Spatial mapping of polymicrobial communities reveals a precise biogeography associated with human dental caries

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Tooth decay (dental caries) is a widespread human disease caused by microbial biofilms. *Streptococcus mutans*, a biofilm-former, has been consistently associated with severe childhood caries; however, how this bacterium is spatially organized with other microorganisms in the oral cavity to promote disease remains unknown. Using intact biofilms formed on teeth of toddlers affected by caries, we discovered a unique 3D rotund-shaped architecture composed of multiple species precisely arranged in a corona-like structure with an inner core of *S. mutans* encompassed by outer layers of other bacteria. This architecture creates localized regions of acidic pH and acute enamel demineralization (caries) in a mixed-species biofilm model on human teeth, suggesting this highly ordered community as the causative agent. Notably, the construction of this architecture was found to be an active process initiated by production of an extracellular scaffold by *S. mutans* that assembles the corona cell arrangement, encapsulating the pathogen core. In addition, this spatial patterning creates a protective barrier against antimicrobials while increasing bacterial acid fitness associated with the disease-causing state. Our data reveal a precise biogeography in a polymicrobial community associated with human caries that can modulate the pathogen positioning and virulence potential in situ, indicating that micron-scale spatial structure of the microbiome may mediate the function and outcome of host-pathogen interactions.

**Significance**

Dental caries remains an unresolved public health problem. The etiology is poorly understood, as the oral cavity harbors diverse communities of microorganisms. Using multiple imaging modalities on human teeth from patients with caries, we discovered a microbial community precisely arranged in a corona-like architecture. Moreover, this organization is mediated by the pathogen *Streptococcus mutans* through production of an extracellular scaffold that directs positioning of other oral microbes. We developed a methodology to quantify the spatial structure of microbial communities at the micron scale and found a precise spatial patterning of bacteria associated with localized caries onset. These findings are relevant as we approach the post-microbiome era, whereby quantifying the community structural organization may be essential for understanding microbiome function.

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of this spatially structured community, we simultaneously analyzed the biofilm architecture, pH microenvironments, and enamel surface properties using a mixed-species biofilm model on human teeth. We found that the rotund architecture created a highly acidic pH region that precisely matched the acute demineralization of the enamel surface, indicating that this highly ordered community is associated with localized caries onset. Mechanistically, construction of this architecture is an active process initiated by the production of an extracellular scaffold by \textit{S. mutans} that directs the positioning of other bacteria by creating physical boundaries to assemble the virulent community arrangement. This structural organization also enhances antimicrobial tolerance and increases bacterial fitness associated with the disease-causing state. Our findings reveal the spatial arrangement of polymicrobial biofilm communities in their native state on diseased human teeth, whereby the biogeography may dictate the positioning of pathogens and the virulence potential in situ associated with severe childhood caries.

\textbf{Results and Discussion}

\textbf{Polymicrobial Community Organization of Biofilm on Teeth in the Disease State.} To analyze the spatial structuring of intact human oral biofilms, it was critical to remove infected teeth from diseased patients without perturbing the biofilm to keep its native state intact (Materials and Methods). Once these teeth were collected, we analyzed the spatial and structural organization of naturally formed biofilms on the noncavitated tooth surfaces using confocal laser scanning microscopy and computational analyses after fluorescent labeling of bacteria via taxonomic ranks (domain-genus-species) (Fig. 1A and SI Appendix, Fig. S1). We then used a fluorescence subtraction method (SI Appendix, Fig. S1) to analyze different bacterial arrangements based on taxa-specific labeling. In brief, we used a sequential imaging approach composed of 1) visualization of the entire tooth surface at low magnification using both confocal and scanning electron microscopy; 2) selection of imaging areas to evaluate the biofilm community structure (termed “architecture”) based on morphology and previously used terminologies (11); 3) high magnification, high-resolution imaging of each biofilm architecture and its spatial organization at micron scale; and 4) quantitative computational analysis to determine the biovolume and microbial positioning within the biofilm. A detailed step-by-step protocol of this imaging procedure and a tutorial guide are provided in SI Appendix, Methods.

Our analysis revealed four types of architecture on the diseased teeth, termed rotund-, corncob-, hedgehog-, and seaweed-like.
architectures based on their visual morphology and the available terminologies (11, 17) (Fig. 1B). These specific terms give an identity to each type of microbial community structure. Rotund was the most commonly detected architecture (found in 21 teeth among 30 samples; 21/30) followed by hedgehog (12/30), corn-cob (11/30), and seaweed (11/30). In addition, we also performed quantitative imaging analysis (COMSTAT) of the micrographs to determine the microbial content based on taxonomic ranks: all bacteria (EUB), Streptococcus (STR), and S. mutans (SMU). Corn-cob and hedgehog were observed previously in plaque samples collected from healthy individuals (11). We also found a seaweed architecture that, similar to corn-cob and hedgehog, was dominated by non-streptococcal bacteria and non-mutans streptococci (Fig. 1B and C). However, the rotund shape was particularly distinctive. Compared with other types, it exhibited a 3D dome-like structure (Fig. 1A and SI Appendix, Fig. S2), typically found in sequence-based analyses of plaque from early childhood caries (ECC) (4–7). Interestingly, the rotund shape harbored a greater abundance of S. mutans compared with other architectures (~68% of the total biomass vs. <6%; P < 0.001) (Fig. 1D). These findings complement a previous study showing the composition and spatial distribution of undisturbed biofilms on natural occlusal caries in which S. mutans clusters were found on cavitated lesion sites but not on noncavitated enamel surfaces (18).

**Spatial Organization of the Community across the Rotund Architecture.** Given the distinctive 3D architecture, high population of S. mutans, and frequency of detection (70% of samples; 21 of 30 teeth), we further examined the spatial structure of the rotund shape (Fig. 2 and SI Appendix, Fig. S1B). Visualization and quantitative image analyses of the rotund architecture revealed a corona-like assembly composed of a densely packed bacterial cluster (inner core) dominated by S. mutans (SMU) that was spatially segregated from outer layers of other oral streptococci (NSMU) and bacteria (NSTR) (Fig. 2). Previous laboratory studies have shown that extracellular polymeric substances (EPS) produced by bacteria can provide structural integrity and scaffolding of 3D biofilm structures (19–21); thus, we next tested for the presence and spatial positioning of bacterial EPS in the rotund architectures. S. mutans is an avid producer of an EPS termed glucan synthesized by glucosyltransferases (Gtfs) using sucrose (a key dietary factor in severe childhood caries) as a substrate (3). Using a glucan-specific labeling technique based on Gtf enzymatic activity (21), we were able to assess the spatial

![Fig. 2. Spatial structuring of the bacterial community across the rotund architecture in its native state. (A) Three-channel imaging (species-genus-domain) of the 3D rotund architecture. SMU, S. mutans; (green); STR, Streptococcus (red); EUB, all bacteria (blue). (B) COMSTAT analysis of bacterial cells across the biofilm thickness. (C) Quantitative analysis of confocal images for determination of the biomass of SMU, STR, and EUB. (D) All merged. (E) Z-stack rendering over a 25-μm-thick rotund-shaped microcolony. (F) Representative confocal images of a corona-like spatial arrangement of intact biofilms (z = 15 μm from the tooth surface) following taxonomic differentiation. Green, SMU; red, NSMU; blue, NSTR. (G) Diagram of a corona-like cell arrangement characterized by computational analysis and 3D image rendering.](https://www.pnas.org/content/early/2020/07/06/2010326117)

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production of EPS by the Gtfs present in the human biofilm samples (on extracted teeth) at the time of collection without labeling bacterial cells or its metabolic activity (Materials and Methods). We detected functional Gtfs across the structured rotund community, showing glucans generated in close association with bacterial cells (SI Appendix, Fig. S2). In contrast, EPS glucans were absent in other architectures. Since segregated lineages usually represent cooperative behaviors of expanding population as clonal clusters (14, 22, 23), corona-like cell segregation of S. mutans in the core is likely associated with expansion of a pathogenic cluster embedded in the EPS matrix (21), which could provide stability for the rotund-shaped architecture.

**Rotund Architecture Creates Localized Acidic pH and Enamel Demineralization.** Next, we asked whether the rotund architecture could be assembled in vitro, which would allow for functional studies with the goal of determining the importance of the spatial structure in cariogenesis. As acidification on the tooth enamel surface is a hallmark of caries onset, we were particularly interested in whether the rotund architecture promotes a more acidic environment on the tooth surface (16). The in vitro model we used was a mixed-species biofilm model in which S. mutans (pathogen) and Streptococcus oralis (commensal) were inoculated on natural human tooth enamel and allowed to form a biofilm in the presence of sucrose (Fig. 3A). We developed a sequential stepwise method for synchronized biofilm structure and enamel surface imaging, followed by image realignment for functional assessment related to spatial localization of caries (Fig. 3 A and B; details provided in Materials and Methods and SI Appendix, Methods). The biofilm structure components were determined via a multilabeling approach using species-specific fluorescent probes and EPS glucan matrix labeling (Materials and Methods). In addition, we used our recently developed in situ fluorescence pH mapping method to measure the spatial distribution of pH values across the biofilm structures using ratio-metric analysis (24). Finally, we performed enamel surface optical/fluorescence imaging and microradiography to determine acid damage of the enamel surface (i.e., caries lesions). A detailed protocol and a tutorial guide, providing a step-by-step workflow and description of procedures, is provided in SI Appendix, Methods.

To precisely match the biofilm architectural features with pH mapping and enamel demineralization, we used a hybrid confocal stereomicroscope system using tiled image acquisition to encompass the entire biofilm and enamel surface (Fig. 3 A and B). This approach allowed synchronized imaging and optical alignment of the biofilm structure formed on the entire enamel block surface with 10-μm-length-scale precision. The human enamel blocks were prepared to have a uniform size (4 × 4 mm²) with distinctive edges as fiduciary marks (SI Appendix, Fig. S3A and Methods) to facilitate optical alignment. In brief, the entire biofilm was first imaged using a hybrid confocal-stereomicroscopy system. Then the biofilm was removed from the enamel block and placed back for surface analysis via optical and fluorescence imaging. The enamel placement was guided by the fiduciary marks under the microscope, allowing spatial mapping and alignment between the community structure and demineralized enamel lesions underneath the biofilm.

The 3D-reconstructed and cross-sectional images of the in vitro model revealed two distinct spatial structures similar to those observed on the extracted human teeth:1) the rotund shape with S. mutans forming a densely packed inner core surrounded by an outer shell of S. oralis cells (Fig. 3 C–E) and 2) a flatter community dominated by S. oralis with intermixed bacterial cells interspersed between rotund architectures (Fig. 3 C and D). The rotund architecture was also characterized by the presence of an abundant EPS glucan matrix both within and surrounding the segregated pathogen cluster (Fig. 3 D and E), suggesting that the production of extracellular matrices in situ is likely an important contributor to the biogeography and structural stability of rotund-shaped architectures.

To determine whether the rotund architecture is associated with cariogenic conditions (i.e., high acidity and localized demineralization), we first mapped the location of mixed-species biofilm architectures on enamel surface and the optical (bright-field)fluorescence image of the surface underneath after biofilm removal (Fig. 3C). The demineralized regions in the brightfield (Fig. 3C, Middle) showed striking similarities to the “white spot” patterns seen in early stages of caries onset in children (16), which appear as localized dark spots (Fig. 3C, Right) under the fluorescence imaging commonly used in the clinic (25). Overall, we found that the position of the rotund-shaped communities matched the location of caries lesions on the enamel surface (SI Appendix, Fig. S4).

We next aligned the biofilm structure with fluorescent pH mapping and enamel surface imaging (Fig. 3F). Close-up images revealed that the rotund-shaped community was associated with highly acidic pH microenvironment (Fig. 3F, Left and Middle in the dotted-line box, and SI Appendix, Fig. S3A), whereas in the flat intermixed community, the pH was close to neutral. The acidic pH in the rotund architecture corresponded to the localized demineralization of the enamel surface (Fig. 3F, Right and SI Appendix, Fig. S3A). In addition, we used the same enamel blocks and positioning to analyze phosphate content on the demineralized areas using a confocal Raman microscope. A polarized Raman spectra heatmap based on phosphate intensity confirmed lower amounts of enamel phosphate minerals in the white spot areas compared with nondemineralized regions (SI Appendix, Fig. S3B).

Following the biofilm-enamel surface analysis, the enamel was retrieved and sectioned transversally for measurement of demineralized lesion depth via quantitative transverse microradiography (TMR). Before sectioning, the region of interest was selected based on the realigned biofilm-surface images encompassing both demineralized and nondemineralized areas (SI Appendix, Fig. S3A and SI Methods, Fig. 2C, and ref. 14). Consistent with physicochemical changes associated with the rotund architecture, the enamel lesion depth and mineral loss were also increased (Fig. 3G and SI Appendix, Fig. S3C). Conversely, areas dominated by S. oralis in flat, nonrotund structures (despite similar bacteria biomass as rotund structures; SI Appendix, Fig. S4) did not cause enamel demineralization (Fig. 3G and SI Appendix, Fig. S3C). The rotund architecture created highly acidic pH regions at the biofilm–enamel interface, resulting in localized surface coarsening and light scattering and leading to delineated areas of eroded enamel with an opaque and white chalky appearance, as typically seen in clinical caries and acid erosion of other mineralized tissues and surfaces (16, 26).

**How Does the Rotund Architecture Form?** To further understand the mechanisms of bacterial positioning and cell arrangement in development of the rotund architecture, we examined the spatiotemporal organization patterns using time-lapse imaging and computational analysis. We observed that the community was predominately intermixed with flat architecture at early stages and progressively underwent structural changes toward the rotund-shaped architecture with spatially segregated communities (Fig. 4 A–C). A 3D analysis of this structure revealed, S. mutans clustered in the center, forming an inner core, with S. oralis surrounding its periphery across the entire depth (Fig. 4D) arranged in a “dome-shaped shell” wrapping the pathogen cluster.

To quantitatively characterize the spatial organization of these two distinct communities (intermixed and segregated) at the micrometer scale, we calculated the proportional occupancy
Fig. 3. Simultaneous analysis of 3D architecture, pH mapping, and surface physicochemistry. (A) Experimental design and workflow of a synchronized analysis for mapping of biofilm architecture, pH distribution, and enamel surface demineralization (optical/fluorescence), followed by lesion depth/mineral loss via TMR (n = 8 experiments). (B) Simultaneous alignment of biofilm architecture and surface features. (C) Confocal image of biofilm 3D architecture on the enamel surface and brightfield and fluorescence images of the surface underneath after biofilm removal. Boundaries (marked with white lines) of rotund-shaped areas are delineated in the confocal image and matched across the enamel surface regions. Demineralized lesions on the enamel surface appear as white spots in the brightfield image and as dark spots in the fluorescence image. (D) An example of orthogonal images of rotund and flat communities. (E) Close-up images of rotund architecture showing a corona-like cell arrangement in an S. mutans-S. oralis biofilm. (F) Close-up images showing the rotund-shaped community matched with an acidic pH microenvironment (Left and Middle in the dotted-line box), whereas in the flat intermixed community, the pH was close to neutral. The acidic pH in the rotund architecture also matched with the localized demineralization of the enamel surface (Right). (G) Quantitative TMR analysis of the depth of demineralized lesion (μm) and integrated mineral loss (vol% × μm) (rotund, n = 7; flat, n = 5). Data are mean ± SD. ***P < 0.001, two-tailed Student’s t test.
Fig. 4. Spatial positioning and 3D structural organization of rotund-shaped architecture. (A) Spatiotemporal changes in the patterns of bacterial cell arrangement. Confocal images show the dynamics of spatial organization and cell arrangement between S. mutans and S. oralis during biofilm development. (B and C) Confocal images of cell intermixing at early stages of community development (B) and the assembly of a spatially segregated corona-like cell arrangement at later stage of the biofilm (C). (D) 3D reconstruction of the rotund-shape architecture. (E and F) PO of S. mutans using S. oralis as a focal point (green) and S. oralis using S. mutans as a focal point (red) in intermixed (E) and segregated (F) phases of biofilm development (n = 6). (G) Core/periphery calculations performed using a centroid of the S. mutans biomass as the focal point and then calculating the PO of S. mutans (red) and S. oralis (green) (n = 6). Shaded areas depict mean ± SD. (H and I) Spatial cell segregation in rotund-shaped architecture with a corona-like cell arrangement via species-specific and EPS glucan labeling (H) and close-up image of EPS-induced segregated linages (I).
(PO) using a customized algorithm (Materials and Methods). PO is defined as the proportion of the volume occupied by a target entity (e.g., bacterial cell) at discrete distance intervals away from a focal entity (e.g., another bacterial cell). These discrete volume intervals are analogous to concentric shells of increased radius centered around a focal point and ultimately characterize how the entire population changes around a focal object as one moves away from it. Calculating the PO for multiple bacteria and EPS in a single image quantitatively characterizes the overall spatial organization of the community. Using this metric to analyze the rotund architecture, we found that the PO of S. oralis increased along with distance when S. mutans was used as the focal point, and that the PO of S. mutans increased with distance when S. oralis was used as the focal point (Fig. 4 E and F). This indicates a nonrandom pattern of spatial organization between S. mutans and S. oralis, demonstrating that S. oralis is physically separated from the S. mutans core (Fig. 4 F and G). Conversely, the PO did not change across distance in the flat-intermixed cell arrangement, indicating that S. mutans and S. oralis were randomly organized and close to each other (intermixing) (Fig. 4E). In addition, we found that S. mutans was closely associated with the EPS matrix in the flat architectures, since PO between both entities decreased with increasing distance (SI Appendix, Fig. S5). However, when the community shifted to the rotund architecture, EPS became more abundant in the community and enmeshed S. mutans, while S. oralis was positioned at the outer layer of the S. mutans-EPS cluster (Fig. 4 H and I). This suggests that S. mutans uses EPS to build a scaffold that subsequently allows for formation of the rotund architecture.

Given the EPS location and structural organization in the mixed community, we assessed the changes in the expression of specific genes associated with biofilm formation over time as the community structure shifts from intermixing to segregation. We focused on key genes involved in glucan production (gtfB) and glucan-binding protein (gpbC) that are essential for 3D scaffolding and structural integrity through physical associations between bacteria and the EPS matrix (27, 28). We found a significant up-regulation of S. mutans gtfB (threelfold increase; \( P < 0.001 \)) in the rotund architecture compared with the flat architecture (SI Appendix, Fig. S6). We then examined whether a strain defective in gtfB can alter the interspecies spatial arrangement normally observed in rotund architecture (SI Appendix, Fig. S7A). Since the gtfB mutant and wild-type S. mutans have similar growth rates (SI Appendix, Fig. S7B), we were able to determine the role of EPS in constructing the rotund and flat architectures. We found that an S. oralis and gtfB-defective S. mutans community was unable to form the rotund architecture and exhibited a disordered intermixing spatial pattern (SI Appendix, Fig. S7 C and D). Conversely, supplementation with GtfB promoted clustering of S. mutans surrounded by outer layers of S. oralis, leading to reestablishment of the rotund architecture with a corona-like arrangement (SI Appendix, Fig. S7C). However, the S. oralis corona-like positioning was dependent on the location on the apatitic surface, whereby in some areas the S. oralis had started to encompass the S. mutans cluster, while in other regions it was fully completed, forming the corona arrangement with the pathogen. Quantitative analysis of the bacterial positioning based on PO confirmed the visual observations, indicating the importance of GtfB in assembling a nonrandom and structured mixed community (SI Appendix, Fig. S7D).

To further validate this GtfB glucan-mediated mechanism, we added EPS-degrading dextranase to digest glucans or a Gtf inhibitor to inhibit glucan synthesis without affecting bacterial viability (29). Dextranase is devoid of antibacterial activity or effects on metabolic activity, as demonstrated previously (24, 30). Exposure to dextranase resulted in degradation of the EPS matrix (SI Appendix, Fig. S8), which disassembled the corona-like layers of S. oralis cells without disturbing the structural integrity of the S. mutans inner core and its cell viability compared with enzyme-buffer treated control (intact corona; SI Appendix, Fig. S8). Close-up images of corona disruption across the biofilm are shown in Fig. 5A (Bottom). Likewise, topical treatment with povidone iodide 0.25% to 0.5%, concentrations that inhibit EPS synthesis without affecting cell viability compared with vehicle (PBS) control, on a mixed-species biofilm reduced glucan synthesis and disrupted the corona-like arrangement with S. oralis (SI Appendix, Fig. S9), demonstrating the importance of EPS glucans for establishing this spatial structure.

**Corona Community Structure on Antimicrobial Tolerance and Bacterial Fitness.** Based on the precise biogeography of the rotund architecture with S. mutans in the core, we assessed whether this spatial structuring would protect the pathogen against antimicrobials. We examined the antimicrobial susceptibility of biofilms with an intact and a disrupted community structure using chlorhexidine (CHX), a clinically used oral antimicrobial agent. Dextranase was used to disrupt the corona-like cell arrangement (as described above), and the efficacy of CHX was compared with intact structure (control) based on viable cell counts and a time-lapse live/dead assay (30). In brief, biofilms with intact (enzyme buffer-treated) or disrupted (dextranase-treated) corona were prepared and then exposed to 0.12% CHX or vehicle control (PBS) for 5 min. Then viable cell counts (CFU) of S. mutans and S. oralis for each of the experimental conditions were determined (Fig. 5B). For S. mutans, intact and corona-disrupted biofilms treated with vehicle (PBS) displayed similar numbers of viable cells. In intact biofilms, CHX treatment was ineffective against S. mutans (vs. vehicle) but was capable of reducing the viability of S. oralis (\( P < 0.05 \)). However, the corona-disrupted biofilm displayed increased susceptibility of S. mutans to CHX treatment (vs. vehicle, \( P < 0.001 \)), resulting in fewer viable cells (vs. intact biofilm treated with CHX, \( P < 0.01 \)) (Fig. 5B). These findings were complemented by cell viability imaging (SI Appendix, Fig. S10). Thus, the intact corona structure can provide enhanced antimicrobial tolerance of S. mutans cells located in the inner core (Fig. 5C).

Since the rotund architecture creates a protective environment, it is conceivable that S. mutans cells in the inner core may also display enhanced fitness in low-acid pH environments. To address this possibility, we used biofilms with intact (buffer-treated) or disrupted (dextranase-treated) architecture and performed simultaneous pH mapping and in situ atpB (a key gene associated with acid tolerance) expression analysis, as described previously (24). Under our experimental conditions, dextranase did not affect in situ pH (compared with intact, buffer-treated control; SI Appendix, Fig. S11) or bacterial activity. In intact biofilm, we found that atpB expression was induced at pH 4.5, compared with pH 7.0 in disrupted biofilm (SI Appendix, Fig. S11), consistent with previous studies (24, 31, 32). Notably, atpB expression was elevated in S. mutans cells located in proximity to the S. oralis outer layer compared with those with no or few S. oralis cells nearby across intact architecture (arrows in Fig. 5D). However, we found a distinctive gene expression pattern when comparing intact (buffer-treated control) and corona-disrupted (dextranase-treated) architectures. The atpB expression by S. mutans was significantly higher within intact rotund architecture compared with dextranase-perturbed architecture at both pH 4.5 and pH 7.0 (Fig. 5D and SI Appendix, Fig. S11). Furthermore, quantitative fluorescence analysis revealed 2.8-fold higher gene expression (Fig. 5E) by S. mutans within the intact corona structure compared with the disrupted structure (arrowheads in Fig. 5D). These data indicate that, in addition to antimicrobial protection, the intact rotund architecture and S. oralis corona promotes acid tolerance of S. mutans, an important fitness trait for creating a virulent (cariogenic) microenvironment (Fig. 5F).
Recently, *S. oralis* and other mitis streptococci have been classified as accessory pathogens (i.e., commensal bacteria that may enhance the virulence of pathogens) in periodontitis (33). However, *S. oralis* and *S. mutans* also have been shown to antagonize each other via bacteriocins and hydrogen peroxide production (34, 35), indicating tightly regulated cooperative and competitive interactions to mediate coexistence and survival within biofilms. Whether its spatial positioning with *S. mutans* influences the virulence in vivo awaits further experimental validation using a rodent caries model.

In summary, spatial mapping of the intact polymicrobial biofilm communities revealed a precise biogeography associated competitive interactions to mediate coexistence and survival within biofilms. Whether its spatial positioning with *S. mutans* influences the virulence in vivo awaits further experimental validation using a rodent caries model.

Fig. 5. Effect of corona community structure on antimicrobial tolerance and fitness of *S. mutans*. (A) Disruption of the corona-like cell arrangement via EPS-degrading enzyme (dextranase) treatment. (B) Viable cell counts (CFU) of *S. mutans* and *S. oralis* recovered from the biofilms with either intact (buffer-treated control) or disrupted (dextranase-treated) corona structure following topical treatment with 0.12% CHX or PBS (as vehicle control vs. CHX) (*n* = 6). Data are mean ± SD. NS, not significant. *P* < 0.05; **P** < 0.01; ***P** < 0.001, two-tailed Student’s *t* test. (C) Proposed “drug-protecting barrier” for antimicrobial tolerance of *S. mutans* afforded by the intact corona structure. (D) In situ atpB gene expression of *S. mutans* within an intact corona structure (buffer-treated control) and a disrupted corona structure (dextranase-treated). Arrowheads indicate the periphery of the *S. mutans* microcolony surrounded by *S. oralis* (Upper) or disrupted physical association with *S. oralis* (Lower). Boxes and arrows on the right (atpB expression) indicate site-specific in situ gene expression of *S. mutans* cells located either in proximity to the *S. oralis* outer ring or next to none or few *S. oralis* cells nearby. (E) Localized gene expression level (arrowheads indicate locations) via measurement of GFP fluorescence intensity using ImageJ (*n* = 15). Data are mean ± SD. ***P*** < 0.001, two-tailed Student’s *t* test. (F) Enhanced fitness of *S. mutans* with an intact corona structure under the acidic microenvironment as determined by increased atpB gene expression.
with human caries. A unique round-shaped architecture composed of multiple species in corona-like arrangement stood out as a landmark communal organization on the infected teeth. Further analysis revealed a dense accumulation of *S. mutans* (a cariogenic pathogen) clustered in the center, forming an inner core encapsulated by outer rings of non-mutans streptococci and other bacteria in a highly ordered manner. Using an experimental biofilm model, we found that this spatial patterning is an active process mediated by the pathogen via production of an extracellular scaffold, which provides a protective milieu whereby *S. mutans* displays enhanced antimicrobial and acid tolerance. In turn, the round architecture creates a localized acidic pH microenvironment at the biofilm-tooth interface that causes caries development in situ. In the structured polybacterial communities, mapping the pathogen positioning and the acidic microenvironments in their native diseased state may reveal unique “virulence hotspots” associated with severe childhood oral disease (Fig. 6). Our findings highlight the importance of biogeography of the human microbiome, whereby the spatial structuring of pathogens and commensal microbiota may dictate the virulence potential in situ. This conceptual framework may be applicable to other biofilm-associated polymicrobial diseases in humans and may provide important insights to aid the development of therapies targeting this precise biogeography.

Materials and Methods

**Sample Collection and Ethics Statement.** To assess the biofilm architecture in disease, we examined the spatial organization of the microbial community using extracted teeth from patients affected by dental caries. Tooth samples (30 teeth) were extracted from children (age 36 to 72 mo) diagnosed with severe ECC as defined by the 2014 Conference Manual of the American Academy of Pediatric Dentistry. Tooth extraction is required if the child has tooth decay extending to the pulp and periapical infection, and if functionality cannot be restored. Primary maxillary anterior teeth with mesial or distal carious lesions were extracted using a pediatric maxillary anterior forceps adapted to grasp mesiodistally so as not to disturb the dental plaque formed on the buccal surface of the tooth enamel. The teeth were luxated and extracted by applying mesiodistal rotational forces, and the intact biofilm was immediately transferred to a custom plate to immobilize the biofilm. Tooth samples that could not be extracted using the foregoing method were not included for further analyses. A highly experienced pediatric dentist who was blinded to the study performed the tooth extractions. For imaging purposes, we excluded the sample if >30% of the enamel surface was cavitied or missing and if the clinician was unable to extract the tooth while preserving the intact biofilm. Whole saliva was collected for the purpose of coating the hydroxyapatite or tooth enamel blocks for the in vitro biofilm studies.

**Bacterial-Derived EPS Analysis of Intact Biofilms.** Major EPS components in cariogenic biofilms are extracellular glucans produced by Gtfs that are secreted extracellularly and bound to microbial surfaces (3). The localized Gtf activity was determined by incorporation of a dextran (10 kDa)-conjugated fluorescent indicator into glucans in the presence of a sucrose substrate (38). The fluorescently labeled dextran acts as a primer and is incorporated into newly formed glucan without staining bacterial cells (38). Freshly extracted teeth were gently immersed in 2 mL of a sucrose substrate (200 mM sucrose, 40 μM dextran 900, and 0.02% NaN3 in a buffer consisting of 50 mM KCl, 1 mM CaCl2, and 0.1 mM MgCl2, pH 6.5) containing a 1 μM Alexa Fluor 467-dextran conjugate (Molecular Probes). The reaction mixture was incubated at 37 °C for 18 h. After incubation, the fluorescently labeled Gtf-EPS glucans were observed using confocal microscopy. The presence of NaN3 in the reaction mixture inhibits bacterial metabolic activity as determined experimentally, allowing visualization and measurement of EPS glucans derived from extracellular activity of Gtf in situ. Since bacterial metabolism is inhibited by the addition of sodium azide, we determined EPS synthesized by the Gtfs present in the sample at the time of collection rather than by de novo secretion from bacteria growing during the incubation period with the sucrose substrate. An EPS glucan-labeled sample was then prepared for FISH procedures for microbial cell labeling as described above.

**Spatial Organization of Microbial Communities in Intact Biofilms on the Tooth Surface.** The intact and undisturbed biofilms formed on noncavitated tooth surfaces were gently washed twice with PBS and fixed with 4% paraformaldehyde (in PBS, pH 7.4) at 4 °C for 4 h. After fixation, the biofilms were washed twice with PBS, then transferred into 50% ethanol in PBS (pH 7.4), and stored at −20 °C. The biofilm 3D architecture was analyzed via fluorescence in situ hybridization (FISH) as detailed previously (36, 37). The following FISH oligonucleotide probes were used in this study: MUT590, 5′-ACT CCAGACCTTTCTGAC-3′ with Alexa Fluor 488 for *S. mutans*; STR405, 5′-TAG CGGTCCCCCTCTGCT-3′ with Cy5 for Streptococcus; and EUB338, 5′-GCTGCC TCCGTAGGAGT-3′ with Cy3 for all bacteria. The sample in the hybridization buffer (25% formalamide, 0.9 M NaCl, 0.01% SDS, and 20 mM Tris-HCl pH 7.2) with the probes was incubated at 46 °C for 4 h. After incubation, the hybridized cells were washed with washing buffer (0.2 M NaCl, 20 mM Tris-HCl pH 7.5, 5 mM EDTA, and 0.01% SDS) and incubated for another 15 min at 46 °C. A detailed step-by-step protocol and procedure are provided in *Si Appendix, Methods.*
In Vitro Biofilm Model. Biofilms were formed on saliva-coated hydroxyapatite (sHA) discs or human enamel blocks vertically suspended in 24- or 48-well plates using a custom-made wire specimen holder, mimicking the smooth surfaces of the pellicle-coated tooth (21, 38). For an in vitro model, well-characterized cariogenic streptococci (S. mutans UA159; ATCC 700651) and early colonizing commensal streptococci (S. oralis; ATCC 35037) were grown in ultrafiltrated tryptone-yeast extract broth (UFTYE; 2.5% tryptone and 1.5% yeast extract, pH 7.0) with 1% glucose at 37 °C and 5% CO2 to exponential phase. Each bacterial suspension was then mixed to provide an initial biofilm community on the apatitic surface. Then the biofilms were transferred to UFTYE containing 1% sucrose to induce environmental changes to simulate a cariogenic challenge at 19 h following the ecological transition. The culture medium was changed twice daily across the biofilm depth (from tooth surface to fluid phase). For spatial cell verification, images were acquired using an LSM 800 confocal laser scanning microscope (SP8; Leica Microsystems) equipped with a 20× objective (1.0 NA) water immersion lens was used. The fluorescently labeled biofilm was excited at 780 nm, and the fluorescence emission was collected with a GaAsP or multi-alkali PMT detector (490 to 550 nm for Alexa Fluor 488, 565 to 620 nm for Cy3, and 645 to 700 nm for Cy5 or Alexa Fluor 561). The pH at the saliva-enamel interface was visualized and measured using a fluorescent probe Lysosensor yellow/blue (Molecular Probes) labeling method using multiphoton confocal microscopy and computational analysis (24). In brief, the biofilms were incubated with Lysosensor yellow/blue-labeled dextran conjugate, and the pH values within intact biofilms were measured based on the fluorescence intensity ratio of the dual-wavelength fluorophore. Lysosensor yellow/blue exhibits a dual-emission spectral peak (450 and 520 nm), and the ratio between the fluorescence intensity of these two spectral peaks is pH-dependent within biofilms (24). The fluorescence intensity of both emission wavelengths and their ratio (I450/I520) within each biofilm image was measured using ImageJ 1.22. The ratios of fluorescence intensity of selected areas within each biofilm image were converted to pH values using the titration curves of ratios vs. pH (ranging from 4.0 to 7.0) as described previously (24). For pH measurement, the ratios of fluorescence intensity of selected areas within each biofilm image were converted to pH values using the titration curves and Imager. For visualization of in situ pH distribution at the biofilm-tooth interface, the fluorescence intensity ratios of confocal images were reconstructed using ImageJ and then rendered using Amira software. The fluorescence intensity was converted into grayscale images using Amira tools (Atlas), and the pH image look-up table to correlate with the pH range of 4.0 (black) to 7.0 (white). Unlabeled biofilms were also imaged to determine whether autofluorescence of biofilm bacterial and EPS components would interfere with in situ pH analyses at the wavelengths and laser intensities used in our study; there was no interference with the measurement based on our image analysis. A detailed step-by-step protocol and procedures are provided in SI Appendix, Methods.

Synchronized Analysis of Biofilm Architecture and Surface Demineralization. We developed a sequential multistep method for synchronized biofilm and enamel surface imaging and realignment for structural and functional assessment related to the spatial localization of caries. In this method, we used our in vitro mixed-species biofilm model (as described above). For simultaneous mapping of the biofilm and the enamel surface underneath, wide-field images of the entire biofilm covering the enamel block surface were acquired using a stereomicroscope (Axis Zoom V16; Carl Zeiss) and a confocal microscope (Carl Zeiss) via high-resolution tile imaging. This approach allowed optical alignment of the biofilm structure formed on the entire enamel block surface with 10-μm length-scale precision. The structural organization was assessed via multilabeling approaches using species-specific fluorescent probes and EPS glucan matrix labeling. The pH at the saliva-enamel interface was calculated and measured using fluorescent pH mapping method and ratiometric analysis as described previously (24). Following biofilm imaging and pH mapping, the biomass was removed using an enzymatic treatment (mixture of 8.75 units of dextranase [Sigma-Aldrich] and 80 units of α-glucan glucosidase from A. niger) to obtain the enamel-dentin interface sample. The crystal structure of the enamel block was imaged using a transmission electron microscope (Quanta 250 FEG; FEI).
and 1.75 units of mutanase [a gift from Johnson & Johnson] at 37 °C for 2 h, followed by water bath sonication for 4 min; this procedure was optimized to prevent structural alterations on the enamel surface integrity as determined by confocal topography and microhardness analyses (36). Then the enamel surface was assessed for demineralized areas with optical and fluorescence imaging complemented by quantitative TMR (25, 36, 40).

We also used Raman spectroscopy (Renishaw) to determine the levels of phosphate minerals on demineralized and nondemineralized areas of the tooth enamel. For Raman spectra measurements, a 785-nm laser was used to collect spectra between 0 and –2,000 wavenumbers (cm⁻¹) with a 5-s dwell time from five repetitions at each spot. Collected spectra were corrected to zero, and solar flare spikes were removed within the Renishaw software. The time from five repetitions at each spot. Collected spectra were corrected to collect spectra between 0 and

1. GBD 2016 Disease and Injury Incidence and Prevalence Collaborators, Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990-2016: A systematic analysis for the global burden of disease study 2016. Lancet 390, 1211–1259 (2017).
2. M. A. Peres et al., Oral diseases: A global public health challenge. Lancet 394, 249–260 (2019).
3. W. H. Bowen, R. A. Burne, H. Wu, H. Koo, Oral biopolysaccharides: Pathogens, matrix, and polymicrobial interactions in microenvironments. Trends Microbiol. 26, 229–242 (2018).
4. E. Hajishengallis, V. Parsaei, M. I. Klein, H. Koo, Advances in the microbial etiology and pathogenesis of early childhood caries. J. Dent. Res. 89, 1224–1229 (2010).
5. J. I. Johansson, E. Wiktowska, B. Kaveh, P. Lif Holgersen, A. C. R. Tanner, The microbiome in populations with a low and high prevalence of caries. J. Dent. Res. 95, 80–86 (2016).
6. J. Xiao et al., Association between oral Candida and bacteriome in children with severe EEC. J. Dent. Res. 97, 1468–1476 (2018).
7. W. H. Bowen, Rodent model in caries research. Odontol. Rev. 101, 9–14 (2013).
8. F. E. Devhirst et al., The human oral microbiome. J. Bacteriol. 192, 5002–5017 (2010).
9. P. L. H. Vandenbroecke, R. J. Palmer J. S. Periasamy, N. J. Jakubovics, Oral multispecies biofilm development and the key role of cell-cell distance. Nat. Rev. Microbiol. 8, 471–480 (2010).
10. J. I. Mark Welch, B. I. Rossetti, C. W. Rieken, F. E. Devhirst, G. G. Borisy, Biogeography of a human oral microbiome at the micron scale. Proc. Natl. Acad. Sci. U.S.A. 113, E791–E800 (2016).
11. V. Zijnge et al., Oral biofilm architecture on natural teeth. PLoS One 5, e9321 (2010).
12. A. Stany, L. Metally, S. E. Darby, S. P. Brown, M. Whiteley, The biogeography of polymicrobial infection. Nat. Microbiol. 14, 93–105 (2016).
13. C. D. Nadell, K. Drescher, K. R. Foster, Spatial structure, cooperation and competition in biofilms. Nat. Microbiol. 14, 589–600 (2016).
14. H. C. Fleming et al., Biofilms: An emerging form of bacterial life. Nat. Microbiol. 14, 563–575 (2016).
15. N. B. Pitts et al., Dental caries. Nat. Rev. Dis. Primers 3, 17030 (2017).
16. J. I. Mark Welch, F. E. Devhirst, G. G. Borisy, Biogeography of the oral microbiome: The site-specialist hypothesis. Annu. Rev. Microbiol. 73, 335–358 (2019).
17. I. Diez, L. Grienkajer, B. Nyvad, Molecular studies of the structural ecology of natural oral biofilms. Caries Res. 48, 451–460 (2014).
18. K. M. Colvin et al., The pel polysaccharide can serve a structural and protective role in the biofilm matrix of Pseudomonas aeruginosa. PLoS Pathog. 7, e1001264 (2011).
19. D. S. Kornbrath, B. J. Rossetti, C. W. Rieken, F. E. Devhirst, G. G. Borisy, Biogeography of the oral microbiome: Molecular architecture and assembly principles of Vibrio cholerae biofilms. Science 337, 236–239 (2012).
20. H. Koo, J. Xiao, M. I. Klein, J. G. Jeon, Exopolysaccharides produced by Streptococcus mutans glucosyltransferases modulate the establishment of microcolonies within multispecies biofilms. J. Bacteriol. 192, 3024–3032 (2010).
21. K. Z. Coyte, J. Schluter, K. R. Foster, The ecology of the microbe: Networks, competition, and stability. Science 350, 663–666 (2015).
23. R. Hartmann et al., Emergence of three-dimensional order and structure in growing biofilms. Nat. Phys. 15, 251–256 (2019).

24. G. Hwang et al., Simultaneous spatiotemporal mapping of in situ pH and bacterial activity within an intact 3D microcolony structure. Sci. Rep. 6, 32841 (2016).

25. B. Angmar-Mansson, J. J. ten Bosch, Quantitative light-induced fluorescence (QLF): A method for assessment of incipient caries lesions. Dentomaxillofac. Radiol. 30, 298–307 (2001).

26. E. A. Masters et al., Evolving concepts in bone infection: Redefining “biofilm”, “acute vs. chronic osteomyelitis”, “the immune proteome” and “local antibiotic therapy”. Bone Res. 7, 20 (2019).

27. J. L. Mieher et al., Glucan binding protein C of Streptococcus mutans mediates both sucrose-independent and sucrose-dependent adherence. Infect. Immun. 86, e00146-18 (2018).

28. J. A. Banas, M. M. Vickerman, Glucan-binding proteins of the oral streptococci. Crit. Rev. Oral Biol. Med. 14, 89–99 (2003).

29. D. Kim et al., Bacterial-derived exopolysaccharides enhance antifungal drug tolerance in a cross-kingdom oral biofilm. ISME J. 12, 1427–1442 (2018).

30. Z. Ren et al., Dual-targeting approach degrades biofilm matrix and enhances bacterial killing. J. Dent. Res. 98, 322–330 (2019).

31. J. A. Lemos, R. A. Burne, A model of efficiency: Stress tolerance by Streptococcus mutans. Microbiology 154, 3247–3255 (2008).

32. W. L. Kuhnert, G. Zheng, R. C. Faustoferrer, R. G. Quivey Jr., The F-ATPase operon promoter of Streptococcus mutans is transcriptionally regulated in response to external pH. J. Bacteriol. 186, 8524-8528 (2004).

33. R. J. Lamont, H. Koo, G. Hajishengallis, The oral microbiota: Dynamic communities and host interactions. Nat. Rev. Microbiol. 16, 745–759 (2018).

34. T. Thurnheer, G. N. Belibasakis, Streptococcus oralis maintains homeostasis in oral biofilms by antagonizing the cariogenic pathogen Streptococcus mutans. Mol. Oral Microbiol. 33, 234–239 (2018).

35. S. Redanz et al., Live and let die: Hydrogen peroxide production by the commensal flora and its role in maintaining a symbiotic microbiome. Mol. Oral Microbiol. 33, 337–352 (2018).

36. Y. Liu et al., Topical ferumoxytol nanoparticles disrupt biofilms and prevent tooth decay in vivo via intrinsic catalytic activity. Nat. Commun. 9, 2920 (2018).

37. T. Thurnheer, R. Gmürr, B. Guggenheim, Multiplex FISH analysis of a six-species bacterial biofilm. J. Microbiol. Methods 56, 37–47 (2004).

38. M. I. Klein et al., Structural and molecular basis of the role of starch and sucrose in Streptococcus mutans biofilm development. Appl. Environ. Microbiol. 75, 837–841 (2009).

39. P. D. Marsh, E. Zaura, Dental biofilm: Ecological interactions in health and disease. J. Clin. Periodontol. 44 (suppl. 18), S12–S22 (2017).

40. T. Buchwald, Z. Okulus, M. Szybowicz, Raman spectroscopy as a tool of early dental caries detection—new insights. J. Raman Spectrosc. 48, 1094-1102 (2017).

41. J. A. Cury, H. Koo, Extraction and purification of total RNA from Streptococcus mutans biofilms. Anal. Biochem. 365, 208–214 (2007).

42. J. He et al., RNA-seq reveals enhanced sugar metabolism in Streptococcus mutans co-cultured with Candida albicans within mixed-species biofilms. Front. Microbiol. 8, 1036 (2017).