Antibacterial Weapons: Targeted Destruction in the Microbiota

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Abstract

The intestinal microbiota plays an important role in health, particularly in promoting intestinal metabolic capacity and in maturing the immune system. The intestinal microbiota also mediates colonization resistance against pathogenic bacteria, hence protecting the host from infections. On the other hand, some bacterial pathogens deliver toxins that target phylogenetically related or distinct bacterial species in order to outcompete and establish within the microbiota. The most widely distributed weapons include bacteriocins, as well as contact-dependent growth inhibition and type VI secretion systems. In this review, we discuss important advances about the impact of such antibacterial systems on shaping the intestinal microbiota.

The intestinal microbiota: our best frenemy

The mammalian intestine is inhabited by a large and diverse community of microbes, referred to as the gut microbiota. The human gut microbiota, also referred to as a "microbial organ", weights 1-2 kg, and consists of approximately 100 trillion (10^{14}) bacteria representing 6-10 phyla, including two predominant phyla – Bacteroidetes and Firmicutes –, and about 500-1000 distinct species [1]. This highly complex **microbial community** (see Glossary) is controlled by various factors, such as host genetics and environmental factors. Moreover, microbiota diversity and composition is influenced by host diet as well as by positive and antagonistic interactions between bacteria within the microbiota.

The intestinal microbiota has an overall beneficial impact on its host, by providing metabolic activities within the intestine and favoring the development of the **intestinal immune system** [2] (**Figure 1**). Exemplifying this notion is the observation that the immune responses in mice housed in germ-free conditions are abnormal compared to conventionally-colonized mice [3, 4]. Therefore, early exposure to microbes in the intestine is a critical factor to modulate intestinal immune responses [5], and a well-documented example of a single microbial member playing a central role in shaping the intestinal immune system is segmented filamentous bacteria (SFB), which can promote the robust differentiation of Th17 cells [6-8]. Moreover, if not well managed, the gut microbiota can become deleterious, for example by inducing uncontrolled intestinal inflammation. In light of the benefits the microbiota confers and on its potential to harm its host, the gut microbiota has previously been referred as the host's best frenemy [9].

Collectively, the microbiota and its derived metabolites are critical components for the maturation of host intestinal immunity, and research has accumulated on the central role played by the intestinal microbiota in the protection of the host intestine against pathogens, a phenomenon called colonization resistance [10-12]. Bacterial competition occurs either by depleting nutrients from the milieu (exploitation competition) or by deploying antibacterial

66 weapons to specifically eliminate target cells (interference competition) (**Figure 1**). Many
67 bacteria can directly prevent intestinal pathogens colonization or overgrowth by consuming
68 common limited resources, hence inducing starvation of competing pathogens. One example
69 highlighting this mechanism of exploitation competition is the finding that the commensal
70 *Bacteroides thetaiotaomicron* consumes carbohydrates used by the pathogen *Citrobacter*
71 *rodentium*, thus leading to a competitive exclusion of the pathogen from the intestine [13, 14]
72 (**Figure 1**). Through the production of specific metabolites, the intestinal microbiota can also
73 modify the host environmental conditions, then compromising pathogen growth and/or
74 virulence. Butyrate, a short-chain fatty acid (SCFA) produced by the intestinal microbiota,
75 downregulates the expression of several virulence genes of *Salmonella enterica* serovar
76 Enteritidis (*S*. Enteritidis) and Typhimurium (*S*. Typhimurium) [15] and inhibits the growth of
77 enterohemorrhagic *Escherichia coli* (EHEC) [16] (**Figure 1**). Finally, members of the
78 microbiota can affect the growth of other cells by producing and releasing inhibitory
79 substances, such as antibiotics and peptide antibiotics, or by injecting antibacterial effectors
80 into target cells. Here we will briefly describe the various mechanisms evolved by bacteria to
81 destroy rivals and we will review recent studies on how these systems contribute to reshaping
82 bacterial communities *in vivo*.
83
84 **Bacterial weapons in the intestine**
85 To combat competitors, bacteria deploy a broad arsenal of antibacterial weapons.
86 These weapons vary in terms of mechanism of action, toxin targets, and mode of penetration,
87 and hence limit the acquisition of mechanism of resistance by competitors. The most widely
88 distributed weapons in Enterobacteria are microcins and bacteriocins [17], contact-dependent
89 growth inhibition (CDI) [18] and Type VI secretion systems (T6SS) [19] (**Figure 2**). Other
90 mechanisms, such as Type I, Type IV and Type VII secretion systems or outer membrane

exchange, have also been recently shown to mediate toxin delivery into competitors or to promote contact-dependent killing [20-25].

Bacteriocins are a group containing a large variety of proteinaceous antibiotics of various lengths: microcins are 15-60 amino-acid peptides, lantibiotics are peptide containing a modified amino-acid (lanthionine), whereas colicin-like proteins are > 40 kDa multidomain proteins and tailocins are high molecular weight multiprotein complexes resembling bacteriophage tails [26-29]. Bacteriocins are produced upon stress conditions, and share a similar mechanism of action. After release, they bind to an outer membrane receptor and parasitize cell envelope components for penetration [26, 27, 30] (**Figure 2**). The exploitation of specific reception and translocation machineries restrict the action of these toxins to the same or phylogenetically related species. Cell toxicity is conferred by pore-forming activity that collapses the membrane potential at the inner membrane, by digestion or cleavage of nucleic acids or of cell wall precursors [26, 30]. Bacteriocins include S-type pyocins liberated by *Pseudomonas* species, and colicins that are produced by enteric strains such as *E. coli*, *C. rodentium* and *Enterobacter* species [26, 30].

By contrast to bacteriocins that are diffusible toxins, CDI and T6SSs are cell-cell contact-dependent mechanisms. CDI is a variant of two-partner secretion, a family of Type V secretion systems widely represented in Proteobacteria [31-33]. It comprises two proteins: the CdiB outer membrane transporter translocates the CdiA protein to the cell surface. CdiA are usually elongated spring-like β-helical structures that carry a C-terminal toxin domain. The C-terminal domain mediates target cell recognition, penetration and toxicity (**Figure 2**). Similar to bacteriocins, the requirements for specific receptors and target cell factors restrict the action of CDI to close relatives [18, 31, 34, 35].

T6SS is a more complex machinery, broadly distributed in Proteobacteria and Bacteroidetes. Its assembly requires at least 13 components to form a speargun-like weapon [19, 36, 37]. T6SS could be considered as a bacteriophage-like contractile tail structure

anchored to the cell envelope [19, 38, 39]. The tail is constituted of an effector-loaded syringe wrapped by the sheath that is built under a metastable, extended conformation [19, 36, 37, 40-42]. Contraction of the sheath presumably propels the syringe towards the rival cell, and thus delivers effectors to cause cell damages [40, 42-46] (**Figure 2**). How the target cell is sensed and how cell-cell contact triggers T6SS assembly or firing is largely unknown but in certain cases, transcriptional, post-transcriptional or post-translational regulatory cascades, cell-envelope damage or the presence of kin cell components in the milieu trigger a T6SS response to attacks [44, 46-52]. By contrast to bacteriocins and CDI, T6SSs do not exploit specific receptors, and hence target a broader range of bacteria, although no T6SS-dependent damages of Gram-positive bacteria have been observed [53]. T6SS antibacterial effectors include peptidoglycan-acting enzymes (amidases, glycosyl hydrolases), membrane-targeting proteins (phospholipases and pore-forming), nucleases and NAD(P)(+) glycohydrolases [53-59]. Interestingly, the T6SS is also capable of delivering effectors into eukaryotic cells, including single-celled microorganisms and animal tissues [54, 60]. Phospholipases and nucleases can act as trans-kingdom effectors, but specialized toxins, such as those interfering with cytoskeleton or tubule dynamics, have been identified and characterized [60-67].

For all these systems, attacker bacteria are protected from self-intoxication and intoxication by kin cells by the production of immunity proteins that usually bind with high affinity and inhibit the catalytic activity of the cognate effector [26, 31, 34, 54, 55, 68, 69].

In addition, some enteric pathogens can produce broadly bioactive small molecules such as antibiotics, non-ribosomal peptide antibiotics, sactibiotics and lantibiotics [70-72], that may act toward other members of the intestinal microbiota and/or toward enteric pathogens. Finally, other extracellular contractile injection systems such as R-pyocins, anti-feeding prophages (Afp) and Afp-like particles have antibacterial or antimicrobial activities [73-75].

Although the role of these systems in antibacterial competition is well documented *in vitro*, studies have only begun to investigate their contribution in *in vivo* animal models.

Role of bacterial competition in the intestinal microbiota

The gut microbiota is a very stable ecosystem [1]. However, the invasion or overgrowth of pathogens induces **dysbiosis**, an instability that may alter the composition of the microbiota but also host physiology [11, 76-78]. It is now well appreciated that interference competition between members of the microbiota, in addition to indirect competition such as exploitation competition, plays a central role in microbiota ecology [78].

Until recently, the role of interbacterial competition in shaping bacterial communities has been underestimated [79]. However, antibacterial weapons are key players in the control of bacterial populations, and a summary of known examples of *in vivo* bacterial competition is presented in **Table 1**. The importance of these weapons *in vivo* is supported by the estimation that more than 10^9 T6SS firing events occur per minute per gram of colonic contents [80]. T6SS gene clusters are highly represented in Bacteroidales strains, which account for a large portion of the gut microbiota [81, 82]. In addition, many Gram-negative enteric pathogens, including *Vibrio cholerae*, *S.* Typhimurium, *C. rodentium,* and *Shigella sonnei* utilize functional T6SSs to fire against other species *in vitro* [83, 84]. In agreement with the concept that T6SSs are important players in bacterial competition within the intestine, a number of enterobacterial T6SS gene clusters are upregulated in conditions encountered in the gut or when a threshold of cell density is reached [85]. The *V. cholerae* T6SS is activated by mucins and microbiota-modified bile salt [86]; the *S.* Typhimurium T6SS is activated by bile salts [87]; and the enteroaggregative *E. coli* Sci-1 T6SS is responsive to iron starvation [88].

V. cholerae, *S.* Typhimurium and *S. sonnei* deploy their T6SSs to kill or displace commensal bacteria, allowing a successful colonization of the host and an increased

persistence in experimental models [89-92]. Using transposon insertion site sequencing (Tn-Seq) to identify *V. cholerae* mutants that exhibit a colonization defect in the rabbit intestine, Fu *et al.* found *tsiV3*, encoding immunity to the T6SS VgrG3 peptidoglycan hydrolase effector [93]. Interestingly, a recent study demonstrated that *V. cholerae* T6SS-mediated colonization specifically occurs in intestinal microenvironments, such as the middle small intestine, suggesting that T6SS effectors might target specific species [94]. The antagonistic behavior of *V. cholerae* in the gut triggers intestinal colonization, virulence gene expression, and host innate immune response [95]. In a recent study, Sana and collaborators demonstrated that the successful establishment of *S.* Typhimurium in the mouse intestine requires the T6SS Tae4 amidase effector [87]. Thus, the observation that bacterial-specific effectors are required for efficient colonization demonstrates that the T6SS mediates antagonistic interbacterial interactions during infection. It is not yet known whether T6SS specifically targets certain species, but the observation that *S.* Typhimurium targets *Klebsiella oxytoca* and has only weak impact on other species suggests that the T6SS does not fire randomly [87]. Interestingly, *S.* Typhimurium and *K. oxytoca* utilize the same carbon sources and thus the specific elimination of a metabolic competitor may provide a better access to the available nutritional resources [79]. Another example of T6SS-mediated metabolic competition is the secretion of manganese- and zinc-scavenging enzymes by pathogens such as *Burkholderia thailandensis* [96, 97]. In addition, EHEC uses its T6SS to secrete catalases, thus providing a higher resistance to reactive oxygen species produced by the host [98]. A recent example of the importance of the T6SS in intestinal colonization is related to the increased prevalence of *S. sonnei* infections over that of the close relative *Shigella flexneri*. By contrast to *S. flexneri*, the genome of *S. sonnei* encodes a functional T6SS that confers a competitive advantage by outcompeting *S. flexneri in vitro* as well as in the mouse gut [90]. Interestingly, *S. sonnei* also encodes the ColE1 colicin that enables *E. coli* elimination [90]. Those recent findings are in agreement with the observation that colicinogenic *E. coli* cells present an increased intestinal

194 persistence compared to the isogenic *E. coli* strain unable to produce colicins [99, 100]. Other
195 bacteriocins and R-pyocins have the ability to destroy rivals in biofilm or mixed communities,
196 and to specifically eliminate bacterial species after therapeutic administration; and hence are
197 proposed to be viable alternatives to antibiotics [101-105].

198 While the above examples showed that antibacterial weapons are used by pathogens to
199 colonize their hosts, the gut microbiota also exerts an important control to prevent
200 colonization by pathogens [106, 107]. Indeed, many commensal strains produce those
201 weapons and therefore protect the niche against the invasion of external microbes or against
202 the overgrowth of indigenous pathogens. An elegant example is the recent observation that
203 the probiotic strain *E. coli* Nissle uses microcins M and H47 to limit the expansion of
204 competing Enterobacteriaceae, including pathogens such as adherent-invasive *E. coli* (AIEC)
205 and *S.* Typhimurium during intestinal inflammation [108]. Another example is *Bacillus*
206 *thuringiensis*, a bacterium able to secrete a bacteriocin (thuricin CD) that directly targets
207 spore-forming Bacilli and Clostridia, including *Clostridium difficile* [109].

208 Other commensals, such as those of the Bacteroidales order, antagonize gut microbiota
209 using secreted antimicrobial proteins or T6SS [56, 110-113]. Interestingly, it has been shown
210 that intense transfer of genetic material occurs between Bacteroidales species in the gut [82].
211 As a consequence, Bacteroidales have accumulated genes encoding immunity proteins to
212 T6SS effectors they do not encode [80], and thus maintain a stable balance in the microbiota
213 by preventing their own elimination. In addition, the symbiotic *Bacteroides fragilis* strain was
214 shown to use the T6SS to harm enterotoxigenic *B. fragilis* cells, demonstrating that the
215 activity of Bacteroidales T6SSs may protect the host against intestinal inflammatory diseases
216 [114]. Altogether, these data demonstrate that a broad range of competitive mechanisms
217 occurs within the intestinal microbiota and plays a role in microbiota composition,
218 establishment, stability, and evolution.

219

Concluding Remarks

Future studies will be needed to further characterize mechanisms by which bacteria compete within the intestinal tract, with a particular focus on long-lasting consequences on microbiota composition and host physiology (see Outstanding Questions). Long-term experiments are also required to understand the real contribution of antibacterial weapons in shaping microbial communities. At present, it is not clear whether being well armed represents a true advantage, as antibacterial weapons have limited impact on well-structured communities [115]. In addition, microbial communities are subjected to a rock-paper-scissor game, in which the production of these weapons is energetically consuming, and hence attacker cells might be defeated by professional cheaters or by strains with a better fitness [116, 117].

Another important field of research that needs further investigation is the impact that such antimicrobial systems may have on both microbiota composition and host physiology in the long term. An example highlighting the importance of interbacterial competition in long term dysbiosis is the observation that intestinal colonization of AIEC, an *E. coli* pathovar associated with Crohn's disease, causes alteration of microbiota composition and chronic colitis in mice, with both phenotypes persisting well beyond AIEC clearance [118]. Hence, in addition to their own virulence potential, AIEC bacteria are able to induce chronic inflammation by detrimentally altering the intestinal microbiota composition [118]. While the precise role played by antibacterial systems in such long-term alterations still needs to be investigated, they may further highlight the unappreciated importance of such bacteria/bacterial competition in microbiota stability and host physiology.

Finally, such antibacterial mechanisms may be tailored in a near future as an alternative or complementary approach to the use of antibiotics. A few bacteriocins, such as nisin, have been validated by the FDA and are used as food preservatives [119]. While such compounds are used to extend shelf life, we still ignore their impact on the intestinal

microbiota. In addition, colicins, R-pyocins, CdiA and T6SS effectors are modular proteins and hence might be genetically engineered to specifically target bacterial populations of interest. Modified colicins, R-pyocins or T6SS effectors have already been demonstrated to be efficiently delivered into target cells or to destroy specific species without affecting the gut microbiota diversity [87, 95, 120-124]. One may predict that these initial attempts will be actively pursued to deliver toxins or CRISPR/Cas system into specific species, with the ultimate goal to prevent intestinal colonization or to beneficially reshape an altered microbial ecosystem.

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533

Glossary

Dysbiosis: microbial population with an imbalanced composition, often associated with deleterious impact for the host. Dysbiosis has been identified as an important player of inflammation in inflammatory bowel diseases.

Intestinal immune system: The complex population of cells and interactions inhabiting our intestine with the dual role of tolerance toward our commensal microbiota and protection against intestinal pathogen. Importantly, the intestine represents the largest compartment of the immune system, with the estimation that 70% of the mammalian immune system is hosted within the intestine.

Microbial community: mixed population of bacteria and single-cell microorganisms. Microbial communities include microbiota, but also biofilms (bacteria that adhere to a support) and consortiums (bacteria that exchange metabolic contents).

Legend to Figures

Figure 1. Commensal microbiota-mediated colonization resistance in the intestine. The intestinal microbiota plays a central role in both intestinal barrier maintenance and immune system maturation. Examples of microbiota-mediated inhibition of intestinal colonization by enteric pathogens are represented: exploitation competition (utilization of nutrient resources), and interference competition (production of virulence gene repressor molecules or antibacterial weapons). EHEC, enterohaemorrhagic *Escherichia coli*.

Figure 2. The arsenal of antibacterial weapons. The major antibacterial weapons used by enteric bacteria and their mechanism of action are schematically represented. Bacteriocins (green) are diffusible multi-domain proteins that are produced and released by the attacker cell. The reception (R) domain binds to the specific receptor and the translocation (T) domain helps the translocation of the activity (A) domain into the target. Contact-dependent growth inhibition (CDI, orange) comprises the CdiB translocator and CdiA toxin. The CdiA C-terminal domain (Ct) binds to a specific receptor and translocates to the target cell. Type VI secretion system (T6SS, blue) is an injection system that uses a contractile mechanism to propel an effector-loaded needle into the target.

565 **Table 1. Summary of known *in vivo* bacterial competition.**

Bacterium	Weapon	Target cell	Toxin / Activity	Reference
Salmonella enterica Typhimurium	T6SS	*Klebsiella oxytoca*	Tae4 (amidase)	[87]
Bacillus thuringiensis	Bacteriocin thuricin CD	Spore-forming Bacilli and Clostridia, including *C. difficile*	pore-forming	[109]
Burkholderia thailandensis	T6SS	unknown	unknown	[96, 97]
EHEC	T6SS	-	Catalases	[98]
Shigella sonnei	T6SS	*S. flexneri*	Unknown	[90]
Shigella sonnei	ColE1 colicin	*E. coli*	Pore-forming	[90]
E. coli Nissle	Microcins M and H47	Enterobacteriaceae, including pathogens such as AIEC and *S.* Typhimuirum	unknown	[108]
Bacteroides fragilis	T6SS	*B. fragilis*, gut microbiota and pathogenic bacteria	Bte2	[56, 110, 114]
V. cholerae	T6SS	commensal *E. coli*	unknown	[95]

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Outstanding Questions Box

- How do antibiotics and antibacterial molecules alter the intestinal microbiota composition and affect colonization resistance?
- What are the direct impacts of antibacterial weapons in the gut?
- What is the contribution of the antibacterial weapons of commensals in the protection of the host against pathogens?
- What is the target range of T6SS and what is the cost to produce antibacterial weapons?
- Is it a real benefit for the bacterium to be equipped with antibacterial weapons?
- How can antibacterial weapons be genetically modified for therapeutic purposes in order to manipulate the intestinal microbiota in beneficial ways?
- What are the long term impacts of such antibacterial systems on the microbiota community and host physiology?

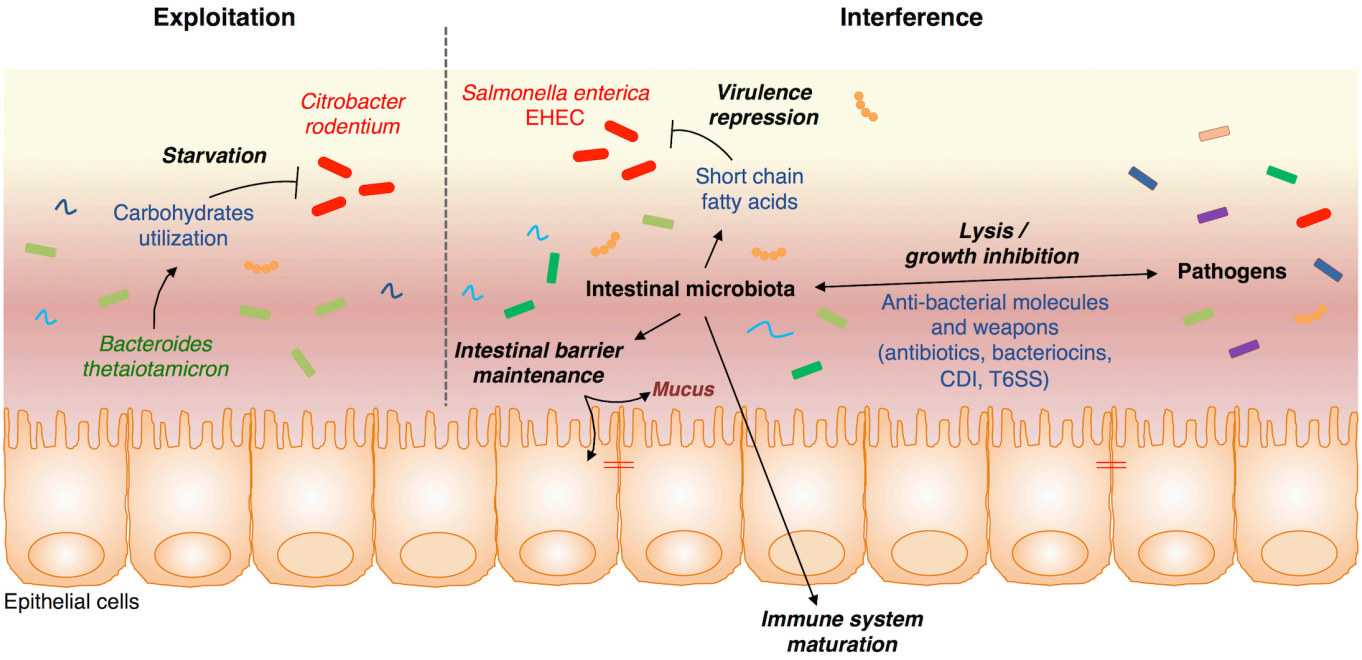
Trends Box

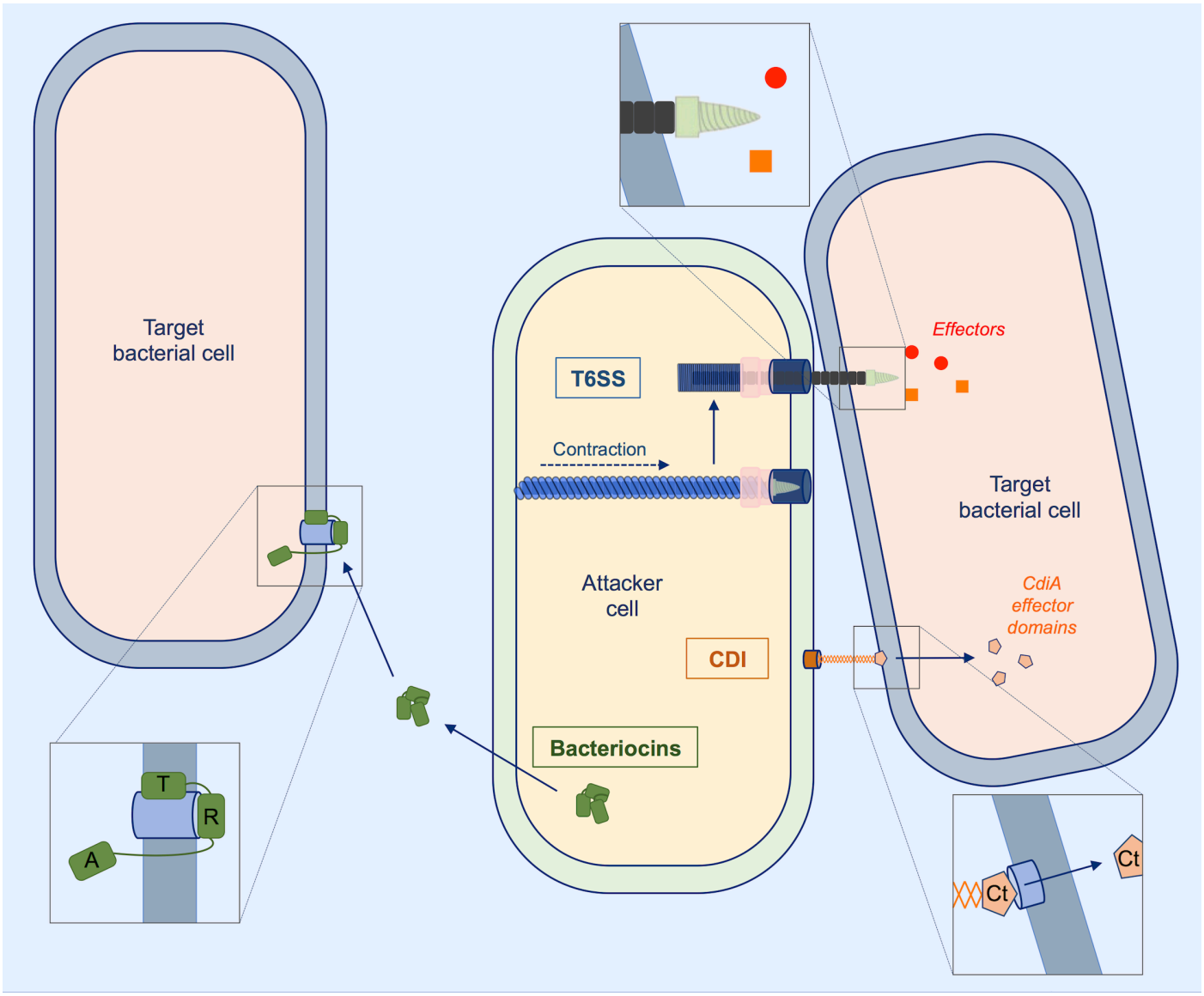
- The intestinal microbiota is a complex but stable ecosystem that plays a central role in human health, and disturbance of its composition and function is associated with many diseases.

- Within the intestinal microbiota, bacteria exchange material and information.

- The microbiota can be peaceful, but many bacteria fight with others to have a better access to their niche or nutrients.

- Different antibacterial weapons have been identified and characterized, and many bacterial pathogens use these weapons to establish themselves in the intestinal environment, whereas some commensals use these weapons to specifically target pathogens, leading to protection of the host.


Exploitation
Interference
Citrobacter rodentium
Starvation
Carbohydrates utilization
Bacteroides thetaiotamicron
Salmonella enterica EHEC
Virulence repression
Short chain fatty acids
Intestinal microbiota
Lysis / growth inhibition
Pathogens
Anti-bacterial molecules and weapons (antibiotics, bacteriocins, CDI, T6SS)
Intestinal barrier maintenance
Mucus
Epithelial cells
Immune system maturation


Target
bacterial cell
T6SS
Contraction
Attacker
cell
CDI
Bacteriocins
Effectors
Target
bacterial cell
CdiA
effector
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