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Multifaceted Interfaces of Bacterial Competition

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Abstract. Microbial communities span many orders of magnitude ranging in scale from hundreds of cells on a single particle of soil to billions of cells within the lumen of the gastrointestinal tract. Bacterial cells in all habitats are members of densely populated local environments that facilitate competition between neighboring cells. Accordingly, bacteria require dynamic systems to respond to the competitive challenges and the fluctuations in environmental circumstances that tax their fitness. The assemblage of bacteria into communities provides an environment where competitive mechanisms are developed into new strategies for survival. In this minireview, we will highlight a number of mechanisms used by bacteria to compete between species. We focus on recent discoveries that illustrate the dynamic and multifaceted functions used in bacterial competition, and discuss how specific mechanisms provide a foundation for understanding bacterial community development and function.

Introduction. Microbes compete to survive in naturally mixed communities and diverse environments. Microbial communities colonize niches as different as the surface of our teeth to the soils beneath our feet. The taxonomic diversity of organisms within these communities is a complex function of differing nutrients, niches, and interactions between species. In general, the abiotic influences on communities are identified through analysis of the chemical, spatial, and other relevant parameters that define local environments. Abiotic factors are varied, affecting microbial growth in many ways, and can often be manipulated in the laboratory to understand their influence on microbial communities. The interactions between species, on the other hand, are functions of a particular community and are a challenge to identify and resolve. Some broad

categorization provides guidelines for outcomes expected during interaction between species. Specifically, when non-neutral interactions occur between species, they are at times cooperative, but this appears to be the exception to the rule (1). More commonly, competition between species appears to define the interactions that may predominate in microbial communities.

Competition is categorized into two modes, exploitative and interference (2). Exploitative competition is passive in the sense that one organism depletes its surroundings of nutrients, thereby preventing competitors from gaining access to those resources. In contrast, interference competition invokes antagonistic factors produced to impede competitors (3). In microbial systems, competition is typically framed in the context of growth limitation or inhibition due to exploitation and interference. However, while species may be sensitive or resistant to growth inhibitory activities, they also may engage in antibiotic synthesis, motility, sporulation, predatory functions, and biofilm formation in response to competition. Though not universal amongst all bacteria, these physiological changes represent the diversity of mechanisms to enhance the competitive fitness of bacterial species equipped with them. The ability of individual species to employ a spectrum of competitive mechanisms and responses to challenges may be essential to their survival in communities of diverse organisms, where competitive stress may take many forms. To better understand the forces that enable bacteria to thrive in communities, we consider numerous competitive functions that determine the relative fitness of different bacteria within a community.

Direct studies on natural communities such as those in soils or plant and animal hosts are notoriously difficult, because they are complex and variable. Also, explanting environmental isolates to the laboratory creates additional complications. For instance, many organisms do not grow under standard laboratory conditions. Recent technological advances such as the iChip (4) enable the growth of many previously uncultured bacteria, but *in situ* manipulation of whole bacterial communities remains challenging. A frequently used approach to study microbial community interactions is to culture two or more species together under defined conditions. By investigating microbial interactions in defined formats, culture-based studies can provide powerful mechanistic insights into competitive functions.

In recent years, competition studies between bacteria have contributed to a more informed view of competitive mechanisms used by different species. We focus this minireview on mechanisms of interference and exploitation competition between species involving specialized metabolites, enzymes, and functions associated with the cell envelope, highlighting interaction outcomes that differ from growth inhibition by classical antibiotics. The cell envelope forms the barrier between a bacterial cell and its surroundings, which include competing bacteria. We will parse different competitive mechanisms into those that occur across the envelope due to exchange of diffusible factors, and those that require contact between cell envelopes, either directly or via their embedded proteins.

Interference and Exploitation At-a-Distance.

Specialized Metabolites. Competition between species is often mediated through bioactive metabolites synthesized by competitors. Specialized metabolites (SMs) are molecules produced by bacteria that are not involved in primary metabolism but are involved in other biological processes. Many specialized metabolites were previously called “secondary” metabolites because their presence is dispensable under laboratory conditions and their production often occurs during late stages of growth (5). However, SMs may be essential for some bacteria to persist in the environment (6) or under competitive stress. In the context of competitive interactions, SMs of primary interest are those affecting the growth and development of competing bacteria. For instance, antibiotics provide some of the clearest mechanistic insights for chemical interactions between competing species of bacteria. However, considering their measurable biological activities at subinhibitory concentrations, even the empirical roles of antibiotics in nature are subject to debate (7–10). Overall, the biological functions of SMs are numerous and, arguably, largely unknown. We will focus, therefore, on several illuminating examples where bacteria use antibiotics and other SMs in precisely targeted mechanisms that affect competing organisms in ways other than inhibition of growth. The abilities of bacteria to respond dynamically to a range of chemical stresses may have profound effects on their fitness in competitive multi-species communities.

Exploitation competition due to SMs. In some cases, clearly self-serving functions of SMs indirectly lead to exploitation of resources, yielding a competitive advantage. Exploitation competition occurs when one organism disrupts the growth of its competitors by using a shared, limited resource (11). Exploitation often occurs when

104 one bacterial species alters its external environment through their various metabolic
105 functions and prohibits the growth of other bacterial species (3). This exploitation can
106 arise from direct consumption of nutrients, buildup of toxic waste products, or the
107 activity of SMs. An example of SM-mediated exploitation is found in siderophores, which
108 are SMs produced for capture of iron (12). Iron is essential for cytochromes and iron-
109 sulfur proteins, and competition for iron is driven by its availability. Siderophores are one
110 mechanism to chelate external iron, which is then imported as a complex into the
111 producer cells (13). Siderophore production thus increases the bioavailability of iron
112 while simultaneously depleting the supply available to competitors. The significance of
113 iron is underscored by the numerous examples of siderophore-mediated competition in
114 different environments, including competition for colonization of the light organ in
115 Hawaiian bobtail squid by different strains of *Vibrio fischeri* (14) and between the human
116 opportunistic pathogens *Staphylococcus aureus* and *P. aeruginosa* (15). Bacteria also
117 acquire iron from their environment and engage in exploitation competition by using
118 other iron uptake systems including transporters (16). However, because siderophores
119 are extracellular SMs, they are also subject to piracy by other species, posing a
120 competitive risk to the producing organism (e.g. (17, 18). These examples of
121 siderophore-mediated interactions illustrate the potential complexity of specialized
122 metabolites and exploitative interactions that are probably pervasive in nutrient-limited
123 environments.
124
125 *Interference competition due to SMs*: *Antibiotic Activity without Antibiosis*. The classic
126 view of antibiotics and other SMs as weapons has guided their isolation and

127 characterization since their discovery. In the process of discovery, antibiotic molecules
128 are isolated from bacterial strains grown in the laboratory and tested for growth
129 inhibition of target organisms (19). This approach has been effective for identifying the
130 majority of antibiotics, but it has left gaps in our understanding of the ecological
131 functions of these molecules. For instance, concentrations of antibiotics sufficient to
132 inhibit growth may be rare in natural environments (20, 21). Do antibiotics at lower than
133 inhibitory concentrations have functions relevant to competitive interactions? This
134 question has inspired investigation into the effects of subinhibitory concentrations of
135 antibiotics on bacteria, where a wide range of responses has been observed among
136 organisms exposed to different antibiotics. For example, subinhibitory concentrations of
137 jadomycin B cause *Streptomyces coelicolor* to prematurely sporulate and produce a
138 pigmented antibiotic prodigiosin (22). Subinhibitory concentrations of kanamycin induce
139 the expression of type VI secretion genes in *Pseudomonas aeruginosa* (23). Numerous
140 other antibiotics induce global transcriptional responses (reviewed in depth, (24)).
141 Cellular stresses from subinhibitory antibiotic concentrations may trigger these
142 responses as early warning systems of chemical warfare. Alternatively, the natural
143 functions of some antibiotics and SMs may be reflected in the subinhibitory responses
144 of competitors, independent of inhibitory activity (10). Clearly delineated mechanisms of
145 concentration-dependent activities and responses during competition are needed to
146 understand the roles of antibiotics and other SMs in community dynamics.
147
148 *Interference competition due to SMs*: *Multifunctional metabolites.* Bacteria produce
149 many SMs, representing an enormous chemical diversity with poorly understood

function (20). Although antibiotic activity is the most common activity ascribed to SMs, many antibiotics also have effects on bacterial competitors that are independent of growth inhibition (see above). There are numerous reports detailing the effects of SMs on the multicellular development of a bacterial species. For example, the soil bacterium *Pseudomonas protogens* produces 2,4-diacetylphloroglucinol, a SM with antifungal activity that is used in biocontrol (25). The cellular differentiation of *B. subtilis* is inhibited by 2,4-diacetylphloroglucinol when cultured with *P. protogens* (26). In contrast, *B. subtilis* biofilm formation is stimulated by the antifungal nystatin (27) and by peptide antibiotics (28). Bacillaene, is a *B. subtilis* produced SM that was originally identified as an antibiotic inhibitor of protein synthesis (29). Bacillaene also interferes with prodigiosin production in *Streptomyces coelicolor* and *Streptomyces lividans* without inhibiting growth (30, 31).

Another mechanism for SM interference in competitor development is to derail normal signaling processes. For example, some marine bacteria produce SMs that interfere with quorum sensing, and thus disrupt subsequent downstream processes reliant on communication between competitor cells (32, 33). One challenge is to understand the fitness benefits of such modulatory activities in competitive interactions between bacteria. However, in many cases the connection between SMs and the responses they elicit in competitors is unknown. Model systems using two or more bacteria cultured together have been developed to investigate how SMs and other factors influence competitive fitness under controlled settings.

Model Systems of SM-Mediated Competition between Species. Multi-species model systems are advantageous because they open the door to the diversity of competitive functions used by a single organism, including production of multiple SMs and different patterns of response to competitor SMs. Soil bacteria provide an illustrative example of diverse competitive functions. Species of *Streptomyces* are ubiquitous in the soil and renowned for their capacity to synthesize SMs (34). In addition, *Streptomyces* species undergo developmental phases of their lifecycle, including aerial growth and sporulation, which may be affected by SM activity (35). For example, sporulation of some streptomycetes depends upon the peptide SapB that acts as a surfactant and lowers surface tension, enabling aerial hyphae to expand upward (36). *Bacillus subtilis* produces its own lipopeptide surfactant, surfactin. *Bacillus subtilis* requires surfactin for biofilm development and some types of motility (27, 37, 38). Intriguingly, surfactin also antagonizes aerial development of many *Streptomyces* species (39, 40). Insight into the mechanism arose from *S. coelicolor,* which when treated with surfactin was unable to process and secrete SapB to support aerial growth (41). When compared to antibiotics that target growth, inhibition of sporulation is a relatively subtle developmental effect that presumably prevents the spread of *Streptomyces*. Although *B. subtilis* does not likely produce multifunctional surfactin explicitly for competition, the inhibition of *Streptomyces* development may enhance competitive fitness in natural environments. Indeed, some species of *Streptomyces* have acquired enzymatic resistance to surfactin, consistent with a natural competitive function. Using imaging mass spectrometry it was demonstrated that *Streptomyces* sp. Mg1 hydrolyzes surfactin (Fig 1A and 1B) (40). The enzyme, surfactin hydrolase, was shown to specifically inactivate surfactin and

plipastatin, another lipopeptide produced by *B. subtilis* (40). Hydrolytic inactivation is a common resistance mechanism for many antibiotics (42). Analogously to the emergence of new β-lactamases, production of surfactin hydrolase and other antibiotic degrading enzymes promotes the competitive fitness of their bacterial producers, although with surfactin the selection is against a developmental process.

FIG 1. Mechanisms of bacterial competition. Top (A,B)- Detecting patterns of SM production and degradation through imaging mass spectrometry. (A) False-colored extracted ion image showing the distribution of surfactin (blue) produced by *B. subtilis* and hydrolyzed surfactin (yellow) resulting from the activity of surfactin hydrolase secreted by *Streptomyces* sp. Mg1. (B) The extracted ion image from (A) overlaid onto a photograph of a culture of *B. subtilis* and *Streptomyces* sp. Mg1 to highlight the localization patterns of each SM during competition. Middle (C,D)- Revealing essential SM functions using predator-prey interactions. (C) Photograph of *M. xanthus* spotted onto the center of a wild-type *B. subtilis* NCIB3610 colony. The colony is mostly opaque due to intact, viable *B. subtilis*. (D) A mutant *B. subtilis* strain deficient in bacillaene production becomes transparent as it is consumed by *M. xanthus*, which forms fruiting bodies on the lysed remains of the *B. subtilis* colony. Bottom (E,F)- Structural features of a contact-mediated competitive apparatus. (E) Cryo-electron micrographs of a T6SS apparatus inside an intact *Vibrio cholerae* cell. Scale Bar is 100 nm. (F) Comparison of flagellum (F) and T6SS sheath (S) isolated from *V. chloerae*. Scale bar Is 100 nm. Panels C and D were provided by John Kirby. Panels E and F were reproduced from (43) with permission.

219

220 Competitive culture models enable us to interpret the functions of SMs in new
221 ways that enhance our view of competition dynamics. Several reports show that SMs
222 provide defense against otherwise overwhelming forces. For instance, laboratory strains
223 of *B. subtilis* are preyed upon by *Myxococcus xanthus,* but the undomesticated *B.*
224 *subtilis* strain NCIB 3610 is resilient (44). Many domesticated laboratory strains of *B.*
225 *subtilis* lack a gene, *sfp*, required for production of several SMs, including bacillaene
226 (45, 46). This defect, which renders domesticated *B. subtilis* susceptible to *M. xanthus*
227 predation, was subsequently shown to be specific to the loss of bacillaene production
228 (44) (Fig. 1C and 1D). Indeed, exogenous application of bacillaene protected sensitive
229 strains of *B. subtilis* and *Escherichia coli* from predation. Thus, under the pressure of
230 predation, bacillaene is essential for defense of *B. subtilis*. Intriguingly this is not the
231 only demonstration of a defensive role for bacillaene. Strains of *B. subtilis* deficient in
232 bacillaene production are also hypersensitive to lysis by linearmycins produced by *S.*
233 Mg1 (47, 48). Bacillaene was originally discovered as an antibiotic inhibitor of protein
234 synthesis, (29) and its function dispensable for growth of *B. subtilis*. However,
235 competition studies expand our view of bacillaene to include essential defensive
236 functions, the precise mechanisms of which are not known. Nevertheless, examples
237 such as bacillaene and surfactin serve to illustrate that SMs provide diverse and
238 important competitive functions for the producer organisms.

239

240 As seen in examples from antibiotics to siderophores, SMs have varied and
241 sometimes essential functions in competition between species. However, aside from

antibiotics, little mechanistic detail is available for the targets and processes affected by SMs (e.g. (32, 33)). The identification of chemically mediated mechanisms of competition will require continued exploration of competitive dynamics between species. An important consideration is how the SMs operate along with other entities that mediate interactions between competing species.

Secreted Enzymes. In addition to SMs, bacteria secrete enzymes that participate in competition. Secreted enzymes that confer antibiotic resistance have a clear competitive benefit (40). Additionally, bacteria benefit by interfering with the development of their competitors, e.g. using enzymes to degrade signaling molecules like acyl homoserine lactones (49–52). However, surprisingly little is known about how bacteria use secreted enzymes to kill or inhibit their competitors. The predatory bacteria *M. xanthus* is a prolific producer of degradative enzymes and encodes in its genome more than 300 degradative hydrolytic enzymes (53, 54). The functions of many of these enzymes are unknown, but bacteriolytic activity has been demonstrated for some (55). An example of competitive enzyme function is found where *Staphylococcus epidermidis* competes with *Staphylococcus aureus* for colonization of the human nasal cavity (56). *Staphylococcus epidermidis* secretes a serine protease, Esp, which inhibits *S. aureus* biofilm formation (57). Esp degrades *S. aureus* biofilms by inactivating autolysins and preventing release of DNA that is an essential component of the biofilm extracellular matrix (58). The presence of *Corynebacterium* spp. in the nasal cavity is often inversely correlated with pathogenic *Streptococcus pneumoniae* (59). Like *S. epidermidis, Corynebacterium accolens* also utilizes a secreted enzyme, LipS1, to interfere with a

265 competitor. LipS1 is a triacylglycerol lipase that produces oleic acid from the hydrolysis
266 of a human-produced triglyceride, triolein (60). Oleic acid and other free fatty acids
267 inhibit the growth of *S. pneumoniae* (60, 61). Esp and LipS1 interfere with bacterial
268 competitors but through fundamentally different mechanisms. Thus, secreted enzymes
269 may have many active roles at or near the cell surface of competitors, although this
270 area is in need of further study.
271
272 **Extracellular Vesicles.** Extracellular vesicles are of great interest for both bacterial and
273 eukaryotic interaction processes. Vesicles are capable of vectoring proteins, lipids,
274 nucleic acids, and small molecules that function in competitive and signaling processes
275 (62). Many bacteria produce extracellular vesicles (EVs) during normal growth. The
276 precise mechanisms of EV biogenesis and cargo loading are beginning to be identified.
277 Gram-negative bacteria produce EVs (also called outer membrane vesicles) when the
278 outer membrane is “pinched,” and the vesicle buds from the cell surface (63). A second
279 vesicle-release mechanism is reported to occur within biofilms of *P. aeruginosa* (64). In
280 this system, prophage-encoded endolysins activate cellular lysis, releasing membrane
281 fragments that form vesicles and permeate the extracellular space. The problem for
282 Gram-positive bacteria is more complicated due to the lack of an outer membrane, and
283 the mechanism of EV generation is currently unknown, although several models have
284 been hypothesized (65). After formation, EVs are released into the environment. When
285 an EV encounters a Gram-negative cell the vesicular membrane and the outer
286 membrane fuse, which delivers the cargo into the recipient’s periplasm (66).

287 Extracellular vesicles have been observed to adsorb to the cell wall of Gram-positive
288 bacteria, thereby delivering their contents to target cells (66).
289
290 Extracellular vesicles are used by bacteria for diverse processes including biofilm
291 formation (67), carbon storage (68), virulence (69), and quorum sensing (70). Bacteria
292 also use EVs for defensive measures against several types of antimicrobial insult. For
293 instance, the EVs of *Prochlorococcus* adsorb phages (68), and EVs from *P. aeruginosa*
294 and *Staphylococcus aureus* protect β-lactamases from proteolytic degradation (71, 72).
295 Though EVs are often characterized for their defensive functions (73), bacteria also use
296 vesicles to deliver antagonistic agents to competing bacteria. These agents can be
297 enzymes, such as the peptidoglycan-degrading hydrolases produced by *P. aeruginosa*
298 (66) and *Lysobacter* sp. XL1 (74), or antibiotic SMs like actinorhodin or prodigiosins
299 found in the EVs produced by *S. coelicolor* (75) and *S. lividans* (76), respectively.
300
301 The EVs of *M. xanthus* are of tour de force in regards to their competitive
302 potential. The EVs produced by *M. xanthus* contain not only 29 predicted hydrolytic
303 enzymes (11 of which were not found in the outer membrane) but also 16 specialized
304 metabolites including the myxalamids, which are known antibiotics, and DKxanthene
305 534 (77). DKxanthene 534 and myxalamids are polyketide and hybrid polyketide-
306 peptide molecules, respectively, both having non-polar hydrocarbon regions. Consistent
307 with membrane localization, both molecules are typically extracted from cell pellets and
308 have low abundance in supernatants (78, 79). These characteristics highlight an

309 important function of EVs to facilitate transfer of hydrophobic molecules, including
310 antibiotics, across aqueous environments (70).
311
312 Extracellular vesicles also intersect with SMs in intriguing patterns that may affect
313 competition between bacteria. Recently, it was shown that *B. subtilis* disrupts its own
314 EVs by secreting surfactin (80). The targeted lysis of EVs by surfactin may serve as a
315 defensive mechanism against antibiotic-laden vesicles produced by competing
316 organisms or as an offensive tool to prevent non-polar signaling molecules, including
317 quorum sensors, from reaching their intended targets. Extending on overlapping
318 functions, bacteria reportedly become reversibly resistant to antibiotics when they
319 swarm (81). In *B. subtilis*, swarming motility requires surfactin (82, 83). As an intriguing
320 hypothesis for niche exploration, *B. subtilis* might produce surfactin not only to promote
321 its movement over surfaces but also as a defense mechanism against EVs produced by
322 other organisms.
323
324 **Contact-Mediated Competition.**
325
326 Different species of bacteria physically interact at high cell densities in ways that
327 promote information exchange, such as plasmid conjugation, or through competitive
328 interaction mechanisms. Some competitive functions appear to have evolved to function
329 specifically in close proximity. In particular, bacteria use membrane and cell envelope
330 embedded functions that are outwardly directed toward competitors. Such mechanisms

are likely to be important for survival under crowded conditions through both their inhibitory functions and their contributions to community structure.

Contact-Dependent Inhibition. As a specific mechanism of interference competition, contact-dependent inhibition (CDI) describes a membrane protein that operates as a delivery system for a cellular toxin. The prototypical CDI system was first described in uropathogenic *E. coli* EC93 and consists of three components: CdiA, CdiB, and CdiI (84). CdiA and CdiB are homologous to the two-partner secretion system proteins TpsA and TpsB, respectively. In two-partner secretion systems, the secreted substrate TpsA is translocated across the outer membrane through its cognate beta-barrel protein TpsB (85). Likewise, in CDI systems the toxin CdiA is attached to CdiB, which is an outer membrane beta-barrel protein that extends away from the cell. This arrangement leads to CDI being referred to as a "toxin on a stick" (86). CdiI provides the producing cell with immunity towards its own toxin by specifically binding to CdiA and inhibiting its activity (87). When a CDI-producing cell (CDI^+) makes direct contact with a susceptible target cell, its CdiA toxin interacts with the outer membrane protein BamA (88). The CdiA protein is then deposited onto the target cell surface and undergoes self-cleavage, which transports the carboxy-terminal (CT) portion of CdiA into the periplasm (89). Many CdiA toxins are nucleases and require entry into the cytoplasm to exert their effects (87). Translocation of the toxin into cytoplasm requires the proton motive force (90) and interaction with toxin-specific inner membrane protein receptors (91). The requirement for a membrane receptor protein on target cells limits CDI to a narrower range of specificity when compared to diffusible agents like antibiotics. This specificity is

due to variability in extracellular loops 6 and 7 of BamA, which form the CdiA-CT-binding site (92). Due to the narrower target range, it has been speculated that CDI systems are a means to inhibit closely related species. This would allow CDI^+ bacteria to inhibit other bacteria that are more likely in direct competition for the same or very similar ecological niches (86).

Biofilms are community structures that form as a result of the concerted effort between many cells. The conditions within a biofilm are inherently stressful to cells. Resources including nutrients, oxygen, and physical space are limiting (93). These conditions breed competition between cells within the biofilm and provide strong selection for competition. For example, growth within a biofilm selects for bacteria that engage in exploitation competition by preferentially occupying biofilm surfaces and gaining access to oxygen (94). Biofilm growth has also selected for cells that are able to engage in inference competition with their neighbors. *Burkholderia thailandensis* illustrates the utility of CDI functions for promoting competitive success in a biofilm. Disruption of the CDI system (CDI^-) of *B. thailandensis* both sensitizes cells to CDI from isogenic siblings and abolishes biofilm formation (95). Both functions are tied to BcpA (homologous to CdiA), but the biofilm functions are independent of CDI activity (96). These observations suggest that CDI systems help to ensure a competitive advantage by supporting biofilm formation while excluding competitors. CDI-dependent cell adhesion and defects in biofilm production for CDI^- strains have also been reported in *E. coli* (97) and *P. aeruginosa* (98), further solidifying the link between CDI and biofilm development.

Aside from the costs of biofilm formation and the cellular challenges within a biofilm, these structures serve to protect bacteria from various external stresses (99, 100). For instance, bacteria have evolved mechanisms, including CDI, to competitively exclude non-sibling cells from biofilms (101). Developing biofilms contain three-dimensional structures called “pillars” for *B. thailandensis* (102). These structures extend outwards from the biofilm attachment site, providing cells within the pillars better access to oxygen and nutrients than the cells in the biofilm substratum (93). The CDI system of *B. thailandensis* excludes CDI-sensitive cells from developing pillars (102). Cells that produce the same CDI system, presumably siblings, are not killed by CDI due to their cognate immunity genes. This selective killing by CDI provides a kin discrimination mechanism for *B. thailandensis* biofilms and likely protects the biofilm from invaders. Taken together, the CDI functions of *B. thailandensis* demonstrate important competitive advantages that arise in close cellular proximity through direct inhibition of competitors and through construction of defensive biofilm structures.

Type VI Secretion Systems. The type VI secretion system (T6SS) was originally identified as a virulence factor produced by *V. cholerae* against amoebae and macrophages (103). Subsequently, genes encoding T6SS were found in roughly one-quarter of Proteobacteria with sequenced genomes (104) including, but not limited to, opportunistic pathogens such as *Acinetobacter baumannii* (105) and *Serratia marcescens* (106). The observation that many of the identified T6SS had no apparent

399 effect on eukaryotic cells and that T6SS gene clusters occurred in non-pathogenic
400 bacteria prompted investigation into potential antibacterial activities (106, 107).

401

402 The T6SS of Gram-negative bacteria have emerged as a powerful weapon in
403 close-quarters interference competition between bacteria. The basic mechanism of
404 function for T6SS is to inject toxic effector molecules directly into the cytoplasm of target
405 cells. Structurally and functionally the T6SS apparatus is homologous to bacteriophage
406 contractile tails (108). The T6SS apparatus is a cylindrical spiked-tipped inner tube that
407 is surrounded by a sheath and anchored to the inner membrane (Fig. 1E and 1F). When
408 the cell is in physical contact with its target, the sheath contracts, and the inner tube is
409 propelled outward and punctures the membrane of a target cell using its spiked tip.
410 Within the target cell the spike disassociates from the tube and the toxic effectors are
411 delivered. Common effectors characterized thus far include phospholipases (109–111),
412 peptidoglycan hydrolases (112–114), and nucleases (115, 116).

413

414 In addition to T6SS being an effective delivery system for toxic payloads, one
415 example demonstrates that the sharpened spike of the T6SS is a potent weapon even
416 in the absence of toxic effectors. Using its TagQRST-PpkA-Fha1-PppA sensing system,
417 *P. aeruginosa* detects cell envelope damage caused by the T6SS of other bacteria
418 (117). This detection or “danger sensing” allows the cell to mount a response against its
419 antagonist and minimize future damage to the cell or its siblings (118). In the case of *P.*
420 *aeruginosa*, the cell retaliates against T6SS-mediated attacks, directing its own T6SS in
421 the same direction as the initial attack in a behavior called “dueling” (119). Duels

damage target cells and can cause membrane blebbing, plasmolysis, and even lysis. Strains of *P. aeruginosa* that are deficient in production of all known T6SS effectors still retaliate against T6SS-mediated attacks and engage in dueling with effective killing activity (117). If *P. aeruginosa* cells lose their duels and are lysed by competitors, they release diffusible danger signals that stimulate T6SS activity and promote the survival of siblings (120).

Like CDI, The T6SS killing mechanism also functions to favor siblings in biofilm formation. Strains of *Proteus* sort self from non-self in mobile multicellular swarms. This kin-discrimination is observed as cell-free zones between swarms called Dienes lines (named for their discoverer Louis Dienes) on agar surfaces. In these zones, opposing swarms of *P. mirabilis* do not intermingle. The establishment of Dienes line formation was found to be due to T6SS (121). At the intersection between opposing swarms, *P. mirabilis* use their T6SS to kill, and in turn are killed by T6SS of competitors, creating a demilitarized zone (DMZ) where the Dienes lines exist between mobile populations. As with *B. thailandensis*, strains join the beneficial swarm when they are not killed by the T6SS. An added benefit of this kin-discrimination arises because swarming provides increased resistance to antibiotics (81). Thus, entry into the swarm promotes competitive fitness of bacteria by excluding unrelated cells and from enhancing defense against antibiotics. Similar boundary formation has also been reported for *M. xanthus* (122) and *B. subtilis* (123). The observation of discrimination in *B. subtilis* demonstrates that CDI and T6SS are not the only mechanisms that bacteria use for kin-discrimination, as *B. subtilis* does not produce CDI or T6SS. The question remains whether *B. subtilis*

445 demarcates Dienes lines through a contact-dependent or –independent mechanism,
446 although evidence suggests combinatorial mechanisms are used (124).
447
448 Both the CDI and T6SS are analogous in that a toxin is delivered directly to a
449 target cell. However, like many antibiotics, these toxins are typically soluble molecules.
450 How then, are insoluble effectors delivered? In one case the T6SS toxin Tse6, produced
451 by *P. aeruginosa*, contains transmembrane domains that are shielded from the aqueous
452 environment by an associated chaperone. The chaperone, EagT6, protects Tse6 until
453 delivery into the target's periplasm (125). This example appears to be the exception,
454 where the majority of membrane-associated effectors lack a chaperone or other clear
455 vectoring mechanism. As described previously, extracellular vesicles are another
456 mechanism for delivery of otherwise insoluble cargo.
457
458 **Outer Membrane Exchange**. In addition to CDI, T6SS, and EVs, Gram-negative
459 bacteria appear to use the outer membrane itself as an effective delivery system for
460 otherwise insoluble toxins. Outer membrane exchange (OME) for *Myxobacteria*, for
461 example, is a contact-dependent mechanism for cells to share membrane components,
462 including phospholipids and insoluble lipoproteins, with other cells (126). OME has been
463 demonstrated to extracellularly complement mutants deficient in production of particular
464 outer membrane products. For example, via OME the gliding motility of non-motile *M.*
465 *xanthus* mutants is stimulated when mutant cells are mixed with wild type cells (127).
466 OME is also intertwined with colony swarming and sporulation (127). Furthermore, a

recent report implicates OME as a powerful defensive mechanism to dilute membrane damage over a population of cells (128).

OME requires the production of an outer membrane protein complex TraAB in both the donor and recipient cell (127). TraAB appears to be the only component necessary to mediate OME (129) and, similarly to the BamA receptor in CDI systems, TraAB contains a polymorphic domain that limits OME to a narrow range of related targets (130). Given the functional similarities to CDI systems and the potential of OME to directly deliver toxic effectors into the envelope of target bacteria, it is not surprising that Myxobacteria use OME to mediate competition and engage in kin recognition. Motile cells of *M. xanthus* are killed when cultured with their non-motile siblings. Killing is dependent upon the presence of TraA in the target motile cell and a polyploid prophage in the killer non-motile sibling (131). Currently, the effector delivered by OME is not known, but it is likely produced from toxin-antitoxin module encoded on the prophage (131). No further examples of OME-mediated competition have been reported thus far. However, as with EVs, new studies will likely uncover fascinating roles for these membrane-derived strategies in bacterial competition.

Conclusions.

Bacteria use competitive mechanisms that are nearly as diverse as the competitors they encounter (Fig. 2). Inherent in each competitive strategy are advantages and disadvantages. When bacteria use secreted effectors like antibiotics,

enzymes, or vesicles, they are able to compete while minimizing the risks of direct damage during contact-mediated competition. Once a cell exports its competitive molecules across its envelope, those molecules are subject to diffusion, which diminishes their growth inhibitory effect on competitors at a distance. However, many of these metabolically expensive products operate between inactive and inhibitory concentrations and may possibly act as chemical cues for competitors (132). Exposure to subinhibitory antibiotic concentrations can induce resistant states (133–135), select for resistant competitors (136), stimulate biofilm formation (137–139), and motility (140). Activation of a resistant state allows a competitor unrestricted access into a previously protected niche. If potential prey senses a cue and escapes predation, then the producer loses nutrients in the form of that lysed cell. Thus, if a competitor senses a cue, the producer may suffer the consequences for competitive fitness. However, it is also important to note that our current understanding of response to subinhibitory concentrations of antibiotics and other SMs in the context of bacterial communities is limited and requires further investigation. The direct delivery of toxins into a target cell by CDI or T6SS circumvents diffusion and the potential costs of subinhibitory antibiotic concentrations. The tradeoff is that contact-mediated competition puts a cell in direct contact with its competitor and allows the risk of retaliation such as in the dueling response (117) or from high concentrations of diffusible SMs.

FIG 2 Summary of mechanisms used in bacterial competition. (A) Contact-mediated mechanisms involve either direct contact between cell envelopes (OME) or are facilitated by protein complexes (CDI and T6SS). In the case of CDI and T6SS, toxic

effectors (square or Pac-Man) are delivered into the target cell. (B) Bacteria compete at-a-distance using SMs (examples shown are bacillaene and streptomycin), secreted enzymes, and extracellular vesicles. CDI, contact-dependent inhibition; EVs, extracellular vesicles; M, membrane; M_T, target cell membrane; IM, inner membrane; OM, outer membrane; PG, peptidoglycan; T6SS, type VI secretion system.

We have emphasized the differences between competitive mechanisms that are contact-mediated and those that occur at-a-distance. However, bacteria are not mutually exclusive in the systems they employ. For example, *Pseudomonas* species use T6SS but are also prolific producers of SMs including antibiotics and siderophores (141). Bacteria also use direct contact to deliver secreted factors at high local concentration. Predatory *Bdellovibrio* species physically collide with target cells, pierce their cell envelope, and digest their prey from within using an impressive cocktail of secreted enzymes that includes nucleases and peptidoglycan hydrolases (142, 143). The differences between contact-mediated and distance approaches may reflect how bacteria use both systems in competition. A cell producing secreted molecules, like antibiotics, creates a chemical or enzymatic protective shell around itself. Within this shell the cell is also able to simultaneously engage in exploitative competition via its exclusive access to nearby nutrients. The spectrum of inhibitory activities, in concert with small size, low charge, and ease of entrance into target cells (144, 145), place antibiotics at the foundation of such protective chemical shells. However, if a competitor breaches the defenses, then the delivery of toxic effectors by CDI or T6SS directly into the target may stop the invasion. A remarkable balance of antibiotic resistance and

536 contact-dependent mechanisms has been shown with *A. baumanii* (146). Several multi-
537 drug resistant *A. baumannii* strains carry a plasmid that provides antibiotic resistance
538 while also inhibiting expression of T6SS systems. However, the plasmid is unstable,
539 and loss of the plasmid provides a mechanism to activate T6SS at the cost of losing
540 antibiotic resistance in some cells. The net result is a population with shared functions in
541 competitive fitness through defense and through close quarters exclusion of
542 competitors. Perhaps contact-mediated mechanisms like CDI, T6SS, or OME are
543 needed to selectively inhibit closely related competitors with the capacity to pass
544 unharmed across a chemical defensive barrier (92, 130, 146).
545
546 Culture-based studies have revealed many mechanistic details of bacterial
547 competition. However, we note that many of the studies highlighted in this minireview
548 used simple, small-scale bacterial communities with minimal mixing. To gain a deeper
549 understanding of bacterial competition in natural communities, systems are needed that
550 combine the use of multiple approaches and expanded knowledge of diverse
551 competitive mechanisms. Although beyond the scope of this minireview, mathematical
552 modeling is a powerful approach to understand how bacterial communities are formed
553 and maintained (e.g. (147, 148)). Mathematical approaches stand to become more
554 powerful as they incorporate diverse competitive outcomes in addition to killing or
555 survival. For instance, what effects does T6SS-mediated retaliation have in a modeled
556 competition? How does SM-mediated developmental inhibition affect a community?
557 What are the consequences of exposure for cells outside the inhibitory ranges of SMs?
558 Using controlled experiments in the laboratory, new mechanistic details of competition

559 are being identified, despite limitations to our understanding of these mechanisms in
560 natural environments. The genomes of many antibiotic producing bacteria contain silent
561 SM gene clusters that are not expressed under laboratory conditions (149). Likewise,
562 many studies with CDI and T6SS require artificial expression conditions (150, 151).
563 These obstacles are a central focus of current efforts to understand competitive
564 mechanisms. Meanwhile, models that better mimic the native environment are being
565 developed to provide a clearer view of bacterial interactions under natural conditions
566 (e.g. (87, 116, 152)) The examples above and many more innovative studies are
567 expanding our views of the interactive interfaces between two bacterial species. The
568 emerging challenge is to build these interfaces into networks, which will represent the
569 many facets of competition within microbial communities.
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578
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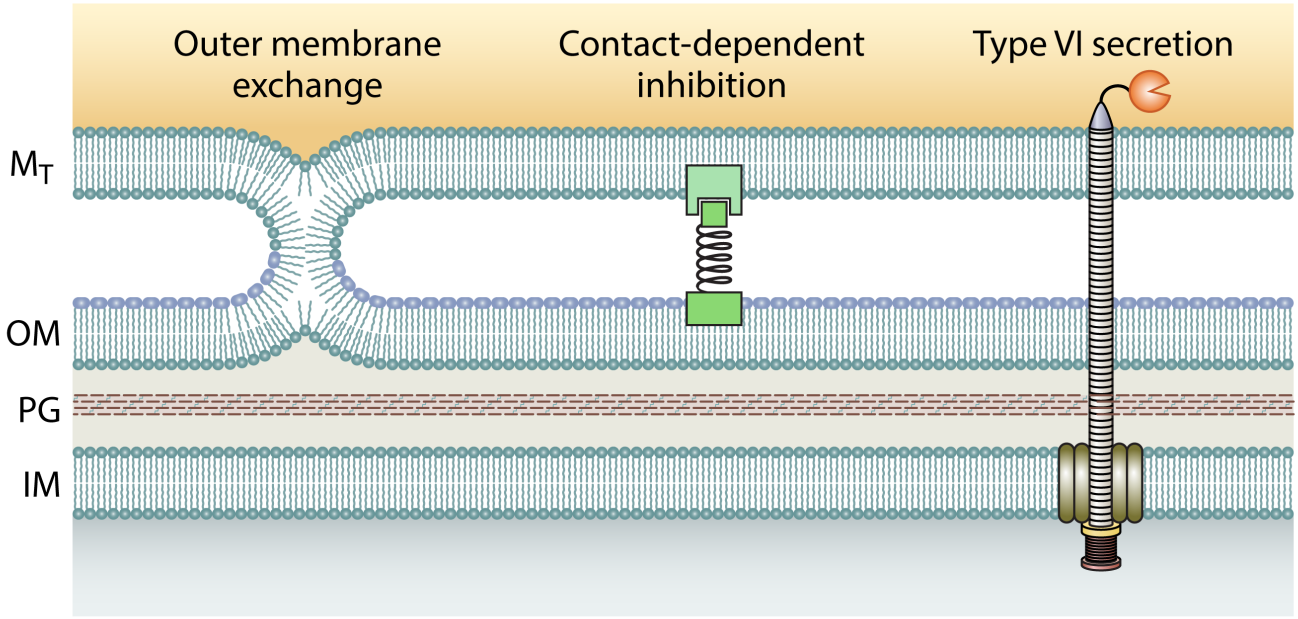
A. Contact-mediated



B. Distance