Getting to Know "The Known Unknowns": Heterogeneity in the Oral Microbiome

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Abstract

Technological advances in DNA sequencing have provided unprecedented insights into the composition of the oral microbiome in health and disease, and RNA-sequencing and metabolomics-related technologies are beginning to yield information on the activities of these organisms. Importantly, progress in this area has brought the scientific community closer to an understanding of what constitutes a health-associated microbiome and is supporting the notion that the microbiota in healthy sites assumes an active role in promoting health and suppressing the acquisition, persistence, and activities of overt and opportunistic pathogens. It is also becoming clear that a significant impediment to developing a conclusive body of evidence that defines a healthy microbiome and the mechanisms by which beneficial bacteria promote health is that an inherent characteristic of the most abundant members of the oral flora, those that potentially play the greatest roles in health and disease, is intraspecies genomic diversity. In particular, individual isolates of abundant commensal and pathogenic streptococci show tremendous variability in gene content, and this variability manifests in tremendous phenotypic heterogeneity. Analysis of the consequences of this diversity has been complicated by the exquisite sensitivity these bacteria have evolved to environmental inputs, inducing rapid and substantial fluctuations in behaviors, and often only within subpopulations of the organisms. Thus, the conditions under which the oral microbiota is studied can produce widely different results within and between species. Fortunately, continually diminishing costs and ongoing refinements in sequencing and metabolomics are making it practical to study the oral microbiome at a level that will create a sufficiently robust understanding of the functions of individual organisms and reveal the complex interrelationships of these microbes ("the known unknowns") in a way that researchers will be able to engage in the rational design of reliable and economical risk assessments and preventive therapies.

Keywords: caries, microbiology, microbial ecology, microbial genetics, genomics, virulence

Introduction

It was not long after the discoveries of restriction enzymes that cleave DNA at specific sequences (Smith and Wilcox 1970) and the ability of DNA ligases to couple 2 strands of DNA that the first recombinant DNA molecules were created in 1972 (Jackson et al. 1972). The first report of a DNA molecule cloned from an oral bacterium was the entire cryptic plasmid (pVA381) from *Streptococcus mutans*, which was cloned onto the *Escherichia coli* plasmid vector pBR322 in 1981 by Roy Curtiss and friends (Hansen et al. 1981). In 1982, the Curtiss group also cloned the first genes from the chromosome of *Streptococcus mutans* and demonstrated that they could be expressed in *E. coli* (Holt et al. 1982; Jagusztyn-Krynicka et al. 1982). In the ensuing 30 y, major strides were made in molecular genetics and DNA-sequencing technologies that led to a much greater understanding of the virulence attributes of *S. mutans*, the most common human dental caries pathogen, how these factors contribute to disease, and how their production was regulated. In 2002, the entire genome sequence of *S. mutans* UA159 was completed (Ajdic et al. 2002) at a cost of more than 3 million U.S. dollars. Today, the cost to sequence an entire bacterial genome can be as low as $50. In 2013, Cornejo and coworkers sequenced nearly 60 genomes of isolates of *S. mutans* from around the globe and performed an analysis that demonstrated the remarkable genomic heterogeneity of this single species of oral streptococcus. While the core genome (i.e., genes present in all isolates) of *S. mutans* was shown to contain about 1,400 genes, the pangenome was estimated at about 3,400 genes and continues to grow because of lateral gene transfer. Viewed another way, each isolate of *S. mutans* carries about 600 genes that are not present in every other isolate of *S. mutans*. Work by Palmer and colleagues (2013) confirmed that the genomic heterogeneity of *S. mutans* manifests in tremendous phenotypic heterogeneity, with individual isolates displaying major differences in properties related to virulence: biofilm formation, acid and oxidative stress tolerance, genetic competence, and production of exopolysaccharides. Emerging genome-scale information and the knowledge that most streptococci can become naturally competent for DNA uptake provide evidence that a similar level of genomic and phenotypic heterogeneity is inherent in oral streptococci (Richards et al. 2014), the most abundant group of

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organisms in the human oral microbiome. Never in the history of oral microbiology and the study of dental caries have we known so much yet has it been so abundantly clear that we now know so little—"the known unknowns"—about the human microbiome and its role in dental caries.

Lessons Learned from a Known Caries Pathogen

Advances in DNA-sequencing technologies in particular, along with some careful clinical and traditional microbiological analyses, have been the primary driving forces that effectively reshaped the view of the etiology of dental caries. As articulated in ICNARA3 presentations and companion papers from Drs. Nascimento's and Mira's research groups, the past 15 y have witnessed the growth of a body of literature that well supports the ecological plaque hypothesis (Marsh 1994; Newbrun 1979). Caries, in 2017, is recognized by the vast majority of the scientific community as a disease of induced-dysbiosis, arising from environmental stresses placed on a health-associated microbiome by increased carbohydrate intake in the diet, with a corresponding increase in the depth and duration of the acid challenge to the tooth and tooth biofilms. While metagenomic studies confirm that *S. mutans* deservedly retains its designation as a primary etiological agent of human dental caries, the sensitivity and specificity of DNA-based analysis of the microbiome has proven that caries can occur in the absence of biologically relevant proportions of this organism (Burne et al. 2012; Nascimento et al. 2017). Moreover, it is interesting to hypothesize that the results of microbiome studies may reflect that there are isolates of *S. mutans* that can be effective pathogens in the early stages of caries but that some of these isolates do not persist as advanced lesions develop. Of course, isolates of *S. mutans* are frequently present in abundance in advanced cavitated lesions and often constitute the majority of the microbes in the aggressive form of the disease known as early childhood caries (Hajishengallis et al. 2017). However, many isolates may simply not persist when the truly aciduric (acid-tolerant) "heavyweights" (e.g., certain lactobacilli, bifidobacteria, and some *Scardovia* spp.) drive the pH of biofilms to values that are lower than can be tolerated by these less-aciduric isolates of *S. mutans.* It should also be recognized that the ability of *S. mutans* to produce a complex exopolysaccharide matrix, when sucrose is present in the diet, can create acidic microenvironments that strongly favor resident aciduric species (Koo et al. 2013).

Are Current Anticaries Strategies Aligned with the Current View of Caries Etiology?

One consequence of the evolution of our view of caries etiology is the recognition that current approaches for caries management do not necessarily address the underlying basis of the problem. In one sense, the most common intervention (i.e., eradication of plaque through mechanical and/or chemical means) is a completely logical approach that in most cases is an effective caries-prevention strategy. However, such an approach generally does not reverse the dysbiosis seen in cariogenic microbiomes, since the susceptible surfaces of the teeth simply become repopulated with a microbiome that is similar in composition to the one that was removed. As noted by Dr. Nascimento, this is why therapeutic strategies that can be combined with traditional methods (dentifrices, rinses), but that are designed to repopulate the teeth with a health-associated microbiome to preserve the integrity of the tissues, is attracting the interest of various research groups (Burne et al. 2012). Such approaches include the use of prebiotics, such as arginine, which appears to inhibit caries and promote remineralization through multiple mechanisms. In particular, arginine metabolism via the arginine deiminase (ADS) pathway expressed by many abundant commensal streptococci produces ammonia and CO_2, which effectively neutralize acids produced from fermentation of carbohydrates. The ADS also influences plaque ecology in a positive way by providing bioenergetic benefits to ADS-positive organisms in the form of elevated cytoplasmic pH, production of adenosine triphosphate (ATP), and generation of a less acidic plaque environment that is favorable for the growth of health-associated species (Nascimento et al. 2014). More recently, arginine has been shown to have detrimental effects on expression of a number of virulence-related attributes of *S. mutans* and to alter the exopolysaccharide matrix produced by *S. mutans* in a way that could reduce the cariogenic potential of oral biofilms (He et al. 2016; Chakraborty and Burne 2017). Notably, a number of probiotic formulations have appeared on the market. Some are based on lactobacilli that presumptively interfere with binding of *S. mutans*, whereas others contain live organisms that have demonstrated some form of in vitro efficacy in interfering with certain pathogens (e.g., Evora). Unfortunately, none of these formulations have been tested in rigorous clinical trials and thus have not received endorsement from reputable regulatory or professional organizations because of a lack of sufficient evidence of efficacy or safety.

Acceptance of the ecological plaque hypothesis as underpinning caries etiology means that one should be able to define a microbiome, either by microbial composition, genomic composition, metatranscriptomic patterns, metabolomic signatures, or some combination of these biomarkers, that will be compatible with sustained health of the underlying tissues. Accepting this tenet, then, one must agree that effectively populating the human oral cavity with health-associated bacteria (either in probiotic or synbiotic formulations) should effectively suppress the emergence of a cariogenic flora and be one possible way to limit the initiation and progression of dental caries. It seems reasonable to predict that, with current technologies, we will soon arrive at some workable definition of a health-associated microflora. Whether, with such information, a probiotic formula can be created and persistently colonize a human host in a cost-effective, or even practical, manner remains to be determined. On one hand, early intervention shortly after tooth eruption with annual or semiannual reinoculation of a probiotic formulation might establish a health-associated flora that persists for a lifetime. Conversely, treatment with a probiotic or

synbiotic may result in only transient colonization with the desired organisms, and the frequency with which one would have to repopulate the oral cavity could render the approach cost prohibitive, impractical, or both. Although important, the potential negative consequences of organisms implanted for caries control finding their way into the circulatory system and organ systems will not be addressed here.

Does a Health-Associated Microbiome Actively Promote Dental Health?

The question posed in this subheading may itself be an overreach. Answering "yes" rejects the notion that the microbiomes that have been associated with health are entirely benign and that they are health associated because they simply do not cause sufficient damage to the tooth to overcome the forces that stabilize tooth mineral. Answering "yes" also posits that a health-associated microbiome plays an active role in the prevention of the initiation and progression of dental caries. Twenty years ago, there was insufficient evidence to make such a claim, but an argument in the affirmative can now be constructed based on a growing body of in vitro and in vivo studies. In fact, there is now ample evidence that commensals perform a number of processes that actively protect the host from the initiation and progression of caries.

First, the microbiome colonizing a healthy tooth actively generates alkaline products from salivary and dietary substrates, neutralizing plaque acids and creating a resting plaque pH that is favorable for remineralization (Burne and Marquis 2000). The elevated pH also provides an environment that favors the growth of health-associated, acid-sensitive organisms over aciduric, cariogenic organisms, which gain their selective advantage at pH values that are generally below 5.5 to 5.8. The bioenergetic advantages of endogenous ammonia production are also substantive and likely influence plaque ecology and competitiveness of commensals. Entry of urea or arginine into the cell does not require ATP or other high-energy equivalents. However, when NH_3 is released in the cytoplasm, as occurs with ureases and the ADS, it can rapidly equilibrate with a proton to yield ammonium ion (NH_4^+), thereby neutralizing the cytoplasm and enhancing the ΔpH component of the proton motive force ($\Delta P = \Delta pH + \Delta \Psi$). Oral streptococci work to keep the intracellular pH some 0.5 to 1.0 pH units higher than the outside of the cell. Practically speaking, NH_3 production inside the cell saves ATP, since the proton-pumping F_1F_O-ATPase is the primary route of extrusion of protons to maintain ΔpH. Notably, the ADS and urease are able to produce excess NH_3, which can leave the cell as an uncharged gas and interact with a proton to form NH_4^+ outside the cell, resulting in an increase in biofilm pH and a decrease in the exposure of the tooth to low pH. The importance of the microbiome actively engaging in pH homeostasis to moderate acidification of plaque cannot be overstated in the context of dental caries (Burne and Marquis 2000). Simply put, caries cannot and does not occur unless the pH is lowered to values that allow for enamel demineralization. Elevated resting plaque pH has been correlated with ammonia levels, and a higher resting plaque pH means that an equivalent amount of acid production from ingestion of fermentable carbohydrates will result in a higher terminal pH in tooth biofilms. Thus, 1 way commensals are actively beneficial to dental health is through alkali generation.

Data are also accumulating that commensals have multiple other "active measures" that they deploy to modify plaque ecology by suppressing the growth of *S. mutans* and even other pathogenic species (Kreth 2011). For a long time, it has been known that oral streptococci and health-associated commensals tend to produce relatively high (mM) amounts of hydrogen peroxide (H_2O_2) when cultivated in the presence of oxygen, at least under certain conditions. The importance of H_2O_2 production in the inhibition of the growth of *S. mutans* by oral streptococcal commensals has been highlighted recently, and the genetics and biochemistry of this process have been further clarified. It had been thought that the H_2O_2-forming NADH oxidases, which are more prevalent in commensal oral streptococci than in many organisms, were a primary route for generation of this inhibitory compound (Marquis 1995). However, more recent analyses have shown that pyruvate oxidase can be the primary pathway to generate H_2O_2 in oral biofilms. In particular, the SpxB enzyme converts pyruvate, molecular oxygen, and free phosphate into the high-energy compound acetyl-phosphate (AcP), plus CO_2 and H_2O_2 (Chen et al. 2011). The ramifications of utilization of pyruvate in this manner are complex. First, pyruvate is directed away from production of the stronger acids (e.g., lactate or formate). Second, the bacteria benefit because AcP serves as a donor, via acetate kinase, to generate ATP. The CO_2 produced provides some buffer capacity when converted to bicarbonate, and of course, the H_2O_2 is secreted into the surroundings where it can inhibit the growth of *S. mutans* and reactive oxygen species–intolerant anaerobes through lipid peroxidation, other damage done directly by H_2O_2, or after conversion to hydroxyl radical (OH·) in the presence of certain transition metals (e.g., iron; Marquis 1995). The regulation of SpxB production is also of interest in that induction is highest under conditions where commensals have an edge over *S. mutans*, in aerobic conditions with relatively lower concentrations of readily fermentable carbohydrates (Zheng et al. 2011). In fact, *spxB* is subject to catabolite repression by physiologically high levels of glucose. Since *S. mutans* growth and biofilm formation are generally inhibited by oxygen, one can envision SpxB-dependent H_2O_2 generation as a mechanism whereby commensals can deal a fatal blow to *S. mutans* in relatively immature, early oral biofilms. Other routes for H_2O_2 generation have emerged; for example, lactate oxidase and L-amino acid oxidases seem to contribute to varying degrees depending on the species of oral streptococcus and the growth conditions (Tong et al. 2007, 2008).

Commensals have other active mechanisms to interfere with the growth and/or expression of virulence of *S. mutans*. Interestingly, a number of processes in *S. mutans* that are directly involved in establishment, persistence, and virulence are regulated by 2 peptides that are externalized and can function as signal molecules to activate a variety of genes (Son et al. 2012). Competence-stimulating peptide (CSP) is derived from the ComC precursor via secretion and processing by a

dedicated ATP-binding cassette (ABC) transported to yield an extracellular 21-aa peptide (Li et al. 2001). Further processing by the SepM protease yields the most-active 18-aa form of CSP (Hossain and Biswas 2012), which is detected by a specific 2 component system composed of the sensor kinase ComD and the cytoplasmic response regulator ComE. In complex media containing peptides, CSP is produced naturally during early exponential growth phase, or it can be synthesized and added to cells, to activate gene expression through ComDE (Son et al. 2012). The primary targets of ComE are genes encoding bacteriocins (mutacins) of the lantibiotic type and nonlantibiotic type, which can kill multiple commensal streptococci and can be active against certain isolates of *S. mutans* (Kreth et al. 2006). A secondary consequence of signaling by CSP through ComDE is activation of natural genetic competence and altruistic cell death pathways; the latter can lead to lysis (Perry et al. 2009) and extracellular DNA (eDNA) release. The CSP-ComDE pathway has also been linked to acid tolerance and biofilm formation. Although some controversy exists as to whether it is truly an intercellular communication signal, the second peptide signaling pathway is XIP (*comX*-inducing peptide), which is a 7-aa peptide produced from the 17-aa ComS peptide (Mashburn-Warren et al. 2010). XIP has been detected outside of cells (Khan et al. 2012) and can be internalized via the Opp ABC peptide transport pathway to form a complex with the ComR transcriptional regulator (Mashburn-Warren et al. 2010). The ComR-XIP complex activates *comX* and *comS*. Addition of nM amounts of XIP to early exponential phase cells causes induction of *comX* and a high level of genetic competence. Higher levels of XIP can induce cell death, possibly through cross-talk with the ComDE system (Son et al. 2015). CSP is not required for induction of competence by XIP in defined medium, but ComR is required for CSP to activate competence.

Kuramitsu and coworkers demonstrated that culture supernates from *Streptococcus gordonii* DL-1 were able to interfere with CSP-mediated signaling. They subsequently identified a protease, designated Sgc or Challisin, that could degrade CSP (Wang and Kuramitsu 2005). Other commensal streptococci, such as the highly arginolytic and antagonistic isolate designated *Streptococcus* A12, produce a Challisin-like protease that is particularly effective at blocking CSP-dependent signaling, with the associated inhibition of activation of bacteriocin gene expression (Huang et al. 2016). Degradation of CSP effectively blocks production of bacteriocins, such that cocultivation of *S. mutans* and A12 results in protection of *S. sanguinis* from killing by mutacins (Huang et al. 2016). Interestingly, *S. gordonii* has no impact on XIP-dependent signaling, whereas A12 is very effective at blocking XIP-dependent activation of *comX* and development of genetic competence (Huang et al. 2016). Collectively, these findings highlight just some of the clever mechanisms commensals have acquired and retained through evolution to disable the primary weapon system of a common human caries pathogen. Likewise, the differences in A12 and DL1 in terms of inhibition of the XIP pathway provide an excellent example of the heterogeneity that exists in commensal oral streptococci in their capacities to antagonize *S. mutans* (Huang et al. 2015). Most recently, and with the advantage of having complete genome sequence of *Streptococcus* A12, functional genomics has identified at least 5 genetic loci that influence the competition of A12 with *S. mutans*, revealing that the complexity of antagonistic strategies used by beneficial commensals may be far greater than previously appreciated (Lee et al., in preparation).

Genomic and Phenotypic Diversity Are a 2-Edged Sword

There is growing acceptance of the notion that examination of the oral microbiome by 16S sequencing alone has some severe limitations (Burne et al. 2012). The primary reasons for these limitations are (1) recent genome sequencing of multiple isolates of a single taxa clearly shows tremendous genomic diversity within species, such that core genomes, particularly in abundant oral streptococci, are but a fraction of the pangenomes; (2) examination of a panel of isolates of *S. mutans* and oral commensals for virulence-related phenotypes and beneficial properties (ADS expression, antagonism of *S. mutans*), respectively, showed that the genetic diversity is correlated with tremendous phenotypic diversity (Huang et al. 2015); and (3) because of this diversity, one cannot know, by 16S sequence alone, if an individual carries particularly virulent or particularly beneficial microbiomes (Palmer et al. 2013; Richards et al. 2014). Collectively, these limitations of 16S-based characterization of the microbiomes have made it especially difficult to identify a truly health-associated microflora and discriminate whether the flora in a given individual is simply benign or is indeed actively engaged in promoting health through mechanisms detailed above.

Fortunately, technologies such as those described by Nascimento, Viera, Koo, and others in this particular issue have evolved to the point where they are sufficiently robust yet economical to tackle the problem of diversity in the microbiome. For example, large-scale sequencing of multiple isolates of the oral microbiome in health and disease not only will give us the ability to correlate phenotypes with gene content (i.e., identifying genetic biomarkers), but this large sequence data set will also allow metatranscriptomic studies to map sequences back to a far greater number of isolates, helping to solve the dilemma of "who is doing what?" in health and disease. These large-scale sequencing efforts, when combined with functional genomic studies, can reveal novel pathways by which truly beneficial bacteria work synergistically to interfere with the establishment, persistence, and virulence of oral pathogens, such as *S. mutans* and *Porphyromonas gingivalis*, but are also very likely to provide microbiologists with new tools to prevent colonization or outgrowth of overtly pathogenic species such as *Streptococcus pyogenes, Streptococcus pneumoniae*, and other respiratory and nasopharyngeal pathogens. In fact, it is not far-fetched to think that commensals may also occlude essential receptors or adhesins for viruses that gain entry to their host via the oral cavity and/or nasopharynx. Thus, the benefits of a health-associated microbiome may extend to protection even from viral infections.

In summary, years of intensive study have brought the oral health research scientific community to a point where we have never understood so much about the organisms, host responses, and interactions that govern the balance between health and disease (both oral and oral:systemic health), yet it has never been so apparent that we have only begun to appreciate the complexities of these diseases and to explore novel, biologically based interventions that can be applied economically and globally to ameliorate oral health and eradicate certain oral diseases. Still, researchers have never been so well positioned to rapidly apply the new technologies at our disposal to make real progress on the aforementioned goals. We finally have gotten to know the known unknowns and realize that we can and will rapidly make these unknowns known.

Author Contributions

R.A. Burne, contributed to conception, design, and data analysis and drafted the manuscript. The author gave final approval and agrees to be accountable for all aspects of the work.

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