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Type VI Secretion Systems and the Gut Microbiota

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ABSTRACT The human colonic microbiota is a dense ecosystem comprised of numerous microbes, including bacteria, phage, fungi, archaea, and protozoa, that compete for nutrients and space. Studies are beginning to reveal the antagonistic mechanisms that gut bacteria use to compete with other members of this ecosystem. In the healthy human colon, the majority of the Gram-negative bacteria are of the order *Bacteroidales*. Proteobacteria, such as *Escherichia coli*, are numerically fewer but confer important properties to the host, such as colonization resistance. Several enteric pathogens use type VI secretion systems (T6SSs) to antagonize symbiotic gut *E. coli*, facilitating colonization and disease progression. T6SS loci are also widely distributed in human gut *Bacteroidales*, which includes three predominant genera: *Bacteroides*, *Parabacteroides*, and *Prevotella*. There are three distinct genetic architectures of T6SS loci among the gut *Bacteroidales*, termed GA1, GA2, and GA3. GA1 and GA2 T6SS loci are contained on integrative and conjugative elements and are the first T6SS loci shown to be readily transferred in the human gut between numerous species and families of *Bacteroidales*. In contrast, the GA3 T6SSs are present exclusively in *Bacteroides fragilis*. There are divergent regions in all three T6SS GAs that contain genes encoding effector and immunity proteins, many of which function by unknown mechanisms. To date, only the GA3 T6SSs have been shown to antagonize bacteria, and they target nearly all gut *Bacteroidales* species analyzed. This review delves more deeply into properties of the T6SSs of these human gut bacteria and the ecological outcomes of their synthesis *in vivo*.

Type VI secretion systems (T6SSs) were first identified and characterized for pathogenic bacteria of the proteobacterial phylum (1, 2). The discovery in 2010 that these secretion systems can target and intoxicate not only eukaryotic cells but also other bacteria (3) revealed that some T6SSs help bacteria compete with other bacteria in a community setting. Indeed, many proteobacterial symbionts, including the plant symbiont *Pseudomonas putida*, the bumble bee gut symbiont *Snodgrassella alvi*, and the squid symbiont *Vibrio fischeri*, all have T6SSs that provide a competitive advantage in their natural ecosystems (4–6). An early *in silico* analysis using clusters of orthologous groups (COGs) models of proteobacterial T6SS proteins against primary sequence databases suggested that T6SSs are largely confined to proteobacterial species, which are minor members of some human-associated microbial communities such as the gut microbiota (7, 8)

In the dense microbial ecosystem of the human colon, contact-dependent mechanisms of antagonism, such as a T6SS, should be an effective means of thwarting competitors. In this microbial ecosystem, the predominant Gram-negative bacteria are of the phylum *Bacteroidetes*, comprising approximately half of the colonic bacteria in many people and vastly outnumbering commensal proteobacteria. Most human gut *Bacteroidetes* species are contained within the order *Bacteroidales*, which includes several different families, each with one predominant genus in the human gut: *Bacteroides* (family *Bacteroidaceae*), the *Parabacteroides* (family *Tannerellaceae*), *Prevotella* (family *Prevotellaceae*), and *Alistipes* (family

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Rickenellaceae), as well as families with more minor representatives. Humans are colonized at high density with numerous *Bacteroidales* species simultaneously (9–11), and the abundant species in one individual may not be the same as in another (9, 10). T6SSs were not discovered in species of the phylum *Bacteroidetes* until recently (12–14), mostly due to the lack of primary sequence or profile sequence similarity of the 13 core proteobacterial T6SS proteins with *Bacteroidetes* proteins. The use of profile-profile analyses revealed that *Bacteroidales* species of the human gut are a rich source of diverse T6SSs (15). As structural and mechanistic properties of T6SSs are discussed elsewhere, here we focus on unique properties of the T6SSs of the human gut *Bacteroidales*, the ecological and functional properties of these T6SSs that are known to date, and the ecological advantages conferred by the T6SSs of human gut proteobacterial strains *in vivo*.

GENETIC CHARACTERISTICS AND PROPERTIES OF THE T6SS OF GUT *BACTEROIDALES*

An analysis of 205 human gut *Bacteroidales* genomes including 35 different species of four genera, *Bacteroides*, *Parabacteroides*, *Prevotella*, and *Alistipes*, revealed the presence of 130 T6SS loci in 115 of these strains, with 15 strains containing two different T6SS loci (15). These T6SS loci were found in the genomes of 19 different species of *Bacteroides*, *Parabacteroides*, and *Prevotella* but were not found in any of the nine *Alistipes* genomes analyzed. A notable feature is that these T6SSs segregated into three very distinct genetic architectures (GA), termed GA1, GA2, and GA3 (15) (Fig. 1A). Within a given GA, the majority of the T6SS genes are of high DNA identity, interspersed with small regions that are variable (Fig. 1A). The variable regions of all three T6SS GAs contain genes encoding effector and immunity proteins, some of which are similar to those previously described for other organisms (15, 16). Most of the GA3 T6SS effector and immunity proteins are unlike those previously described and function by mechanisms currently unknown. On the basis of comparison of the divergent regions within a GA, there are predicted to be at least 30 different variable regions in the 48 GA1 loci analyzed, 21 in the 9 GA2 regions analyzed, and 17 in the 56 GA3 regions analyzed, with each divergent region likely encoding at least one effector immunity pair. Therefore, the T6SSs of gut *Bacteroidales* encode numerous distinct toxins, many of which operate via unknown mechanisms.

An analysis of the predicted proteins produced by these loci showed that four of the conserved T6SS proteins of proteobacterial species are missing in gut *Bacteroidales* T6SSs: TssA, TssJ, TssL, and TssM. TssJ, -L, and -M are components of the transmembrane complex (17), and TssA binds this complex and likely recruits the baseplate assemblage and coordinates tail tube and sheath biogenesis (18, 19). Instead of encoding these proteins, all three GAs of *Bacteroidales* T6SS loci encode four conserved proteins not present in proteobacterial T6SS loci, namely, TssO to TssR (13, 15). These likely serve as functional orthologs of the proteobacterial proteins comprising the transmembrane complex.

All three T6SS GAs encode multiple TssD (Hcp) needle proteins, with GA2 and GA3 loci encoding six and five distinct TssD proteins, respectively. In the GA2 loci, one of the six TssD proteins has a C-terminal extension, likely conferring toxin activity on this protein (Fig. 1A). In the GA3 locus, the main structural TssD protein was identified among the five TssD proteins (Fig. 1A) (20). The function of the accessory TssD proteins may be to bind effector proteins and incorporate them into the needle structure, as in both the GA2 and GA3 loci, four of the *tssD* genes flank the effector and immunity gene variable regions (Fig. 1A).

An important feature of the three different GAs is their distribution among gut *Bacteroidales* species. GA1 and GA2 T6SS loci are present in numerous species of *Bacteroides*, *Parabacteroides*, and *Prevotella*, whereas GA3 T6SSs are present exclusively in *Bacteroides fragilis*. The wide distribution of GA1 and GA2 in at least three distinct families of *Bacteroidales* is due to their presence on integrative and conjugative elements (ICE) (12, 15) (Fig. 1B). ICE containing GA1 T6SSs are in the range of 120 kb and ICE containing distinct GA1 T6SSs are extremely similar to each other. Excluding the T6SS divergent regions, the DNAs of ICE containing distinct GA1 T6SS loci are approximately 95% similar over their entire lengths. Although very dissimilar to the GA1 ICE, GA2-containing ICE are on the order of 100 kb and share approximately 75% to 99% DNA identity over their lengths with other GA2-containing ICE. As ICE are chromosomal self-transmissible mobile elements (21, 22), they have the ability to move between species. In fact, the GA1-containing ICE have been found to be transferred extensively between *Bacteroidales* species that are coresident in the human intestine (12, 15) (Fig. 1C). Examination of hundreds of genomes of gut *Bacteroidales* has indicated that GA2 T6SSs are not present in a strain that has either a GA1 or GA3 T6SS locus. However, *B. fragilis* strains can have both GA1 and GA3 T6SS

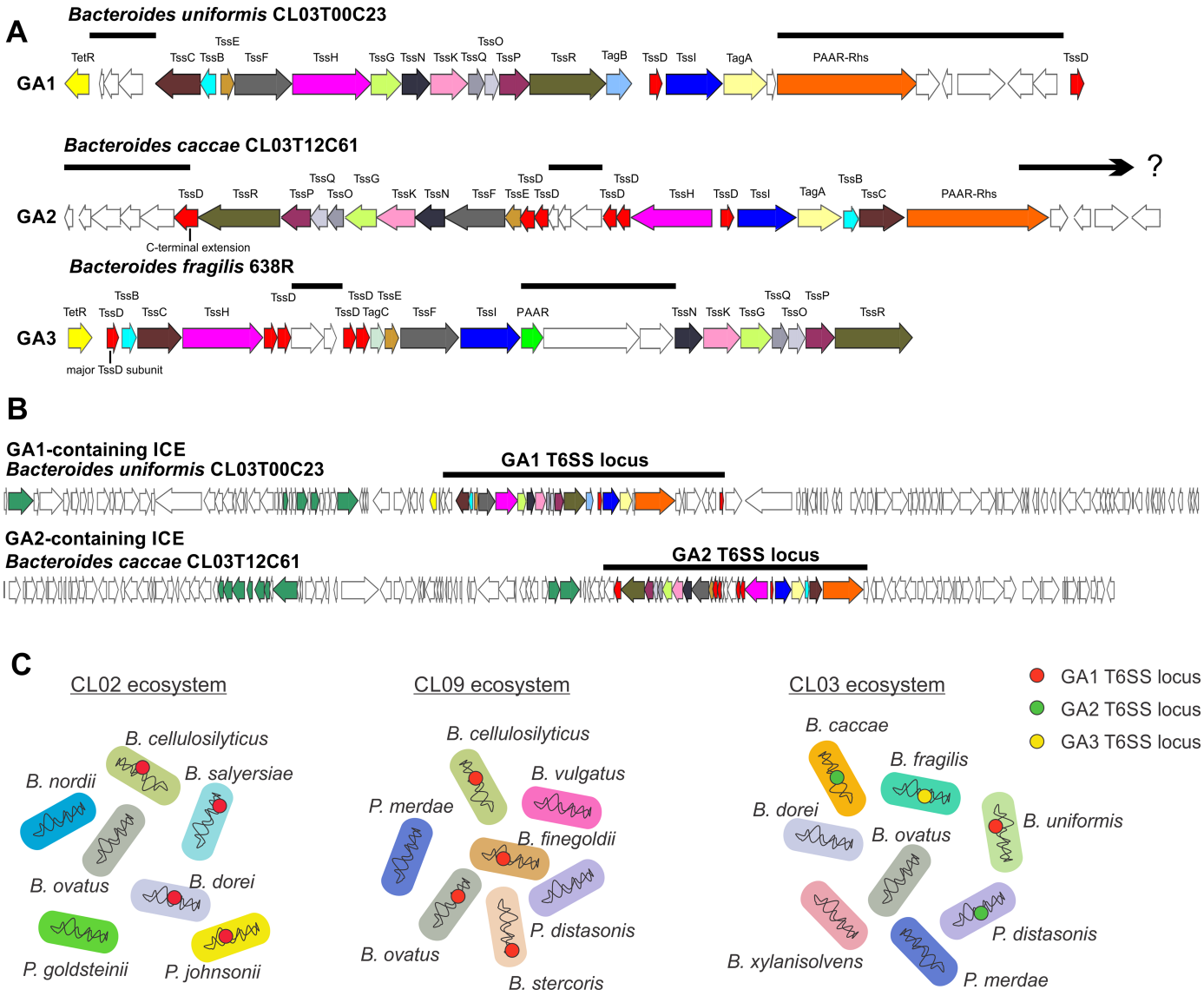

FIGURE 1 **(A)** Open reading frame (ORF) maps of one representative locus of each of the three genetic architectures (GA) of T6SS loci of gut *Bacteroidales*. T6SS loci of GA1 and GA2 are present in diverse *Bacteroidales* species, whereas GA3 T6SS loci are confined to *B. fragilis*. T6SS loci of a given GA are extremely similar to each other except for the divergent regions noted by lines above the genes, which encode known or putative effector and immunity proteins. The major TssD protein of GA3 is noted, as is the TssD protein of the GA2 loci that have C-terminal extensions likely conferring toxin activity. The ends of the GA1 and GA2 loci have not been precisely determined. **(B)** ORF maps of ICE containing GA1 and GA2 T6SS loci of two *Bacteroides* species. The T6SS loci are designated by a line above the map. Genes involved in conjugative transfer (*tra* genes) are colored green (15). **(C)** The abundant fecal gut *Bacteroidales* from three different healthy humans (CL02, CL09, and CL03) were analyzed for the presence of T6SSs. Seven *Bacteroidales* species were isolated and sequenced from subject CL02 and from subject CL09. Four of the seven species harbor nearly identical GA1 T6SSs loci within a subject, demonstrating transfer of these ICE between these strains in their gut (12, 15). In contrast, of the eight species isolated and sequenced from human subject CL03, two contain GA2 T6SS loci, albeit with different divergent regions. Therefore, these GA2 ICE were not transferred between these species. In addition, one species contains a GA1 T6SS locus and the *B. fragilis* strain from this individual contains a GA3 T6SS locus (15). Red, green, and yellow dots represent the GA1, GA2, and GA3 T6SS loci.

loci in their genome. The reason for the apparent lack of GA2 T6SS loci in the same chromosome with GA1 or GA3 T6SS loci is currently unknown. *Bacteroidales* species present in the gut of an individual can, however, collectively contain all three different T6SS GAs (15) (Fig. 1C).

ECOLOGICAL CONSEQUENCES OF GUT *BACTEROIDALES* T6SS ANTAGONISM

The fitness benefits of theT6SSs of the gut *Bacteroidales* are still incompletely understood. The best studied are the GA3 T6SSs of *B. fragilis*. GA3 T6SSs were found in approximately 86% of *B. fragilis* strains based on genome or metagenome analyses including hundreds of strains (15, 23), making them widely distributed in the species. The GA3 T6SSs of two different *B. fragilis* strains were found to antagonize all gut *Bacteroidales* species tested, including *Bacteroides* and *Parabacteroides* species, but not the one *Prevotella copri* strain analyzed or other *B. fragilis* strains with the same GA3 T6SS region or immunity genes (20, 24). In addition, no killing was evident against any gut proteobacterial species analyzed (20), suggesting specificity for *Bacteroidales*. The TssD needle protein of the *B. fragilis* 638R GA3 T6SS is present in the supernatant of actively growing cells *in vitro* as well as in the feces of monoassociated gnotobiotic mice (20), suggesting that this antagonistic system is constitutively synthesized and firing, rather than only responding to specific external signals or threats. The number of GA3 T6SS transmission events in a human gut colonized at typical levels with *B. fragilis* was predicted to be on the order of 6×10^{10} to 10^{11} per day (24).

Effector and immunity proteins were identified in three different GA3 T6SS loci (20, 24, 25). These toxic effector proteins are not similar to other known proteins and therefore intoxicate by as-yet-unidentified mechanisms. The toxicity of two of these effectors requires an added N-terminal periplasmic targeting sequence when they are produced inside a sensitive cell from an inducible promoter (26), suggesting that they may need to be localized to the periplasm for toxicity. In a few strains, genes encoding immunity proteins to GA3 effectors were found outside of the T6SS regions, in some cases in strains that did not have a GA3 T6SS locus (24). These immunity genes were found to confer protection from attack by a *B. fragilis* strain synthesizing the T6SS effectors to which the immunity proteins are directed, suggesting that acquisition of these immunity genes confers an advantage on an organism by protecting it from GA3 T6SS-mediated antagonism (24). A recent study analyzing human gut metagenomic data revealed arrays of orphan immunity genes, which were termed acquired interbacterial defense gene clusters (27). These orphan immunity islands reside on predicted mobile elements and include immunity genes likely derived from *B. fragilis* GA3 T6SS loci and disseminated by lateral transfer.

Although the GA3 T6SSs are very effective at antagonizing *Bacteroidales* species *in vitro*, the effects *in vivo* are more variable. As *B. fragilis* is known to coexist in the human gut with numerous other *Bacteroidales* species that are susceptible to its GA3 T6SS, the spatial organization of the microbiota likely dictates the effectiveness of this weapon. In a gnotobiotic mouse competitive colonization model, an isogenic T6SS$^+$ wild-type strain outcompetes an isogenic mutant strain lacking the T6SS effector and immunity genes (20, 24). As isogenic strains should share the same spatial and nutritional niche and therefore should make frequent contacts, a strong antagonistic effect is expected. The ability of GA3 T6SSs of *B. fragilis* to antagonize other wild-type nonisogenic *B. fragilis* strains *in vivo* was also demonstrated (24, 25). However, the effectiveness of the GA3 T6SS was found to be lower when analyzing antagonism of different *Bacteroides* species (Fig. 2B). A significant effect was observed with a *Bacteroides vulgatus* strain (24) but not with *Bacteroides thetaiotaomicron* (20, 24). It may be that in this model, in which the mice are provided ample nutrients and likely utilize different nutrients in the gut (28, 29), *B. fragilis* and *B. thetaiotaomicron* would make infrequent contacts (Fig. 2B).

Analysis of metagenomic data sets of human gut samples revealed a link between the presence of *Bacteroidales* T6SSs and the composition of the microbiota, especially with regard to the GA3 T6SSs of *B. fragilis* (23). The presence of GA3 T6SS genes correlated significantly with an abundance of *Bacteroides* and a decrease in specific *Firmicutes*. Moreover, the microbiota of the developing infant gut is significantly more likely to contain GA3 T6SSs than that of adults. Also, *B. fragilis* strain replacement in the infant microbiota is more pronounced than in the adult microbiota, which appears to be dominated by a single strain of *B. fragilis*. These findings suggest that competition among *B. fragilis* strains for dominance is fiercest early in life and that the ultimate microbiota composition may be influenced by GA3 T6SSs (23).

The functions of the GA1 and GA2 T6SSs have not yet been elucidated. There has been no demonstration that these T6SSs target bacteria as do the GA3 T6SSs. Many of the identifiable effectors are predicted nucleases

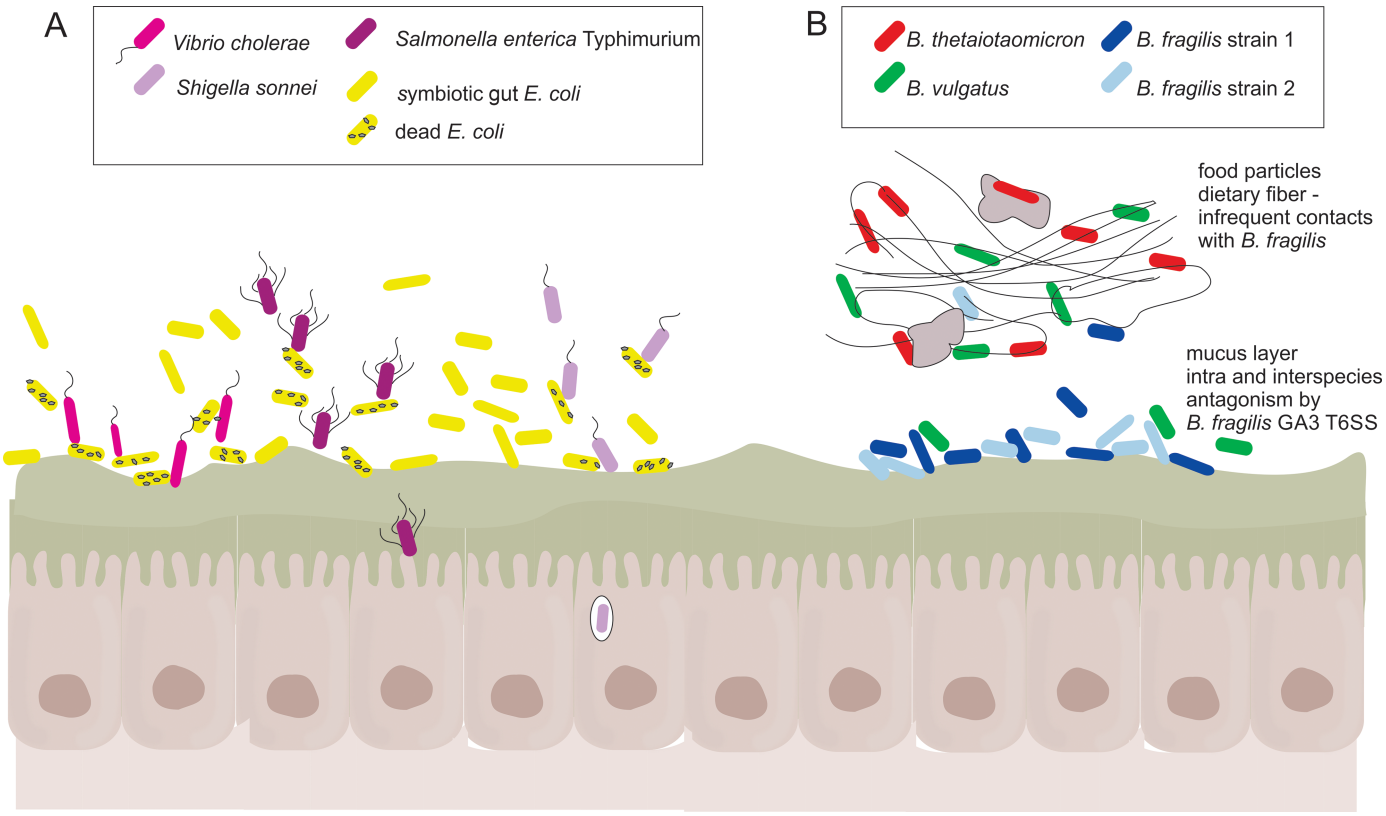

FIGURE 2 T6SS-mediated antagonism in the mammalian gut. **(A)** Three different proteobacterial enteric pathogens, *Vibrio cholerae*, *Salmonella enterica* Typhimurium, and *Shigella sonnei*, use T6SSs to target resident gut *E. coli* to overcome colonization resistance and cause disease in animal models (32–36). In the case of *V. cholerae*, the lysed *E. coli* organisms initiate innate immune responses that upregulate virulence factors and increase dissemination (32). **(B)** *Bacteroides fragilis* GA3 T6SS antagonize nearly all gut *Bacteroidales* species *in vitro*. *In vivo*, strong antagonistic effects are seen between two distinct *B. fragilis* strains likely due to their localization at the mucosal surface, where they will make frequent contacts. This intraspecies antagonism may lead to the dominance of one strain. *B. vulgatus* was also significantly antagonized by a *B. fragilis* GA3 T6SS, possibly due to overlapping nutritional niches. In contrast, a significant antagonistic effect by the GA3 T6SS of *B. fragilis* was not observed when this organism was coinoculated with *B. thetaiotaomicron*. These varied effects may be due to the substrate preferences of these species, which may spatially segregate them under normal dietary conditions.

or other nucleic acid-targeting enzymes (15) that could function in bacteria, archaea, or eukaryotes. The fact that the ICE containing these T6SSs are shared between different coresident species of *Bacteroidales* in the human gut suggests that they likely do not have a *Bacteroidales* target and may instead provide defense against a common enemy. It is also possible that these T6SSs may allow for nutrient acquisition, or protection from environmental stressors, as has been shown for T6SSs in other organisms (30, 31). The prevalence and transfer of these systems among human gut *Bacteroidales* species make them intriguing secretion systems for continued analysis.

EFFECTS OF PROTEOBACTERIAL T6SSs IN THE MAMMALIAN GUT

Although less abundant in the healthy human gut microbiota than *Bacteroidales*, *E. coli* gut symbionts play a crucial role in colonization resistance against enteric pathogens of the proteobacterial phylum. Enteric pathogens such as *Vibrio cholerae*, *Shigella sonnei*, and *Salmonella enterica* have all been shown to utilize T6SSs to overcome colonization resistance by antagonizing resident gut *E. coli* (32–36) (Fig. 2A). What is less studied is whether symbiotic gut *E. coli* have T6SSs that function in colonization resistance against enteric pathogens.

Antagonism by the diffusible microcin toxin produced by *E. coli* Nissle was shown to function as a colonization barrier to enteric pathogens (37), providing precedent for such an effect. Queries of a set of 1,267 human gut metagenomes consolidated into the "three cohorts gene catalog (3CGC)" (38) for matches to Pfam models identifying the four conserved proteobacterial T6SS proteins absent in *Bacteroidales* T6SSs (TssA, -J, -L, and -M) revealed that 174 of these metagenomes encoded proteins with motifs that met or exceeded the gathering threshold of all four of the proteobacterial models used (M. J. Coyne and L. E. Comstock, unpublished data). These findings suggest that resident gut *E. coli* strains likely harbor T6SSs, the *in vivo* effects of which remain to be determined.

CONCLUDING REMARKS

The discovery of T6SS loci and their prevalence in diverse human gut *Bacteroidales* species has revealed that the composition of the human gut microbiota is likely significantly influenced by these secretion systems. We still know very little about these secretion systems, including the targets of the GA1 and GA2 T6SSs, the advantage of transfer of these regions to coresident *Bacteroidales* species, and the mechanisms of action of many of the toxic effectors. In addition, it will be interesting to determine the prevalence of T6SSs in human gut *E. coli* strains and whether these antagonistic systems contribute to their ability to affect colonization by enteric pathogens.

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