparations. To isolate inner and outer membranes (IM and OM, respectively), a stationary-phase culture of the indicated B. thetaiotaomicron strain was centrifuged at 13,000 × g for 10 min. The resulting pellet was resuspended in buffer (10 mM Na2HPO4 and 5 mM MgSO4) and incubated with sonicating for 10 min. Total membrane (TM) was isolated by centrifuging the supernatant at 135,000 × g for 40 min. To separate inner and outer membranes, the total membrane pellet was resuspended in 1% (wt/vol) N-lauroyl sarcosine (Sarkosyl) and incubated with rocking at room temperature for 1 h. To separate IM from OM, the sample was centrifuged at 135,000 × g for 40 min. The Sarkosyl-soluble supernatant, which contained the inner membrane fraction, was collected. The Sarkosyl-insoluble pellet, which contained the outer membrane, was washed with 1% Sarkosyl, centrifuged at 135,000 × g for 40 min, and then resuspended in water for downstream applications.

Quantification of membrane proteins. For quantification of protein, total membranes, outer membranes, or OMVs were resuspended in 0.5% Triton X-100, while inner membranes were resuspended in 1% N-lauroyl sarcosine. Protein concentration was quantified by the DC protein assay (Bio-Rad) per the manufacturer’s instructions. For all experiments involving OMVs, OMV concentrations are normalized by the total protein concentration of the vesicles.

TEM. Five microliters of OMVs that had been diluted 1:10 in saline were added to a glow-discharged 200-mesh Formvar/carbon grid (Electron Microscopy Sciences; FCF200-Cu) and incubated for 5 min. Excess sample was wicked off, and the grid was washed once with a drop of water and stained for 1 min with 2% uranyl acetate before being air dried and imaged. Imaging was performed on an FEI Tecnai Spirit transmission electron microscope (TEM) at the Center for Biomedical Research Support Microscopy and Imaging Facility at UT Austin (RRID no. SCR_021756).

Expression of His-tagged proteins in B. thetaiotaomicron. Bacteroides expression vector pFD340 (85) expressing His-tagged membrane proteins was transformed into the donor strain E. coli s17-1 λpir and conjugated into B. thetaiotaomicron as previously described (86). Briefly, pellets from overnight cultures of E. coli s17-1 λpir expressing the tagged gene of interest and B. thetaiotaomicron were pooled, plated on BHI agar plates, and grown aerobically overnight at 37°C without selection. The resulting lawn was resuspended in a small volume of BHIS. To recover single colonies of B. thetaiotaomicron contaminants, dilutions were plated on BHI agar containing gentamicin and erythromycin and incubated anaerobically at 37°C for 24 h.

OMV uptake assay. To assess fusion of B. thetaiotaomicron OMVs by S. flexneri, a slight modification of a previously published protocol was used (87). Briefly, 50 μg of purified B. thetaiotaomicron OMVs in
250 μL of saline was stained with 5 μg/mL of FM4-64 FX at room temperature for 15 min. To eliminate excess unbound dye, the stained OMVs were washed three times with a 20-fold excess volume of saline on a centrifugal filter (100-kDa-molecular-weight cutoff; Amicon). To control for dye carryover, the staining and washing procedure described above was also performed on 250 μL of saline in the absence of OMVs. To measure OMV uptake, S. flexneri from a late-log-phase culture was adjusted to an OD600 of 1.0 in 1 mL of saline and coincubated, with rocking at room temperature, with either 50 μg of stained OMVs or an equal volume of stained saline in the absence of OMVs. At the indicated time points, 500 μL of sample was removed, washed three times with saline, fixed for 10 min in 4% paraformaldehyde, and washed three times more. The fluorescence (Ex/Em of 510/640 nm; SpectraMax M3) and OD600 (FlexStation 3) of S. flexneri that had been coincubated with stained OMVs were read on the respective microplate readers. To control for dye diffusion from OMVs, the OMVs were stained as described above and then soaked in 1 mL of saline for 30 min. After 30 min the soaked OMVs were separated by centrifugation at 135,000 × g for 1 h. The supernatant and soaked OMVs were each collected and used in an OMV uptake assay as described above.

SUPPLEMENTAL MATERIAL
Supplemental material is available only online.

FIG S1, DOCX file, 0.5 MB.
FIG S2, DOCX file, 0.03 MB.
FIG S3, DOCX file, 0.5 MB.
FIG S4, DOCX file, 0.03 MB.
TABLE S1, DOCX file, 0.02 MB.
TABLE S2, DOCX file, 0.02 MB.

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