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Solving the etiology of dental caries

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For decades, the sugar-fermenting, acidogenic species *Streptococcus mutans* has been considered the main causative agent of dental caries and most diagnostic and therapeutic strategies have been targeted toward this microorganism. However, recent DNA- and RNA-based studies from carious lesions have uncovered an extraordinarily diverse ecosystem where *S. mutans* accounts only a tiny fraction of the bacterial community. This supports the concept that consortia formed by multiple microorganisms act collectively, probably synergistically, to initiate and expand the cavity. Thus, antimicrobial therapies are not expected to be effective in the treatment of caries and other polymicrobial diseases that do not follow classical Koch's postulates.

Microbiology of dental caries

Classical Koch's postulates contend that a specific microorganism can be found to be responsible for an infectious disease when it invades a host, a principle that has been assumed to be correct for most microbial infections. Although the identification of asymptomatic carriers readily showed that the postulates have important limitations and the original formulation has been modified with the introduction of genetic techniques [1], the principles proposed by Koch have remained a cornerstone in microbiology. Probably due to this, when the sugar-fermenting, acidogenic species *S. mutans* was isolated in the 1920s from carious lesions, it was considered to be the etiological agent of dental caries [2]. Dental caries is considered the most prevalent human disease, affecting 80–90% of the world population [3]. In children, it appears to have a fivefold higher prevalence than asthma, which is the second most prevalent disease. For decades, mutans streptococci have been considered the main causative agent of the disease [4] and most diagnostic, preventive, and therapeutic strategies have been targeted toward this microorganism (see, for example, [5–7]). However, other microbial species were also isolated from carious lesions and have been related to the process of tooth decay, including lactobacilli [8] and bifidobacteria [9]. The introduction of molecular approaches to study the human microbiome revealed that the oral ecosystem is inhabited by hundreds of bacterial species [10], most of which are considered commensals, and that species regarded as pathogens are frequently found in healthy individuals, although at lower levels than in diseased subjects [11]. An important hurdle in determining the etiology of tooth decay is that many samples were not taken from the disease site itself but from other, noninvasive samples such as saliva, which does not represent the cariogenic microbiota (Box 1). However, in a seminal work, Aas and collaborators obtained over 1200 clones of the 16S rRNA gene from dental plaques and carious lesions at different stages of the disease [12]. This work showed that *S. mutans* could not be PCR amplified in a significant proportion of samples and other bacteria such as *Atopobium*, *Prevotella*, and *Propionibacterium* appeared to be associated with the disease. Recent work added *Scardovia wiggsiae* as a new etiological agent of severe early childhood caries [13]. In recent years, the use of second-generation sequencing and metagenomic techniques has uncovered an extraordinarily diverse ecosystem where *S. mutans* accounts only for 0.1% of the bacterial community in dental plaque and 0.7–1.6% in carious lesions [14,15]. When the DNA of samples from dentin caries was directly sequenced, obviating cloning or PCR techniques, *Veillonella* appeared as the most common genus [16], underlining the varying nature of microbial composition in cavities. However, these DNA-based studies may quantify dead, transient, or inactive microorganisms that do not contribute to the disease, inflating estimates of diversity and introducing noise in the analysis [17]. Thus, the application of high-throughput sequencing to the RNA extracted from oral samples finally provides an opportunity to identify the metatranscriptome; that is, the active microbial composition and expressed genetic repertoire underlying disease initiation and progression.

The first of these RNA-based studies on the surface of teeth [18] studied the active microbial communities in oral biofilms before and after a meal, identifying the bacteria that increase their activity after food ingestion, with the premise that these organisms may be involved in sugar fermentation and acid formation. Metatranscriptomic data indicate that the active microbiota is a subset of the total microbial composition in oral biofilms [19] but is still extraordinarily diverse. In addition, the RNA-based estimates of diversity indicate that different microbial consortia are formed in the dental plaque of different individuals. Thus, determining the active microbiota in carious lesions may finally unravel the elusive etiology of the disease, paving the way for diagnostic and preventive tools.

The active microbiota of cavities

The first RNA-based estimates of bacterial diversity in cavities are shown in Figure 1, putatively representing the microbial consortia that are actively contributing to the disease. This approach shows an average of eight active

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genera at a presence of over 1% in both enamel and dentin lesions. However, each lesion appears to harbor a different combination of bacteria. There was only one case in which the lesion was dominated by a single bacterial genus (marked as sample CA085, tooth 47 in the figure). This exceptional instance involved *Lactobacillus*, which was found to represent 99% of the RNA-based population in a hidden dentin cavity (55% of the reads had maximal similarity to *Lactobacillus gasseri*, but three other species of lactobacilli were present). However, all of the other samples showed diverse bacterial compositions that varied dramatically between individuals, even within the same carious lesion type (Figure 1). The estimated bacterial diversity was lowest for enamel carious lesions, with an average of 177 species-level phylotypes per sample, and highest for 'open' dentin cavities that were exposed to the oral cavity, which had an average of 251 species per sample. This suggests that open dentin cavities have an input of microorganisms from saliva, even if the biofilm formed on top of the lesion is eliminated from the sample, as was the case here. 'Hidden' dentin cavities, which have almost no contact with the oral cavity except for a minimal lesion through the enamel, had an estimated number of species-level phylotypes of 201 [20]. The observation that multiple species are detected by their RNA in carious lesions unequivocally demonstrates that they are alive and supports the concept that consortia formed by multiple microorganisms act collectively to initiate and expand the cavity. It has been shown that several oral species can act synergistically to increase their pathogenic effect [21]. In a fascinating example of metabolite cross-feeding, *Streptococcus gordonii* was shown to increase the virulence of *Aggregatibacter actinomycetemcomitans* in an animal abscess model [22]. Mixed cultures of *S. mutans* and *Veillonella alcalescens* were also found to produce higher acid levels than biofilms containing only one of these species [23]. In addition, *Veillonella* may favor acid-producing bacteria in caries through nitrate reduction [24], given that low concentrations of nitrite killed several cariogenic organisms. Finally, it must be remembered that not only bacterial interactions but also bacterial–fungal associations can be vital for promoting virulence in disease-associated consortia [25]. For instance, *S. gordonii* modulates biofilm formation in *Candida albicans* and *Candida* load influenced oral bacterial diversity and antibiotic resistance (reviewed in [26]). Also, viruses may play a vital role in shaping microbial populations [27], but this has been poorly studied in oral environments [27].

In the putative list of caries-associated bacteria revealed by this metatranscriptomic approach, *S. mutans* accounts for 0.02% of the active microorganisms in hidden dentin cavities, 0.48% in open dentin cavities, and 0.73% in enamel carious lesions [20]. Thus, although there is substantial evidence that *S. mutans* is associated with caries risk, other species clearly arise as main players in the microbial community, including *Veillonella*, *Rothia*, and *Leptotrichia* in enamel caries and *Streptococcus sanguinis*, *Atopobium*, *Schlegelella*, *Pseudoramibacter*, and *Lactobacillus* in dentin caries. Some of these bacteria are poorly characterized, as exemplified by the genus *Schlegelella*, in which the 16S rRNA sequences identified in this and other studies [28] indicate that this caries-associated oral microbe is a different species from the only two isolated organisms in this genus that are currently described, both in nonhuman niches. The polymicrobial nature of carious lesions implies that animal models are probably not representative of human oral disease, especially in cases where single bacterial species are inoculated in the animal [29,30].

A revealing aspect of RNA-based studies is that the composition of active bacteria in initial, enamel lesions appears to be different from that found in more advanced dentin cavities. This observation holds even in cases where enamel and dentin cavities from the same tooth were sampled and analyzed. An example is shown in Figure 2, where the hidden dentin cavity of a molar tooth had high frequencies of *Neisseria*, *Lactobacillus*, *Megasphaera*, and *Rothia*, whereas a non-cavitated enamel caries lesion had high frequencies of *Haemophilus* and *Gemella*. In addition, the streptococci were dominated by *S. sanguinis* in the dentin cavity, while *Streptococcus mitis* was at significantly higher levels in the enamel caries lesion, which also showed a higher streptococcal diversity [20]. A previous metagenomic study demonstrated that

Box 1. Targeting the causative agents of dental caries

DNA-based studies of microbial diversity in the oral cavity have estimated that the human supragingival dental plaque (the biofilm formed on tooth surfaces) contains between 500 and 700 bacterial species [10,12,16]. These estimates are even higher in saliva, probably because this oral fluid is in contact with all niches in the mouth, reaching values between 1000 and 2000 species in stimulated saliva [52]. In carious lesions, however, the number decreases dramatically to 100–200 species-level phylotypes, both in initial, enamel caries lesions and in dentin or deep-dentin cavities [14,15,35,53], but because these studies are based on PCR amplification of DNA many of the organisms detected may be inactive and not contributing to lesion progression. The recent RNA-based data (see Figure 1 in main text) identify bacteria that are actively involved in translation processes, narrowing the list of caries-related organisms to 40–160 per sample, which are presumably those active in individual cavities. It has been assumed for years that the bacteria involved in the disease should also be present in saliva, which has been the preferred oral sample collected in etiological and epidemiological studies of dental caries due to its noninvasive nature (see, for instance, [54,55]). However, when saliva, dental plaque, and carious lesions from the same individuals are analyzed, it is readily observed that saliva is not representative of the bacterial diversity located at the disease site (Figure I). The microbial composition of the enamel lesion under study (orange circle) appears to be dominated by *Veillonella*, *Fusobacterium*, and *Porphyromonas*, whereas in the saliva sample (blue, outer circle) from the same individual *Streptococcus*, *Neisseria*, and *Prevotella* are the genera found at the highest proportions. This is in agreement with other studies that strongly suggest that saliva samples are not appropriate for studying the microbiology of oral diseases [52,56]. Although the dental plaque samples (green circle) are more similar in bacterial composition to that found in their respective carious lesions, several genera decrease and other increase in proportion in the cavity, as a consequence of the more specialized niche. Thus, even dental plaque will not accurately show the bacterial communities responsible for dental caries and the data strongly recommend the use of carious lesion samples with RNA-based approaches (pink, inner circle) to determine the active etiological agents of the disease.