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REVIEW

Mechanisms of Synergy in Polymicrobial Infections

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Communities of microbes can live almost anywhere and contain many different species. Interactions between members of these communities often determine the state of the habitat in which they live. When these habitats include sites on the human body, these interactions can affect health and disease. Polymicrobial synergy can occur during infection, in which the combined effect of two or more microbes on disease is worse than seen with any of the individuals alone. Powerful genomic methods are increasingly used to study microbial communities, including metagenomics to reveal the members and genetic content of a community and metatranscriptomics to describe the activities of community members. Recent efforts focused toward a mechanistic understanding of these interactions have led to a better appreciation of the precise bases of polymicrobial synergy in communities containing bacteria, eukaryotic microbes, and/or viruses. These studies have benefited from advances in the development of *in vivo* models of polymicrobial infection and modern techniques to profile the spatial and chemical bases of intermicrobial communication. This review describes the breadth of mechanisms microbes use to interact in ways that impact pathogenesis and techniques to study polymicrobial communities.

Keywords: polymicrobial, synergy, infection, metatranscriptomics, quorum sensing, imaging mass spectrometry, 3D printing

Introduction

Virtually no microbes live in isolation. Instead, they inhabit complex polymicrobial communities where interactions between individuals shape the composition and biological activities of the population. Whether in the environment or associated with the human body, microbial communities are often highly diverse. For instance, a gram of soil is estimated to contain more than one million different bacterial species, while the human gut and oral microbiota contain between 700 and 1000 bacterial species, with a single individual carrying over 150 species (Gans *et al.*, 2005; Paster *et al.*, 2006; Qin *et al.*, 2010). As in macroscopic communities, interactions between individuals within polymicrobial communities play an integral part in shaping the landscape of the environments they inhabit.

While the idea that microbes inhabit diverse communities in nature has been appreciated since the time of Pasteur (Pasteur and Joubert, 1877), most laboratory studies have focused on a single microbe grown in isolation. This reductionist approach has proven powerful in the study of microbial pathogenesis, where molecular and biochemical studies have enhanced our knowledge of single species pathogenesis both *in vitro* and in model infections. The guiding principles of these studies are the famous Koch's postulates, which specify criteria for declaring a pathogen responsible for a disease: the putative pathogen must be isolated and cultured from a diseased host; when introduced into a model host it must replicate the disease; and the same putative pathogen must be isolated and cultured from this model infection (Koch, 1882). However, it is increasingly clear that these criteria are insufficient to identify the microbial bases for a number of infectious diseases (Table 1). While this is due in part to difficulty in cultivating many human-associated microbes, it is also likely that for many of these diseases there is no single etiological agent responsible for pathogenesis.

The realization that not all infections are caused by a single species has proved extremely valuable in furthering our understanding of the nature of these polymicrobial infections. Among the most significant advances made under this framework is the appreciation that polymicrobial infections are often worse than similar monomicrobial infections, and can display enhanced pathogen persistence in the infection site, increased disease severity, and increased antimicrobial resistance (Kaplan *et al.*, 1989; Chen *et al.*, 1996; Kesavalu *et al.*, 1998; Nagashima *et al.*, 1999; Ramos *et al.*, 2001; Brogden and Guthmiller, 2002; Bakaletz, 2004; Kozarov *et al.*, 2005; Mastropaolo *et al.*, 2005; Dalton *et al.*, 2011; Peters *et al.*, 2012a), in a phenomenon known as polymicrobial synergy. The term 'synergy' was initially applied to microbiological systems in 1924 by Kämmerer (1924), defining synergy as 'the sum activity of two or more microbes'. This definition was expanded on in 1982 by Bjornson to define synergy as a 'cooperative interaction of two or more bacterial species that produces a result not achieved by the individual bacteria

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Table 1. The utility of Koch's postulates in determining an etiology for infectious diseases

Infections for which Koch's postulates apply	References	Infections for which Koch's postulates do not apply	References
Tuberculosis	Koch (1882)	Wound infections	Koch (1878), Price *et al.* (2009), Percival *et al.* (2010), Dalton *et al.* (2011), Peters *et al.* (2012a), Korgaonkar *et al.* (2013)
Anthrax	Koch (1876)	Periodontitis	Kesavalu *et al.* (1998), Socransky *et al.* (1998), Yamada *et al.* (2005), Orth *et al.* (2011)
Cholera	Koch (1893)	Cystic fibrosis	Rogers *et al.* (2004), Sibley *et al.* (2008b), van der Gast *et al.* (2011), Moree *et al.* (2012), Nelson *et al.* (2012), Zhao *et al.* (2012)
Typhoid fever	Eberth (1881), Gaffky (1884)	Pneumonia	Craven and Steger (1995), Safdar *et al.* (2005), Wong *et al.* (2013)
Polio	Rivers (1937)	Otitis media	Bakaletz (2009), Armbruster *et al.* (2010), Laufer *et al.* (2011)
SARS	Fouchier *et al.* (2003)	Peritonitis	Holley *et al.* (1992), Mashburn *et al.* (2005)
Malaria	Laveran (1880), Ross (1898)	Device-related infections	Darouiche (2001), Costerton *et al.* (2005), Lynch and Robertson (2008)
Gastric cancer	Watanabe *et al.* (1998)	Vaginosis	Darouiche (2001), Ling *et al.* (2010), Twin *et al.* (2013)
Mad cow disease	Aguzzi *et al.* (2008)	Urinary tract infections	Ronald (2002), Aguzzi *et al.* (2008)
Rice seedling blight	Partida-Martinez and Hertweck (2005)	Abscesses	Nagashima *et al.* (1999), Ramsey *et al.* (2011)

acting alone' (Bjornson, 1982). In regards to infection, this 'result' refers to enhanced disease symptoms. Based on recent data that not all interactions leading to synergy in infections are cooperative and that synergy is not exclusive to bacteria, we propose a definition for synergy during infection as 'an interaction of two or more microbes in an infection site that results in enhanced disease compared to infections containing the individual microbe acting alone'.

It is important to note that not all polymicrobial infections display synergy, a distinction noted by Rotstein *et al.* in an excellent review of polymicrobial surgical infections in 1985 (Rotstein *et al.*, 1985). Here, we consider three types of polymicrobial infections: (1) those that result from changes in the relative composition of individual species comprising the microbiota (referred to as dysbiosis, e.g., periodontitis) (Domann *et al.*, 2003; Imirzalioglu *et al.*, 2008; Ling *et al.*, 2010; Ravel *et al.*, 2011; Wang *et al.*, 2013), (2) those that result when a pathogenic microbe colonizes an infection site already containing commensal microbes (e.g., cholera), and (3) those that result when microbes colonize a body site they normally do not inhabit (e.g., chronic wound infections) (Dymock *et al.*, 1996; Marra *et al.*, 2005; Price *et al.*, 2009). In this review, we describe the range of mechanisms that can contribute to polymicrobial synergy in infection and modern techniques to manipulate and profile polymicrobial communities.

Etiology of polymicrobial infections

Even during the time of Koch, the polymicrobial nature of certain infections was not unappreciated. In 1890, the microbiologist Miller noted bacterial cell morphologies under the microscope that were not cultivable from patient samples (Miller, 1890). In this study, Miller attempted to culture pathogenic bacteria from the gangrenous pulp of an oral infection. First, he inoculated pus into mice, transferred the pus over the course of several generations from one mouse into another, and then, in accordance with Koch's postulates, cultured the bacteria from the infection (Miller, 1890). However, he noted that when he infected a mouse with only the cultivable bacteria, the infection was less severe than when he infected them with pus directly. This result suggested to Miller that multiple species, some perhaps uncultivable, could contribute to pathogenesis in an infection. Yet for many decades, the microscope was the best tool available to survey uncultivable microbes. It was not until nearly a century later when the advent of sequence-based methods for identifying and quantifying bacteria in natural populations heralded a new culture-independent molecular era in microbial ecology, both outside and inside the human body (Pace *et al.*, 1986).

Metagenomic analyses of disease-associated microbial populations have been instrumental in our understanding of the microbial ecology of the human body during infection. One disease for which this is especially true is the human oral disease periodontitis. Sequence-based diversity profiling methods have revealed extensive microbial diversity associated with both health and disease in the subgingival crevice (the site of infection in periodontitis) (Socransky *et al.*, 1998; Paster *et al.*, 2001). Similar studies of chronic lung infections in cystic fibrosis (CF) patients, which had long been associated with only a handful of pathogens, including *Burkholderia cepacia*, *Haemophilus influenzae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (Rogers *et al.*, 2003), have revealed a highly diverse microbial community including many obligate anaerobes, suggesting the presence of previously unappreciated nutritionally, distinct microenvironments in infection (Rogers *et al.*, 2003, 2004; Sibley *et al.*, 2008b; van der Gast *et al.*, 2011; Zhao *et al.*, 2012). Ultimately, through ambitious collaborative efforts, such as the Human Microbiome Project (Turnbaugh *et al.*, 2007), we will have a full picture of the microbial diversity at healthy and diseased sites across the human body at single base pair resolution. Yet just as the cataloging of animal specimens for a natural history museum cannot reveal how key interactions between different species impact ecosystem function in nature, metagenomic studies of polymicrobial infections must be coupled with further study both in model infections and "in the wild" (i.e., in human infections).

Metatranscriptomics - behavior in the wild

Modern metagenomic sequencing allows us to understand

diversity in complex microbial ecosystems at the genomic scale. Yet advances in sequencing technology allow us to profile the transcriptional activity of members of those ecosystems as well. All cells respond to their environment through transcription regulation, and the bacteria that inhabit infections are no exception. Sequencing of RNA-derived cDNA (RNA-seq) provides a highly sensitive assessment of the transcriptional activity of each member of the microbial community within an infection site. Initially applied to environmental samples such as ocean water (Frias-Lopez *et al.*, 2008), this approach has proven highly successful in profiling gene expression in disease-associated microbial populations. A recent study profiled both gene expression and genetic diversity from bacteria, viruses, and the human host in CF patients with chronic lung infections, and found that while taxonomic diversity is high between patients, the metabolic capabilities and activities of the microbial community as determined by RNA-seq is highly conserved (Lim *et al.*, 2012).

A key challenge in applying transcriptional profiling experiments to the discovery of common features in polymicrobial infections lies in identifying appropriate healthy control samples against which to compare gene expression in disease, especially in infections resulting from dysbiosis of the normal flora. This difficulty arises from two complementary factors: (1) the high person-to-person diversity in microbiota composition, and (2) the often progressive nature of these diseases. A recent study on the dysbiotic disease bacterial vaginosis (BV) makes an important first step towards addressing these concerns (Macklaim *et al.*, 2013). The authors of this study used RNA-seq to profile gene expression and abundance of vaginal flora microbes in both healthy and diseased individuals. They found that the transition from acidic conditions in the healthy vagina to basicity in BV was associated with decreased expression of lactate fermentation genes and increased expression of short-chain fatty acid fermentation genes at the community level. Importantly, while the identity of certain members of the healthy and diseased vaginal ecosystems varied between individuals, the function that those members fulfilled in the community (i.e., expression of fermentative pathway genes) remained constant. This phenomenon may underlie the high species-level diversity found in other dysbiotic diseases as well. This does not address whether certain species or disease-associated gene expression profiles are a cause or an effect of disease, however. We have not discussed this issue here, as it is complex and has been addressed in a recent review (Zhao, 2013).

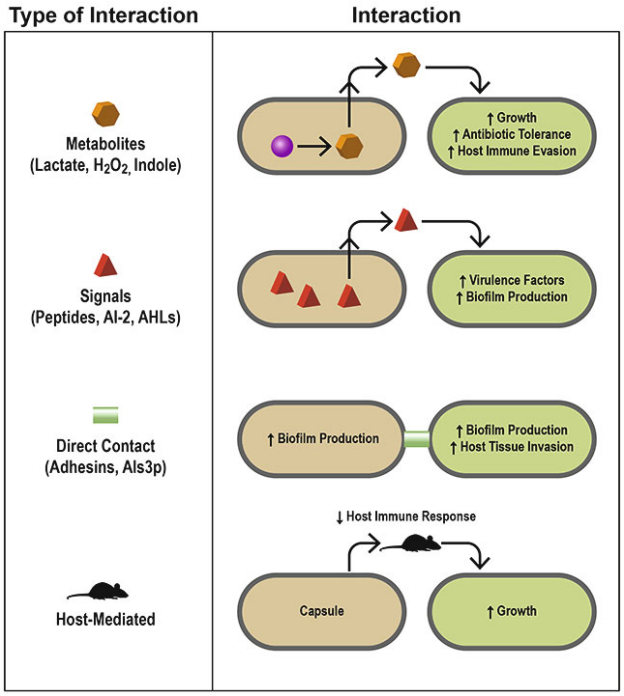

Fig. 1. Mechanistic bases of polymicrobial interactions. Different interactions discussed in this review are summarized here. The left column lists the types of interactions and how they are mediated with specific examples, some of which are discussed in the text. The right column demonstrates how the interaction occurs and the response of the different microbes involved and the infected host. Signals, proteins, metabolites, and even the host immune system serve as liaisons between different microbes allowing complex interactions to occur that impact the environments in which they live. During infection, these interactions ultimately lead to polymicrobial synergy and are therefore detrimental to the host (AI-2, autoinducer-2; AHLs, acyl-homoserine lactones).

Cues and signals that promote synergy

Metagenomic, metatranscriptomic, and ecological studies have suggested that polymicrobial communities exhibit complex, multispecies metabolic interactions and that the structure of these "food webs" can serve as markers for health and disease. These interactions have been shown to ultimately impact synergistic pathogenesis in a number of cases, largely in studies of model two-species communities (Fig. 1). Recent work has shown that metabolite cross-feeding, in which a bacterium within a population consumes the metabolic end-products produced by a second community member, can mediate synergy in multi-species infections including those caused by the oral pathogen *Aggregatibacter actinomycetemcomitans* (Brown and Whiteley, 2007; Ramsey and Whiteley, 2009; Ramsey *et al.*, 2011). *A. actinomycetemcomitans* is a member of the oral microbiota and an opportunistic pathogen often found in high abundance in patients with localized aggressive periodontitis. Most infections caused by *A. actinomycetemcomitans* are polymicrobial, and common co-infecting bacteria are the commensal oral streptococci including *Streptococcus gordonii*. Interestingly, *A. actinomycetemcomitans* preferentially catabolizes lactate over other carbon sources despite its ability to grow faster and reach higher cell densities when growing on carbohydrates (Brown and Whiteley, 2007). Since *S. gordonii* as well as many other oral streptococci produce large amounts of lactate as the primary metabolite during catabolism of carbohydrates, it was hypothesized that *A. actinomycetemcomitans* is cross-feeding on *S. gordonii*-produced lactate during co-culture infection. To test this hypothesis, an *A. actinomycetemcomitans* strain unable to catabolize lactate was constructed by disruption of the enzyme lactate dehydrogenase, and polymicrobial synergy was assessed using a murine abscess model of infection. While the *A. actinomycetemcomitans* mutant unable to catabolize lactate was able to form mono-culture infections

similar to that observed with the wild-type bacterium, the inability of this mutant to utilize lactate eliminated synergy in co-culture infections (Ramsey *et al.*, 2011). These results demonstrate that cross-feeding is critical for *A. actinomycetemcomitans* synergy during co-culture infection with *S. gordonii*, supporting the idea that metabolites secreted by commensal bacteria impact virulence of opportunistic pathogens.

While metabolites are clearly cues that impact synergy in polymicrobial infections, dedicated signaling systems are also important. Many bacteria have evolved mechanisms to communicate and coordinate their activities, a process referred to as quorum sensing. The quorum sensing paradigm involves secretion of low molecular weight signals that are produced at constant levels by bacteria during growth. As the population density increases, the levels of signal increases proportionally to the number of bacteria. At a specific density, the signal induces transcriptional changes that allow bacteria to coordinate their activities as a group. Most studies have focused on quorum sensing as a means for individual bacterial species to modulate their behavior, although it is now clear that other bacteria within polymicrobial communities may 'eavesdrop' and respond to these signals in defined ways. For example, it was recently demonstrated that several streptococcal species can sense and respond to quorum-sensing signals produced by other species (Cook *et al.*, 2013). This study examined quorum sensing in group A streptococci (GAS), group B streptococci (GBS), and *Streptococcus dysgalactiae* subsp. *equisimilis*. These streptococci produce peptide signals 22–23 amino acids long that share 55–86% identity. Since these bacteria often reside in the same location within the host, the authors hypothesized that these signals may facilitate bidirectional communication between these distinct *Streptococcus* species. To test this hypothesis, expression of known quorum sensing-controlled genes was assessed in the presence of peptide signals produced by other species. The result of these studies revealed each of these streptococci can perceive these non-cognate signals and that upon sensing these signals, GAS enhances biofilm formation. This latter finding suggests that the ability to perceive non-cognate signals may have relevance during infection.

Interspecies quorum sensing-mediated communication also occurs between the opportunistic pathogens *Haemophilus influenzae* and *Moraxella catarrhalis* in otitis media infections. A recent study demonstrated a beneficial relationship between these two organisms that leads to robust biofilm formation and antibiotic resistance (Armbruster *et al.*, 2010). These phenotypes are mediated by the quorum sensing signal autoinducer-2 (AI-2). In monoculture, even though it cannot produce AI-2, *M. catarrhalis* responds to an exogenous AI-2 precursor and produces thicker biofilms as a result. *H. influenzae* produces AI-2 in co-culture with *M. catarrhalis*, which promotes biofilm thickness and therefore antibiotic resistance in *M. catarrhalis*. In order to test the effects of this relationship *in vivo* this study employed a chinchilla otitis media model of infection. The bacterial loads of *M. catarrhalis* increased significantly in the presence of *H. influenzae* during co-infection, but these effects were abolished during *M. catarrhalis* co-infection with a *luxS* mutant strain of *H. influenzae*, which cannot produce AI-2. Recent studies also show that *P. aeruginosa* responds to AI-2 producing oropharyngeal flora which co-infect the CF lung (Duan *et al.*, 2003). Similar to *M. catarrhalis*, *P. aeruginosa* is incapable of generating AI-2 but has the capacity to sense and respond to this signal. Co-culture with AI-2-producing oropharyngeal flora resulted in enhanced production of several *P. aeruginosa* virulence factors. These studies indicate that in addition to metabolites, quorum sensing signals are also important chemical mediators of synergy in polymicrobial infections.

Promoting synergy by enhancing resistance to the immune system

In addition to metabolites and quorum signals, microbes in complex communities are exposed to a range of small molecules produced by their microbial neighbors, including antimicrobials. While microbiologists have traditionally considered antimicrobials as defensive agents produced by other microbes, a recent study of *A. actinomycetemcomitans* and *S. gordonii* showed that they can also serve as cues to promote synergy by stimulating resistance to the immune system. In addition to lactate, *S. gordonii* produces high levels of the antimicrobial hydrogen peroxide (H_2O_2) during normal growth. While *A. actinomycetemcomitans* is relatively resistant to H_2O_2 due to its production of the H_2O_2-degrading enzyme catalase, this bacterium displays a unique transcriptional response to H_2O_2. Upon exposure to H_2O_2, *A. actinomycetemcomitans* enhances expression of *apiA,* a gene that encodes an outer membrane protein involved in evasion of the immune response (Ramsey and Whiteley, 2009). ApiA functions by binding to the human serum protein Factor H, which protects *A. actinomycetemcomitans* from killing by inhibiting the alternative pathway of complement activation (Asakawa *et al.*, 2003). These results demonstrate that *A. actinomycetemcomitans* resists killing by host innate immunity during co-culture and suggest that this bacterium uses a streptococcal antimicrobial as a cue to an impending immune response.

In addition to stimulating resistance of a pathogen to the immune system, synergy can also result via suppression of the immune system by commensal bacteria. Urinary tract infection (UTI) is a prevalent human infectious disease that primarily affects women. The most common cause of UTI is ectopic colonization of the urinary tract by the gut commensal bacterium uropathogenic *Escherichia coli* (UPEC). Once in the urinary tract, UPEC encounters a variety of commensal bacteria including the opportunistic pathogen GBS. GBS are common members of the gastrointestinal and urinary tract microbiota and do not cause infection in immunocompetent individuals. Recently, Kline *et al.* (2012) reported that co-inoculation of UPEC with GBS in a mouse UTI model resulted in enhanced survival of UPEC in the bladder lumen despite the rapid clearance of GBS from these infections. Interestingly, both the TLR4-driven host response and GBS capsular sialic acid residues were required for this effect, suggesting that the GBS capsule enhances UPEC survival in the bladder via modulation of the host innate immune response to UPEC lipopolysaccharide. The

authors propose that by suppressing the immune response, GBS promotes a more hospitable growth environment for pathogens such as UPEC. Thus, during infection, the host can be an active participant in synergistic polymicrobial interactions.

Promoting synergy via direct contact

Microbes not only interact through chemicals, but also through direct contact. This has been extensively studied in the human oral cavity, where direct interactions between oral community members are essential for the formation of biofilms (Yamada *et al.*, 2005; Zhu *et al.*, 2013). These interactions are mediated by membrane-bound structures, known as adhesins, that recognize specific receptors on microbial or host-associated surfaces (Kolenbrander and London, 1993). Adhesins often facilitate binding between different genera, and historically, these partnerships have been discovered through *in vitro* coaggregation assays (Kolenbrander *et al.*, 2010), wherein two species are said to coaggregate if they can be made to fall out of suspension by vortexing. To date, every oral bacterial strain tested [~1,000 (Gordon and Pesti, 1971)] has been found to coaggregate with at least one partner, suggesting that intergeneric binding is a beneficial trait for life in the oral cavity. While these partnerships are often highly specific, a notable exception is the *Fusobacteria*, which can attach to many different members of the oral community. Thus, species such as *F. nucleatum* are often described as "bridging organisms" because they potentially bring community members together that normally cannot bind to each other (Kaplan *et al.*, 2009). Microscopic examination of dental plaque has revealed that intergeneric partnerships also form *in vivo* (Valm *et al.*, 2011; Schillinger *et al.*, 2012) (Fig. 2), so a major biological role for coaggregation is likely to promote mixed-species biofilm development. For example, the periodontal pathogens *Porphyromonas gingivalis* and *Treponema denticola* are known coaggregation partners (Grenier, 1992), and when co-cultured in a flow cell, they form morphologically distinct, thicker biofilms than when cultured alone, a phenotype that depends on expression of the *P. gingivalis* gingipains, extracellular proteolytic enzymes with adhesin domains (Chen and Duncan, 2004; Yamada *et al.*, 2005; Orth *et al.*, 2011; Zhu *et al.*, 2013). Interestingly, *P. gingivalis* and *T. denticola* display synergistic virulence in both murine periodontitis (Orth *et al.*, 2011) and lesion (Kesavalu *et al.*, 1998) models, and in the lesion model, removing expression of trypsin-like protease activity in *P. gingivalis* mitigates synergistic virulence with *T. denticola* (Kesavalu *et al.*, 1998). These results are intriguing, but further experiments are required to discern a direct role for receptor-mediated coaggregation in stimulating *P. gingivalis-T. denticola* virulence.

Direct interactions are also critical for synergy in infections caused by the bacterium *S. aureus* and the opportunistically pathogenic fungus *Candida albicans*. These microbes are often co-isolated from both chronic and acute infections, where they can physically interact. An investigation of the mechanisms of adhesion between these two organisms in co-culture led to the discovery of a specific *C. alibicans* protein, Als3p, involved in mediating direct interactions with *S. aureus* (Peters *et al.*, 2012b). After proteomic screening for differentially expressed proteins involved in biofilm formation in co-culture, it was confirmed that Als3p mediates the adherence of *S. aureus* to *C. albicans* hyphae. Scanning electron and confocal microscopy visually demonstrated a lack of adherence between *S. aureus* and a *C. albicans* mutant that cannot produce Als3p. A mouse tongue epithelium *ex vivo* model was then used to test the effect of Als3p on colonization and penetration into the mouse tongue subepithelium by co-infecting with *S. aureus* and the wild-type *C. albicans* or the Als3p mutant *C. albicans*. Interestingly, both wild-type and mutant *C. albicans* were able to penetrate into the subepithelium, but *S. aureus* was seen in the subepithelium only when adherence to *C. albicans* was possible through functional Als3p.-Because *S. aureus* infections usually proceed via tissue invasion, this is an example of polymicrobial synergy that leads to increased *S. aureus* virulence in co-culture with *C. albicans*. Other studies have shown that in co-culture with *C. albicans* in a biofilm, *S. aureus* is more antibiotic resistant, though the mechanism responsible is not fully elucidated (Harriott and Noverr, 2009).

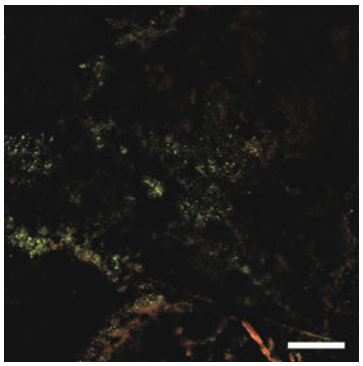

Fig. 2. Confocal micrograph of a murine abscess co-infected with the periodontal pathogen *Aggregatibacter actinomycetemcomitans* and the oral commensal *Streptococcus gordonii*. *A. actinomycetemcomitans* (labeled red) and *S. gordonii* (labeled green) display synergistic virulence in a murine abscess model (Ramsey *et al.*, 2011). Direct imaging of labeled bacteria in co-infected abscesses reveals extensive co-localization, suggesting a possible link between close physical contact and virulence (scale bar, 200 μm).

Invertebrate models of polymicrobial infection

Model infections have long been used to study the precise molecular bases for pathogenesis in a controlled fashion. However, as interspecies microbial interactions can greatly impact pathogenesis in polymicrobial infections, models of these infections must include a great deal of control of the host microbiota to avoid complications arising from unwanted microbe-microbe interactions. There are several strategies that have been used to develop such models: (1) gnotobiotic (germ-free) animals can be used (reviewed in (Gordon and Pesti, 1971)), (2) the animal can be treated with antimicrobials to eliminate unwanted microbes from the infection site, or (3) an infection can be initiated at a host site that does not harbor microbes, such as the formation

of intramuscular abscesses (Brook *et al.*, 1984).

Bacterial infections in insects and nematodes provide relatively inexpensive and rapid models of key features of infections in humans, including cytotoxic and proteolytic virulence factors produced by bacteria and the host innate immune response (Mahajan-Miklos *et al.*, 1999; Jander *et al.*, 2000; Mylonakis *et al.*, 2007). The fruit fly *Drosophila melanogaster* is particularly useful in this regard because of extensive knowledge about its development, anatomy, and immune system. Another advantage of *D. melanogaster* infection models is the genetic tractability and wide availability of many mutant strains of fruit flies. Studies of the interactions between *P. aeruginosa* and *S. aureus* have benefited from using *D. melanogaster* to demonstrate synergistic virulence during a polymicrobial infection due to *P. aeruginosa* sensing peptidoglycan shed by *S. aureus* (Korgaonkar *et al.*, 2013). Interestingly, this effect by *S. aureus* was only observed when the Gram-positive *D. melanogaster* gut flora was cleared with antibiotic treatment, further underscoring the need for control of commensal organisms in infection models. The main strength of *D. melanogaster*-based models in the study of polymicrobial infections is that their low cost and ease enable high-throughput studies. For example, a screen of 40 commensal oropharyngeal isolates in co-infection with *P. aeruginosa* in *D. melanogaster* revealed that certain isolates that were beneficial to the fly in single-species infection significantly enhanced *P. aeruginosa* virulence in co-infection (Sibley *et al.*, 2008a).

While the nematode *Caenorhabditis elegans* is evolutionarily more distant from humans than *D. melanogaster*, it has shown predictive power in the identification and characterization of both microbial and host factors in pathogenesis for mammalian infections (Jander *et al.*, 2000). *C. elegans* has also been used to study polymicrobial interactions during infection (Lavigne *et al.*, 2008; Peleg *et al.*, 2008; Tampakakis *et al.*, 2009; Vega *et al.*, 2013). In an interesting example of intermicrobial communication resulting in decreased rather than increased virulence, both the bacterium *Acinetobacter baumanii* and the bacterium *Salmonella enterica* serotype Typhimurium were found to inhibit the virulence of the fungus *C. albicans* in a *C. elegans* oropharyngeal model of infection (Peleg *et al.*, 2008; Tampakakis *et al.*, 2009). These bacteria did so by inhibiting filamentation of *C. albicans*, a key virulence determinant, and in the case of *A. baumanii*, a putative two-component system sensor kinase was required for this effect. This suggests that defined signaling pathways control this intermicrobial signaling, though the identity or nature of the signals involved is not clear. *S.* Typhimurium has also been shown to participate in synergistic interactions with other microbes in the *C. elegans* gut. A recent study showed that *S.* Typhimurium can sense indole, a metabolite produced by catabolism of tryptophan by *E. coli*, and increase its antibiotic tolerance during *C. elegans* infection, even though *S.* Typhimurium itself is incapable of producing indole (Vega *et al.*, 2013). The use of a *C. elegans* model was key to this study, as the authors were able to easily manipulate the availability of tryptophan to *E. coli* and *S.* Typhimurium in the animal to support their model. Thus, invertebrate models of polymicrobial infection are widely accessible, offer excellent experimental manipulability, and will continue to provide insight into the mechanisms of polymicrobial interactions *in vivo*.

Mammalian models of polymicrobial infection

While mammalian models of infection involve more ethical concerns, higher cost, and often more complicated procedures than invertebrate models, they are undoubtedly a much closer proxy for human infection, and have been used for this purpose for centuries. In modern history, as the role of polymicrobial interactions in pathogenesis became clearer, methods were developed for controlling confounding factors arising from the mammalian host microbiota at the site of infection. For example, a rat oral model of periodontitis was used to demonstrate the synergistic virulence of the periodontal pathogens *P. gingivalis*, *T. denticola*, and *Tannerella forsythia* after using antimicrobials to suppress the native oral flora (Kesavalu *et al.*, 2007).

Infections can also be established in body sites that are normally sterile to avoid the influence of the host microbiota. A mouse thigh abscess model is relevant for periodontal infections because dental bacteria form polymicrobial abscesses in the mouth and other locations in the body (Williams *et al.*, 1983; Kaplan *et al.*, 1989). An advantage of this abscess model is the ease and reliability of administering the infection and monitoring disease progression by abscess weight and bacterial viability counts, and it has been used to investigate the molecular bases for synergy between the oral bacteria *A. actinomycetemcomitans* and *S. gordonii* (discussed above) (Ramsey *et al.*, 2011). Similarly, a chinchilla model of otitis media has proven to be an excellent system for investigating middle ear infections, as the chinchilla develops an infection from a very low inoculum that remains localized to the middle ear (Giebink *et al.*, 1976; Laufer *et al.*, 2011). The chinchilla ear canal is both highly accessible and anatomically similar to that of humans, especially children, in whom otitis media is most common (Jurcisek *et al.*, 2003). Finally, and most importantly for the study of polymicrobial interactions during infection, the middle ear is largely free of endogenous flora (Giebink *et al.*, 1976). Among the first observations gleaned from this model involved polymicrobial synergy: the opportunistic pathogen *Streptococcus pneumoniae* was more infective in the chinchilla middle ear when co-inoculated with influenza A virus (Giebink *et al.*, 1980). More recently, this model has proven useful in examining interspecies quorum sensing interactions in the opportunistic oropharyngeal pathogens *H. influenzae* and *M. catarrhalis* (discussed above) (Armbruster *et al.*, 2010).

Soft tissue infections such as those found in chronic wounds are often polymicrobial (Price *et al.*, 2009; Percival *et al.*, 2010; Peters *et al.*, 2012a), and mammalian models of these infections have proven highly successful in recapitulating key features of human wound infections, including polymicrobial synergy. By surgically inflicting a wound dorsally on a mouse, a recent study was able to show that a four-species polymicrobial wound infection led to attenuated wound healing and enhanced antimicrobial resistance in comparison to monomicrobial infections (Dalton *et al.*, 2011). Yet differences between mice and humans in body size and the immune

system represent challenges for murine models of infection (Mestas and Hughes, 2004). Models involving wounds inflicted on larger mammals such as pigs can offer several advantages over those in mice, including highly prolonged infection trajectories and greater similarity to human wounds both anatomically and with respect to the pharmacokinetics of systemic and topical treatments (Wright *et al.*, 2002; Jacobsen *et al.*, 2011; Roth *et al.*, 2013). So far, work on porcine wound models has largely focused on the efficacy of experimental treatments in single species infections with great success, and this system has strong potential as a model for polymicrobial wound infections in humans (Wright *et al.*, 2002; Jacobsen *et al.*, 2011).

Manipulating polymicrobial communities

Microbial landscapes in the environment and in infection are spatially and temporally dynamic, and cells use intricate sensing mechanisms to survey a myriad of environmental factors to adapt to their surroundings. Spatial organization and chemical gradients within a community mediate intra- and interspecies interactions at the global, local, and individual cell levels. To truly uncover how microbial interactions enable pathogens to thrive and cause infection, *in vitro* experiments need to represent physiologically relevant physical and chemical conditions. In this section, we briefly discuss some of the modern analytical tools that offer a means to probe bacterial interactions in a more meaningful context for polymicrobial infections.

Microorganisms live in high-density microcolonies, or aggregates, and share these small spaces with many different community members. Microfluidic devices have emerged as a powerful platform for studying small populations of bacteria in a controlled microenvironment. Microfluidic channels are useful for defining the spatial distribution and size of bacterial microcolonies as well as delivering chemical gradients to precise locations, and numerous applications of microfluidics in microbiology have been reviewed elsewhere (Weibel *et al.*, 2007; Connell *et al.*, 2012; Wessel *et al.*, 2013). Here, we focus on several specific examples that employ microfluidic strategies and microfabricated materials to explore spatial structure and chemical interactions among neighboring groups of microbes. Microfluidic devices have been demonstrated as a versatile tool for creating synthetic multispecies bacterial communities with defined spatial structure by patterning separate colonies on a shared membrane (Kim *et al.*, 2008) or extruding different species in a core-shell fiber (Kim *et al.*, 2011). Controlling the spatial structure and chemical communication between the separate species enabled the bacteria to engage in interactions that stabilized the global community relative to a mixed-species population (Kim *et al.*, 2008, 2011).

It is common for microbial communities to exhibit complex three-dimensional (3D) spatial structure. A recent study describes an aqueous two-phase system for building polymicrobial biofilm communities with 3D structure by patterning one species on top of the other (Yaguchi *et al.*, 2012). This system showed that two populations could interact with one another, but is restricted to patterning droplet geometries and has very limited 3D capabilities (Yaguchi *et al.*, 2012). These strategies supply the ability to form microscale arrangements of cells; however, it is challenging to confine individual cells or small, dense aggregates with as few as several thousand cells, much like those commonly observed in nature. Recently, a micro-3D printing technique based on multiphoton lithography was reported that has the capacity to arrange picoliter-sized polymicrobial communities with arbitrary 3D structure *in situ* by crosslinking porous protein walls directly around bacteria embedded in a thermally gelled matrix (Connell *et al.*, 2013). This study demonstrated that polymicrobial interactions enable a picoliter-sized aggregate of *S. aureus* confined within a shell of *P. aeruginosa* to exhibit increased resistance to β-lactam antibiotics and established gelatin-based micro-3D printing as a flexible tool for organizing multiple bacterial populations with sub-micrometer resolution in three dimensions (Fig. 3) (Connell *et al.*, 2013).

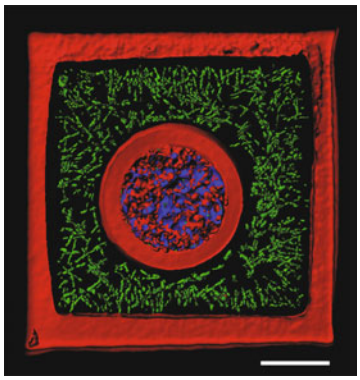

Fig. 3. Gelatin-based micro-3D printing of a spatially structured polymicrobial community. A confocal fluorescence isosurface with the roof of each microtrap cut away shows a spatially organized polymicrobial community constructed using gelatin-based micro-3D printing, where a nested population of *S. aureus* (blue) is surrounded on all sides (except on the coverglass) by *P. aeruginosa* cells (green). The photocrosslinked walls printed to confine each population appear red (scale bar, 10 μm).

Profiling intermicrobial signals

Microfluidic and microfabrication tools provide a means to precisely define the spatial distribution and control the chemical properties of a microbial microenvironment. Although these technologies allow researchers to evaluate how spatial structure and molecular transport shape microbial interactions, characterizing the molecular details of these behaviors and determining how they can subsequently modify the local and global environment requires different tools. Various analytical methods have been adapted to address this problem. Here, we discuss two emerging techniques for characterizing microbial environments and interactions at the molecular level – scanning electrochemical microscopy (SECM) and imaging mass spectrometry (IMS).

SECM is an electrochemical technique that can be used to gather spatial and chemical information about a biological system with microscale precision by scanning the tip of an

ultramicroelectrode over the substrate surface in the x-y plane (Liu *et al.*, 2011). The tip is positioned at a defined distance above the sample using a feedback approach-curve prior to scanning; therefore, this process creates a 3D spatial map of the local concentration of the redox-active molecule of interest. This technique has been used successfully to create a map of the hydrogen peroxide concentration profile over a polymicrobial biofilm containing *S. gordonii* and *A. actinomycetemcomitans* as well as each species individually, and showed that the hydrogen peroxide concentration fluctuates locally depending on the species composition of the bioflm (Liu *et al.*, 2011). SECM is a powerful technique that provides quantitative information about the concentration and reactivity of an analyte within a biological system in real-time with high 3D spatial resolution.

In contrast to SECM, which can probe multiple target molecules individually in a sequential manner, IMS is a high-throughput analytical technique with multiplex capabilities to profile thousands of molecules simultaneously (Watrous *et al.*, 2011; Watrous and Dorrestein, 2011; Phelan *et al.*, 2012; Yang *et al.*, 2012; Wessel *et al.*, 2013). IMS can be used to characterize the chemical environment of a polymicrobial community by mapping the spatial distribution of secreted molecules, such as signals, metabolites, products, and antibiotics (Watrous *et al.*, 2011; Watrous and Dorrestein, 2011; Phelan *et al.*, 2012; Yang *et al.*, 2012). In a typical IMS setup, the microbial sample is mounted on an x-y stage, an ionization source is used to bombard the surface of the sample with ions, and the secondary ions produced by this process are detected by a mass analyzer (Watrous and Dorrestein, 2011). The mass spectra collected as the stage is scanned across the sample in a raster pattern create a spatial map of the molecular characteristics of the microbial environment. IMS has already proven to be a useful platform in microbiology for identifying unknown microbes as well as profiling the molecular interactions and metabolic output within a microbial population (Watrous *et al.*, 2011; Watrous and Dorrestein, 2011; Phelan *et al.*, 2012; Yang *et al.*, 2012; Wessel *et al.*, 2013).

Traditionally, ionization occurs under vacuum, which requires biological specimens to be dehydrated, and often, coated with an organic matrix to aid in ionization (Phelan *et al.*, 2012). However, various alternative ionization methods have now been developed that do not require the samples to be ionized under vacuum, allowing for much less invasive profiling of metabolites. The most popular IMS methods for biological applications, secondary-ion mass spectrometry (SIMS), matrix-assisted laser desorption-ionization mass spectrometry (MALDI), desorption electrospray ionization mass spectrometry (DESI), and nanospray DESI (NanoDESI), have been reviewed extensively (Watrous and Dorrestein, 2011). Here, we will highlight some emerging IMS techniques using MALDI and NanoDESI that have been applied to investigate polymicrobial interactions.

Most IMS platforms are adaptable to a range of workflows, which enables researchers to further characterize microbial interactions at the molecular level by combining IMS analysis with other analytical techniques, including tandem mass spectrometry (MS/MS) (Watrous *et al.*, 2011; Watrous and Dorrestein, 2011; Moree *et al.*, 2012; Rath *et al.*, 2012; Watrous *et al.*, 2012; Nguyen *et al.*, 2013; Rath *et al.*, 2013), light microscopy (Hsu *et al.*, 2013), and atomic force microscopy (Ovchinnikova *et al.*, 2014). For example, a MALDI-IMS study exploring interspecies interactions between *B. subtilis* and *S. aureus* showed that two *B. subtilis*-secreted antibiotics, surfactin and plipastatin, were present at the *B. subtilis*-*S. aureus* interface when colonies of the two species were cultured next to each other (Gonzalez *et al.*, 2011). Images obtained from mapping colonies positioned at different orientations and distances relative to each other also revealed that direct contact between the two species was not required for this interaction (Gonzalez *et al.*, 2011). In addition to interspecies interactions, MALDI-IMS has been used to probe interkingdom interactions in a study tracking phenazine production by *P. aeruginosa* in co-culture with *Aspergillus fumigatus*, a fungal pathogen that is often found in cystic fibrosis infections with *P. aeruginosa* (Moree *et al.*, 2012). This study coupled MALDI-IMS with MS/MS networking to characterize the conversion of *P. aeruginosa*-produced phenazines by *A. fumigatus* into phenazine dimers (Moree *et al.*, 2012).

Recent advances in microbial IMS have enabled researchers to gather mass spectral data directly from living microbial samples using NanoDESI (Watrous *et al.*, 2012). In this ionization technique, molecules are desorbed directly from the surface of the microbial sample at the interface of a liquid bridge that forms between a solvent-filled capillary and a self-aspirating capillary, and injected into the mass spectrometer (Watrous *et al.*, 2012). NanoDESI sampling can be done quickly (~20–30 sec), and the small two-capillary probe (10–500 μm) is minimally disruptive to the biological sample; therefore, this method is capable of visualizing how polymicrobial interactions proceed over time on a single sample (Watrous *et al.*, 2012). This flexible, ambient ionization strategy has been applied to many types of microbial samples, including MS/MS studies to construct molecular networks and metabolic profiles of numerous bacterial genera (Watrous *et al.*, 2012), untargeted mapping of metabolite transformations among complex gut microbial populations containing 500–1000 species (Rath *et al.*, 2012), and metabolomic studies of metabolic exchange interactions between *P. aeruginosa* and *B. subtilis* (Rath *et al.*, 2013).

IMS techniques boast a number of advantages that make them attractive for exploring polymicrobial systems: (1) strains can be identified and the molecular environment can be characterized simultaneously, (2) no labeling chemistry is required and in some cases no sample preparation is required, which could provide a means to analyze organisms that are uncultivable in the lab, and (3) IMS is a multiplex tool that can gather information about thousands of molecules simultaneously, and can be used to build metabolic networks (Watrous *et al.*, 2011; Watrous and Dorrestein, 2011; Phelan *et al.*, 2012). A number of strategies are already being explored to develop integrative methods to extend the utility of IMS, including sectioning MALDI samples to image the substrate in slices and create 3D profiles of metabolic exchange involved in bacterial-bacterial interactions and bacterial-fungal interactions (Watrous *et al.*, 2013), forming networks of fragmentation patterns to identify gene cluster

and molecular families (Nguyen *et al.*, 2013), coupling atomic force (Ovchinnikova *et al.*, 2014) and light microscopy (Hsu *et al.*, 2013) with IMS to improve spatial resolution and gather topographic information as well as enable microscopy-guided MS molecular analysis of biological samples, and isotope labeling to visualize subcellular localization within bacterial membranes (Doughty *et al.*, 2014). Although IMS is still in its infancy as a tool for examining microbial interactions, it is clear that this technique has the potential to uncover valuable information to correlate the chemistry of a microbial microenvironment at the molecular level with phenotype. IMS is a powerful hypothesis-generating discovery tool (Gonzalez *et al.*, 2011), but other complementary approaches and technologies are required to further characterize the chemical environment of microbial habitats and the impact of that environment on populations in polymicrobial infections.

Conclusions

Because microbes rarely act alone and are often much more harmful in an infection in combination, research into the bases for polymicrobial interactions has the potential to guide novel treatment strategies that would not be found by studying infections in monoculture. Based on the studies mentioned in this review, it is clear that many more microbes in a population can contribute to infection than was thought in the time of Koch. Interactions that lead to polymicrobial synergy in infection are complex and not only occur between different pathogens, but also between commensals and pathogens, between microbes and the host, and amongst the microbiota writ large. It is likely that a complex web of interactions enhances pathogenicity in polymicrobial infections, with contributions from multiple species impacting the ultimate fate of the microbiota and of the disease itself. Therefore, a better mechanistic understanding of the complicated interplay between all community members with an increased emphasis on the precise molecular bases for polymicrobial interactions will provide a better picture of microbial communities and lead to the improvement of infection treatment.

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