Klebsiella and Providencia emerge as lone survivors following long-term starvation of oral microbiota

Jonathon L. Bakera, Erik L. Hendricksonb, Xiaoyu Tangc, Renate Luxb, Xuesong Hed, Anna Edlundb, Jeffrey S. McLeanb, and Wenyuan ShiId,1

*Genomic Medicine Group, J. Craig Venter Institute, La Jolla, CA 92037; bDepartment of Periodontics, School of Dentistry, University of Washington, Seattle, WA 98195; cDepartment of Oral Biology, School of Dentistry, University of California, Los Angeles, CA 90095; and dDepartment of Microbiology, The Forsyth Institute, Cambridge, MA 02142

Edited by Jeff F. Miller, University of California, Los Angeles, CA, and approved March 20, 2019 (received for review December 3, 2018)

It is well-understood that many bacteria have evolved to survive catastrophic events using a variety of mechanisms, which include expression of stress-response genes, quiescence, necrotrophy, and metabolic advantages obtained through mutation. However, the dynamics of individuals leveraging these abilities to gain a competitive advantage in an ecologically complex setting remain unstudied. In this study, we observed the saliva microbiome throughout the ecological perturbation of long-term starvation, allowing only the species best equipped to access and use the limited resources to survive. During the first several days, the community underwent a death phase that resulted in a ~50–100-fold reduction in the number of viable cells. Interestingly, after this death phase, only three species, Klebsiella pneumoniae, Klebsiella oxytoca, and Providencia alcalifaciens, all members of the family Enterobacteriaceae, appeared to be transcriptionally active and recoverable. Klebsiella are significant human pathogens, frequently resistant to multiple antibiotics, and recently, ectopic colonization of the gut by oral Klebsiella was documented to induce dysbiosis and inflammation. MetaOmics analyses provided several leads for further investigation regarding the ecological success of the Enterobacteriaceae. The isolates accumulated single nucleotide polymorphisms in known growth advantage in stationary phase alleles and produced natural products closely resembling antimicrobial cyclic depsipeptides. The results presented in this study suggest that pathogenic Enterobacteriaceae persist much longer than their more benign neighbors in the salivary microbiome when faced with starvation. This is particularly significant, given that hospital surfaces contaminated with oral fluids, especially sinks and drains, are well-established sources of outbreaks of drug-resistant Enterobacteriaceae.

Significance

This study illustrates the dynamics of the oral microbiome during long-term starvation. After an initial ecological collapse, only three species were recoverable and displayed significant transcriptional activity: Klebsiella pneumoniae, Klebsiella oxytoca, and Providencia alcalifaciens. Klebsiella spp. are significant human pathogens and are frequently resistant to multiple classes of antibiotics. In addition to its status as a clinical scourge in its own right, K. pneumoniae has emerged as a chief facilitator in the transfer of drug resistance genes from the environment to pathogens. Hospital surfaces contaminated with oral fluids are well-documented sources of outbreaks of drug-resistant Enterobacteriaceae; therefore, the ability of Klebsiella to outcompete its neighbors during starvation and survive long-term in saliva is particularly noteworthy.

Author contributions: J.L.B., X.H., J.S.M., and W.S. designed research; J.L.B., E.L.H., X.T., A.E., and J.S.M. performed research; J.L.B., E.L.H., R.L., X.H., A.E., J.S.M., and W.S. analyzed data; and J.L.B. wrote the paper.

Conflict of interest statement: J.L.B. is a part-time consultant for uBiome, Inc. W.S. is a part-time chief science officer of C3J Therapeutics, Inc., which has licensed technologies from the University of California Regents that could be indirectly related to this research project.

This article is a PNAS Direct Submission.

This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

Data deposition: The raw sequencing files used in the genomic, metagenomic, and transcriptomic analyses in this study have been deposited in the Sequence Read Archive, https://www.ncbi.nlm.nih.gov/sra [accession nos. PRJNA525688 (genomes) and PRJNA525517 (metagenomes/transcriptomes)].

1To whom correspondence should be addressed. Email: wshi@forysth.org.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1802059116/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1802059116
oral microbiome (11–14). One of the major impediments to the study of complex, human-associated microbial communities is the difficulty cultivating such diverse ecologies in a well-controlled laboratory setting. The oral microbiota was chosen as a model system for this pilot study because of the existence of a well-established in vitro culture system using media that allows for the growth of a diversity of species approaching that of an in vivo human mouth (15).

### Results

**Long-Term Starvation of a Complex Oral Community Results in a Death Phase, Followed by a Long-Term Stationary Phase.** The starting community in this study was derived from a previous study that optimized media (SHI media) and growth conditions to stably maintain the highest diversity of oral bacteria achievable in vitro to date (15). The community was generated from the pooled saliva of six healthy adults, had a microbial diversity approaching that of human oral cavity, and responded to a carbohydrate pulse in a manner similar to in vivo dental plaque (15). After overnight batch growth in SHI media, the community was harvested and subsequently starved in aliquots of either PBS or a 1:1 mixture of PBS and cell-free saliva. Reminiscent of monospecies cultures of *E. coli* (4), the communities (both in PBS and PBS:saliva) first experienced a death phase with a rapid decrease in colony-forming units per milliliter over time, followed by a stabilization, a long-term stationary phase (Fig. 1A). It is important to note that many taxa that are viable in the liquid culture of the community cannot grow as isolated colonies on solid media, and thus are not measured in Fig. 1A. Therefore, although the colony-forming unit measurement is useful for illustrating the overall trend of viable cells in the community, the results are likely an underestimate, making it critical to complement this assay with mRNA-based taxonomic profiling, as described below, to determine the living members of the community during starvation. Illumina sequencing of 16S rDNA V3-V4 amplicons revealed that alpha diversity was higher in the PBS:saliva community than the PBS community, reflective of the supplementary substrates provided by the sterilized saliva at the start of the starvation (Fig. 1B). Intrasample diversity (alpha diversity) of the community increased across the first several days of starvation and then decreased to levels similar to that of the starting community after day 32 (Fig. 1C). Intercommunity diversity (beta diversity) also shifted over time, particularly during the first 12 d (Fig. 1D), followed by relative stabilization. The presence of saliva in the starvation medium had an effect on the community, as the two starvation communities separated in Principal Coordinate Analysis (PCoA) space over time (Fig. 1D). There were shifts in the relative abundances of many taxa, with by far the most notable change being an increase in the number of Enterobacteriaceae, particularly *Klebsiella*, at the expense of *Streptococcus*, which had been the most abundant genus in the starting communities (Fig. 1 E and F). Because the DNA harvested from the starving community was likely to contain the DNA of dead cells, aliquots of the starvation culture from various points were used to inoculate fresh SHI media, and the outgrowth culture was investigated with 16S rDNA sequencing after overnight growth to observe the “live” species from the initial starvation experiment (Fig. 1 E and F). The increase in Enterobacteriaceae 16S rDNA observed during long-term starvation was much more dramatic in the outgrowth communities. After only 4 d of starvation, the proportion of *Klebsiella* in the communities had increased dramatically, and by day 12, Enterobacteriaceae accounted for more than 90% of the relative abundance of the 16S rDNA in the outgrowth communities (Fig. 1 E and F). Although *Klebsiella* was the genus with the highest relative abundance, species of “unclassified” Enterobacteriaceae (highly likely to be *P. uto* or *P. alcalifaciens*) increased in relative abundance during the later points of the outgrowth. The community starving in PBS underwent a transition similar to that of the community starving in PBS:saliva, albeit with a reduced abundance of *Neisseria* and *Porphyromonas* (Fig. 1F), indicating that components in the filtered saliva made these genera more competitive.

**Shifts in diversity and relative abundances of constituents of the saliva microbiome during long-term starvation.** (A) Number of viable cells in the community, as determined by colony-forming units during starvation in PBS:saliva or PBS over the course of 100 d. (B) Alpha diversity of the PBS:saliva or PBS communities across 84 d of starvation. (C) Beta diversity of the PBS:saliva and PBS communities across 100 d. (D) PCoA plot of unweighted UNIFRAC distances illustrating beta diversity of the PBS:saliva or PBS over the course of 100 d. (E) Principal Coordinates Analysis (PCoA) space over time (Fig. 1D). There were shifts in the relative abundances of many taxa, with by far the most notable change being an increase in the number of Enterobacteriaceae, particularly *Klebsiella*, at the expense of *Streptococcus*, which had been the most abundant genus in the starting communities (Fig. 1 E and F). Because the DNA harvested from the starving community was likely to contain the DNA of dead cells, aliquots of the starvation culture from various points were used to inoculate fresh SHI media, and the outgrowth culture was investigated with 16S rDNA sequencing after overnight growth to observe the “live” species from the initial starvation experiment (Fig. 1 E and F). The increase in Enterobacteriaceae 16S rDNA observed during long-term starvation was much more dramatic in the outgrowth communities. After only 4 d of starvation, the proportion of *Klebsiella* in the communities had increased dramatically, and by day 12, Enterobacteriaceae accounted for more than 90% of the relative abundance of the 16S rDNA in the outgrowth communities (Fig. 1 E and F). Although *Klebsiella* was the genus with the highest relative abundance, species of “unclassified” Enterobacteriaceae (highly likely to be *P. uto* or *P. alcalifaciens*) increased in relative abundance during the later points of the outgrowth. The community starving in PBS underwent a transition similar to that of the community starving in PBS:saliva, albeit with a reduced abundance of *Neisseria* and *Porphyromonas* (Fig. 1F), indicating that components in the filtered saliva made these genera more competitive.

**After 20 d of Starvation, Only Enterobacteriaceae Are Recoverable on Solid Media.** When plated on SHI media agar, the day 0 community produced colonies with a wide variety of morphologies, reflective of the diversity of species present (Fig. 2). During the death phase, the relative number of the large mucoid colonies obtained increased dramatically, such that by day 20, and all following times, all the colonies obtained were large and mucoid.
A selection of diverse colony morphologies across three ages was isolated and identified, using Sanger sequencing of full-length 16S rDNA amplicons. At day 1, several species of Streptococcus were recovered, as were Gamella sanguinis, Klebsiella pneumoniae, and Klebsiella oxytoca. At day 20, K. pneumoniae, K. oxytoca, Enterobacter homaechii, and Providencia alcalificiens were the only recoverable species. Finally, at day 84 and day 100, only K. pneumoniae and P. alcalificiens were recoverable under the conditions tested. This is a particularly interesting result because all the surviving organisms are documented pathogens and are frequently drug resistant.

RNA Sequencing Confirms That Enterobacteriaceae Are the Dominant (and Likely Only Living) Community Members After Long-Term Starvation. To obtain a finer resolution of the viable and actively transcribing species, shotgun sequencing of cDNA from the transcripome of the community at five points during the long-term starvation was performed, followed by MetaPhlAn2 analysis to calculate the relative abundances of taxa. By day 20, P. alcalificiens increased in number such that the genus represented 46% of all RNA. This increase was largely at the expense of Firmicutes, which decreased to <5% of RNA. By day 20, Providencia accounted for 69% of the RNA, whereas Neisseria accounted for 20%. Ultimately, after 100 d of starvation, Providencia accounted for 90% of the RNA, indicating that it was the major living organism remaining in the community. As with the 16S rDNA profiling, the changes in abundance of taxa in the PBS:saliva community were similar to the major trends of the PBS:saliva community at five points during the long-term starvation. Collection and visualization using Revigo (25) and a custom R script (SI Appendix, Fig. S1). Eleven, 14, 15, and 5 nonredundant biological processes exhibited decreased expression at days 4, 20, 84, and 100 compared with day 0, respectively. Meanwhile, 22, 10, 20, and 40 nonredundant biological processes exhibited decreased expression on days 4, 20, 84, and 100, respectively. Interestingly, 20 of these Enterobacteriaceae during long-term starvation, the fact that these variants increased in frequency several times, independently, highlights a need for further investigation.

Enterobacteriaceae Exhibit Shifts in the Transcriptome During Long-Term Starvation. To obtain information about transcriptional activity among the Enterobacteriaceae during the long-term starvation, the cDNA Illumina sequencing reads were mapped to the assembled genomes of K. pneumoniae, K. oxytoca, and P. alcalificiens. Genes that were differentially abundant between day 0 and a subsequent time were identified using DESeq2 (Dataset S4). Gene ontology terms for each differentially abundant gene were summarized and visualized using Revigo (25) and a custom R script (SI Appendix, Fig. S1). Eleven, 14, 15, and 5 nonredundant biological processes exhibited increased expression at days 4, 20, 84, and 100 compared with day 0, respectively. Meanwhile, 22, 10, 20, and 40 nonredundant biological processes exhibited decreased expression on days 4, 20, 84, and 100, respectively. Interestingly, day 20, the point at which Enterobacteriaceae had become the most abundant members of the community, was the only observed point at which the number of biological processes with increased expression exceeded the number of biological processes with decreased expression. Negative regulation of flagellum motility, negative regulation of biofilm formation, and positive regulation of carbohydrate metabolic processes were the biological processes with the most highly elevated expression at days 20, 84, and 100 compared with day 0. By day 100, these three biological processes, along with positive regulation of catalytic activity, and oxidation-reduction process were the only biological processes that were up-regulated compared with day 0. This may suggest that the Enterobacteriaceae may be attempting to conserve energy and passively transport to a location with a novel food source. Xylose metabolism, valine biosynthetic process, and xylose transport were the three most highly up-regulated biological pathways at day 4, indicating that these pathways may be important to the Enterobacteriaceae early during long-term starvation, and that they may play a role in adapting to new conditions. Collectively, pathway analysis provides

Whole-Genome Sequencing of Enterobacteriaceae Isolates Reveals Single Nucleotide Polymorphism Accumulation During Starvation. DNA obtained from Enterobacteriaceae isolates from days 0, 20, and 84 (Dataset S1) was subjected to Illumina shotgun sequencing. Genomes were assembled from each point and compared with the earliest available point for each species (in the PBS community). There were six nonsynonymous single nucleotide polymorphisms (SNPs) observed in the K. pneumoniae and P. alcalificiens strains that increased in prevalence to >95%, in both PBS and PBS:saliva long-term starvation communities (Dataset S3). In K. pneumoniae, these SNPs occurred in the topB topoisomerase, the allS_2 LysR family transcriptional regulator, the glyS glycine tRNA ligase, and the tamB_1 maltoporin. Most intriguingly, both topoisomerase and LysR-type regulators were previously identified as GASP alleles, although the mechanism of the contribution of mutations in these genes to the GASP phenotype remains unknown. In K. pneumoniae and Salmonella enterica, it is currently unclear whether these SNPs contributed to the success of these Enterobacteriaceae during long-term starvation, the fact that these variants increased in frequency several times, independently, highlights a need for further investigation.

Fig. 2. Colony morphology during long-term starvation. Representative image of the PBS:saliva community on SHI agar after the indicated number of days of long-term starvation.
leads for further research into the mechanism behind the success of the Enterobacteriaceae during long-term starvation.

**Natural Products Analysis Indicates Generation of Cyclic Depsipeptides by Enterobacteriaceae.** To explore the dynamics of the small molecules produced by the communities during long-term starvation, harvested cells, as well as culture supernatants, of both the community and the isolated strains were analyzed by liquid chromatography mass spectrometry, followed by spectral analysis using Global Natural Products Social Molecular Networking (GNPS) (26). As is the present case with most metabolomics analysis, the majority of MS spectra were unannotated/unknown, with only ∼40% of the spectral clusters mapping to known annotations.

Twenty-five unknown spectral clusters were assigned a putative molecule class based on molecular networking analysis. The complete molecular network was visualized using Cytoscape (27) and is complete. A large number of MS spectra that networked with known lipid spectra were found only in the cell pellets, congruent with the concept that these are membrane-associated molecules and are unlikely to be secreted. There were a large number of MS spectra that only appeared in the community, either because the species synthesizing them were not species that were isolated or because isolates that were analyzed did not make the natural products in a single-species culture. There were more spectra associated with the Enterobacteriaceae species than the Streptococci, which could be expected given that the Enterobacteriaceae quickly increased in relative abundance in the communities during starvation. Interestingly, valine was significantly more abundant at later points (Fig. 4A and SI Appendix, Fig. S2), in agreement with up-regulation of transcription of valine biosynthetic processes among the surviving Enterobacteriaceae (SI Appendix, Fig. S1; day 20). The GNPS Dereplicator identified two spectral clusters with a GNPS library hit to a cyclic depsipeptide with a parent mass of 1,124.6 and a cyclic peptide sequence of MeGlu-ξIle-Phe-Pro-Gly-MeVal-MeGlu-ξIle-Pro-Val (Fig. 4B and C). Cyclic depsipeptides are a fascinating class of small molecules that frequently have antimicrobial and/or anticancer activity and are the subject of ongoing research (28–32). These two spectral clusters networked with two other unidentified spectral clusters with a parent mass of 1,025.5, which may indicate loss of a valine residue from the structure of the known cyclic depsipeptide (Fig. 4B). All four cyclic depsipeptide spectral clusters appeared to be associated with Enterobacteriaceae, based on the number of spectra associated with single-species isolates. This family of molecules also appeared in the community samples during early times, when the main shift in species abundance was occurring (∼day 4; Fig. 4A). These molecules may be bactericidal compounds secreted by the Enterobacteriaceae to kill the neighboring species during starvation for use in necrotrophy, and further study of these compounds is warranted.

**Enterobacteriaceae Increase in Abundance During Starvation in Additional Communities from Individual Donors.** To ensure that the phenomenon of the Enterobacteriaceae species becoming the dominant, living members of the starving community was not unique to this consortium of bacteria, the same long-term starvation experiment (using PBS as starvation media) was performed on five additional salivary communities, each isolated from the saliva of a single, healthy individual. 16S rDNA PCR-denaturing gradient gel electrophoresis was used to monitor taxonomic profiles of the five communities during the first 20 d of starvation (SI Appendix, Fig. S4) The denaturing gradient gel electrophoresis profiles of Community numbers 1 and 5 contained bands representing Enterobacteriaceae, as determined by Sanger sequencing of...
Fig. 4. Accumulation of valine and production of cyclic depsipeptides during long-term starvation. (A) Heat map illustrating the number of liquid chromatography mass spectrometry spectra associated with the indicated spectral cluster after the indicated number of days of starvation. Spectral clusters are grouped into molecule classes (e.g., amino acids) based on molecular networking to GNPS library hits. Row clustering within molecule classes was performed using a Pearson similarity distance matrix. Spectral clusters are named using the GNPS library hit where applicable; otherwise, clusters are named using the liquid chromatography mass spectrometry spectra associated with the indicated spectral cluster after the indicated number of days of starvation. Spectral clusters are grouped into clusters. Black outlines denote nodes which mapped to the GNPS library hit, cyclo-MeGlu-O-ξ-Ile-Phe-Pro-Gly-MeVal-MeGlu-ξ-Ile-Pro-Val.

Discussion

This study provides an account of a complex, human-associated microbial community experiencing the ecological perturbation of long-term starvation. The finding that Klebsiella and Providencia species were the apparent sole survivors in a community after long-term starvation is significant and highly intriguing. K. pneumoniae, K. oxytoca, and P. alcalifaciens are all members of the Enterobacteriaceae family of Proteobacteria. K. pneumoniae is a significant pathogen and represents the ‘K’ in the ESCKAPE pathogens, a group of organisms frequently resistant to multiple antibiotics (16). K. oxytoca and P. alcalifaciens are also opportunistic pathogens and are frequently drug resistant (17, 18). Oral K. pneumoniae was recently shown to induce inflammation and dysbiosis in the gut after ectopic colonization, and it was hypothesized that the oral cavity provides a reservoir for would-be intestinal pathogens, such as Klebsiella (33). Furthermore, aside from being a substantial pathogen in its own right, evidence is accumulating that K. pneumoniae serves as a key trafficker of drug resistance loci from the environment to human pathogens (34).

The mechanisms employed by these Enterobacteriaceae to outlast their neighbors during long-term starvation await investigation. The Enterobacteriaceae encode among the largest genomes in the oral microbiome (35) and, as such, have added metabolic flexibility compared with Streptococci and other common constituents of the oral cavity (36). It is likely that during long-term starvation, species with reduced genomes have less metabolic flexibility and are at a significant disadvantage to Enterobacteriaceae (37). The mechanisms employed by these Enterobacteriaceae to outlast their neighbors during long-term starvation await investigation. The Enterobacteriaceae encode among the largest genomes in the oral microbiome (35) and, as such, have added metabolic flexibility compared with Streptococci and other common constituents of the oral cavity (36). It is likely that during long-term starvation, species with reduced genomes have less metabolic flexibility and are at a significant disadvantage to Enterobacteriaceae (37).

Klebsiella are diazotrophs, and all Enterobacteriaceae are capable of using nitrate, S-oxides, and N-oxides as terminal electron acceptors (36). Thus, the concept that these abilities were advantageous during long-term starvation remains an attractive hypothesis. In addition, the MetaMics analyses performed in this study provided several additional hypotheses for further investigation. The increased abundance of SNPs in several genes in K. pneumoniae and P. alcalifaciens may represent GASP mutations, which were originally discovered in E. coli, another member of the family Enterobacteriaceae (4). Overall analysis of the Enterobacteriaceae transcriptome indicated that the species may be attempting to conserve energy and use passive transport to locate a novel food source. Meanwhile,

the DNA contained within the excised band. Most importantly, the density of the Enterobacteriaceae bands increased during starvation, concurrent with a decrease in the density of most other bands within the denaturing gradient gel electrophoresis profile. This finding signifies an increase in the relative abundance of Enterobacteriaceae at the expense of other taxa, indicating that the phenomenon observed in the main experiment was not exclusive to that starting community of bacteria. The other three communities did not appear to have significant numbers of Enterobacteriaceae at any point (SI Appendix, Fig. S4).
several intriguing cyclic depsipeptides may have been employed by the Enterobacteriaceae to kill their neighbors.

The results presented here also illustrate the value of RNA-based detection methods. Although a large number of taxa were present at all points, according to sequencing of 16S amplicons, as well as metagenomes, sequencing of mRNA revealed a much more drastic reduction of species during and after the death phase. This loss of diversity was also reflected in the sequencing of the outgrowth communities and the plating assay, from which only the three Enterobacteriaceae species were recoverable at day 20 under the conditions tested, despite the presence of DNA from a multitude of species in the community at that time. Because some bacteria are known to enter a viable but not culturable state during adverse growth conditions, the lack of recovery of diversity at the transcriptional level during the later points of starvation serves as an important validation that the colony-forming units per milliliter and recoverable species on solid media reported here are not largely underestimated.

The ability of the Enterobacteriaceae to survive longer than other members of the saliva microbial community may have a great deal of clinical significance. Although the long-term starvation model used in this study is unlikely to simulate the oral cavity, where periods of starvation are much shorter, it is presumably analogous to the sucrose-less agar under microaerophilic conditions. 16S rDNA taxonomic profiling was performed by illumina sequencing of V2-V4 amplicons, followed by analysis using QIIME. Metagenomes were assembled de novo using SPAdes and aligned using Mauve. Metatranscriptomic reads were mapped to Enterobacteriaceae genomes using Burrows-Wheeler alignment, and genes that were significantly differentially expressed were identified using DeSeq. Natural products analysis was performed using reverse-phase high-pressure liquid chromatography followed by tandem mass spectrometry, as detailed in the SI Appendix. Raw sequencing data have been deposited in the Sequence Read Archive (SRA) (40, 41).

Materials and Methods

More detailed methods with additional references are available in the SI Appendix. The starting bacterial community (S-mix), derived from the saliva of six healthy subjects, ages 25–35 y, has been described previously (15). After overnight growth in ShI medium in microaerophilic conditions (2%O2, 5%CO2, 93%N2), 1 mL aliquots of 5-mix were starved in either 1x PBS or a 1:1 mixture of 1x PBS and cell-free saliva. Colon-forming units per milliliter was determined by growth for 72 h on SSI motility agar under microaerophilic conditions. 16S rDNA taxonomic profiling was performed by illumina sequencing of V2-V4 amplicons, followed by analysis using QIIME. Metagenomes were assembled de novo using SPAdes and aligned using Mauve. Metatranscriptomic reads were mapped to Enterobacteriaceae genomes using Burrows-Wheeler alignment, and genes that were significantly differentially expressed were identified using DeSeq. Natural products analysis was performed using reverse-phase high-pressure liquid chromatography followed by tandem mass spectrometry, as detailed in the SI Appendix. Raw sequencing data have been deposited in the Sequence Read Archive (SRA) (40, 41).

ACKNOWLEDGMENTS. The authors thank Roberta Faustoferri for helpful proofreading of the manuscript. This study was supported by National Institutes of Health/National Institute of Dental and Craniofacial Research Grants F32 DE026947 (to J.L.B.), R00 DE024543 (to A.E.), and R01 DE020102 and R01 DE026186 (to X.H., J.S.M., and W.S.).