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Bacterial Defense against the Type VI Secretion System

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Bacteria have evolved systems dedicated to interbacterial competition. Here we highlight defenses utilized by Gram-negative cells against type VI secretion system (T6SS)-wielding competitors, including physical barriers, genetically encoded antidotes, and stress responses. Further investigation of specific and general defenses will reveal the interbacterial selective pressures impacting bacterial survival in nature.

Introduction

Type VI secretion systems (T6SS) are used by diverse Gram-negative bacteria to deliver effector proteins to target cells. While targeting of eukaryotic cells by the T6SS of some pathogens is linked to virulence, many bacteria use the T6SS to deliver bacteriostatic or bacteriolytic effector proteins to other bacterial cells [1,2]. T6SS effectors exert their inhibitory activity on other bacteria via the targeting of inherent vulnerabilities of the recipient cell, including membranes, the peptidoglycan layer, and nucleic acids [2]. Kin cells are protected from T6SS intoxication by the production of cognate immunity proteins that typically neutralize toxic cognate effector activity through highly specific protein–protein interactions. The mechanisms by which non-kin cells defend against T6SS intoxication are just beginning to be revealed.

Shoring up the Wall: Physical Protection from the T6SS

Intoxication by the T6SS requires prolonged cell–cell contact, as occurs when bacteria grow on solid surfaces or in highly viscous liquids. Recent evidence suggests that physical structures that prevent intimate association between competing non-kin bacteria can neutralize T6SS-dependent killing. In *Vibrio cholerae*, exopolysaccharide (EPS) production facilitates adhesion to surfaces and provides a physical structure that encloses bacteria into a matrix and facilitates biofilm formation [3]. Genetic ablation of EPS production in cells targeted by T6SS-wielding competitors confers increased susceptibility to exogenous T6SS attacks from varied antagonists, indicating that the physical elaboration of EPS structures provides defense against the T6SS (Figure 1A). Notably, the EPS-dependent protective effect does not inhibit endogenous T6SS functionality, suggesting that EPS structure modulates passage and allows T6SS egress, but not foreign T6SS ingress. Biofilm matrix production is also linked to pleiotropic effects, including resistance to antibiotics, colonization resistance, and defense against phage [4,5]. In this manner, matrix production may serve as a flexible defense mechanism that may be particularly useful when bacteria inhabit different environments or interface with diverse competitors. Other physical structures can also serve in defense against the T6SS, including dead bacterial cells [6]. Extension of this concept raises the possibility of additional physical defenses that may protect cells against the T6SS, like liquid crystalline sheaths made by filamentous phages or paracrystalline surface layers [7,8].

The bacterial cell wall itself can also provide defense against T6SS attack. The peptidoglycan (PG) layer is a common target of T6SS-delivered effectors that act through the hydrolysis of PG peptide crosslinks [2]. Unique T6SS PG hydrolase effector families have distinct specificity for different bonds in PG crosslinks. Recent evidence demonstrates that this specificity can be leveraged to avoid killing by the T6SS. *Acinetobacter baumanii* can escape intoxication by T6SS PG hydrolases through the incorporation of noncanonical D-amino acids into PG muropeptides, thereby preventing peptide cleavage and lysis [9]. Similar modifications to the glycan strands, such as O-acetylation, may protect cells from PG-targeting glycoside hydrolases [10]. Together, these findings implicate structures like EPS and the PG layer of the cell envelope as key physical bulwarks for neutralization of the T6SS.

Genetic Antidotes

Effector toxins delivered by the T6SS and their cognate immunity factors can vary dramatically between strains and species [2]. This diversification is predicted to be the result of an evolutionary arms race between bacteria and their antagonists [11]. In this scenario, selective pressure favors the acquisition of new immunity genes by target cells for their protection. In line with this prediction, recent studies from diverse taxa have discovered aggregated clusters of horizontally acquired 'orphan' immunity genes by bacteria that lack the corresponding cognate effector genes (Figure 1B). For example, *Vibrio cholerae* genomes harbor clusters of orphan immunity genes that reside downstream of intact effector–immunity bicistrons that are predicted to evolve via the recombination of horizontally acquired modules into existing T6SS loci where they might confer protection against intraspecies competitors [12].

One context in which orphan immunity genes appear to be under strong selection is the human gut microbiome. Found within the genomes of diverse *Bacteroides* species are consolidated clusters of orphan immunity genes on mobile elements termed acquired interbacterial defense (AID) systems [13]. AID systems neutralize T6SS effectors delivered by *Bacteroides fragilis* during contact-dependent interactions both *in vitro* and in dual-colonized gnotobiotic mice. The consequences of *B. fragilis* T6SS neutralization by AID-encoded orphan immunity appear profound, since many human gut microbiomes with a high

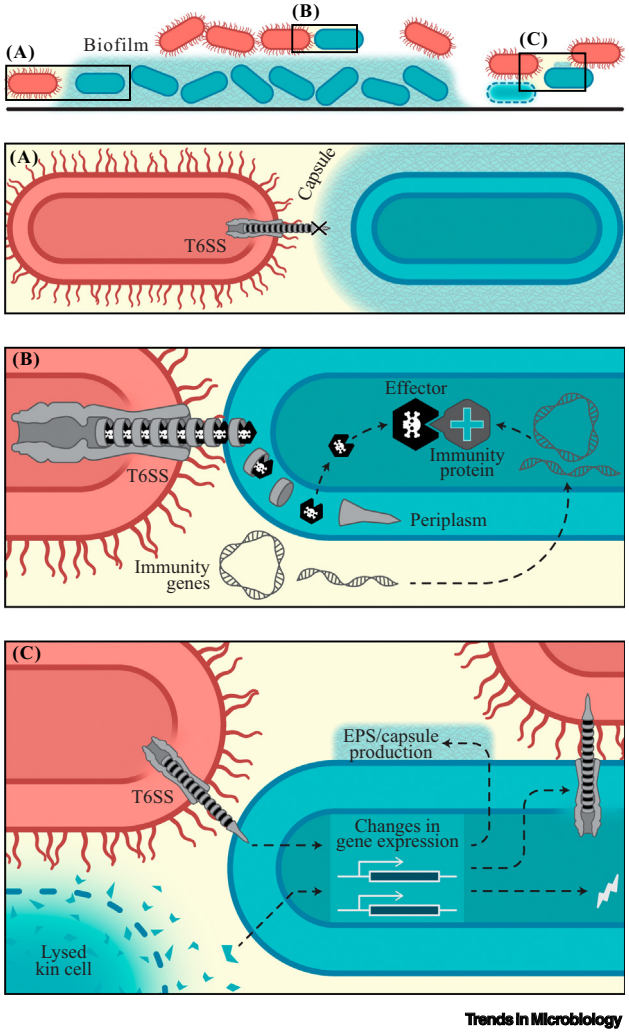

Figure 1. Bacterial Defenses against Type VI Secretion System (T6SS) Attack. Overview of select bacterial strategies for defense against T6SS antagonism. (A) Extrapolysaccharide (EPS)/capsule and biofilm matrix production defends target cells (blue) from T6SS-wielding cells (red). (B) Orphan immunity genes acquired by target cells through horizontal gene transfer produce immunity factors that can neutralize T6SS-delivered effectors. While only cytoplasmic-acting effectors and orphan immunity factors are shown here, periplasmic localization and activity is also common. (C) Intoxication or physical penetration of target cells by the T6SS of antagonists, or the sensing of lysed kin cells, can induce altered gene expression that increases target cell fitness. Size scaling of figure components is illustrative only. Not all defensive mechanisms described in the text are depicted in the figure.

abundance of orphan immunity lack *B. fragilis*, suggesting that orphan immunity genes (and, by inference, the T6SS) are a critical determinant of strain composition in the gut microbiome. Further evidence reinforces the pervasiveness of orphan immunity in the gut. Bacteroidales genomes harbor clusters of orphan immunity genes found adjacent to genes predicted to encode tyrosine recombinases. These loci, termed recombinase-associated AID (*r*AID) systems, feature intergenic sequence motifs and genetic organization that is reminiscent of integrons, implicating active gene acquisition as a mechanism by which bacteria aggregate orphan immunity genes.

Because the relationship between T6SS effector and immunity proteins is so highly specific, many Gram-negative bacteria may face strong selection as a result of repeated ecological encounters with antagonistic strains or species that wield unique T6SS effectors. Repeated exposure of target cells to specific antagonists could thereby favor the accretion of repertoires of orphan immunity genes due to their capacity to neutralize discrete T6SS effectors that exert persistent selective pressure. Some orphan immunity genes may also encode broad specificity that permits cross-neutralization of nonhomologous T6SS effectors, as has been demonstrated for immunity factors that defuse the toxicity of T6SS-delivered ADP-ribosyltransferase effectors [14]. Understanding to what extent orphan immunity factors are common features of Gram-negative bacterial defenses will require the careful detection and curation of T6SS effector and immunity genes and the systematic analysis of high-quality genome assemblies across diverse taxa.

Stress Is Protective

Intoxication occurs when target bacteria are not protected from T6SS attack, either by insufficient physical barriers or the lack of appropriate orphan immunity genes. However, under conditions of direct intoxication by the T6SS, or in proximity to intoxicated cells, bacteria can mount concerted physiological responses that promote their survival (Figure 1C). *Pseudomonas aeruginosa* cells detect the lysis of neighboring kin cells and alter gene expression via post-transcriptional regulation, leading to increased activity of the T6SS, among other pathways, in a process termed danger sensing [15]. In parallel, *P. aeruginosa* cells can directly sense intoxication and physical damage caused by the T6SS of competitors, such as *V. cholerae*, leading to the post-translational activation and firing of the T6SS and 'tit-for-tat' counterattack [16,17]. In a similar way, *Serratia marcescens* cells can tune expression of the T6SS in response to the degree of competitor aggression they experience [18]. Other bacteria, such as *Escherichia coli*, that are intoxicated by the *V. cholerae* T6SS, can promote their own survival (after said intoxication) through the concerted action of specific envelope stress-response pathways that induce upregulation of capsule biosynthesis, osmotic-response genes, and endogenous T6SS genes [19]. Finally, *Salmonella enterica* serovar Typhimurium strains inhabiting mixed biofilms can induce cellular stress responses following T6SS intoxication by competitor cells [20]. Target

cell stress-response pathway activation, in intoxicated *Salmonella* cells, includes RpoS- and SoxRS-mediated pathways leading to the upregulation of biofilm matrix production and antibiotic export pumps. These examples of T6SS attack resulting in physiological stress responses in target cells appear to be parallel examples of a bacterial collective behavior termed competition sensing [21]. Notably, these pleiotropic stress responses are hard-wired into the genetic circuitry of bacteria and may be agnostic to the specific identity of the antagonist. Further investigation of T6SS-induced stress responses may reveal the extent to which cellular defenses identified to date are interconnected. For example, *Bacteroides ovatus* cells intoxicated by the T6SS of *Bacteroides fragilis* specifically upregulate orphan immunity gene expression [13].

Concluding Remarks

Our detailed understanding of the inner workings of the T6SS apparatus and the vital cellular epitopes targeted by effectors is due to productive efforts focused on the aggressor-side of the T6SS-mediated interbacterial interaction. In comparison, relatively little is known about the role of the T6SS in natural settings, including the identity of bacteria sharing overlapping niches that are targets of the T6SS, and the consequences of T6SS-mediated antagonism on bacterial coexistence within microbial communities. We propose that the investigation of specific (e.g., orphan immunity) or general (e.g., EPS or stress response) defense strategies that are utilized by bacteria to increase fitness in the face of T6SS attack will yield insight into these mysteries. An understanding of the mechanisms that resident bacteria use for defense against T6SS-facilitated invasion could assist development of improved strains that can surmount those defenses to increase their capacity for colonization. Similarly, efforts to harness the T6SS for microbiome editing might take into account the capacity for target bacteria to mount defenses that render the weapon ineffective. Finally, there are striking parallels between the defenses described in this study and those used by bacteria to protect from antibiotics or phage attack. Future work should reveal the commonalities and idiosyncrasies of bacterial defense against varied threats.

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