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REVIEW ARTICLE

Interbacterial mechanisms of colonization resistance and the strategies pathogens use to overcome them

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The communities of bacteria that reside in the intestinal tract are in constant competition within this dynamic and densely colonized environment. At homeostasis, the equilibrium that exists between these species and strains is shaped by their metabolism and also by pathways of active antagonism, which drive competition with related and unrelated strains. Importantly, these normal activities contribute to colonization resistance by the healthy microbiota, which includes the ability to prevent the expansion of potential pathogens. Disruption of the microbiota, resulting from, for example, inflammation or antibiotic use, can reduce colonization resistance. Pathogens that engraft following disruption of the microbiota are often adapted to expand into newly created niches and compete in an altered gut environment. In this review, we examine both the interbacterial mechanisms of colonization resistance and the strategies of pathogenic strains to exploit gaps in colonization resistance.

INTRODUCTION

The intestinal microbiota is composed of diverse communities of microorganisms that colonize different regions of the gastro-intestinal tract. The composition of these communities varies along the length of the intestine, in addition to variation from the lumen to the mucous and enterocyte barrier.[1] Dramatic transitions in the microbiota occur with age, and a major shift from the microbes present following initial colonization in the neonatal period to the mature state has been well documented.[2–5] Although the microbiota is highly complex, at higher taxonomic levels consistent patterns exist between healthy individuals.[1] For example, the healthy colon is dominated by strict anaerobes in the Bacteroidetes and Firmicutes phyla.[6,7] Furthermore, significant similarity between individuals is observed if diversity is assessed on the basis of the conservation of functional pathways present in the microbiota.[6,8]

Expansion of some opportunistic species including Enterococcus faecalis, Enterococcus faecium and members of the Enterobacteriaceae family that are typically present in low abundance to very high levels can have negative consequences for the host. For pathogenic taxa, such as Salmonella enterica serovar Typhimurium (Salmonella) or Clostridium difficile, expansion of these species in the gut triggers an inflammatory response and diarrhea (reviewed in refs.[9,10]). For other species, including Klebsiella pneumoniae, a member of the Enterobacteriaceae, and Enterococcus faecium, expansion in the gut does not trigger overt intestinal inflammation but is associated with an increased risk for patients to subsequently develop bacteremia.[11,12] Increasingly, the strains that dominate the gut in clinical settings are resistant to one or more antibiotics. Indeed, in the United States, the Center for Disease Control has classified vancomycin-resistant Enterococcus (VRE), carbapenum-resistant Enterobacteriaceae, and extended spectrum β-lactamase producing Enterobacteriaceae (ESBLs) as serious or urgent threats.[13]

In mouse models and in patients, expansion of these potential pathogens can be triggered by disruption of the microbiota. The ability of the healthy microbiota to prevent expansion of these potential pathogens is termed colonization resistance, and is a critical function of the healthy microbiota under homeostatic conditions. The mechanisms of microbiota-mediated colonization resistance are diverse. In general, resistance mechanisms prevent expansion of pathogenic bacteria by either direct, bacteria-to-bacteria pathways, or by activating host immune defenses. Conversely, opportunistic bacterial species that dominate the gut use diverse strategies to exploit breakdowns in colonization resistance, leading to their expansion into newly created niches.

In this review, we examine direct interbacterial mechanisms of colonization resistance, as well as the strategies used by opportunistic species to compete and take advantage of the breakdown in colonization resistance that enables their expansion.

DIRECT MECHANISMS OF COLONIZATION RESISTANCE

The important function of the microbiota in providing colonization resistance has been recognized for decades.[14,15] Indeed, work from Bohnhoff et al. in 1954 demonstrated that antibiotic-mediated disruption of the microbiota leads to a dramatic increase in susceptibility to Salmonella infection.[15] These early pioneering studies and others generated interest in understanding the mechanisms of colonization resistance. Here we highlight some of the recent advances in our understanding of this critical function.
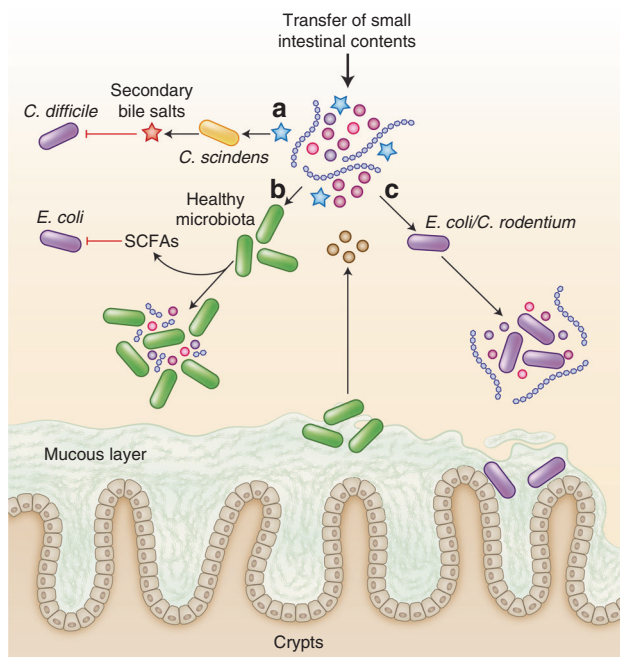
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Fig. 1 Microbiota metabolism contributes to colonization resistance. **a** Members of the microbiota, such as *C. scindens*, convert primary bile salts (blue stars) into secondary bile salts (red stars) that inhibit the vegetative growth of *C. difficile*. **b** The healthy microbiota ferments diet-derived simple sugars (purple/pink circles), complex polysaccharides (blue lines), and microbiota-liberated metabolites from the mucous layer (brown circles) to produce inhibitory SCFA. **c** In the absence of competing strains, *E. coli* and *C. rodentium* utilize the increased availability of simple sugars for replication

Metabolic

Nutrient limitation. When food is consumed, host mechanical and chemical processes function to break down large complexes of protein, fat, and carbohydrates, thereby enabling absorption of nutrients. In addition to host-directed pathways, the microbiota plays an important role in digestion by expressing a complementary suite of catabolic pathways (Fig. 1). Microbiota-dependent digestion supports the growth of the bacterial community and generates byproducts that are utilized by the host, such as short-chain fatty acids (SCFAs). Competition for nutrients in the gut is intense, and evolution has shaped the cross-feeding patterns and substrate preferences of the microbiota to maximize utilization of the available substrates.[16–18] Therefore, under homeostatic conditions, an exogenous species is unlikely to find an uncontested niche, and will be forced to compete with the established microbiota for nutrients.[19,20] Interestingly, the odds of success for an exogenous species in establishing residence in the gut is increased if the microbiota of the gut harbors related taxa to the new species, suggesting that appropriate nutrient conditions exist.[21]

The abnormal expansion of *Citrobacter rodentium* (*C. rodentium*) or *Escherichia coli* (*E. coli*) in the gut is fueled by utilization of simple sugars.[22–24] Kamada et al. found that *C. rodentium* competes for simple sugars with the endogenous microbiota.[22] Furthermore, shifting the diet to only simple sugars forced competition with strains like *Bacteroides thetaiotaomicron* that normally occupy different nutritional niches.[22] *E. coli* is able to utilize a variety of sugars present in the mucous layer simultaneously, including galactose, fucose, mannose, *N*-acetyl glucosamine, arabinose, and ribose,[23] and commensal strains of *E. coli* can provide colonization resistance against pathogenic *E. coli* O157 based on overlapping sugar utilization.[25,26] Together, these results highlight the potential importance of carbon and energy

source availability. However, additional studies are required to determine the contribution of nutrient limitation in the context of a normal diet and a complex, unperturbed microbiota in providing colonization resistance. In line with this, studies in both mice and humans have demonstrated that substrate availability and competition for those substrates along with subsequent microbiota structure is strongly influenced by diet and the presence of appropriate substrate utilization pathways.[27–29]

In addition to competition for carbon and energy sources, invading bacteria compete with the microbiota for trace metals. For example, the probiotic Nissle 1917 *E. coli* strain protects mice from pathogenic *Salmonella* infection in manner that is dependent on iron uptake.[30] Furthermore, zinc uptake pathways in *Vibrio cholerae* and *Campylobacter jejuni* are critical to the ability of these pathogens to compete with the densely colonizing microbiota.[31] The importance of this competition for trace metals is highlighted by observations demonstrating that immunization of mice to generate antibodies against iron-scavenging siderophores is sufficient to reduce the burden of *Salmonella* infection.[32]

Bile salts. Bile salts are secreted into the small intestine by the host and function to increase fat solubilization and digestion.[33] Bile salts are generated in the liver from enzymatic conversion of cholesterol, and the majority are conjugated to glycine or taurine through an amide linkage.[34] The majority of secreted bile salts are reabsorbed in the small intestine; however, unabsorbed bile salts continue to the colon where they are metabolized by the microbiota into secondary bile acids. Microbiota-driven metabolism of bile salts begins with deconjugation from glycine or taurine, in a reaction catalyzed by bile salt hydrolases.[35] These hydrolases are expressed by a large number of taxa within the normal microbiota.[35] Further metabolism of bile salts is encoded in bile-acid-inducible (Bai) operons by a small subset of commensal species of the microbiota that includes *Clostridium scindens* and catalyze 7α/β-dehydroxylation[35,36] (Fig. 1). Interestingly, secondary bile salts have been reported to induce the germination of *C. difficile* spores and inhibit the replication of vegetative cells in a concentration-dependent manner.[37] Transfer of *C. scindens* restores colonization resistance against *C. difficile* in a bile-salt-dependent manner.[38] In agreement, the kinetics of *C. difficile* germination and the initiation of pathology correlate with antibiotic-induced changes in bile salt levels and compositions,[39] and varying antibiotic regimens leading to different bile salt levels correlated with susceptibility to *C. difficile* expansion.[40] The relative resistance of various clinical *C. difficile* isolates to the secondary bile-salt lithocholic acid positively correlates with disease severity in murine infection models.[41,42]

SCFA-mediated inhibition. The dominant phyla of the large intestine, *Bacteroidetes* and *Firmicutes*, are anaerobic bacteria that metabolize diverse substrates including complex polysaccharides, simple sugars, and glycans released from ingested fiber and also host mucus using fermentation pathways.[43,44] This fermentation process leads to the generation of SCFAs (Fig. 1). The most abundant SCFA in the gut are acetate, propionate, and butyrate and the total concentration of SCFA in the proximal colon can reach concentrations of 70–140 mM.[45,46] The majority of the SCFA are produced in the proximal colon and are absorbed by the host to support enterocyte metabolism.[47] In the proximal colon, high levels of SCFA production contribute to acidification of the lumen.[45] For example, using a wireless motility capsule approach in human subjects, Farmer et al. reported average pH values immediately distal to the ileal:caecal junction of 6.16 in healthy individuals.[48] In vitro, numerous studies have demonstrated that SCFAs at an acidic pH are able to inhibit the replication of *E. coli* and *Salmonella*,[49–51] and loss of SCFA production and acidity were correlated with in vivo susceptibility to *Salmonella* following streptomycin treatment.[14] However, other work has argued
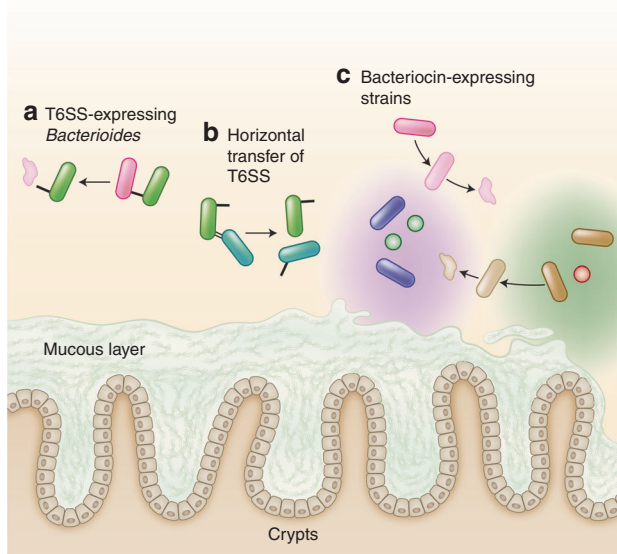
Fig. 2 Mechanisms of active antagonism contribute to colonization resistance: a Constituents of the microbiota can express contact-dependent secretion systems that deliver antagonistic effector proteins. In Gram-negatives, such as *Bacteroides*, delivery is driven by the T6SS, while the Esx system has recently been identified as having an analogous role in Gram-positive bacteria. **b** In *Bacteroides*, the GAI-1 and GAI-2 variants of the T6SS are encoded on conjugative plasmids enabling horizontal gene transfer to other strains in the microbiota. **c** Short peptide antimicrobials, termed bacteriocins, are secreted by the microbiota and have activity against both related non-immune commensal strains and exogenous species

against a significant role for direct SCFA-mediated inhibition in vivo.[19,52] During homeostasis, the production of SCFAs by the microbiota also plays a critical role in maintaining the limited availability of oxygen and nitrates in the lumen of the gut.[53,54] Recent work has demonstrated that butyrate production by *Clostridia* activates PPAR-γ in epithelial cells resulting in the repression of *Nos2* expression, and activation of oxygen-consuming β-oxidation as opposed to anaerobic glycosis.[54–56] Loss of butyrate production triggers increased oxygen and nitrate release into the lumen of the gut, which can be exploited by *Enterobacteriaceae*, as described below. In addition, several studies have demonstrated that the detection of SCFAs triggers a complex modulation of virulence factor expression in *Salmonella*.[57–60] Therefore, the relative contribution of direct SCFA-mediated antagonism to colonization resistance compared with other mechanisms of colonization resistance or, indeed, other indirect effects of SCFAs remains unclear.

Active antagonism
Established members of the microbiota are also able to inhibit the expansion of new species through mechanisms of active antagonism. In the context of an exogenously introduced pathogen, these pathways contribute to colonization resistance. However, in many cases these mechanisms of antagonism can target the same or closely related species, suggesting that, in the absence of exogenous pathogenic bacteria, these pathways function to allow expressing strains to compete with other commensal strains capable of utilizing similar nutrients.

Bacteriocins. Microbiota-derived antimicrobials have been identified that have activity against both Gram-positive and Gram-negative pathogens. The original natural variants of clinical

antibiotics are produced through complex and diverse biosynthetic pathways, and include polyketides (erythromycin, tetracycline),[61] non-ribosomally produced glycopeptides (vancomycin),[61] aminoglycosides (neomycin),[62] and lincosamides (clindamycin).[63] Within the gut, the microbiota-produced antimicrobials are most frequently ribosomally synthesized and post-translationally modified peptides (RiPPs), also termed bacteriocins, which are processed and secreted from the cell[64] (Fig. 2).

Some of the best-characterized bacteriocins are those produced by members of *Lactobacillales* order. Nisin is a prototypical member of the lantibiotic class of bacteriocin that is produced by *Lactococcus lactis* that inhibits a broad range of Gram-positive bacteria including *Staphylococcus aureus*, and *Listeria monocytogenes*.[65] Nisin functions by binding to Lipid II leading to pore formation and has bactericidal activity at nanomolar levels in Gram-positive bacteria where the peptidoglycan layer is exposed.[66] As a result of its potency, Nisin has been used as a food preservative for decades to prevent the expansion of Gram-positive pathogens.[65] Beyond Nisin, bacteriocin production in *Lactobacillales* is widespread. For example, *Lactococcus salivarius* UCC118 production of the bacteriocin Abp118 is able to protect mice from *L. monocytogenes* infection.[67] In comparison to nisin, some bacteriocins target a narrower range of species.[68] Thuricin CD is a bacteriocin produced by commensal *Clostridium thuringiensis* that has potent antimicrobial activity against *C. difficile* but does not target other members of the microbiota including *Bifidobacteria* or *Lactobacillus*.[68]

Although bacteriocins produced by Gram-positive bacteria can have high levels of activity against Gram-positive pathogens, most of these peptides lack significant activity against Gram-negative bacteria. However, recent work examining the bacteriocin NAI-107 found that this lantibiotic has activity against a small number of fastidious Gram-negative pathogens, and synergizes with polymyxin to inhibit a broader range of Gram-negative pathogens including *Klebsiella pneuomoniae*, and *E. coli*.[69] Several groups have also attempted to artificially broaden the range of targets of bacteriocins.[70,71] For example, Nisin that had been complexed to carbon and gold nanoparticles with a net positive charge had increased access to the periplasm of Gram-negative bacteria and antibacterial activity against *E. coli* and *Pseudomonas aeruginosa*.[70] Similarly, fusion of Nisin to truncations of Gram-negative targeting antimicrobial peptides led to increased efficacy against *E. coli*.[71]

Using a bioinformatics approach, Donia et al. demonstrated that a large number of biosynthetic gene clusters are widely distributed among the different microbiota communities, and include pathways for the production of lantibiotics, thiazole/oxazole-modified microcins, and thiopeptides.[72] In support of this bioinformatic data, the authors demonstrated that *Lactococcus gasseri* produces lactocillin, a thiopeptide with activity against a range of Gram-positive pathogens.[72]

Some strains of commensal *Enterococcus* express the bacteriocins AS-48, bac-31, and bac-21 from the pheromone-responsive conjugative plasmids pAD1, pYI17, and pPD1, respectively.[73–75] Conjugative transfer of these plasmids is triggered by recognition of peptide pheromones secreted by recipient cells,[76] which would allow for rapid horizontal propagation of the bacteriocin-producing phenotype. Interestingly, production of the pheromone cOB1 by commensal strains of *Enterococcus* can trigger cell death of VRE strains with cOB1 responsive mobile elements on the pTEF2spc plasmid and an IS-like elements in the main chromosome.[77] In vivo, expression of bac-21 increases the long-term colonization of commensal *E. faecalis* through increased competitiveness with other related strains.[78] Furthermore, the high-density environment of the gut promotes conjugative transfer of bacteriocin-expressing plasmids from positive to negative strains.[78] Finally, by engineering conjugation-defective pPD1 plasmids, Kommineni et al. were able to demonstrate that commensal *E. faecalis* carrying the engineered plasmids is able

to de-colonize mice of pPD1-negative VRE.[78] Potential probiotic *Enterococcus* strains have been identified that express bacteriocins targeting Gram-positive species including *L. monocytogenes*.[79]

The nasal microbiota provides colonization resistance against *S. aureus*, and *Staphylococcus epidermidis* or *Streptococcus mitis* have been negatively correlated with *S. aureus* abundance.[80,81] To examine the mechanism of protection, Zipperer et al. screened nasal isolates for the ability to inhibit *S. aureus* and demonstrated that a commensal *Staphylococus* strain, *Staphylococcus lugdunensis*, is able to directly inhibit the growth of *S. aureus* through the production of a peptide antimicrobial, termed lugdunin.[82] In addition to targeting MRSA, lugdunin also inhibits the growth of *Listeria monocytogenes*, VRE, and *Streptococcus pneuomoniae*.[82]

Members of the Gram-negative *Enterobactericeae* family can produce microcins, a class of short peptide bacteriocins, that have activity against closely related strains of bacteria.[83] A large number of microcins have been identified in commensal strains of *E. coli*, including the probiotic Nissle 1917 strain.[84] Recent work from Sassone-Corsi et al. demonstrated that probiotic *E. coli* Nissle 1917 could compete with both *E. coli* and *Salmonella* in a manner dependent on microcin expression.[85] Interestingly, this successful competition occurred only in inflamed conditions but not in mice treated only with streptomycin. Under inflamed conditions the competition for iron becomes critical for *Enterobacteriaceae* expansion.[30] By linking these observations, Sassone-Corsi et al. demonstrated that microcin expression is induced by iron deprivation, and that microcin-dependent competition in the gut depends on an iron-limited environment.[85]

Antimicrobial proteins. In contrast to shorter peptides, some species of the microbiota also produce larger proteins with antimicrobial activities. For example, some members of the *Bacteroidales*, including *Bacteroides fragilis* and *Bacteroides uniformis*, can produce *Bacteroidales*-secreted antimicrobial proteins that inhibit related species and increase competitiveness in the gut.[86,87] In addition, some *B. fragilis* strains can secrete a ubiquitin-like protein termed BfUbb that has antimicrobial activity against related strains.[88] Similarly, some strains of *Enterobacteriaceae* carry plasmids with genes encoding colicins, a class of antimicrobial proteins that target related species lacking the appropriate immunity proteins.[89] Unlike the best-characterized peptide antimicrobials which often target the cell wall, colicins must be transported by specific receptors on target Gram-negative cells into the periplasmic space and can have diverse activities inside the cell.[89] Interestingly, work examining the dynamics of competition between colicin-resistant, colicin-sensitive, and colicin-producing *E. coli* strains has found that colicin production can actually promote diversity of related strains in the gut as equilibrium is reached.[90]

Type VI secretion systems. Type VI secretion systems (T6SS) are complex structures that allow for export of effector molecules across the inner and outer membranes of Gram-negative bacteria and into adjacent bacteria or eukaryotic cells.[91] Therefore, unlike antimicrobial compounds or toxic byproducts of metabolism, T6SS activity requires cell-to-cell contact for antagonism (Fig. 2). The best-characterized T6SS are those expressed by pathogenic Gamma*proteobacteria*.[92] The delivered effectors antagonize neighboring cells through varied mechanisms of action, including degradation of NAD(P)+ and peptidoglycan hydrolysis.[91,93–95] Critically, T6SS+ strains must encode an immunity protein that negates the activity of the effector to avoid self-targeting.[93]

In healthy adult individuals, *Bacteroidetes* are typically a major constituent of the anaerobic community in the large intestine. Interestingly, orthologs of the well-characterized Proteobacterial T6SS gene clusters are widespread in *Bacteroidetes* taxa,[96] and can be horizontally transferred between strains[97,98] (Fig. 2). T6SS in *Bacteroidales* are present in three forms termed GAI-1, GAI-2, and

GAI-3.[98] GAI-1 and GAI-2 are widely distributed among *Bacteroidales* while GAI-3 is restricted to *B. fragilis*.[98,99] In vivo, an individual's microbiota tends to harbor single variants of GAI-1/GAI-2 immunity/effector genes suggesting that a stable consortium of *Bacteroidales* in a gut share T6SS by horizontal gene transfer and subsequently resist colonization from new strains.[99] On the basis of initial in silico identification of these orthologs, Russell et al. examined the expression of T6SS in vivo and found robust expression of T6SS components by both *B. fragilis* and *Bacteroides eggerthii*.[96] In vitro, *B. fragilis* was able to inhibit the growth of a T6SS- strain of *Bacteroides thetaiotaomicron* in a T6SS-dependent manner.[96] More recent reports have shown that *B. fragilis* T6SS are functional in vivo and mediate competition with related strains including T6SS- *B. fragilis* and *Bacteroides vulgatus*.[100,101] Interestingly, both studies found that target strains with susceptibility to in vitro T6SS-antagonism are not necessarily susceptible to in vivo T6SS-antagonism, and species such as *B. thetaiotaomicron* can be unaffected by the presence of a T6SS-expressing competitor despite in vitro susceptibility.[100,101] Importantly, T6SS-expressing *B. fragilis* can provide colonization resistance against pathogenic enterotoxigenic *B. fragilis* (ETBF).[102]

Recent work from Whitney et al. has demonstrated the existence of a cell-to-cell contact-dependent mechanism of antagonism in Gram-positive bacteria, including in the genomes of gut resident species.[103] In this pathway, Gram-positive bacteria are able to secrete members of the LxG group of proteins, including toxins, through the Esx secretion system.[103,104] For example, TelC is a cell wall targeting effector of the Esx secretion system,[103] and putative NAD(P)+ hydrolyzing toxins with LXG domains have recently been identified.[105] This suggests that, similar to Gram-negative T6SS, contact-dependent secretion systems in Gram-positive bacteria might also secrete a diverse group of effectors. However, it remains unclear if the Esx-type secretion system is capable of delivering toxins across the thick peptidoglycan layer of target Gram-positive cells in a manner analogous to intracellular delivery toxins by a T6SS.[103]

Consortia of bacteria can restore colonization resistance
Under homeostatic conditions, the microbiota is a highly complex community that as a whole provides colonization resistance to exogenously introduced species. Clinically, fecal microbiota transplantation has been used successfully to treat recurrent infection with *C. difficile*.[106,107] Therefore, transferring a complex community of bacteria can restore colonization resistance. As specific pathways of colonization resistance are identified, along with the microbiota constituents responsible for those pathways, it will likely be possible to design minimal consortia of bacteria that provide colonization resistance or clearance against specific clinically relevant species. In agreement, several papers have already identified small consortia that provide full or partial colonization resistance against VRE, *L. monocytogenes*, *Salmonella*, or *C. difficile*.[38,108–112]

STRATEGIES TO PATHOGENS TO EXPLOIT GAPS IN COLONIZATION RESISTANCE
As described above, under steady-state conditions, the healthy microbiota effectively excludes newly introduced potential pathogens through an array of mechanisms. In agreement, clinically relevant expansion of potential pathogens often occurs after the microbiota is perturbed with antibiotics (reviewed in ref. [113]). Unfortunately, common nosocomial pathogens, including members of the *Enterobacteriaceae*, VRE, and *C. difficile* are particularly adept at dominating the gut following perturbation of the microbiota.

Perturbation of the healthy gut microbiota creates an opportunity for expansion, and drives intense competition between related strains to expand into those niches. The idea that species

which expand into dysbiotic gut niches exhibit their own forms of colonization resistance and competition is demonstrated by the observation that initial colonization of the gut by a *Salmonella* strain prevents subsequent establishment of other isogenic wild-type strains.[114] In this section, we examine the strategies utilized by these pathogens to exploit gaps in colonization resistance, compete for newly created niches, and subsequently maintain dominance in the dysbiotic gut.

Antagonism
Recent work has demonstrated that pathogens that expand into dysbiotic conditions utilize a variety of mechanisms to antagonize both the normal microbiota and other species or strains that are also seeking to rapidly expand. For example, as described above, the inflamed gut is an iron-limited environment. This environment can signal to initiate production of microcins in probiotic *E. coli*;[85] however, pathogenic members of the *Enterobacteriaceae* such as *Salmonella* also use these environmental signals to upregulate expression of their own antimicrobial proteins, including Col1b.[115] In line with this, pathogenic strains of *E. coli* causing urinary tract infections carry a greater number of microcin genes, and have higher prevalence of microcin H47 and colicin E1 compared to nonpathogenic *E. coli* strains.[116] The gut microbiota has a high level of colonization resistance against *Listeria* expansion.[109] Likely in response to this selective pressure, epidemic *Listeria* strains have virulence factors that target the microbiota.[117,118] In the gut, *Listeria* expresses Listeriolysin S, which is able to inhibit the growth of members of the microbiota, including *Lactococcus lactis*, in order to promote infection.[117,118]

Many Gram-negative pathogens, including *V. cholerae*, *P. aeruginosa*, and *S. typhimurium*, have T6SS that are expressed during colonization and deliver a wide range of effectors to give a fitness advantage in a dysbiotic gut[119–121] (Fig. 3). Strikingly, among *Proteobacteria* harboring a T6SS and an identifiable

effector, 40% of strains had two effectors with different predicted activities, and 25% had three or more effectors.[122] Work from LaCourse et al. found that *P. aeruginosa* secretes multiple effectors whose activities can be both synergistic and dependent on environmental conditions.[122] In addition to its well-characterized Type III secretion systems SPI-1 and SPI-2, recent work has demonstrated that *Salmonella* also utilizes a T6SS to promote its expansion in the gut.[121] The *Salmonella* T6SS is able to target commensal strains in vitro and ΔT6SS strains are deficient in in vivo colonization.[121] Both *Salmonella* and *Pseudomonas* tightly regulate the activity of the T6SS and recognize cues present in the gut to activate the T6SS. These cues include both host-derived factors such as bile salts, as well as cell stress or kin cell lysis.[120,121,123]

Exploitation of nutrients available during inflammation and dysbiosis
Enterobacteriaceae can rapidly expand following initiation of either dysbiosis or inflammation. In 2007, two reports demonstrated that *Salmonella* and *Citrobacter* both drive host-driven inflammation in order to expand to high levels and compete with the microbiota.[124,125] Reducing inflammation using avirulent *Salmonella* or chemically inducing inflammation with DSS is sufficient to prevent or enable expansion in the gut, respectively.[124,125] Subsequent work has found that the ability to utilize nutrients uniquely present in these conditions is essential for *Enterobacteriaceae* (Fig. 3). Importantly, and unlike the anaerobic fermenting bacteria of the microbiota, *Enterobactericeae* are able to perform both aerobic and anaerobic respiration, utilizing either O_2 or other molecules as terminal electron acceptors in a membrane electron transport chain that generates proton motif force to produce ATP.

Two critical components of the inflammatory response to infection are the activation of inducible nitrate oxide synthase, iNOS, encoded by *Nos2*, and NADPH oxidase encoded in part by $gp91^{phox}$. iNOS generates nitric oxide through the oxidation of L-arginine and nitric oxide,[126] and NADPH oxidase generates reactive oxygen species.[127] The production of these reactive oxygen and nitrogen species has long been recognized as two of the primary antibacterial effector mechanisms of the myeloid compartment.[128] Importantly, the release of these reactive molecules also creates an oxidative environment, and reactive oxygen and nitrogen species can oxidize molecules present in the gut.

Tetrathionate was the first inflammation-associated terminal electron acceptor identified for *Salmonella*.[129] Although tetrathionate is not present in the healthy gut, the microbiota produces hydrogen sulfide which is converted to thiosulfate by the host.[130] iNOS-generated nitric oxide is capable of oxidizing thiosulfate to tetrathionate in the lumen.[129] In this environment, *Salmonella* capable of respiring tetrathionate outcompete respiration-deficient mutants.[129] The presence of tetrathionate as an anaerobic electron acceptor also enables *Salmonella* to utilize ethanolamine as a carbon source both in vivo and in vitro.[131,132]

Released nitric oxide can also react to form nitrate, and both NO and NO_3 increase in the gut lumen during inflammation.[133,134] *E. coli* are able to utilize nitrates, S-oxides, and N-oxides as terminal electron acceptors under anaerobic conditions through the production of molybdenum-containing reductases.[135] As a result, *E. coli* with nitrate reductase activity have a competitive advantage over deficient strains in conditions of intestinal inflammation in DSS-treated or Il10$^{fl/fl}$ CD4-cre mice, as well as in mice with low-level inflammation following streptomycin treatment.[54,133,136]

The importance of anaerobic electron acceptors for *Salmonella* replication is highlighted by the observation that *Salmonella* expresses two chemotactic receptors Aer and Tsr that enable chemotaxis toward nitrate and tetrathionate in vitro and are critical for *Salmonella* to take advantage of the available electron acceptors in vivo.[137,138] Furthermore, treatment with tungsten to
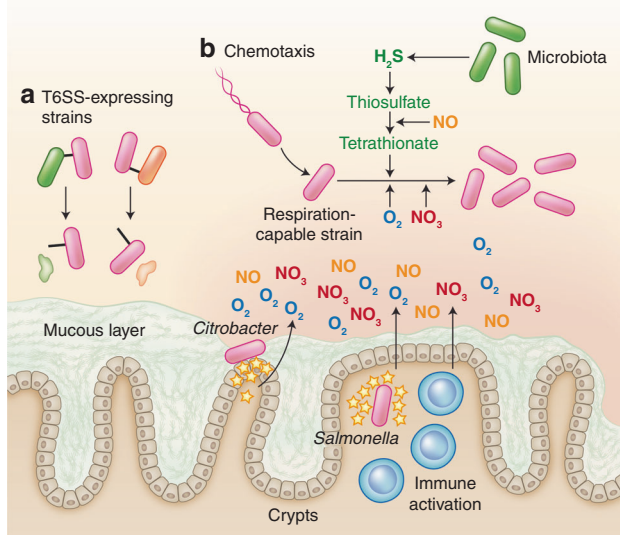
Fig. 3 Expansion and engraftment strategies of invading species. **a** Some pathogens, such as *Salmonella* and *P. aeruginosa*, target either the microbiota or other opportunistic species through using T6SS's and bacteriocin production. **b** Pathogens can drive host inflammatory responses (yellow stars) in order to trigger the release of nitrates or oxygen into the lumen of the gut (shaded gradient), and the formation of an oxidative environment. Together with novel metabolites formed by oxidation, such as tetrathionate, O_2 and NO_3 are used in anaerobic and aerobic respiration pathways by pathogens able to use these electron acceptors

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inhibit the formation of the molybendum cofactor that is necessary for anaerobic reductases counters the competitive advantage gained by *Enterobacteriaceae* in the inflamed gut environment.[139]

The oxidative environment generated by iNOS also leads to the oxidation of carbon sources in the gut and generation of carbon sources that are absent under homeostatic conditions.[140] In an in vitro assay, reactive nitrogen oxidizes galactose to galactarate and glucose to glucarate. In vivo, galactarate and glucarate increase in the lumen following streptomycin treatment, and this increase is *Nos2* dependent.[140] Both *E. coli* and *S. Typhimurium* are able to use glucarate and galactarate as carbon and energy sources, and mutants that are deficient in this pathway are outcompeted in the gut following antibiotic treatment.[140]

As described above, the normal microbiota plays an essential role in maintaining an anaerobic environment in the gut and depletion of *Clostridia* taxa leads to increased oxygen availability in the lumen of the gut.[53,54] Under microaerophilic conditions, *Enterobacteriaceae* express cytochrome oxidase variants with increased oxygen affinity that enable them to utilize oxygen as a terminal electron acceptor at low oxygen tensions.[141] *Salmonella* and *E. coli* with the ability to utilize oxygen in these conditions have a competitive advantage compared to deficient strains.[53,54] Similarly, *Citrobacter* actively promotes oxygenation of the lumen by anchoring to enterocytes and promoting crypt hyperplasia using T3SS-delivered effectors.[142] This functions to create an inflammatory environment where wild-type strains able to utilize oxygen outcompete deficient strains.[142] Together, the presence of both oxygen and other anaerobic electron acceptors promotes *Enterobacteriaceae* expansion. Indeed, the loss of both pathways triggers a synergistic colonization defect in *E. coli*,[54] and both pathways enable the usage of alternate carbon sources including ethanaloamine or 1,2-propanediol.[131,143]

Disruption of the microbiota can trigger transient increases in metabolites that are normally present but typically at low levels owing to utilization by the microbiota.[144–147] For example, both *Salmonella* and *C. difficile* can utilize sialic acid that is cleaved from host mucus by microbiota-encoded sialidase enzymes during their enteric expansion.[144,148] Alternatively, *C. difficile*, *C. rodentium*, and *Salmonella* can utilize microbiota-derived succinate during expansion.[145,147,149] Work from Maier et al. found that *Salmonella* is able to utilize microbiota-generated H_2 as an electron donor in the anaerobic gut to drive anaerobic respiration.[150] In addition, *Salmonella* can also utilize fucose that is liberated in a microbiota-dependent manner.[144] Fucose is a deoxyhexose sugar that is produced by the mammalian host, and, in the adult gut, glycans containing α(1-2)-linked fucose are exposed to the intestinal lumen.[151] Members of the microbiota, such as *Bacteroides acidifaciens* or *Bacteroides thetaiotaomicron*, can cleave fucose from the conjugated proteins, thus enabling utilization by the microbiome.[152,153] Following LPS stimulation of the immune system, fucosylation in the gut is increased in an *fut2*-dependent manner.[152] Unexpectedly, increased availability of fucose increases tolerance of *C. rodentium*, without decreasing pathogen burden and correlating with reduced virulence gene expression.[152] As described above, *Citrobacter* actively drives inflammatory responses partly in order to support its oxidative metabolism. Therefore, the release of fucose by fucosidase activity of the microbiota could provide substrates for *Citrobacter*, counteracting the importance of pathogen-driven intestinal inflammation. Overall, pathogens that expand in the gut are well adapted to take advantage of transient increases in microbiota-derived metabolites to fuel their expansion.

CONCLUSIONS
Under homeostatic conditions, the normal constituents of the gut microbiota exist in a constant state of competition for nutrients and niches. The intense competition that results has likely driven evolution of the bacteria present in this environment to both cooperate and compete. In most cases, the mechanisms of colonization resistance against pathogenic species described in this review are also active against commensal members of the microbiota and likely help to shape the equilibrium that exists between these species. Therefore, a newly introduced potential pathogen encounters a pre-existing web of interacting bacteria occupying all available niches and successfully competing with related strains. As a result, in most cases, the microbiota provides effective colonization resistance, and newly introduced pathogens are unable to gain a foothold and expand. If they are able to gain a foothold in the gut, often due to perturbation of the microbiota, pathogens that can expand in that environment use a similar collection of antagonistic pathways as the microbiota in addition to metabolic flexibility to successfully engraft.

In this review, we have focused on interbacterial mechanisms of colonization resistance; however, fungi, protozoa, helminths, and bacteriophage can also be present in the gut. Recent studies have highlighted the fact that non-bacterial gut residents including protists and helminths can influence mucosal immune responses or cell differentiation in the epithelial barrier,[154–156] and it has been suggested that bacteriophage may play an important role in shaping the bacterial community.[157,158] Therefore, it will be interesting to determine to what extent these non-bacterial gut residents contribute to colonization resistance through either direct or indirect mechanisms.

A potential layer of complexity in understanding colonization resistance is that the distribution of bacteria in the gut is not homogenous,[159] and, therefore, the distribution of different effector mechanisms of colonization resistance is also likely to vary. To understand how potential pathogens try to establish themselves within the web of the microbiota, it will also be critical to understand the spatial organization of the gut microbiota and how the pathways of metabolic competition or exclusion and antagonism are distributed across the normal biogeography of the gut. Understanding the arrangement of both pathogens and the recovering microbiota following antibiotic treatment will likely be critical in predicting colonization outcomes. For example, our laboratory has demonstrated that *Klebsiella* and VRE reside in close proximity to the mucous layer, and that despite being physically close, *K. pneuomoniae* and VRE occupy different niches in the mouse gut.[160] Recent work from Whitaker et al. demonstrated an elegant system for engineering *Bacteroides* species to express fluorescent proteins driven by promoters with varying activities to allow for the simultaneous imaging of multiple species in the gut.[161] These engineered strains can be coupled with high-throughput imaging platforms designed for the microbiota.[162] Expansion of these and similar techniques to other phyla will likely play a critical role in understanding colonization resistance in the complex spatial organization of the gut.

The multiple mechanisms of colonization resistance can function simultaneously in the gut. As a result, evaluating the relative importance of each individual pathway is difficult in this environment. While gene knockout strategies have been widely utilized in Gram-negative members of the microbiota, genetic manipulation of the Gram-positive taxa common in the gut remains more difficult. Progress has been made in improving the genetic tractability of Gram-positive members of the microbiota.[163–165] Alternatively, pathways and genes of interest from the microbiota can also be expressed in genetically tractable species.[166] Recent work from Lim et al. introduced a system that allows tunable expression of a desired protein in *Bacteroides* in vivo.[167] Overall, these advances in genetic manipulation of the microbiota will enable studies with microbiota compositions either lacking or overexpressing different components of colonization resistance.

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