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Polymicrobial Interactions: Impact on Pathogenesis and Human Disease

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INTRODUCTION

Microbes rarely exist as single-species planktonic forms; the majority are found thriving in complex polymicrobial biofilm communities attached to biotic and abiotic sites. Polymicrobial biofilm communities may be defined as a varied collection of organisms (fungi, bacteria, and viruses) that exist at a phase or density interface and are coated in a self- and/or host-derived hydrated matrix, often consisting of polysaccharide (26). The gastrointestinal (GI) tract and the oral cavity harbor a tremendous amount of microbial diversity, where an estimated 600 to 1,000 unique bacterial species have been identified as either permanently or transiently colonizing these human mucosal sites (1, 126). Because of the large variety and concentration of microbes present and the relatively minute amount of space available, species-specific physical and chemical interactions have developed over thousands of years of coevolution. Some microbes have evolved mutualistic or even synergistic relationships to facilitate cohabitation on epithelial surfaces and to efficiently utilize metabolic by-products, while others have developed competitive antagonistic approaches during cocolonization. These relationships are manifested by contact-dependent attachment, cell-cell communication via quorum-sensing cross talk, an enhancement of colonization, augmented virulence phenotypes *in trans*, immunomodulation, or a combination of these events (162).

Observations using the earliest microscopes revealed the colonization of multispecies communities on human tissues. However, we still do not have a solid understanding of how multispecies interactions govern the scope, progression, and severity of human disease, and even less is known regarding how the host responds to polymicrobial infection compared to monomicrobial infection (86). It was previously believed that a single virulence factor sufficiently mediated disease caused by a single organism. While this is true for some human infections, immunization against single virulence factors of other organisms (i.e., *Staphylococcus aureus*) has proven much more difficult. Just as virulence can no longer be associated with a single virulence factor for some organisms, some diseases can no longer be defined as an infection by a single species (Fig. 1) (42).

Most diseases were previously characterized as being monomicrobial in nature, likely due to the extensive use of culture-dependent isolation techniques. However, with the advent of culture-independent community analysis methodologies, several are becoming increasingly recognized as true polymicrobial infections, including diseases of the oral cavity, otitis media, diabetic foot wound infections, and chronic infection in the cystic fibrosis lung. In these cases, the composition of microbial populations predicts disease severity and outcome. Therefore, epidemiologic identification and comprehensive pyrosequencing surveys during human infection coupled with mechanistic studies of derived novel interspecies cross-kingdom microbial relationships should lead to the increased surveillance of potential disease risk factors. In addition, innovative preventative strategies and therapeutic measures for combating polymicrobial diseases may also result.

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doi:10.1128/CMR.00013-11

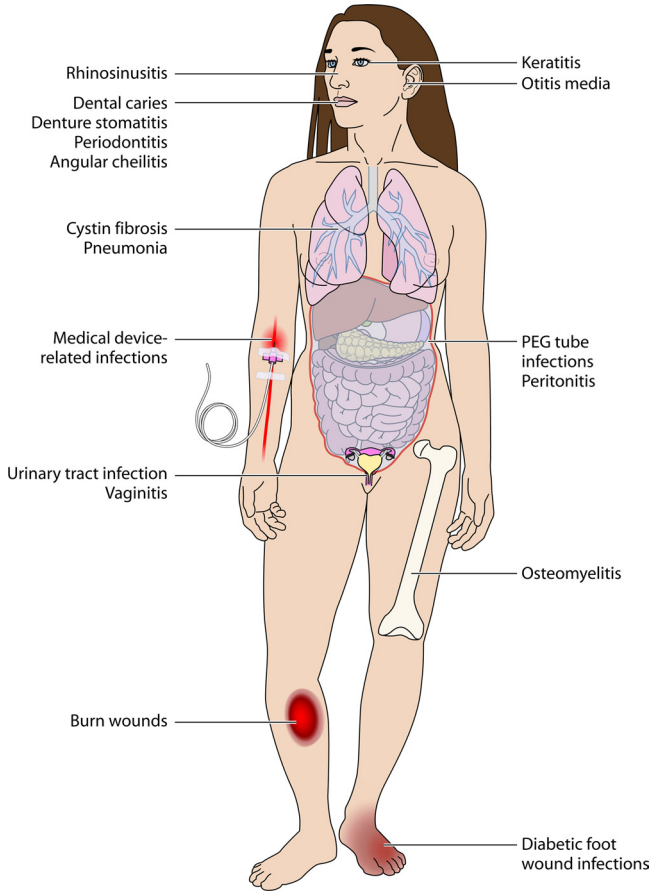

FIG 1 Schematic highlighting the sites and types of polymicrobial diseases commonly located throughout the human body.

POLYMICROBIAL BIOFILM FORMATION

Our initial understanding of and appreciation for the complexity of polymicrobial biofilm communities are represented most commonly by the microbial populations existing in the oral cavity. The colonization of tooth surfaces and oral tissue occurs in a temporal manner such that the attachment of one species becomes the scaffolding to which other species may adhere. As a result, the composition of early colonizers determines which microbes colonize at later time points. This process of sequential attachment is commonly referred to as coaggregation (176). Coaggregation is believed to be mediated in two distinct ways: either a secondary colonizer in suspension binds to specific molecules on the surface of a biofilm and begins the coaggregation cascade, or several bacteria form an aggregate that results in phenotypic changes promoting further coaggregation on the biofilm exterior. After the transition from the planktonic (or free-floating) state to the sessile state, attached microorganisms begin to radically change their gene and protein expressions (41). One common phenotype of attached communities is the elaboration of a biofilm "matrix" into the extracellular environment. This matrix, often composed of DNA, carbohydrate polymers, and/or proteins, encases the microbial communities, increasing both surface adhesion and protection from host damage and antimicrobial therapy (4, 213). In an environment that is highly competitive for space and nutrients, biofilm formation and coaggregative effects allow competing microbes to maximize the colonization surface area and intimately position themselves near potential sources of nutrition (108).

An example of the complexity of coaggregation may be the range of intergeneric coaggregations occurring between the oral fungal pathogen *Candida albicans* and other oral species that may play an important role in the colonization of the oral cavity by *C. albicans*. Although streptococcal species, namely, *Streptococcus gordonii*, *Streptococcus oralis*, and *Streptococcus sanguinis*, exhibit the highest affinities for *C. albicans*, *C. albicans* as well as *Candida dubliniensis* have been shown to coaggregate with *Fusobacterium* species in suspension (75, 76). The latter interactions were inhibited by mannose and therefore were thought to involve a protein component of *Fusobacterium* binding to a carbohydrate (mannan) receptor on the *Candida* cell surface (96). In contrast, a study demonstrating the ability of *Actinomyces* to coaggregate with *C. albicans in vitro* identified the receptors to be a protein moiety on the *Candida* surface, interacting with a carbohydrate-containing molecule on the surface of *Actinomyces* (76). These two examples demonstrate the diversity of ligand-receptor interactions that govern coaggregation on both bacterial and fungal surfaces. The most serious ramifications of these fungal-bacterial interactions, with clinical implications, are the findings that the physical interactions of *C. albicans* yeasts and hyphae with oral cocci lead to an increased tolerance of the polymicrobial biofilm to antimicrobial agents and enhanced polymicrobial biomass (25).

The most well-defined bacterial-fungal relationship is that which exists between *S. gordonii* and *C. albicans*. Holmes et al. initially demonstrated that fungal binding was mediated by a carbohydrate molecule on the bacterial surface, because alkali-extractable carbohydrate moieties from *S. gordonii* could be used to block coaggregation *in vitro* (90). Later, it was elucidated that these interactions were much more complex than initially thought; streptococcal surface proteins A and B (SspA/B), along with cell surface hydrophobicity proteins A and B (CshA/B), were demonstrated to be important for binding *C. albicans* yeast cells because antiserum raised against these cell wall proteins inhibited this candidal-streptococcal interaction (91). These interactions can be further enhanced 2- to 3-fold by the addition of sterilized human parotid saliva (155). Most recently, the heterologous expression of the *C. albicans* surface proteins Als3p and Eap1p in *Saccharomyces cerevisiae* was able to induce yeast binding to *S. gordonii* cells, while untransformed *S. cerevisiae* cells were unable to bind (147). More specifically, it was further demonstrated by heterologous expression in *Lactococcus lactis* that streptococcal SspB could interact directly with candidal Als3p, and this interaction partially stimulates polymicrobial biofilm formation (197). Unfortunately, there has been no *in vivo* modeling of these interactions, nor have the clinical ramifications of the coaggregation and colonization of epithelial and tooth surfaces been clarified.

After a partial clearance of polymicrobial biofilms by physical removal, such as toothbrushing or normal salivary flow, the colonization cycle repeats itself in the same general spatiotemporal progression until a mature community of microbes is repopulated (109). Studies examining the composition and colonization rates of sterile enamel chips implanted into the mouths of human volunteers demonstrated that early colonization (within 4 h) was dominated by *Streptococcus* spp. belonging to the *Streptococcus oralis-Streptococcus mitis* group (52). Other commonly identified genera were *Actinomyces*, *Gemella*, *Granulicatella*, *Neisseria*, *Prevotella*, *Rothia*, and *Veillonella*. While the specific composition

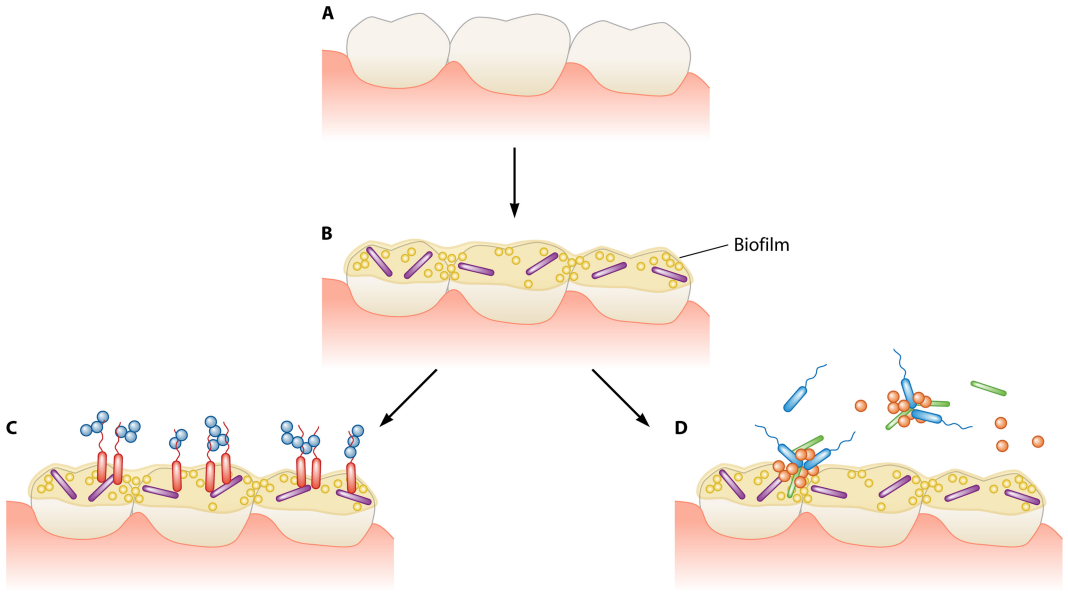

FIG 2 Polymicrobial biofilm formation is thought to proceed in several distinct phases. (A) Uncolonized biotic surface (e.g., teeth) lacking any biofilm formation. (B) Deposition of a conditioning layer promotes the adherence of early colonizers that begin the coaggregation cascade. From here, the coaggregative development of the polymicrobial biofilm can occur via two possible methods. (C) Early colonizing bacteria may directly support the binding of late colonizers that then facilitate the attachment of several other microbial species. (D) Specific planktonic intermicrobial interactions can lead to phenotypic changes that support the attachment of preaggregated clusters of cells; however, nonaggregated cells remain unable to attach.

and repopulation of microbial communities vary among individuals, the most abundant genera are usually conserved. Whether shifts in species composition are radical or subtle, these changes are believed to help promote disease-associated phenotypes. An excellent example of this is exposure to broad-spectrum antibiotics that reduce global populations of bacterial communities in the host. This sudden and sharp decrease in protective polymicrobial commensals may allow the growth of nontargeted or resistant organisms and is a predisposing factor for subsequent infection (50).

While the complexity, progression, and kinetics of oral colonization by diverse groups of bacterial, fungal, and viral pathogens have not been entirely explicated, several oral diseases have been extensively described to be polymicrobial in nature.

EXAMPLES OF HUMAN POLYMICROBIAL DISEASES

Diseases of the Oral Cavity

The most common oral infection, affecting 60 to 90% of school-aged children and nearly all of the adult population, is dental caries (164). Dental caries, or tooth decay, can be defined as the breakdown of the hard tooth surfaces, including the enamel, dentin, and cementum (92). The tooth surface becomes coated with a conditioning layer of salivary and host proteins, termed the acquired pellicle, which provides a substratum for the attachment and coaggregation of microbial species to develop into matrix-producing polymicrobial biofilm communities, commonly referred to as dental plaque (Fig. 2) (159). Tooth surface destruction is facilitated by the fermentation of carbohydrates, mainly sucrose and fructose, into lactic acid by several species of organisms, such as *Streptococcus mutans*, *Lactobacillus acidophilus*, *Actinomyces viscosus*, *Nocardia* spp., and *Candida albicans* (106, 138). The tooth surface is constantly undergoing demineralization and remineralization, a process that can only occur homeostatically at a pH of >5.5. Microbially derived lactate drives down the microenvironmental tooth surface pH and significantly slows the remineralization process, leading to demineralized surfaces that become weakened and subsequently decayed (128). Saini et al. examined bacterial isolates from healthy individuals and those with deep-seated dental caries and found that 100% of all cariogenic samples harbored a polymicrobial infection; it was also noted that the microbial populations of healthy teeth shifted from being dominated by Gram-positive anaerobic cocci to being dominated by Gram-positive anaerobic bacilli (183). Furthermore, the ability of *C. albicans* to cocolonize with streptococci and to grow and survive at low pH (<4.5) suggests that *C. albicans* may colonize sites of active caries formation (33, 101). In fact, evidence shows that there is a higher incidence of *Candida* spp. in patient populations with higher levels of susceptibility to caries (48). As described above, the mechanisms of coaggregation of streptococci and *C. albicans* may help exacerbate caries development.

Several recent studies using culture-independent techniques have more comprehensively uncovered the polymicrobial nature of dental caries. Li et al. used denaturing gradient gel electrophoresis (DGGE) to assess microbial population shifts specific to the cariogenic state and identified increased *S. mutans* colonization to be associated with active dental caries compared to healthy controls (119). There were also several DGGE bands that were of higher abundance during the caries-free states. However, these bands were not sequenced for further analysis. Additional DGGE profiling work by Li et al. did show differences in genera isolated from caries in early childhood and from caries-free children, suggesting cariogenic roles for *Fusobacterium* and *Neisseria* and protective roles for *Bacteroidetes*, *Treponema*, *Prevotella*, and *Corynebacterium* (118). 16S rRNA sequencing analysis of the oral microbiota from teeth of cariogenic and healthy children attributed a role in caries formation to several bacterial species: *S. san-*

guinis was correlated with health, while *Actinomyces gerencseriae*, *Bifidobacterium*, *S. mutans*, *Veillonella*, *Streptococcus salivarius*, *Streptococcus constellatus*, *Streptococcus parasanguinis*, and *Lactobacillus fermentum* (listed in order of decreasing community numbers) were associated with caries formation (18). Interestingly, *Veillonella* was originally thought to be associated with health due to its ability to readily degrade lactate and succinate, thereby negating the effects of *S. mutans* caries-associated lactate production (99). However, Arif et al. demonstrated that only certain *Veillonella* species exhibited this protective effect (6). Surprisingly, 16S rRNA sequencing of clone libraries generated from cariogenic and healthy tooth root plaques of older individuals differed still (169). Healthy individuals showed higher numbers of *F. nucleatum*, *Leptotrichia*, *Selenomonas noxia*, *Streptococcus cristatus*, and *Kingella oralis* bacteria, while cariogenic samples demonstrated increased numbers of *Actinomyces*, lactobacillus, *S. mutans*, *Enterococcus faecalis*, *Atopobium*, *Pseudoramibacter*, and *Propionibacterium* bacteria. While culture-dependent techniques accurately predicted a role for *S. mutans* in caries formation, culture-independent methods have elucidated a much greater complexity of microbial compositions promoting cariogenesis. Combined, these data suggest that interactions between microbes and species composition may significantly affect the predisposition to dental caries or disease outcomes.

Denture stomatitis is another well-studied example of a polymicrobial biofilm-mediated oral disease. Denture stomatitis refers to erythema and edema of the soft palate and tissues of the oral cavity that are in close contact with the denture surface, as prosthetic placement restricts the lubrication achieved by normal salivary flow; redness, swelling, and burning sensations on the upper palate are most commonly caused by infections with the fungus *C. albicans* (219). A recent 16S ribosomal DNA (rDNA) survey and amplification of the fungal 18S-28S internal transcribed spacer region of biofilm material recovered from the palates of 10 denture stomatitis subjects as well as 10 healthy controls demonstrated the presence of *Candida* spp. in all biofilms (28). However, that study also identified 82 species of bacteria in both subject groups: 27 that were common to both groups, 29 that were specific to denture stomatitis, and 26 that were specific to healthy controls. Therefore, specific bacterial populations may exacerbate *Candida*-induced denture stomatitis. Further studies by Baena-Monroy et al. examined saliva and culture swabs of denture surfaces from 105 subjects fitted with dentures (12). Using culturing techniques, it was found that *C. albicans* and *S. aureus* could be recovered from the oral mucosa and denture surfaces of both denture stomatitis patients and healthy controls. However, more *C. albicans* cells were recovered on the denture surface, while *S. aureus* was found in the oral mucosa of denture stomatitis cases, suggesting that *C. albicans* may facilitate colonization by *S. aureus*, enabling the staphylococci to mediate the inflammation of host tissue and bacterial superinfection.

Indeed, unique associations between *C. albicans* and *S. aureus* in several different *in vivo* and *in vitro* models have been described. A series of experiments by Carlson demonstrated the ability of *C. albicans* to decrease the 50% lethal dose (LD_{50}) of *S. aureus* 200- to 70,000-fold when coinoculated into the peritoneal cavity of immunocompetent mice (29). More strikingly, the coinfection of mice with sublethal monomicrobial infectious doses resulted in 100% mortality (30). Quantitative CFU counts from organ homogenates demonstrated the systemic spread of staphylococci to various organs, including a polymicrobial-induced increase in staphylococcal loads in kidney, liver, pancreas, and spleen. Histological analysis revealed that staphylococci could always be found associated within the fungal growth rather than at the periphery, and it was hypothesized that this coassociation somehow protected the bacteria. In addition, when these pathogens were injected at proximal but nonoverlapping sites, the bacteria always associated at the fungal interface and not vice versa. Decades later, work by Harriott and Noverr showed that *C. albicans* and *S. aureus* could form polymicrobial biofilms *in vitro* and that *S. aureus* could be protected from vancomycin treatment, presumably through encapsulation by the *C. albicans* biofilm matrix (82). Peters et al. demonstrated that *S. aureus* preferentially binds to the hyphal form of *C. albicans* during polymicrobial biofilm growth and that this association is nonantagonistic (Fig. 3). By use of two-dimensional in-gel electrophoresis, it was also shown that metabolic, stress, and virulence factor protein expressions could be significantly altered due specifically to polymicrobial growth conditions (163). Therefore, based on findings with these disparate model systems, the colonization of the denture surface with *C. albicans* may promote the deposition of other bacterial species (or vice versa) and enable specific bacterial populations to uniquely manipulate the microenvironment via direct or cooperative efforts, leading to exacerbated disease.

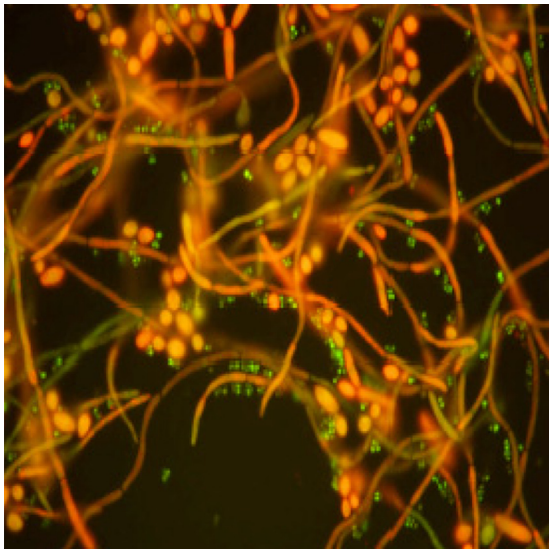

FIG 3 Peptide-nucleic acid fluorescent *in situ* hybridization (PNA-FISH) microscopy image depicting *S. aureus* (green) preferentially attached to the hyphal cells of *C. albicans* (red) during polymicrobial biofilm growth *in vitro*. (Reprinted from reference 163 with permission of John Wiley & Sons.)

Periodontitis may be the most classically defined polymicrobial biofilm-mediated disease of the oral cavity and results from a chronic inflammation of the gums that leads to the damage of structural tooth support, resorption of bone, and eventual tooth loss (189). Symptoms of the disease include inflamed gum tissue, bleeding from the gums, gingival recession, deep-pocket formation between the gum and tooth surface, and loose teeth. Aside from dental caries, periodontitis is considered to be the second most common cause of worldwide infectious diseases, affecting nearly half of the U.S. population alone (165). Culture-dependent analyses of biofilm plaques found on inflamed gum surfaces dem-

onstrated that periodontal patients could be categorized into two unique disease factions, "adult periodontitis" (patients >35 years old) and a rapidly progressing form of "early-onset periodontitis" (patients <35 years old) (68). Organisms thought to be involved in adult periodontitis included *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, while early-onset periodontitis more commonly involved *Aggregatibacter actinomycetemcomitans*, which was often coisolated with *P. gingivalis*, *Prevotella intermedia*, and *Capnocytophaga sputigena* in the periodontal plaque. However, the increased use of culture-independent techniques has led to the finding that the microbial compositions identified for each periodontal disease type were not as clearly defined as previously thought (8). It was determined that healthy individuals often harbored these periodontal "pathogens" in low numbers as part of the normal flora, without any signs of overt disease; differences in virulence factor expressions of individual strains may partly explain this discrepancy (85, 112). Moreover, many cases of periodontitis were mediated in the absence of the major contributing species formerly believed to facilitate disease onset (139). Thus, the nonvalidated age-dependent terms "early-onset" and "adult" peritonitis were more broadly defined as "localized aggressive" and "chronic" peritonitis, respectively (8). One interesting observation resulting from culture-independent analyses of the subgingival microbiota revealed the presence of *Archaea* in subgingival pockets (115). Archaea are prokaryotes that physically resemble bacteria but are more genetically similar in their 16S rRNA sequences to eukaryotes. Indeed, as a function of progressing disease, increasing numbers of *Archaea* (including *Methanobrevibacter oralis*) can be isolated from subgingival pockets. Despite the lack of any direct cause-effect *in vivo* studies assessing the role of *Archaea* in periodontitis, strikingly, these organisms have never been found in the subgingival microflora of healthy individuals or at healthy sites in patients with periodontitis (115, 116). The difference between the onsets of chronic and localized aggressive forms of periodontitis is unclear at this time but is thought to result from a host genetic predisposition to colonization, a hyperaggressive immune response to oral bacteria involving increased levels of polymorphonuclear infiltrate, differences mediated by the microbial composition itself, or a combination of these effectors (44, 58, 84).

While not a comprehensive analysis of the microbial interactions governing periodontal infections *in vivo*, several *in vitro* studies have revealed interesting associations, with possible pathogenic ramifications. *F. nucleatum*, a filamentous Gram-negative anaerobic bacterium, supports *P. gingivalis* during polymicrobial biofilm growth, leading to synergistic increases in biomass (186). Furthermore, *F. nucleatum* was shown to increase the penetration of *P. gingivalis* during coinfection of human gingival epithelial cells *in vitro* via an undefined mechanism (185). While it is unclear at this time, invasive *P. gingivalis*-mediated human periodontitis may be enhanced by interactions with *F. nucleatum*. Additional studies have examined the fungal colonization of the periodontal pocket and have found that only *C. albicans*, a filamentous fungal species, is harbored in both the chronic and localized aggressive forms of periodontitis (209). It is interesting to hypothesize whether *P. gingivalis* virulence is enhanced in the presence of invasive microbial species. However, comprehensive culturing and limited sequencing analyses of microbiotas obtained from periodontitis patients and healthy subjects have not yet defined these clinical polymicrobial associations.

Taken together, these diseases represent the diversity and complexity of oral polymicrobial biofilm communities and highlight the unique associations that lead to complex disease phenotypes. Although the oral cavity has been the most extensively researched polymicrobial environment, significant advancements in the mechanistic explanations of clinically relevant coassociations and the development of *in vivo* model systems to test these hypotheses are critical for developing therapeutic strategies against oral polymicrobial biofilm infections.

Otitis Media

Otitis media (OM), a common childhood disease, is an infection of the middle ear, often involving the Eustachian tube between the tympanic membrane and the inner ear (191). While rarely associated with mortality, OM symptoms often include ear pain, fever, and middle ear effusion. In severe cases, a perforation of the tympanic membrane can result in purulent discharge from active chronic infection. Most cases of OM spontaneously resolve within a few weeks. However, chronic infections can lead to partial or total hearing loss (105). The microbial species responsible for the vast majority of OM are the normally commensal bacterial species *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae* (NTHi), and *Moraxella catarrhalis* along with upper respiratory viruses, including influenza A virus, respiratory syncytial virus (RSV), adenoviruses, and human rhinovirus (142). Not only can these pathogens cause significant morbidity during monomicrobial infection, they also are often coassociated as polymicrobial biofilm complexes, where combinations of pathogens enhance disease or predispose the host to colonization by coinfecting microbes. In fact, the increased use of culture-independent techniques suggests a very diverse microbial etiology of OM pathogenesis (114). It is important to note that during commensal colonization, none of the aforementioned bacterial species cause inflammation or breach epithelial barriers; it is only during times of epithelial rupture or immune dysfunction that they cause infection.

Interestingly, it appears that upper respiratory viral infections predispose the host to and enhance OM pathogenesis (81). It has been well noted that viral infection of certain cell types enhances the bacterial colonization of the cellular surface (Fig. 4) (80, 102, 187). Preinfection with various viruses changes the physiological properties of infected airways, alters susceptibility to antibiotics, and modulates the innate and adaptive immune responses resulting in proinflammatory signaling events (2, 32, 38, 217). In fact, experimental human challenge with influenza A virus leads to increases in the isolation of *S. pneumoniae* and *S. aureus* from the nasopharynges. Also, viral preinfection has been correlated with OM in children (160, 214).

Giebink et al. used an animal model of OM in which chinchillas were infected with influenza A virus and subsequent pressure and inflammation of the middle ear were monitored (69). Viral infection alone led to increases in negative pressure, inflammation, and epithelial damage in the Eustachian tube proximal to the nasopharynx; these symptoms closely mimic those of human infections and demonstrate the ability of viral infection to enhance OM pathology. In order to more closely mimic the polymicrobial nature of human disease, Krishnamurthy et al. utilized a mouse nasal colonization model of infection and pretreated animals with Sendai virus prior to inoculation with bacterial species (110). Viral pretreatment led to significant increases in bacterial burdens re-

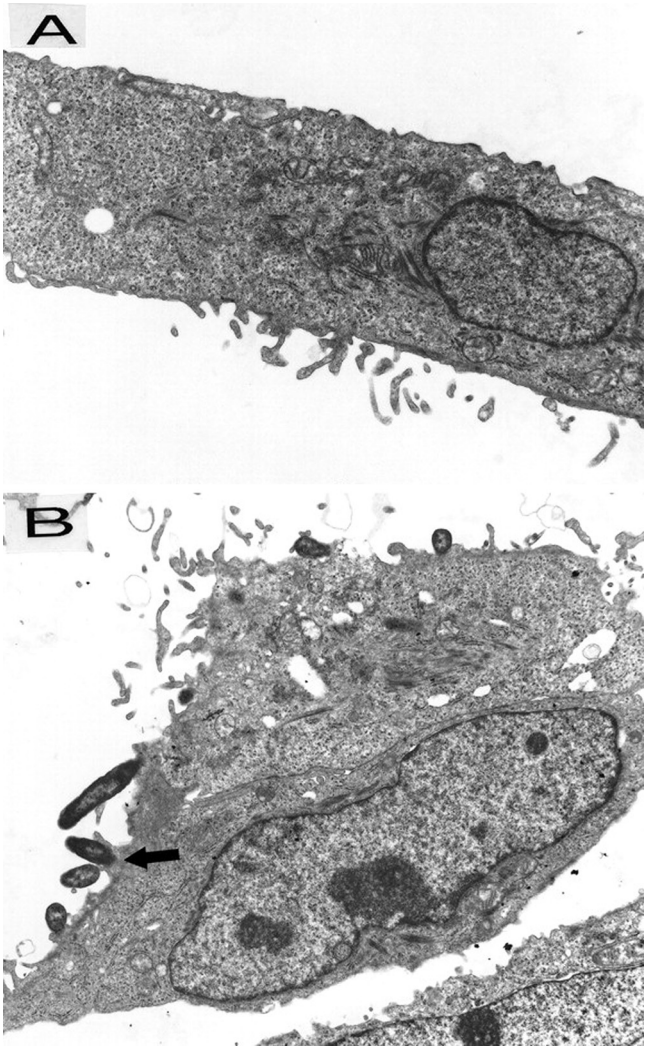

FIG 4 Preinfection of the respiratory epithelial cell line A549 with RSV enhances adherence of P5-fimbriated NTHi (B, black arrow) compared to non-virus-infected control cells (A). (Reprinted from reference 102.)

gardless of the coinfecting bacterial strain. When *S. pneumoniae* and *M. catarrhalis* were used together as infecting species, *S. pneumoniae* was the most abundant bacterial species present during experimental OM. However, *M. catarrhalis* significantly increased the pneumococcal OM bacterial load, incidence, and time to infection. NTHi had a similar effect on *S. pneumoniae* during coinfection but to a lesser degree. In all cases of polymicrobial disease, inflammatory responses peaked at day 1 postinfection, but in monomicrobial infections, inflammatory markers were not maximally upregulated until day 3. Recently, a chinchilla model of coinfection using *M. catarrhalis* and NTHi showed that polymicrobial infection resulted in increased microbial resistance to antibiotic treatment and clearance from the host (Fig. 5) (7). *In vitro* imaging revealed a physical coassociation of these two pathogens and a synergistic increase in the biofilm mass during polymicrobial growth. Interestingly, an NTHi *luxS* mutant deficient in the production of the quorum-sensing molecule autoinducer-2 could no longer induce immunoavoidance or antimicrobial resistance mechanisms in *M. catarrhalis*. These data provide evidence for interspecies microbial cross talk via quorum-sensing communication to enhance infection during polymicrobial biofilm-mediated OM.

Viral infection of the upper respiratory tract can lead to immunological changes that further enhance OM pathogenesis. Antimicrobial peptides, including defensins, lysozyme, lactoferrins, and surfactants A and D, are secreted in the middle ear and have been shown to be effective at clearing OM pathogens at micromolar concentrations (120). McGillivary et al. demonstrated that infection of chinchilla respiratory epithelial cells *in vitro* with RSV led to significant decreases in the levels of transcription of the innate antimicrobial peptide chinchilla beta-defensin 1 (cBD1), of which human beta-defensin 3 (hBD3) is an ortholog (133). Furthermore, *in vivo* RSV infection of the chinchilla nasopharynx led to nearly a 40% decrease in cBD1 transcript levels when matched to those of mock-infected controls. Consequently, *in vivo* coinfections with NTHi and RSV resulted in a 10- to 100-fold increase in the level of NTHi recovered from nasopharyngeal lavage fluids of infected chinchillas compared to those infected with NTHi alone. Therefore, the modulation of innate immune effectors, such as defensins, by viral respiratory pathogens may predispose the host to more severe infection by OM pathogens.

In summary, these studies, although limited by culture-dependent techniques, identified the major microbes involved in the pathogenesis of OM and demonstrated the importance of polymicrobial interactions and cooperative viral and bacterial effects during disease predisposition and progression. Further work on a more comprehensive disease model utilizing *S. pneumoniae*, *M. catarrhalis*, NTHi, and an upper respiratory virus in concert would be extremely useful in mimicking *in vivo* infection and determining the potential roles that each microbe assumes during polymicrobial biofilm growth. Moreover, an increased surveillance of potential pathogenic microbial associations, by use of culture-independent techniques, will further enhance our understanding of OM pathogenesis.

Diabetic Foot Wound Infections

Diabetes mellitus, commonly referred to as diabetes, is a disease involving defects in the bodily response to insulin and affects nearly 6.4% of the population worldwide; this number is expected to double by the year 2030 and has prompted the Centers for Disease Control and Prevention (CDC) to classify diabetes as a current epidemic (61). Diabetes can manifest itself in several forms. However, two of the most common forms are type 1 and type 2 (3). Type 1 diabetes is caused by a lack of insulin production and is thought to be caused by the autoimmune-mediated destruction of pancreatic beta-cells, which secrete insulin. Type 2 diabetes refers to a group of metabolic disorders characterized by various levels of insulin resistance suspected to be caused by defects in or the expression of the insulin receptor (107). In addition, impaired insulin secretion and increased hepatic glucose production are also implicated. Type 2 diabetes is thought to be mediated by genetic and environmental triggers, including obesity, diet, low activity level, and high blood pressure. The effects of low levels of insulin production or insensitivity to insulin result in high glucose blood levels, osmotic imbalance, dehydration of body tissues, and, if not treated properly, eventual organ damage (3).

Another damaging effect of high glucose levels mediated by an insulin deficiency or resistance is the development of peripheral neuropathy and poor blood circulation, especially in extremities

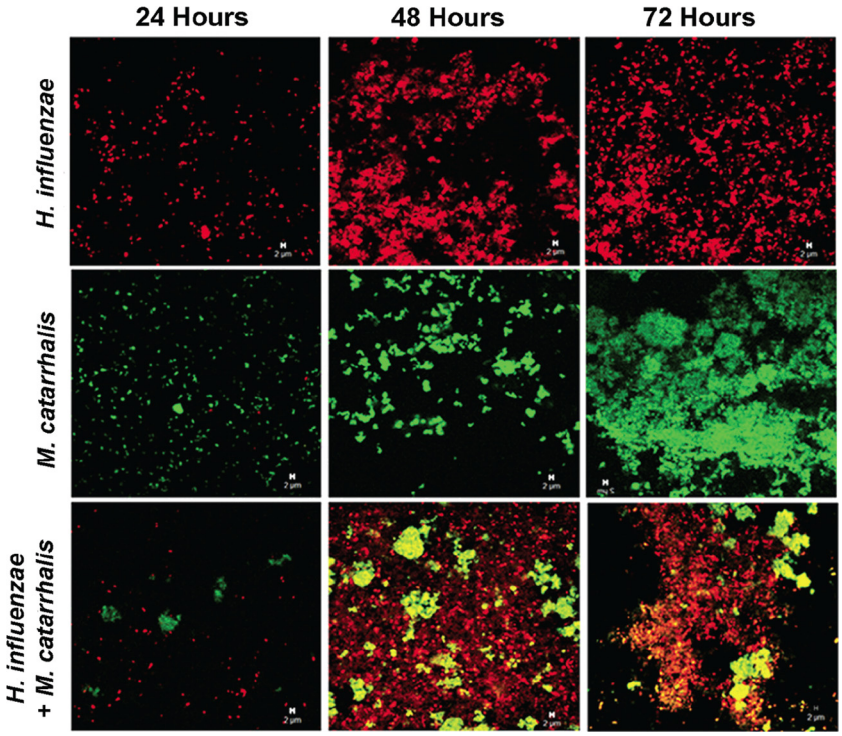

FIG 5 *In vitro* biofilm formation of single-species or polymicrobial inocula composed of NTHi (red) and/or *M. catarrhalis* (green), assessed over a time course by immunostaining and confocal laser scanning microscopy (CLSM). While single-species biofilms appeared to be composed of smaller aggregates of cells, polymicrobial biofilm growth resulted in larger aggregates of *M. catarrhalis* interspersed among lawns of NTHi. (Reprinted from reference 7.)

such as the hands and feet. Combined, these symptoms predispose diabetic individuals to an increased risk of infection, and if not identified or treated early, these infections may fulminate into chronic ulcerating polymicrobial biofilm-mediated wounds that often do not resolve with standard therapies and result in eventual limb amputation (222). The development of such ulcers is often the synergy of two contributing factors, e.g., decreased neurosensory perception and stepping on a sharp object; it is this critical breach of the epithelial surface, coupled with neurological abnormalities, cardiovascular aberrations, and immune dysfunction, which facilitates polymicrobial colonization and subsequent pathogenesis. Due to the inability of subjects with this disease to feel cuts and irritations on visually obscured areas of the feet, these infections often go unnoticed and progress to more serious illness (21). While not only extremely uncomfortable and cumbersome for the patient, diabetic foot wound infections also contribute to significant yearly costs of medical care, as these infections often require several rounds of therapeutic treatment and surgical debridement. In 1997, average inpatient costs for lower limb complications were $16,580 for foot ulcers, $25,241 for toe or toe and other distal amputations, and $31,436.33 for major amputations; the total estimated yearly costs are in the billions (172).

As stated above, diabetic foot wounds are often mediated by a mixture of several species of microbes coexisting as complex biofilm communities. A large multicenter analysis of 454 individual diabetic foot wound infection swabs and aspirates resulted in the identification of over 1,600 organisms by aerobic and anaerobic culturing techniques (40). Interestingly, of the specimens tested, 48.9% were infected with aerobic bacteria only, 1.3% were infected with anaerobic bacteria only, and 43.9% contained a mixture of aerobic and anaerobic bacteria. Bacterial growth was not identified in 5.9% of the samples. Of the positive cultures identified, 16.2% harbored one bacterial isolate, 20.4% contained two bacterial isolates, 19.7% had three bacterial isolates, 13.3% demonstrated four bacterial isolates, and 30.4% supported the growth of five or more bacterial isolates. Of these, the most abundant aerobic isolates recovered were *Corynebacterium* spp., *Enterococcus* spp., *Escherichia coli*, *Staphylococcus epidermidis*, and *S. aureus*; among the most commonly isolated anaerobic bacteria were *Fusobacterium* spp., *Porphyromonas* spp., *Prevotella* spp., *Bacteroides* spp., and *Clostridium* spp. A smaller-scale study using DGGE, 16S rRNA gene sequencing techniques, and microscopy to examine debrided tissues from diabetic foot wounds resulted in the identification of highly polymicrobial communities and the detection of several species unidentifiable by standard culturing techniques (98). Notably, DGGE analysis demonstrated the presence of several unique bands (corresponding to unique species) for each sample tested, and banding patterns differed between individual samples. While no distinct relationships between coisolated organisms can be derived from these analyses, these studies demonstrate the tremendous diversity in microbial composition and the true polymicrobial nature of diabetic foot wound diseases. As of now, it is unclear whether diabetic foot wounds arise from specific combinations of pathogens or if a simple increase in the microbial loads of any opportunistic microbes can sustain infection (67).

Another potential mechanism for the chronic infection of diabetic foot wounds is the inability of wound healing to proceed properly. Diabetic patients have been shown to have several defects in wound healing, including decreases or impairments in angiogenic factors, growth factors, epidermal barrier function, fi-

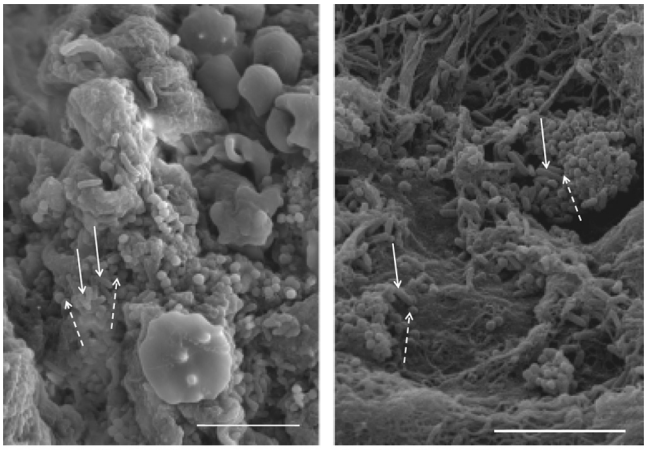

FIG 6 Scanning electron micrograph comparing *in vivo* polymicrobial diabetic foot wound growth with *in vitro* growth obtained by using the Lubbock chronic wound model system. Note the complexity of the adherent microorganism composition growing within close proximity (solid and dashed arrows depict rod- and coccus-shaped bacteria, respectively). "Host" substrata and the elaboration of microbial biofilm matrix also appear similar in both growth systems. (Reprinted from reference 203 with permission of John Wiley & Sons.).

broblast migration, and macrophage function (59, 65, 66, 130). Specifically, at the edge of diabetic foot wound infections, keratinocytes demonstrate an absence of migration and the inability to differentiate completely, while unaffected cells from adjacent sites appear normal but are physiologically impaired. However, these "normal" cells can become functional by the addition of specific growth factors (201). A recent study by Schierle et al. showed for the first time that murine cutaneous wounds cannot be resolved by the host with infection by biofilm-forming *S. aureus* strains (190). Remarkably, when a *traP S. aureus* mutant defective in biofilm formation or biofilm-inhibiting peptides was used, the wound healed normally. This finding suggests that biofilm formation may specifically inhibit wound-healing mechanisms during infection in the host. Because *S. aureus* is one of the bacterial strains most commonly isolated from diabetic foot wound infections, its ability to modulate wound healing and avert localized immunity may enable other microbes to colonize the wound and exacerbate disease (22).

While there has not been an extensive development of *in vitro* or *in vivo* modeling systems to study the polymicrobial environment of diabetic foot wound infections, there has been some recent progress. Sun et al. devised an *in vitro* system, dubbed the Lubbock chronic wound biofilm model, consisting of a series of glass tubes filled with appropriate media (replete with damaged tissue, red blood cells, and plasma) to support multispecies growth and abiotic plastic surfaces to enhance biofilm formation (203). Both microscopically and macroscopically, the resulting polymicrobial growth strikingly resembles chronic diabetic foot wound biopsy specimens (Fig. 6). This model has extreme usefulness for the rapid screening of compounds for antimicrobial efficacy against diabetic foot wound infections during the growth of mixed microbial populations. Perhaps one of the most important studies demonstrating the effects of polymicrobial infection in a mouse model of type 2 diabetes was performed by Mastropaolo et al. Leptin receptor-deficient mice (BKS.Cg-$m^{+/+}$ $Lepr^{db}$/J) were injected into the inner thigh with either *E. coli* alone, *Bacteroides fragilis* alone, *Clostridium perfringens* alone, all combinations of two of the aforementioned pathogens, or all pathogens simultaneously. Infected abscesses were removed at several time points and assayed for microbiological enumeration (131). It was found that injection with all three bacteria simultaneously led to the highest rate of mortality. When combinations of two pathogens were used, certain microbial pairings resulted in vast differences: *E. coli* bestowed strong synergy to *B. fragilis* but not to *C. perfringens* during early infection (day 1), *B. fragilis* and *C. perfringens* provided moderate synergy but only in infections of young mice, and *B. fragilis* reacted antagonistically during polymicrobial growth with *E. coli* at later time points during infection (day 22). Most importantly, when age-matched nondiabetic C57BLKS/J mice were infected with these pathogens, they harbored anywhere from 5- to 35-fold-fewer bacterial CFU than their diabetic counterparts, demonstrating the contribution of the diabetic state to the severity of the polymicrobial infection. While only speculative at this time, the progression of human diabetic foot wound infections may be partially dependent on the species initially colonizing the wound. Furthermore, archived clinical data should be analyzed, or new studies aimed at determining polymicrobial compositions that predict disease severity or worsen disease outcome should be undertaken.

Infection of the Cystic Fibrosis Lung

Cystic fibrosis (CF) is an autosomal recessive genetic disorder, most common among Caucasians, and is caused by an inherited mutation in a specific chloride ion channel named the cystic fibrosis transmembrane conductance regulator (CFTR) (47). Mutations in phenylalanine residue 508 of the CFTR are responsible for the majority of cystic fibrosis cases; this precise mutation leads to improper protein folding and subsequent cellular degradation. However, there are nearly 1,900 documented mutations associated with the disease (http://genet.sickkids.on.ca/app). Regardless of the source of genetic polymorphism, the lack of functional CFTR molecules on the surface of mucosal tissues severely affects the production of sweat, components of the digestive juices, and mucous composition, as imbalances in chloride ion secretion outside the cell lead to cationic influx, osmotic imbalance, and eventual dehydration (135). Early symptoms of cystic fibrosis are increased susceptibility to lung infections, persistent coughing, and heightened sputum production. As infecting microbes fail to be cleared, uncontrolled inflammation begins to cause permanent damage to the lung architecture, resulting in bronchiectasis, pulmonary hypertension, and hypoxia (19). End-stage cystic fibrosis requires the use of positive-pressure air masks or ventilators to mechanically assist breathing. Besides destructive effects on the airway, cystic fibrosis can also lead to nutrient loss by the progressive scarring of the pancreas, which becomes dehydrated in a fashion similar to that of the lungs (54).

One of the most notable features of CF is the loss of normal mucociliary clearance, resulting in extensive mucous buildup and excessive lung inflammation. Not only is the mucous unable to be cleared efficiently, dehydration results in thick and adhesive secretions; these conditions are ideal for supporting robust bacterial growth (140). Individuals with functional CFTR receptors inhale potentially pathogenic microbes on a routine basis, but they are efficiently trapped in the respiratory tract and eliminated by the mucociliary escalator. However, individuals suffering from cystic

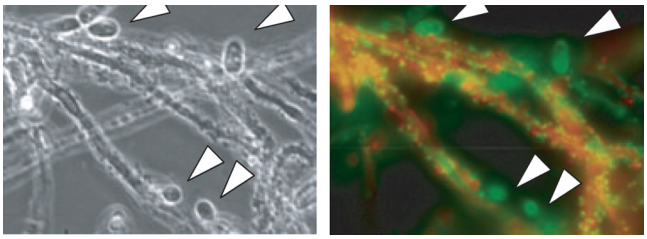

FIG 7 Phase-contrast microscopy image showing the attachment of *P. aeruginosa* to the hyphal filaments of *C. albicans* (left, white arrowheads). Epifluorescence microscopy utilizing the Live/Dead staining system (live cells are stained green, while dead or dying cells appear red) demonstrates the ability of *P. aeruginosa* to specifically kill the hyphae of *C. albicans*, while the yeast form remains unharmed (right, white arrowheads). (Reprinted from reference 89 with permission of John Wiley & Sons.)

fibrosis are unable to clear such organisms, and these organisms often develop into polymicrobial biofilm-mediated infections in the lower airways (194). Bacterial lung infection still remains the primary cause of morbidity and mortality in CF patients. Some of the most common isolates from human bronchiolar lavage samples of CF patients are *Pseudomonas aeruginosa*, *S. aureus*, *Streptococcus milleri* group (SMG) pathogens, *Burkholderia cepacia*, *Stenotrophomonas maltophilia*, *H. influenzae*, and *C. albicans*. However, this list ignores members of the normal flora that are not regularly screened in the clinical laboratory (143, 179, 182, 194). Several interesting findings and trends from coinfections with several of these pathogens will be discussed.

Due to undergoing repeated rounds of antimicrobial therapy and relatively high levels of colonization in the nasopharynx, *C. albicans* is often found in the lower airways of individuals with cystic fibrosis (39). *C. albicans*, a dimorphic yeast, can switch from a usually commensally associated round yeast form to an elongated hyphal form, a transition central to its pathogenesis. These hyphae are important for the progression of biofilm formation and are also needed to puncture the epithelial and endothelial layers to gain access to deep tissue (100, 175). A series of eloquent experiments demonstrated that *P. aeruginosa*, one of the organisms most often cultured from the lower airways of infected CF patients, is able to inhibit candidal germination by the secretion of the molecule 3-oxo-C_{12} homoserine lactone (89). Furthermore, *P. aeruginosa* can attach to localized areas of the hyphal surface and induce cell lysis, thereby effectively killing the hyphal cell, but are unable to attach to or kill the round yeast form of *C. albicans* (Fig. 7); mutants lacking the production of this quorum-sensing molecule were unable to propagate hyphal lysis (88). Although the molecules that enable pseudomonal binding to hyphal surfaces were not entirely elucidated, work by Brand et al. demonstrated that binding requires both contact dependence and soluble factors (24). *P. aeruginosa* culture supernatants were capable of inducing lysis independent of cellular contact, but cocultured cells displayed only localized points of *C. albicans* cell wall weakening. It was also noted that *C. albicans* mutants with severely truncated O-linked, but not N-linked, cell wall glycans were hypersusceptible to killing by *P. aeruginosa*. The truncation of fungal O-linked glycans may cause cell wall rearrangements that are more amenable to *P. aeruginosa* binding or result in the exposure of high-affinity binding sites.

However, this association is not unilaterally antagonistic. *C. albicans* produces the quorum-sensing molecule farnesol, a sesquiterpene alcohol, which inhibits the transition of yeast to hyphae. As discovered by Cugini et al., farnesol is toxic to *P. aeruginosa* by significantly downregulating the transcription of the *pqsA* gene, an important mediator of the expression of the pseudomonal iron-scavenging virulence factor pyocyanin (43). Interestingly, pyocyanin production is upregulated when *P. aeruginosa* is cultivated in sputum from CF patients compared to that of growth in standard culture (156). Pyocyanin is also toxic to *C. albicans*. Therefore, the secretion of farnesol may be a protective mechanism to reduce pyocyanin levels during the intimate cocolonization of the CF lung (103). Farnesol secretion has also been shown to inhibit swarming motility in *P. aeruginosa*; this may slow the initial deposition of pseudomonads onto the hyphal surface and result in less lytic activity (132).

Another clinically relevant polymicrobial association in the CF lung is that which exists between *S. aureus* and *P. aeruginosa*. Liou et al. analyzed data obtained from 5,820 randomly selected patients to develop a multivariate 5-year survivorship model and to partially identify predictors of disease outcomes (122). While the model accurately predicted several known risk factors for CF disease, such as decreased forced expiry volume, increased age, and decreased weight, one interesting result was uncovered: infection with *S. aureus* resulted in increased survivorship over a 5-year period, independent of age, while patients infected with *B. cepacia* were predicted to have the worst outcome. In fact, it has been shown that CF patients often initially become colonized early with *S. aureus* or *H. influenzae*, followed by a lengthy colonization with *P. aeruginosa*, and are then terminally colonized with *B. cepacia* (83). The reason for the increased 5-year survivorship associated with *S. aureus* infection is unknown, but it was hypothesized that chronic low-level lung inflammation induced by *S. aureus* may be partially protective against *P. aeruginosa* colonization and progressive disease. It could also be due to the staphylococcal production of an unidentified pseudomonicidal compound.

While potentially helpful for the CF patient, infection with *S. aureus* assumes both antagonistic and beneficial roles when cocultured with *P. aeruginosa*. During *in vitro* polymicrobial growth in CF sputum, *S. aureus* growth is repressed by *P. aeruginosa* within 5 h (156). This growth inhibition could be due to the production of staphylolytic secreted compounds or 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) (125). Hoffman et al. demonstrated that the coculturing of *S. aureus* and *P. aeruginosa* protected staphylococci from killing by the antibiotic tobramycin via the temporary inhibition of staphylococcal respiration, thereby impeding rapid growth (87). Incubation and the prolonged exposure of *S. aureus* to purified HQNO led to the development and recovery of small-colony-variant (SCV) phenotypes. SCVs are often morphologically and phenotypically distinct from their parental generations, with the most common change being defects in the electron transport chain. Therefore, exposure to respiratory inhibitors, such as HQNO, naturally selects for clones that are inherently resistant to such compounds. Because SCV phenotypes do not respond to commonly used antibiotics, they can serve as sources of chronic infection. Indeed, HQNO was found to be present in the airways of the CF lung but not in those of matched healthy controls, which may partially explain the relatively high incidence of SCV identification in CF patients. Additionally, Duan et al. used a rat lung infection model to demonstrate that the coinfection of *P. aeruginosa* with both

Staphylococcus spp. and *Streptococcus* spp. resulted in increased lung histopathology. In addition, a luciferase reporter promoter library screen showed the modulation of *P. aeruginosa* virulence and metabolic genes during coculture (56). Although not fully elucidated, clearly, *S. aureus* and *P. aeruginosa* have the potential to serve both antagonistic and synergistic roles during polymicrobial infection of the CF lung.

Burkholderia cepacia complex infection, consisting of several phenotypically indistinguishable genomovars, is often associated with high mortality rates in CF patients (49). The coinfection of *B. cepacia* with *P. aeruginosa* was reported to result in a more rapid decline in pulmonary function and worse clinical outcomes (97). Preliminary *in vitro* experiments have shown that coculture with *P. aeruginosa* upregulates putative burkholderial virulence factors and increases epithelial adhesion (134). Using the Calgary biofilm device (CBD), Tomlin et al. demonstrated the ability of *B. cepacia* and CF *P. aeruginosa* isolates to form mixed polymicrobial biofilm communities. However, when pseudomonal laboratory strain PAO1 was used, *B. cepacia* was not incorporated into the developing biofilm (205). The detection of copious pyocyanin production in strain PAO1 was found to inhibit *B. cepacia* growth. By utilizing green fluorescent protein (GFP)-based sensor plasmids capable of recognizing specific *N*-acylhomoserine-lactone quorum-sensing molecules, Riedel et al. determined that *B. cepacia* could respond to quorum-sensing signals of *P. aeruginosa* both *in vitro* and during *in vivo* infection of mouse lungs, but this chemically mediated exchange was not reciprocated by *P. aeruginosa* (177). Unfortunately, the target effectors of this quorum-sensing cross talk have not yet been identified, requiring the need for further studies of this interesting and clinically relevant polymicrobial interaction.

Members of the SMG, composed of *Streptococcus constellatus*, *Streptococcus intermedius*, and *Streptococcus anginosus*, common members of the airway microflora, have recently been identified as clinically relevant causes of pulmonary exacerbations in CF patients (193). The coinfection of rat lungs (agar-bead model of CF) with streptococci and *P. aeruginosa* led to increased microbial burden and percent lung damage compared to infection with either microbe alone. Importantly, *in vitro* studies using a promoter-reporter plasmid harboring the *luxABCDE* cassette revealed that several *P. aeruginosa* genes, including virulence factors and drug efflux pumps, could be differentially expressed during coculture with streptococcal flora (56). Furthermore, it was demonstrated that some of these same gene modulations could be mediated by the quorum-sensing molecule AI-2, which can accumulate to high levels in the sputum of CF patients, suggesting that intermicrobial communication between microbes of the "normal" flora may exacerbate CF disease caused by classical pathogens. A culture-independent analysis of a small cohort of CF patients revealed that SMG organisms not only were found during episodes of CF airway exacerbations but also were the numerically dominant species present (195). While the virulence mechanisms of SMG are unclear at this time, future studies should determine whether the increased infectious burden, the quorum-sensing-enhanced virulence of other species present, or a combination of these effects results in enhanced CF disease.

Tunney and colleagues recently reported stark differences between the composition of anaerobic species found in CF patients and that of anaerobic species found in healthy controls by using anaerobic culturing techniques (206). Sputum samples from adult CF patients contained increased numbers and various compositions of anaerobic organisms, including *Prevotella*, *Veillonella*, *Propionibacterium*, and *Actinomyces*. A similar composition of microbial isolates was obtained from the bronchoalveolar lavage fluid of pediatric patients. Interestingly, the identification of *P. aeruginosa* positively predicted increased numbers of anaerobic bacteria in the sputum. Later studies using culture-independent techniques confirmed previous findings but revealed an even more diverse composition of anaerobic flora, with significant patient-to-patient variation (207). Despite the microbial community composition remaining relatively stable, it was shown that antimicrobial therapy resulted in a decreased overall microbial burden that was more pronounced in aerobic than in anaerobic flora. If anaerobic flora do indeed play a critical role in mediating the severity of disease, current antimicrobial therapies may be ineffective at targeting the reduction of this crucial group of organisms.

While not classically identified as confounding causes of CF chronic infection, the presence of a variety of viruses infecting the upper respiratory tract results in an exacerbation of CF symptoms. Mice coinfected intranasally with RSV and *P. aeruginosa* developed more severe disease than mice infected with *P. aeruginosa* alone (51). Even more drastic was the nearly 2,000-fold increase in CFU counts from lung homogenates after coinfection compared to monomicrobial infection. Differences in inflammatory mediators were negligible between the two groups, except that coinfection slightly induced the expression of MIG (monokine induced by gamma interferon), a T-cell chemoattractant. Van Ewijk et al. showed that preinfection and coinfection of epithelial cell monolayers with RSV strongly increased the adherence of *P. aeruginosa* to the epithelial surface; a direct binding of *P. aeruginosa* and RSV virus particles was also observed (210). Therefore, viral particles may serve as a bridge between contact points on the bacterial and host surfaces. Similarly, using PCR detection with nasal swabs, Wat et al. showed that children afflicted with CF at the time of temporary exacerbated pathology were colonized with an upper respiratory virus 46% of the time, compared to unexacerbated controls (16.9%); rhinovirus, influenza A virus, and influenza B virus were the most commonly isolated viruses (218). In support of these findings, Oliver et al. showed that *in vitro* infection of alveolar macrophages with rhinovirus resulted in a replication-dependent release of tumor necrosis factor alpha (TNF-α) and the neutrophil chemokine interleukin-8 (IL-8) (152). However, the preinfection of alveolar macrophages with rhinovirus which were then challenged with either lipopolysaccharide or lipoteichoic acid, major constituents of bacterial Gram-negative and Gram-positive cell walls, respectively, failed to induce the secretion of TNF-α or IL-8. Therefore, viral coinfection with bacterial pathogens may lead to increased colonization or immunomodulatory effects that repress bacterial clearance mechanisms in the CF lung.

The overproduction of mucous and reduced ciliary clearance predispose the CF lung to infection with a variety of organisms. While not fully appreciated, the clinical significance of microbial associations and polymicrobial infection in the lower airways will lead to a better understanding of CF disease progression and selective therapies to eradicate potentially hypervirulent pathogenic microbial compositions.

Parenteral Nutrition Feeding Tubes

While the polymicrobial diseases discussed above are commonly associated with biofilm formation on human mucosal tissues or

epidermal layers, an overwhelming number of human polymicrobial diseases are propagated on abiotic surfaces, such as intravenous and urinary catheters, cosmetic and cochlear implants, stents, artificial lenses, internal nondissolving stitches, tympanostomy tubes, artificial heart valves, ventilator tubes, cerebrospinal shunts, pacemakers, and orthopedic devices, including prostheses, intramedullary rods, external fixation devices, screws, and plates (113, 146, 180). These infected implants may serve as sources of chronic infection and can potentially serve as a source of inoculation into the bloodstream, leading to sepsis. One such example of an implanted medical device-related polymicrobial biofilm infection is the biofilm contamination of parenteral nutrition feeding tubes.

Patients unable to masticate—due in most cases to neurological or pharyngeal disease—may require nutritional support for extended periods. For such individuals, enteral nutrition (EN) is preferred over the parenteral route because it is both associated with a lower risk of serious sequelae and more physiologically relevant in that it preserves the barrier, absorptive, and immunological functions of the gut (31, 94, 117, 226). Percutaneous endoscopic gastrostomy (PEG) tube feeding involves the delivery of nutrients via a silicone tube directly into the stomach and is usually begun after patients have been received EN nasogastrically (NG). PEG feeding is preferred over NG feeding because NG tubes are uncomfortable and easily displaced by the patient (202). Either type of EN bypasses many of the mechanisms preventing microbial colonization of the upper gut, and the feeding tube itself acts both as a conduit through which microorganisms can migrate into the stomach from the external environment and as a convenient surface upon which biofilms can accumulate. The result of these ruptures in the body's innate defenses is the development of an abnormal microflora in the stomach and duodenum and, inevitably, the formation of a polymicrobial biofilm on the surface of the PEG tube.

In immunocompetent individuals, the upper gastrointestinal (GI) tract is sparsely colonized by microorganisms. Based on culture-dependent analyses, the stomach is generally devoid of significant microbiota other than *Helicobacter pylori* and some lactobacilli that are present in low numbers (ca. 10^1 to 10^3 CFU per ml contents) (78, 174). In contrast, the duodenum contains a resident microbiota in which lactobacilli and streptococci are the main culturable species, reaching population densities of approximately 10^2 to 10^4 CFU per ml contents (148). The microbial density in the intestinal tract increases with proximity to the rectum; colonic contents contain up to 10^{12} CFU per gram, principally comprising obligately anaerobic genera such as *Bacteroides* (93).

Low gastric pH is thought to be a major factor suppressing the microbial colonization of the stomach (220). However, some enteric bacteria possess acid resistance mechanisms that may confer protection in the GI tract (34). Additionally, a number of innate defense mechanisms break down in patients with PEG tubes. The absence of the sensory stimuli associated with food intake inhibits saliva production and peristalsis, while reduced swallowing increases pH and reduces gastric nitrite concentrations. The net effect is a greater susceptibility to microbial overgrowth in the stomach and duodenum. The most common pathology in such patients is chronic diarrhea, although more serious complications, such as malabsorption and sepsis, also occur (27). The formation of microbial biofilms on PEG tubes is an unavoidable consequence of bacterial overgrowth. Such microcommunities are highly recalcitrant to antibiotic therapy (200, 216). Moreover, biofilms can harbor pathogens and/or microorganisms carrying antibiotic resistance genes and often cause problems with indwelling devices (17, 151, 158). *Candida* spp. are known to colonize PEG tubes, a phenomenon that can lead to tube deterioration (72–74). There is a consistent overlap of microorganisms found on PEG tube biofilm samples, whether they are obtained from pediatric or from adult patients, where enterococci, staphylococci, *Candida* spp., pseudomonads, and bacilli predominate (46, 137). This is similar to the microbiota present in the upper GI tract of critically ill patients in surgical intensive care units (129). Such colonization has been shown to result most commonly in chronic diarrhea but also poses an increased risk of microbial translocation across the epithelial barrier, potentially leading to bacteremia—the "gut-origin-of-sepsis" hypothesis (124). Thus, the potential clinical consequences of the long-term colonization of the upper GI tract are obvious, especially where the PEG tube surface-associated biofilm acts as a potential refuge and locus of recolonization after antibiotic treatment.

A comprehensive study of the microbiota of patients receiving EN via a PEG tube was carried out by O'May et al. An array of 11 selective solid media was used to isolate microorganisms from the luminal surface of PEG tubes and from gastric and duodenal aspirates from the same individuals (154). The genera isolated were similar to those isolated in previous studies, comprising mainly *Candida* spp., *Enterobacteriaceae*, streptococci, staphylococci, and lactobacilli. Data suggested that the gastric pH had no significant effect on the density of colonization in the stomachs and duodena of patients on EN, although it did affect the composition of the microbiota: *Bifidobacterium*, *Klebsiella*, and *Staphylococcus* spp. were detected only in aspirates with a pH of >3. *Candida* spp., *E. coli*, and streptococci were detected in aspirates with a pH of 1 to 7. PEG tube surface-associated microbiota were similar in composition to those detected in aspirates; thus, it seems likely that these communities are able to seed the lumen of the stomach, thereby allowing recolonization after antibiotic treatment. Significantly, *E. coli*, staphylococci, and *Candida* spp. were detected only in aspirates from patients who had received antibiotic treatment during their stay in the hospital. The same researchers developed a chemostat-based model system using a defined microbiota composed of the microorganisms isolated most commonly from patients on EN (153).

This model system was used to investigate empirically the effect of pH on planktonic and PEG tube surface-associated microbiota. In general, *in vitro* data mirrored those obtained from the abovementioned *ex vivo* studies. The lowering of the pH from 6 to 3 had no significant effect on the density of planktonic or biofilm communities and a significant (circa 10^7 CFU/ml) microbiota was detected at pH 3. It is important that because of the continuous-culture methods employed in that study, these recovery data must represent cells actively multiplying at such low pH values and not merely dormant microorganisms surviving. These data are in stark contrast to those from other studies which suggested that *E. coli* is unable to multiply in environments with pH values of <4.4 (121). However, the lowering of the pH altered markedly the composition of the recovered microbiota: *Candida* spp. and lactobacilli were aciduric, while the numbers of *E. coli* and *Klebsiella pneumoniae* bacteria detected decreased steadily with decreasing pH. Visualization by fluorescent *in situ* hybridization of PEG tube surface-associated biofilms revealed microcolonies surrounded

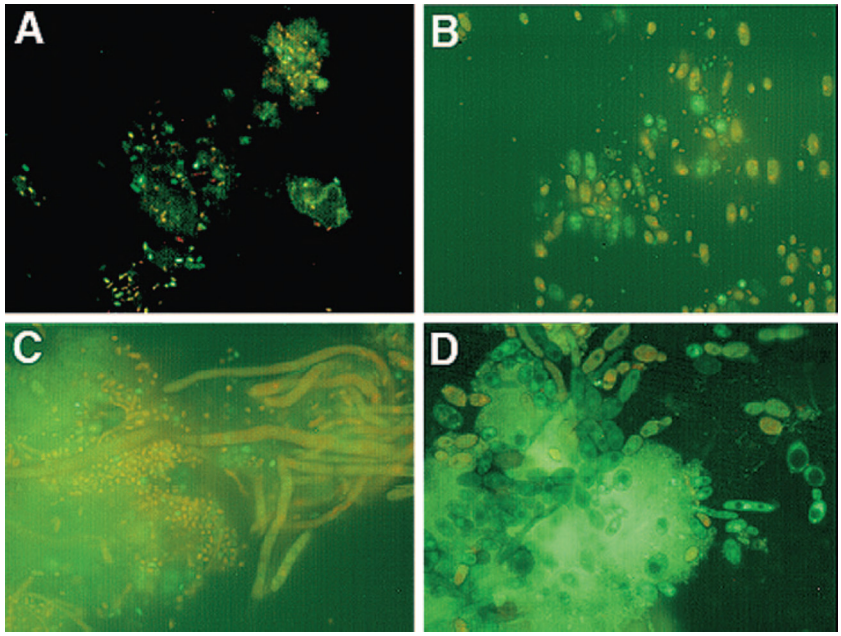

FIG 8 Live/Dead-stained fluorescence microscopy images of *in vitro* PEG tube surface biofilms grown in chemostats at pH 6.0 (A), pH 5.0 (B), pH 4.0 (C), and pH 3.0 (D). Note that as the pH decreases, the yeast cell mass and pseudohyphal growth increase. Frequently, bacterial cells surrounding these filamentous fungi were stained red, indicating cell death, while nearby microbial community members not in contact with the pseudohyphae appeared healthy (green). (Reprinted from reference 153.)

by significant areas of sparsely colonized interstitial space. Microcolonies were comprised of both live and dead cells; in many cases, yeast pseudohyphae were found to be invading the interior of the microcolonies. Where this occurred, any bacterial cells surrounding the pseudohyphae were invariably dead, as determined by BacLight Live/Dead staining (Fig. 8). The mechanism behind this killing remains unknown and requires further research but could be due to the bactericidal *Candida* quorum-sensing compound farnesol, as described above.

The increasing use of EN in the nosocomial setting renders an understanding of the mechanisms behind and consequences of microbial colonization in such patients increasingly important. Biofilm formation is inevitable when the upper GI tract becomes overgrown and when the stable nonshedding surface of the tube luminal surface is present for long periods. Data suggesting that the use of antibiotics in such patients may actually increase the probability of colonization by potentially pathogenic microorganisms, such as *S. aureus* and *C. albicans*, make the search for alternative therapies ever more urgent.

NOVEL APPROACHES TO POLYMICROBIAL DISEASE

Although many common infectious diseases can be initiated by a single pathogen or virulence factor, others can originate from or be attributed to a complex milieu of microorganisms. These consortia of microbes typically coexist as combinations of highly structured communities of bacteria, viruses, protozoans, and fungi attached to biotic and abiotic surfaces, with their architectures facilitated by specific intermicrobial and host interactions (14, 111, 212). Many of these interactions are mutually beneficial for both the host and the microorganism (e.g., the gastrointestinal and oral microbiota). However, microbial species population shifts and waning host immunity can mediate colonization and subsequent infection by opportunistic pathogens that exploit unique niches in the polymicrobial environment (198). Although it has been established that polymicrobial infections significantly alter treatment therapies and patient outcomes, necessary criteria for characterizing and diagnosing these diseases are still ill defined (170, 211).

Considerations for Design and Use of Polymicrobial Vaccines

Despite the challenges of implementing polymicrobial vaccines, several have been attempted and proven successful, while others have resulted in unexpected findings. The reasons for polymicrobial vaccine failures and considerations for their design and future success will be discussed further. Traditionally, the guidelines for vaccine development for monomicrobial infections often rely heavily on molecular Koch's postulates such that directing an immune response against a single virulence or colonization factor will provide protection against disease (60). Although these rules have proven invaluable for vaccination against several diseases (e.g., *Corynebacterium diphtheriae*), they do not adequately consider the pathogenesis of microbes with multiple virulence factors or polymicrobial infections. It has been well documented that biofilm communities demonstrate a significantly different repertoire of gene and protein expression compared to that of their planktonic counterparts (57, 215). However, little is known about the transcriptomic and proteomic profiles of multispecies biofilms assessed against monomicrobial communities. The pleiotropic effects of intermicrobial interactions on the individual disease-causing pathogens and the infected host are only now being appreciated. A recent study by Sibley et al. used a *Drosophila melanogaster* polymicrobial disease model and luciferase reporter assay analyses to examine the effects of common human oropha-

ryngeal commensal isolates in coculture with *Pseudomonas aeruginosa* during infection (192). The results from that study demonstrated that the virulence of *P. aeruginosa* could be substantially enhanced or reduced dependent upon the coinfecting microbe present. Even more surprising was the modulation of host antimicrobial and innate immunity genes due specifically to polymicrobial infection versus monomicrobial infection. These altered microbial and host profiles are likely due to the unique physical interactions and chemical signaling events that occur during the development of polymicrobial communities (15, 89). Therefore, the selection of antigenic targets should be screened for *in vivo* via biologically relevant routes of infection or colonization to ensure that immunogenic proteins of interest are expressed during infection and in the context of a polymicrobial environment, as was previously described (23, 79, 181).

The impact of the polymicrobial nature of a disease on colonization and infection should also be considered during vaccine development. A disease must first be classified as being truly polymicrobial based on sufficient data from clinical studies and epidemiological records. Important criteria regarding the time course, composition, abundance, and consistency of microbes present throughout the course of the disease, from colonization to fulminant infection, should be closely considered (178, 204). One must also be careful to distinguish contaminating microbes (pathogens or commensals) from those that initiate and propagate infection. If, in fact, a disease is considered to be of a polymicrobial nature, a vaccine composed of a multivalent cocktail of antigenic proteins from all microbes involved in disease pathology may be warranted. Although seemingly trivial, these defining criteria are crucial to an understanding of the pathogenesis and the development of successful vaccines for multimicrobial diseases.

Polymicrobial infections can result in several different outcomes in the human host that may affect microbe targeting for vaccine design. Two (or more) microbes may act synergistically or antagonistically to mediate disease, although either microbe in isolation is differentially virulent or benign (29, 53). Periodontal disease, perhaps the most well-studied polymicrobial disease, is an infection of the gums mediated by chronic infection and subsequent host inflammatory responses resulting in the resorption of oral bone tissue. Although the entire consortium of microbial species responsible for periodontitis is unknown, the Gram-negative bacterial pathogen *P. gingivalis* is believed to exacerbate disease pathology by the production of lactic acid, which increases inflammation at the gum interface, and by the direct penetration of the oral mucosa (145, 224). A study by Saito et al. showed that *F. nucleatum*, a resident of the oral cavity, enhances the transportation of *P. gingivalis* inside epithelial cell monolayers *in vitro*. However, coincubation with another species, *A. actinomycetemcomitans* or *T. forsythia*, resulted in the abrogation of *P. gingivalis* invasion (184). The enhanced invasion of the epithelium may promote the further degradation of gum tissue and increase host inflammation. Furthermore, studies by Kesavalu et al. demonstrated that periodontal infection with *P. gingivalis*, *T. denticola*, and *T. forsythia* resulted in increased alveolar bone resorption during polymicrobial disease compared to infection with either microbe alone or healthy controls; the addition of *F. nucleatum*, a known etiological agent of periodontitis, was not required for enhanced disease (104). Importantly, these results suggest that specific polymicrobial associations may exacerbate periodontal disease severity or progression. A current recombinant DNA vaccine directed toward a *P. gingivalis* outer membrane protein has demonstrated promising results in a mouse model of infection, but a successful vaccine for chronic periodontitis has yet to be licensed for human use (225). It is debatable whether a successful vaccine for periodontitis should include antigens against *F. nucleatum* as well as *P. gingivalis*. The failure to reduce levels of *F. nucleatum* may facilitate the increased invasion of other microorganisms that can result in the same disease phenotype. However, due to the complexity and variation of cariogenic microbial communities between individuals, characterizing a discrete set of antigens against caries-related microorganisms suitable for a comprehensive vaccine would be challenging.

Further considerations arise when designing polymicrobial vaccines by the current vaccinology dogma. Even if a vaccination attempt successfully negates a necessary virulence factor for one pathogen (i.e., a toxin), virulence could potentially be complemented in *trans* by another factor produced by a neighboring species in the polymicrobial community. One example of virulence factor sharing has been observed for *Bordetella pertussis*, the causative agent of whooping cough. *B. pertussis* encodes the 105-kDa A-B-type pertussis toxin, which it utilizes to deplete impending host innate immune responses encountered in the human respiratory tract (5). Bacterial superinfection with *S. aureus*, *H. influenzae*, or *S. pneumoniae* is quite common during and after infection with *B. pertussis* (188). Studies by Tuomanen demonstrated that these coinfecting microbes bound poorly to ciliated epithelia, the primary site of *B. pertussis* colonization. However, the pretreatment of ciliary epithelial surfaces with pertussis toxin greatly enhanced their adherence (208). These important results show that microbes can benefit from virulence factors of other organisms or strains during coinfection to enhance their own pathogenesis and colonization. Therefore, attempts at vaccination against an *S. aureus* or *S. pneumoniae* colonization factor may have no effect in cases of coinfection with *B. pertussis* due to virulence factor hijacking. Vaccine strategies can be optimized by directing antigen selection toward shared microbial determinants to simultaneously attenuate the potential virulence of coinfecting species that pose significant threats for secondary infections with similar disease phenotypes.

An additional concern for polymicrobial vaccine design is that the eradication of one species from the polymicrobial community may be insufficient to reduce overall disease, as another organism present may fill the niche left behind. Otitis media (OM), a spectrum of infections of the middle ear, is one of the most common worldwide pediatric diseases, affecting nearly 80% of children before the age of 3 years. Pathological symptoms include fever, localized infection of the area between the tympanic membrane and the inner ear, increased pressure in the middle ear associated with mild to severe pain, and difficulty hearing; untreated cases can lead to complete hearing loss (62). OM is responsible for yearly health care costs upwards of $5 billion in the United States alone due to recurrent infectious episodes, lengthy antibiotic therapies, and the installation of preventative tympanostomy tubes (71). Clinical evidence suggested that OM is a polymicrobial disease mediated by infection primarily with *S. pneumoniae*, NTHi, *M. catarrhalis*, and respiratory syncytial virus (13). Recent attempts to limit middle ear infections in infants resulted in the development of the heptavalent pneumococcal conjugate vaccine (PCV7), which was made available for use in the year 2000. Since then, it has been effective at reducing bilateral and recurrent cases

of OM in high-risk populations but has failed to reduce the overall burden or time to development of OM (144). PCV7 is composed of capsular poly- and oligosaccharide antigens from the most common strains of *S. pneumoniae* infecting young children. PCV7 has failed to induce high levels of protection against OM due to its ineffectiveness against the polymicrobial contributions of *H. influenzae* and *M. catarrhalis* to disease pathology (167). Furthermore, recent epidemiological data suggested that the administration of PCV7 has led to increases in the colonization of the nasopharynx by *S. aureus* and nonvaccine strains of *S. pneumoniae*, which may contribute to vaccine-resistant OM and other infections (20, 149). In spite of this, a PCV13 vaccine, containing additional antigens for six other common pneumococcal strains, has recently been cleared for use in the United States and several other countries (223). Initial studies have shown similar safety and efficacy in priming antibody responses to the same degree as the PCV7 vaccine; follow-up studies will further clarify the success of this current vaccine design in reducing overall invasive pneumococcal morbidity (157). Therefore, it is imperative that vaccines be designed with expectations that the elimination of a single microbial species or strain may be insufficient to prevent infection and that a subsequent colonization of microbes may exacerbate or facilitate other infectious processes.

Synbiotic Use

Besides vaccination, several other therapeutic measures have been undertaken to prevent polymicrobial infection. In the case of PEG-mediated polymicrobial disease, a failure of antimicrobial therapy requires an alternative treatment strategy, given the evidence that the administration of antibiotics may actually increase overgrowth by potentially pathogenic microorganisms (154). Smith et al. used the above-mentioned *in vitro* model to investigate the use of a synbiotic for just such a purpose (A. R. Smith, S. Macfarlane, G. A. O'May, N. Reynolds, and G. T. Macfarlane, submitted for publication). A synbiotic is defined as the combination of a probiotic and a prebiotic (an oligosaccharide indigestible by humans but able to be fermented by beneficial gut bacteria such as *Lactobacillus* and *Bifidobacterium* spp., therefore promoting their growth). The synbiotic was composed of *L. acidophilus*, *Bifidobacterium bifidum*, *Bifidobacterium lactis*, and Synergy 1 prebiotic and had been used in a previous study for the treatment of ulcerative colitis and Crohn's disease (64, 199). In this study, the synbiotic was administered either 48 h before or after the insertion of the PEG tube into the chemostat. The addition of the synbiotic post-PEG insertion reduced biofilm formation on the PEG tube, with a significant reduction in numbers of *E. coli* and *K. pneumoniae* bacteria. When the synbiotic was added prior to the PEG tube insertion, the colonization of the tube surface by *Candida albicans* and *Candida famata* was also inhibited. These preliminary data suggest that the administration of a synbiotic to PEG patients may be an alternative to antibiotic therapy.

Diabetic Foot Wound Treatment

Diabetic foot wound infections are often resistant to traditional antimicrobial regimens and, due to the immunocompromised nature of such individuals, can actually increase the risk of certain infections, like clostridial colitis (77). As such, several alternate treatment strategies have been developed, including the use of vacuum-assisted closure (VAC), hyperbaric oxygen (HBO) facilities, and maggot debridement therapies (MDTs). VAC therapy is utilized to apply negative pressure evenly across a wound surface via an electronic vacuum pump, which leads to the removal of interstitial fluid from the wound site while simultaneously increasing vascularity for enhanced tissue repair and microbial clearing (150). Along with conventional surgical treatments, VAC therapy has been shown to aid in the clearance of various polymicrobial wound infections and reduce the time to complete wound closure (9, 10, 150). Hyperbaric oxygen treatment involves the intermittent inhalation of 100% pure oxygen at pressures greater than 1 atm (16). Increased oxygen levels are then delivered to anoxic tissues found at the locations of the diabetic foot wound infection; benefits of enriched oxygen tension at these sites are multifold. HBO treatment leads to increased host angiogenesis, neutrophil function, and fibroblast regeneration, while it also encourages the respiration of aerobic microorganisms and the simultaneous inhibition of anaerobic bacterial growth (221). The rationale behind these benefits is that increased respiration leads to increased growth and higher susceptibility to commonly used broad-spectrum antimicrobial agents, enhancing the killing of previously dormant biofilm communities (171). The reduction of anaerobic bacterial populations may reduce the complexity of polymicrobial biofilm composition-mediated infectious synergism, enabling an increased efficacy of standard treatment therapies. Although HBO treatment has been shown to significantly increase oxygen tension in anoxic tissues, it is most advantageous when used in conjunction with antimicrobial therapy and surgical debridement (166).

MDT is a rediscovered treatment for diabetic foot wound infections. Prior to the widespread use of antibiotics, fly larvae (e.g., *Lucilla sericata*) were commonly used for the debridement of decaying host tissue (141). MDT involves an on-and-off cycle of the addition of maggots to infected wounds. It has been shown that during the maggot feeding process, physical mechanisms and the release of antimicrobial and lytic enzymes result in the killing of infecting microbes and the breakdown of devitalized host tissue; antibiofilm properties and the *in vitro* upregulation of fibroblast growth and stimulation by larval secretions may partly explain their usefulness during infection (36, 127, 168). While potentially useful, MDT for the management of chronic wounds has mixed outcomes; essentially, the maggot-facilitated debridement of diabetic foot wounds results in clinical success rates similar to those of traditional surgical debridement but may enhance the efficacy of commonly administered antimicrobials (35, 161). However, MDT is considerably less expensive than surgical debridement and may be a good treatment option in developing countries. Again, the removal of dead host tissue eliminates substrata to which polymicrobial biofilm communities may attach, and attachment prevention may significantly reduce the complexity of coaggregating disease-contributing microbial communities.

Phage Therapy

Phage therapy may be an additional intriguing alternative treatment strategy for complicated polymicrobial biofilm-mediated infections (55). Phages are viruses with a specific tropism for bacterial cells which can infect and reduce bacterial populations by undergoing rounds of phage replication typically followed by bacterial lysis. While bactericidal activity was thought to primarily be a consequence of lytic events, it has been shown that phage particles encode depolymerases that demonstrate enzymatic activity against bacterial matrices, including exopolymeric compounds

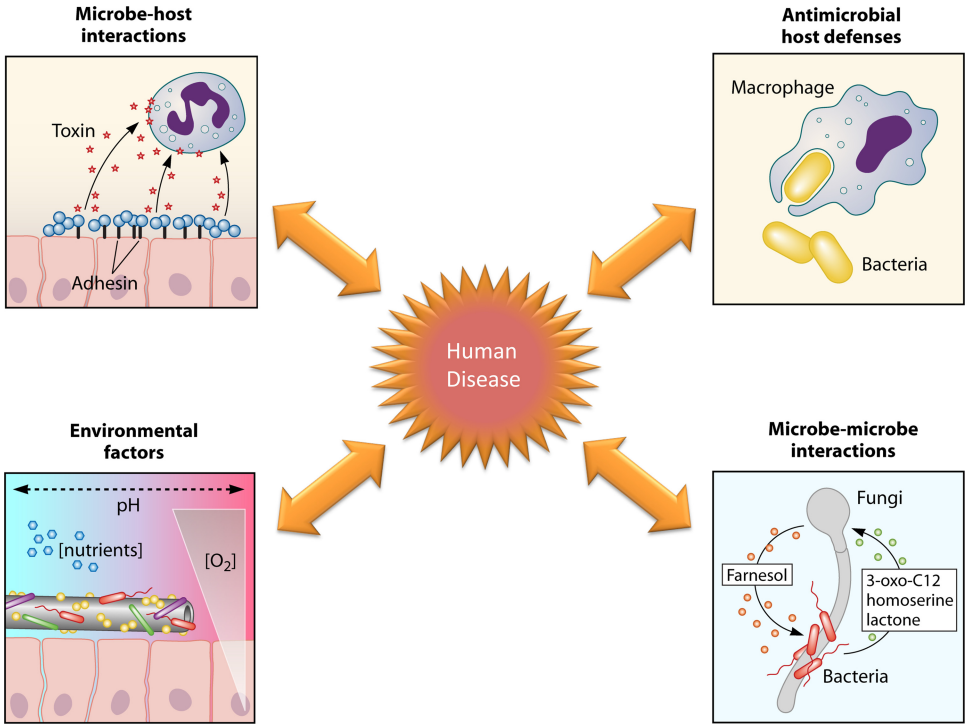

FIG 9 Schematic showing the interdependent relationships required for development of human disease. Infection is influenced by microbe-microbe interactions, microbe-host interactions, antimicrobial host defenses, and environmental factors. Significant changes in any of these factors can lead to the development of or predisposition to infection. For example, microbes lacking virulence factors may become apathogenic. Similarly, host immunodeficiencies will encourage infectious processes. It is now becoming increasingly appreciated that intermicrobial interactions and environmental cues also determine infection outcomes such that specific microbial populations under certain conditions may enhance or predict disease progression.

(95, 173). For example, Glonti et al. have shown that bacteriophage PT-6 produces an alginase capable of breaking down the thick polysaccharide alginate matrix elaborated by *P. aeruginosa* during lower airway infection of the CF lung (70). Additionally, Cerca et al. demonstrated that the application of bacteriophage K to planktonic and biofilm cultures of *S. epidermidis* led to significant reductions in bacterial populations (37). Kinetic analysis showed that while logarithmic-phase planktonic cultures were lysed more rapidly than biofilm cultures, stationary-phase planktonic cultures were cleared at the same rate as that of biofilm clearance. Because some phages are strain and species specific or because bacteria may become resistant to bacteriophage treatment, the use of phage cocktails that are bactericidal to multiple bacterial populations may be warranted (55). As such, phage cocktail-impregnated hydrogels were effective in reducing numbers of *P. aeruginosa* bacteria on catheter surfaces during *in vitro* infection and may have potential applications for the prevention of medical-device-related infections (63). The immediate intraperitoneal delivery of a phage cocktail after *P. aeruginosa* infection of murine burn wounds conferred a nearly 90% increase in the survival of mice compared to those which did not receive phage therapy (136). Lu and Collins also used bacteriophages to overexpress *lexA*, a suppressor of the bacterial SOS response (123). The infection of susceptible *E. coli* cells led to a thousandfold-increased sensitivity to treatment with several different types of fluoroquinolones, demonstrating synergistic potential between phage therapy and standard antibiotic treatment. Combined, these studies have important applications for reducing the biofilm colonization of biotic and abiotic surfaces during infection.

Furthermore, Sillankorva et al. demonstrated that bacteriophages can discriminately destroy target cells during polymicrobial biofilm growth with nontarget bacteria (196). *Pseudomonas fluorescens-Staphylococcus lentus* dual-species biofilms were grown such that *P. fluorescens* comprised most of the biomass. The application of a *P. fluorescens*-specific bacteriophage not only effectively reduced pseudomonal populations but also caused significant changes in the dual-species biofilm architecture, which facilitated the release of staphylococci into the planktonic phase of growth. Although initially tested on environmental organisms with implications for the control of biofouling, it serves as a proof-of-concept for the targeting of pathogenic or coaggregative organisms during polymicrobial biofilm growth. Most importantly, sporadic recommendations for the treatment of various human diseases have demonstrated that phage therapy is seemingly safe, effective, and relatively inexpensive and, as a single treatment, should theoretically self-propagate biofilm clearance; these properties make phage therapy an extremely attractive option for therapy against polymicrobial biofilm-mediated diseases (11).

Therefore, vaccine design and other nonstandard treatments for polymicrobial infections should adequately consider the consortia of microbes responsible for disease, potential intermicrobial interactions resulting in the modulation of *in vivo*-expressed antigens, and the strategic elimination of microbes that enhance or contribute to pathogenesis. It may also be necessary to use small-scale pilot studies and closely tracked follow-up examinations to elucidate which pathogenic microbes may subsequently colonize newly created niches, prior to large-scale treatment deployment. Future strategies may be to target vaccination or treatment against

seemingly nonpathogenic organisms that facilitate the increased pathogenicity and colonization of virulent microbes. Of course, the suppression of "commensals" may have deleterious immunological and microbiological consequences in the host and should be rigorously tested prior to utilization. The use of combinational therapies, including novel synergistic antimicrobial permutations or phage therapy and HBO treatments with chemotherapeutic adjuvants, may be warranted for the eradication of polymicrobial biofilm-mediated infections.

CONCLUSIONS AND FUTURE DIRECTIONS

Bacteria, fungi, and viruses are often coisolated together from complex polymicrobial biofilm communities *in vivo*. Polymicrobial diseases represent the clinical and pathological manifestations induced by the presence of multiple infectious agents and are referred to as being complex, complicated, mixed, dual, synergistic, or concurrent. The presence of a polymicrobial infection has important implications for management because it will modify the clinical course of the disease, impacting the selection of antimicrobial therapy and the anticipated response to treatment, especially when it involves pathogens commonly exhibiting antimicrobial resistance. However, despite the gravity of such infections, polymicrobial disease research is in its infancy.

The biological relevance of microbial interactions remains largely unknown. A deeper understanding of the mechanisms of adhesion and signaling involved in polymicrobial interactions will provide a new perspective on the role of known virulence determinants and the factors relevant to polymicrobial disease. Instead of infection being thought of as a defined host-pathogen relationship, it should be envisioned as a spectrum of host-microbe pathogenic mechanisms, microbe-microbe interactions, host immunity-mediated antimicrobial defenses, and environmental factors. Imbalances in any arm of this interdependent disease continuum may significantly affect disease outcomes (Fig. 9). It may be possible to modify colonization by specific microbes and thus impede the development of disease by the manipulation of adhesion interactions, interference of cell-cell communication mechanisms, or targeted augmentation of host immunity. As such, future studies should focus on implementing animal model systems to study *in vivo* polymicrobial biofilms to investigate the complex dynamics within mixed microbial communities and their importance during interactions with the host.

The key challenges now are to determine mechanistically precise details of the unique biology of polymicrobial interactions under conditions of coexistence. With the application of powerful DNA microarray, proteomic, and metabolomic technologies, the tools are now available to undertake such efforts. The ultimate aim will be to use the knowledge of these processes to develop novel therapeutics, vaccines, and other potential biotechnological applications. The identification of potential targets for the inhibition of coadhesion and biofilm development may ultimately provide the means to modify microbial colonization and thus reduce the impact of polymicrobial diseases on human health.

ACKNOWLEDGMENTS

These studies were funded by the National Institute of Allergy and Infectious Diseases, National Institutes of Health grant R01 AI69568, and the National Institute of Dental and Craniofacial Research, National Institutes of Health, grants IR01DE20939, IR01DE14158, and 5T32DE007309.

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