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Tip extension and simultaneous multiple fission in a filamentous bacterium

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Abstract

Organisms display an immense variety of shapes, sizes, and reproductive strategies. At microscopic scales, bacterial cell morphology and growth dynamics are adaptive traits that influence the spatial organization of microbial communities. In one such community—the human dental plaque biofilm—a network of filamentous *Corynebacterium matruchotii* cells forms the core of bacterial consortia known as hedgehogs, but the processes that generate these structures are unclear. Here, using live-cell time-lapse microscopy and fluorescent D-amino acids to track peptidoglycan biosynthesis, we report an extraordinary example of simultaneous multiple division within the domain *Bacteria*. We show that *C. matruchotii* cells elongate at one pole through tip extension, similar to the growth strategy of soil-dwelling *Streptomyces* bacteria. Filaments elongate rapidly, at rates more than five times greater than other closely related bacterial species. Following elongation, many septa form simultaneously, and each cell divides into 3-14 daughter cells, depending on the length of the mother filament. The daughter cells then nucleate outgrowth of new thinner vegetative filaments, generating the classic “whip handle” morphology of this taxon. Our results expand the known diversity of bacterial cell cycles and help explain how this filamentous bacterium can compete for space, access nutrients, and form important interspecies interactions within dental plaque.

Significance

The shape of bacterial cells and the way that bacteria maintain their shape through cellular reproduction are fundamental biological characteristics that link form, physiology, and environment in the microbial world. While most bacteria divide by binary fission, we discovered a remarkable example of simultaneous multiple fission in a filamentous bacterium that has a key structural role within human dental plaque. This study expands our understanding of the oral microbiome, where hundreds of bacterial species compete for space and nutrients, forming biofilms that have direct impacts on human health. And our findings extend beyond the oral microbiome, revealing a unique bacterial cell cycle and an example of how cell morphology and reproductive strategy can influence the spatial organization of microbial communities.

Main text:

Introduction

All organisms have a growth form adapted to the environment in which they evolved (1). For bacteria, cellular morphology and reproduction set constraints and open new possibilities in nutrient acquisition, motility, attachment, and dispersal (2, 3). Typical bacteria are rods or cocci 0.5 to 5 micrometers long that divide by binary fission, with one mother cell giving rise to two daughter cells (4). While binary fission is the traditional understanding of bacterial reproduction extensively studied in model species like *Escherichia coli* and *Bacillus subtilis*, there are a few reports of non-binary lifecycles (5-7). Multiple fission—when a large polyploid mother cell divides into more than two

daughter cells—occurs in isolated instances, including in *Streptomyces* species as aerial hyphae separate into spores (8), in pleurocapsalean cyanobacteria (9), and during one stage of the predatory *Bdellovibrio bacteriovorus* lifecycle (6, 10). Non-binary cell division is more complex than binary fission because several septa form simultaneously and many progeny are generated at once (10).

Beyond the implications for the lifestyle and fitness of the individual species (11), bacterial cell morphology and growth dynamics shape spatial patterns within microbial communities (2, 3, 12, 13). Some of the most simple and effective ways that a bacterium can structure a microbial community are by outcompeting other taxa for space (14), and/or by adopting an elongated or other form that facilitates interspecies interactions (15). Therefore, microbial ecology and taxon-taxon interactions depend on cell cycle and physiological growth dynamics of individual taxa within microbial communities (16). This is particularly true for keystone taxa that have an outsized consequence on community structure (17, 18).

Human oral microbial communities are ideal for studying the cellular morphogenesis and growth dynamics of keystone bacterial species. Dental plaque is a polymicrobial biofilm composed of hundreds of bacterial taxa of varying cell shape and size, from ultra-small cocci 200 nanometers wide to filamentous organisms hundreds of microns long (19, 20). Among this diversity, multiple lines of evidence have converged to show that the filamentous bacterium *Corynebacterium matruchotii* is a key plaque taxon. Metatranscriptomic and 16S rRNA gene sequence analyses indicated that *C. matruchotii* is one of the most prevalent bacterial species in dental plaque, and that it is present at high abundance relative to other taxa (15, 21-24). This species has been

isolated from humans across the world (25) and is closely related to other human commensal *Corynebacterium* species and pathogenic mycobacteria (including *Mycobacterium tuberculosis*, all within the order Mycobacteriales) (26). *Corynebacterium matruchotii* lives in dental plaque both below (subgingival) and above (supragingival) the gumline and is associated with a healthy oral microbiome (15, 23, 27, 28). Spectral imaging fluorescence *in situ* hybridization (CLASI-FISH) revealed that filamentous *C. matruchotii* cells are a primary structural component of dental plaque (15). The filaments interact physically and chemically with small cocci (usually *Streptococcus* and *Porphyromonas* species) to form corncobs (15, 29-31) which are nested within larger hedgehog consortia (15). The *C. matruchotii* cells are hypothesized to act like a scaffolding spanning the biofilm, attached to initial colonizers such as *Actinomyces* species at the base, with a central zone where *Fusobacteria*, *Capnocytophaga* and *Leptotrichia* cells localize, and terminating at an aerobic peripheral region dominated by *Streptococcus*, *Haemophilus* and *Neisseria* species (15).

Although filamentous *C. matruchotii* cells are a main structural component of the natural dental biofilm, the process by which these forms are generated via the cellular growth and reproductive cycle is unclear (15). For decades, *C. matruchotii* has been noted for its pleomorphic cell morphology (32-35). An assortment of cellular forms is routinely observed upon microscopic observation of liquid cultures of this bacterium, including filaments of various lengths and “whip handles” that appear as a wider base from which a thinner filament extends (32). In this study, we characterized the cellular growth cycle of *C. matruchotii* using live-cell confocal time-lapse microscopy, nucleoid staining, and

111 spatiotemporal tracking of peptidoglycan biosynthesis. We report *C. matruchotii* growth
112 dynamics at the single-cell level, including rapid polar growth by tip extension and
113 simultaneous multiple fission. These basic aspects of *C. matruchotii* biology are likely
114 critical to its structural role in the formation of natural plaque biofilm.

115

116 Results

117 ***Corynebacterium matruchotii*** **has a Cell Cycle Based on Simultaneous Multiple**
118 **Division**

119 We tracked growth kinetics and morphometrics of individual isolated cells fixed in place
120 under an agarose pad as they developed into microcolonies to characterize the general
121 phases of the *C. matruchotii* cell cycle (Fig. 1). Filaments elongated without visible
122 septa or other internal divisions (Fig. 1A and Movie S1). Filamentous growth appeared
123 in transmitted light images to be by tip extension, unidirectionally at one cell pole (Fig.
124 1A, phase 1). Septa then appeared simultaneously at multiple sites along the
125 filamentous cell (Fig. 1A, arrows in phase 2 panel). Following septal formation,
126 cytokinesis occurred, and the filament fractured into many cells during a simultaneous
127 multiple division event (Fig. 1, phase 3). Cell division occurred rapidly, resulting in
128 daughter cells with squared edges that were oriented relative to adjacent cells at 30° to
129 180° angles. Daughter cells were often partially attached by a transient hinge-like
130 structure (Fig. 1A, arrow in phase 3 panel). After multiple division, new filamentous
131 outgrowths emerged from daughter cells (Fig. 1A, arrows in phase 4 panels), with any
132 new poles acting as potential initiation sites, and the original growth tip of the mother
133 filament also continuing to grow. Outgrowth of new filaments coincided with complete

separation of cells and release from transient hinge-like attachments. New filaments elongated and thickened over time, and subsequent multiple division events repeated the cell reproduction cycle, driving microcolony development (Fig. 1A, phase 5).

Within individual microcolonies, growing cells of the type strain fell into two categories based on final length and how long they elongated prior to dividing. Most cells elongated for 2-4 hours, then septated and divided upon reaching an intermediate length of 25-40 μm (Fig. 1B). These cells contributed to the exponential increase in the number of cells and total area of the microcolony. Another group of cells elongated for 6 h or longer, reaching lengths of 45 to 70 μm or more. Some of these longer cells were over 120 μm and still elongating at the end of experiments. Extra-long cells include the progenitor cell tracked in Fig. 1, and many others that grew along the periphery of microcolonies at later stages of development (Movie S1).

We tracked cell cycle dynamics for 40 cells within microcolonies formed by two different strains, the type strain ATCC 14266 and ATCC 43832 (Fig. 2A), and for >500 cells recorded across different experiments seen in Movies S1-S4. The same overall growth dynamics occurred in microcolonies grown in Brain Heart Infusion medium (BHI; Fig. 1, Fig. 2A, Movies S1 and S2), within multispecies microcolonies grown in saliva medium as a simplified mock supragingival plaque condition (Fig. 2A, Movie S3), and both with (Movies S1, S2, S3) and without (Movie S4) 5% CO2 supplementation of the growth atmosphere.

Cellular Growth and Division are Heterogeneous and Asymmetric

We found heterogeneity among many aspects of the *C. matruchotii* cell cycle (Fig. 2). In both strains and in both media (BHI and saliva), new outgrowths that emerge from daughter cells were thinner relative to the mother filaments prior to multiple fission (Fig. 2B). In the type strain, newly emerging filaments had a mean width one-half that of the mother filaments (0.78 vs. 1.56 μm). Outgrowth of new filaments occurred from most daughter cells within 10-30 minutes after cytokinesis. We also observed lag periods of no growth for some daughter cells amidst actively growing microcolonies, followed by the delayed emergence of a new filament (SI Appendix, Fig. S1).

Filament length at septation ranged from 6 to over 70 μm, with mean of 38.2 μm (SD = 17.0) for the type strain ATCC 14266 and 25.2 μm (SD = 7.7) for ATCC 43832. The number of daughter cells resulting from multiple fission varied from 3 to 14, with a median of 7 cells for the type strain ATCC 14266 and 5 for ATCC 43832. A Pearson correlation indicated a significant positive relationship between length at septation and the number of daughter cells, where longer mother cells gave rise to more daughter cells in both strains (Fig. 2C; $r = 0.864$ for ATCC14266 and 0.888 for ATCC 43832, with p values <0.001). Daughter cells varied in length from 1 to 19 μm (Fig. 2D), with a mean of 5.70 μm for the type strain (SD = 3.66) and 5.58 μm for ATCC 43832 (SD = 2.74).

Cell division in *C. matruchotii* was asymmetric (Fig. 2E). We calculated division symmetry values for daughter cells after simultaneous multiple division events (36). In this type of analysis, bacteria that reproduce by binary fission with septa aligned at mid-cell yield division symmetry values at or near 0.5, because one daughter cell is exactly or approximately half of the combined length of both sisters (36). Of 256 newly

generated daughter cells that we analyzed, only 13 cells (5.28%) were at or near symmetric (having symmetry values 0.4-0.5; Fig. 2E). Rather, most nascent daughter cells were asymmetric, having division symmetry values in the range of 0.05-0.2 (Fig. 2E). Mean division symmetry values were 0.137 (SD = 0.102) for the type strain and 0.200 (SD = 0.108) for strain ATCC 43832.

Daughter Cells Elongate Rapidly, with Rates Increasing with Filament Length and Subapical Width

We tracked cellular elongation rates in several conditions for cells that continued to grow for 6-8 hours without dividing. For the type strain in BHI medium and in the saliva medium-based multispecies condition, newly divided cells elongated at 1.5-7.5 $\mu m.h^{-1}$ in the first hour after cell birth, and their elongation rate increased over time, reaching a maximum of 15-19 $\mu m.h^{-1}$ (Fig. 2F). Spearman's rank correlation measurements of the BHI medium and saliva medium data sets (with sample sizes of 43 and 48 paired values, respectively) shown in Fig. 2F indicated a significant positive relationship between elongation rate and cell length (r_s = 0.890 for cells in BHI medium and 0.952 in saliva medium, with p-values <0.001). In BHI, cells reached higher elongation rates at shorter cell lengths on average relative to cells in saliva medium, and then remained at a steady state of rapid elongation once cells reached 50 μm in length, with an average maximum of 16.4 $\mu m.h^{-1}$ (SD = 0.60; Fig. 2F). Cells in the saliva medium condition reached a slightly higher average maximum of 17.5 $\mu m.h^{-1}$ (SD = 0.91). A two-tailed T-test indicated that there was no significant difference between the average maximum elongation rates in BHI medium and the multispecies saliva condition (p >0.05).

As cells elongated, subapical width of the growth tip increased, while the non-growing end of filaments remained at the same width (SI Appendix, Fig. S2). Spearman's rank correlation of growth tip width and total cell length yielded r_s values of 0.906 for the BHI medium data set and 0.935 for the saliva condition, with p-values <0.001. Applying the same statistical analysis using subapical width values of the non-growing end of filaments resulted in non-significant, weak relationships with cell length for both culture conditions (r_s values <0.3 with p-values >0.1). We found a significant positive relationship between elongation rate and subapical width of elongating growth tips (SI Appendix, Fig. S2). Spearman's rank correlation of the BHI medium and saliva condition data sets yielded r_s values of 0.970 and 0.860, respectively (p-values <0.001). Most measured filaments that reached elongation rates over 10 $\mu m.h^{-1}$ were at least 20 μm in length and had growth tips with subapical widths above 1.2 μm.

Filaments Elongate at One Pole by Tip Extension, and Develop Many Septa Simultaneously During Cell Division, with Condensed Nucleoids

In transmitted light images, *C. matruchotii* cells appeared to grow from one end of the filament, with visible septa forming simultaneously prior to multiple division (Fig. 1A, Movie S1). We extended these observations using fluorescent D-amino acids (FDAAs). FDAAs have emerged as powerful tools for tracking bacterial cell morphogenesis because they are covalently incorporated into the cell wall by transpeptidases without interfering with the growth of live cells (37-39). A range of FDAAs have been designed with different spectral properties, enabling multicolor "virtual time-lapse" experiments that show sequential periods of incorporation (40, 41). By growing cells in the presence

224 of FDAAs throughout their cell cycle, one can label the entire cell wall, whereas a
225 shorter “pulse” of FDAA exposure reveals specific regions of active peptidoglycan
226 biosynthesis (40).

227 To image spatiotemporal dynamics of cellular elongation and division, we
228 conducted experiments with one or multiple FDAAs (Fig. 3). Exposing growing cells to a
229 single pulse of Rf470DL for 15 minutes labeled a short region of the cell wall at a single
230 growth tip (Fig. 3A). Cells with an overall length of 10-20 μm showed a 1-2 μm apical
231 region of FDAA signal, corresponding to an estimated elongation rate of 4-8 μm·h-1.
232 Consistent with direct measurement of cell length during conventional timelapse
233 experiments (Fig. 2F), a 72-micron-long filament had a 4 μm region of FDAA signal,
234 indicating that it was elongating at a greater rate of ~16 μm·h-1 during the labeling
235 experiment. We also imaged cells that were developing >5 septa simultaneously during
236 the 15 min FDAA exposure (Fig. 3A). In separate virtual timelapse experiments, we
237 exposed growing cells to HADA for 4 h, followed by a 45 m pulse of NADA. This
238 resulted in signal from HADA across most of the cell length, with NADA signal localized
239 at a single growth tip and at the sites of multiple developing septa (Fig. 3B). As further
240 confirmation of these elongation dynamics, we conducted experiments with sequential
241 exposure to 3 different FDAAs. The result was elongated cells, including those with
242 whip handle morphology, with unidirectional regions of signal from each FDAA (Fig.
243 3C). We also imaged a less common double-ended whip handle. The double-ended
244 whip handle was divided by an older septum (formed during incubation with the first
245 FDAA), representing two daughter cells that failed to separate during multiple division,
246 each elongating in one direction (Fig. 3C, arrow). These images demonstrate that single

growing cells (not divided by septa) elongate at one pole by tip extension and establish that new peptidoglycan was synthesized at multiple septa simultaneously within single filaments.

We stained nucleoids at the end point of FDAA experiments with SYBR green, a stain that preferentially labels double-stranded DNA. We found that nucleoids are condensed between developing septa within cells undergoing multiple division. Each nascent daughter cell contained one or more nucleoid bodies, with evidence of sister nucleoids and occlusion from the septal region (Fig. 4 and SI Appendix, Fig. S3). In many actively elongating cells that we observed (defined as cells lacking septa and having a region of recently added FDAA at the growth tip), nucleoids were decondensed, present in a diffuse state throughout the filament (Fig. 4A). Other long cells were elongating at the growth tip and forming septa further back in the filament at the same time. These cells were imaged in a heterogenous state, with regions of uncondensed and condensed nucleoids (Fig. 4B). Filaments with the most condensed nucleoids were in an advanced state of multiple division, with developed septa, and partially separated cells attached by the hinge-like structure (Fig. 4C). The difference in nucleoid condensation between these cell states is further visualized through fluorescence intensity plots (SI Appendix, Fig. S4). We also observed several long, septating filaments with highly condensed nucleoids that did not have HADA incorporated at a growth tip, indicating they were not actively elongating prior to initiating cell division (Figs. S5-S6). One cell was 93 microns long, forming 28 septa simultaneously and poised to divide into 29 daughter cells (SI Appendix, Fig. S5).

Ex-vivo Dental Plaque Biofilm Contains Filamentous *C. matruchotii* Cells with Heterogenous Lengths, Widths, Morphologies, and Orientations

We expanded on our previously published findings (15) by analyzing a representative CLASI-FISH image that highlights heterogenous growth of *C. matruchotii* within the natural dental plaque biofilm (Fig. 5). Within the dense network of *C. matruchotii* filaments, we measured cells from 1 to over 30 μm long, in line with the wide range observed within in vitro microcolonies grown in the saliva-based simplified mock supragingival plaque community (Figs. 2E-F). Filaments extended from the core of the biofilm to the periphery where corncobs formed at the distal ends of the apparent growth tip (Fig. 5, arrow). Within the ex-vivo plaque image, we measured filament widths ranging from 0.6 to 1.3 μm, matching heterogeneity in width that we recorded in vitro within the saliva condition (Fig. 2B). We also measured single ex-vivo cells that increased in width across the length of the filament, like those measured in vitro (SI Appendix, Fig. S2). Finally, individual filaments were not always straight, and instead often showed sharp breaks or turns in direction. This tangled mass of filaments, extending at all angles, was conceivably the result of microcolony growth driven by multiple division as seen in Movies S1-S4.

Discussion

New Cell Cycle Biology and Comparison with Other Bacteria

The cellular growth and division cycle in *C. matruchotii* has unique qualities that go beyond features shared with other Gram-positive bacteria. In many Gram-positive rod-

shaped bacteria such as *B. subtilis*, new peptidoglycan is synthesized dispersively along the sidewall (42, 43). In contrast, cellular elongation in *C. matruchotii* occurs via polar growth (44). The feature of polar growth is shared with *Corynebacterium glutamicum* and mycobacteria, except that in these taxa, growth is bipolar with cell wall synthesis occurring at both poles (42, 43, 45-48), whereas in *C. matruchotii*, peptidoglycan synthesis occurs unidirectionally at one cell pole (Fig. 6). This type of unipolar elongation has previously been described in more distantly related streptomycetes and in some alphaproteobacteria (42, 49, 50).

We observed that *C. matruchotii* divides through a form of V-snapping as seen in other Actinobacteria (46, 48, 51), resulting in partial attachment of daughter cells by a hinge-like structure (48, 51). Despite the overall commonality of V-snapping-like division, *C. matruchotii* does not divide by binary fission like other species in the genera *Corynebacterium* and *Mycobacterium* (48, 52-54). Rather, *C. matruchotii* divides through simultaneous multiple fission (Fig. 6). Outgrowth of new filaments half the width of the daughter cells that they emerge from is another variance from other bacteria in the order Mycobacteriales, which continue to grow after binary fission at or near the same width as the mother cell (46, 52).

The hyphal lifestyle of the genus *Streptomyces* parallels most aspects of the *C. matruchotii* growth cycle, including elongation by tip extension, V-snapping, and a multiple division-based reproductive stage. In *Streptomyces*, hyphae are a vegetative growth phase, whereby filamentous cells elongate by tip extension (50, 55). Then aerial hyphae develop, septate at many sites simultaneously (referred to as sporulation septa) and divide into unigenomic prespore compartments (8, 55, 56). Zhou, Halladin and

Theriot described this process in *S. venezuelae* as a “chain reaction,” during which hyphae snapped at once or in quick succession into many spores (51). Images of sporulation septa in *S. venezuelae* or sporangia in *B. subtilis* can appear superficially similar to the fluorescence micrographs of multiple division that we captured (55-57), including nucleoid condensation and occlusion from septal regions (58). However, *Corynebacterium* species are not known to form endospores (32, 59). Instead, multiple division in *C. matruchotii* gives rises to new vegetative cells that immediately elongate.

We identified predicted orthologs of bacterial polar elongation and division proteins that are attractive targets for future investigation of tip extension and multiple fission in *C. matruchotii* (SI Appendix, Table S1). Based on studies of *C. glutamicum* (60, 61), we expect that DivIVA is essential for polar elongation. DivIVA (known as Wag31 in mycobacteria) localizes to growing poles in actinobacteria, where it acts as a scaffold that recruits other components to the polar elongation complex (or polarisome) (60, 62-64). Distinct dynamics of the contractile ring-forming protein FtsZ and FtsZ-interacting proteins of the division complex (or divisome), such as SepF and SepH (65, 66), are also likely at play. The synchronous assembly of Z-ladders during the development of sporulation septa in *S. coelicolor* serves as one model for how FtsZ may localize during multiple division (56). We anticipate that the corynebacterial cytoskeletal element RsmP (Rod-Shaped-Morphology Protein) is involved as well. Like Scy and FilP in *Streptomyces* species (67-71), RsmP is an intermediate-filament-like protein that self-assembles into filaments in vitro and within living cells (61). Mutation, depletion or overexpression of several other genes in *Corynebacterium* and *Mycobacterium* species that are normally short rods resulted in a filamentous or multiseptate phenotype (72).

Mutating the gene encoding lipoprotein LpqB in *M. smegmatis* resulted in a hyphal, polyploid cellular phenotype resembling the natural morphology of streptomycetes (73). Likewise, depletion of WhiB2 in *M. smegmatis*, a homolog of transcriptional regulator WhiB essential for septation of aerial hyphae in streptomycetes, caused a filamentous, branching phenotype (72, 74). We found homologs of all these proteins in *C. glutamicum,* a taxon with a short rod morphology that divides by binary fission. This suggests that the *C. matruchotii* growth form results from modifications to the structure and function, localization and/or expression of these proteins rather than their presence or absence. Considering that researchers continue to find proteins with new functions in actinobacterial growth and division (66-68, 75), it is plausible that unknown molecular factors will be discovered while elucidating the *C. matruchotii* cell cycle.

Cell Division is Heterogenous and Exceeds the Level of Asymmetry Observed in Mycobacteria

Bacteria from the genus *Mycobacterium* reproduce through an asymmetric form of binary fission (47). A wider distribution of division symmetries has been recorded among populations of mycobacteria cells relative to *E. coli* and vegetative *B. subtilis* cells that typically divide into two cells of equal length (36, 52, 76). Despite this distinction, only a minority of sister pairs in *M. smegmatis* and *M. tuberculosis* are asymmetric, with most daughter cells being near symmetric (36). In contrast, over 90% of daughter cells in *C. matruchotii* were asymmetric, consistent with a lack of binary fission. Heterogeneity in *C. matruchotii* cellular growth is also seen in the wide range of daughter cell lengths after multiple division and in lag periods before new outgrowth that occurred in a

minority of daughter cells. These growth lags resembled “new end take off” dynamics in *M. smegmatis and M. tuberculosis* (52).

In mycobacteria, physiological heterogeneity and asymmetry can enhance the ability of a population of cells to cope with environmental stress and variable microenvironments (36, 46, 77), and future studies may test whether the same is true for *C. matruchotii.* Mycobacterial cells with variable sizes and growth rates have survival advantages during antibiotic treatment and within host tissues (46, 77). This relationship between heterogeneity at the population level and differential survival of individual cells likely extends to the oral cavity, where *C. matruchotii* encounters a variety of physical, chemical, and biological conditions (15, 78).

Spatial Context for the Whip Handle Morphology, and a Constitutive, Natural Filamentous Lifecycle

Our time-lapse imaging of immobilized microcolonies adds spatiotemporal context to the pleomorphic forms seen in shaken liquid or otherwise disrupted cultures (32). Whip handles are the shorter cells generated from simultaneous multiple division of a filament, with outgrowths of the new thinner filaments. Unlike conditional filamentation induced by SOS responses (79), or filamentous phenotypes of genetically engineered bacterial strains (58, 80), the filamentous lifecycle is natural and constitutive in *C. matruchotii*. Our experiments show a filamentous growth cycle in two undomesticated, genetically wild type human isolates, without antibiotic treatment, DNA damage, or other experimental stress, in rich and reduced strength saliva media, in monoculture and multispecies microcolonies, and throughout 24-hour growth experiments, at low and

high cell densities. This filamentous lifecycle is also unlike that of the so-called cable bacteria and giant bacteria like *Thiomargarita* species, which grow as chained cells that are intrinsically multicellular as they elongate (7, 81).

Corynebacterium matruchotii is Among the Most Rapidly Elongating Bacteria

Corynebacterium matruchotii is capable of sustained cell elongation rates above 16 microns per hour, greater than most other bacteria and all close relatives in the genera *Corynebacterium* and *Mycobacterium* with known elongation rates. Wild type *C. glutamicum* cells elongated at maximum of 2.6 $\mu m \cdot h^{-1}$ in BHI medium in one study (53), and a few cells reached 3 $\mu m \cdot h^{-1}$ in another study, with the average under 2 $\mu m \cdot h^{-1}$ (47). Maximum elongation rates within *Mycobacterium smegmatis* cell populations in rich medium were reported under 2 $\mu m \cdot h^{-1}$ (36) and pathogens like *M. tuberculosis* have much lower maximum elongation rates (36, 52). Among model Gram negative and Gram positive bacteria, *E. coli* elongated at 3.72 $\mu m \cdot h^{-1}$ (36) and *B. subtilis* at a maximum of 3.5 $\mu m \cdot h^{-1}$ in an intermediate growth condition and 8 $\mu m \cdot h^{-1}$ in a fast growing condition (82). In summary, *C. matruchotii* cells elongate twice as fast as *B. subtilis*, 4 times faster than *E. coli*, and more than 5 times faster than other *Corynebacterium* and *Mycobacterium* species.

One bacterial genus with measured cellular elongation rates that do match *C. matruchotii* is *Streptomyces*, the same group that we argue shares the most in common with the *C. matruchotii* filamentous lifecycle. Several hyphal *Streptomyces* species elongate by tip extension at rates of 10 $\mu m \cdot h^{-1}$ (83), or above 20 $\mu m \cdot h^{-1}$ in some culture

conditions (84). An increase in elongation rate as cells lengthen has also been reported for *Streptomyces* species (83). The increase we measured may be due to an accumulation of proteins that mediate cell wall biogenesis at the growth tip, and was correlated with an increase in subapical width, presumably expanding the growth zone. In mycobacteria, accumulation of the polar growth scaffold protein Wag31 resulted in increased polar elongation rates over time for individual cells (52).

Significance for the Spatial Organization of the Natural Plaque Biofilm

We propose that rapid growth by tip extension and simultaneous multiple division explain how *C. matruchotii* outcompetes other taxa to form filamentous networks at the core of the dental plaque biofilm (15). At each multiple division event, cells nucleate outgrowth of many additional growth tips extending at different angles relative to the axis of the mother cell. Based on the elongation rates that we report, a colony of filamentous *C. matruchotii* cells could theoretically advance 0.3-0.4 millimeters in favorable growth conditions during a 24-hour period. Like sessile *Streptomyces* bacteria and mycelial fungi that evolved a hyphal lifecycle adapted to utilizing nutrients among particles of soil or decaying plant material (85, 86), we speculate that a filamentous mode of growth, the capacity for rapid tip extension, and reproduction by multiple division provide selective advantages as *C. matruchotii* competes for space and nutrients within the heterogeneous, biodiverse dental plaque environment.

Considering that *C. matruchotii* is incapable of swimming motility, the ability to elongate rapidly by tip extension may enable a form of exploratory growth that

enhances cell fitness in certain conditions. At high cell density and favorable growth conditions, multiple division may occur more frequently, promoting a rapid increase in the number of cells and growth tips that is associated with exponential growth of a microcolony. Long filaments (50 to >120 μm) that elongate for extended periods of time could favor mutualistic interspecies interactions. For example, corncobs may develop at the edge of hedgehog consortia through the coupling of *C. matruchotii* tip extension with streptococcal reproduction. Tip extension would expand the attachment substrate and interface for chemical interaction (30) for growing streptococci. Beyond the significance for dental plaque, insights from this study provide a striking example of how bacterial cell morphology and growth dynamics can structure the spatial organization of microbial communities.

Materials and Methods

Strains, Growth Media and Culture Conditions

The strains used in this study (SI Appendix, Table S2) were cultured in BHI medium (37 g in 1L, BD Cat. No. B11059) with 0.5% (w/v) yeast extract. Cells were used for experiments after a single growth passage from frozen glycerol stocks inoculated on BHI medium containing 1.5% agar. Cultures were incubated at 37°C, shaking at 180 rpm. Our protocol for producing saliva growth medium was adapted from previous studies (87, 88). 5 ml of unstimulated saliva was collected from four volunteers who self-identified as having good oral health and combined. The pooled saliva was centrifuged at 1500 rpm for 10 m and the supernatant was diluted with phosphate-buffered saline

(PBS) to a final concentration of 25%. The 25% saliva solution was supplemented to a final concentration of 0.25% BHI powder and 0.25% yeast extract and passed twice through a 0.22-micron filter. This saliva medium is ~1/15 strength BHI relative to full strength BHI medium. Protocols involving human saliva including volunteer consent were approved by an Institutional Review Board (WCG IRB Protocol #20232102).

Preparation of Bacteria for Time-lapse Microscopy

Overnight cultures of *C. matruchotii* were diluted 1:50 in BHI medium and incubated for ~8 h until they reached an optical density at 600 nm of 0.4-0.5. These mid-exponential phase cultures were centrifuged at 10,000 rpm for 2 m, resuspended in fresh medium, mechanically homogenized by pipetting, and diluted 1:10 in fresh growth medium. 10 µl of diluted culture was then aliquoted onto the bottom surface of a chambered coverglass (Ibidi Cat. No. 80807). An agarose pad (growth medium supplemented with 1.5% agarose) was then placed and gently pressed on top of the cell culture suspension to confine cellular growth within a narrow focal range. The lid was added to the chambered coverglass and the vessel was immediately placed within the microscope incubator system.

Multispecies Mock Supragingival Plaque Community

Multispecies biofilms were established to test *C. matruchotii* growth dynamics in a system that more closely resembled the natural condition. *Actinomyces naeslundii* and *Streptococcus cristatus* (SI Appendix, Table S2) were chosen for coculture with *C.*

matruchotii because they are prevalent and abundant in supragingival plaque (15, 24). *A. naeslundii* and *S. cristatus* were grown in BHI medium and prepared through the same process described for *C. matruchotii*. Mid-exponential phase cultures of the 3 species were centrifuged at 10,000 rpm for 2 m, washed by resuspension in PBS, centrifuged again, and resuspended in the 25% saliva medium. A mixture of the cultures of each species in saliva medium at equal volumes was used to inoculate chambered coverglass wells. The coverglass was coated with saliva as an attachment substrate intended to mimic the salivary pellicle of teeth. Saliva coating was performed by adding 50 µl of 25% sterile saliva to each well, rotating to coat the entire surface, and drying uncovered in a laminar flow hood for 1 hour.

Labeling Experiments with Fluorescent D-amino Acids and Nucleoid Stains

Fluorescent D-amino acid experiments were conducted as previously described (37, 39, 40) using FDAA molecules HADA, NADA green and Rf470DL (SI Appendix, Table S3). Overnight cultures were diluted 1:50 in BHI, grown until mid-exponential phase and then diluted again in fresh medium containing FDAA. The duration and number of FDAA exposures, whether a single pulse or sequential exposures (virtual timelapse), are indicated in Figs. 3-4. All exposures occurred at a working FDAA concentration of 0.25 mM, incubated at 37°C in BHI medium.

For the single 15 m pulse of Rf470DL (Fig. 3A), cultures were immediately washed after FDAA exposure by centrifugation at 10,000 rpm for 2 m and resuspension in cold PBS.

Cold ethanol was added to a final concentration of 70% and cells were fixed on ice for 1 hour. Cells were then washed twice in PBS and resuspended in PBS prior to imaging.

For virtual time-lapse experiments, cells were washed twice with PBS after the initial exposure to remove the first FDAA. After washing, cells were resuspended in 1 ml of pre-warmed growth medium containing a second FDAA and returned to the incubator. For 2-color experiments (Fig. 3B), cells were washed and fixed after the second exposure. For 3-color experiments (Fig. 3C), washing was repeated after the second exposure, and cells were resuspended in medium containing a third FDAA, then incubated, washed and fixed.

For experiments when FDAA labeling was combined with nucleoid staining (Fig. 4), cells were exposed to FDAAs within growth medium as described above, washed, and then incubated at room temperature with SYBR green for 15 m in PBS at a working concentration of 2x according to manufacturer instructions. Following SYBR labeling, cells were washed twice with PBS and immediately imaged without fixation. We imaged these cells without fixation to avoid loss of SYBR signal due to interaction with ethanol (89). Ethanol fixation reportedly causes a hollowing (phase-transparency) of some bacterial cells (39), which could perturb the natural organization of nucleoids.

To collect reference spectra for linear unmixing of multi-label experiments, cells were labeled at the same working concentrations with each FDAA individually for 4 h, and with SYBR green alone for 15 m. For imaging FDAA experiments, cell suspensions were mounted on a 1.5% agarose pad (prepared with PBS). The agarose pad was

covered by a No. 1.5 coverglass within a 125 µl Gene Frame (Thermo Scientific AB-0578) adhered to a standard microscope slide (90).

CLASI-FISH of Ex-vivo Dental Plaque

The dental plaque sample shown in Fig. 5 was collected, fixed, hybridized, mounted, imaged, and analyzed through the methods detailed in Mark Welch et al. 2016 (15). Fluorescence signal for the five species in Fig. 5 corresponds to hybridization with probes Cor633, Str405, Cap371, Fus714 and Lep568 as previously described (15).

Microscopy Acquisition and Image Analyses

Images for time-lapse experiments were captured using a Zeiss Plan-Apochromat 63×/1.4 NA oil lens on a Zeiss LSM 980 microscope system configured on an inverted Axio Observer Z1, with Pecon incubation system. The system included a heated stage insert, heated insert lid, and light-tight incubated chamber, set to 37°C and 5% CO2 (except for the experiment shown in Movie S4 when cells were grown without CO2). Transmitted light images for time-lapse series were captured at 10 m intervals through T-PMT with excitation from the 639 nm laser at 0.2% power, automated through the Zeiss ZEN software. Cell length and width were measured using Fiji (91). Length was measured along the center line of filaments, accounting for cell curvature. See the SI Appendix for additional detail on image analyses.

Virtual time-lapse imaging experiments with FDAAs were imaged with a Zeiss Plan-Apochromat 40×/1.4 NA oil lens on a Zeiss LSM 780 system, configured on an inverted

Axio Observer, and equipped with a 34-channel spectral array module. Images for multi-label experiments were captured in lambda mode with 405 nm, 458 nm and 488 nm laser excitation. Within the Zeiss ZEN software, a median filter was applied with a kernel size of 3 for X and Y and reference spectra were used for weighted linear unmixing.

Data, Materials, and Software Availability

All data are included in the main article and/or supporting information files.

Acknowledgements

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Figure legends

Fig. 1. Microcolony development is driven by filament elongation and simultaneous multiple division.

(A) The general phases of the *C. matruchotii* cell cycle highlighted by tracking a single cell as it develops into a microcolony. In phase 1, the progenitor cell elongates and forms a filament. The outline of the progenitor cell at $t = 0$ (purple) is overlaid on the 3.5 h image. Multiple septa then form along the filament (phase 2, arrows), and it divides into many daughter cells simultaneously, with some daughter cells transiently attached by a hinge-like structure (phase 3, arrow). After multiple division, new thinner filaments emerge from daughter cells (phase 4, arrows). After the first division event, continued cycles of filament elongation, multiple division, and daughter filament outgrowth drive exponential growth of a microcolony (phase 5). (Scale bars for top panels and detail panels below are 20 μm and 5 μm, respectively.) (B) Growth dynamics of the microcolony imaged in (A). Black lines track elongation and division events (black boxes) for individual cells, including the progenitor cell and some of its offspring. Overall growth of the microcolony is expressed by the total number of cells (blue line) and % area of the field of view covered by cells (orange line).

Fig. 2. Single-cell growth kinetics and micron-scale morphometrics reveal rapid elongation, heterogeneity, and asymmetry in the *C. matruchotii* cell cycle.

(A) Representative images of microcolonies developing in several conditions, with cells dividing by multiple division. (Scale bars: 10 μm.) (B) Width of mother cell filaments and new filaments that emerge after multiple division, for 15 cells per condition. (C) The number of daughter cells resulting from multiple division for 20 cells per strain, plotted according to the length of the mother cell. (D) Violin plots of daughter cell lengths, with mean shown as circles. (E) Daughter cell lengths plotted according to the length of the mother cell at septation. Each vertical series represents one of 40 total cells. Most cells divided after reaching lengths of 5-40 μm (Group 1), while other cells divided at lengths ranging from 45-75 μm (Group 2). Below: a histogram of division symmetry values, calculated for a total of 256 daughter cells resulting from multiple division events. (F) Elongation rates of 12 individual cells (6 per condition) over an extended period of growth (6-8 h), plotted according to cell length.

Fig. 3. New peptidoglycan is synthesized at one pole of elongating filaments and at the sites of multiple septa developing simultaneously within dividing cells.

(A) Eight representative cells imaged following a single 15 min pulse of the fluorescent D-amino acid Rf470DL. Outlines show the entire cell from transmitted light images. (B) Maximum intensity projection images acquired from a 2-color virtual time-lapse experiment, showing a dividing cell grown in the presence of the fluorescent D-amino acid HADA for 4 hours, then washed and grown with NADA for 45 mins. The left panel is the transmitted light image. Bottom image panels are maximum intensity views of the X, Z plane showing 6 z-slices, representing 1.6 μm of depth. A fluorescence intensity profile of each FDAA along the cell length is also shown. (C) Representative images of elongating cells having a whip handle morphology from a 3-color virtual time-lapse experiment. Cells were grown with sequential exposure to HADA for 4 h, followed by 30 min pulses of Rf470DL and NADA. A less common double-ended whip handle is also highlighted (arrow), composed of two elongating cells divided by a septum. (Scale bars for A-C: 10 μm.)

Fig. 4. Nucleoids are condensed between multiple developing septa in dividing cells.

Columns A-C show cells representing three cell cycle stages: elongating, elongating and septating, and elongating, septating and dividing. Cells were grown with a long exposure (6 h) of Rf470DL, followed by washing and a 45 min HADA exposure. Then cells were washed in PBS and labeled with the double-stranded DNA stain SYBR green. Transmitted light images (top row) are single z-slices. Lower rows are maximum projection images of 3 z-slices (showing a depth of 1 μm) for the fluorescence channels corresponding with the experiment overview schematic. (Scale bar: 10 μm.)

840 **Fig. 5. Ex-vivo dental plaque biofilm contains a core of filamentous *C. matruchotii***
841 **cells with heterogenous lengths, widths, morphologies, and orientations,**
842 **consistent with in vitro experiments.**

843 A supragingival plaque sample imaged with the CLASI-FISH technique (15). Arrow
844 highlights a corncob formed by interaction between *C. matruchotii* and *Streptococcus*
845 cells. (Scale bar: 20 μm.)

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Fig. 6. Tip extension and simultaneous multiple fission add special features relative to other characterized bacterial reproductive modes

A comparison of one generation of cell division for bacteria that undergo: (A) symmetrical binary fission after adding new cell wall material along the sidewalls, as seen in *E. coli* and vegetative *B. subtilis*, (B) exact or approximate symmetric polar elongation and binary fission as seen in *C. glutamicum*, (C) asymmetric elongation and binary fission as in *Mycobacterium* species, and (D) a model for tip extension and simultaneous multiple fission in *C. matruchotii*, showing a filament dividing into 7 daughter cells (the median from our experimental data). Actively growing areas of the cell are shown in lighter shades.