Polyphosphate-accumulating Bacteria: Potential Contributors to Mineral Dissolution in the Oral Cavity

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J.V.B., B.E.F., and R.S.J., conceived the study and designed the research with input from A.A.B.; Experimental data was generated by A.A.B. with help from J.N.; sample collection was conducted by J.B., M.H., T.R., and R.S.J.; Genome research and analysis was conducted by B.E.F.; A.A.B. and J.V.B. wrote the paper with input from other authors.
ABSTRACT

Bacteria that accumulate polyphosphate have previously been shown to dynamically influence the solubility of phosphatic minerals in marine settings and wastewater. Here we show that dental plaque, saliva, and carious lesions, all contain abundant polyphosphate-accumulating bacteria. Saturation state modeling results, informed by phosphate uptake experiments using the model organism Lactobacillus rhamnosus, which is known to inhabit advanced carious-lesions, suggest that polyphosphate accumulation can lead to undersaturated conditions with respect to hydroxyapatite under some oral cavity conditions. The cell densities of polyphosphate-accumulating bacteria we observed in some regions of oral biofilms are comparable to those that produce undersaturated conditions (i.e., those that thermodynamically favor mineral dissolution) in our phosphate uptake experiments with L. rhamnosus. These results suggest that the localized generation of undersaturated conditions by polyphosphate-accumulating bacteria constitutes a new potential mechanism of tooth dissolution that may augment the effects of metabolic acid production.

IMPORTANCE

Dental caries is a serious public health issue that can have negative impacts on overall quality of life and oral health. The role of oral bacteria in the dissolution of dental enamel and dentin that can result in carious lesions, has long been solely ascribed to metabolic acid production. Here we show that certain oral bacteria may act as a dynamic shunt for phosphate in dental biofilms via the accumulation of a polymer known as polyphosphate - potentially mediating phosphate-dependent conditions such as caries (dental decay).
Introduction

The localized ionic saturation state of oral fluids with respect to the thermodynamic solubility product of dental mineral phases influences mineral solubility and the likelihood of enamel dissolution at the tooth/plaque interface or within existing caries lesions (1,2). Ion exchange between salivary fluids and the tooth surface has widely been accepted as a “chemical” pathway by which Ca\(^{2+}\) and PO\(_4^{3-}\), the primary ions in equilibrium with calcium phosphate minerals, is modulated by human-induced, dietary, and non-microbial factors in the oral cavity (3). Here, we provide evidence in support of the novel hypothesis that certain oral bacteria may play a considerable role in dynamically modulating the ion concentrations of PO\(_4^{3-}\), and thus the saturation state/solubility of calcium phosphate minerals at the tooth/plaque interface, through intracellular polyphosphate (polyP) accumulation. While all bacteria make short chains of polyP as labile metabolites, polyP-accumulating bacteria (PAB) store substantial intracellular inclusions of polyP in response to specific environmental conditions.

Polyphosphates are linear polymers of orthophosphate residues linked by high energy phosphaonyhydride bonds. PolyP accumulation has long been known to be associated with the ability of certain microbes to resist physical and chemical stressors, as well as provide an alternative source of energy under unfavorable or variable environmental conditions (4–8). The metabolic processes of PAB have been extensively investigated in environmental systems such as enhanced-removal of phosphorus from wastewater, and marine calcium phosphate mineral deposits that are thought to be mediated by PAB (9–12). It has been demonstrated that PAB are capable of modulating the ionic constituents in equilibrium with apatite-group minerals in pore waters and subsequently altering the saturation state of the surrounding fluids resulting in microenvironments that are thermodynamically favorable for mineral precipitation (12–16).
study of metabolic processes of PAB in these systems have achieved a new paradigm in our understanding of the modulation of $\text{PO}_4^{3-}$ and $\text{Ca}^{2+}$ activities and their relationship to the solubility of calcium phosphate minerals. While previous researchers proposed that the synthesis of polyphosphate by *Streptococcus* may play a role in caries (17–19), the influence of diverse polyphosphate-accumulating bacteria on mineral saturation state has not, to our knowledge, been applied to the oral environment, where we show that biofilms in plaque and saliva contain abundant polyphosphate-accumulating bacteria that may chemically exchange ions with the calcium phosphate minerals that comprise the inorganic portion of the tooth.

The oral microbiome is a dynamic and diverse community that develops under a wide variety of environmental conditions. Although oral biofilm research has spanned over a hundred years, little work has been done to investigate the role microbes may play as dynamic mediators of ion concentrations. The current and widely accepted model of the caries process is based on the ability of cariogenic plaque microbiota to establish and thrive in low pH environments in which the metabolic production of mixed acids contributes to enamel demineralization (20–23). Although localized acid production in cariogenic biofilms undoubtedly impacts mineral solubility, biological influence on chemical saturation of $\text{Ca}^{2+}$ and $\text{PO}_4^{3-}$ may present an additional component to the development and rapid progression of carious lesions. In order to address several different facets of the hypothesis that PAB may affect localized chemical saturation in the oral cavity, we analyzed genomic databases of oral taxa, we quantified PAB in clinical samples of plaque, saliva, and dentinal lesions, we conducted phosphate uptake experiments using a defined *in vitro* single-species model, and we modeled the potential impact of polyP-accumulation on the saturation state of saliva.
Results

Potential for Polyphosphate Metabolisms in Genomes of Oral Taxa. The main enzymes responsible for synthesizing polyP and subsequently hydrolyzing polyP in bacteria are polyphosphate kinase (ppk1, ppk2) and exopolyphosphatase (ppx) respectively (6, 24, 25). We included these well-studied genes for hydrolyzing polyP in our search of genomic databases in oral taxa in the HOMD database (Dataset S1) (26, 27). In general, the genetic potential to accumulate polyP was found broadly across the oral microbiome (Figure 1). Multiple caries associated clades strongly demonstrated the genetic potential to accumulate polyP, such as Propionibacterium (n = 140/142), Lactobacillus (n = 606/842), Rothia (n=12/12), Actinomyces (n=41/76), and Bifidobacterium (291/292). Notably absent were the Streptococci, including S. sorbinus SL-1 ATCC 33478 which was reported to accumulate polyP (18) (only 29 non-oral isolates out of 2875 genomes). In addition to querying for the primary genes responsible of synthesizing and hydrolyzing polyP, we queried the annotated genomes for the gene polyphosphate glucokinase (ppgk) (EC 2.7.1.63) (28). This gene is found in order Actinomycetales, which includes many clades of caries associated bacteria (e.g. Actinomyces, Corynebacterium, Rothia). In this clade PPGK phosphorylates glucose during its degradation via the Embden-Meyerhof-Parnas pathway. What makes PPGK particularly interesting here is that its phosphoryl donor is often polyphosphate. Our gene survey detected ppgk broadly throughout the Actinomycetales as well as some strains of one other known polyphosphate accumulator, Bradyrhizobium sp.

Oral Biofilms Contain Abundant Polyphosphate-Accumulating Bacteria. Our microscopy observations show that plaque (Figure 2a), dentinal lesions (Figure 2c), and our model organism,
L. rhamnosus (Figure 3), contain abundant intracellular polyp inclusions that can be visualized with the DNA stain DAPI. Binding of polyP to DAPI, shifts its peak emission wavelength from 475 nm (blue for DNA) to 525-550 nm (excitation at 360 nm), resulting in the DAPI-polyP complex to appear yellow and inclusions can be observed as discrete yellow/green spheres within the cell (Figure 2) (29). We found that the dental plaque samples from all 30 patients sampled (60 samples in total) contained polyP inclusion bodies in morphologically diverse, and spatially heterogeneous oral biofilms. Staining of dentinal lesions, from five extracted teeth, also revealed abundant polyP inclusion bodies. As expected, the bacterial morphotypes in the dental plaque samples include long filamentous organisms as well as small cocci and bacillus-shaped cells. The morphologic diversity of the dentin samples appeared to be less than that of the dental plaque, and primarily consisted of small cocci and bacillus shaped organisms, while filamentous bacteria were absent.

We used spectral-scanning confocal microscopy to resolve polyP inclusions in complex three-dimensional oral biofilms by linearly unmixing the spectral signal for polyP-DAPI from that of DNA-DAPI (Figs. 1B, C; Fig. S1). PolyP inclusions were not homogenously dispersed throughout the biofilms and appeared to be denser within certain regions of the sample. In regions of high cell density, polyP-containing cells exceeded the cell densities observed in our model organism L. rhamnosus phosphate uptake experiments and reached up to 1.06x10^{12} inclusions/cm^3, leading us to hypothesize that these dense assemblages can conceivably modulate localized saturation gradients to create undersaturated conditions within discrete areas of the biofilm.
Polyphosphate Accumulation in *Lactobacillus rhamnosus*. We selected the caries-associated bacterium *Lactobacillus rhamnosus* as a model organism to manipulate and observe polyP metabolisms in response to nutritional limitations and dynamic environmental conditions because of its ability to accumulate polyP and because it is associated with the progression of dental caries (25). When grown on semi-defined media, *L. rhamnosus* accumulated polyP within a 24-hour period but only when manganese (0.05 g/l) was included. By comparing a Mn²⁺-amended culture to an un-amended culture, we were able to quantify the influence of polyP accumulation on extracellular phosphate concentration.

**Colorimetric Inorganic Phosphate Quantification.** By developing a media that enables us to grow *L. rhamnosus* under conditions that allow for polyP accumulation versus conditions that do not, we were able to spectrophotometrically quantify the relative concentration of phosphorus from our media that is being incorporated as intracellular polyP inclusions. Using a colorimetric assay (30), we compared the supernatant of our *L. rhamnosus* cultures to that of a known set of phosphate standards to quantify the depletion of total inorganic phosphate during the growth cycle.

Since bacteria use phosphate for a variety of different purposes other than polyP accumulation, it is important to take into consideration the quantity of phosphorus necessary for cell growth. Figure 4 illustrates inorganic phosphate change, over the course of 24 hours, between our Mn²⁺-deficient glucose medium (negative polyP accumulation) and “standard” Mn²⁺ glucose medium (positive polyP accumulation). *L. rhamnosus* grown under conditions that allow for polyP accumulation (“standard” Mn²⁺) show a decrease in media PO₄³⁻ concentrations of approximately 1.3 mM after 18 hours incubation. *L. rhamnosus* grown in Mn²⁺-deficient
glucose media, i.e. Mn$^{2+}$ limited conditions, that prevent polyP accumulation, show a decrease of approximately 0.38 mM PO$_4^{3-}$ after 18 hours incubation. We make the assumption that, most, if not all, of the difference in the total PO$_4^{3-}$ concentration results from P$_i$ uptake and storage in the cells as the intracellular polyP granules that we observed in the standard Mn$^{2+}$ cultures. Since the cell densities between the two media were similar (Figure 4), with the Mn$^{2+}$-deficient culture being slightly lower, the concentration of phosphate was adjusted to reflect equal cell densities among the two media. Using this assumption, we calculate a maximum net change of phosphate, via polyP accumulation by *L. rhamnosus* to be approximately 0.91 (± 0.12) mM after 18 hours incubation. If polyP-accumulating bacteria in plaque or dentinal lesions are accumulating similar amounts of polyP as our model organism, *L. rhamnosus*, is capable of accumulating, then it is probable that oral biofilms influence the saturation chemistry of the saliva/mineral interface through the metabolism of polyP. To assess the potential impact polyP accumulation and/or release may have on mineral solubility, we used a web-based geochemical modeling program, WEB-PHREEQ (https://www.ndsu.edu/webphreeq/) based on the program PHREEQC (31), to calculate changes that would result in supersaturated or undersaturated conditions with respect to the apatite group mineral, hydroxyapatite.

**Saturation State Response to Polyphosphate Accumulation.** One of the primary factors controlling the solubility of hydroxyapatite in dental enamel or dentin, is the saturation state of saliva with respect to its primary ionic constituents, calcium and phosphate (32). Saturation state refers to the thermodynamic dependence of the mineral’s solubility on the product of the activities (i.e. functional concentrations) of the constituent ions in equilibrium with the solid phase (e.g. the inorganic portion of the tooth enamel). For undersaturated conditions (Ω<1), the
dissolution of the mineral is thermodynamically favored, while under supersaturated conditions 
(Ω<1), mineral precipitation is favored. Dissolving table salt (NaCl) in a dilute solution, is an 
example of mineral dissolution in an undersaturated solution. Despite the fact that saliva is 
generally supersaturated with respect to hydroxyapatite (32, 33), some individuals experience 
extensive mineral dissolution while others accumulate dental calculus (mineralized plaque).

We modeled the mineral saturation state of saliva using three different salivary 
concentrations of calcium and phosphate (34) and compared these saturation states with those in 
which we subtracted the 0.91 mM phosphate derived from our L. rhamonosus P-uptake 
experiments (Figure 5). From these results, we conclude that when salivary calcium and 
phosphate concentrations are high (4.2 and 12.6 mM respectively), the impact on saturation state 
resulting from a phosphate drawdown of 0.91 mM is minimal. However, when calcium and 
phosphate concentrations in the saliva are relatively low (1.1 and 2.0 mM respectively), a 
drawdown of 0.91 mM phosphate can have a sizable effect on mineral solubility. If this 
magnitude of change were to occur in saliva at a pH of 5.85 (assuming a stable calcium 
concentration of 1.0 mM and an initial phosphate concentration of 2.0 mM) then the system 
would go from saturated with respect to hydroxyapatite (Ω=−1) to undersaturated (Ω=−0.50), a 
condition that thermodynamically favors mineral dissolution.

Discussion

Dental caries is a dynamic and multi-factorial disease whose etiology is thought to be 
based largely on the ability of the bacterial community to produce acid and survive in decreasing 
pH conditions (35). Tooth enamel, primarily composed of hydroxyapatite (Ca$_5$(PO$_4$)$_3$(OH)), is 
highly susceptible to demineralization from prolonged exposure to organic acids, by-products of
bacterial carbohydrate fermentation (22). *Streptococcus mutans* was established early on as a key player in the caries process, and remains a focus of investigation due to its acidogenic and aciduric properties (35, 36). However, recent studies have shown that, although *S. mutans* is associated with both enamel and dentinal carious lesions, it is part of a larger consortia of cariogenic bacteria that thrive in low pH conditions as a result of frequent carbohydrate exposure (20, 22, 23). Recent developments in community characterization using 16S rRNA gene based amplicon sequencing and metagenomics has made it possible to identify and study the microbiota associated with cariogenic plaque. Along with *S. mutans*, cariogenic plaque is comprised of a community of diverse microbial species including *Rothia*, *Actinomyces*, *Bifidobacterium spp.*, *Lactobacilli*, and other non-mutans streptococci (20, 35, 37–39). Among the microbiota identified as key players in the development of carious lesions, our genome analysis results show that several clades possess the genetic potential to accumulate polyP. In most of the caries-associated clades, the capacity to accumulate polyP has also been demonstrated experimentally (25, 40, 41). Whether strains of *Streptococci*, in particular *S. mutans*, has the capacity to accumulate polyP by a yet to identified genetic pathway remains to be explored. Regardless, the capacity to accumulate and release polyP appears in many cases to be highly variable within a clade down to the strain level and may contribute to the phenotypic variability within caries microbiomes resulting in the modulation of disease progression. For example, the acid producing fermentation of sugar, the canonical primary driver of caries, when directly coupled to polyphosphate metabolism, in strains with polyphosphate-glukokinase, could result in the concomitant production of acid and reduction of the saturation state of oral fluids, via phosphate uptake.
Our preliminary DAPI staining of 60 plaque, five carious dentin, and 60 saliva samples demonstrates that PAB are ubiquitous in the oral cavity (n=123/123). Even though many of the organisms we observed to contain accumulations of polyP in the oral cavity may not be associated with the development and progression of dental caries (i.e. certain plaque and salivary microbiota), the mere presence of heterogeneous dense assemblages of PAB in the oral community conceivably introduces a new paradigm in the realm of dental disease and oral microbial ecology. PAB present in our clinical samples of dentinal lesions provide suggestive evidence that polyP accumulation may play a role in Pi modulation between bacteria and dental enamel. Since we were unable to identify which species of PAB were present within these dentinal lesions, we utilized in vitro culturing of L. rhamnosus, an organism known to be associated with caries progression, to determine if polyP accumulation may have an effect on the saturation state of the surrounding fluids in an environment where PAB are well established.

Our results demonstrate that L. rhamnosus accumulates concentrations of polyP that could result in changes in saturation state to the surrounding oral environment in situations where PO₄³⁻ concentrations are naturally low or depleted at the tooth/biofilm interface. Unrecognized factors such as dietary nutrient sources, continued organic acid exposure, and oxygenation conditions may initiate bacterial polyP uptake and reduce (or increase) localized concentrations of Ca²⁺ and PO₄³⁻. The uptake and subsequent release of inclusions of polyP may significantly alter the saturation chemistry of the fluids surrounding the tooth surface by shifting the chemical equilibrium and result in dissolution of apatite-group minerals in the form of dental caries.

In a scenario where nutrient availability is limited, pH is relatively acidic, and oxygen levels are depleted, PAB bacteria such as L. rhamnosus may exist as opportunists that establish themselves in an exclusive ecological niche in which their polyP metabolisms may provide a...
competitive edge amongst other oral microbiota. Carious lesions that had initially demineralized from exposure to mixed organic acids may become even more susceptible to mineral loss as aciduric/acidogenic PAB, such as *L. rhamnosus*, establish themselves in the vicinity of dissolution. As tooth enamel demineralizes, PAB may increase dissolution by further disrupting the chemical balance of Ca^{2+} and PO_{4}^{3-} by accumulating P_{i} from the dissolved enamel thus creating a demineralization environment that results both from acid production and phosphate depletion (Figure 6).

The sequestration of PO_{4}^{3-} by PAB has the potential to alter the chemical conditions of the oral environment that promote mineral dissolution under certain conditions in the mouth, leading to dental decay. Alternatively, the concentrated release of PO_{4}^{3-} from PAB could lead to the precipitation of dental calculus (mineralized dental plaque) under a different set of oral microenvironmental conditions. These ions can also be incorporated into various other phases of apatite such as fluorapatite (Ca_{5}(PO_{4})_{3}F) and carbonate-hydroxyapatite (Ca_{5}(PO_{4},CO_{3})_{3}(OH)). These substitutions are common in the oral cavity and vary from individual-to-individual, as well as from tooth-to-tooth. Mineral solubility may increase or decrease depending on the substitutions in the lattice structure (42). In order to assess the controls on PAB metabolisms and their potential roles in altering the saturation chemistry of the saliva/mineral interface, a comprehensive understanding of the ecology and physiology of PAB in the oral environment is needed. Clinical assessments and in situ taxonomic identification of PAB in oral biofilms will aid us in understanding their ecophysiology, as well aid in our ability to treat oral diseases such as dental caries that remain incompletely understood.

**Materials & Methods**
Genomic Identification of Candidate Isolates. An initial list of 1362 microbial genomes of oral taxa in the Human Oral Microbiome Database (HOMD) was used for detecting genes related to polyP accumulation in oral microbes. Besides one strain of Aggregatibacter, genomes for each HOMD taxon were available and annotated by DOE’s Integrated Microbial Genome pipeline (IMG/M) (43). The genomes were queried (find function search) for genes classified as coding for the enzymes PPK1 (EC:2.7.4.1), PPX (EC:3.6.1.11), PPGK (EC:2.7.1.63) and as COG PPK2 (COG2326). For a better context of the likelihood that a clade may or may not be a polyP accumulator, genomes of all other strains within the same clade were included in our search for the presence or absence of the queried genes. The results are presented in Dataset S1. A few HOMD strains from the list were determined to be duplicate strains and are reported as such. When available, peer-reviewed studies were utilized in the interpretation of genetic potential. Complete references cited in Dataset S1 are located in the Supplemental Material.

Lactobacillus rhamnosus Phosphate Uptake Experiments. Lactobacillus rhamnosus ATCC 7469 DSM 20021, obtained from the USDA Agricultural Research Station Culture Collection, was used in our single species model to assess polyP metabolic potential in a caries-associated organism. Specifically, growing L. rhamnosus under conditions that allow it to accumulate polyP vs. those that do not, allowed us to quantify PO₄³⁻ uptake specific to polyP that we then used to model saturation state changes under conditions in which fluctuations in intracellular polyP accumulation may have on oral saturation state chemistry. Two mediums modified after a Lactobacillus MRS growth medium (44), were developed containing the following (g/L): 20 D-(+)-Glucose Monohydrate, 99%; 10 peptone type 1; 5 yeast extract; 5 sodium acetate; 0.1 MgSO₄; 2 K₂HPO₄; 0.05 MnSO₄ · H₂O; 1mL Tween 80. The second medium, designated Mn²⁺-deficient glucose medium, contained the same chemical proportions of the medium described above (“standard” Mn²⁺ glucose medium), with the exception of MnSO₄ · H₂O, which was excluded from the medium. Triplicate cultures of L. rhamnosus (starting inoculum adjusted to 0.2 OD) were cultivated in an aerobic environment for 24 hours at 37°C with orbital shaking at 90
rpm. One milliliter of culture was collected from each replicate every three hours, optical density readings were collected and samples were centrifuged at 10,000 g for 10 min at 4°C. The supernatant was transferred to a separate centrifuge tube for further chemical analysis and the cell pellet was resuspended in one milliliter of 50% ethanol for fluorescence microscopic examination after staining for polyP (as described below). After testing for its suitability to preserve polyP granules, ethanol fixation was used to keep cellular structures intact and metabolisms inert.

**Plaque and Dentin Sampling/Collection.** Plaque samples were collected from male or female children between ages 4-18 years old who satisfied one of the following inclusion criteria: (1) oral health with an absence of dental caries or hardened dental plaque, (2) dental caries or recent history of dental caries, (3) hardened dental plaque with an absence of dental caries. The process of collecting the dental plaque followed Institutional Review Board (IRB) procedures at the University of Minnesota (IRB#1507M75441). Plaque samples consisted of two separate samples collected from the anterior and posterior dentition using a sterile dental scaler. Two samples were taken from 30 patients for a total of 60 samples.

Prior to the appointment, subjects were expected to have fasted one hour prior to sampling as well as refrained from brushing their teeth the morning of sampling. Each sample was placed into separate test tubes containing 1 mL of 50% ethanol. The test tubes were immediately placed into ice for transport and stored at -20°C for future microscopic analysis. Five dentin samples were collected from extracted teeth that were to be discarded as pathologic waste. Once the teeth were extracted, gross debris consisting of heme, remnants of PDL/gingival attachments, and any granulomatous tissue was removed chairside with 2x2 cotton gauze. Dentin was extracted from each of the carious teeth with sterile hand instrumentation under a Biosafety
Cabinet Class II/Type A2. The dentin samples were immediately stained for microscopic visualization and the extracted teeth were placed into individual tubes containing 50% ethanol.

**Polyphosphate Identification via Fluorescence Microscopy.**

DAPI (4',6-diamidino-2-phenylindole) binds to both polyp and DNA, the corresponding complexes, polyP-DAPI complex and the DNA-DAPI complex have a distinct emission spectrum (461-nm and 525-nm) when excited by 360-nm light. To resolve the polyP-DAPI complex, custom band-pass filters (Chroma), were employed (DNA-DAPI excitation/emission (nm) 345/455 and polyp-DAPI excitation/emission (nm) 415/550). This emission wavelength shift results in the emission of a distinct yellow color that can be used to differentiate the polyP-DAPI complex from the DNA-DAPI complex (45). Nine samples of *L. rhamnosus*, collected every three hours over the course of a 24 hour period, as well as 60 plaque and five dentin samples, were collected and fixed for staining. Each ethanol fixed sample was placed in a designated well on a Teflon-printed microscope slide and allowed to air-dry until the cells were adhered to the slide. Eight microliters of 5 µg/mL DAPI was pipetted onto each sample containing well and left to incubate in the dark for 30 minutes in a hybridization chamber. Imaging was performed on an Olympus BX61 fluorescence microscope equipped with an XM10 CCD camera and cellSens Dimensions Imaging Software (Version 1.13). Photoshop CS5 (ver. 12.0.4) was used to adjust brightness and contrast uniformly across the entire image in Figure 3 (a, b).

**Confocal Spectral Imaging.**

Confocal spectral imaging was employed using an Olympus Fluoview FV1000 IX2 inverted confocal microscope. Olympus Fluoview (ver. 04.01.01.05) was employed for image acquisition. Linear spectral unmixing of the polyP-DAPI from the DNA-
DAPI signal was performed by designating regions of interest based on emission properties obtained from a spectral wavelength lambda scan of the sample. The sample was scanned from 450nm to 580nm in increments of 10nm. Ethanol-fixed oral biofilm samples were stained with DAPI and analyzed under a 60X oil objective with a numerical aperture of 1.42. PolyP and DNA signals were separated based on a standard polyP emission wavelength (550 nm) and DNA emission wavelength (461.0 nm).

Images were analyzed with AutoQuant X (ver. X3.0.4) and Imaris x 64 (ver. 7.5.2). Blind 3D deconvolution with default settings (adaptive PSF, 10 iterations, and medium noise) was employed in AutoQuant X. After image deconvolution, the files were exported to Imaris x 64 for 3-dimensional analysis and polyP quantification. PolyP inclusions were quantified by creating a three-dimensional surface characterized by the emission spectra of polyP-DAPI. Designated regions of interest (ROI) with substantial densities of polyP inclusions were selected to quantify the amount of polyP containing cells within a dense region. Regions of interest ranged in size from 5 μm$^3$ to 10 μm$^3$ (example ROI surface estimation located in Fig. S2).

**Inorganic Phosphate Quantification.** An inorganic phosphate quantification method (30) was adapted (100μL ascorbic acid/mixed reagent to 1.0 mL of sample) to assess the influence of polyP accumulation on the P$_i$ concentration in the extracellular medium in our model organism, *L. rhamnosus*. In order to account for the amount of phosphate that would have been taken up from the media for purposes other than polyP accumulation, our semi-defined medium was modified to inhibit cellular polyP accumulation while maintaining similar cell densities to those of the polyP-accumulating culture. Cell counts from three experimental replicates were used to
normalize the slightly different cell densities between the two culture types when calculating phosphate change/cell.

**Geochemical Analysis and Saturation Calculations.** To assess the potential impact cellular polyP accumulation/release has on enamel mineral solubility (i.e. hydroxyapatite), we employed WEB-PHREEQ: Aqueous Geochemical Modeling (version 2) to model chemical saturation state fluctuations in response to fluctuating phosphate concentrations. A range of salivary phosphate and calcium concentrations (mM) reported in the literature (34) were used as a series of arbitrary, but patient-derived, starting values in determining chemical saturation of hydroxyapatite in response to fluctuating pH and phosphate concentrations. Net polyP accumulation (0.91 mM), as previously determined from our *L. rhamnosus* phosphate uptake experiments, was subtracted from literature reported phosphate values to assess saturation index fluctuations during our maximum observed cellular polyP accumulation and release.

**ACKNOWLEDGEMENTS.** We thank Chris Crosby, Thomas Pengo, Dan Jones, Mark Sanders and Guillermo Marques for training, advice and assistance. We acknowledge funding support from the Office of the Vice President for Research Minnesota Futures grant program. Image acquisition and analysis support was provided by the University of Minnesota Imaging Center. We thank the editor and two anonymous reviewers for their comments that greatly improved the manuscript.

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**Fig. 1.** Putative genetic potential to synthesize polyP as defined by the presence of specific genes (see Methods) in most of the available genomes for that clade (green); some of the available
genomes for that clade (yellow) or few/none of the available genomes for that clade (red). See Dataset S1 for more details. The filled circle indicates literature reports of polyP accumulation in that clade.

**Fig. 2.** Staining of clinical samples with DAPI (4′,6-diamidino-2-phenylindole). Fluorescence microscopic examination demonstrates that plaque (a,b) and dentinal lesions (c) contain abundant, morphologically diverse, and spatially heterogeneous bacteria that accumulate polyP that can be stained with DAPI (yellow/green inclusions).

**Fig. 3.** Polyphosphate accumulation in *L. rhamnosus* in response to nutritional limitations. (a) *L. rhamnosus* cultured in Mn$^{2+}$-deficient glucose medium for 21 hours exhibited no accumulation of polyP. (b) While *L. rhamnosus* cultured in “standard” Mn$^{2+}$ glucose medium for 21 hours were replete with inclusions of polyP (yellow/green spheres).

**Fig. 4.** Inorganic Phosphate Quantification using the oral isolate *L. rhamnosus*. Growing *L. rhamnosus* under conditions that allow for polyP accumulation (“standard” Mn$^{2+}$) resulted in a decrease in media P$_i$ of approximately 1.3 mM at 18 hours. *L. rhamnosus* grown without the addition of Mn$^{2+}$ conditions, which prevents polyP accumulation (Mn$^{2+}$-deficient), resulted in a decrease in media P$_i$ of approximately 0.4 mM. Initial media P$_i$ concentration is approximately 10.8 mM. Error bars indicate ± one standard deviation from the mean.
Fig. 5. Modeled shift in relative saturation of salivary fluids in response to bacterial polyP accumulation. (a) Solid lines represent saturation state modeled on values derived from literature salivary concentrations of PO$_4^{3-}$ and Ca$^{2+}$ (34). High PO$_4^{3-}$ and Ca$^{2+}$ values of 12.6 and 4.2 mM respectively (black curve), medium PO$_4^{3-}$ and Ca$^{2+}$ values of 7.3 mM and 2.65 mM respectively (red curve), and low PO$_4^{3-}$ and Ca$^{2+}$ values of 2.0 mM and 1.1 mM respectively (blue curve). (b) Corresponding dashed lines represent a shift in relative saturation in response to polyP accumulation resulting in a ~0.91 mM PO$_4^{3-}$ concentration change (arrow), as determined by our single species phosphate uptake model of *L. rhamnosus*. A PO$_4^{3-}$ drawdown of 0.91 mM changes the saturation state to one in which dissolution becomes thermodynamically favorable at a higher pH than without the drawdown in a scenario where PO$_4^{3-}$ and Ca$^{2+}$ concentrations are low in the surrounding fluids. Relative saturation equilibrium ($\Omega=1$) is represented by the solid horizontal line. Omega values >1 represent saturated conditions while omega values <1 represent undersaturated conditions.

Fig. 6. Schematic of polyphosphate-accumulating bacteria take up inorganic phosphate to synthesize poly-P potentially resulting in undersaturated conditions that lead to mineral dissolution and caries progression. Polyphosphate-accumulating bacteria may also acquire PO$_4^{3-}$ from acid-induced dissolution of the enamel.
| Strong Genetic Potential Clades               | Highly Variable Clades                                  | Low Genetic Potential Clades                  |
|----------------------------------------------|---------------------------------------------------------|-----------------------------------------------|
| Achromobacter●                              | Actinomyces●                                             | Gemella                                       |
| Acinetobacter●                              | Anaerococcus●                                            | Granulicatella                                 |
| Actinobaculum                               | Bacteroides                                              | Haemophilus                                    |
| Afipia                                       | Bartonella                                               | Jonquettella                                   |
| Agrobacterium●                              | Chloroflexi                                              | Lactococcus●                                  |
| Alloiococcus●                                | Desulfobulbus                                             | Leptotrichia                                   |
| Anaerooglobus●                              | Desulfovibrio●                                           | Listeria●                                     |
| Arsenicicoccus●                             | Escherichia coli●                                         | Mycoplasma●                                   |
| Bacillus anthracis●                         | Synergistetes                                            | Oribacterium                                   |
| Bifidobacterium●                            | Lachnospiraceae●                                          | Parvimonas                                    |
| Bordetella●                                 | Methanobrevibacter●                                      | Peptostreptococcaceae●                         |
| Bradyrhizobium●                             | Olsenella                                               | Prevotella                                     |
| Brevundimonas●                              | Ottowia                                                 | Pseudoramibacter                              |
| Burkholderia cepacia●                       | Paenibacillus●                                           | Pyramidobacter                                 |
| Campylobacter●                              | Peptoniphilus                                            | Selenomonas                                   |
| Capnocytophaga●                             | Sangibacter                                              | Shuttleworthia                                |
| Comamonas●                                  | Sarothrix                                                | Sneathia                                      |
| Corynebacterium●                            | Carnobacter                                              | Solobacterium                                 |
| Cronobacter●                                | Cryptobacterium●                                         | Streptococcus●                                |
| Cryptobacterium●                            | Cutibacterium                                            | Tannerella                                    |
| Cutibacterium●                              | Delphia                                                  | Candidate division TM7                       |
|                                             | Dialister                                                | Treponema                                     |
|                                             | Eikenella                                                |                                               |
|                                             | Enterobacter●                                            |                                               |
|                                             | Finegoldia                                               |                                               |
|                                             | Gardnerella                                              |                                               |
|                                             | Helicobacter pylori●                                     |                                               |
|                                             | Johnsonella                                              |                                               |
|                                             | Kingella                                                 |                                               |
|                                             | Klebsiella●                                              |                                               |
|                                             | Kluvera                                                  |                                               |
|                                             | Kytococcus●                                              |                                               |
|                                             | Lachnospiraceae●                                          |                                               |
|                                             | Lautropia                                                |                                               |
|                                             | Lysinibacillus●                                          |                                               |
|                                             | Megasphaera                                              |                                               |
|                                             | Mesorhizobium●                                           |                                               |
|                                             | Microbacterium                                           |                                               |
|                                             | Mitsukella                                               |                                               |
|                                             | Mobiluncus                                               |                                               |
|                                             |                                                                 |                                               |
|                                            |                                                                 |                                               |
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