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A Type VI Secretion-Related Pathway in Bacteroidetes Mediates Interbacterial Antagonism

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SUMMARY

Bacteroidetes are a phylum of Gram-negative bacteria abundant in mammalian-associated polymicrobial communities, where they impact digestion, immunity, and resistance to infection. Despite the extensive competition at high cell density that occurs in these settings, cell contact-dependent mechanisms of interbacterial antagonism, such as the type VI secretion system (T6SS), have not been defined in this group of organisms. Herein we report the bioinformatic and functional characterization of a T6SS-like pathway in diverse Bacteroidetes. Using prominent human gut commensal and soil-associated species, we demonstrate that these systems localize dynamically within the cell, export antibacterial proteins, and target competitor bacteria. The Bacteroidetes system is a distinct pathway with marked differences in gene content and high evolutionary divergence from the canonical T6S pathway. Our findings offer a potential molecular explanation for the abundance of Bacteroidetes in polymicrobial environments, the observed stability of Bacteroidetes in healthy humans, and the barrier presented by the microbiota against pathogens.

INTRODUCTION

The Bacteroidetes are a phylum of Gram-negative bacteria that can be isolated from diverse natural habitats (Thomas et al., 2011). Though they include agriculturally and medically relevant pathogens, as well as representatives that play important roles in critical environmental processes such as bioremediation, the phylum is relatively poorly studied. Bacteroidetes are perhaps most appreciated for their intimate association with humans and other mammals, as abundant residents of the gastrointestinal (GI) tract. In this ecosystem, bacteria form dense microbial communities that can exceed 10^{13} cells per milliliter (Lozupone et al., 2012; Qin et al., 2010; Smith et al., 2006). Bacteroidetes constitute 20%–80% of the fecal microbiota of most adult humans and are largely represented by two genera, *Bacteroides* and *Prevotella* (Human Microbiome Project Consortium, 2012). Members of this phylum generally act as mutualists by aiding in the digestion of complex carbohydrates, promoting gut development, modulating the immune system, and protecting against colonization by pathogens (Round and Mazmanian, 2009; Smith et al., 2006; Thomas et al., 2011). As metabolically pliable organisms, Bacteroidetes also help to support a diverse gut community through syntrophic interactions with other microbes (Fischbach and Sonnenburg, 2011; Rey et al., 2010).

Evidence suggests that the capacity of a bacterium to survive in a polymicrobial environment is related to the elaboration of interbacterial antagonistic factors. Studies performed primarily on Proteobacteria have shown that Gram-negative organisms can utilize soluble products as well as contact-dependent mechanisms to compete with other bacteria (Hayes et al., 2014; Riley and Wertz, 2002). Although Bacteroidetes occupy numerous polymicrobial niches, including the human gut, contact-dependent antagonistic pathways are poorly understood in this phylum.

The type VI secretion system (T6SS) is a pathway that grants Gram-negative bacteria the capacity to translocate substrates to a wide range of recipient cells (Coulthurst, 2013). Initially speculated to participate strictly in host cell interactions, it is now clear that the more common function of the system is to deliver proteins from the cytoplasm of a donor cell to the periplasm of a Gram-negative recipient (Schwarz et al., 2010a). Substrates transported in a T6S-dependent manner include antibacterial effectors with diverse activities such as phospholipases, peptidoglycan hydrolases, nucleases, and membrane pore-forming proteins (Benz and Meinhart, 2014; Russell et al., 2014). The pathway appears to lack a mechanism for discriminating self from nonself; thus, bacteria with active T6SSs possess immunity proteins that interact with, and inactivate, the effector molecules (Hood et al., 2010; Russell et al., 2011). These interactions are allele specific, and cognate effector-immunity (E-I) pairs are generally encoded adjacently within predicted operons (English et al., 2012; Russell et al., 2012).

The T6S pathway requires the functions of 13 core proteins; unique subsets of these appear to have evolutionary relatedness

to type IV secretion system (T4SS) components or to bacteriophage (Boyer et al., 2009; Cascales and Cambillau, 2012). Proteins within the subsets are generally encoded adjacent to each other and interact extensively, suggesting that although each of the 13 core genes is essential, the complete system may be composed of modular, functionally distinct subcomplexes. The T4S-related components, TssL and TssM, are integral membrane proteins that form a trans-envelope complex with an outer-membrane lipoprotein, TssJ (Aschtgen et al., 2010; Felisberto-Rodrigues et al., 2011; Ma et al., 2009). The bacteriophage-like protein TssC, in conjunction with TssB, forms a dynamic filamentous assembly with gross structural similarity to the bacteriophage sheath complex (Basler et al., 2012; Bönemann et al., 2009). Two other bacteriophage-related proteins, VgrG and Hcp, interact with nonoverlapping sets of effectors, forming the basis for genetically distinct pathways for T6S-dependent substrate export (Shneider et al., 2013; Silverman et al., 2013; Whitney et al., 2014). VgrG is a phage tail spike-like protein that interacts with effectors via conserved adaptor domains, whereas Hcp is ring-shaped, bears structural homology to the major phage tail tube protein gpV, and interacts with effectors within its pore. Supporting the relationship of Hcp to gpV, Hcp rings have been observed to form higher-order head-to-tail stacked structures in vivo, analogous to those observed in bacteriophage (Brunet et al., 2014).

Here, we report the bioinformatic and functional characterization of a T6S-like pathway in the phylum Bacteroidetes. We demonstrate that this pathway has the capacity to mediate cell contact-dependent intra- and interphyla bacterial antagonism. Although the pathway lacks conserved elements essential to the well-characterized Proteobacterial T6SS, and components that are shared with Proteobacteria display considerable sequence divergence, we provide evidence that they function in a mechanistically similar manner. Several genera that possess the Bacteroidetes T6S-like pathway, including *Bacteroides*, *Prevotella*, and *Porphyromonas*, are abundant members of human-associated polymicrobial communities, suggesting that the pathway may have an important role in defining the composition of the microbiome (Falagas and Siakavellas, 2000).

RESULTS

Bioinformatic Characterization of a T6S-like Gene Cluster in Bacteroidetes

A generally applicable diagnostic secretion signal for T6S effectors is not available; however, genes encoding these proteins can often be identified by sequence-based approaches. Our group and others have noted that in many cases T6S effector and *vgrG* genes occur proximally and codirectionally on bacterial chromosomes (Barret et al., 2011; Russell et al., 2013; Zhang et al., 2012). We recently exploited this observation to identify a large superfamily of T6S-exported phospholipases. Interestingly, a search for homologs of this class of effectors revealed their presence in Bacteroidetes, a bacterial phylum that does not possess a characterized T6S pathway (Russell et al., 2013). Moreover, like the Proteobacterial effectors, those found in the Bacteroidetes reside in close proximity to apparent *vgrG* genes and adjacent to open reading frames (ORFs) encoding predicted periplasmic immunity determinants (Figure S1). Our observations concerning phospholipase effector distribution are supported by an exhaustive study of polymorphic toxin domains conducted by Aravind and colleagues, which found genes encoding putative T6 effectors of various catalytic classes represented in Bacteroidetes (Zhang et al., 2012).

Given the abundance and widespread nature of antibacterial T6S effector genes in Bacteroidetes, we postulated that these proteins participate in interbacterial interactions via a yet uncharacterized T6S-like pathway. Proteobacterial T6S gene clusters often include effector loci; thus, to identify a T6S-like pathway in Bacteroidetes, we searched in the vicinity of putative effector genes for elements that could constitute a secretion system. This led to the identification of a cluster of 12 genes, including *vgrG*, with orthologs invariantly found in species with predicted effectors (Figures 1A–1C and Table S1). Supporting the hypothesis that this gene cluster encodes a T6-like pathway, among the products of the 12 genes, we found a predicted ATPase with domain architecture similar to the Proteobacterial T6S core ATPase, ClpV (Figure 1D) (Schlieker et al., 2005).

Homology searches of the remaining conserved elements of the Bacteroidetes gene cluster failed to identify corresponding Proteobacterial T6S proteins. Given the evolutionary distance between Bacteroidetes and Proteobacteria, we posited that conservation between the constituents of this putative secretion system and the Proteobacterial T6SS might be below the detection limit of noniterative approaches. By applying iterative search algorithms such as jackHMMER and PSI-BLAST (Altschul et al., 1997; Johnson et al., 2010), we found that six additional genes in the Bacteroidetes cluster encode proteins bearing distant homology to core elements of the Proteobacterial T6SS: TssB, TssC, TssE, TssF, TssG, and TssK (Figure 1C and File S1).

In total, our sequence-based searches identified 8 of the 13 putative functional orthologs of the Proteobacterial T6SS encoded within the Bacteroidetes gene cluster (Table S1). Included in these eight components are each of the identified proteins of the bacteriophage-like module of the T6SS, with the exception of Hcp. In the Proteobacterial T6SS, Hcp proteins are required for effector recognition and export; thus, the apparent absence of this conserved component was unexpected (Silverman et al., 2013). To ensure we had not overlooked a cryptic Hcp functional ortholog, we turned to structural prediction algorithms, which can identify highly divergent proteins, or convergent proteins, by their common folds. Indeed, using the Phyre remote homology modeling server, we found that one of the remaining four conserved genes in the Bacteroidetes cluster is predicted to encode a protein that adopts a structure with a high degree of similarity to Proteobacterial Hcp proteins (>90% confidence) (Kelley and Sternberg, 2009). To further probe this predicted relatedness, we heterologously expressed and purified a member of this putative Hcp-like protein family encoded within the T6S-like gene cluster of *Flavobacterium johnsoniae*, a soil-dwelling member of the Bacteroidetes phylum (Figure 1E) (McBride et al., 2009). Visualization of this protein by negative stain transmission electron microscopy demonstrated that it adopts the characteristic ring shape and approximate dimensions of Proteobacterial Hcp proteins (Figure 1F). Together, our data suggest that a conserved gene cluster within the Bacteroidetes phylum encodes a T6S-like pathway. Henceforth, we refer to this pathway as T6SS subtype 3 (T6SS^{iii}), with the intent to

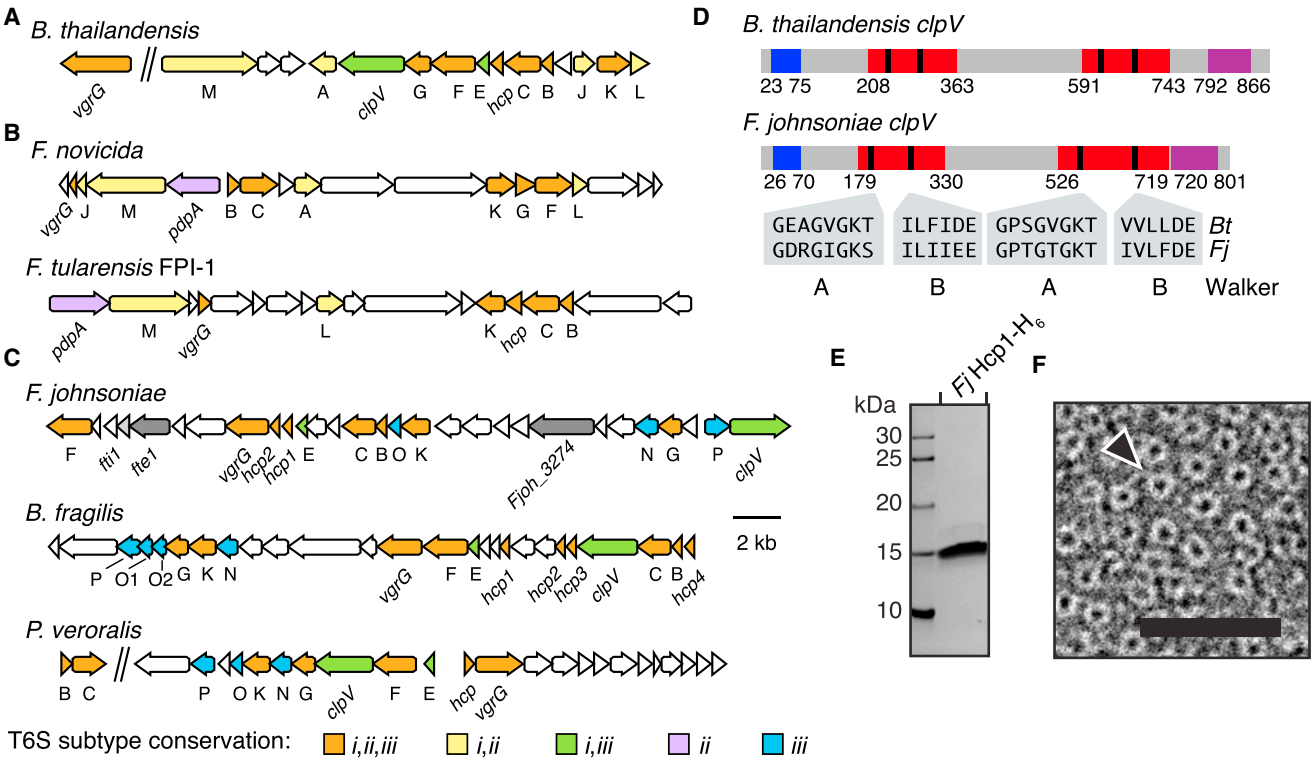

Figure 1. T6S-like Gene Clusters Are Found within the Bacteroidetes

(A–C) Gene content and conservation both within and between selected representative members of T6SS^{i} (A), T6SS^{ii} (B), and T6SS^{iii} (C). Genes with commonly used *tss* nomenclature are abbreviated to a single letter. The *Francisella tularensis* FPI is depicted owing to its status as the only T6SS^{ii} to be characterized; however, this system lacks clear homologs of *tssA* and *tssJ*, which are present in representative T6SS^{ii} systems such the *F. novicida* gene cluster shown. Locus tags of the regions shown: *B. thailandensis* E264 BTH_I2705 and BTH_I2954-2968 (A); *F. novicida* U112 FTN_0037-0054, *F. tularensis* SCHU FTT_1344-1361c (B); *F. johnsoniae* UW101 Fjoh_3254-328, *B. fragilis* NCTC 9343 BF9343_1918-1943, *P. veroralis* F0319 HMPREF0973_02422-02423 and HMPREF0973_02441-02466 (C). Genes encoding *F. johnsoniae* T6SS^{iii} substrates identified in this study (dark gray) and a validated immunity locus (light gray) are labeled. For sequence alignments of T6SS^{iii} TssB, TssC, TssE, TssF, TssG, and TssK proteins, including those depicted, see File S1.

(D) Comparison of domain organization of ClpV homologs from T6SS^{i} (*B. thailandensis*, *Bt*) and T6SS^{iii} (*F. johnsoniae*, *Fj*) pathways. Colors denote homologous domains: blue, ClpN; red, AAA+; purple, ClpB D2. Sequences highlight the conservation of motifs implicated in ATP binding and hydrolysis within the Walker A and B motifs.

(E) SDS-PAGE analysis of purified Fjoh_3262 bearing a C-terminal hexahistidine tag (*Fj* Hcp1-H_6). Proteins were visualized by Coomassie blue staining.

(F) *F. johnsoniae* Hcp1 is a ring-shaped molecule with dimensions similar to Proteobacterial Hcp proteins. Transmission electron micrograph of purified *Fj* Hcp1-H_6 (E) negatively stained with uranyl formate is shown. The arrowhead indicates a typical ring-like assembly. Scale bar, 40 nm.

distinguish it from the general Proteobacterial and Francisella pathogenicity island-like systems, herein constituting subtypes 1 and 2 (T6SS^{i} and T6SS^{ii}), respectively (Figures 1A–1C) (Boyer et al., 2009; Bröms et al., 2010).

In order to better understand the relationship of the three T6SS groups, we performed phylogenetic analyses on the shared elements TssC and TssF. Phylogenetic trees generated from conserved regions of these proteins exhibited similar topologies, suggesting that the genes encoding them have been coinherited (Figures 2A and S2A and Files S1, S2, and S3). In each tree, all Bacteroidetes components comprise a unique clade, distinct from Proteobacterial T6S homologs. While some proteins encoded by species in the phylum Acidobacteria are also found in this clade, analysis of the genomic context of these homologs indicates they reside in gene clusters that lack conserved Bacteroidetes components (Figure S2B). We therefore restrict our definition of T6SS^{iii} to those systems that reside in Bacteroidetes (Figure 2B and Files S1 and S4). Interestingly, T6SS^{iii} gene clusters lack homologs of the T6SS^{i} proteins TssA, TssL, TssM, and TssJ. A gene encoding a putative TssM protein was recently suggested to reside in a *B. cellulosilyticus* T6S gene cluster (Coyne et al., 2014). However, we note that homologs of this gene are neither generally found within or adjacent to T6SS^{iii} clusters across the Bacteroidetes phylum, nor are they encoded in the genomes of all organisms that possess the T6SS^{iii} pathway (data not shown). Our data suggest the entirety of the T6SS^{i} trans-envelope subcomplex—including *tssM*—is absent from T6SS^{iii}. In summary, our data suggest that a phylogenetically distinct T6S-like pathway—composed of an assemblage of proteins distinct from those required for the function of Proteobacterial T6SSs—is found within members of the phylum Bacteroidetes.

A T6SS^{iii} Pathway Exports Antibacterial Effectors

T6SSs are functionally versatile and can deliver effectors to other bacteria, to eukaryotic host cells, or to both of these cell types. We identified a number of predicted antibacterial effectors

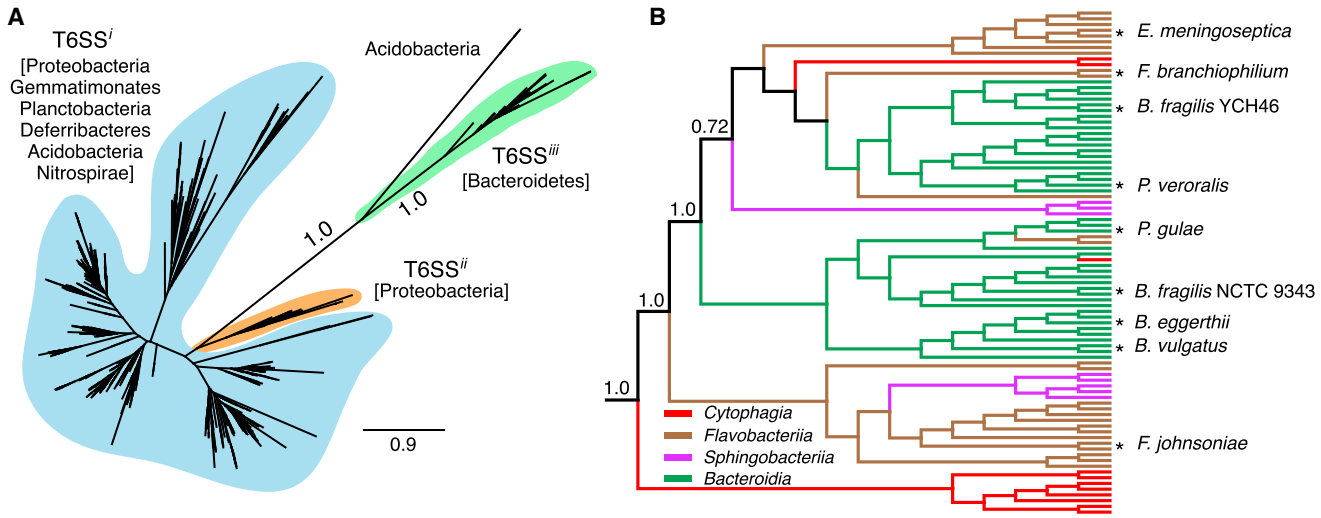

Figure 2. T6SS^{iii} Is Phylogenetically Distinct from T6SS^{i} and T6SS^{ii}

(A) Maximum likelihood (ML) phylogenetic tree generated from a partial alignment of 686 representative TssC sequences spanning the diversity present in T6SS^{i}, T6SS^{ii}, and T6SS^{iii} gene clusters. Phyla represented by each system are indicated. Branch support values derived from aBayes analysis for the T6SS^{iii} clade are shown. Scale bar represents amino acid changes per site. A similar tree for TssF is provided in Figure S2A.

(B) ML tree created from a partial alignment of TssC sequences found within T6SS^{iii} gene clusters. The tree is rooted with Acidobacterial TssC sequences. Lengths do not reflect evolutionary distance. Colors trace the class from which each sequence is derived. Nodes representing TssC sequences of organisms discussed in the text and those of particular significance are marked with an asterisk. Branch support values were generated by aBayes analysis. Partial sequence alignments and branch supports corresponding to phylogenetic trees in (A) and (B) and Figure S2 are provided in Files S1, S2, S3, and S4.

associated with T6SS^{iii} gene clusters in Bacteroidetes, suggesting that this system might possess the capacity to mediate interbacterial antagonism (data not shown). To first establish whether the T6SS^{iii} gene cluster encodes a secretory pathway responsible for the export of effector proteins, we conducted secretome measurements using *F. johnsoniae*. We chose this organism because it is genetically tractable, it is easily maintained under aerobic conditions in the laboratory (McBride et al., 2009; Rhodes et al., 2011), and it possesses a T6SS^{iii} gene cluster that is highly representative of the system in other Bacteroidetes, including the human gut-associated commensal species *B. fragilis*, *B. vulgatus*, and *B. eggerthii* (Figure 2B).

To determine the contribution of the T6SS^{iii} pathway to the secretome of *F. johnsoniae*, we compared the culture supernatant of a strain bearing an in-frame deletion of its predicted *tssC* homolog, Fjoh_3266, to the wild-type parental strain using mass spectrometry. This analysis identified six proteins that were undetected in *F. johnsoniae* Δ*tssC* but met our criteria for inclusion in the wild-type secretome (Table 1 and Table S2). Strikingly, the two most abundant of these proteins were Fjoh_3262 and Fjoh_3260, the Hcp and VgrG homologs encoded within the T6SS^{iii} gene cluster, respectively (Figure 1C). This finding parallels similar secretome studies of T6SS^{i} pathways, which generally find Hcp- and VgrG-family proteins as the major components of the T6SS-dependent substrate pool (Fritsch et al., 2013; Hood et al., 2010; Russell et al., 2012).

Of the remaining four proteins, two are encoded by hypothetical ORFs present within the T6SS^{iii} gene cluster (Fjoh_3257 and Fjoh_3274), whereas the remaining two are implicated in gliding motility and possess predicted signal sequences (Figure 1C and Table 1) (Rhodes et al., 2011). While the latter may have yet unrecognized roles relevant to T6SS^{iii}, for the purposes of identifying secreted effectors we focused on Fjoh_3257 and Fjoh_3274, which do not contain predicted signal peptides. Notably, these proteins both possess domains found in known or predicted T6SS^{i} effectors. Fjoh_3257 contains an HEXGH motif found in zinc metalloproteases fused to T6SS^{i}-exported VgrG proteins, and Fjoh_3274 contains both a central glycoside hydrolase domain and a C-terminal zinc-dependent peptidoglycan endopeptidase motif (Figure 3A) (Pukatzki et al., 2007; Russell et al., 2012). Based on these data, we hypothesized that Fjoh_3257 and Fjoh_3274 are T6SS^{iii}-exported effectors that exert toxicity in the periplasm of target cells.

To test the hypothesis that the T6SS^{iii} pathway exports antibacterial effectors, we further investigated Fjoh_3257. When directed to the periplasm of *Escherichia coli*, Fjoh_3257 induced significant cell lysis, whereas the native protein, predicted to localize to the cytoplasm, did not exhibit apparent toxicity (Figures 3B and S3). T6SS^{i} delivers effectors directly to the periplasm of recipient cells. If T6SS^{iii} functions similarly, our data suggest Fjoh_3257, henceforth referred to as *Flavobacterium* type VI secretion effector 1 (Fte1), could promote intercellular toxicity and thus necessarily associate with a cognate immunity determinant. Moreover, we would expect this immunity protein to reside in the periplasmic compartment, as T6SS^{i} effector inactivation invariably occurs via direct interaction with immunity (Benz et al., 2012; Dong et al., 2013a; Russell et al., 2011; Zhang et al., 2013). We identified a gene encoding a protein matching the predicted immunity criteria directly downstream of *fte1*, Fjoh_3256 (hereafter referred to as *Flavobacterium* type VI secretion immunity 1, or *fti1*). Coexpression of Fti1 specifically abrogated the lytic effects of Fte1, indicating that Fte1-Fti1 comprise an antibacterial effector-immunity pair of T6SS^{iii} in *F. johnsoniae* (Figure 3C).

Table 1. Secreted Proteins Not Detected in the *F. johnsoniae* ΔtssC Secretome

Locus Tag[a]	Name	Abundance[b]	Unique peptides detected	Signal peptide[c]	T6SS^{iii} cluster[d]	Molecular weight	Predicted/determined function
Fjoh_3262	Hcp	133 ± 3.5[e]	10	N	Y	14.5	Secreted T6S structural component
Fjoh_3260	VgrG	13.3 ± 1.5	5	N	Y	66.0	Secreted T6S structural component
Fjoh_3206	RemH	11.7 ± 2.3	4	Y	N	16.9	Gliding motility
Fjoh_0984	RemF	5 ± 1.0	4	Y	N	16.8	Gliding motility
Fjoh_3274	–	4 ± 1.0	3	N	Y	102.0	T6SS^{iii} effector
Fjoh_3257	Fte1	3 ± 1.7	2	N	Y	62.7	T6SS^{iii} effector

[a]Locus tags derived from *F. johnsoniae* UW101 (NCBI Accession NC_009441.1).
[b]Average spectral counts of three technical replicates.
[c]Signal peptide predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP).
[d]The T6SS^{iii} gene cluster comprises two apparent divergently transcribed operons (Fjoh_3254-Fjoh_3281), with the terminal gene of each operon encoding a conserved T6SS^{iii} element.
[e]Standard deviation of three technical replicates.

To test whether Fte1 exerts antibacterial activity in a T6SS^{iii}-dependent manner, we measured the cellular integrity of *F. johnsoniae* strains lacking *fti1*. When propagated on a solid substratum, a condition conducive to prolonged cell contact, we observed increased membrane permeability in the Δ*fti1* strain (Figure 3D). This phenotype was abrogated by concomitant deletion of *tssC*, *fte1*, or by growth in liquid media. Together with our secretome studies, these data strongly suggest the capacity of T6SS^{iii} to participate in interbacterial interactions through the export of antibacterial effectors.

T6SS^{iii} Mediates Interspecies Bacterial Antagonism

Interbacterial T6SS^{i} has been observed to be a crucial determinant of fitness during polymicrobial growth. Under contact-promoting conditions, its inactivation generally leads to significant defects in the capacity to outcompete other organisms in coculture. To determine whether T6SS^{iii} also functions in interspecies antagonism, we grew wild-type *F. johnsoniae* and derivative strains with either *Burkholderia thailandensis* or *Pseudomonas putida* under T6-conducive conditions. The inactivation of T6SS^{iii} by a deletion of *tssC* in *F. johnsoniae* greatly impacted the outcome of these growth competitions, allowing for significant expansion of the competitor population (Figures 4A and 4B). This phenotype could be complemented by the introduction of an extrachromosomal copy of *tssC*, demonstrating that the observed change in fitness was not due to mutant polarity. Moreover, wild-type and Δ*tssC* displayed equal fitness in liquid growth medium, consistent with the known requirement for intimate cell-cell contact in T6S-dependent interactions. We further examined interspecies cocultures containing *F. johnsoniae* lacking the T6SS^{iii}-restricted component, *tssN* (Fjoh_3277, Table S1). *F. johnsoniae ΔtssN* antagonized *B. thailandensis* and *P. putida* to an equivalent degree as Δ*tssC* or a strain bearing deletions in both *tssC* and *tssN*, consistent with our hypothesis that these genes encode essential elements of the same pathway (Figures 4A and 4B). Overall, our data strongly suggest that the T6SS^{iii} pathway mediates interbacterial antagonism in a manner analogous to T6SS^{i}, yet using a distinct complement of proteins.

The T6SS^{iii} Apparatus Exhibits Dynamic Behavior

Antibacterial effectors released by the T6SS^{i} pathway are operative on Gram-negative recipients only if they are delivered across the outer membrane by the translocation machinery. Owing to this feature of the system, the apparatus must behave dynamically in order to sample localizations that orient the system toward competitor cells. Green fluorescent protein (GFP) fusions to the C terminus of ClpV proteins have served as a convenient means to visualize this behavior of T6SS^{i} systems. Since an apparent ClpV ortholog is identifiable in T6SS^{iii} gene clusters, we sought to monitor the subcellular localization and dynamic behavior of this protein as a way to further interrogate the mechanistic similarity between T6SS^{i} and T6SS^{iii} pathways. We began by generating a strain of *F. johnsoniae* bearing a functional *clpV-gfp* fusion at the native *clpV* locus (Figure S4). Visualization of this strain using time-lapse fluorescence microscopy revealed punctate foci appearing, disappearing, and frequently reappearing at different subcellular locations on a rapid time scale (Figure 4C and Movies S1 and S2). Inactivation of the system in *F. johnsoniae* through the deletion of *tssC* abrogated these foci, similar to what has been observed in the T6SS^{i} pathway. It is worth noting that T6SS^{iii} ClpV exhibits punctate localization and dynamic behavior in the absence of an apparent TssM homolog, whereas in T6SS^{i} systems, TssM proteins are required for ClpV dynamics. Taken together with our bioinformatic, secretomic, and phenotypic data, these findings strongly suggest that the T6SS^{iii} pathway functions in a manner mechanistically similar to T6SS^{i} despite a highly divergent and unique assemblage of core components.

Bacteroides fragilis Targets *B. thetaiotaomicron* via T6SS^{iii}

Motivated by our characterization of the T6SS^{iii} pathway in *F. johnsoniae*, we sought to explore the relevance of our findings to human-associated Bacteroidetes. Our analyses indicate T6SS^{iii} gene clusters are present in many members of the genus *Bacteroides*, including numerous prominent human gut residents (Figure 2B and File S1). To probe the potential for the T6SS^{iii} pathway to influence the behavior of these organisms in a physiological setting, we colonized germ-free mice with a community containing *B. fragilis*, *B. eggerthii*, and the Proteobacterium *E. coli* and measured T6SS^{iii} expression. Quantitative RT-PCR of the cecal contents from these mice revealed the *tssC* gene in both *Bacteroides* species is expressed, at levels approaching (*B. eggerthii*) or exceeding (*B. fragilis*) the housekeeping transcript *rpoD* (Figure 5A).

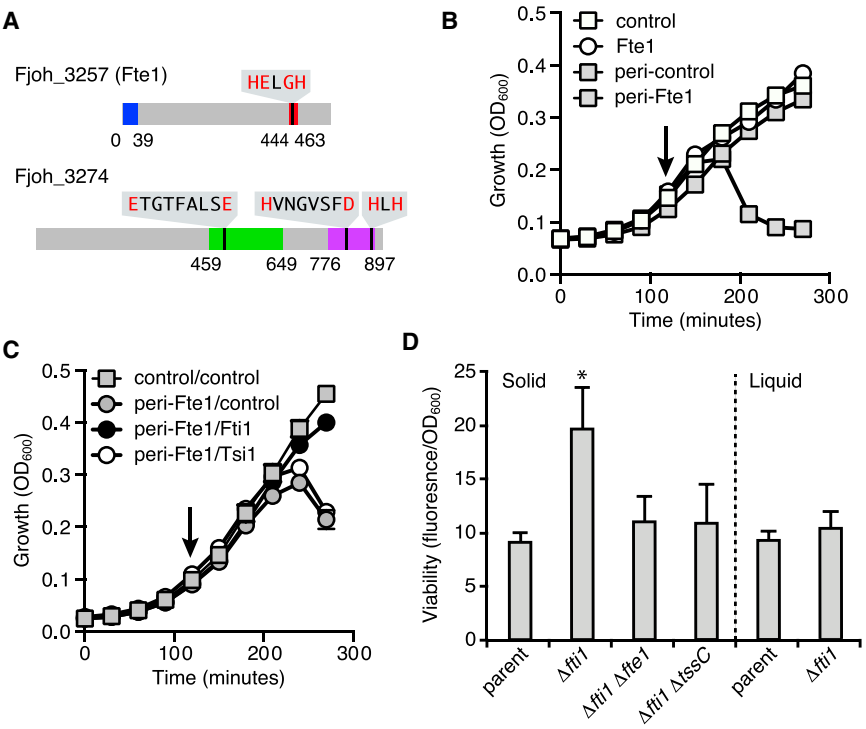

Figure 3. *F. johnsoniae* T6SS^{iii} Exports an Antibacterial Protein that Is Encoded Adjacent to a Cognate Immunity Determinant

(A) Domain organization of the putative substrates of *F. johnsoniae* T6SS^{iii}. PAAR-like (blue), zinc-dependent metalloprotease (red), glycoside hydrolase (green), and zinc-dependent peptidoglycan amidase (purple) domains are indicated. Expanded amino acid sequences in each domain correspond to conserved motifs and invariant or critical catalytic residues (red).

(B and C) Growth of *E. coli* strains harboring the indicated expression vectors. Empty vectors (control) and vectors that introduce an N-terminal Sec signal peptide (peri) are indicated. Cells were induced to express predicted immunity proteins (C) at time 0 and Fte1 at the indicated time point (arrow). Type VI secretion immunity protein 1 (Tsi1) is used as a noncognate immunity control. Error bars represent ± standard deviation (SD) (n = 3). Expression data for (B) are provided in Figure S3.

(D) Intercellular self-intoxication of the indicated *F. johnsoniae* strains as measured by propidium iodide staining. Liquid cultures were grown with vigorous shaking, which inhibits the formation of the prolonged cell-cell contacts required for T6-mediated interactions (Hood et al., 2010; LeRoux et al., 2012). Error bars represent ± SD (n = 3). Samples differing significantly from parent as measured by a two-tailed t test are indicated by asterisks ($p < 0.01$).

Expression of *tssC* in the mammalian gut environment led us to hypothesize that the T6SS^{iii} pathway could be employed by *Bacteroides* to target other Gram-negative human gut microbes, including other species of *Bacteroides*. To test this hypothesis, we assessed the ability of wild-type *B. fragilis* to inhibit growth of the prominent human gut commensal *B. thetaiotaomicron*, which lacks a T6SS. Growth competition experiments revealed that *B. fragilis* reduces *B. thetaiotaomicron* growth by approximately two orders of magnitude (Figure 5B). Strikingly, this activity is almost entirely T6SS^{iii} dependent, as an in-frame, unmarked deletion of *tssC* (BF9343_1941) in *B. fragilis* renders this species largely unable to reduce *B. thetaiotaomicron* growth. These data demonstrate the capacity of T6SS^{iii} to act between prominent human gut-associated members of the genus *Bacteroides*.

DISCUSSION

With the bioinformatic and functional characterization of T6SS^{iii}, it is now evident that the Bacteroidetes possess a means for contact-dependent interbacterial antagonism. This is consistent with the observation that Bacteroidetes frequently occupy contact-promoting, polymicrobial niches (Thomas et al., 2011). Indeed, many of the organisms we identify the T6SS^{iii} pathway within, including *Porphyromonas*, *Prevotella*, and *Bacteroides* spp., are highly adapted host-associated obligate anaerobes that predominate, as pathogens or commensals, within the most densely populated polymicrobial sites in the human body (Falagas and Siakavellas, 2000; Smith et al., 2006). Thus, within sites such as the GI tract, oral cavity, and the vagina, where bacteria with T6SS^{iii} are abundant, the pathway may play a broad role in defining community composition.

By analogy with T6SS^{i} and T6SS^{ii}, it is reasonable to speculate that T6SS^{iii} has the capacity to mediate host cell interactions in addition to its now established role in interbacterial antagonism. Certain T6SS^{i} and T6SS^{ii} pathways appear to specialize in either bacterial or host cell targeting, whereas others can act on both cell types (Hood et al., 2010; MacIntyre et al., 2010; Pukatzki et al., 2007; Schwarz et al., 2010b). Target range appears to be dictated, at least in part, by the specific complement and corresponding activities of the effectors transported by a system. For example, recent reports suggest that by virtue of structural conservation among the phospholipid constituents of cellular membranes, T6S effectors belonging to the Tle phospholipase superfamily can confer both antibacterial and antieukaryotic activity (Dong et al., 2013b; Jiang et al., 2014; Russell et al., 2013). While members of the Tle superfamily are among the many apparent effectors of T6SS^{iii}, the preponderance of predicted effectors that target peptidoglycan, a molecule found exclusively in bacteria, indicates that interbacterial antagonism is likely the basal function of the T6SS^{iii} pathway.

Despite lacking several T6SS^{i} core components, including TssJ, TssL, and TssM, our observations suggest that T6SS^{iii} functions in a fundamentally analogous manner. Both systems exhibit dynamic behavior, target effectors to the periplasm of recipients, and abundantly export VgrG- and Hcp-family proteins. There are several conceivable explanations for these observations. One possibility is that the unique T6SS^{iii} components, TssN, TssO, and TssP, functionally substitute for the missing components. This model is supported by the prediction that these components, like TssL and TssM, are integral membrane proteins (Ma et al., 2009; Aschtgen et al., 2012). However, TssJ is a predicted lipoprotein that requires localization to the outer

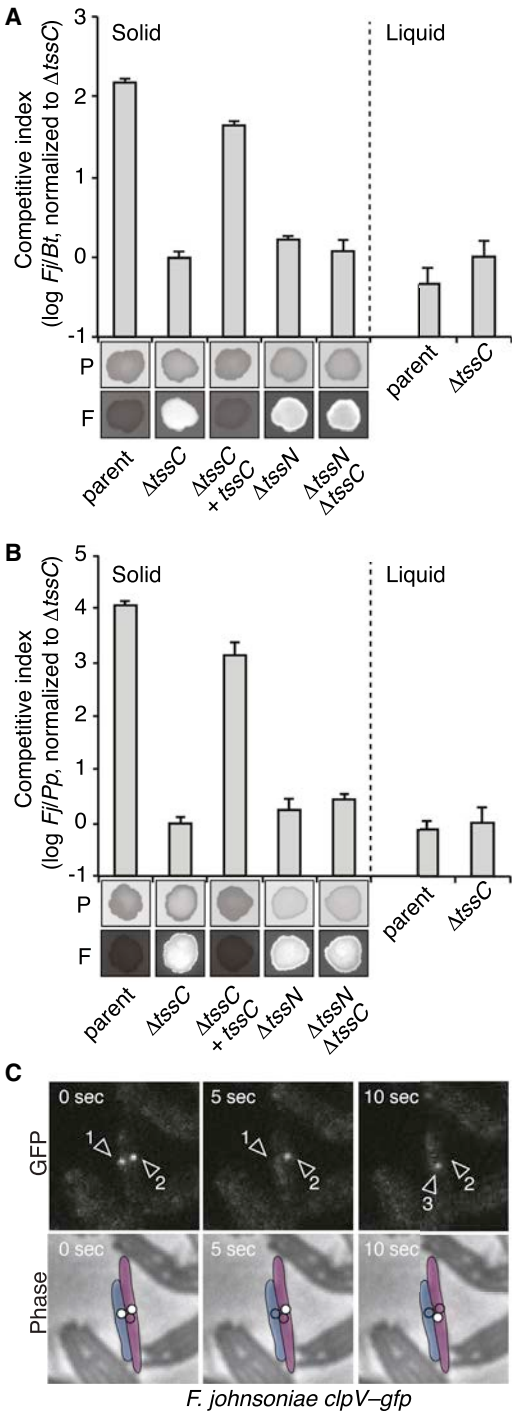

Figure 4. *F. johnsoniae* Utilizes a Dynamic T6SS^{iii} Apparatus to Target Competitor Organisms

(A and B) Growth experiments measuring fitness of the indicated *F. johnsoniae* (*Fj*) strains in coculture for 20 hr with fluorescently labeled competitors *B. thailandensis* (A, *Bt*) or *P. putida* (B, *Pp*). Competitive index is defined as the change in *F. johnsoniae*/competitor colony forming units between initiation and harvest of the coculture. Experiments were performed under contact-promoting (solid) and contact-inhibiting (liquid) conditions. Qualitative analysis of competition outcome by photographic (P) and fluorescence (F) imaging is shown for corresponding samples grown under contact-promoting conditions after 48 hr of coculture. Error bars represent ± SD (n = 3).

(C) Micrograph series depicting a 10 s time course of wild-type *F. johnsoniae clpV-gfp*. Phase and GFP channels are presented separately. Three dynamic foci were observed over the duration of the experiment (arrowheads), and the presence or absence of each focus in each frame is schematized in the phase micrographs with white or unfilled circles, respectively. Full-length movies that include the region represented are available as Movies S1 and S2. See also Figure S4.

membrane for function, and so far a T6SS^{iii}-conserved predicted outer membrane-localized protein has not been identified. It is worth noting that TssJ, TssL, and TssM interact stably to form a trans-envelope complex (Cascales and Cambillau, 2012). While it has been postulated that this complex facilitates the passage of bacteriophage-like proteins and effectors out of the recipient cell, there are little experimental data to support this notion. It is therefore not yet possible to rule out a model whereby the components shared between T6SS$^{i–iii}$—namely, those belonging to the bacteriophage-like subcomplex—represent the minimal structural assemblage of the T6SS. Distinguishing essential structural components from proteins with critical regulatory roles, for example, can be challenging (Hsu et al., 2009; Silverman et al., 2011). Understanding the functional significance of the varied complement of core elements associated with T6SS$^{i–iii}$ will ultimately require both detailed biochemical approaches aimed at defining more precisely the roles of the individual proteins and ultrastructural characterization of the system.

While T6SS^{i} and T6SS^{iii} are divergent, predicted effector proteins in T6SS^{iii}-encoding organisms are often closely related to homologs in organisms possessing T6SS^{i}. For example, homologs of Fte1 are readily identified in *Acinetobacter* spp. as well as strains of *E. coli*. These homologs, like Fte1, are encoded adjacent to predicted periplasmic immunity proteins as well as VgrG, suggesting that they likely possess common modes of toxicity and export. The relative ease with which homologs of effectors that transit the T6SS^{i} and T6SS^{iii} pathways can be identified is further indicative of the similarity between these two systems and suggests they might share a common pool of potential effectors exchanged through horizontal gene transfer.

The gene encoding one of the putative substrates identified in our study, Fjoh_3274, is found adjacent to a locus that encodes a small protein possessing a DUF4280 domain (Fjoh_3275). In Fjoh_3274 homologs found in other species, these open reading frames are often fused, suggesting their function is linked. Structure prediction algorithms indicate a strong likelihood that DUF4280 adopts a fold closely related to the PAAR domain, which forms a pyramidal structure that is thought to recruit effector proteins to the apparatus via interaction with VgrG (Shneider et al., 2013). Interestingly, proteins bearing DUF4280 are found in Gram-positive bacteria, a division of bacteria not known to possess a T6S-like pathway. Moreover, the genes encoding these proteins are often found adjacent to VgrG-like proteins. Our finding herein that the T6S pathway extends to the Gram-negative phylum Bacteroidetes raises the possibility that other organisms, even Gram-positive bacteria, may also possess related systems that have yet to be identified. By analogy, the antibacterial nature of the C-terminal polymorphic toxin domains of YD-repeat proteins was initially discovered in Gram-negative bacteria; however, more recent studies have found homologs of these toxin domains participate in interbacterial

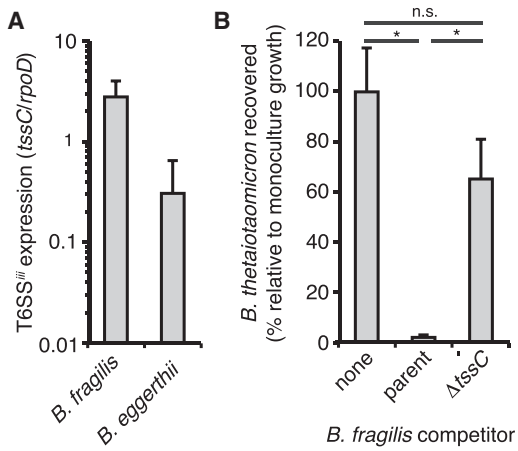

Figure 5. The T6SS^{iii} Pathway Is Expressed and Active in the Genus Bacteroides

(A) Quantitative RT-PCR analysis of the in vivo expression of *tssC* for the indicated bacteria. Expression was measured in cecal samples from germ-free mice colonized for 1 week with *B. fragilis*, *B. eggerthii*, and *E. coli* and normalized using species-specific primers for the housekeeping sigma factor, *rpoD*. Error bars represent ± SEM (n = 3).

(B) Growth competitions measuring viability of *B. thetaiotaomicron* after 24 hr in the presence of the indicated *B. fragilis* strains on solid media. *B. thetaiotaomicron* growth was normalized to values obtained in the absence of *B. fragilis*. Viability was determined by colony forming units. Error bars represent ± SD (n = 3). Significance as indicated by asterisks was measured by a two-tailed t test ($p < 0.002$). n.s.; no statistical difference.

antagonism in Gram-positive organisms (Koskiniemi et al., 2013).

Colonization resistance is a property of the gut microbiota whereby it acts as a coherent, resilient entity that exhibits resistance to invading microbes (Stecher and Hardt, 2011). The importance of this property is exemplified by the enhanced susceptibility to pathogens observed following either depletion or dysbiosis of the gut microbial community during antibiotic treatment (Lawley and Walker, 2013). Notably, recent studies have also shown that individuals carry the same commensal strains in their gut microbiomes for years or decades, and that members of the Bacteroidetes exhibit the greatest stability (Faith et al., 2013). A complete molecular explanation for these observations will likely include metabolic exclusion (Turnbaugh et al., 2009), colonization of critical niches (Lee et al., 2013), and the production of diffusible antimicrobials such as bacteroicins (Pujol et al., 2011). We posit that antagonistic contact-dependent interactions mediated by the T6SS^{iii} pathway are another important contributor to colonization resistance and commensal stability. Interestingly, Turnbaugh and colleagues recently identified a transcript corresponding to a core T6SS^{iii} element (*vgrG*) derived from Bacteroidales in a metatranscriptome of fresh human fecal samples (Maurice et al., 2013). Moreover, another study showed that the physical environment of the gut is conducive to T6S-mediated interbacterial interactions (Fu et al., 2013). While experiments involving T6SS^{iii} mutants within gut colonization models will be needed in order to directly establish its role in this environment, taken together with our demonstration of the antibacterial nature of the system, these findings are consistent with the hypothesis that contact-dependent interbacterial interactions occur among commensals in the human gut.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions

F. johnsoniae, *B. thailandensis*, *P. putida*, *B. fragilis*, *B. eggerthii*, and *B. thetaiotaomicron* used in this study were derived from the sequenced strains UW101, E264, KT2440, NCTC 9343, ATCC 27754, and VPI-5482, respectively. *E. coli* strains used in this study included DH5α for plasmid maintenance and triparental conjugation of plasmids into *F. johnsoniae* and *B. fragilis*, Rosetta 2(DE3) (EMD Millipore) for toxicity experiments, BL21(DE3) pLysS for the expression and purification of Fjoh_3262, and Nissle 1917 for mouse colonization experiments. Growth conditions for all strains and plasmid and strain construction details are described in Supplemental Experimental Procedures.

Informatics Analyses

ClpV- and VgrG-like proteins were identified by automated annotation from NCBI blast servers. Hcp-like proteins were initially found within *F. johnsoniae* by PHYRE 2.0 (Kelley and Sternberg, 2009) and were thereafter identified by homology using blastp analysis. Other T6SS homologs were identified in T6SS^{iii} gene clusters by the application of the iterative search algorithm jackHMMER on the RefSeq protein database using seed proteins obtained from *F. johnsoniae* (Finn et al., 2011). Alignments, domain prediction, phylogenetic trees, and subcellular localization were determined as described in Supplemental Experimental Procedures.

Secretome Preparation and MS Analysis

The *F. johnsoniae* secretome was obtained using previously described methods with modifications indicated in Supplemental Experimental Procedures (Hood et al., 2010). The UniProt *F. johnsoniae* UW101 database was used as a reference for peptide identification using MaxQuant v1.4.1.2 (Cox and Mann, 2008). Relative abundance of proteins was assessed using spectral counting (Liu et al., 2004). Proteins were filtered such that all had at least two unique peptides detected and possessed an average of three spectral counts in wild-type replicates.

Cellular Toxicity Assays

E. coli toxicity assays were performed as described previously with minor modifications (Russell et al., 2011). For the analysis of T6SS^{iii}-dependent Fte1 toxicity in *F. johnsoniae*, strains were grown on a nitrocellulose surface as monocultures for 20 hr before analysis by propidium iodide staining. Full details are provided in Supplemental Experimental Procedures.

Bacterial Competitions

Bacterial cocultures were prepared as described in Supplemental Experimental Procedures and either spotted on nitrocellulose, placed on solid media, or subinoculated into liquid media. After 20 hr (*F. johnsoniae*) or 24 hr (*Bacteroides*) of competition, cells were harvested and plated on selective media for quantification of each organism. Fluorescence images and photographs were acquired for the *F. johnsoniae* experiments after 48 hr. Full details are provided in Supplemental Experimental Procedures.

Fluorescence Microscopy

Microscopy was performed as described previously (LeRoux et al., 2012). *F. johnsoniae* cells were prepared for microscopy after growth in conditions similar to bacterial competition experiments and were visualized on 1.5% w/v agarose phosphate-buffered saline pads. Automated image acquisition was performed at 5 s intervals for 6 min. Full details are provided in Supplemental Experimental Procedures.

In Vivo Expression of T6SS^{iii}

All animal experiments were performed using protocols approved by the Yale University Institutional Animal Care and Use Committee. Germ-free Swiss Webster mice were maintained in flexible plastic gnotobiotic isolators with a 12 hr light/dark cycle. Mice (n = 5/group) were individually caged and were

provided with standard autoclaved mouse chow (5K67 LabDiet, Purina) ad libitum. On day 0, mice were gavaged orally with 2×10^8 cfu of each strain (*B. fragilis*, *B. eggerthii*, and *E. coli*). Animals were sacrificed on day 7 and samples were collected along the length of the gut. All samples were snap-frozen in liquid nitrogen and stored at −80°C.

RNA extraction from mouse cecal samples, cDNA synthesis, and quantitative RT-PCR were performed using standard methods. Expression for each T6SS gene was normalized to *rpoD* expression levels in the same organisms using species-specific primers. Genomic DNA samples used to generate standards for quantitative RT-PCR were obtained by published methods (Degnan et al., 2014). Full details are provided in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, two tables, two movies, and four additional files and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2014.07.007.

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REFERENCES

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. *25*, 3389–3402.

Aschtgen, M.S., Gavioli, M., Dessen, A., Lloubès, R., and Cascales, E. (2010). The SciZ protein anchors the enteroaggregative Escherichia coli Type VI secretion system to the cell wall. Mol. Microbiol. *75*, 886–899.

Aschtgen, M.S., Zoued, A., Lloubès, R., Journet, L., and Cascales, E. (2012). The C-tail anchored TssL subunit, an essential protein of the enteroaggregative Escherichia coli Sci-1 Type VI secretion system, is inserted by YidC. Microbiologyopen *1*, 71–82.

Barret, M., Egan, F., Fargier, E., Morrissey, J.P., and O'Gara, F. (2011). Genomic analysis of the type VI secretion systems in Pseudomonas spp.: novel clusters and putative effectors uncovered. Microbiology *157*, 1726–1739.

Basler, M., Pilhofer, M., Henderson, G.P., Jensen, G.J., and Mekalanos, J.J. (2012). Type VI secretion requires a dynamic contractile phage tail-like structure. Nature *483*, 182–186.

Benz, J., and Meinhart, A. (2014). Antibacterial effector/immunity systems: it's just the tip of the iceberg. Curr. Opin. Microbiol. *17*, 1–10.

Benz, J., Sendlmeier, C., Barends, T.R., and Meinhart, A. (2012). Structural insights into the effector-immunity system Tse1/Tsi1 from Pseudomonas aeruginosa. PLoS ONE *7*, e40453.

Bönemann, G., Pietrosiuk, A., Diemand, A., Zentgraf, H., and Mogk, A. (2009). Remodelling of VipA/VipB tubules by ClpV-mediated threading is crucial for type VI protein secretion. EMBO J. *28*, 315–325.

Boyer, F., Fichant, G., Berthod, J., Vandenbrouck, Y., and Attree, I. (2009). Dissecting the bacterial type VI secretion system by a genome wide in silico analysis: what can be learned from available microbial genomic resources? BMC Genomics *10*, 104.

Bröms, J.E., Sjöstedt, A., and Lavander, M. (2010). The Role of the Francisella Tularensis Pathogenicity Island in Type VI Secretion, Intracellular Survival, and Modulation of Host Cell Signaling. Front Microbiol *1*, 136.

Brunet, Y.R., Hénin, J., Celia, H., and Cascales, E. (2014). Type VI secretion and bacteriophage tail tubes share a common assembly pathway. EMBO Rep. *15*, 315–321.

Cascales, E., and Cambillau, C. (2012). Structural biology of type VI secretion systems. Philos. Trans. R. Soc. Lond. B Biol. Sci. *367*, 1102–1111.

Coulthurst, S.J. (2013). The Type VI secretion system - a widespread and versatile cell targeting system. Res. Microbiol. *164*, 640–654.

Cox, J., and Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat. Biotechnol. *26*, 1367–1372.

Coyne, M.J., Zitomersky, N.L., McGuire, A.M., Earl, A.M., and Comstock, L.E. (2014). Evidence of Extensive DNA Transfer between Bacteroidales Species within the Human Gut. MBio *5*.

Degnan, P.H., Barry, N.A., Mok, K.C., Taga, M.E., and Goodman, A.L. (2014). Human gut microbes use multiple transporters to distinguish vitamin B_{12} analogs and compete in the gut. Cell Host Microbe *15*, 47–57.

Dong, C., Zhang, H., Gao, Z.Q., Wang, W.J., She, Z., Liu, G.F., Shen, Y.Q., Su, X.D., and Dong, Y.H. (2013a). Structural insights into the inhibition of type VI effector Tae3 by its immunity protein Tai3. Biochem. J. *454*, 59–68.

Dong, T.G., Ho, B.T., Yoder-Himes, D.R., and Mekalanos, J.J. (2013b). Identification of T6SS-dependent effector and immunity proteins by Tn-seq in Vibrio cholerae. Proc. Natl. Acad. Sci. USA *110*, 2623–2628.

English, G., Trunk, K., Rao, V.A., Srikannathasan, V., Hunter, W.N., and Coulthurst, S.J. (2012). New secreted toxins and immunity proteins encoded within the Type VI secretion system gene cluster of Serratia marcescens. Mol. Microbiol. *86*, 921–936.

Faith, J.J., Guruge, J.L., Charbonneau, M., Subramanian, S., Seedorf, H., Goodman, A.L., Clemente, J.C., Knight, R., Heath, A.C., Leibel, R.L., et al. (2013). The long-term stability of the human gut microbiota. Science *341*, 1237439.

Falagas, M.E., and Siakavellas, E. (2000). Bacteroides, Prevotella, and Porphyromonas species: a review of antibiotic resistance and therapeutic options. Int. J. Antimicrob. Agents *15*, 1–9.

Felisberto-Rodrigues, C., Durand, E., Aschtgen, M.S., Blangy, S., Ortiz-Lombardia, M., Douzi, B., Cambillau, C., and Cascales, E. (2011). Towards a structural comprehension of bacterial type VI secretion systems: characterization of the TssJ-TssM complex of an Escherichia coli pathovar. PLoS Pathog. *7*, e1002386.

Finn, R.D., Clements, J., and Eddy, S.R. (2011). HMMER web server: interactive sequence similarity searching. Nucleic Acids Res. *39* (Web Server issue), W29-37.

Fischbach, M.A., and Sonnenburg, J.L. (2011). Eating for two: how metabolism establishes interspecies interactions in the gut. Cell Host Microbe *10*, 336–347.

Fritsch, M.J., Trunk, K., Diniz, J.A., Guo, M., Trost, M., and Coulthurst, S.J. (2013). Proteomic identification of novel secreted antibacterial toxins of the Serratia marcescens type VI secretion system. Mol. Cell. Proteomics *12*, 2735–2749.

Fu, Y., Waldor, M.K., and Mekalanos, J.J. (2013). Tn-Seq analysis of vibrio cholerae intestinal colonization reveals a role for T6SS-mediated antibacterial activity in the host. Cell Host Microbe *14*, 652–663.

Hayes, C.S., Koskiniemi, S., Ruhe, Z.C., Poole, S.J., and Low, D.A. (2014). Mechanisms and biological roles of contact-dependent growth inhibition systems. Cold Spring Harb Perspect Med *4*.

Hood, R.D., Singh, P., Hsu, F., Güvener, T., Carl, M.A., Trinidad, R.R., Silverman, J.M., Ohlson, B.B., Hicks, K.G., Plemel, R.L., et al. (2010). A type VI secretion system of Pseudomonas aeruginosa targets a toxin to bacteria. Cell Host Microbe *7*, 25–37.

Hsu, F., Schwarz, S., and Mougous, J.D. (2009). TagR promotes PpkA-catalysed type VI secretion activation in Pseudomonas aeruginosa. Mol. Microbiol. *72*, 1111–1125.

Human Microbiome Project Consortium (2012). Structure, function and diversity of the healthy human microbiome. Nature *486*, 207–214.

Jiang, F., Waterfield, N.R., Yang, J., Yang, G., and Jin, Q. (2014). A Pseudomonas aeruginosa type VI secretion phospholipase D effector targets both prokaryotic and eukaryotic cells. Cell Host Microbe *15*, 600–610.

Johnson, L.S., Eddy, S.R., and Portugaly, E. (2010). Hidden Markov model speed heuristic and iterative HMM search procedure. BMC Bioinformatics *11*, 431.

Kelley, L.A., and Sternberg, M.J. (2009). Protein structure prediction on the Web: a case study using the Phyre server. Nat. Protoc. *4*, 363–371.

Koskiniemi, S., Lamoureux, J.G., Nikolakakis, K.C., t'Kint de Roodenbeke, C., Kaplan, M.D., Low, D.A., and Hayes, C.S. (2013). Rhs proteins from diverse bacteria mediate intercellular competition. Proc. Natl. Acad. Sci. USA *110*, 7032–7037.

Lawley, T.D., and Walker, A.W. (2013). Intestinal colonization resistance. Immunology *138*, 1–11.

Lee, S.M., Donaldson, G.P., Mikulski, Z., Boyajian, S., Ley, K., and Mazmanian, S.K. (2013). Bacterial colonization factors control specificity and stability of the gut microbiota. Nature *501*, 426–429.

LeRoux, M., De Leon, J.A., Kuwada, N.J., Russell, A.B., Pinto-Santini, D., Hood, R.D., Agnello, D.M., Robertson, S.M., Wiggins, P.A., and Mougous, J.D. (2012). Quantitative single-cell characterization of bacterial interactions reveals type VI secretion is a double-edged sword. Proc. Natl. Acad. Sci. USA *109*, 19804–19809.

Liu, H., Sadygov, R.G., and Yates, J.R., 3rd. (2004). A model for random sampling and estimation of relative protein abundance in shotgun proteomics. Anal. Chem. *76*, 4193–4201.

Lozupone, C.A., Stombaugh, J.I., Gordon, J.I., Jansson, J.K., and Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. Nature *489*, 220–230.

Ma, L.S., Lin, J.S., and Lai, E.M. (2009). An IcmF family protein, ImpLM, is an integral inner membrane protein interacting with ImpKL, and its walker a motif is required for type VI secretion system-mediated Hcp secretion in Agrobacterium tumefaciens. J. Bacteriol. *191*, 4316–4329.

MacIntyre, D.L., Miyata, S.T., Kitaoka, M., and Pukatzki, S. (2010). The Vibrio cholerae type VI secretion system displays antimicrobial properties. Proc. Natl. Acad. Sci. USA *107*, 19520–19524.

Maurice, C.F., Haiser, H.J., and Turnbaugh, P.J. (2013). Xenobiotics shape the physiology and gene expression of the active human gut microbiome. Cell *152*, 39–50.

McBride, M.J., Xie, G., Martens, E.C., Lapidus, A., Henrissat, B., Rhodes, R.G., Goltsman, E., Wang, W., Xu, J., Hunnicutt, D.W., et al. (2009). Novel features of the polysaccharide-digesting gliding bacterium Flavobacterium johnsoniae as revealed by genome sequence analysis. Appl. Environ. Microbiol. *75*, 6864–6875.

Pujol, A., Crost, E.H., Simon, G., Barbe, V., Vallenet, D., Gomez, A., and Fons, M. (2011). Characterization and distribution of the gene cluster encoding RumC, an anti-Clostridium perfringens bacteriocin produced in the gut. FEMS Microbiol. Ecol. *78*, 405–415.

Pukatzki, S., Ma, A.T., Revel, A.T., Sturtevant, D., and Mekalanos, J.J. (2007). Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin. Proc. Natl. Acad. Sci. USA *104*, 15508–15513.

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., et al.; MetaHIT Consortium (2010). A human gut microbial gene catalogue established by metagenomic sequencing. Nature *464*, 59–65.

Rey, F.E., Faith, J.J., Bain, J., Muehlbauer, M.J., Stevens, R.D., Newgard, C.B., and Gordon, J.I. (2010). Dissecting the in vivo metabolic potential of two human gut acetogens. J. Biol. Chem. *285*, 22082–22090.

Rhodes, R.G., Pucker, H.G., and McBride, M.J. (2011). Development and use of a gene deletion strategy for Flavobacterium johnsoniae to identify the redundant gliding motility genes remF, remG, remH, and remI. J. Bacteriol. *193*, 2418–2428.

Riley, M.A., and Wertz, J.E. (2002). Bacteriocins: evolution, ecology, and application. Annu. Rev. Microbiol. *56*, 117–137.

Round, J.L., and Mazmanian, S.K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. Nat. Rev. Immunol. *9*, 313–323.

Russell, A.B., Hood, R.D., Bui, N.K., LeRoux, M., Vollmer, W., and Mougous, J.D. (2011). Type VI secretion delivers bacteriolytic effectors to target cells. Nature *475*, 343–347.

Russell, A.B., Singh, P., Brittnacher, M., Bui, N.K., Hood, R.D., Carl, M.A., Agnello, D.M., Schwarz, S., Goodlett, D.R., Vollmer, W., and Mougous, J.D. (2012). A widespread bacterial type VI secretion effector superfamily identified using a heuristic approach. Cell Host Microbe *11*, 538–549.

Russell, A.B., LeRoux, M., Hathazi, K., Agnello, D.M., Ishikawa, T., Wiggins, P.A., Wai, S.N., and Mougous, J.D. (2013). Diverse type VI secretion phospholipases are functionally plastic antibacterial effectors. Nature *496*, 508–512.

Russell, A.B., Peterson, S.B., and Mougous, J.D. (2014). Type VI secretion system effectors: poisons with a purpose. Nat. Rev. Microbiol. *12*, 137–148.

Schlieker, C., Zentgraf, H., Dersch, P., and Mogk, A. (2005). ClpV, a unique Hsp100/Clp member of pathogenic proteobacteria. Biol. Chem. *386*, 1115–1127.

Schwarz, S., Hood, R.D., and Mougous, J.D. (2010a). What is type VI secretion doing in all those bugs? Trends Microbiol. *18*, 531–537.

Schwarz, S., West, T.E., Boyer, F., Chiang, W.C., Carl, M.A., Hood, R.D., Rohmer, L., Tolker-Nielsen, T., Skerrett, S.J., and Mougous, J.D. (2010b). Burkholderia type VI secretion systems have distinct roles in eukaryotic and bacterial cell interactions. PLoS Pathog. *6*, e1001068.

Shneider, M.M., Buth, S.A., Ho, B.T., Basler, M., Mekalanos, J.J., and Leiman, P.G. (2013). PAAR-repeat proteins sharpen and diversify the type VI secretion system spike. Nature *500*, 350–353.

Silverman, J.M., Austin, L.S., Hsu, F., Hicks, K.G., Hood, R.D., and Mougous, J.D. (2011). Separate inputs modulate phosphorylation-dependent and -independent type VI secretion activation. Mol. Microbiol. *82*, 1277–1290.

Silverman, J.M., Agnello, D.M., Zheng, H., Andrews, B.T., Li, M., Catalano, C.E., Gonen, T., and Mougous, J.D. (2013). Haemolysin coregulated protein is an exported receptor and chaperone of type VI secretion substrates. Mol. Cell *51*, 584–593.

Smith, J.C., Rocha, E.R., and Paster, B.J. (2006). The Medically Important Bacteroides spp. in Health and Disease. Prokaryotes *7*, 381–427.

Stecher, B., and Hardt, W.D. (2011). Mechanisms controlling pathogen colonization of the gut. Curr. Opin. Microbiol. *14*, 82–91.

Thomas, F., Hehemann, J.H., Rebuffet, E., Czjzek, M., and Michel, G. (2011). Environmental and gut bacteroidetes: the food connection. Front Microbiol *2*, 93.

Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., Sogin, M.L., Jones, W.J., Roe, B.A., Affourtit, J.P., et al. (2009). A core gut microbiome in obese and lean twins. Nature *457*, 480–484.

Whitney, J.C., Beck, C.M., Goo, Y.A., Russell, A.B., Harding, B.N., De Leon, J.A., Cunningham, D.A., Tran, B.Q., Low, D.A., Goodlett, D.R., et al. (2014). Genetically distinct pathways guide effector export through the type VI secretion system. Mol. Microbiol. *92*, 529–542.

Zhang, D., de Souza, R.F., Anantharaman, V., Iyer, L.M., and Aravind, L. (2012). Polymorphic toxin systems: Comprehensive characterization of trafficking modes, processing, mechanisms of action, immunity and ecology using comparative genomics. Biol. Direct *7*, 18.

Zhang, H., Zhang, H., Gao, Z.Q., Wang, W.J., Liu, G.F., Xu, J.H., Su, X.D., and Dong, Y.H. (2013). Structure of the type VI effector-immunity complex (Tae4-Tai4) provides novel insights into the inhibition mechanism of the effector by its immunity protein. J. Biol. Chem. *288*, 5928–5939.