THE UNIVERSITY OF CHICAGO

# PATHOGENIC NICHE ESTABLISHMENT OF *BACTEROIDES FRAGILIS*

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AARON LAWSON HECHT

CHICAGO, ILLINOIS

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## ABSTRACT

Eukaryotic organisms are, in part, defined by their associated microbiota, composed of a complex series of relationships between species. *Bacteroides fragilis* is a ubiquitous anaerobic bacterium in the mammalian intestinal tract. The range of symbiosis and pathogenic factors produced by this organism provide a fascinating window into host-microbe interactions. Non-toxigenic *B. fragilis* (NTBF) strains are symbiotic in the intestinal environment, providing protection from inflammatory disease while Enterotoxigenic *B. fragilis* (ETBF) strains induce colitis through a toxin called *B. fragilis* toxin (BFT). This dichotomy allows for detailed study of the interactions between these bacteria and their effect on the environment in which they live.

The studies contained herein explore the relationships between a virulence factor, molecular cues and bacterial competition in the context of the mammalian intestine. We find that toxigenic strains of *B. fragilis* transcriptionally regulate production of BFT in response to environmental and growth conditions, partially mediated by a two-component system response regulator. Additionally, we demonstrate that a host factor protects from lethal BFT-dependent ETBF infection, illustrating the close relationship between these species and the role of bi-directional signaling molecules in maintaining homeostasis.

The effect of colonization with NTBF differs drastically from that of infection with ETBF. We found strain-specific exclusion of ETBF by NTBF from the microbiota in a type VI secretion system-dependent manner. A putative type VI secretion effector/immunity pair confers protection against pathogen exclusion, increasing host disease burden. Resistance against acquisition of a second non-toxigenic strain was also observed, indicating a potential function of type VI secretion systems in microbiota composition. This resistance did not apply across species, however, suggesting a complex environment- and regulation-dependent interaction in

the colon. Our studies demonstrate a novel role of type VI secretion systems in host-protective colonization resistance against a pathogen. This understanding of bacterial competition may be utilized to define a molecularly targeted probiotics strategy.

# Chapter I

## Introduction

It is increasingly appreciated that multi-cellular, eukaryotic organisms are shaped not only by their own genetic makeup, but also that of their associated microbes, called the microbiota. Work and theory within the last decade has produced the concept of 'holobionts', on which evolutionary pressure is applied (Zilber-Rosenberg and Rosenberg 2008). The concept of a holobiont entails consideration of both the host and all of the microbes comprising the microbiota as one organism, wherein an interdependent relationship develops for the survival of all involved species. Expansion of this theory into testable hypotheses is ongoing and remains controversial (Bordenstein and Theis 2015). Recent studies support these concepts, including the predominance of indigenous microbes over environmental selected microbiota during sequential colonization of mice germ-free mice (Seedorf et al. 2014). The principles resulting from the holobiont concept provide a useful tool for considering host-microbial interactions. For instance, consideration of single organisms as analogous to a gene on the level of holobiont evolution allows testing of single bacteria for their role in host development. Our studies focus on the bacterium *Bacteroides fragilis*, an organism with a complex, multifaceted host interaction, including both protective and injurious lifestyles. Molecules produced from both bacteria and the intestine mediate this relationship. This chapter will introduce current knowledge within the field on the signals that allow both the host and bacteria to control their environment.

**Intestinal homeostasis through capsular polysaccharide A**

The human microbiota is predominantly comprised of two phyla: Bacteroidetes and Firmicutes. Within the Bacteroidetes, the Bacteroidales is the most predominant order in the human microbiota (Faith, Colombel, and Gordon 2015; Faith et al. 2013; Comstock et al. 1999; Coyne, Roelofs, and Comstock 2016). This includes the genus *Bacteroides*, the most studied of the Bacteroidetes. The *Bacteroides* are a genus of gram-negative anaerobic organisms, without a known extra-intestinal lifecycle. Within the *Bacteroides* are multiple prominent species for human health, including *B. fragilis* and *B. thetaiotaomicron*, comprising up to 2.5% and 10% of the microbiota, respectively (Ley, Peterson, and Gordon 2006; Ley et al. 2008; Eckburg 2005). The lifecycles of these organisms has become increasingly well understood, showing many important factors produced by the host and bacteria.

One molecule of particular interest is polysaccharide A (PSA), a capsule generated and released by *B. fragilis.* This capsule was originally discovered for its ability to cause abscess formation when injected into the intraperitoneal space and the requirement of PSA for *B. fragilis* to form extra-intestinal abscesses (Tzianabos, Onderdonk, and Rosner 1993; Tzianabos et al. 1994; Comstock et al. 1999). Indeed, multiple capsule types have been found to cause abscesses when produced by *B. fragilis*, each of which can be turned on or off through reversible promoters (Liu et al. 2008; Kuwahara et al. 2004; Coyne et al. 2003).

This capsule repertoire is also required for colonization of the host where elimination of certain capsule types diminishes competitive niche establishment. In particular, PSA mediates close contact, allowing for colonization of the mucus layer produced by the epithelium (Round et al. 2011). This occurs in a targeted manner: PSA capsule is delivered through secretion of outer

membrane vesicles, which traverse the mucus layer to access the tissue through sulfatase expression in the bacteria (Shen et al. 2012; Hickey et al. 2015). PSA induces T-regulatory cells (Tregs) and the anti-inflammatory cytokine IL-10 through uptake by dendritic cells. These effects are TLR2-dependent on both cell types and require MyD88 signaling in the T-cells (Round and Mazmanian 2010; Round et al. 2011; Kubinak et al. 2015). The induction of Tregs stops rejection of *B. fragilis* in close contact with the host epithelium and increases the recovered colony forming units (CFU) from the colonic mucosa-associated compartment. This Treg-inducing response corrects a deficiency in the germ-free mice, normalizes the disequilibrium between $T_h1$ and $T_h2$ cells, reduces the $T_h17$ response against *B. fragilis* and protects the host from inflammatory bowel disease and autoimmune encephalomyelitis in animal models (Round and Mazmanian 2010; Q. Wang et al. 2006; Mazmanian, Round, and Kasper 2008; Round and Mazmanian 2009; Mazmanian et al. 2005; Y. K. Lee, Menezes, and Umesaki 2011). PSA is therefore considered to be a molecule that signals symbiosis between *B. fragilis* and the host and may be a co-evolved mechanism of allowing for commensal colonization in close approximation to the host tissue (Mazmanian, Round, and Kasper 2008; Y. K. Lee and Mazmanian 2010).

**Host-*Bacteroides* symbiosis through carbon metabolism**

The *Bacteroides* serve other commensal and symbiotic roles in the microbiota, predominately revolving around digestion of complex plant polysaccharides. Multiple *Bacteroides* species are able to digest plant polysaccharides, otherwise indigestible by the host (McNeil 1984). This can be considered an exporting of critical digestion functions to the microbiota, supporting the holobiont model. Digestion of these carbohydrates is not possible by

many other organisms in the intestine, but *Bacteroides*, in particular *B. thetaiotaomicron*, *B. ovatus* and *B. fragilis* are able to utilize these carbon sources for survival in the gut (Flint et al. 2008; Salyers et al. 1977; Martens, Chiang, and Gordon 2008). Survival of the *Bacteroides* species is determined by the ability to digest various molecules within the colonic environment (Sonnenburg et al. 2010).

The *Bacteroides* are able to catabolize these carbohydrates through the function of various polysaccharide utilization loci (PULs). These are multiple-gene operons, called starch utilization system (Sus)-like genes, which encode secreted, inner- and outer-membrane proteins, conveying the ability to digest and import these complex carbohydrates (Martens et al. 2011; Koropatkin, Cameron, and Martens 2012; Koropatkin et al. 2008; Comstock 2009). Different *Bacteroides* species have evolved a wide array of systems, numbering in the hundreds of genes per organism (Martens et al. 2011; Xu et al. 2003; Xu and Gordon 2003). As such, complex carbohydrate metabolism genes can comprise up to 10% of the bacterial genome. Through this divergence, the *Bacteroides* have developed systems for digestion of host-produced glycans.

**Host-generated oligosaccharides and anti-bacterial factors serve as mediators of *Bacteroides* symbiosis**

The host is known to control its microbiota in many ways, including generation of an inflammatory response, secretion of anti-microbial peptides and selection of glycans from which select organism can forage. *Bacteroides* species are able to avoid most of these defenses, indicating their adaptation of an interaction in the mammalian gut. Mucus is a critical barrier produced by goblet cells in the colonic epithelium. This provides lubrication and protection from

material moving through the lumen, including microbes. One mechanism of selecting species for colonization of the mucus layer is through glycosylations (Koropatkin, Cameron, and Martens 2012; Koropatkin et al. 2008). Mucus is composed of a mix of amino acids and carbohydrates; the predominant form of mucus in the colon is the protein Muc2, which is heavily O-glycosylated. These O-liked carbohydrates are digestible by particular PULs in the *Bacteroides*, upregulated in their presence and enhancing survival/transmission of the organism (Martens, Chiang, and Gordon 2008). Digestion of mucin-linked oligosaccharides by *B. thetaiotaomicron* releases fucose into the surrounding environment. During mouse infection modeling, this metabolism protects the host from damage by *Citrobacter rodentium* through virulence factor downregulation by available fucose. In the absence of *B. thetaiotaomicron*, these sugars are inaccessible to the *C. rodentium* and thus the mouse experiences disease (Ng et al. 2013; Pacheco et al. 2013; Pickard et al. 2014). Human milk oligosaccharides have a similar structure to mucus, including glycosylations that are indigestible to the host cells. The PUL loci that break down mucus can similarly be upregulated to utilize the milk oligosaccharides, thereby enhancing colonization during early life (Marcobal et al. 2011; Martens et al. 2009). Indeed, of the entire microbiota, supplementation of milk oligosaccharides into the diet of a mice alters the *B. fragilis* expression profile more significantly than any other bacteria (Charbonneau et al. 2016). Therefore, the host utilizes glycosylations of its own secreted proteins to provide a barrier that is only survivable by particular species of bacteria and excludes pathogens.

Mucus provides a second layer of protection by serving as a reservoir for anti-microbial peptides, released by the host to dictate the composition of microbes in that space (Antoni et al. 2013). *Bacteroides* species are remarkably resistant to high concentrations of polymyxin B (PMB), an anti-bacterial agent that mimics the structure of human antimicrobial peptides. PMB

is able to kill most bacteria through binding to the negatively charged phosphate of LPS, but *Bacteroides* species avoid this binding via a phosphatase enzyme called LpxF, which removes the charged group (Cullen et al. 2015). This allows for resistance to PMB and colonization of mice; consistent with this finding, a mutant in the *lpxF* gene results in decreased CFU relative to the wildtype (WT) clone. Moreover, this effect is enhanced when the mice are treated with a pro-inflammatory molecule or bacteria, stimulating the release of antimicrobial peptides. The effective loss of this advantage causes elimination of the clone when co-colonized with other species.

*B. fragilis* likely has a significant requirement for LpxF. The lifecycle of this species particularly involves the mucus, as *B. fragilis* specifically localizes to the inner mucus layer of the colon, distinct from many other organisms (Huang, Lee, and Mazmanian 2011). This is possible for several reasons: 1. direct ability to bind to the mucus, thereby providing a stable adhesive site for attachment; 2. capability of digesting host mucus oligosaccharides as its sole carbohydrate source; 3. ability to survive host antimicrobial peptides; and 4. suppression of the host immune response through PSA signaling to decrease rejection (Huang, Lee, and Mazmanian 2011; Salyers et al. 1977; Cullen et al. 2015; Round et al. 2011).

These traits allow for *B. fragilis* to colonize a rare niche in the colonic mucosal crypt (Lee et al. 2013). Through expression of a novel PUL, named the commensal colonization factor (*ccf)* locus, *B. fragilis* is able to colonize up to 10μm below the surface of the colonic epithelium, likely due to either binding or breakdown of a particular oligosaccharide. The localization to this physical space allows for stable colonization of the intestine in mouse modeling and resistance to both antibiotic elimination and pathogen invasion: while a WT clone is able to fully recover after

these microbiota disturbances, the mutant is defective. Other *Bacteroides* species encode loci that are similar, but significantly diverged from that of *B. fragilis* and therefore do not compete for the same niche space. These observations led to the hypothesis that this *B. fragilis ccf* locus provides the capability to form a 'stem cell'-like niche, which then seeds the remainder of the intestine. The mucus layer is a critical part of the host-bacterial interaction within the *Bacteroides* genus, and acts as a signaling molecule to the microbiota, selecting for certain species of benefit to the organism as a whole. Loss of the mucus layer in mice, through mutation of Muc2, results in significant disturbance of homeostasis, leading to severe pathogen infection, spontaneous colitis and colon cancer (Bergstrom et al. 2010; Velcich et al. 2002; Van der Sluis et al. 2006).

### *B. fragilis* toxin is a pro-inflammatory signal from Enterotoxigenic *B. fragilis*

While most of the *Bacteroides* species are thought to be intra-intestinal symbionts, required for the host and microbial lifecycles, a set of strains within the *B. fragilis* species produce a toxin, known as *B. fragilis* toxin (BFT). These strains are referred to as Enterotoxigenic *B. fragilis* (ETBF), while the remainder of the species is referred to as non-toxigenic *B. fragilis* (NTBF). Myers, *et al*. were the first to detect ETBF, originally in farm animals, where dense packing and poor sanitation resulted in spread of disease (Myers et al. 1984). The first case was found in a sick lamb: isolation from diarrheal samples and reinoculation confirmed that a *B. fragilis* strain was the cause. Studies also found ETBF strains in other livestock such as calves, young pigs and rabbits causing similar diarrheal symptoms (Myers and Shoop 1987; Myers et al. 1985; Myers et al. 1989; Myers, Shoop, and Collins 1990).

Quickly thereafter, ETBF was associated with human diarrheal cases, both adult and infant, where the supernatant from the isolates caused a positive reaction in a rabbit ileal loop model (Myers et al. 1987; Sack et al. 1992). Further studies found that supernatant from the toxigenic strains caused a loss of epithelial integrity, decreased intercellular junctions, cell rounding, detaching of epithelial cells into the lumen and neutrophil recruitment (Myers, Collins, and Shoop 1991; Collins, Bergeland, and Myers 1989; Duimstra et al. 1991). It was, therefore, theorized that these strains encoded a toxin generating these disease phenotypes.

Eight years after the initial identification of ETBF as a diarrheal-causative agent, BFT was first purified by the Wilkins group (Van Tassell, Lyerly, and Wilkins 1992). The toxin was found to have a molecular weight of approximately 20kD and was able to induce a similar response in the ileal loop model to ETBF supernatant. Further work by this group and others showed that toxin is present in the membrane fraction of ETBF, but is not cytotoxic and has a molecular weight of approximately 45kD, suggesting the presence of an inhibitory pro-domain (Van Tassell, Lyerly, and Wilkins 1994; Franco et al. 1997). A portion of the toxin was subsequently cloned from ETBF and enzymology studies found that it functions as a metalloprotease (Moncrief et al. 1995). Future work would prove these predictions correct, as crystallography of the toxin reveals an inhibitory pro-domain and structural homology to the A Disintegrin And Metalloproteinase proteins from eukaryotic organisms (Goulas, Arolas, and Gomis-Rüth 2011). The mechanism of pro-domain removal and secretion was unknown until work from our laboratory uncovered Fragipain (Fpn), a novel cysteine protease that cleaves and activates BFT *in vitro* (Choi et al. 2016).

Upon advances in sequencing technology, the toxin was found to be encoded within a pathogenicity island (BFPAI), along with a second metalloprotease-encoding gene, called *mpII*, which has a distinct target cleavage profile from BFT (Moncrief et al. 1998; Claros et al. 2006; Shiryaev et al. 2013). The *bfpai* locus is flanked by mobilization genes and is present within a broader context of a large, conjugative transposon, variably present in NTBF strains, suggesting that the *bfpai* may be transferred horizontally to NTBF strains (Franco et al. 2004).

Through the utilization of cultured colonic epithelial cells, it was found that the toxin causes significant rearrangement of the cytoskeleton and alters cell morphology (Donelli, Fabbri, and Fiorentini 1996; Saidi and Sears 1996; Saidi et al. 1997; Koshy, Montrose, and Sears 1996). These cellular effects occur through binding of BFT to an unidentified proteinaceous receptor, present on the basolateral side of epithelial cells, and cleaves the adherens junction protein E-cadherin (Wu et al. 2006; Wu et al. 1998). Concomitant with E-cadherin cleavage, the cells secrete IL-8 and TGF-β in response to NFκB signaling (Sanfilippo et al. 2000; Wu et al. 2004). Human studies confirmed these effects, where fecal IL-8 and TNF-α levels are elevated during ETBF infection (Sears et al. 2008).

Cases of diarrheal disease in humans have long been associated with ETBF. ETBF has been isolated from both inflammatory and watery diarrhea, where other known enteric pathogens are absent from the stool (Sears et al. 2008; Sears 2009; Zhang et al. 1999; Rabizadeh et al. 2007; Obuch-Woszczatyński et al. 2004; Pituch et al. 2002; Albert et al. 1999; Niyogi et al. 1997; San Joaquin et al. 1995). ETBF is also associated with cases of active inflammatory bowel disease (IBD), but not patients with IBD in remission or healthy controls (Prindiville et al. 2000). Additionally, increased colon cancer rates have been associated with ETBF infection, while

ETBF has been isolated from 100% of late stage colon tumors in a recent study (Ulger Toprak et al. 2006; Boleij et al. 2014). These findings have been confirmed in mouse models, where ETBF exacerbates IBD and increases colon tumor formation in susceptible mice (Rabizadeh et al. 2007; Wu et al. 2009; Rhee et al. 2009). All of these phenotypes are attributable to BFT and are not observed in response to NTBF strains.

BFT-driven colon cancer is dependent upon an increased IL-17/$T_h$17 response from colonic lymphocytes (Wu et al. 2009). Stat3 induction of intraepithelial and lamina proprial lymphocytes occurs within 6 hours of intoxication, followed by epithelial cell Stat3 expression (Housseau and Sears 2010; Wick et al. 2012). This is proceeded by IL-17 generation, predominantly by gamma delta T cells, which have the greatest impact on colon tumorigenesis of the IL-17 sources (Housseau et al. 2016). A reduced burden of ETBF through antibiotic treatment, within the first two weeks of inoculation, substantially decreases tumorigenesis (DeStefano Shields et al. 2016).

**Colonization of *B. fragilis***

*B. fragilis* is a significant part of the microbiota, but has an outsized influence on disease and the microbes around it. Modeling from human microbiota samples identify *B. fragilis* as a keystone organism, indicating its presence is critical in determining the other bacteria comprising the community (Fisher and Mehta 2014). While ETBF is associated with disease, up to 20% of the population is colonized asymptomatically, suggesting an ability to live at homeostasis with its host (San Joaquin et al. 1995; Zhang et al. 1999). Once colonized, strains of *B. fragilis* are very stable, remaining for up to a year (Zitomersky, Coyne, and Comstock 2011). This occurs

through the aforementioned *ccf* locus, which conveys colonization resistance against challenge strains after initial colonization (Lee et al. 2013). The following chapters document studies on the role of bacterial and host factors in the colonization of *B. fragilis*, focusing on BFT regulation mechanisms, competitive colonization through type VI secretion and colonization resistance in excluding ETBF from the microbiota.

# Chapter II

## Regulation of signaling molecules in host-bacterial homeostatic interactions

### Introduction

Co-evolution of bacterial communities and their mammalian hosts has produced varied and complex relationships between these species (Lee and Mazmanian 2010). The close proximity of these organisms and their reliance on one another necessitates communication, mediated by a range of microbial and mammalian molecular cues. *B. fragilis* serves as a fascinating model organism for the study of host-bacteria interactions, due to its identification as symbiont, commensal, keystone organism and pathogen, dependent upon both strain- and host-specific variables. The close relationship between *B. fragilis* and its host is exemplified by the multitude of factors produced by each organism that mediate this interaction.

Capsule generated by *B. fragilis*, named polysaccharide A (PSA), alters the host immune response in mouse models (Mazmanian et al. 2005). Release of PSA from outer membrane vesicles causes a TLR2-dependent increase in FoxP3+ regulatory T cells (Tregs) and proper development of the immune system in germ-free mice, including increased numbers of CD4+ T-cells and a decrease in the $T_h2$ population (Round and Mazmanian 2010; Shen et al. 2012). These immunomodulatory effects provide anti-inflammatory effects in the intestine, generating host-protection against models of inflammatory bowel disease (IBD) through suppression of the $T_h17$ response (Mazmanian, Round, and Kasper 2008). Moreover, decreased $T_h17$ cells induced by

PSA allows for mucosal-associated colonization of *B. fragilis* within the intestine. *B. fragilis* is thus considered a symbiotic organism, with PSA serving as a critical mediator of the interaction.

Similarly, the host modulates its interaction with *B. fragilis* through both anatomy and secreted products. These features form the core of the intestinal biogeography, which is of critical importance to the *B. fragilis* lifecycle (Donaldson, Lee, and Mazmanian 2016). The nutritional landscape for organisms in the intestine is divided into two parts: lumenal and mucosal. The lumenal region is filled with rich carbohydrates, from which bacteria can forage, including host-indigestible plant and milk oligosaccharides along with microbe-produced saccharide sources (Martens et al. 2011; Martens, Chiang, and Gordon 2008; Marcobal et al. 2011; Martens et al. 2009). While nutrient dense, this is also the most competitive space for carbon, given the high concentration of organisms. In the mucosal-associated compartment, the carbon source is predominately comprised of glycosyl residues on the host-produced mucus. Specifically, the inner mucus layer is structured by the host protein Muc2; the heavy O-linked glycosyl residues are relatively inaccessible to bacteria, selecting for a particular set of organisms that can thrive in this region (Velcich et al. 2002; Martens, Chiang, and Gordon 2008; Van der Sluis et al. 2006). Additionally, the presence of anti-microbial peptides secreted by the host reduce the bacterial load in this space (Cullen et al. 2015).

*B. fragilis* is able to overcome both of these barriers to inhabit the inner mucus through anti-microbial resistance, immune suppression, mucus binding and mucin-oligosaccharide degradation for carbon (Huang, Lee, and Mazmanian 2011; Cullen et al. 2015; Round et al. 2011). Mucin-degradation is a central part of the *Bacteroides* lifecycle, occurring through numerous diverse polysaccharide utilization loci (PULs), of which *B. fragilis* encodes over 30

(Martens et al. 2009; Martens, Chiang, and Gordon 2008). As *Bacteroides* have unique access to this nutrient space, it is possible that the relative nutrient-accessible density for *B. fragilis* is highest in the inner mucus (Pacheco et al. 2013). The mucus serves as a platform for *Bacteroides* species to colonize separate niches, within colonic crypts, through differential encoding of PULs. *B. fragilis*, through the commensal colonization factor (*ccf*) locus is able to stably form a 'stem cell-like' population, serving as a stable niche to survive pathogen infiltration or antibiotic treatment (Lee et al. 2013). The stringent environment generated by the host allows for selection of organisms in close proximity with the epithelium and thereby is a central mediator of symbiosis with *B. fragilis*.

While much of the interaction between *B. fragilis* and its mammalian host is symbiotic, the strain and environment can dictate a more harmful, pathogenic relationship. When *B. fragilis* is found in extraintestinal spaces, due to dysbiosis or physical disruption of the epithelial barrier, it is the leading cause of anaerobic abscess and sepsis (Wexler 2007). Interestingly, the same molecule that determines symbiosis in the intestine, PSA, is critical for these extra-intestinal diseases, likely due to the same immunomodulatory effects (Coyne et al. 2001).

Within the colon, a sub-group of *B. fragilis* called Enterotoxigenic *B. fragilis* (ETBF) induces acute intestinal disease in mammals, including humans (Myers et al. 1987; Myers et al. 1989; Myers et al. 1984). This disease was attributed to a secreted protein, called *B. fragilis* toxin (BFT), produced exclusively by ETBF strains. Through cleavage of the adherens junction protein, E-cadherin, BFT induces non-cytolytic damage to the intestinal epithelium, causing an increased $T_h17$ response (Wick et al. 2012). The increased inflammation causes tumor formation in colon-cancer susceptible mice and exacerbates IBD symptoms in the dextran sulfate sodium

(DSS)-colitis mouse model (Wu et al. 2009; Rhee et al. 2009; Housseau et al. 2016). ETBF is physically associated with colon cancer in human patient populations, particularly in the mucosa of the cancerous tissue (Boleij et al. 2015). It is also correlated with activation of inflammatory bouts in patients with IBD (Rabizadeh et al. 2007). Despite the association with disease states, ETBF readily colonizing up to 20% of healthy human populations (Zhang et al. 1999; San Joaquin et al. 1995; Sears 2009).

The pathogenic potential of ETBF strains raises questions about the origin of this subgroup and its relationship with non-toxigenic *B. fragilis* (NTBF) strains. BFT is genomically encoded in a small (~6kB) pathogenicity island (*bfpai*), which is only known to encode one other gene, *mpii* (Moncrief et al. 1998). Both BFT and MPII are metalloproteinases, though only BFT has any ascribed role during disease (Shiryaev et al. 2013). Mobilization genes flank the pathogenicity island, indicating it is a transferable genetic element (Franco et al. 1999). This site is similarly present in NTBF strains, but remains as a small, non-coding sequence in the absence of the *bfpai*. A putative conjugative transposon contains the integration site in ~50% of NTBF strains (Franco 2004). This data suggests that the toxin is potentially mobilized to NTBF strains through multiple mechanisms and that some strains may be more susceptible to insertion than others.

The consistent presence of BFT within the pan-genome of *B. fragilis* indicates that the toxin is in some way part of the ETBF lifecycle and likely provides some benefit to the organism. Logically, however, the secretion of a pro-inflammatory molecule in an organism that is otherwise symbiotic and anti-inflammatory is paradoxical. The specifics further raise questions about the utility of BFT in light of the pro-$T_h17$ features of the toxin and anti-$T_h17$ effects of

PSA. The seemingly contradictory nature of these molecules provides a model for understanding the core interaction between host and bacteria during a commensal relationship. As both NTBF and ETBF strains asymptomatically colonize mice at similar rates, these strains provide a window into the role of bacterial toxins in commensal homeostatic states. This understanding may shed light on a set of pathogens that more commonly associate with humans as commensals, despite the encoding of toxins, such as *Staphylococcus aureus*, *Clostridium difficile*, *Escherichia coli* and *Streptococcus pneumoniae*.

One approach to understanding the role of a toxin in the lifecycle of an organism is to describe the conditions under which that toxin is produced. Previous studies have provided limited insight into the context of BFT secretion. The secreted toxin is maximally produced in early-stationary phase of growth but is decreased ten-fold when excess glucose is supplemented into the media (Van Tassell, Lyerly, and Wilkins 1992). While proposed to result from catabolite repression, this phenomenon has never been described in *B. fragilis*. Expression of plasmid-encoded BFT in two strains of NTBF, with and without the flanking conjugative transposon shows that only the strain encoding the flanking region was able to fully produce active toxin, theorized to be caused by the presence of a transcriptional regulator in this region (Franco et al. 2002). Work from our lab has since shown that BFT is also regulated through activation by the *B. fragilis* cysteine protease, Fragipain (Fpn), and through glycosylation of the pro-domain (Choi et al. 2016, Choi and Bubeck Wardenburg, unpublished).

In this chapter, we show that BFT is regulated on a transcriptional level by its environment, including both fermentable carbohydrate availability and growth phase. We additionally delineate the toxin promoter and through pulldown studies identify a novel

transcriptional regulator of BFT, the two-component system response regulator, RprY. RprY binding to the promoter is enhanced by phospho-activation and overexpression of either RprY or its cognate histidine kinase, RprX, significantly reduces BFT expression. Finally, we find that disruption of host mucus in a mouse colonization model causes BFT-dependent lethality, possibly related to toxin regulation. These data reveal novel mechanisms of BFT regulation and host mucus as mediators of host-pathogen homeostasis. Further understanding of these topics will provide insight into multi-species interactions within the microbiota and shed light on the relationship of pathogens with their hosts.

## Results

### BFT is transcriptionally regulated by environmental cues

There are no known transcriptional regulators of BFT and the environment in which the toxin is released has not been studied. We wanted to determine if the aforementioned glucose-suppression phenotype is specific to glucose or extends more broadly to other carbohydrates. To accomplish this, ETBF was grown overnight with a panel of supplemental carbohydrates and the supernatant screened for toxin production through anti-BFT immunoblot. Comparison of active toxin in the supernatant reveals that, in addition to glucose, multiple carbohydrates suppress toxin output relative to the no supplement control, while others have no effect (Figure 1a). This phenotype correlates with fermentation, as *B. fragilis* can only metabolize the suppressive carbohydrates, indicating that toxin is co-regulated with simple carbohydrate metabolism (Cato and Johnson 1976).

Toxin production can be broken down into three phases: transcription of the full-length toxin, translation of the preprotoxin, and cleavage of pro-toxin into its active form by Fpn. To determine the point of toxin regulation in response to carbohydrates, a time course was performed with and without supplemental glucose, isolating supernatant to probe for active toxin, cell pellet to test pro-toxin concentrations and RNA at multiple time points during a growth curve. Each fraction was tested for toxin expression via immunoblot (supernatant, cell pellet) or QRT-PCR (RNA). This reveals several key pieces of information: first, toxin expression is minimal in all three fractions before early stationary phase, despite the large majority of the total cell growth already occurring (Figure 1b,c). Second, glucose suppresses toxin expression at every time point, indicating that this phenotype is not sensitive to time (Figure 1c). Third, without glucose supplementation, BFT production increases during early stationary phase in all fractions, with the majority of expression occurring within a 1-2 hour period, confirming and extending data from previous literature (Van Tassell, Lyerly, and Wilkins 1992). Finally, glucose suppression of supernatant toxin observed in Figure 1a extends to the pro-toxin in the cell pellet fraction and BFT mRNA (Figure 1c,d). Together, this data suggests that toxin is produced under specific conditions *in vitro*, dependent upon environment and growth phase, regulated through a transcriptional mechanism.

**Putative promoter P4 is the RNA polymerase binding site for BFT transcription and is required for the glucose suppression phenotype**

Transcriptional regulation of virulence factors in response to environmental cues is a common trait among pathogens (Miller, Taylor, and Mekalanos 1987; Mani and Dupuy 2001;

Pacheco et al. 2013). As BFT is regulated in response to both growth phase and environment, we sought to use ETBF as a model for the role of a toxin during host-pathogen commensalism. To further explore the context in which *bft* is expressed, we pursued studies to define its promoter structure and transcriptional regulators.

Previous work on the structure of promoters in the *Bacteroides* genus reveals a variation from that of *E. coli* (Bayley, Rocha, and Smith 2000). Specifically, the *Bacteroides* have a consensus sigma factor binding site at -7/-33 as opposed to the -10/-35 canonical sequence. Additionally, the sequences differ significantly, with *Bacteroides* having a TAnnTTTG/TTTG consensus sequence. Previous work on BFT and its potential promoter structure revealed that BFT expression is dependent upon the pathogenicity island, specifically ~800bp upstream of the toxin translational initiation site (Franco et al. 2002). Additionally, this work revealed five putative promoter sequences, named P1-5, which have the approximate configuration of the aforementioned consensus sequence (Figure 2a). Finally, it was shown that P1 alone was not sufficient to drive toxin production.

Plasmid-borne BFT with the full 800bp upstream sequence intact produces toxin in both the pro-form in the pellet and the active form in the supernatant (Figure 2b). Serial truncation of the upstream region, corresponding to the putative promoter sites, revealed that loss the region upstream of P5, and P5 itself had no effect on toxin production, but removal of P4 had a striking impact, with no detectable toxin remaining by immunoblot (Figure 2b). Subsequent mutation of the -7 and -33 sites independently, within the context of the full 800bp upstream region, each produced a similar phenotype to the complete removal of P4 (Figure 2b; data not shown), demonstrating the importance of the P4 promoter alone for transcription of the toxin.

To further define the BFT promoter structure and confirm P4 as the sigma factor binding site, we utilized 5' rapid amplification of cDNA ends (5'RACE) to determine the transcriptional start site. As tri-phosphate groups are present on the 5' end of bacterial full-length mRNA transcripts, removal of this group with tobacco acid pyrophosphatase (TAP) was used to differentially resolve the non-degraded mRNA (Figure 2c). This procedure revealed a single band visible after agarose gel separation, exclusively present in the +TAP lane. Sequencing of this band shows that the cDNA matches the upstream region of BFT up to 6bp downstream of the -7 site of P4 (Figure 2d), matching expectations of transcriptional start site locations with respect to sigma factor binding sites in *B. fragilis* (Bayley, Rocha, and Smith 2000; Smith, Rogers, and McKee 1992). To determine if P4 is required for glucose regulation of BFT, the toxin was deleted from the ETBF strain through allelic exchange and a copy of BFT was inserted in the genome, with the P4 promoter replaced with a constitutive promoter for RpoD from *B. thetaiotaomicron*. While the P4 promoter allowed for a 30-fold decrease in active toxin produced, the RpoD promoter only had a two-fold change, indicating that the majority of glucose regulation on BFT is P4 promoter-specific (Figure 3a). Removal of the toxin and its P4 promoter from the context of the surrounding genome via plasmid-based expression generated the same glucose-responsive phenotype, which was lost when replaced with a constitutive promoter (Figure 3b). This work confirms that the sequence containing P4 is critical BFT promoter and is both necessary and sufficient for environmental regulation in response to carbohydrate.

**RprY, a two-component response regulator, binds to the P4 promoter**

The importance of the P4 promoter suggests that DNA-binding transcriptional regulators modulate toxin production. In order to determine the genetically-encoded factors involved in this process, we utilized a DNA-pulldown approach. Using the P4 promoter as bait we identified the proteins pulled down from ETBF lysate, with either no DNA or an irrelevant DNA from within the coding region of BFT as differential comparison samples. After elution, bands were compared by silver stain gel and sent for mass spectrometry analysis (Figure 4a,b). Five bands were visibly distinct between the conditions: RNA polymerase (RNAP) complex components 1. RNA polymerase alpha (RpoA), 2. RpoB, 3. RpoC; 4. Rho transcriptional terminator and; 5. RprY, a two-component response regulator (Figure 4a-c). The presence of the RNAP complex components indicates that a promoter is present, further supporting P4 as the sigma factor binding site for BFT. A sigma factor, however, was not present in the elution and further attempts to identify this transcription factor were unsuccessful. Based on previously mentioned literature that the flanking region of the BFPAI is thought to be important in regulation of toxin, we mutated the only sigma factor identified by gene homology contained in this region. However, mutation of this sigma factor had no effect on toxin expression (data not shown). Given the importance of sigma factors in *Bacteroides* gene regulation, future discovery of this factor may be critical to understanding the environment in which BFT is regulated and the genes that are co-transcribed with the toxin.

In addition to the RNA polymerase complex, the other notable discovery from pulldown of the BFT promoter is RprY, the transcriptional regulator of a two-component system (Figure 4a,c). RprY has been previously identified in an organism related to *B. fragilis*, *Porphyromonas*

*gingivalis* (Duran-Pinedo, Nishikawa, and Duncan 2007; Krishnan and Duncan 2013). In this organism, it is an orphan response regulator: that is to say it is not genetically coupled to a cognate activator histidine kinase. Additionally, it has been tied to environmental response, particularly sodium and oxidative stress. Finally, in *B. fragilis*, RprY and its cognate histidine kinase, RprX, were found to alter *E. coli* tetracycline resistance when heterologously expressed, mediated by change in levels of the OmpC and OmpF (Rasmussen and Kovacs 1993).

**Activated RprY binds specifically to the BFT promoter**

To confirm that RprY is a DNA-binding transcriptional regulator of BFT, we generated RprY antiserum in a rabbit. Repeat of the pulldown from Figure 3, followed by an anti-RprY immunoblot confirmed that RprY is specifically pulled down by the P4 promoter and not irrelevant DNA or no DNA controls (Figure 5a). To further test the specificity of RprY binding to the BFT promoter, we performed electrophoretic mobility shift assays (EMSA) with recombinant RprY protein. Increased quantities of RprY caused a shift of the promoter at 93 pmol of protein (Figure 5b), similar to that of other two-component system EMSAs (Wen et al. 2006). To determine if RprY binding to the BFT promoter is enhanced or inhibited by phosphorylation of the regulator domain, a high-energy phosphodonor, acetyl-phosphate, was added before testing binding affinity. Phosphorylation of RprY increased binding to the P4 promoter, as indicated by the DNA shift at lower concentrations of the protein (Figure 5b). Surprisingly, phosphorylated RprY produced increasing shift at various molecular concentrations, suggesting a possibility of multiple binding sites on the DNA or multimer formation. RprY, in its native state or phosphorylated, had no binding affinity to irrelevant DNA

control, as there was no shift detected under these conditions (Figure 5b). Finally, to definitively show the interaction of RprY and the BFT promoter, the promoter DNA was bound to phosphorylated RprY and competed with either cold-promoter or cold-irrelevant DNA control. Competition with the cold-BFT promoter precluded DNA shift while cold-irrelevant DNA had no effect (Figure 5c). This data demonstrates the strong affinity of the transcriptional regulator RprY for the P4 toxin promoter, implicating its involvement in toxin modulation.

**RprY and its cognate histidine kinase downregulate BFT**

Specific binding of RprY to the P4 promoter site strongly suggests that BFT transcription is modulated by this interaction. Ideally, deletion of RprY in ETBF would provide insight into its role in toxin regulation. However, saturating transposon mutagenesis in *B. fragilis* revealed *rprY* as an essential gene for *in vitro* growth (Veeranagouda et al. 2014). Our results mirrored these findings, as we were unable to generate an *rprY* mutant through allelic exchange, despite identification of multiple wildtype (WT) sensitive clones. While the transposon mutagenesis was also unable to find *rprX* mutant clones within their library, the authors suggest this may be due to polar effects on the downstream, essential *rprY*. Through allelic exchange, we were able to isolate a clean deletion mutant of *rprX*, however, this did not generate noticeable changes in BFT production *in vitro* (data not shown).

As previously mentioned, RprY is an orphan transcriptional regulator, lacking RprX, in other species within the Bacteroidetes phylum. To determine if RprY has an effect on BFT production, we generated overexpression clones of both RprY and RprX, driven by a highly active constitutive promoter, glyceradehyde-3-phosphate dehydrogenase (GAPDH)

(Veeranagouda et al. 2014). Overexpression of either RprY or RprX independently produced significantly decreased toxin production in the cell pellet, without altering cell growth or RpoA loading control (Figure 6a). QRT-PCR for BFT confirms that RprX and RprY overexpression significantly dampen toxin on a transcriptional level (Figure 6b). This work defines the two-component system of RprX/Y as a toxin suppressor, working either in tandem or independently to decrease toxin transcription (Figure 7a,b).

Immunoblot of ETBF cell pellet with antiserum generated against RprX and RprY reveals that overexpression of RprX increases its concentration by over 10-fold, while overexpression with the same promoter only provides a ~2-fold increase in RprY (Figure 6a). This confirms previous RNA-sequencing (RNA-Seq) data, demonstrating that RprY is expressed *in vitro* at much higher levels than RprX and perhaps explains the aforementioned lack of BFT regulation in the RprX mutant (Veeranagouda et al. 2014).

**Muc2 is required for protection from BFT-induced lethality**

Mucus is a critical part of the host-bacterial interface, providing protection from exposure to potential pathogens and limiting close contact to particular organisms. One of the major monosaccharide components of the predominant extracellular mucin, Muc2, is galactose, which suppresses BFT (Figure 1a) (Kunz et al. 2000; Ninonuevo et al. 2006; Koropatkin, Cameron, and Martens 2012). We predicted that mucus downregulates toxin, thus providing protection to the host from ETBF and BFT despite the localization of the organism to the inner mucus layer. To test this, we bred Muc2 mutant mice lacking a key exon of the gene (Velcich et al. 2002). Infection of these mice with WT ETBF caused 100% lethality within 48 hours of oral inoculation

whereas WT mice had minimal negative health outcomes and no death by 120 hours (Figure 8a). Importantly, infection of both WT and Muc2 KO mice with ETBF lacking BFT produced no lethality in either group, thus showing the centrality of toxin in the phenotype (Figure 8b). This demonstrates that the mucus layer provides a critical platform for ETBF-host interface and is a central part of the normal homeostatic interaction in this host-pathogen interaction.

**Discussion**

Understanding the interface of bacteria and their hosts is only a recently explored frontier in microbiology, cell biology and immunology. As our knowledge of the microbiota grows, so too does our appreciation of the importance of these organisms in human health and disease. Maintaining homeostasis in the context of thousands of bacterial species living on and within us requires both host and bacterial factors. *B. fragilis* provides a novel model organism for exploring multiple aspects of these relationships simultaneously. Additionally, as a prominent member of the microbiota, it stands as an important bacterium for human health. NTBF and ETBF are separated, in part, by the production of a disease-causing toxin providing a dichotomy between their lifecycles. Understanding how this toxin is regulated will provide critical insight into the context of its expression and its signaling role in the host-pathogen relationship.

In this chapter, we have defined environmental and transcriptional signals that regulate BFT. The identification of a two-component system that binds to the toxin promoter and regulates its expression provides key insight into the context of BFT-relevance in the host-bacterial relationship. Several questions remain about the RprX/Y system and its role in toxin regulation. The essentiality of RprY *in vitro* and simultaneous dispensability of RprX argues that

RprY is activated independently of RprX and perhaps serves to integrate multiple cues. This is supported by the previously discussed status of RprY as an orphan response regulator in other organisms (Duran-Pinedo, Nishikawa, and Duncan 2007). Possible alternative means of RprY activation include phosphorylation by a separate histidine kinase or direct activation by a high-energy phosphodonor within the cell (Figure 7b). For classical two-component systems within the *Bacteroides* genus, the promiscuity of histidine kinases and response regulators outside of the cognate pair is low, as opposed to hybrid two-component systems (Townsend et al. 2013). This decreases the likelihood of a non-RprX histidine kinase activating RprY; instead, RprX could serve to enhance RprY signaling under particular conditions. High-energy phosphodonors such as acetyl-phosphate play a role in activation states of two-component systems in bacteria, providing a crossover point between carbon metabolism and two-component signaling events (Wolfe 2005; Fredericks et al. 2006). Considering the phenotype of the RprY overexpression and glucose suppression, RprY may mediate toxin regulation in response to carbohydrates (Figure 7b). Future work will be required to understand the environmental cues that activate or repress this signal and to determine the genes that are co-regulated with BFT, so as to define the conditions of toxin expression.

Loss of the host-signaling molecule, Muc2, results in lethality during mouse colonization. A similar phenotype has been shown with the mouse pathogen *Citrobacter rodentium*; however, as an organism that requires physical contact with the epithelium to cause disease, a loss of mucus enhancing pathology and death is not surprising (Bergstrom et al. 2010). Indeed, microcolony formation was observed in the Muc2 KO mice, indicating significant mucosal overgrowth of the pathogen. As *B. fragilis* is a normal commensal with a secreted toxin as its key virulence factor, the cause of lethality in this model is not readily apparent. We propose that loss

of the normal *B. fragilis* niche in the inner mucus layer relieves toxin repression by carbohydrates and/or RprX/Y signaling, thereby causing increased toxin expression and intestinal damage (Figure 9a,b). This may be exacerbated by increased toxin exposure to the epithelium due to loss of the sieve-like mucus or decreased separation between the bacteria and host-cells. Alternatively, provided our findings on BFT-mediated sepsis lethality, the loss of Muc2 may increase systemic exposure *B. fragilis* (Choi et al. 2016).

This work provides the first evidence that mucus acts as an intermediary of host-pathogen homeostasis between ETBF and the colon. Ongoing studies will focus on the molecular mechanisms of this homeostatic interaction through further investigation of toxin regulation and colonic cell signaling. This work will provide necessary insight into understanding the normal interaction between a model pathobiont and its host, perhaps applicable to other commensal organisms with pathogenic capabilities.

# Chapter III

## A type VI secretion system mediates colonization resistance against pathogenic *Bacteroides fragilis*

This chapter is adapted from a version of the forthcoming manuscript 'A type VI secretion system mediates colonization resistance against pathogenic *Bacteroides fragilis*' currently in revision. The authors on this manuscript are as follows: Aaron L. Hecht, Zachary M. Earley, Benjamin W. Casterline and Juliane Bubeck Wardenburg. A.L.H performed all experiments alone or in collaboration with Z.M.E or B.W.C.

### Introduction

Bacterial antagonistic relationships are appreciated as a critical factor in defining the dense ecosystem of the intestinal microbiota (Chatzidaki-Livanis, Coyne, and Comstock 2014; Kommineni et al. 2015). Pathogen exclusion through competition with the microbiota is a long-understood strategy of disease prevention, indicating that these relationships may, in part, underpin individual susceptibility to disease (Sassone-Corsi and Raffatellu 2015; Buffie and Pamer 2013; Lawley and Walker 2012; Sekirov et al. 2008; Leatham et al. 2009; Corr et al. 2007; Endt et al. 2010). Enterotoxigenic *Bacteroides fragilis* (ETBF) is a known causative agent of inflammatory diarrhea (Sears et al. 2008; San Joaquin et al. 1995; Wick et al. 2012; Wick and Sears 2010). In humans, toxinogenic strains are associated with colitis, inflammatory bowel disease (IBD) and colon cancer (Prindiville et al. 2000; Ulger Toprak et al. 2006; Sears 2009;

Rabizadeh et al. 2007; Holton 2008; Boleij et al. 2015). Infection of mice with ETBF induces an IBD-like colitis, dependent upon its toxin BFT (Rhee et al. 2009). ETBF also leads to increased tumor formation in susceptible $APC^{min}$ mice through $T_h17$ inflammation (Wu et al. 2009; Housseau et al. 2016; Goodwin et al. 2011). Finally, our work has shown that ETBF sepsis causes lethality via BFT (Choi et al. 2016).

Conversely, NTBF acts as a symbiont, protecting against inflammatory disease (Mazmanian et al. 2005; Mazmanian, Round, and Kasper 2008; Round and Mazmanian 2010; Round et al. 2011). This occurs through the release of the capsule polysaccharide A (PSA) from outer membrane vesicles, signaling to increase Foxp3+ Tregs and dampen $T_h17$ inflammation. In this way, PSA has contradictory function to BFT. NTBF is able to restore normal colon architecture in two IBD mouse models and while ETBF also encodes the locus for producing PSA, BFT secretion clearly overrides this effect (Mazmanian, Round, and Kasper 2008; Wu et al. 2009).

Analogous to other toxin-producing intestinal pathogens, human fecal samples suggest a competitive interplay between ETBF and non-toxigenic *B. fragilis* (NTBF) strains (Leatham et al. 2009; Nagaro et al. 2013; Zitomersky, Coyne, and Comstock 2011). *Bacteroides* species prove to be particularly stable over time in the microbiome (Faith et al. 2013). Longitudinal human studies show that the *B. fragilis* population in an individual microbiota is stably predominated by either ETBF or NTBF. Fifteen volunteers were monitored for fecal *B. fragilis* composition, using BFT as a strain marker (Zitomersky, Coyne, and Comstock 2011). This experiment found that every individual had a *B. fragilis* community predominantly composed of either ETBF or NTBF, not an even mix of both. Moreover, monitoring of the individual over

time revealed that those colonized with NTBF initially remained so by the end of the study, one year later. The same was true for those with ETBF at the initial collection. The determinants of this competition and susceptibility to ETBF colonization remain unknown, representing a unique model for the study of microbiota-pathogen interactions. In mouse modeling, antibiotic treatment five days or two weeks-post colonization eliminates ETBF from the microbiota and protects against colon tumor formation, indicating that reduced host-exposure to ETBF decreases the risk of disease (DeStefano Shields et al. 2016). The risk of using antibiotics to eliminate ETBF, however, may outweigh the benefits of decreased colon cancer risk. Therefore, the interplay between NTBF and ETBF strains may be critical in the long-term health outcomes of the host, and could lead to probiotics based targeted therapeutic strategies.

To determine the mechanisms of strain stability in the colonic niche, we took a candidate approach focusing on known competition factors in *B. fragilis*. One prominent determinant of contact-dependent inhibition is the type VI secretion system (T6SS) in which the attacking cell injects effector proteins through the membrane of the target organism (Pukatzki et al. 2006; MacIntyre et al. 2010). The secretion system utilizes a needle complex to deliver effectors into the periplasm of an adjacent bacterium or the cytosol of a eukaryotic cell. T6SSs are composed of multiple units including a needle complex, a membrane-spanning base and a force-generating sheath. The needle complex is predominantly composed of the protein Hcp, which forms a hexameric ring structure and is capped by the protein VgrG (Brunet et al. 2014). The needle is forcefully ejected from the attacking cell through a firing mechanism, driven by the sheath. The sheath functions as a homolog of phage contractile apparatus and is composed of the proteins TssB and TssC, which form dimers around the Hcp ring structure within the cytoplasm (Basler et

al. 2013). The force is generated against large membrane-spanning structures of the inner and outer membranes.

Effectors are injected into the target cell through four mechanisms: 1. encoding at the C-terminus of the tip protein VgrG, 2. binding to VgrG, 3. binding to adaptor PAAR-motif proteins or 4. binding directly to the needle. Effector-neutralizing immunity proteins are encoded in the genome of the attacking strain, typically directly downstream of the effector, to prevent self-intoxication (Brooks et al. 2013; Dong et al. 2013; Fu, Waldor, and Mekalanos 2013). In *Vibrio cholerae*, it was discovered that regions of non-homology are encoded within common T6SS loci of the species. These regions contained differentially encoded effector-immunity pairs, which contributed to strain competition within the species (Unterweger et al. 2014). Effector-immunity combinations defined compatibility groups, allowing for co-existence and growth on agar plates. Combinations of incompatible strains cause significant T6SS-dependent killing.

Broad conservation of T6SS loci was recently uncovered in the Bacteroidetes phylum (Russell et al. 2014). This included *B. fragilis*, for which the locus had not previously been discovered due to the lack of obvious homology to multiple conserved T6SS genes. *B. fragilis* is able to kill the closely related *B. thetaiotaomicron in vitro* during plate competitions assays. The authors found this to be T6SS dependent, as mutation of *tssC* in *B. fragilis* relieved the growth inhibition of *B. thetaiotaomicron*. The Bacteroidetes comprise up to 50% of the human microbiota; T6S may play a key role in determining its composition (Coyne, Roelofs, and Comstock 2016). The complex biogeography of the intestinal ecosystem necessitates examination of putative competitive factors *in vivo* (Donaldson, Lee, and Mazmanian 2016). The distinct biological properties of non-toxigenic and toxigenic *B. fragilis* provide a model to

examine both symbiont-pathogen competitive colonization and bacterial antagonism in composition of the microbiota. Moreover, the competition between *B. fragilis* strains provides a proof of concept for targeted probiotics in disease modification.

## Results

### Type VI secretion limits colonization of an ETBF strain *in vivo*

To understand competitive dynamics within the *B. fragilis* species, we utilized a co-colonization system in specific pathogen-free (SPF) C57BL/6J mice. Specifically, mice were pre-treated with clindamycin for one day, followed by continuous antibiotic treatment in the water for the duration of the experiment. Two strains of *B. fragilis*, one ETBF and one NTBF were marked with antimicrobial resistance conferred by a plasmid. While both plasmids encode a clindamycin resistance cassette, maintained in the organisms through antibiotic treatment in the water, they are distinguished through expression of either a chloramphenicol resistance cassette (chloramphenicol acetyl transferase; CAT) or tetracycline resistance cassette (TetQ gene; Tet) (Lee et al. 2013). Orogastric gavage was performed on the antibiotic-treated mice, delivering a 1:1 ratio of the marked strains. Colonization was monitored over time by fecal colony forming unit (CFU) recovery on selective media. Co-colonization of mice with NTBF strain NCTC 9343 (N1) and ETBF strain ATCC 43858 (E1; Table 1) resulted in N1 predominating in the microbiota, having a significantly higher rate of colonization from day 5-post gavage to 4 weeks-post colonization (Fig. 10a).

We hypothesized that competition between these strains may be mediated by T6S, with N1 killing E1 *in vivo* through differential encoding of effector/immunity pairs. To examine the role of T6S in *B. fragilis* competition, we generated an N1 mutant harboring a genomic deletion of the *tssC* locus (N1 Δ*tssC*). This gene encodes an essential machinery component of the T6SS, one of the two members of the heterodimeric pair TssB/TssC, which forms the sheath (Leatham et al. 2009; Russell et al. 2014; Nagaro et al. 2013). Co-colonization of N1 Δ*tssC* with E1 caused a loss of E1 repression, resulting in non-significant differences between the two strains (Figure 10b). E1 was found to overtake N1, displacing the non-toxigenic strain from the $10^{10}$ level it typically achieves. The E1 suppression phenotype produced by wildtype (WT) N1 was regained by plasmid complementation of *tssC* (N1 Δ*tssC*::*tssC*), expressed under control of the highly active GAPDH promoter (Figure 10c).

To understand how N1 functions to control E1 colonization, E1 fecal CFU levels were compared between groups in competition with N1 WT, N1 Δ*tssC* and N1 Δ*tssC*::*tssC*. E1 competition with N1 Δ*tssC* produced toxigenic strain colonization approximately 100 fold higher than competition with N1 WT or N1 Δ*tssC*::*tssC* (Figure 10d). E1 colonization in the context of *tssC* mutant N1 was approximately equivalent to mono-infection of E1 at $10^{10}$ CFU per gram feces (Figure 11). The N1 Δ*tssC* clone experienced a concomitant decrease in colonization, corresponding to the level of E1 colonization during WT co-infection, but is restored to WT levels when complemented (Figure 12a). As expected, this switch in colonization between the strains leads to a dramatic alteration in competitive index, changing from a negative value (E1 over N1; $\log_{10}$ transformed) to a positive value when *tssC* is deleted from N1 (Figure 12b). Importantly, N1 and E1 fecal CFU along with competitive index are not significantly different from one another one day-post co-colonization (Figure 13a-c). This indicates that competition is

occurring within the intestine, not before the organisms are inoculated or during passage through the stomach or small intestine. The competition, instead, evolves over time, likely within the confines of the colonic architecture.

To test if T6S is required for colonization in the absence of E1, we performed mono-colonizations of mice with N1 WT, N1 Δ*tssC* or N1 Δ*tssC*::*tssC*. Loss of T6SS function had no effect on N1 colonization, as WT and mutant clones both displayed consistent fecal CFU levels at $10^{10}$ per gram (Figure 14a). Additionally, four weeks post-oral gavage no difference was observed between the groups (Figure 14b). *In vitro* growth curves confirm that the mutant and complemented clones display no gross defect (Figure 14c). This data indicates that the defect in N1 Δ*tssC* growth during co-colonization with E1 is dependent upon the interaction between the two strains. While non-competition roles have now been ascribed to T6SSs in other organisms, this work demonstrates that the importance in strain competition is mediated by the interaction between the two organisms.

**N1 directly kills E1 through T6S during *in vitro* competition**

To determine if the N1 dominance phenotype is a feature of *in vivo* competition, dependent upon the colonic environment or other organisms in the intestine, *in vitro* plate competitions were performed. During 1:1 competitions, $10^8$ CFU of each strain were mixed, spotted on a rich media plate and incubated together overnight. When N1 WT was competed against E1 WT, both strains suffered significant death, resulting in $10^5$ and $10^6$ organisms recovered, respectively (Figure 15a,b). E1 killing was relieved when N1 Δ*tssC* was competed with E1 WT, leaving E1 at the level of input, but resulting in a $10^5$ CFU recovered from N1. This

indicates that both strains are able to kill the other, partially through T6S. As most studies performing T6SS-dependent competition will use a 1:10, attacker:pray ratio, we input $10^8$ N1 and $10^7$ E1 via the same protocol. This produced strikingly different results, with N1 growing up to $10^9$ CFU in either the WT or Δ*tssC* condition (Figure 15c,d). E1 was killed in both conditions, but was diminished below the limit of detection when competed with N1 WT and recovered at around $10^5$ CFU in the N1 Δ*tssC* competition. Both of these experiments confirm that, *in vitro*, the N1 T6SS plays a major role in killing E1 directly. This work has been confirmed by recent publications, showing similar competition between *B. fragilis* strains *in vitro* and in mouse modeling (Wexler et al. 2016; Chatzidaki-Livanis, Geva-Zatorsky, and Comstock 2016).

This data confirms that T6S is a critical factor in competition between *B. fragilis* strains *in vitro* and provides evidence of interaction in the intestinal environment. Moreover, co-colonization of E1 with WT N1 effectively reduces the load of host exposure to toxigenic organism. Understanding the detailed molecular mechanisms of this competition is key to determining how the microbiota can be rationally manipulated for therapeutic purposes.

**Identification of a novel effector/immunity pair defines the *in vivo* competition between *B. fragilis* strains**

Both N1 and E1 encode a T6SS locus, falling into the category of *Bacteroides* T6SSs called Genetic Architecture 3 (GA3) (Coyne, Roelofs, and Comstock 2016). GA3 encodes all of the known machinery components of the *B. fragilis* T6SS in a single operon, including the *tssC* gene (Russell et al. 2014). Alignment of the N1 and E1 T6SS loci reveals that the majority of these loci are conserved between the strains (Figure 16a). In particular, the machinery

components are completely conserved at near 100% identity on the nucleotide level. One sizeable region, however, is highly variable between the strains. This region begins in the 3' end of the VgrG gene and ends at the 3' terminus of a gene approximately 6kb downstream. Homology search of the N1 site reveals that the gene just downstream of VgrG encodes a PAAR-motif, known as the cap of the VgrG needle complex that acts as a delivery mechanism for diverse effectors (Shneider et al. 2013). The remaining genes within this region of non-conservation did not yield hits of any known effector or immunity genes, even after iterative homology search.

The presence of distinct regions between strains downstream of VgrG is reminiscent of strain competition observed *Vibrio cholerae* (Unterweger et al. 2014). Indeed, the region also has a low GC content, similar to the *V. cholerae* loci, suggesting they are elements of the genome that have been more recently integrated. Finally, the location within the VgrG coding region wherein percent strain identity drastically drops is the site of a recombination hot spot. This indicates that strains may exchange this region through horizontal transfer within the species. Recent work supports this concept, as the evolutionary tree of this region does not converge with genome alignment phylogeny (Wexler et al. 2016).

To determine if any effectors are produced by N1, the strain was grown in minimal media and the proteins in the supernatant tested by mass spectrometry analysis. The secretome of N1 WT was compared to N1 Δ*tssC*, sorted by the proteins most distinct between the clones. All of the top hits aligned to the T6SS locus, most of which are known machinery components (Table 2). These include VgrG, TssC, TssB, Hcp-2, Hcp-3, Hcp-4, and the aforementioned PAAR-domain protein. In addition, two proteins of unknown function are significantly decreased in the

N1 Δ*tssC* supernatant: *BF9343_1928* and *BF9343_1937*. While *BF9343_1937* is not a known machinery component and was recently identified as a putative effector, *Bacteroides* T6S effector 1 (Bte1), its presence in both N1 and E1 excluded it from our immediate interest (Wexler et al. 2016). Instead, we focused our attention on *BF9343_1928*, which is not present in strain E1. We therefore considered *BF9343_1928* as encoding a putative effector and hypothesized that the open reading frame immediately downstream, *BF9343_1927*, encodes its cognate immunity protein (Figure 16 a). Concurrent with our work, *BF9343_1928* has now been renamed Bte2 and *BF9343_1927* has been renamed *Bacteroides* T6S immunity 2a (Bti2a). For consistency with the literature, we will incorporate this nomenclature throughout the remainder of the chapter.

To determine if Bte2 is an effector that mediates N1/E1 competition, *bte2* was mutated in N1 (N1 Δ*bte2*). N1 Δ*bte2* was not able to outcompete E1 during co-colonization, and was actually outcompeted by E1 at certain time points (Figure 16b,c). This confirms that this single effector mediates some, if not all, of the T6S-dependent competition of N1 against E1. While two potential cognate immunity proteins are encoded downstream of *bte2*, we cloned *bti2a* onto a plasmid for heterologous expression in WT E1 (E1 *bti2a*). *Bti2a* provided protection to E1 against N1 T6S attack, as E1 *bti2a* was no longer outcompeted at 3- and 4-weeks post co-colonization (Figure 16d). Additionally, this clone displayed enhanced colonization relative to E1 WT when competed against N1 WT at every time point (Figure 16b,d). Moreover, at 4-weeks post-inoculation, E1 was able to achieve colonization levels similar to mono-colonization during competition with N1 Δ*tssC*, N1 Δ*bte2* or when provided *bti2a* (Figure 16e). In aggregate, this is the first direct evidence of *in vivo* strain competition by differential T6S effector/immunity pairs.

**N1 T6S protects its host from ETBF-mediated disease**

To protect the host from BFT exposure and the associated pro-inflammatory disease, NTBF competition can be used to decrease ETBF bacterial load. We hypothesized that this reduced exposure to ETBF, as quantified by fecal CFU, would similarly decrease the host's contact with BFT, thereby leading to lower intestinal inflammation. To test this theory, we first determined the quantity of toxin expressed in E1 competition with N1 WT. RNA was extracted from the feces of mice one week after co-colonization with E1 and N1 WT. QRT-PCR was then performed, testing the level of toxin transcription relative to 16s rRNA. While BFT mRNA was expressed to detectable levels, they were relatively low (Figure 17a). Co-colonization of E1 against N1 Δ*tssC* caused a 75-100-fold increase in toxin transcript, in agreement with the differences in ETBF fecal CFU at that time point. This demonstrates that, as expected, decreased colonization capability by ETBF due to co-colonization with a superior competitor protects the host from toxin.

Next, we explored whether fecal toxin transcript translates into the level of host BFT exposure. In human populations, active infection with ETBF causes a four-fold increase in serum anti-toxin IgG at 21 days-post infection (Sears et al. 2008). To test this in our mouse model, co-colonization studies of E1 with either N1 WT or N1 Δ*tssC* were performed. Four weeks after inoculation, the mice were euthanized, blood was extracted and serum harvested. Using purified, recombinant BFT, an ELISA was performed on serial dilutions of mouse serum to detect serum anti-BFT IgG. End point titers were determined for each sample and compared between groups (Figure 17b). Anti-BFT IgG is 75-100 fold higher in mice co-colonized with E1 and N1 Δ*tssC*

relative to E1 and N1 WT, commensurate with the BFT transcript found in the fecal samples of the same groups.

Colonic tissue damage is a hallmark of BFT-induced disease in mouse modeling. Combination of an epithelial injurious agent, dextran sodium sulfate (DSS), with ETBF infection has been shown to cause an IBD-like phenotype (Rhee et al. 2009). To test if competition with NTBF can reduce this colonic damage, mice were pre-treated with DSS followed by either inoculation with no organisms, ETBF only, ETBF with WT NTBF or ETBF with NTBF Δ*tssC*. Enteric pathogens are noted to cause gross architectural changes to the cecum through virulence factors (Barthel et al. 2003). Infection with ETBF alone caused a four-fold decrease in cecal size, relative to sham-inoculation, indicating significant inflammation (Figure 17c,d). Colon histology revealed that mice colonized with ETBF only had significant ulcerations with complete loss of crypt architecture and immune cell infiltration (Figure 17e). Competition with WT NTBF protected from ETBF disease, revealing no sign of similar lesions. We thus conclude that N1 WT is able to outcompete E1, thereby providing the host with a significant measure of protection from toxin exposure and disease.

**Discussion**

The intestinal microbiota has been linked to a multitude of diseases, ranging from obesity to colitis, inflammatory bowel disease to autism spectrum disorders, heart disease to cancer, and immunotherapy to type II diabetes (Turnbaugh et al. 2006; Gevers et al. 2014; Hsiao et al. 2013; Wang et al. 2015; Gilbert et al. 2013; Vetizou et al. 2015; Sivan et al. 2015; Zeevi et al. 2015; Fischbach and Segre 2016). As our understanding of the species, strains and molecules involved

in these diseases come into view, a requirement for targeted manipulation of the microbiota will emerge. The microbiota matures through the first two-three years of life, likely affected by nutrition, family members and the environment (Subramanian et al. 2014; Yatsunenko et al. 2012; Faith et al. 2013). Once established, many species and strains within the community become stable, particularly true with *Bacteroides* species, thereby losing a window of opportunity for long-term manipulation and intervention to prevent the emergence of chronic diseases later in life (Faith, Colombel, and Gordon 2015).

In this chapter, we present data demonstrating that a mouse colonization model mirrors the aforementioned human study of *B. fragilis* strain stability; we find that strain competition leads to the dominance of one clone within the population. We go on to show that this strain dominance is, in part, mediated by type VI secretion in the dominant NTBF strain, repressing ETBF from full colonization. This, in turn, is due to an effector/immunity pair located in a region of non-homology between the strains within the GA3 T6SS locus. Protection of the ETBF strain through expression of the immunity gene provides direct evidence of interaction within an *in vivo* environment.

Understanding the genes and factors that mediate colonization is of critical importance to deriving molecularly targeted probiotic therapy. Multiple questions remain unanswered in this chapter, including the location of the strain-strain competition *in vivo*. *In vitro* data suggests that co-localization allows for bi-directional killing, where the total bacterial load between N1 and E1 is decreased (Figure 15a,b). However, when N1 outnumbers E1 1:10, the killing is seemingly unidirectional. In both contexts, mutation of the T6SS allows for higher growth of E1, but the input ratio determines which strain wins out. It is thought that killing through T6S requires cell-

cell contact for a significant period of time, and may explain the mixed killing during 1:1 competition, with a cleaner phenotype during 1:10 plating (LeRoux, De Leon, and Kuwada 2012). During 1:10 competition, N1 generated an almost sterilizing anti-E1 response, with all samples falling below the limit of detection. With a significantly enhanced proportion of N1, it is imaginable that the surface of each E1 bacterium is covered by N1, providing little opportunity for counter attack.

Curiously, co-colonization of N1 and E1 at a 1:1 ratio quickly produces a dominant strain. Within five days post-inoculation, N1 has already gained a two-log CFU advantage, which is maintained for the duration of the experiment. Three rationales may explain why the plate competitions do not match the *in vivo* colonization experiments. First, the constant flux of the intestine may prove a critical factor during competition in the colon, sweeping away dead organisms and allowing live ones to thrive. This is supported by a recent model, demonstrating that the combination of mucus binding and flux is a critical host-based factor for determining microbiota composition (McLoughlin et al. 2016). On a plate, dead bacteria likely interfere with both growth and competition, limiting the outgrowth of dominant clones. Second, the presence of other microbes within the microbiome alters the rate of killing between *Bacteroides* (Wexler et al. 2016). Therefore, the dynamics *in vivo* are likely distinct from those *in vitro*. Third, the structures on which *in vivo* interaction occurs are likely critical determinants of the competitive outcome. *B. fragilis* forms biofilms within the colonic mucus layer and the cecum (Swidsinski et al. 2005). It is difficult to imagine incompatible strains stratifying into coherent biofilms; likely single strains or strains from the same compatibility group form biofilms and exclude competitors through T6S (Schwarz et al. 2010; Russell, Peterson, and Mougous 2014). The feces is a second probable location of co-localization and killing, where blooms of each strain would

cause overlap in physical space (Sonnenburg et al. 2010; Martens et al. 2011). Whether the competition occurs within a 'stem cell-like niche' of *B. fragilis* or simply after seeding on the feces could fundamentally alter approaches to defining probiotic strategies (Lee et al. 2013).

Though fecal ETBF load correlates with host exposure to toxin, the location of BFT production is unexplored; understanding the relevance of mucosal and fecal populations in host exposure to BFT will shed light on the dynamics of host-pathogen interaction in the colon. While N1 efficiently eliminates E1 at a level of ~100 fold, it remains unknown how/where the remainder of the E1 population survives and is maintained. Ongoing work will strive to determine the anatomical location of these populations both during competition and maintenance.

Throughout all of the *in vivo* experiments, *B. fragilis* load remains constant at approximately $10^{10}$ CFU per gram of feces, even in the context of the E1/N1 T6SS mutant competition, wherein N1 and E1 bacterial load is roughly similar (Figure 10 b). This would argue that in a strain-independent manner, there is a set CFU of *B. fragilis* achievable in the colon. This has been previously observed for *Bacteroides* species in germ-free mice, arguing architectural and nutrient features of the intestine dictate a *B. fragilis* 'compartment' (Lee et al. 2013).

The strain composition of the *B. fragilis* compartment is of great importance to human health. Our model provides the first evidence of a T6SS endowing protection against an enteric pathogen. Exposure to BFT is associated with colitis, IBD, and colon cancer in human populations and is the causative molecule in mouse models of those diseases. Reduction of ETBF colonization leads to a concomitant decrease in colon tumor formation in a susceptible

mouse model (DeStefano Shields et al. 2016). This chapter demonstrates that use of rationally selected or designed probiotics have the potential to prevent or ameliorate intestinal disease. While most individuals colonized with ETBF are symptom-free, the low penetrance may be due to increased disease burden through chronic, low-level inflammation or enhanced disease in otherwise susceptible individuals. For instance, ETBF is strongly associated with the mucosa of late-stage colon tumors: this could be due to increased tumor formation from constant BFT exposure or an increased rate of growth once pre-formed for unrelated reasons (Boleij et al. 2015). In either case, the presence of ETBF in the human likely increases the risk of poor health outcomes and its elimination would either be beneficial to the population as a whole or to susceptible individuals.

Through comparative genetics and reductionist approaches, we have isolated a specific effector/immunity locus that mediates the competition between an NTBF/ETBF pair. As sequencing technology becomes price-efficient, the identification of particular species, strains and genes within the microbiota may become a normal part of healthcare. This type of 'personalized medicine' will only lead to benefits if there is a detailed understanding of the molecular mechanisms underlying microbial colonization. The NTBF/ETBF paradigm we demonstrate herein may serve as a model for human testing in targeted probiotics. As ETBF colonization rates are as high as 20% in children under the age of one and the microbiota is malleable during this period of life, sequencing for *bft* and the co-encoded *t6ss* locus may allow for competitive, targeted elimination of this pathogen (San Joaquin et al. 1995; Kato et al. 1999).

# Chapter IV

## Invasion of the microbiota is mediated by type VI secretion and the *Bacteroides fragilis* pathogenicity island

This chapter is adapted from a version of the forthcoming manuscript 'A type VI secretion system mediates colonization resistance against pathogenic *Bacteroides fragilis*' currently in revision. The authors on this manuscript are as follows: Aaron L. Hecht, Zachary M. Earley, Benjamin W. Casterline and Juliane Bubeck Wardenburg. A.L.H. performed all experiments presented in this chapter.

### Introduction

Colonization resistance is a phenomenon by which the endogenous microbiota of a host organism restricts super-colonization by invading bacteria. Often, this is mentioned in the context of host protection from pathogen invasion. For over five decades, this phenomenon has been recognized in mouse modeling, wherein treatment with antibiotics substantially reduces resistance to *Salmonella* infection (Bohnhoff and Miller 1962). This phenomenon has been confirmed for a wide range of both gram-negative and gram-positive pathogens, including *Escherichia coli*, *Shigella flexneri*, *Clostridium difficile* and *Enterococcus faecalis*, where colonization with one strain restricts secondary challenge of the same strain (Buffie and Pamer 2013; Buffie et al. 2015; Wilson and Sheagren 1983; Hentges and Freter 1962; Kommineni et al. 2015; Lewis et al. 2015). While the mechanisms of colonization resistance are specific to each

organism and interaction, they generally fall into a few categories. These include competition for nutrients, host binding, and production of anti-bacterial factors, both direct and indirect.

Nutrient-mediated competition is one of the most highly studied mechanisms of colonization resistance. Utilization of common carbon sources is a long-understood means of *S. flexneri* exclusion from the microbiota through competition with similar microbes (Hentges and Freter 1962; Freter 2016). By-products produced from bile breakdown by *Clostridium scindens* causes the death and elimination of *C. difficile* from the microbiota (Buffie et al. 2015). Fucose has been a focus of multiple studies: host fucosylation of secreted mucus, subsequently released by commensal bacteria, can either lead to a decrease in virulence factor expression or outgrowth of the pathogen through carbon utilization (Ng et al. 2013; Pacheco et al. 2013; Pickard et al. 2014). In particular, *B. thetaiotaomicron* decreases the virulence and colonization of *Citrobacter rodentium* through increasing accessibility to host-generated fucose (Pacheco et al. 2013).

Factors other than nutrient sequestration are similarly critical to colonization resistance. Secondary challenge is unsuccessful when the primary colonizing strain attaches to the common sites on the host tissue or mucus (Freter, Brickner, Fekete, et al. 1983; Freter, Brickner, Botney, et al. 1983; Freter 1983; McLoughlin et al. 2016). Direct competition for organisms that co-localize within the intestine can be mediated by anti-bacterial factors produced from the challenge strain (Kommineni et al. 2015). This can also be accomplished indirectly by stimulation of the host to reject a second organism (Brandl et al. 2008; Buffie and Pamer 2013).

The *Bacteroides* species comprise a large part of the microbiota and are stable in a given individual on the strain level. The nature of this stability, however, is not well understood. Primary colonization of gnotobiotic mice with *B. fragilis* does not fill all of the possible niches

within the colon, as the bacterial load is below the capacity of the intestine (Lee et al. 2013). However, secondary challenge with the same strain is rapidly eliminated from the microbiota. This is, in part, determined by the commensal colonization factor locus, a starch utilization system-like locus, which allows for colonization of the colonic crypt mucus and saturation of the *B. fragilis* niche. It is unknown if other strains within the species, in particular toxigenic strains, are able to overcome this colonization resistance.

In this chapter, we determine that colonization resistance within the *B. fragilis* species is strain-dependent. We additionally develop a new paradigm for understanding strain-specific colonization, defined by three genomically encoded properties of the organisms. We find that this colonization resistance phenotype is partially mediated by both type VI secretion (T6S) in the primary colonizing strain, restricting invasion of a second organism. The *B. fragilis* pathogenicity island (*bfpai*) locus is necessary, but not sufficient, for enhanced secondary colonization of an ETBF strain. The T6S-mediated colonization resistance does not extend to other species, despite killing *in vitro*. The specificity of competition for colonization within the intestinal environment strongly argues for testing of competitive factors in an environment native to the organisms. Our work demonstrates that T6S is a major factor for stability of the *Bacteroides*; future manipulation of the microbiota for therapeutic purposes will require detailed molecular understanding of these bacterial interactions.

## Results

### An NTBF strain fails to provide colonization resistance against an ETBF challenge

Colonization of gnotobiotic mice with N1 saturates the *B. fragilis* intestinal niche and prevents secondary challenge by the same strain (Lee et al. 2013). To determine if colonic establishment of the NTBF strain N1 provides colonization resistance against the ETBF strain E1 (Table 1), we colonized mice initially with N1 containing an antibiotic resistance-encoding plasmid. One-week post-primary colonization, the mice were divided into two groups: one secondarily challenged with N1 and the other with E1, each encoding an antibiotic resistance marker that is distinct from the primary colonizing strain. Primary colonization with N1 caused restriction of secondary N1 challenge, while E1 challenge produced stable colonization (Figure 18a). Results in SPF mice phenocopied the gnotobiotic model (Figure 18b). This data suggests strain-specific colonization resistance within the *B. fragilis* species.

### Properties of intraspecific colonization resistance

To define the traits associated with strain-specific colonization resistance, we performed a broader pair-wise analysis of five *B. fragilis* strains, three NTBF and two ETBF (N1, N2, N3, E1, and E2; Table 1) to examine *B. fragilis* competition behavior. While each strain exhibited similar primary colonization (Figure 19a-j), we observed a distinct pattern of intra-specific niche competition between strains (Summarized in Figure 18c). As *Bacteroides* species readily transfer antibiotic resistance horizontally, we wanted to test the identity of successful secondary colonizing strains. To do so, the genomes were compared and regions of difference used to

distinguish between the strains. PCR from colonies of successful secondary strains confirmed that the resistant strains were the challenge organisms (Fig. 20a-e). This work confirms the validity of the mouse model for testing of sequential colonization phenotypes and strain-dependent colonization resistance characteristics in *B. fragilis*.

Completion and organization of the sequential colonization experiments reveals three distinct properties of strain competition and colonization resistance. First, secondary challenge of mice with the same strain used for initial colonization results in complete restriction: a self-restriction phenotype previously observed in N1, extended to four other strains (Fig. 18c, grey box) (Lee et al. 2013). Second, challenge by strains other than self resulted in a strain-specific colonization resistance. Particular strains including N2, N3, and E1 exhibit a dominant exclusion phenotype, restricting non-self organisms from successful secondary challenge (Fig. 18c, red boxes). Finally, our data shows a strong priority effect of *B. fragilis* intestinal niche establishment that can be overcome when challenged by E2, which we call enhanced secondary colonization fitness (Fig. 18c, dashed box). We therefore propose three features of *B. fragilis* colonization resistance: self-restriction, non-self exclusion and enhanced secondary colonization fitness. Notably, these traits are not correlated, as certain strains display non-self restriction but lack enhanced secondary colonization fitness and vice-versa. For instance, E2 is able to colonize after all three NTBF strains, but provides no resistance to non-self secondary colonization. These properties suggest that modular, genetically encoded factors and environmentally driven gene regulation mediate complex strain-strain interactions. Importantly, host protection against ETBF colonization is dependent upon strain of initial exposure.

**Self-colonization resistance requires the *ccf* locus**

One factor involved in self-colonization resistance has already been identified in the *Bacteroides* genus. The commensal colonization factor (*ccf*) is a locus encoding starch utilization system-like genes, thought to be involved in the breakdown of complex carbohydates (Lee et al. 2013). The *ccf* locus allows for inhabitation of the colonic crypt by *B. fragilis* and distinguishes the niches of *Bacteroides* species from one another. Mutation of *ccf* in N1 allows for success of a secondary challenge, thus relieving self-colonization resistance. Mutation of the *ccf* locus in N2 confirmed that this principle is applicable more broadly within the species, whereby this clone was no longer able to exclude WT N2 from secondary colonization (Figure 21 compared to Figure 19c,d). Genetically identical organisms competing for niche establishment on the basis exposure order implicates a regulatory process in the priority effect. Indeed, secondary challenge with organisms isolated directly from a colonized mouse overcomes the colonization barrier (Lee et al. 2013). Our results corroborate this finding and simultaneously imply that genetic strain differences are the basis for variable non-self colonization resistance.

**Type VI secretion mediates non-self colonization resistance**

The T6SS has been implicated in colonization resistance against pathogen invasion (Pukatzki et al. 2006; Russell et al. 2014; MacIntyre et al. 2010); however, *in vivo* molecular evidence for this is lacking. The role of T6S observed in our co-colonization study suggested that this system might govern intra-specific competition in secondary challenge. To examine this hypothesis, we generated a *tssC* deletion mutant in N2 (N2 Δ*tssC*), a strain that demonstrates broad colonization resistance, specifically against self and strains N1/E1 (Figure 19c). *TssC*

deletion relieved N2 colonization resistance against N1, which was restored upon plasmid-based complementation (Figure 22a,b).

To show that *tssC* mutation did not alter N2 colonization resistance through non-competitive means, primary colonization was compared, showing no defect of *in vivo* growth (Figure 22 and figure 23a,c,e). Challenge with N2 after primary colonization with N2 WT or Δ*tssC* confirmed that the encoding of T6S in the primary strain had no impact on self-secondary colonization. This substantiates the concept that effector injection is the mechanism of T6S exclusion during secondary challenge. While N2 Δ*tssC* retains colonization resistance against E1, the rate of elimination was significantly reduced compared to wild type N2 (Fig. 22e,f). Secondary strain recovery one day-post challenge was not significantly different between groups, emphasizing the importance of the colonic environment in mediating competition (Fig. 23b,d,f). These data show that T6S is important for non-self colonization resistance *in vivo*, indicating that direct bacterial antagonism may occur through strain-specific effector-immunity pairs (Brooks et al. 2013; Unterweger et al. 2014; Dong et al. 2013; Fu, Waldor, and Mekalanos 2013).

**Sequential colonization is not altered by an *in vitro*-identified competition factor**

T6S alone cannot explain the complex strain-strain interactions we observed (Fig. 18c). To date, one other antibacterial factor has been identified in *B. fragilis*. Bacteroidales secreted antimicrobial protein 1 (BSAP-1) is a membrane attack complex/perforin (MACPF)-containing protein produced by *B. fragilis* strains for intraspecific competition on plate assays (Chatzidaki-Livanis, Coyne, and Comstock 2014). N2 encodes BSAP-1 and is able to kill the N1 strain, which is missing the *bsap-1* locus, *in vitro*. Mutation of *bsap-1* in N2 (N2 Δ*bsap-1)* had no effect

on N2 colonization resistance against N1 *in vivo* (Figure 24), despite the validity of this model for finding colonization resistance factors (Figure 22a,b). This data demonstrates the importance of the *in vivo* niche in defining factors that mediate intra-specific colonization resistance and stresses the requirement for modeling suspected colonization factors within a native context.

**The *Bacteroides fragilis* pathogenicity island is a putative locus for enhanced secondary colonization**

Our work shows that one strain, E2, displays enhanced ability to colonize in high exclusion strains (Figure 18c). E2 is able to successfully invade the microbiota after primary colonization with all three of the NTBF strains (N1-3), from which we conclude that this strain possesses enhanced secondary colonization fitness. This phenotype provides an opportunity to test the relevance of the *bfpai* locus in secondary colonization. Deletion of the *bfpai* locus results in an approximate five-log reduction of fecal CFU relative to WT E2 after primary colonization with the strain N2 (Figure 25a,b). The phenotype emerges over time, not present one day-post challenge, indicating a dependence on the *in vivo* environment for this defect. We found this to be strain-dependent, not appearing in challenge of N1 or N3 primary colonization (Data not shown).

Future work will be required to determine which elements in the pathogenicity island are required for enhanced secondary colonization and if the factors directly impact on the competing organism or have an indirect effect through host signaling. It is also notable that E1 does not display this phenotype, showing that the *bfpai* locus is not sufficient for enhanced secondary colonization and must require other loci to uncover its effect. This data demonstrates the high

degree of strain specificity in sequential colonization and illustrates that differential encoding of genetic elements in the secondary strain can have an impact on colonization outcomes.

**Type VI secretion competition is species-dependent *in vivo***

The commensal colonization factor (*ccf*) locus of *B. fragilis* enables niche occupancy within the colonic crypt and is required by N1 for self-colonization resistance (Lee et al. 2013). Diverged *ccf* loci of B. *thetaiotaomicron* and *B. vulgatus*, two species closely related to *B. fragilis*, are suggested to define separate niches for those organisms, supported by the observation that N1 does not exhibit colonization resistance against either species (Lee et al. 2013). It is unknown if these niches are spatially distinct from the *B. fragilis* niche or if these *Bacteroides* species interact in the colon. N1 kills *B. thetaiotaomicron* through T6S *in vitro* (Russell et al. 2014), which we confirmed for N2 (Figure 26a). N2, however, did not confer resistance against secondary challenge by *B. thetaiotaomicron in vivo* (Figure 26b), indicating the species-specificity of colonization resistance. Mutation of the T6SS of N2 had no effect on fecal recovery of *B. thetaiotaomicron* after secondary challenge (Figure 26b-d). Similarly, despite T6S-dependent killing of *B. vulgatus in vitro* (Figure 27a), N2 did not restrict *B. vulgatus* in secondary challenge (Figure 27b-d). As T6S is contact-dependent, this data suggests a physical niche separation between these *Bacteroides* species and implies that intra-species competition is a primary function of *Bacteroides* T6S *in vivo*.

**Discussion**

Our data illustrate the importance of bacterial antagonistic factors in the composition and stability of the microbiota, providing direct demonstration of T6S-mediated competition in the intestine. In the context of both co-colonization and colonization resistance, T6S is a mechanism of strain dominance. The Bacteroidetes are a resilient phylum within the human microbiota (Faith et al. 2013). Broad conservation of T6SSs within this phylum suggests that specific effector-immunity proteins are critical mediators of *in vivo* competition (Russell et al. 2014; Coyne, Roelofs, and Comstock 2016). The uneven distribution of T6S within particular species (i.e. present in the majority of *B. fragilis* but absent in *B. thetaiotaomicron*) (Coyne, Roelofs, and Comstock 2016) highlights the importance of microbial lifecycle and ecosystem, and underscores the need to define the molecular mechanisms by which strain-specific effector modules govern competition (Unterweger et al. 2014). While plate-based studies have identified antagonistic factors in Bacteroidetes (Russell et al. 2014; Chatzidaki-Livanis, Coyne, and Comstock 2014), our observations reveal that the biological relevance of competitive factors is exhibited in the context of the intestinal niche. This native environment is essential to provide the appropriate regulatory cues, biogeography, niche, and colonization order.

Membership in a predominant phylum and limitation of the *B. fragilis* lifecycle to the intestine make this organism an ideal model for microbiota competition. Strain-dependent virulence properties of *B. fragilis* associated with human disease further heighten the import of understanding competitive dynamics within this species. Our findings reveal the strong influence that competitive factors have on host exposure to toxigenic organisms and uncover a putative effector-immunity pair mediating this outcome. Non-conservation of this region between *B.*

*fragilis* strains suggests that this may be a critical component in determination of the strain-level composition of the microbiota. Examination of these loci in the native microbiota of human populations may reveal strains that provide enhanced protection against ETBF.

The principles stated within this chapter provide a necessary framework in defining targeted probiotics for rational, therapeutic manipulation of the microbiota, which may apply to other pathogenic bacteria with non-toxigenic strains, including *Clostridium difficile* and *Escherichia coli* (Nagaro et al. 2013; Leatham et al. 2009). Previous work with *C. difficile* shows that pre-colonization in hamsters or mice with a non-toxigenic strain of *C. difficile* leads to both colonization- and disease-prevention of the toxigenic forms (Sambol et al. 2002; Merrigan et al. 2009; Chen et al. 2008). The level of colonization is, likewise, strain dependent (Nagaro et al. 2013). It is now accepted that fecal microbiota transplant (FMT) results in reduced recurrence of *C. difficile* associated diarrheal infection (van Nood et al. 2013). Consortia of bacteria are able to clear the organism and protect from disease in mouse modeling (Lawley et al. 2012; Lawley and Walker 2012). Selection of a particular *Clostridial* species, *C. scindens*, is able to exclude virulent *C. difficile* from the microbiota through production of a toxic metabolite derived from host bile (Buffie et al. 2015). These data illustrate the importance of species, strain and order of colonization in protection against *C. difficile*-related illness. Future work may produce more specific molecular mechanisms of toxigenic *C. difficile* by non-toxigenic strains, allowing for potential prevention of microbiota invasion.

Similar observations have been made in other intestinal organisms including *E. coli* and *Salmonella enterica* serovar Typhimurium (Leatham et al. 2009; Lam and Monack 2014). While the use of a lab-domesticated strain of *E. coli* is not able to produce self-colonization resistance,

WT human commensal and pathogenic strains uniformly do (Lee et al. 2013; Leatham et al. 2009). Additionally, commensal *E. coli* strains are variable in their ability to exclude a hemorrhagic, O157:H1 strain, while collections of strains are able to fully provide colonization resistance. Oral gavage of eight identical, tagged *S. Typhimurium* clones results in a bottleneck for intestinal dominance, resulting in a one-two clone outgrowth (Lam and Monack 2014). Systemic dissemination offered less of a bottleneck phenotype in this same oral gavage model, with an average of three-four clones surviving in a single mouse. Intestinal colonization resistance was also observed in reseeding of the intestine, once primarily infected with the same strain. The features of both of these organisms match those observed for *B. fragilis* strains, suggesting a broad applicability of the principles. Combining the information gleaned from these separate bacteria, colonization of the intestine involves both spatial and regulatory factors that mediate stable population of the host.

Speculatively, we propose the following model for *B. fragilis* colonization: initial inoculation of the host allows for a large population to infiltrate a range of spaces within the intestine. One or two single organisms reach the *B. fragilis* niche, upregulate the genes required for inhabitation of that space and seed the entire cecum and colon. Once established, a challenge with the same strain is unsuccessful due to the lack of gene regulation already attained by the pre-existing clone. Secondary colonization with other strains may or may not be successful, dependent upon differentially encoded factors. Our work has shown that this includes T6S loci and the *bfpai* locus. However, our work cannot explain all of the complexities implied within Figure 18c. Factors likely to impact on this competitive colonization include carbohydrate digesting Sus-like loci, host-targeted factors to induce a favorable environment, bacterial-targeted factors to out-compete other strains and species in the microbiota, and binding factors to

enhance flow-resistant flushing from the intestine (Winter et al. 2010; Martens, Chiang, and Gordon 2008; Kamada et al. 2012; Freter, Brickner, Fekete, et al. 1983; Freter 1983; McLoughlin et al. 2016; Huang, Lee, and Mazmanian 2011).

Future work will be required to continue exploration of the mechanisms involved in the phenotypes presenting within this chapter and the consequences of competitive outcomes between the non-toxigenic and toxigenic strains of *B. fragilis*. Several key remaining questions are as follows: where is the T6S competition occurring within the sequential colonization model? While this may relate to the future work of Chapter III in a technical sense, the actual localization of this competition may be distinct from that of the co-colonization model. Moreover, for successful secondary challenge strains, do they co-localize with the initial strain? Our work would suggest that homeostatic co-existence is difficult to imagine for the tested strains, as most are diverged within the T6S effector/immunity locus. From our five-strain set, only N2 and N3 encode matching T6SS loci and only E2 does not encode a locus at all (data not shown), while the remainder of combinations would likely be competitive. We, therefore, theorize that differential localization of the challenge strain would allow for survival at a lower CFU than the primary strain. Localization data may also shed light on the possibility of T6S preventing a challenge strain from invading a pre-formed biofilm of the primary colonizer. This has been previously theorized for intestinal bacteria and confirmed in flow-modeling for *Burkholderia thailandensis* (Russell, Peterson, and Mougous 2014; Schwarz et al. 2010). As *B. fragilis* is known to form biofilms in the intestine, this is a well-supported hypothesis for future studies.

Understanding the lasting outcomes of long-term colonization with ETBF remains to be determined. However, clinical studies and mouse modeling have consistently associated ETBF with diarrhea, colon cancer and active inflammatory bowel disease (Myers et al. 1989; Pituch et al. 2002; San Joaquin et al. 1995; Wick and Sears 2010; Myers et al. 1984; Albert et al. 1999; Akpinar et al. 2010; Boleij et al. 2015; Ulger Toprak et al. 2006; Rabizadeh et al. 2007; Wick et al. 2012; Sears 2009; Wu et al. 2009). *B. fragilis* is also the number one causative agent of anaerobic sepsis in human populations (Redondo et al. 1995; Ngo et al. 2013; Snydman et al. 2010). Strains encoding the *bfpai* locus are enriched in bloodstream infection, suggesting a causative role in sepsis disease (Kato et al. 1996; Claros et al. 2006; Franco et al. 1999). Indeed, sepsis modeling in mice shows that BFT is a required factor for mortality (Choi et al. 2016). Anaerobic infection is often a result of intestinal homeostasis disruption associated with acute injury or disease, with the microbiota composition during this event likely playing an integral role in health outcomes. Elimination of ETBF from colonizing the host may lead to protection from systemic disease; without the toxigenic organism present, the opportunity to escape into the blood stream is eradicated. Understanding how selective probiotics colonization of mammals alters long-term health consequences will be important for determining an ideal microbiota composition.

Our work demonstrates that molecular factors such as T6S and the *bfpai* locus influence competitive colonization of the intestine. While it had been previously speculated that T6S is involved in colonization resistance against pathogens, our work is the first concrete evidence of this phenomenon *in vivo* (Russell, Peterson, and Mougous 2014). Virulence factors also have a demonstrated role in colonization of other organisms (Winter et al. 2010; Kamada et al. 2012). Future work will be required to determine which modules of the *bfpai* are necessary for E2

colonization and if those factors directly impact the host, bacteria or both. These studies will produce a more comprehensive understanding of colonization resistance and provide targets for future therapeutics preventing host exposure to ETBF.

# Chapter V

## Conclusions

The holobiont theory necessitates complex relationships between host and microbial cells, with interdependence at the center of the concept. *B. fragilis* presents as a unique model for exploring this exchange through a range of molecules produced by both the host and the microbe in this interaction. Some of these molecules are demonstrably beneficial to both organisms, yielding improved health for the host and unique access to food sources for the bacterium. Virulence factors are difficult to fit into this framework, yet they remain encoded by colonizing organisms. The universal detection of BFT around the world in various mammals argues that the expression of this toxin is not a simple anomaly, however, its presence in an otherwise symbiotic bacterium is challenging to reconcile.

The work presented herein provides a new look at the regulation of BFT in response to environmental cues. Our studies illustrate that the toxin is only produced under certain conditions and growth phases, which can be suppressed through addition of simple carbohydrates. This was found to be dependent upon the promoter of the toxin, which we newly identified. Through delineation of the BFT promoter, we were able to uncover the first DNA-binding protein of the BFT promoter, a two-component system response regulator, RprY. We find that overexpression of this response regulator or its cognate histidine kinase produces decreased toxin expression, implicating this system as a toxin suppressor mechanism. While the system may or may not be related to the carbohydrate suppression phenotype, these regulators suggest a tight control on

toxin expression and led us to hypothesize that BFT is regulated so as to maintain close association with the host.

Secreted products from colonic epithelial cells are critical in maintaining the barrier and mediating its interaction with the microbiota. *B. fragilis* has a particularly close association with the mucus layer of the intestine, enabling this organism to colonize a niche within the crypts and create a stable host-associated population. We theorized that toxin regulation in combination with the mucus allows for homeostasis and precludes toxin-induced injury. Loss of the host mucus layer led to lethal infection during ETBF colonization, dependent on BFT. This data suggests an important interplay between these signaling molecules, where mucus protects from toxin-mediated death, the mechanism of which will the subject to future work. Possible explanations include both intra- and extra-intestinal effects. Increased toxin expression by ETBF or increased host exposure to the toxin due to a lost physical barrier could cause increased intestinal damage and mortality through fluid loss. Alternatively, increased toxin expression may induce systemic pathology through aberrant immune signaling, thus causing a sepsis-type response, in the absence of actual bloodstream infection. Finally, mucus defects would allow for ETBF to localize more proximate to the epithelium, possibly causing increased invasion into colonic tissue and egress into the systemic circulation. As ETBF sepsis lethality is toxin-dependent, this possibility is well founded. Further studies will be required to understand the mechanistic interaction of BFT and mucus.

*B. fragilis*, including ETBF, is a long standing co-evolutionary partner of mammalian hosts. As *B. fragilis* has no known lifecycle outside of host-host transmission, these species are likely dependent upon one another for survival. Understanding the role of this toxin in the life of

the host and ETBF would shed light on the interaction between these species and more broadly on the interaction between toxin-encoding bacteria and the host. We will consider the following scenarios with respect to the effect of BFT on both organisms: parasitism, commensalism and symbiosis.

The concept of parasitism entails a relationship in which the host is harmed and the parasite benefits. Toxins are often thought of through this lens, inflicting harm on host cells to protect against phagocytosis, increase replication or cause for dissemination. ETBF could fall into the category of parasite, causing minor host damage for enhanced microbial survival. We propose three theories for BFT-mediated parasitism. First, BFT could stimulate the immune system of the host in order to eliminate competitor bacteria in the same niche space and prevent utilization of necessary carbohydrates. One piece of corroborating evidence for this is that increased carbohydrate decreases toxin production; thus, in a low-nutrient environment, ETBF may be stimulated to increase toxin expression, which in turn causes a release of antimicrobial factors and elimination of competitor organisms. A strategy of increased host inflammation for bacterial benefit is a well understood paradigm of *Salmonella* pathogenesis, stimulating reactive oxygen species through virulence factors and utilizing these as electron donors for enhanced growth (Winter et al. 2010). Second, BFT may stimulate a host response that allows for enhanced replication of ETBF. For instance, toxin may increase the output of nutrients from the host through stimulation of mucus secretion or altered glycosylation of the mucus. There already exists some evidence for this possibility: BFT stimulates the Stat3 signaling pathway in innate immune cells, causing release of IL-22, thereby increasing mucus production from goblet cells in the epithelium (Wick et al. 2012; Sugimoto et al. 2008). Enhanced mucus production may in turn increase the viability of ETBF in its colonic crypt niche, as it would provide a greater access to a

key carbon source. Third, the toxin could stimulate host injury for the purpose of increasing transmission to the next host. ETBF is found commonly in sewage and is prevalent in regions with poor sanitation. Thus, toxin expression, in a more classic sense, could cause diarrhea for enhanced release into the environment and increased fecal-oral transmission.

However, in considering the close ties between *B. fragilis* and its host, it stands to reason that BFT, while deleterious under certain conditions (such as loss of Muc2 or long-term exposure), might be either host-neutral or host-beneficial in evolutionary timescales. In the case of commensalism, a relationship of neutrality to the host but benefit to the organism, one would hypothesize that the pan-genome of *B. fragilis* includes BFT as an advantageous factor in competition for niche acquisition and nutrients. These include the same possibilities described previously for parasitism, but highlight that in an evolutionary sense, low level, local toxin expression may not produce significantly negative outcomes for the host. The maintenance and mobilization of BFT within the *B. fragilis* population strongly argues for a selective advantage for the organism within its native, intestinal environment.

A final possibility is that BFT serves as a symbiotic molecule. Horizontal transmission of an A Disintegrin and Metalloprotease (ADAM) family gene from a mammalian host is the likely derivation of BFT, further emphasizing the close ties between this molecule and host cells (Goulas, Arolas, and Gomis-Rüth 2011). Moreover, BFT can be activated in the presence of mucus alone, without the need for Fpn (Choi et al. 2016). The vast presence of ETBF in mammalian populations argues that BFT may provide host-benefit, perhaps in maintaining intestinal homeostasis. While the toxin can cause clear disease phenotypes in susceptible humans and mouse models, BFT signaling to the host may boost the inflammatory response during times

of dysbiosis or loss of normal environmental conditions. In this way, altered carbohydrate availability and/or RprX/Y signaling could preclude pathogenic invasion by other microbes through immunological priming. Organisms such as segmented filamentous bacteria (SFB) are able to train the immune response so as to exclude deleterious infection (Ivanov and Littman 2010; Ivanov et al. 2009). In a more nuanced way, ETBF may be selected by the host to serve as an extension of the immunological sensing system, with BFT as the signal molecule to bridge the species divide. In this way, as a proxy detector of homeostasis, ETBF may serve as a 'canary in the coal mine' or a 'sentinel bacteria' for the presence of oncoming disruptions, signaling to the host through BFT.

Defining the role of one toxin in a host-pathogen interaction provides insight more broadly into the human co-evolved relationship with the microbiota. While some organisms such as *Vibrio cholerae* quite clearly utilize toxins for the intentional detriment of the host, many others are seemingly more prominent as asymptomatic colonizers. For instance, *S. aureus*, while a harmful and often deadly pathogen, is present on the skin and in the anterior nares of a majority of humans, without causing overt disease. Yet an arsenal of extracellular toxins is broadly conserved, even within these 'commensal' strains. For instance, alpha hemolytic toxin (Hla) is encoded by almost 100% of isolates in the current epidemic strain USA-300 (DeLeo et al. 2011). The maintenance of these virulence factors in the context of health defies simple explanation and belies the close association and co-evolution with the host. For both ETBF and *S. aureus*, survival of the host is integral to their lifecycle, as it is likely required for maximal viability and transmission.

While the co-evolution of toxic molecules may provide some host protection under certain conditions, in the current state of human health, BFT has a demonstrably deleterious effect. This may, in part, be owing to the dysbiosis caused by poor diet or antibiotic treatment in the modern world. Defining the role of this toxin in the lifecycle of this pathogen opens a window to understand mechanisms of host-bacterial homeostasis and may provide insight into future treatment for microbiota-dependent diseases.

The studies within this dissertation demonstrate the importance of microbial interactions within the microbiota for the health of the host. We have shown that *B. fragilis* strains compete for colonization through T6S. The outcome of these interactions determines the composition of the *B. fragilis* compartment; in particular, we find that one NTBF strain has a T6S-dependent protective effect against host exposure to ETBF and BFT, resulting in a decreased host inflammatory response. This data corroborates that of limited human studies, suggesting these principles hold in the human microbiota.

Through systematic description of sequential colonization dynamics between five strains of *B. fragilis*, we propose three properties of strain colonization: self-colonization resistance, non-self colonization resistance and enhanced secondary colonization fitness. These properties are determined by separate genetic loci, producing a complex network of interactions at the strain level in the microbiota. The encoded factors identified in these studies include a carbohydrate utilization operon, a contact-dependent inhibition locus and a pathogenicity island. The multifaceted nature of competitive colonization within a single species exposes the incredible complexity of stable microbiota composition and factors that contribute to dynamic changes within it.

The requirement of T6S in primary colonization for exclusion of other strains from the microbiota indicates that T6SSs are likely a major actor in which species and strains colonize. However, we find that primary colonization of a strain with high non-self colonization resistance properties is unable to exclude the closely related species type strains *B. vulgatus* and *B. thetaiotaomicron*, despite T6S-dependent killing *in vitro*. Moreover, while this same strain is able to kill a separate NTBF strain *in vitro*, we find no role for this factor in sequential colonization. These data demonstrate the high level of specificity for *in vivo* competition factors, which likely depend upon physical approximation, factor regulation, and timing. Further, these studies suggest a requirement for testing of putative colonization and competition factors in a biologically relevant.

One central finding of the work presented herein is that order and strain of colonization matter substantially for the composition of the microbiota and the potential pathogenicity of those microbes. Particular NTBF strains are able to limit host exposure to certain ETBF clones, partially through T6S, while other combinations allow for significant colonization of pathogenic *B. fragilis*. These organisms are associated with a large set of diseases, ranging from colitis to sepsis, diarrhea to IBD, colon cancer to focal inflammation. We speculate that long-term exposure to ETBF and BFT results in disease susceptibility. Chronic inflammation is a known risk factor for both colorectal cancer and IBD; this toxin may be one mechanism of inflammatory stimulation. ETBF in the microbiota also likely increases the risk of anaerobic sepsis secondary to intestinal disruption in select populations of patients. We propose that targeted colonization of these at-risk populations with genetically well-defined strains of NTBF could ameliorate short-term colitis and prevent long-term disease risk through exclusion or dampening of ETBF (Figure 28). As personalized medicine strategies incorporate microbiota sequencing, specifying the

presence of BFT and the co-encoded T6SS locus becomes possible. As the future of targeted probiotics unfolds, understanding the loci involved in colonization will allow for improved and deliberate control of the microbiota.

*B. fragilis* serves as a flexible model for understanding the multifaceted nature of the host-bacterial interaction between mammals and the microbiota. Spanning symbiosis to pathogenesis through discrete molecules provides a clear and unique opportunity for studying the cross-species signals within the holobiont. As these relationships come into view, we gain invaluable insight on human biological function and disruptions of homeostasis that trigger disease.

# Chapter VI

# Materials and methods

## Bacterial culture

*Bacterial strains, culture conditions, and antibiotics*. *Bacteroides* strains used in these studies include *B. thetaiotaomicron* ATCC 29148 and *B. vulgatus* ATCC 8482. *B. fragilis* strains are noted in Extended Data Table 1. *B. fragilis*, *B. thetaiotaomicron* and *B. vulgatus* were grown in Brain Heart Infusion (BHI) broth anaerobically at 37°C with a gas mix of 5% $H_2$, 10% $CO_2$ and 85% $N_2$. BHI was supplemented with 0.0005% hemin and 0.5 μg/mL vitamin K1 for optimal growth (BHIS). *E. coli* S17-1 was used for cloning of shuttle and suicide plasmids and conjugation into *B. fragilis*. *E. coli* strains were grown in LB aerobically at 37°C. Antibiotics used were as follows: ampicillin (100 μg/mL), kanamycin (50 μg/mL), gentamicin (200 μg/mL), clidamycin (5 μg/mL), tetracycline (10 μg/mL), chloramphenicol (10 μg/mL) and rifampicin (20 μg/mL). Carbohydrates for toxin suppression testing were utilized at final concentration of 0.5% (weight/volume). These included Dextrose, L-Arabinose, Fructose, Galactose, L-Rhamnose, Lactose, Sorbitol and Mannitol.

*Conjugations*. A method modified from previous studies was used (Comstock et al. 1999): appropriate *B. fragilis* and *E. coli* strains were grown to mid-log phase in BHIS anaerobically and LB aerobically, respectively. Equal volumes of these cultures were sedimented into a single

tube, resuspended in BHI, pooled onto a BHIS plate and grown aerobically at 37°C overnight. This mix was spread onto a selective BHIS plate with gentamicin and clindamycin and grown anaerobically at 37°C.

*Growth curves*. For testing of toxin production during various growth phases of ETBF, overnight cultures of stationary phase overnight cultures were diluted 1:50 into fresh BHIS. Samples were removed from the culture each hour until stationary phase was reached. For growth testing of N1 WT and the *tssC* mutant, clones were grown overnight, diluted 1:50 in fresh BHIS and grown to mid-log phase ($OD_{600}$= 0.5). The cells were pelleted (5,000g, 5 minutes, 4°C), resuspended in 10mL of PBS and normalized to $OD_{600}$= 0.55 at a 1:5 dilution. The cells were pelleted and resuspended in 1.8mL of PBS. These were diluted 1:100 in fresh BHIS in a 96-well plate in the anaerobic chamber, sealed with optically clear adhesive and tested for $OD_{600}$ absorbance every 20 minutes in a Tecan Infinite M200pro plate reader.

**Cloning**

*Plasmids.* pRK231 is a conjugation helper vector, used to increase transfer from *E. coli* to *B. fragilis*. Allelic exchange mutagenesis was completed with the suicide vector pKNOCK. The shuttle vector pFD340 provided antibiotic resistance against clindamycin (all pFD340-based vectors), tetracycline (pFD340-Tet) and chloramphenicol (pFD340-CAT), as in previous studies.(S. M. Lee et al. 2013) The pFD340-based vectors were used in the *B. fragilis* strains N1, N2, N3 and E1 along with *B. thetaiotaomicron* and *B. vulgatus*. As we could not recover

successful E2 conjugates with the pFD340-based vectors, we generated the shuttle vector pAH2 (see below). The chloramphenicol resistance cassette with associated promoter and ribosomal binding site, called IS4351-CAT, was cloned from pFD340-CAT into the KpnI restriction enzyme site of pAH2 to generate pAH2-CAT. Inserts for all newly generated constructs were sequence verified.

*Generation of pAH1 and pAH2*. While the pFD340 plasmid was usable for strains N1-3 and E1, we were not able to isolate pFD340 positive clones from conjugation and selection. This may be due to the strain-specific restriction systems encoded in phage elements. To complement mutants and use plasmid-based expression in this strain, we generated two new vectors, pAH1 and pAH2. pAH1 is a genome-inserting vector used for single copy expression of genes. The genome insertion has been confirmed through PCR (test primers; Table 3). To generate pAH1, multiple possible insertion sites were identified with the characteristics as follows: downstream of genes on either side, not evidently upstream of any ORFs, greater than 750bp between genes and present in strains N1-3 and E1. Three regions were tested: insertion site 1 (IS1), IS2 and IS10. PCR was performed from E2 genomic DNA, of which only IS2 produced a band of the approximate expected size. This region was cloned into the XbaI site of the suicide vector pKNOCK and transformed into S17-1 *E. coli* (this strain encodes the λ-pir element required for R6K replicon of pKNOCK). pAH2 is a replicating vector for use in strain E2. We predicted that pFD340 rejection from E2 was due to DNA sequence resisted by this strain. As pKNOCK and pAH1 are readily taken up by E2, we cloned the pFD340 replicon in the NotI site of pKNOCK. We were able to generate clindamycin resistant E2 clones after conjugation with pAH2-

containing *E. coli*. We confirmed the identity of E2 through PCR genotyping (See genotyping method) and the status of pAH2 as a replicating vector through plasmid recovery, transformation of competent *E. coli* and sequencing of the vector.

*Determination of BFT promoter and cloning of constitutively active BFT*. To delineate the BFT promoter, the toxin with its upstream region were cloned into the pFD340 vector in the KpnI and BamHI sites with a 6xHistidine tag encoded at the 3' end of the gene. The upstream region was serially shortened with primers around the putative promoters P1-5. These primers are provided in Table 3. Constitutively active BFT was generated in two manners: first, before the discovery of P4 as the BFT promoter, the constitutive promoter IS4351 already encoded in the pFD340 vector was used to drive BFT. The region upstream of BFT previously described to be insufficient for toxin production, which only includes the P1 site, was cloned with the BamHI cut site at the 5' end and KpnI at the 3' end. This allowed for successful driving of toxin expression *in vitro* and is called 'constitutive promoter'. To generate the RpoD-BFT clone, after the discovery of P4 as the promoter, the RpoD promoter was cloned in place of the P4 promoter, leaving intact the normal transcriptional start site. The primers used are shown in Table 3.

*Cloning of RprX,Y and protein purification*. To generate recombinant protein for antiserum production, RprX and RprY were cloned into an expression vector. PCR was performed to amplify RprY from the E1 strain with a nucleotide sequence appended to the 3' terminus encoding a 6xHis tag. The DNA sequence encoding the C-terminal region of RprX, downstream of the putative transmembrane domain, was similarly amplified with a 6xHis tag appended. Both

were cloned into the pET28b vector into the NcoI and XhoI sites. These were then sequence verified and transformed into BL21 cells for protein expression. Overnight cultures of these clones were then diluted 1:50 into fresh LB media and grown to $OD_{600}$= 0.5 and induced for 4 hours with 1mM IPTG. The cells were pelleted and resuspended in buffer composed of 50mM Tris-base, 500mM NaCl, 20mM imidazole, pH 7.4. The resuspended cells were French pressed 3 times, causing lysis and release of soluble protein. After centrifugation for 30 minutes at 12,000g, the supernatant was incubated with nickel-NTA beads, rocking at 4°C for one hour. The column was washed with the resuspension buffer and eluted with the same buffer, supplemented with 250mM imidazole. Elution fractions above a concentration of 1mg/mL were dialyzed against PBS overnight at 4°C.

*Mutants and complementation.* In-frame deletions were generated through allelic exchange using a protocol modified from previous studies (Lee et al. 2013). To generate *tssC* mutants, 1kb upstream and 1kb downstream of *tssC* were amplified from strains of interest and fused via overlap PCR. This construct was cloned into pKNOCK and conjugated into strains N1 and N2. Single clones resistant to clindamycin, indicating genomic integration, were passaged (1:100) daily without antibiotics. After 5-10 passages, single clones were patched onto selective (clindamycin) and non-selective plates. Sensitive colonies were PCR screened for loss of *tssC*. *Bsap-1*, *ccf* locus, *bte2* and *bfpai* locus mutations was generated in the same manner for the N2 strain (*bsap-1* and *ccf*), N1 strain (*bte2*) and E2 strain (*bfpai*).

*TssC* mutation was complemented on pFD340 and pFD340-CAT for N2 and N1, respectively. This was fused downstream of the *B. fragilis* glyceraldehyde-3-phosphate dehydrogenase

(GAPDH) promoter and RBS and cloned into the KpnI restriction enzyme site of the aforementioned vectors. The resulting vectors were named pFD340::*tssC* and pFD340-CAT::*tssC*. Overexpression of *bte2a* was accomplished via fusion to the GAPDH promoter and RBS and was cloned into the KpnI restriction enzyme site of pFD340 Tet.

**Molecular biology**

*B. fragilis pellet and supernatant fraction preparation*: For detection of BFT, RprX, RprY or RpoA in the cell pellet or supernatant fraction, the samples were prepared as follows: 1mL of culture at the indicated time point was pelleted at 5,000g for 5 minutes at room temperature, directly after removal from the anaerobic environment. The supernatant was removed and precipitated in a final concentration of 10% TCA. This was incubated for 1 hour on ice. The cell pellet was resuspended in 2x Laemmli sample buffer and heated to 95°C for 10 minutes. The precipitated supernatant was spun at maximum speed on the bench-top centrifuge for 30 minutes. The supernatant of this spin was removed and washed with 100% acetone to resuspend the pellet. The washed pellet was then spun for 10 minutes at maximum speed, followed by removal of the supernatant, a repeat wash and spin. After removal of the final supernatant, the pellet was air-dried for 30 minutes and resuspended in 2x Laemmli sample buffer.

*Immunoblots*. For all immunoblots, samples were suspended in 2x Laemmli sample buffer run on SDS-PAGE gels (10% for pro-toxin detection, 15% for active toxin, RprX, RprY and RpoA) and transferred onto PVDF membrane. This was blocked with 5% skim milk in TBS buffer

supplemented with 0.1% Tween-20 (TBST). Membranes were subsequently incubated with primary antibody for one hour, followed by three washes in TBST for 5 minutes each. Secondary antibody was incubated with the membrane for one hour, proceeded by three TBST washes. This was subsequently imaged on a Li-Cor Odyssey system.

*Antibodies*. For immunoblots, primary antibody generation and usage were as follows: rabbit anti-BFT antibody was generated, as previously reported.(V. M. Choi et al. 2016) The rabbit anti-RprX and anti-RprY antibodies were created as a service from Pocono Rabbit Farm and Laboratory, under IACUC approved protocol PRF2A. Mouse monoclonal Anti- *E. coli* RNA-polymerase α (RpoA) antibody was obtained from Biolegend, which we found reactive against *B. fragilis* RpoA. Primary antibodies were used at the following dilutions in TBST: BFT-1:2000, RprY- 1:2000, RprX- 1:1000, RpoA-1:2000. Secondary antibodies were obtained from Life Technologies and used at a 1:10,000 concentration in TBST.

*Quantitative Reverse Transcription PCR*. To test the transcription levels of BFT, quantitative reverse transcription PCR (qRT-PCR) was used. RNA was collected from cell culture using the RNeasy kit and RNA protect (Qiagen), according to manufacturers instructions. For fecal pellets, RNA was collected with the ZR soil/fecal RNA microprep kit (Zymo Research). RNase-free DNase (Fisher) was used to digest contaminating genomic DNA in the samples. First strand cDNA synthesis was performed with iScript cDNA synthesis kit (Bio-Rad) and qPCR was performed with SYBR Green (Bio-Rad) on a Bio-Rad CFX96 machine. BFT transcript was

quantified with BFT-specific primers (Table 3) and normalized to *B. fragilis* 16s rRNA. Efficiency for each primer set was determined to calculate accurate fold-differences and melt curves were used to test the specificity of each reaction.

*5'RACE*. To determine the transcriptional start site of BFT, 5' Rapid Amplification of DNA Ends (5'RACE) was performed. To accomplish this, early stationary phase ETBF was pelleted and RNA extract as previously described. 5'RACE was performed with the FirstChoice RLM-RACE kit (Invitrogen) according to manufacturers instructions with two alterations. In brief, Tobacco Acid Pyrophosphatase (TAP) either was or was not added to ETBF RNA. Adaptor DNA was then ligated to the 5' end of the mRNA. cDNA was generated as previously described, but a BFT-specific primer (Table 3) was used instead of random priming, in order to increase the signal. PCR was performed with this same BFT-specific primer and the adaptor-specific primer. This was run on a 2% agarose gel and imaged. One band was found specific to the +TAP lane and was sequenced through sanger sequencing.

*Pulldown*. To determine the binding proteins of the BFT promoter from ETBF lysate, we utilized a modified protocol from a previously published method (Jutras, Verma, and Stevenson 2012). In brief, a 5' biotin-tagged primer with an untagged primer was used to amplify a ~300bp sequence flanking the P4 toxin promoter. This product was column purified to a total of 50µg of DNA per reaction. The same approach was taken with irrelevant DNA control of the same length, amplified from within the BFT coding region. Both of these primer sets are found in Table 3.

Strepavidin-agarose beads (Thermo-Fisher) were washed two times with 2x B/W buffer (10mM Tris pH 7.5, 1mM EDTA, 2M NaCl). The amplified products were bound to beads for 1 hour, rotating at room temperature and washed thrice with TE buffer. The beads were then washed with BS/THES twice, followed by one wash with BS/THES supplemented with 10µg/mL salmon sperm DNA (Fisher).

E1 was grown overnight, diluted 1:50 into fresh BHIS and grown to early stationary phase, the point of maximal toxin induction. 500mL of the culture was pelleted and resuspended in 1xBS/1xTHES buffer (5x BS buffer: 50mM HEPES, 25mM $CaCl_2$, 250mM KCl, 60% glycerol; 2.25x THES: 50mM Tris pH 7.5, 10mM EDTA, 20% sucrose, 140mM NaCl) supplemented with complete, EDTA-free protease inhibitor (Roche). These cells were French pressed four times and spun at 20,000rpm for 30 minute to clear cell debris. 100µg of salmon sperm DNA was added to supernatant, which was then incubated, rotating for one hour. The beads were then washed five times with BS/THES supplemented with 10µg/mL of salmon sperm DNA. Finally, the beads were washed twice more with BS/THES and the supernatant discarded.

Elution was performed with elution buffer (25mM Tris pH 7.5, varying NaCl), increasing NaCl during each elution to serially remove more tightly bound proteins. Each elution was performed through rolling incubation for 5 minutes, followed by spin and storage of the supernatant. This was performed with 100, 200, 300, 500, 750mM and 1M NaCl concentrations. Each fraction was run on an SDS-PAGE gel (15%), comparing promoter DNA to irrelevant DNA and no DNA conditions. Silver stain (Pierce) was performed, bands of interest excised and sent for mass spectrometry peptide sequencing (Taplin Biological Mass Spectrometry Facility, Harvard University).

*Eletrophoretic mobility shift assay*. To determine the specificity of RprY binding to the toxin promoter, we performed an electrophoretic mobility shift assay (EMSA). A protocol was adapted from a previously published method.(Hellman and Fried 2007) Briefly, promoter or irrelevant DNA was radiolabeled with $^{32}P$ using ATP-$\gamma^{32}P$ (Perkin Elmer) and T4 polynucleotide kinase (NEB). The DNA was column purified and diluted to a concentration of 5fmol/µL. RprY was prepared via incubation with or without acetyl phophate in buffer (2x buffer: 50mM Tris pH 7.5, 20mM $MgCl_2$, 0.1mM DTT, +/- 20mM acetyl phosphate, frozen in aliquots at -20°C immediately after making). Incubation of various concentrations of RprY with 5fmol of DNA was performed for 1 hour in 1x binding buffer (5x binding buffer: 100mM HEPES pH 7.9, 300mM KCl, 25mM MgCl, 5mM EDTA, 5mM DTT, 1.5mg/mL BSA, 1mg/mL salmon sperm DNA, 50% glycerol). After 1 hour of binding, the samples were run immediately on a 5% TAE acrylamide gel. The gels were dried, exposed overnight and imaged.

*Secretome mass spectrometry: peptide preparation*. The secretome of WT and Δ*tssC* N1 was analyzed by mass spectrometry as follows: overnight cultures were grown in BHIS, pelleted and washed in minimal media followed by 1:50 dilution into minimal media for overnight growth. Supernatant was collected and TCA precipitated. After a one-hour incubation on ice, the precipitated protein was spun and washed twice with cold acetone. The dried pellet was resuspended in 7.5mM TCEP, 8M Urea, 100mM $NH_4HCO_3$ solution and incubated for one hour. This was spun on a 3K MWCO, mixed with 8M Urea, 100mM $NH_4HCO_3$, 50mM iodoacetamide

solution and incubated in the dark for one hour. 500mM DTT was added to inactivate the iodoacetamide and the filter washed four times with 50mM $NH_4HCO_3$. The remaining solution was trypsin treated overnight, acidified to 1% TFA and dried.

*Secretome mass spectrometry: mass spectrometry.* Note: this section was provided by Dr. Young Ah Goo and was performed at the University of Maryland School of Pharmacy, Mass Spectrometry Center. Peptide (5 µL) was introduced to a hybrid quadrupole-orbitrap mass spectrometer (Orbitrap Elite, Thermo Fisher, San Jose, CA) coupled to a nanoflow LC system (NanoAcquity; Waters Corporation, Milford, MA). A 100 µm i.d. x 20 mm pre-column was in-house packed with 200Å, 5µm C18AQ particles (Michrom BioResources Inc., Auburn, CA, USA). A 75 µm i.d. x 180 mm analytical column was pulled using a Sutter Instruments P-2000 $CO_2$ laser puller (Sutter Instrument Company, Novato, CA) and packed with 100 Å, 5 µm C18AQ particles. Mobile phase A was composed of 0.1% formic acid in water. Mobile phase B was 0.1% formic acid in acetonitrile. For each injection, an amount of 5 µL of sample was loaded on the pre-column at 4 µl/min for 10 min, using loading buffer of 5% acetonitrile and 0.1% formic acid. Peptide separation was performed at 250 nL/min flow rate in a 95 min gradient, in which mobile phase B started at 5%, increased to 35% at 60min, 80% at 65min, followed by a 5 min wash at 80% and a 25 min re-equilibration at 5%.

Mass spectrometry data were collected in positive ionization mode using a data dependent acquisition method with a full MS scan for *m/z* range 350-2000 in orbitrap at 120K resolution. Consecutive MS/MS scans selected top 15 abundant ions in ion trap by rapid scan mode with a

dynamic exclusion of 30 seconds. Precursor ions selected from the MS scan were isolated with an isolation width of 2 *m/z* for collision induced dissociation (CID) energy, NCE, at 35.

*Secretome mass spectrometry: data analysis.* Note: this section was provided by Dr. Young Ah Goo and was performed at the University of Maryland School of Pharmacy, Mass Spectrometry Center. MS data were analyzed by MaxQuant (version 1.5.0.25)(Cox and Mann 2008) using standard settings and a UniprotKB database of Bacteroides fragilis. Peptide-spectrum matches (PSM) and protein identifications were filtered at a false discovery rate of 0.01. Label-free quantification, MS/MS spectral counts, were extracted and used for statistical analysis of differential expression by using QSpec tool (version 1.2.2)(H. Choi, Fermin, and Nesvizhskii 2008). Results are averaged from three technical replicates.

**Mouse modeling**

All animal studies were conducted in accord with ethical regulations under protocols approved by the University of Chicago Animal Care and Use Committee and Institutional Biosafety Committee. SPF C57BL/6 mice were bred in-house from mice originally purchased from Jackson Laboratory or purchased from Jackson Laboratory and maintained under SPF conditions for use in experimentation at 4 weeks of age. Gnotobiotic C57BL/6J mice were also bred in-house, maintained in germ-free conditions and used at 6 weeks of age. Muc2 knockout (KO) breeder mice were a generous gift from the Chang lab and were bred in-house as Muc2 (+/-) x (+/-), heterozygous x heterozygous pairs. All pups were genotyped with both KO mice and WT

controls used from the same litters. All experiments in SPF mice were performed with males, the gnotobiotic experiment with females and Muc2 KO mice with both sexes. Sample size estimates for animal experiments were based on prior animal modeling studies utilized within the laboratory for investigation of colonization. At the time of weaning, animals were randomly distributed for use in experimentation. There was no investigator blinding in animal experimentation, and no animals were excluded from analysis.

*Mono-colonization and co-colonization*. SPF mice were pre-treated with 100 mg/L clindamycin in their drinking water for one day prior to and throughout the course of infection to ensure plasmid retention. To prepare inocula, overnight cultures of *B. fragilis* were subcultured at a 1:50 ratio into fresh BHIS and grown until they reached $OD_{600} \approx 0.5$. Bacteria from 50 mL culture were sedimented and resuspended in 10 mL PBS. These suspensions were adjusted by the addition of PBS until a 1:5 dilution yielded an $OD_{600} = 0.55$. Bacteria from 9 mL of suspensions were sedimented and resuspended in 1.8 mL 0.1N sodium bicarbonate to yield a concentration of $10^{10}$ CFU/mL. For mono-colonization mice were inoculated via oral gavage with $10^8$ CFU of E1 or N1 clones. For co-colonization, mice were co-colonized with $10^8$ CFU of E1 and N1 via oral gavage. E1 was marked with pFD340-Tet, N1 WT and Δ*tssC* with pFD340-CAT and N1 Δ*tssC*::*tssC* with pFD340-CAT::*tssC*. To analyze fecal CFU following oral inoculation, fecal pellets were collected from individual mice, weighed, and vortexed in 1 mL PBS to achieve homogenization. Serial 10-fold dilutions were plated on BHIS agar containing gentamicin and clindamycin plus either tetracycline, to monitor E1 recovery, or chloramphenicol, to determine

N1 clone recovery. CFU/g feces for each clone was calculated, $\log_{10}$ transformed and plotted over time.

*Sequential colonization*. Similar to co-colonization experiments, SPF and gnotobiotic mice were pre-treated with 100 mg/L clindamycin in drinking water for one day prior to and throughout the course of infection to ensure plasmid retention. Inocula were prepared as in co-colonization. Sequential colonization was performed with a modified protocol from previous studies.(S. M. Lee et al. 2013) Primary colonization was achieved with $10^8$ CFU of *B. fragilis* strains (N1, N2, N3, E1) containing pFD340 or E2 containing pAH2 via oral gavage. After colonization for 7-10 days, secondary challenge was performed via oral gavage with $10^8$ CFU of *B. fragilis* strains (N1, N2, N3, E1), *B. thetaiotaomicron*, or *B. vulgatus* containing pFD340-CAT or E2 containing pAH2-CAT. Fecal pellets were handled as noted in co-colonization. Inherent antibiotic resistance (Extended Data Table 1) was used to determine primary colonization fecal CFU; homogenized fecal pellets were plated onto BHIS plus gentamicin and clindamycin only (N1, E1), with tetracycline (N3, E2) or with rifampicin (N2). Secondary challenge CFU was determined by plating onto BHIS with gentamicin, clindamycin and chloramphenicol. CFU/g feces was determined as noted in co-colonization. Limit of detection is dependent upon the weight of each fecal pellet, indicated based on average fecal pellet weight at ~$10^{3.5}$.

*B. fragilis genotyping*. The identity of each strain was determined via PCR with three primer sets (Table 3), which distinguish the five *B. fragilis* strains (Fig. S5). PCR was performed on

successful secondary challenge strains from single colonies of each mouse. The identity of the strains was confirmed by banding pattern.

*Serum ELISA*. Four-weeks post colonization, mice were euthanized and blood was obtained via cardiac puncture. Blood was spun in serum collection tubes and serum stored for ELISA testing. Recombinant, purified BFT was coated onto Maxisorp plates (Nunc) at concentration of 2μg per well overnight in bicarbonate buffer (0.1M) at 4°C. The plate was washed with ELISA buffer and blocked for one hour with 2% BSA in PBS. Serum samples were diluted 1:10 and serially 1:4 to generate a dilution curve in PBS. After blocking, the serum samples were incubated for 1 hour. After 3 washes with elution buffer, secondary HRP-conjugated anti-mouse IgG antibody (Southern Biotech) was applied (1:10,000) for 1 hour. After washing, the elisa was developed for 10 minutes and absorbance recorded on the aforementioned Tecan 96-well plate reader. Data was analysed on Prism to determine the limiting dilution through sigmoidal curve fit, with a cutoff set at 2x background.

*DSS colitis*. To test the impact of *B. fragilis* competition on the health of the mouse, we used Dextran Sodium Sulfate (DSS) as an inducer of colitis susceptibility. Mice were pre-treated with DSS (2%, Fisher) and clindamycin in the drinking water for 5 days pre-colonization. Mice were then either sham-infected, colonized with E1 alone, a competition of E1 and N1 WT or a competition of E1 and N1 Δ*tssC*. Four days post-colonization, the mice were euthanized, the

ceca imaged and weighed and the colons dissected. The colon tissue was fixed in 10% formalin, formed into a swiss roll, paraffin embedded, sectioned and H&E stained.

### *In vitro* competition

*B. fragilis* N2 clones (WT, Δ*tssC*, and Δ*tssC*::*tssC*), *B. thetaiotaomicron*, and *B. vulgtus* inocula were prepared in a similar manner to co-colonization, with the final resuspension in PBS instead of sodium bicarbonate. N2 WT and Δ*tssC* were marked with pFD340 and N2 Δ*tssC*::*tssC* with pFD340::*tssC*, while *B. thetaiotaomicron* and *B. vulgatus* were marked with pFD340-CAT. $10^8$ CFU of N2 was competed against $10^7$ CFU of either *B. thetaiotaomicron* or *B. vulgatus* in a 10 μL spot on a BHIS plate with gentamicin and clindamycin. After overnight competition, these spots were resuspended in 1 mL of PBS and serial 10-fold dilutions plated on BHIS with gentamicin, clindamycin and chloramphenicol to quantify *B. thetaiotaomicron* or *B. vulgatus* recovery. *B. thetaiotaomicron* and *B. vulgatus* competition was compared between N2 WT, Δ*tssC*, and Δ*tssC*::*tssC* groups, normalized as a percentage to the N2 Δ*tssC* group.

### Bioinformatic analysis

T6SS loci of N1 and E1 were aligned in Geneious version 6.0.5. The alignment was performed with Geneious alignment software (cost matrix: 65% similarity, gap open penalty: 12, gap extension penalty: 3) and visualized in Geneious.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism software. Pairwise comparisons were performed using the Student's unpaired two-sided t test. Sets of three groups were tested via one-way ANOVA, with Tukey's multiple comparisons test used to compare between groups. Bonferroni correction was applied to account for multiple time points in colonization experiments. Comparison of variances was performed in GraphPad utilizing the F test, providing documentation of similar variance between groups.

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## Appendix A

## Figures

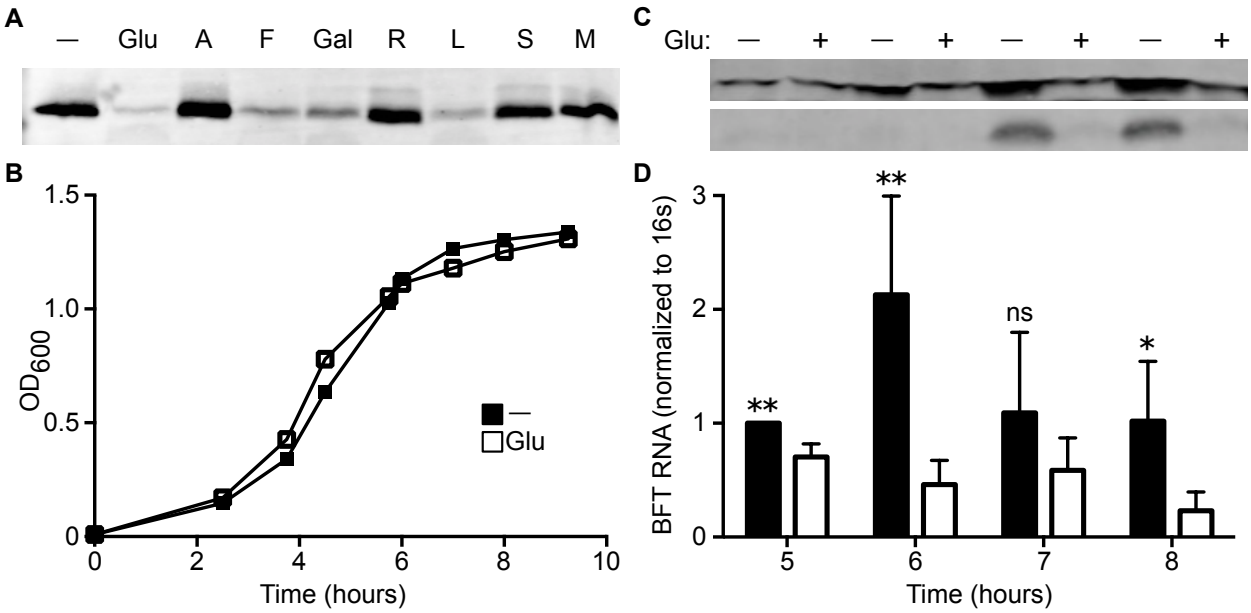



**Figure 1**. Fermentable carbohydrates suppress BFT expression. A. Immunoblot was performed with anti-BFT serum on ETBF overnight cultures, grown in BHIS supplemented with PBS (-) or 0.5% of various carbohydrates (Glu=glucose, A= arabinose, F= fructose, Gal= galactose, R= rhamnose, L= lactose, S= sorbitol, M= manitol). B. ETBF was grown with or without glucose supplementation and growth tested through $OD_{600}$ over time. C,D. At various time points during the growth curve from B, BFT from the (C) cell pellet (protoxin; upper image) and supernatant fractions (active toxin; lower image) or (D) RNA from the cells were extracted and tested via immunoblot against BFT or qRT-PCR respectively. *$p<0.05$, ** $p<0.01$; ns, not significant.

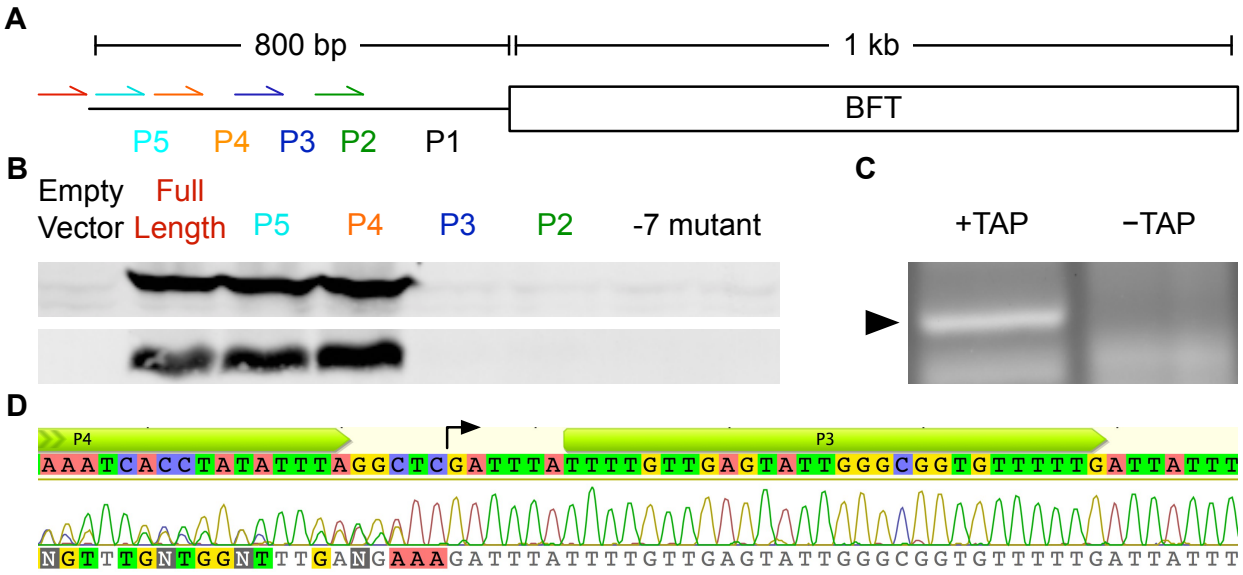



**Figure 2.** The putative promoter P4 is the RNA polymerase binding site for *in vitro* toxin transcription. A. Schematic of the BFT possible promoter structure with five putative promoters (P1-5) identified from homology to approximate sigma factor binding site consensus sequence. Upstream primers used for cloning region around P1-5 are shown. B. Immunoblot against anti-6xHis tag of BFT from cell pellet (protoxin) and supernatant (active toxin) from overnight culture of ETBF encoding plasmid-borne BFT with various lengths of upstream region, matching the color scheme from A. Mutation of the P4 site in the -7 location was tested in the context of the full 800bp upstream region. C. Agarose gel from 5'RACE reaction with and without Tobacco acid pyrophosphatase (TAP). The only band identified as specific to the +TAP lane is indicated (arrowhead). D. DNA sequence of the BFT upstream region encompassing the P3 and P4 sites (above) and sanger sequencing results from the band indicated in C. Mismatch occurs upstream of the guanine base highlighted by the arrow, after which is the adapter sequence. The arrow indicates the transcriptional initiation site. Results are representative of two (C,D) or three (B) independent experiments.

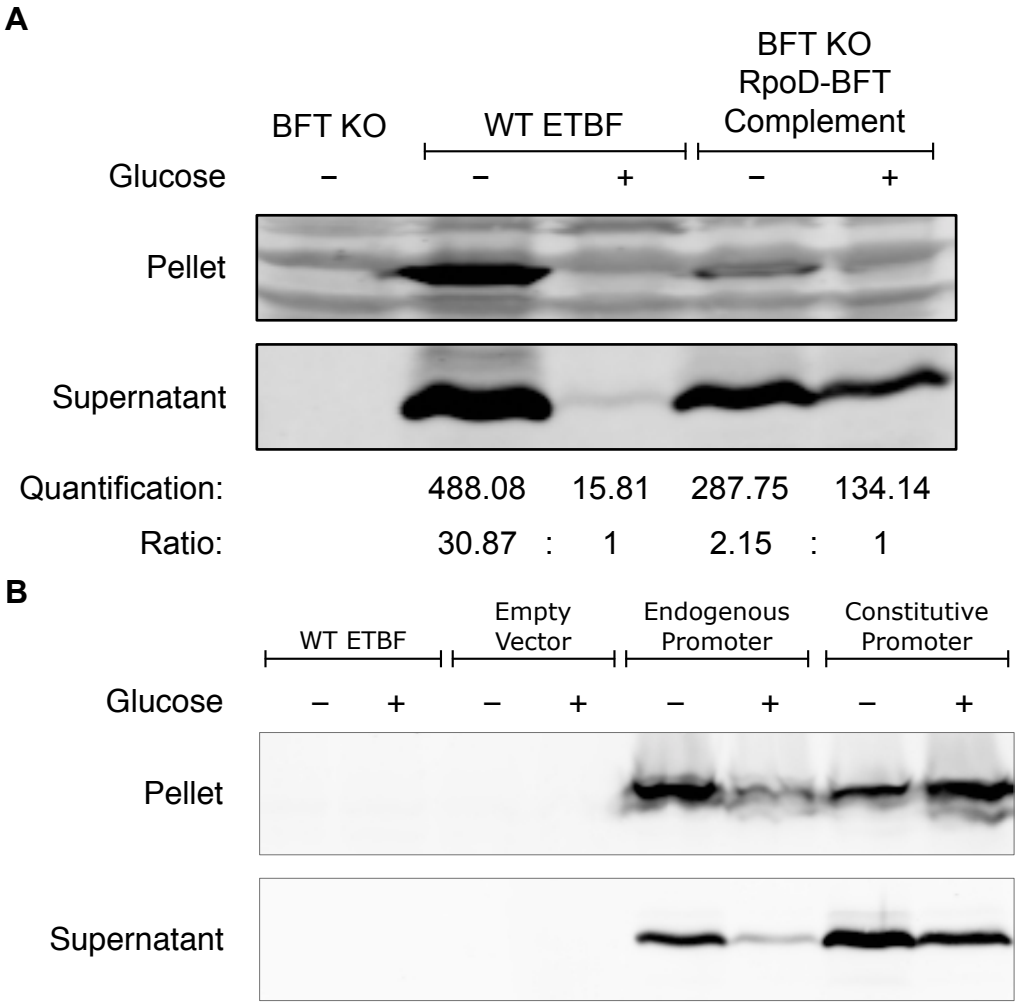
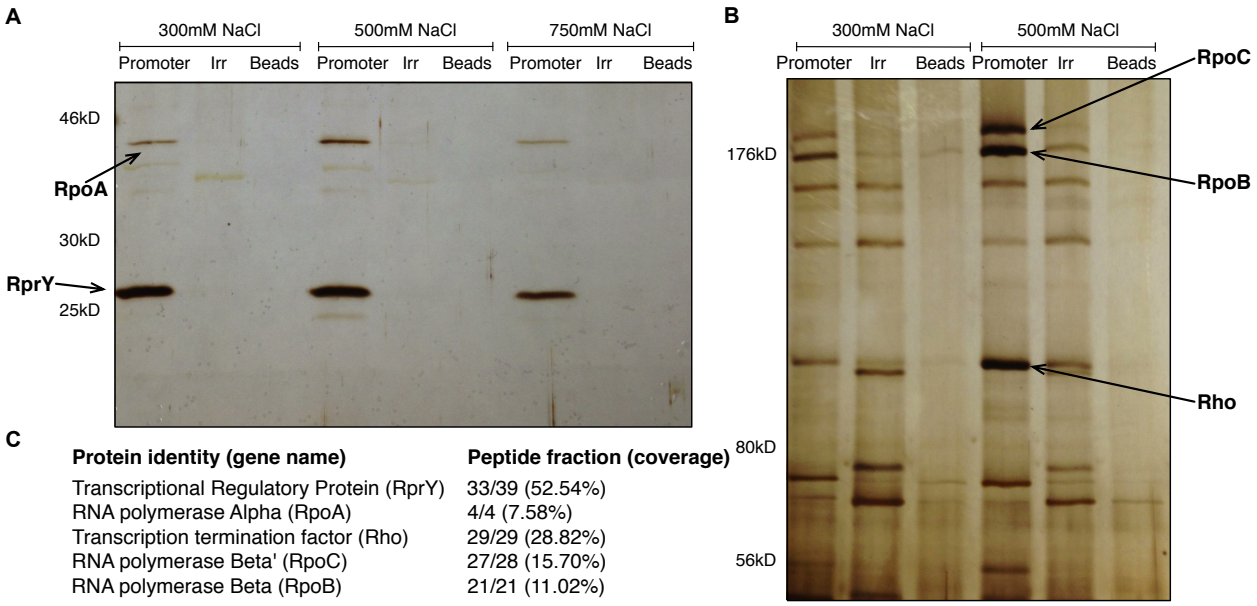



**Figure 3.** The P4 promoter is required for the glucose suppression phenotype. A. Immunoblot against BFT from overnight culture of cell pellet (protoxin) or supernatant (active toxin) of WT ETBF or ETBF mutated for *bft*, followed by in-genome complementation on pAH1 with the P4 promoter switched to a constitutive RpoD-promoter. Glucose supplementation is indicated and the supernatant toxin was quantified on the Li-Cor Odyssey system, setting the glucose conditions as 1 when comparing the fold reduction of the two promoters. B. Immunoblot from cell pellet and supernatant against anti-6xHis tagged BFT from plasmid-encoding ETBF with the P4 promoter upstream of BFT or the constitutive IS4351 promoter in its place. Results are representative of three independent experiments.




C

| Protein identity (gene name) | Peptide fraction (coverage) |
|---|---|
| Transcriptional Regulatory Protein (RprY) | 33/39 (52.54%) |
| RNA polymerase Alpha (RpoA) | 4/4 (7.58%) |
| Transcription termination factor (Rho) | 29/29 (28.82%) |
| RNA polymerase Beta' (RpoC) | 27/28 (15.70%) |
| RNA polymerase Beta (RpoB) | 21/21 (11.02%) |

**Figure 4.** DNA pulldown reveals multiple proteins that bind specifically to the BFT promoter. A,B. DNA pulldown was performed with BFT-promoter DNA or irrelevant control DNA as bait from ETBF lystate. The elutions were run on 15% (A) or 5% (B) polyacrylamide gels. Multiple specific bands appeared in the promoter lane and not the irrelevant DNA or no DNA (beads) at high concentrations of NaCl elution. C. The indicated bands (arrows) were excised and sent for mass spectrometry peptide analysis. The results are shown with the name of the identified protein and the peptide faction and coverage of that sequencing from the band. Results are representative of three independent experiments (A,B) or a single trial (C).

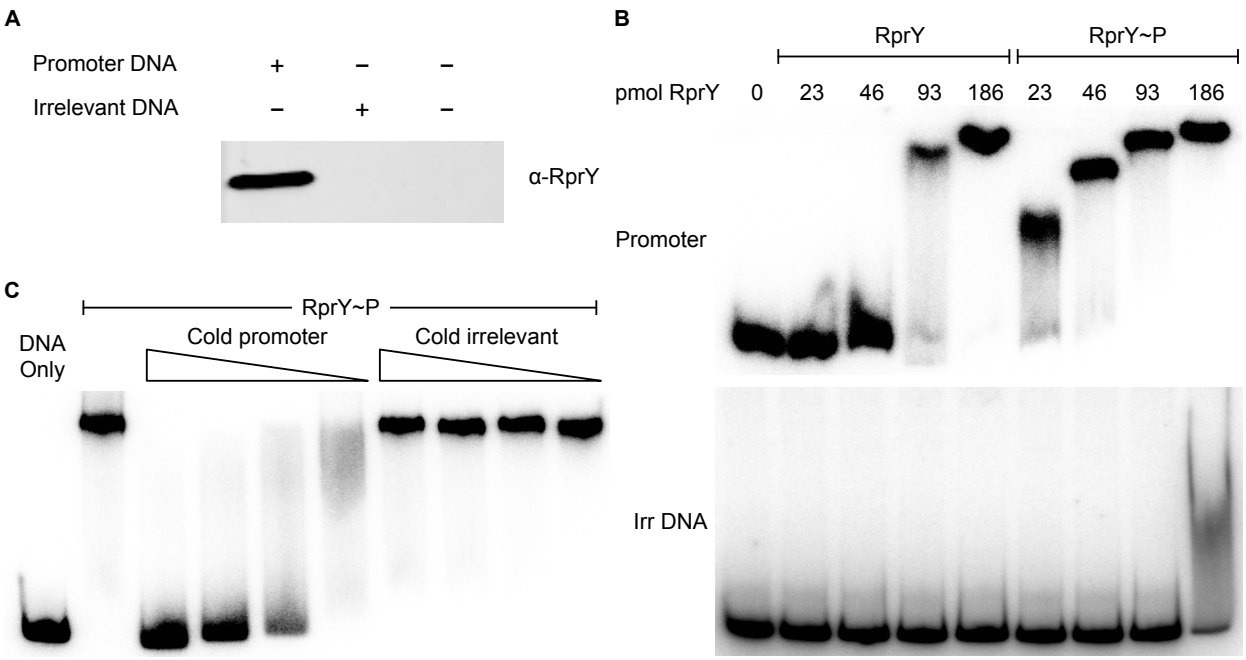



**Figure 5**. RprY specifically binds to the BFT promoter region. A. Immunoblot with anti-RprY serum after repeating the pulldown shown in Figure 4. B. EMSA of labeled BFT promoter (top) or irrelevant DNA (bottom) with increasing concentrations of RprY with (RprY~P) or without acetyl-phosphate treatment. C. EMSA of labeled BFT promoter with RprY~P followed by competition with cold promoter DNA or irrelevant DNA. The high concentration competition is a 1000x cold competitor, tested to a low of 100x. Results are representative of three independent experiments.

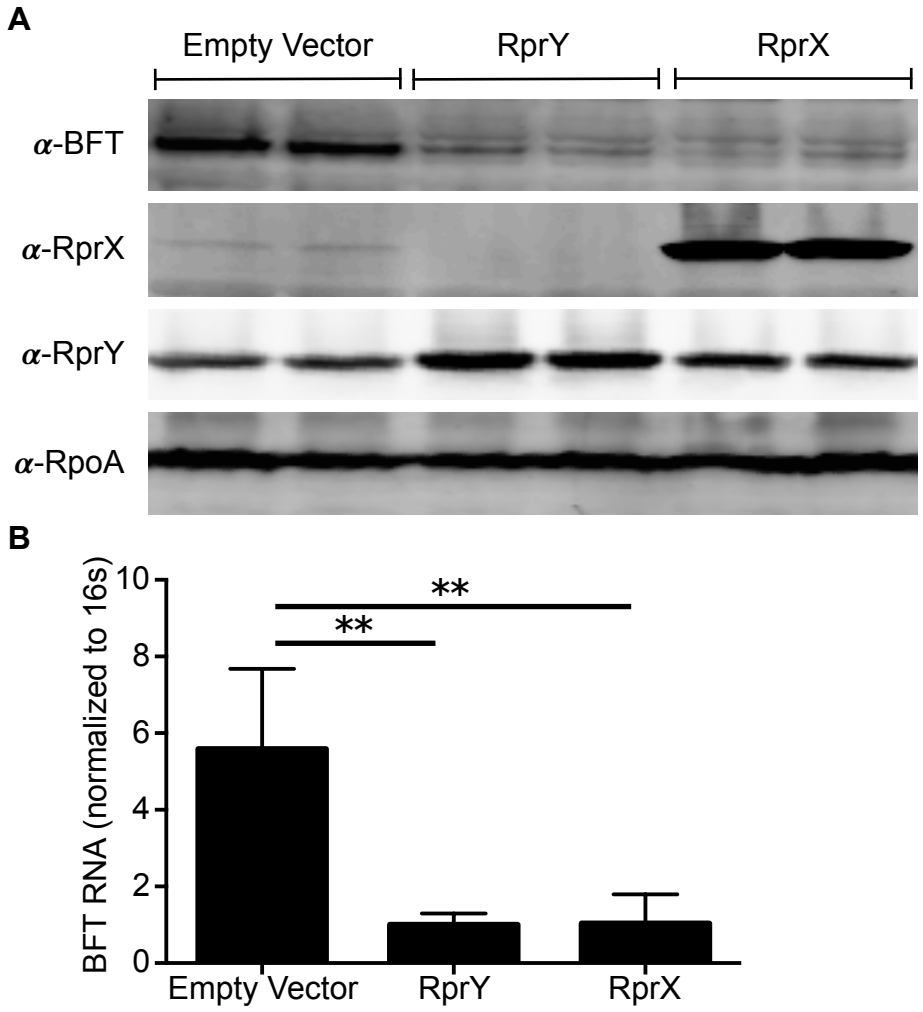



**Figure 6.** RprX and RprY overexpression downregulates BFT expression. A,B. RprY or RprX were overexpressed downstream of the GAPDH promoter on a plasmid. Detection of various proteins was performed via immunoblot, utilizing anti-BFT, RprX, RprY and RpoA (loading control) antibodies from the cell pellet of ETBF encoding the overexpression constructs (A). RNA was extracted from the ETBF overexpression cells and BFT transcript quantified using qRT-PCR (B). **$p<0.01$. Results are representative of three independent experiments (A) or are pooled from four biological replicates of independent trials.

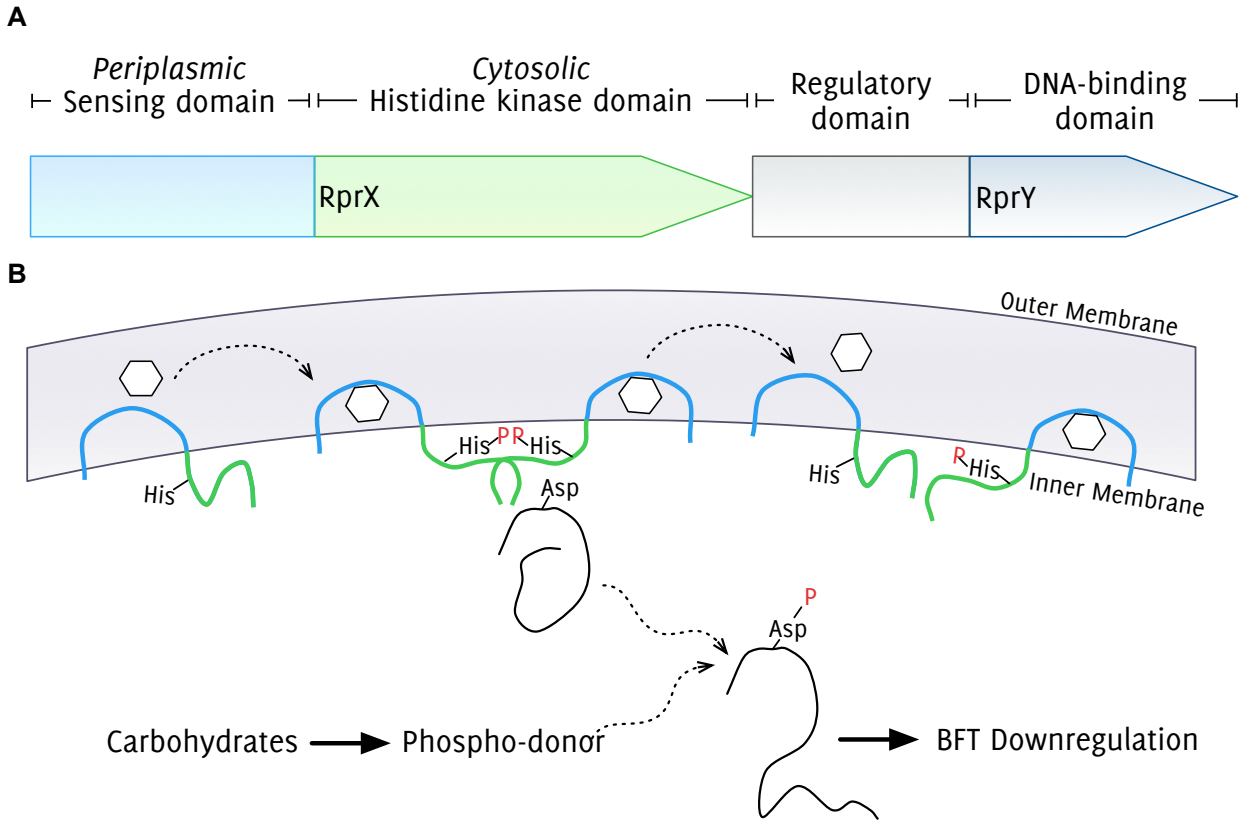



**Figure 7.** RprX and RprY may relay environmental signals to downregulate BFT. A. Locus schematic of *rprX* and *rprY*, including recognized domains based on sequence homology to known two-component system genes. B. Diagram of possible signaling interaction between RprX, RprY and BFT transcription. RprX may sense a signal in the periplasm of ETBF, detecting an environmental cue for toxin regulation. The activating phosphorylation may then be accepted by RprY to upregulate its activity. Carbohydrates may act through an intermediary high-energy phospho-donor to relay other environmental signals to RprY. These activations may result in BFT downregulation through promoter binding.

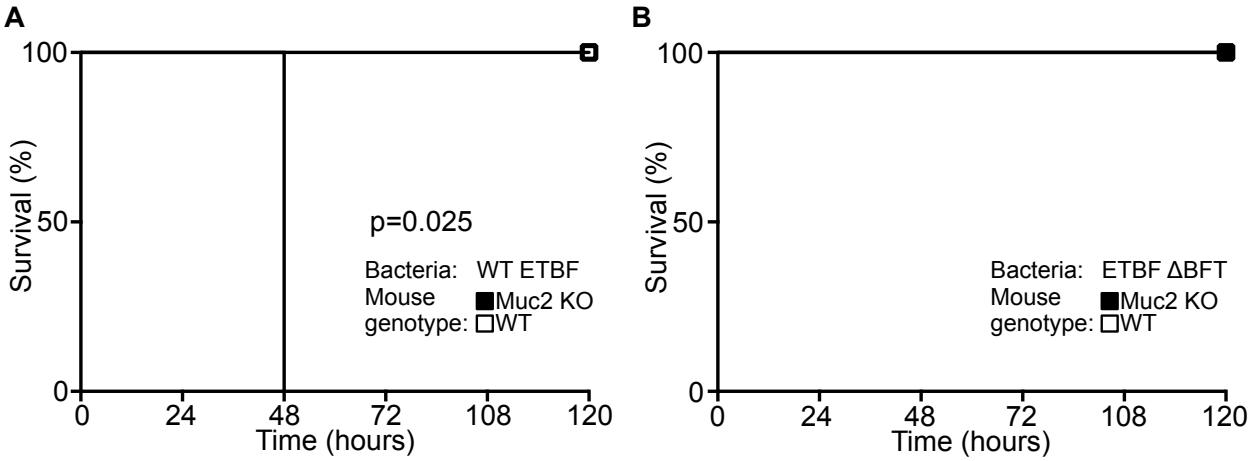



**Figure 8.** Colonization of mucus-deficient mice with ETBF results in BFT-dependent death. A. Muc2 KO (closed squares; n=3) and WT (open; n=5) mice were bred and after a single day of antibiotic pre-treatment were orally gavaged with WT ETBF. Mice were monitored for 5 days for moribund status. B. Muc2 KO (closed squares) and WT (open) mice were colonized after a single day of antibiotic pre-treatment with ETBF Δ*bft*. Mice were monitored as stated in A. Results are representative of three independent experiments.

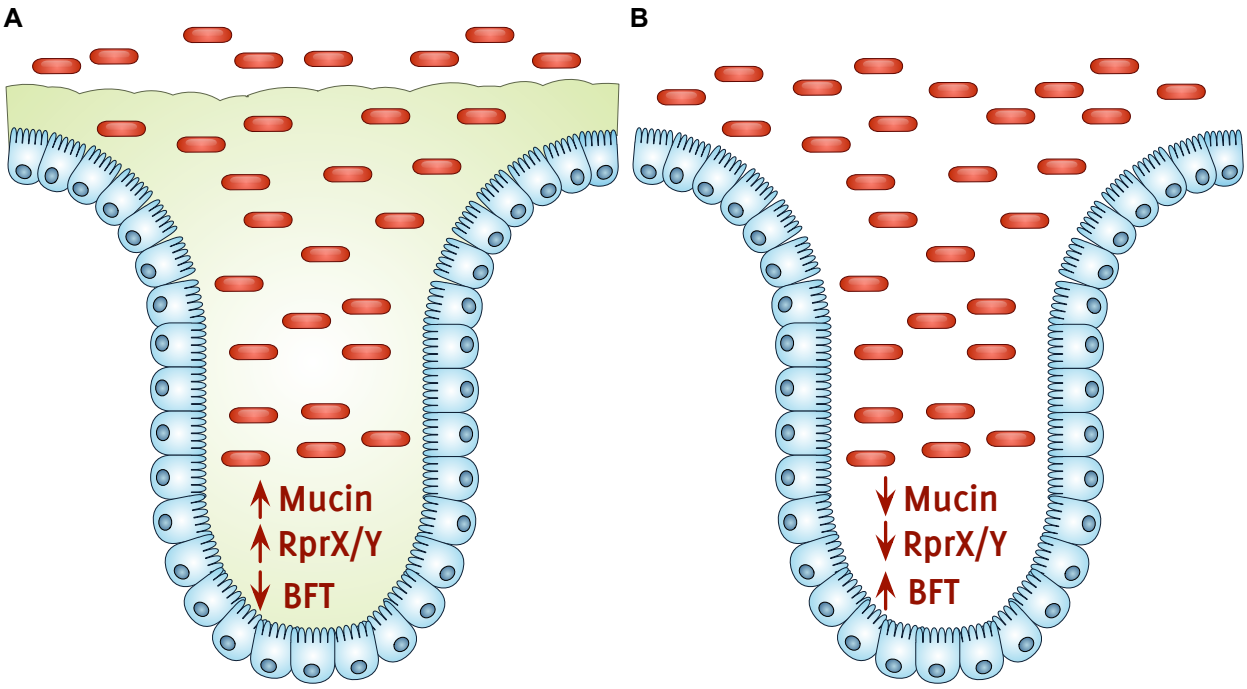



**Figure 9.** Muc2 may be a critical factor for BFT regulation *in vivo*. A,B. Schematic representation of a colonic crypt colonized with ETBF. Normal colonic tissue (A) includes a mucus layer, composed of Muc2, that may be used as a signal to dampen BFT expression through mucin oligosaccharides or RprX/Y signaling. Mutations in the Muc2 gene (B) lead to loss of the mucus layer, perhaps causing toxin upregulation due to lack of carbohydrates or RprX/Y stimulation.

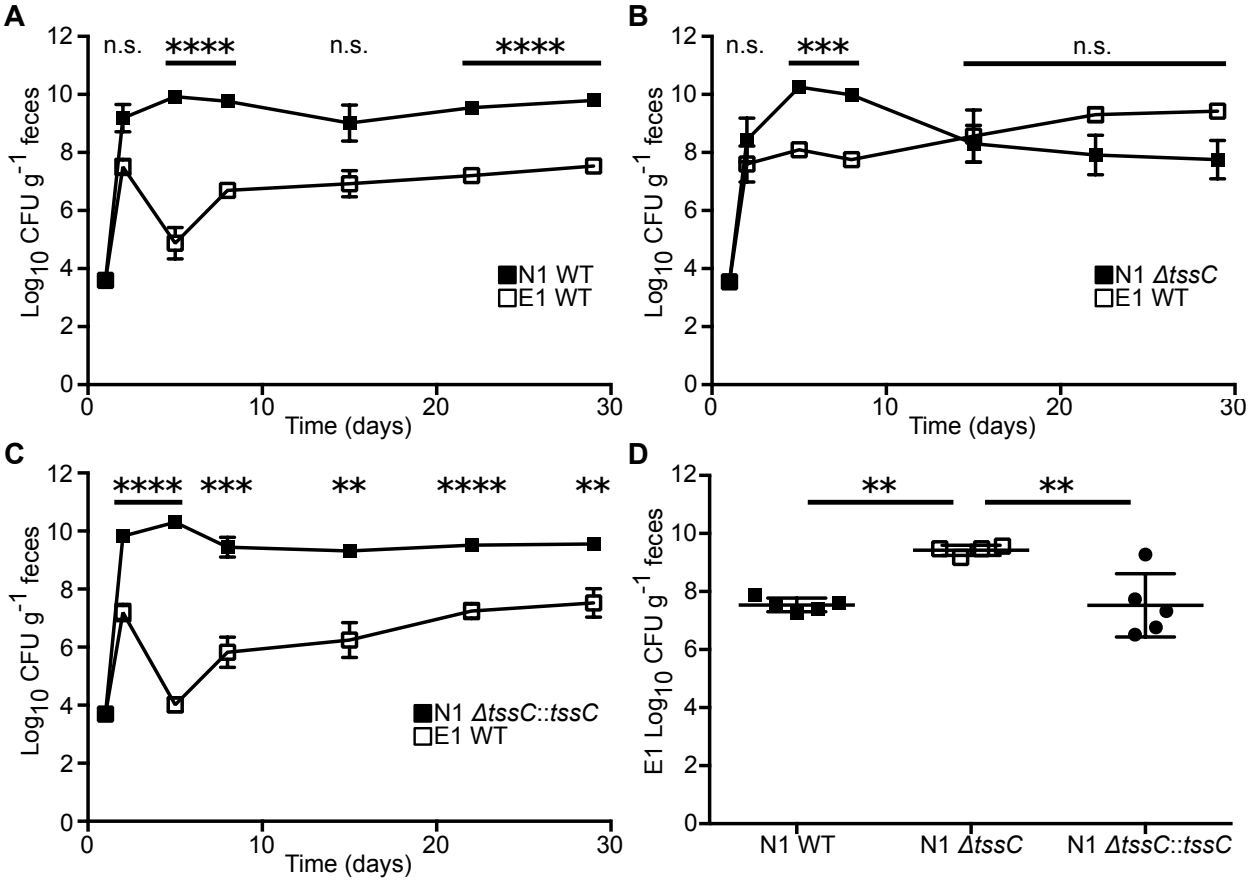



**Figure 10.** NTBF strain dominance over ETBF through T6S. A to C. SPF C57BL/6J mice were co-colonized with E1 and N1 wild type, (WT, n=5) (A) N1 T6SS mutant, (Δ*tssC*, n=4) (B) or N1 complemented (Δ*tssC*::*tssC*, n=5) (C). Fecal CFU was quantified for E1 (closed squares) and N1 (open squares) weekly. D. Four weeks post-colonization, E1 fecal recovery was compared between the N1 WT, Δ*tssC*, and Δ*tssC*::*tssC* groups. Results are representative of three independent experiments. Error bars: mean ± s.e.m (A to C), mean ± s.d. (D). n.s., not significant; **p<.01, ***p<.001, ****p<.0001.

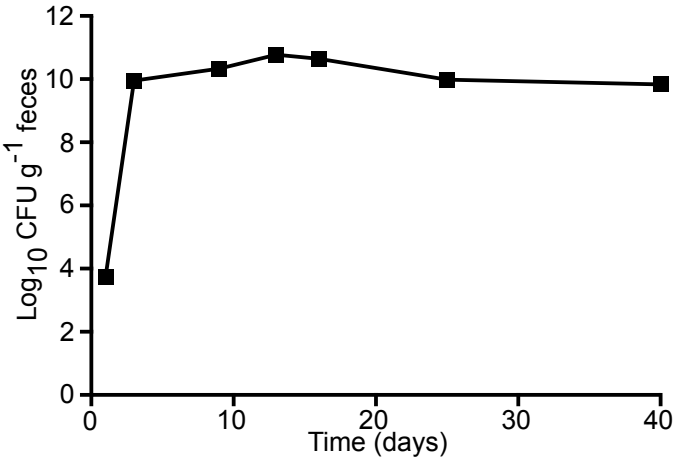



**Figure 11.** E1 mono-colonization results in stable colonization. SPF mice (n=4) were mono-colonized with E1 and fecal CFU determined over time. Results are representative of three independent experiments. Error bars: mean +/- s.e.m.

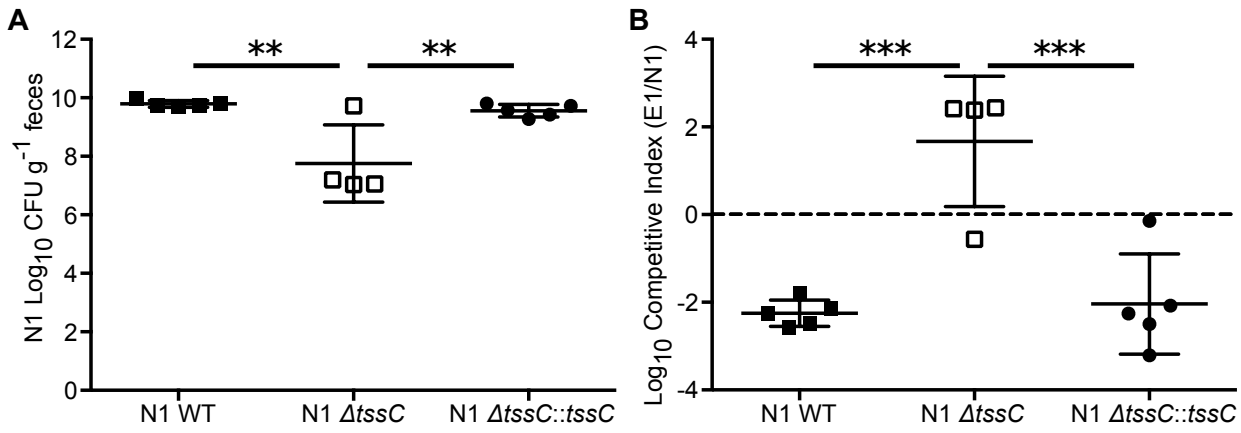



**Figure 12.** N1 T6SS is required for strain dominance of E1. A,B**.** SPF mice were co-colonization with E1 WT and N1 WT (n=5), T6SS mutant (Δ*tssC*, n=4), or complemented (Δ*tssC*::*tssC*, n=5). Fecal CFU was monitored for 4 weeks post-colonization. N1 clone fecal CFU was compared between groups at the 4-week time point (A) and the competitive index of E1 over N1 was determined for each mouse (B). Results are representative of three independent experiments. Error bars: mean +/- s.d. **p<.01, ***p<.001.

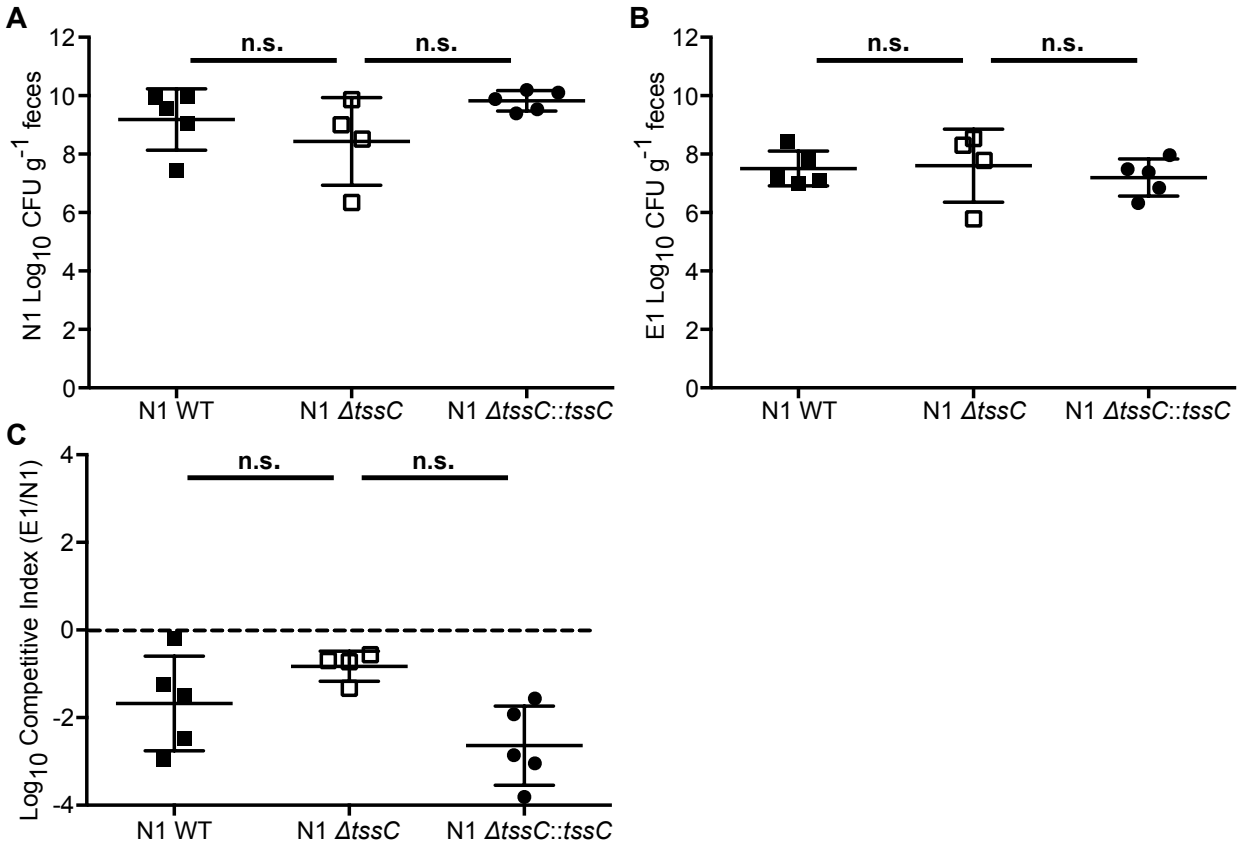



**Figure 13.** N1 and E1 colonization and competitive index is not significantly different between groups one day post-colonization. A-C. SPF mice were co-colonized with E1 and N1 WT (n=5), T6SS mutant (Δ*tssC*, n=4) or complemented (Δ*tssC*::*tssC*, n=5). Fecal CFU at one-day post co-colonization was determined for N1 (A) and E1 (B) and competitive index of E1 over N1 was calculated for each mouse (C). Results are representative of three independent experiments. Error bars: mean +/- s.d. n.s., not significant.

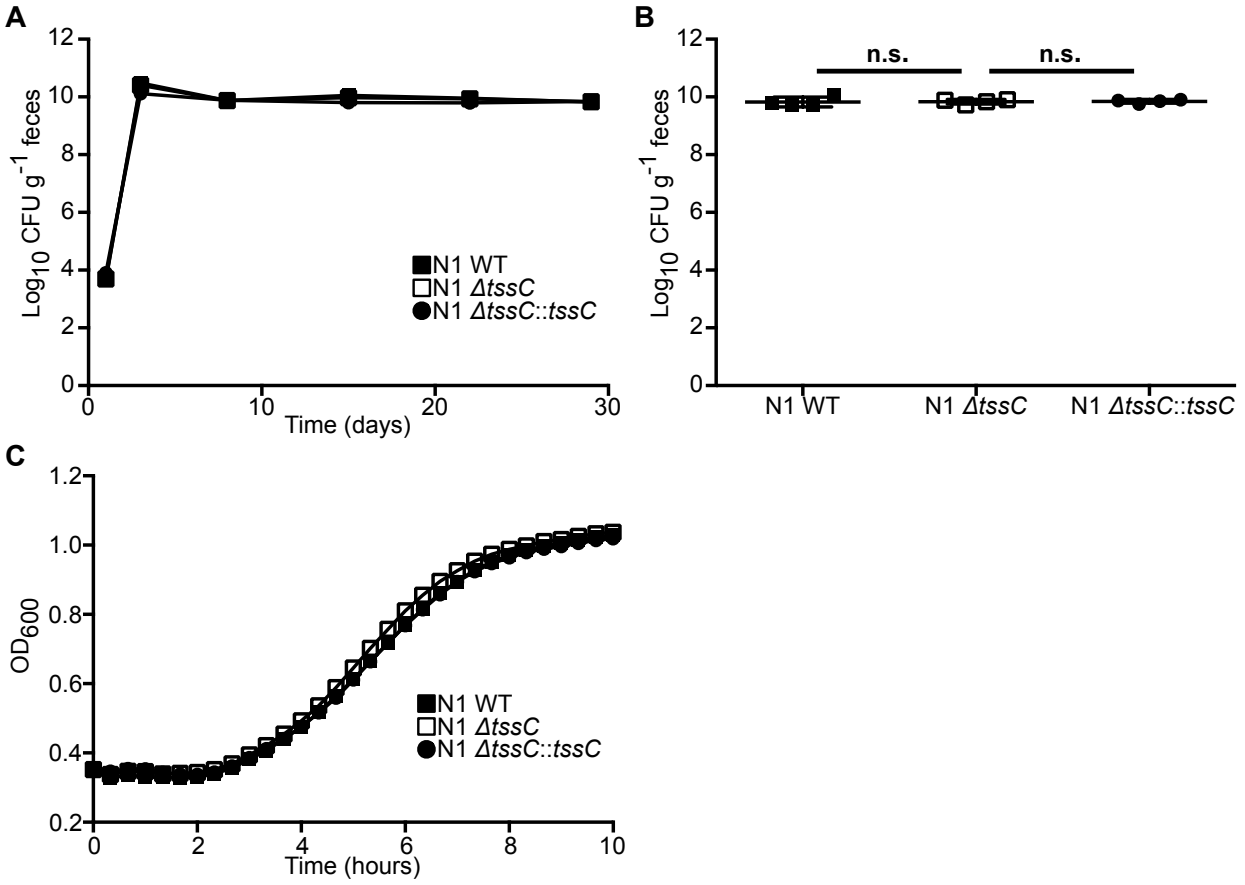



**Figure 14.** N1 T6SS mutant displays no defect during mono-colonization or growth. A,B. Mice (n=4) were mono-colonized with N1 WT, T6SS mutant (Δ*tssC)* or complemented (Δ*tssC*::*tssC*) and fecal CFU was determined for four weeks (A). Comparison of fecal CFU was made between groups after four weeks, each point representing one mouse (B). C. Growth of N1 WT, Δ*tssC* and Δ*tssC*::*tssC* were observed over time *in vitro*. Results are representative of three independent experiments. Error bars: mean +/- s.e.m. (A,), mean +/- s.d. (B). n.s., not significant.

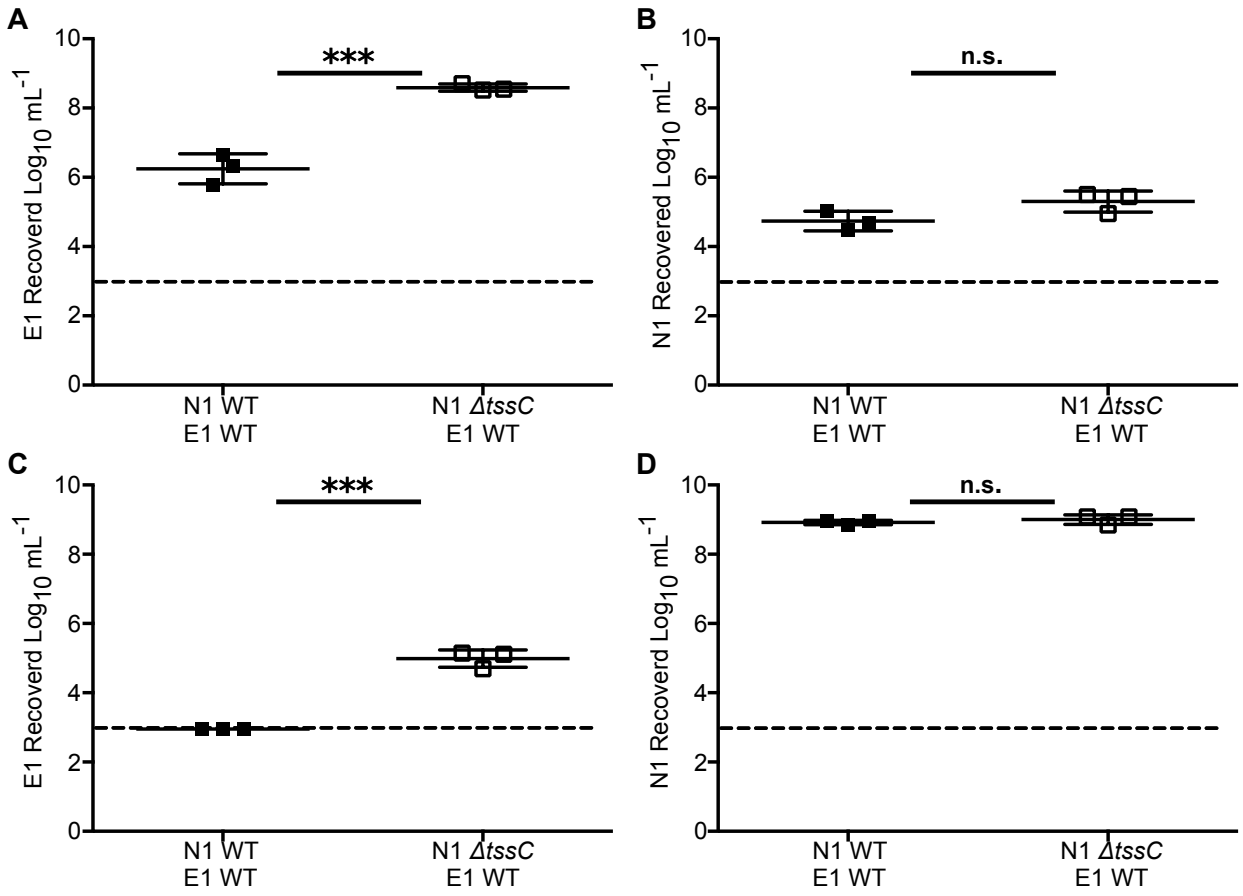



**Figure 15.** *In vitro* strain competitions between N1 and E1 result in T6S-dependent killing. A-D. *In vitro* strain competitions were performed with N1 and E1 at a 1:1 ratio (A,B) or a 1:10 ratio (C,D) respectively. Quantification of E1 (A,C) and N1 (B,D) after the competition is shown. Error bars: mean +/- s.d. ***p<.001; n.s., not significant.

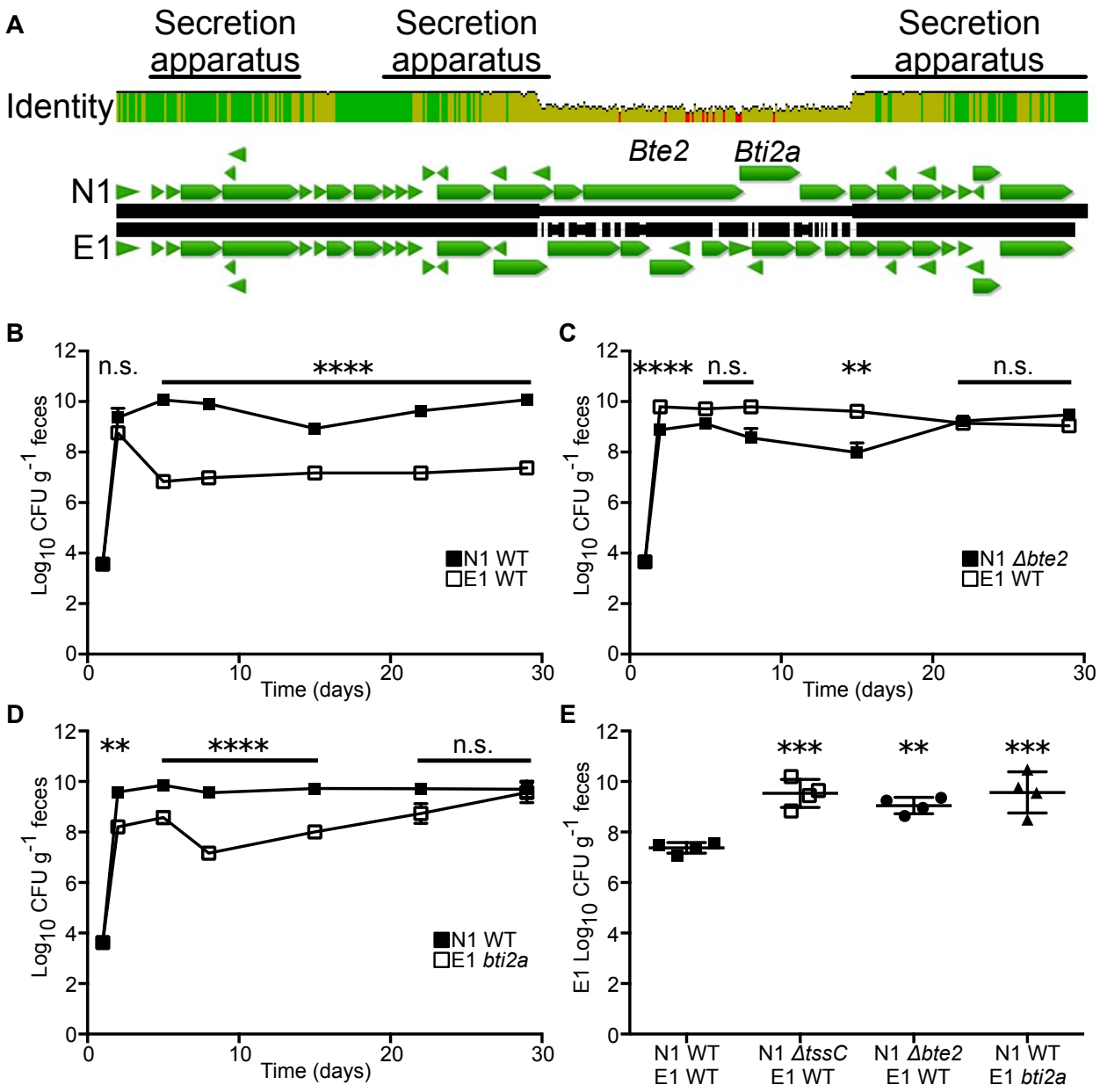



**Figure 16.** A putative effector-immunity pair mediates E1 colonization resistance. A. Nucleotide alignment of the T6SS locus from N1 and E1. Percent identity is indicated as height, green representing high homology with red highlighting non-conserved regions. B-E**.** Co-colonization of N1 WT (B,D) or N1 Δ*bte2* (C) with E1 WT (B,C) or E1 overexpressing *bti2a* (E1 *bti2a*, D). Fecal CFU was monitored over time (B-D) with E1 compared between groups at four-weeks post colonization (E). Results are representative of two independent experiments. Error bars: mean ± s.e.m (B-D), mean ± s.d. (E). **p<.01, ***p<.001, ****p<.0001; n.s., not significant.

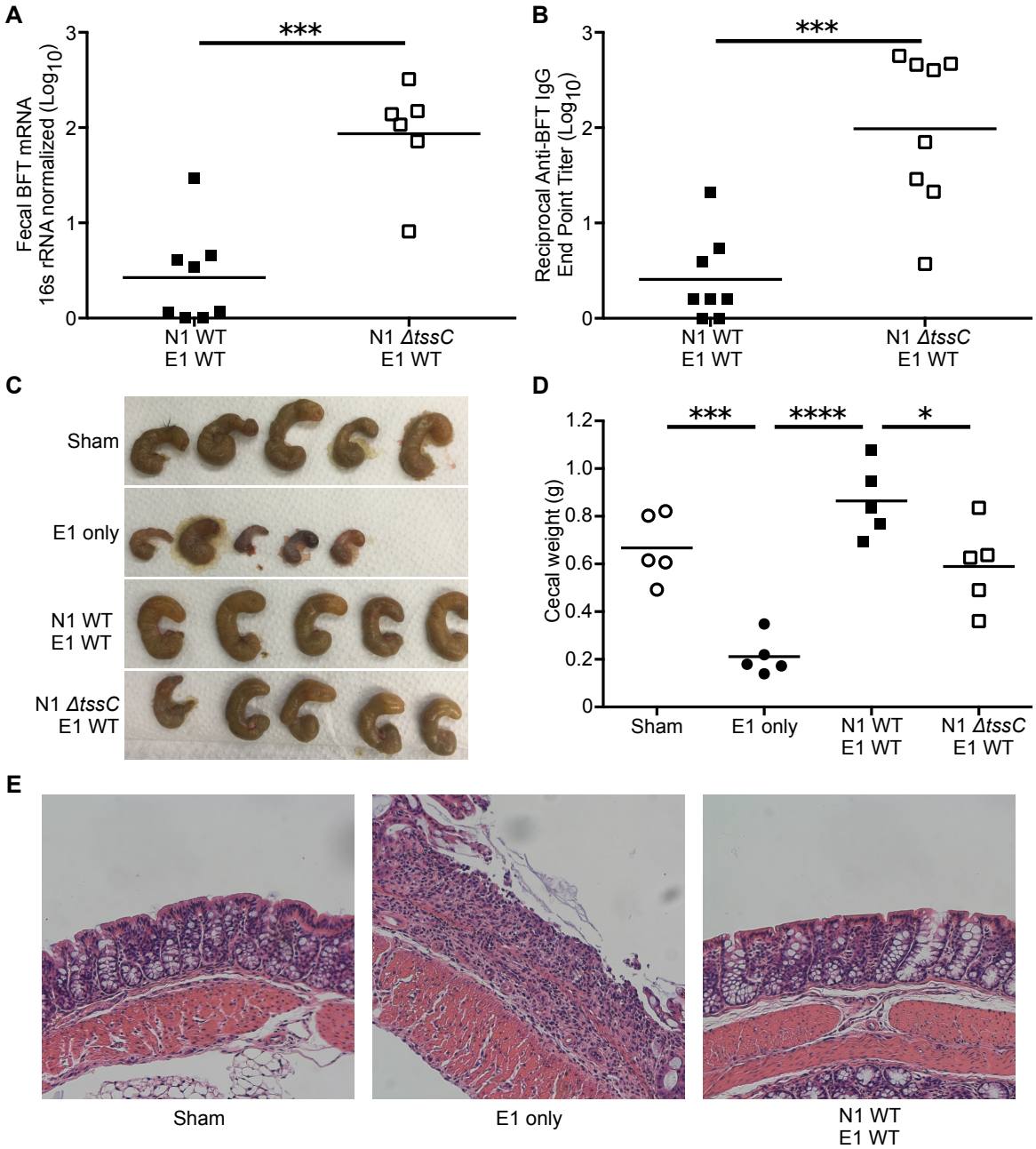



**Figure 17**. The N1 T6SS protects against ETBF-mediated disease. A,B. Mice were co-colonized with E1 WT and either N1 WT (n=4 mice per group) or N1 Δ*tssC* (n=3 mice per group). Five days-post inoculation, fecal RNA was extracted and tested for BFT expression via qRT-PCR (A). Twenty eight days post-inoculation, the serum from co-colonized mice were tested for anti-BFT IgG levels. C-E. Mice pre-treated with DSS were inoculated with no organisms, E1 only, or E1 in the competitions indicated. Five days post-inoculation, the ceca were dissected (C) and weighed (D) and the colons fixed for histopathological examination. Experiments are a pooling of two independent repeats (A,B) or are representative to two independent trials (C-E). Error bars: mean ± s.e.m (B-D), mean ± s.d. (E). *p<.05, ***p<.001, ****p<.0001.

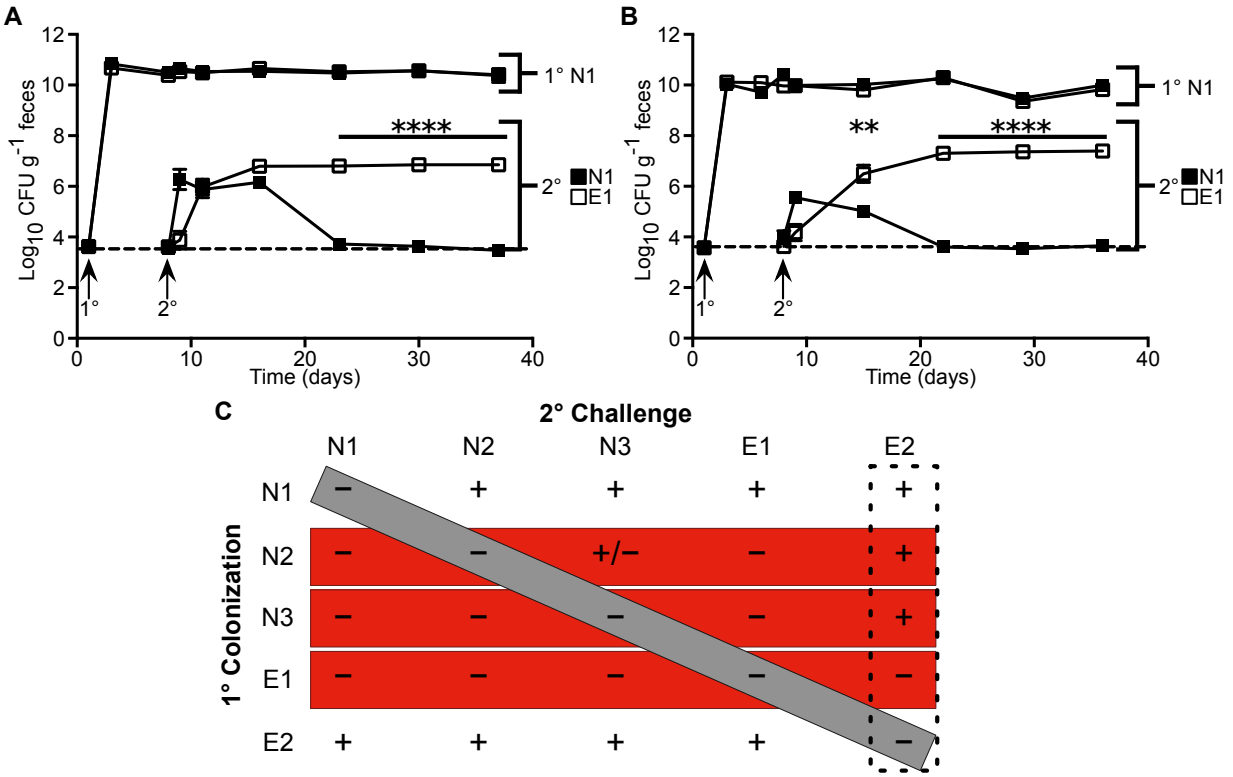



**Figure 18.** *B. fragilis* provides strain-specific colonization resistance. A,B. Initial colonization of gnotobiotic (A) or SPF (B) mice (n=4) with N1 followed by secondary challenge with N1 (closed squares) or E1 (open squares). Fecal CFU was determined for the primary and secondary colonization strains through four weeks-post secondary challenges. Statistical difference between E1 and N1 secondary challenge is noted. Error bars: mean ± s.e.m. Arrows denote day of primary colonization and secondary challenge. Limit of detection is denoted by a dashed line. **p<.01, ****p<.0001. C. All primary colonization and secondary challenge pairs were tested with 3 NTBF and 2 ETBF strains. Stable colonization of the secondary challenge strain significantly above the limit of detection is denoted as a '+' while failure is denoted as a '−'. The diagonal gray bar indicates the self secondary challenge, the horizontal red bars show strains that provide broad colonization resistance against other strains and the vertical dashed box indicates strains that have an enhanced secondary colonization phenotype. Results illustrate a single experiment (A) or are representative of at least two independent experiments (B,C).

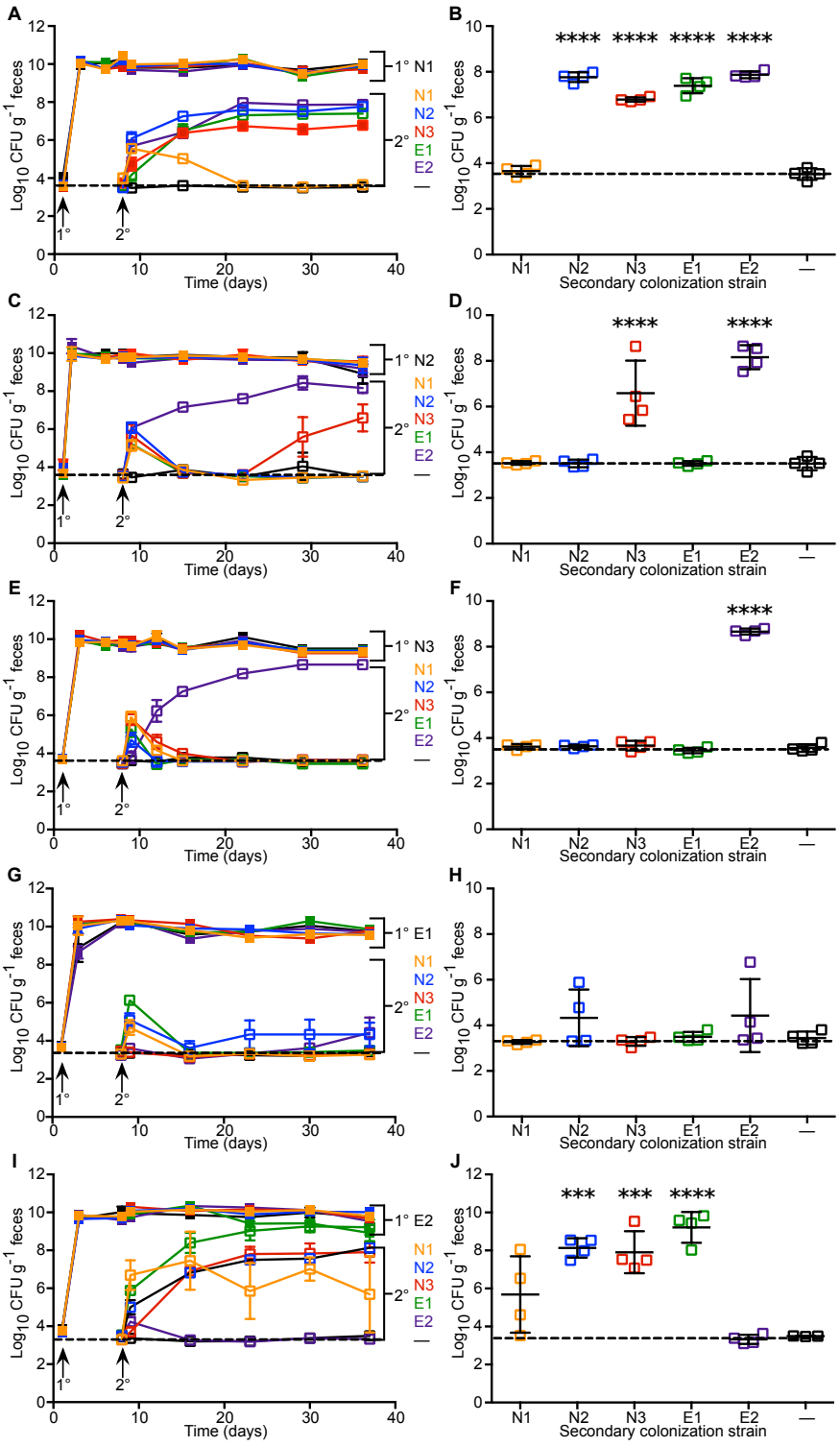



**Figure 19.** Successful *B. fragilis* secondary challenge is strain-dependent. A-J. Six groups of 4 mice were initially colonized with N1 (A,B), N2 (C,D), N3 (E,F), E1 (G,H) and E2 (I,J) followed by secondary challenge by all strains or a mock-inoculum at one week post-primary colonization.

Fecal CFU of primary and secondary strains were monitored for four weeks post-secondary challenge (A,C,E,G,I). At the last time point, the secondary challenge strain was tested for statistical significance above the mock-inoculated group, which is at the limit of detection (B,D,F,H,J). Results are representative of at least two independent experiments. n=4 mice per group. Error bars: mean +/- s.e.m. (A,C,E,G,I), mean +/- s.d. (B,D,F,H,J). Limit of detection is denoted by a dashed line. n.s., not significant; ***p<.001, ****p<.0001.

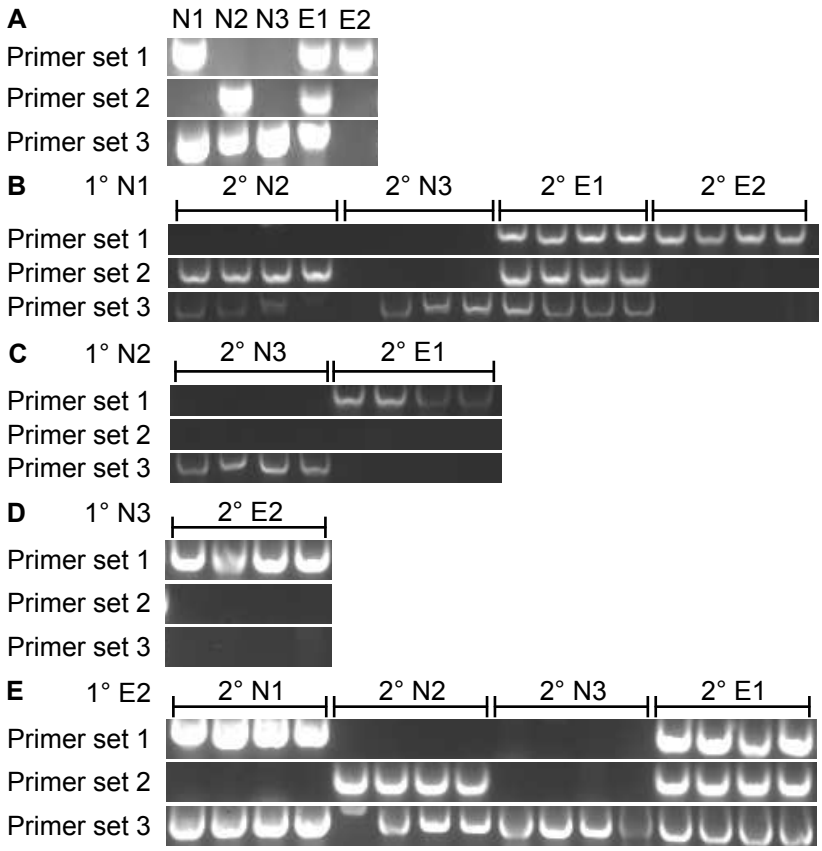



**Figure 20.** Successful secondary challenge strain identities are confirmed via colony PCR. A. Colony PCR with three primer sets to distinguish the strains was performed on the five *B. fragilis* strains in this study. B-E. Colony PCR was performed on the successful secondary challenge strains four weeks post-challenge. Each column of lanes represents a colony from each mouse in the indicated groups (n=4).

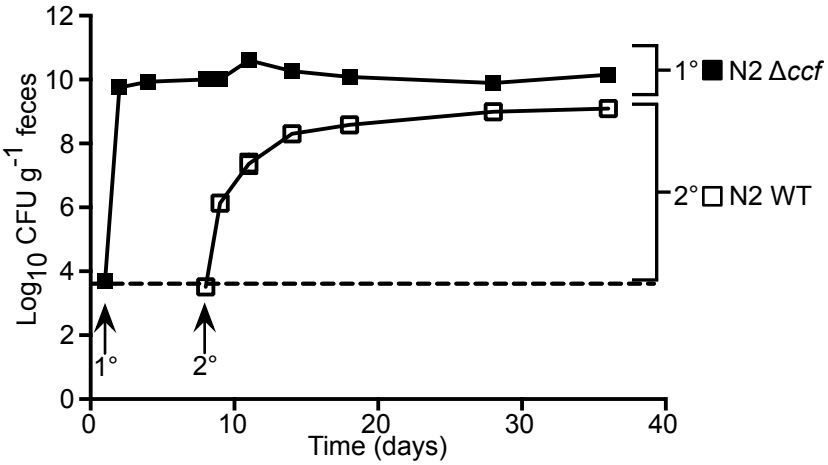



**Figure 21.** The *ccf* locus is required for self-colonization resistance of N2. Mice were primarily colonized with N2 mutated for the *ccf* locus followed by a secondary challenge of N2 WT. Fecal CFU was monitored for four weeks-post challenge. Error bars: mean +/- s.e.m.

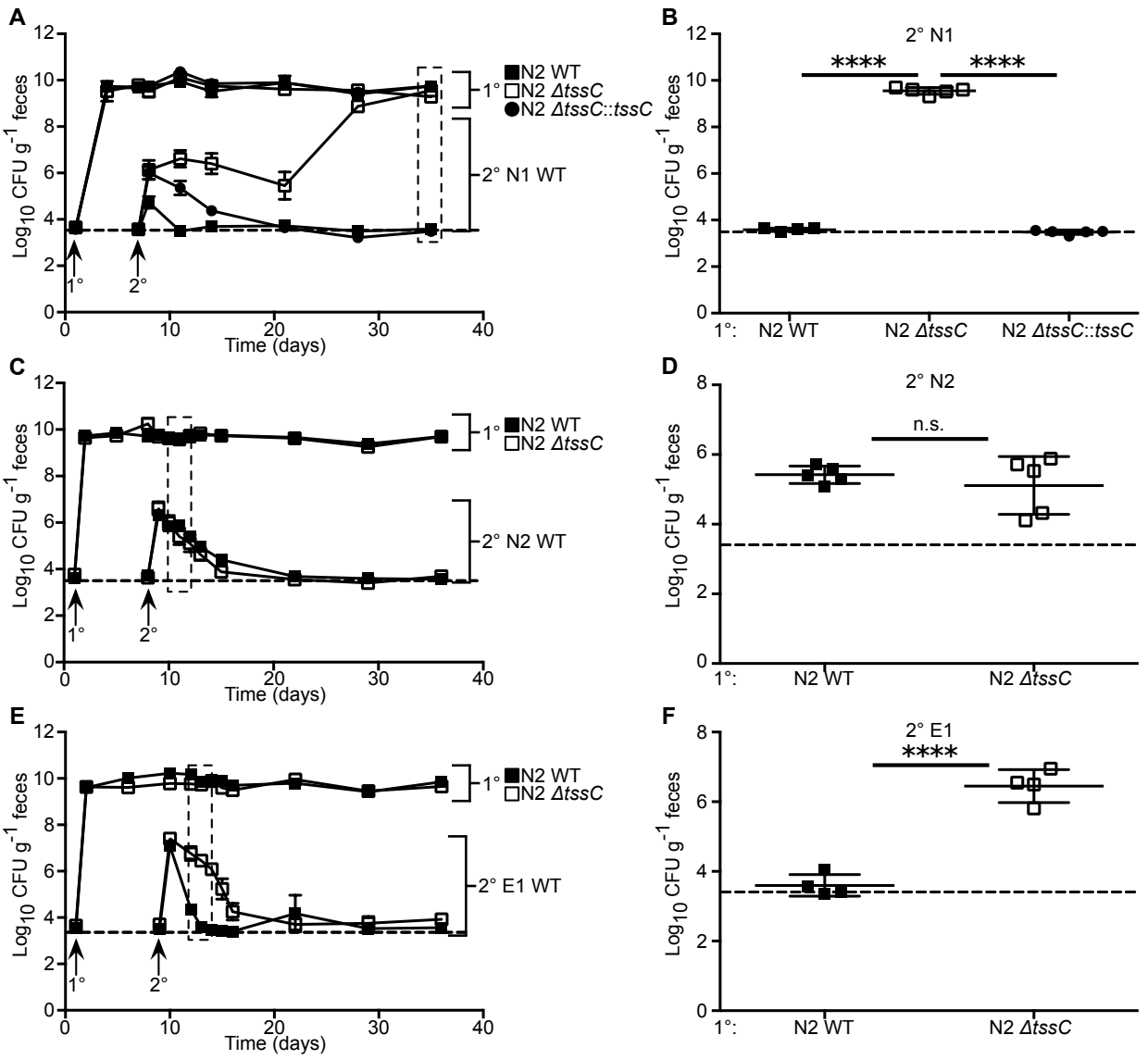



**Figure 22.** T6S is required for strain-specific colonization resistance. A-F. Primary colonization of SPF mice with N2 WT, T6SS mutant (Δ*tssC*) and complemented (Δ*tssC*::*tssC*) followed by secondary challenge with N1 WT (A,B) N2 WT (C,D) or E1 WT (E,F) was performed. A,C,E. Fecal CFU for primary and secondary strains was determined for four weeks post-secondary challenge. B,D,F. Selected time points were tested for statistical difference of secondary challenge between groups. This includes four weeks post-secondary challenge (B) and three days post-challenge (D,F). Results are representative of at least three independent experiments. n=4 (E,F) or n=5 (A-D). Error bars: mean ± s.e.m. (A,C,E), mean ± s.d. (B,D,F). Limit of detection is denoted by a dashed line. n.s., not significant; ****p<.0001.

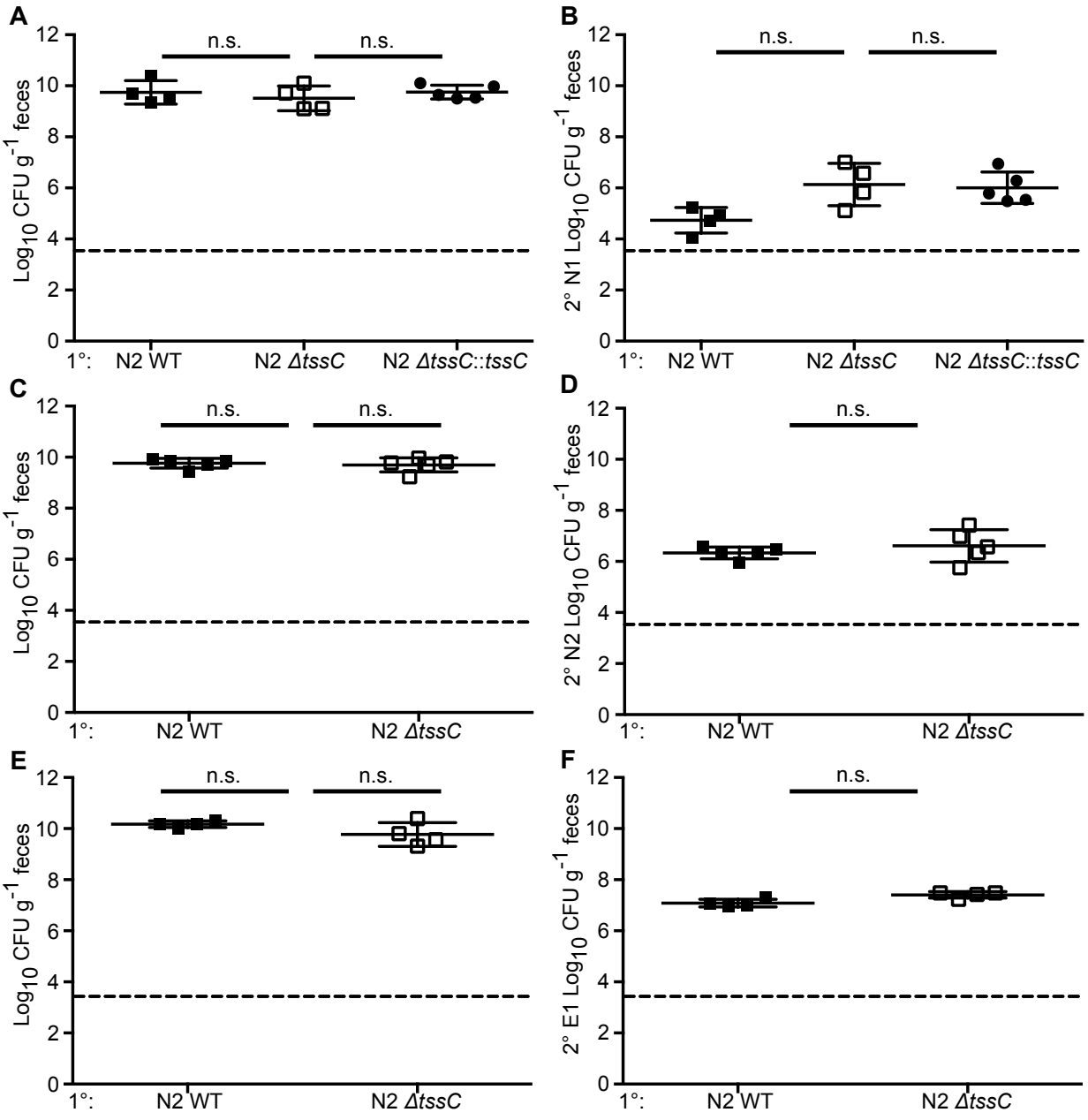



**Figure 23.** T6S has no effect on primary colonization of N2 nor secondary challenge strain one day post-secondary challenge. A to F. Primary colonization of SPF mice with N2 WT, T6SS mutant (Δ*tssC*) and complemented (Δ*tssC*::*tssC*) followed by secondary challenge with N1 WT (A and B) N2 WT (C and D) or E1 WT (E and F) was performed. Fecal CFU for primary (A, C and E) and secondary strains (B, D and F) were determined one day post-secondary challenge. Results are representative of at least three independent experiments. n=4 (E and F) or n=5 (A to D). Error bars: mean +/- s.d. Limit of detection is denoted by a dashed line. n.s., not significant.

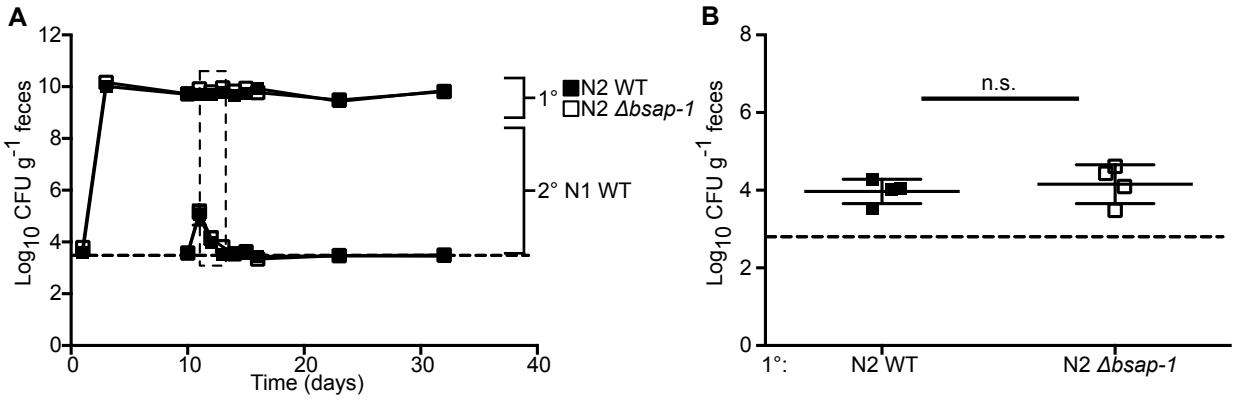



**Figure 24.** BSAP-1 has no effect on N2 colonization resistance against N1. A. Fecal CFU was determined after primary colonization with N2 WT or N2 *bsap-1* mutant (Δ*bsap-1*) followed by secondary challenge with N1 WT. B. Comparison of secondary challenge fecal CFU two days post-challenge between N2 WT and Δ*bsap-1* groups. Results representative of two independent experiments with four mice per group. Error bars: mean +/- s.e.m (A), mean +/- s.d. (B). Dashed lines indicate the limit of detection. n.s., not significant.

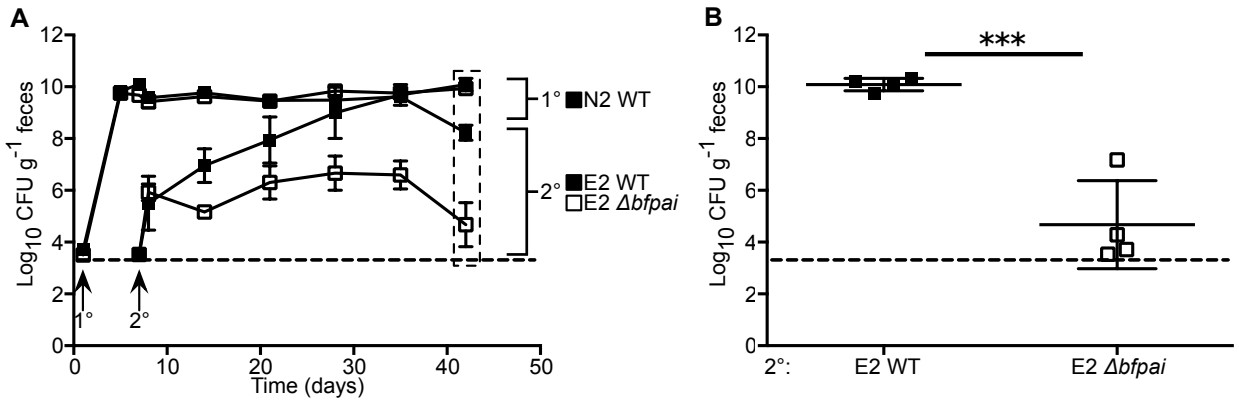



**Figure 25.** The *bfpai* locus is required for enhanced secondary colonization fitness of E2 after N1 primary colonization. A,B. Mice were primarily colonized with N2 WT and subsequently challenged with either E2 WT or E2 Δ*bfpai*. Fecal CFU was monitored for five weeks post-challenge. N=4 mice per group. Figures are representative of three independent experiments. Error bars: mean +/- s.e.m (A), mean +/- s.d. (B). Dashed lines indicate the limit of detection. n.s., not significant.

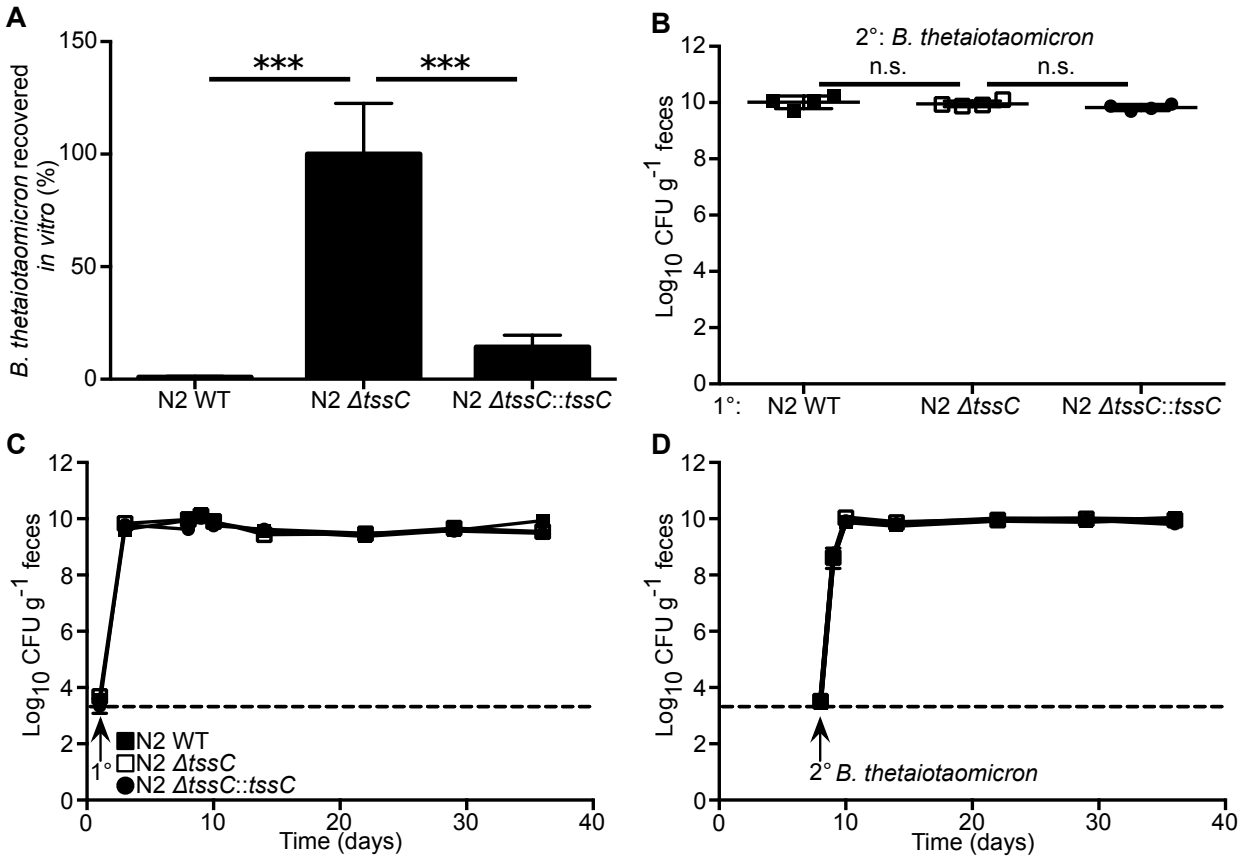



**Figure 26.** T6SS-dependent colonization resistance does not extend to related *Bacteroides* species *in vivo*. A. *B. thetaiotaomicron* recovered after *in vitro* competition with N2 WT, Δ*tssC* or Δ*tssC*::*tssC* in a 1:10 ratio (*B. thetaiotaomicron*:N2). B-D. SPF mice (n=4) were sequentially colonized with N2 WT, T6SS mutant (Δ*tssC*) or complemented (Δ*tssC*::*tssC*) strains, followed by secondary challenge of *B. thetaiotaomicron* one week after primary colonization. Four weeks post-secondary challenge, fecal CFU was determined for *B. thetaiotaomicron* (B). Fecal CFU was determined over time for primary colonizing strains (C) or secondary (D). Results are representative of three independent experiments. Error bars: mean ± s.d. (A,B), mean ± s.e.m. (C,D). n.s., not significant; ***p<.001.

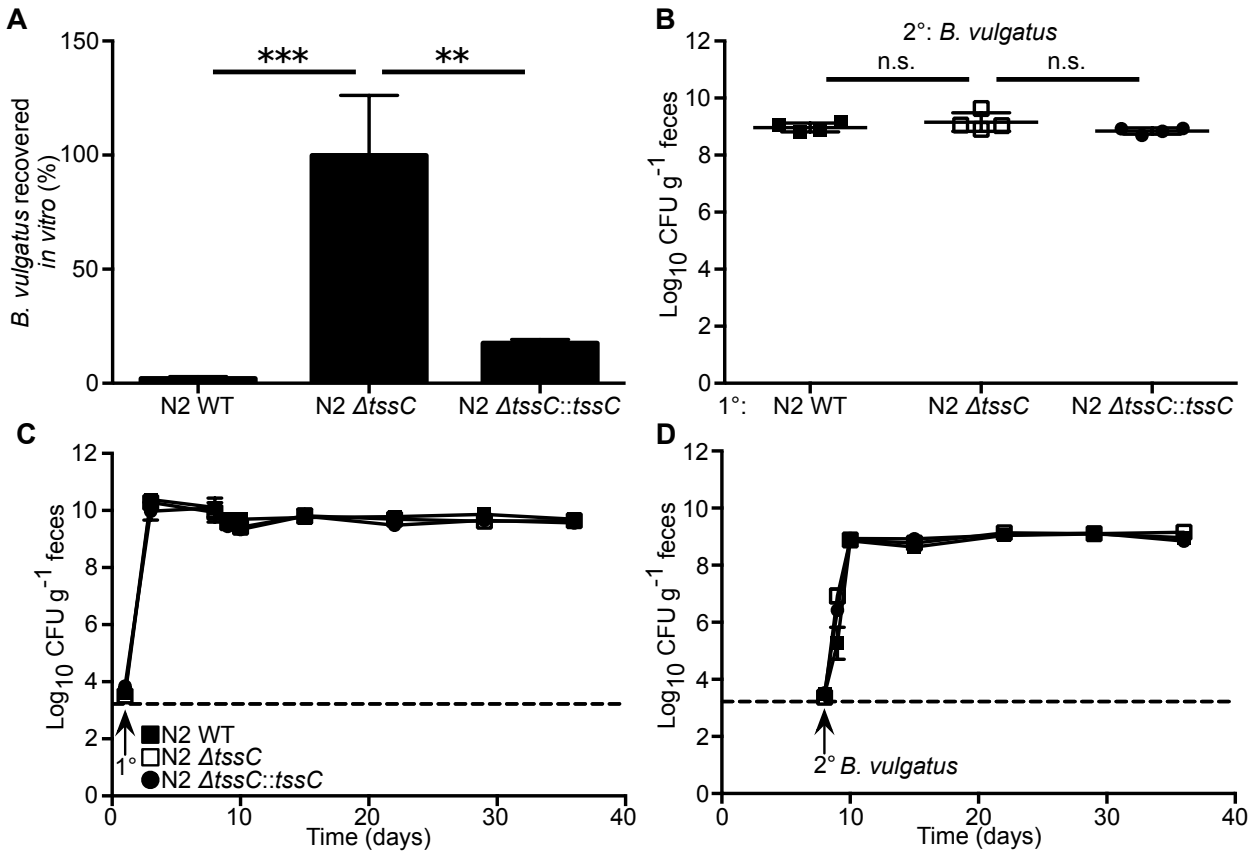



**Figure 27.** Enhanced N2 T6SS-dependent colonization resistance does not extend to *B. vulgatus*, despite *in vitro* killing. A. *B. vulgatus* recovered after *in vitro* competition with N2 WT, *ΔtssC* or *ΔtssC*::*tssC* in a 1:10 ratio (*B. vulgatus*:N2). B and D. Primary colonization of SPF mice with N2 WT, T6SS mutant (*ΔtssC*) and complemented (*ΔtssC*::*tssC*) followed by secondary challenge with *B. vulgatus* was performed. *B. vulgatus* recovery was compared between groups four weeks post-secondary challenge (B). Fecal CFU for primary (C) and secondary strains (D) were determined over four weeks post-secondary challenge. Results are representative of three independent experiments with four mice per group. Error bars: mean ± s.d. (A,B), mean ± s.e.m. (C,D). Limit of detection is denoted by a dashed line. n.s., not significant; **p<.01, ***p<.001.

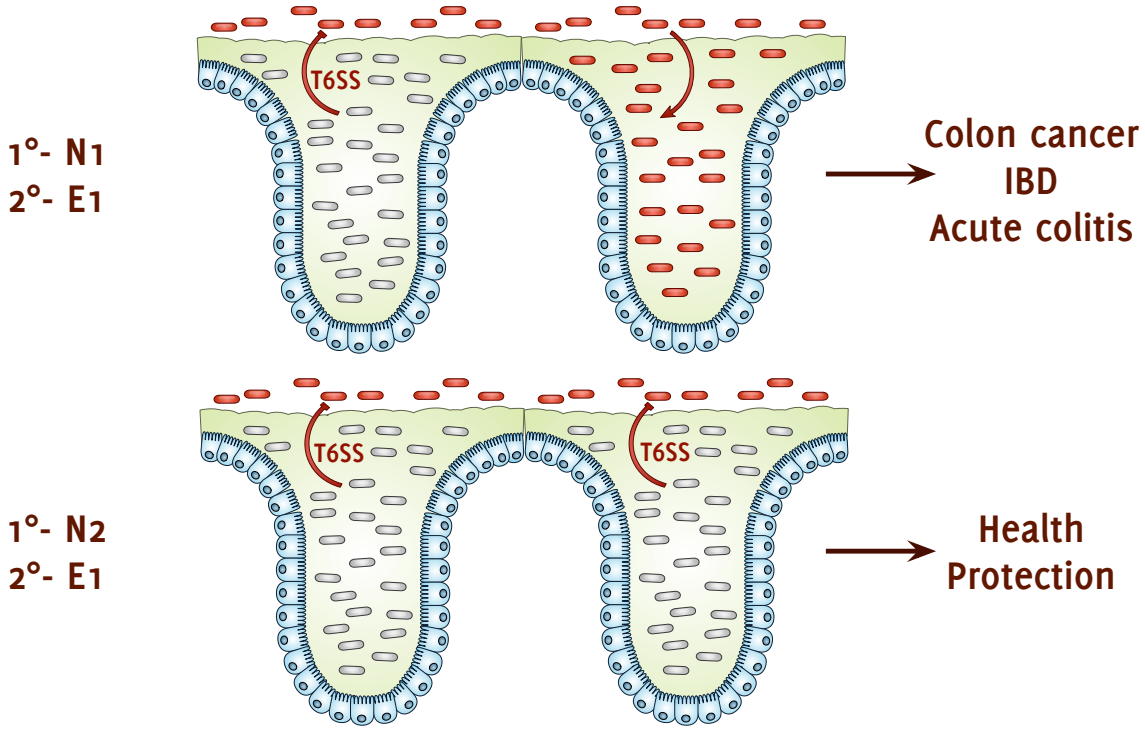



**Figure 28.** Strain-specific protection of the host from long-term exposure to ETBF is mediated by T6S. Schematic representation of competition between *B. fragilis* strains in the sequential colonization model. N1 primary colonization (top) partially restricts E1, but not entirely. This chronic exposure to ETBF and BFT may result in poor health outcomes. Pre-colonization with N2 (bottom), however, completely eliminates E1, partially mediated by T6S. This could lead to less inflammatory stimulus to the host over time, thereby protecting from disease.

## Appendix B

### Tables

**Table 1.** Strain names, abbreviations and inherent antibiotic resistances

| Strain abbr. | Reference name | Tetracycline | Rifampicin | Chloramphenicol |
|---|---|---|---|---|
| N1 | NCTC 9343<br>ATCC 25285 | S[a] | S | S |
| N2 | TM4000, 638R | S | R | S |
| N3 | YCH46 | R[b] | S | S |
| E1 | ATCC 43858 | S | S | S |
| E2 | ATCC 43859 | R | S | S |

a. S indicates inherent sensitive to the antibiotic at the concentration used in these studies.
b. R indicates inherent resistance to the antibiotic at the concentration used in these studies.

**Table 2.** Mass spectrometry peptide analysis of T6S-dependent secretome.

| Locus | Name | Abundance WT | Abundance Δ*tssC* | Fold Change ($log_2$) | MW | Sequence coverage |
|---|---|---|---|---|---|---|
| BF9343_1928 | Bte2 | 38.3 +/- 2.1 | 1 +/- 1 | 3.354 | 112.69 | 45.8 |
| BF9343_1930 | VgrG | 66 +/- 2 | 2.3 +/- 0.6 | 3.153 | 67.894 | 76.5 |
| BF9343_1941 | TssC | 10 +/- 3 | 0 +/- 0 | 2.321 | 52.269 | 49.2 |
| BF9343_1939 | HCP-3 | 6.3 +/- 0.6 | 0 +/- 0 | 1.903 | 16.093 | 75.4 |
| BF9343_1942 | TssB | 6.3 +/- 1.2 | 0 +/- 0 | 1.889 | 16.692 | 56.4 |
| BF9343_1943 | HCP-4 | 87 +/- 5.6 | 12.3 +/- 2.1 | 1.841 | 14.489 | 97.7 |
| BF9343_1929 | PAAR-repeat protein | 3.7 +/- 1.2 | 0 +/- 0 | 1.359 | 23.703 | 15.8 |
| BF9343_1938 | HCP-2 | 3 +/- 0 | 0 +/- 0 | 1.092 | 15.376 | 50.4 |
| BF9343_1937 | Bte1 | 2 +/- 0 | 0 +/- 0 | 0.671 | 32.841 | 13.2 |

**Table 3**. Primers

| Purpose | Direction | Sequence |
|---|---|---|
| Generation of pAH1 | F | ATAGCGTCTAGATTTTCTTGGGCATACTGCGTT |
| | R | ATAGCGTCAGACATCTTACACCAAAATTGCACA |
| Validate pAH1 | F | CGCGGTGGCGGCCGCT |
| | R | AAGGAGCAATTTAAATTTAAAATAG |
| Generation of pAH2 | F | GCGGCCGCGAATTCGATAACAGCCGGTG |
| | R | GCGGCCGCCGCTCCCCGACCGATGAT |
| Generation of pAH2-CAT | F | GGTACCAAAGATCTGAAAGAGAGACAATG |
| | R | GGTACCCGAATTTCTGCCATTCATCCG |
| N-term 6xHis-BFT | F | GCACGTGGTACCGGTTTTGCATTTAGAAGT |
| | R | TGCGGATCCCTAGTGATGATGATGATGATGATCGCCATCTGCTATTTC |
| RpoD-BFT | F | CTTTGCTATATTTTATATAACGTATAAGTTAAAAACAGATTTGGAGTGCAAAG |
| | R | CTTTGCTATATTTTATATAACGTATAAGTTAAAAACAGATTTGGAGTGCAAAG |
| | F | CTTTTGCACTCCAAATCTGTTTTTAACTTATACGTTATATAAAATATAGCAAAG |
| *TssC* mutant construct | US F | GGTACCGGTATTCGTCCACCGTAGTG |
| | US R | AGACAATAAGAACTATGGAAGATCAGGCGTAATAAGAATAAAATATG |
| | DS F | CATATTTTATTCTTATTACGCCTGATCTTCCATAGTTCTTATTGTCT |
| | DS R | GGTACCTCTTGTAGTCTGTATTAAACAAAT |
| *Bsap-1* mutant construct | US F | GGATCCATAAGGAGTTGAAAGATATGAATC |
| | US R | ATAATTTCAAGAGGTTATGGTCTGAATAGCATTAGTAAAAGTCCAA |
| | DS F | TTGGACTTTTACTAATGCTATTCAGACCATAACCTCTTGAAATTAT |
| | DS R | GGATCCAAAAGATTCAGCCACAGCAATG |

**Table 3. Continued.**

| Purpose | Direction | Sequence |
|---|---|---|
| *TssC* complementation GAPDH promoter | US F | GGTACCCTTTTTTTATATTTAATATGAATTTAAT |
| | US R | TAAAACCCTTAAAAGTTTAAACAAAATGGAAGATAATAAAAATAAGTCTG |
| | DS F | CAGACTTATTTTTATTATCTTCCATTTTGTTTAAACTTTTAAGGGTTTTA |
| | DS R | GGTACCTTACGCCTGTTCGTAATTTGTA |
| *B. fragilis* genotyping primers | Set 1 F | CCTAGATTGAATCATATTCTATAC |
| | Set 1 R | AATCTCAAACTTGGAAGGCTG |
| | Set 2 F | GAGTGCAGTAGTTTTGATAGT |
| | Set 2 R | CAAGCGACTGCTCCAAAAGC |
| | Set 3 F | ACGCCCTATTAAAGTGATTATTT |
| | Set 3 R | TCAGCCCTGCTCTCCGATT |