ORIGINAL RESEARCH–GENERAL OTOLARYNGOLOGY

Tonsillolith: Not just a stone but a living biofilm

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ABSTRACT

OBJECTIVE: To study the morphology and activity of tonsilloliths, demonstrating oxygen respiration, denitrification, and acidification on exposure to sucrose.

STUDY DESIGN: Tonsilloliths were extracted in atraumatic conditions during tonsillectomy from 16 adults and sent to two different laboratories for histological, bacteriological, and biofilm studies under sterile conditions.

SETTING: Multicenter laboratory study.

SUBJECTS AND METHODS: Multiple tonsilloliths from two patients examined by confocal microscopy and microelectrodes were used to measure aerobic/anaerobic respiration and acid production (dissolved oxygen, nitrous oxide, pH) when exposed to saliva following addition of sucrose and fluoride.

RESULTS: Morphologically, tonsilloliths were similar to dental biofilms, containing corncob structures, filaments, and cocci. Microelectrodes showed that the microorganisms respired oxygen and nitrate. The oxygen concentration in the center of the tonsillolith was depleted to approximately one-tenth of that of the overlying fluid. The addition of sucrose resulted in acid production within the tonsillolith, dropping the pH from 7.3 to 5.8. The data showed stratification with oxygen respiration at the outer layer of tonsillolith, denitrification toward the middle, and acidification toward the bottom. The depletion of oxygen and acid production following addition of sucrose may allow the proliferation of anaerobic/acidophilic bacteria. Fluoride suppressed acid production in the presence of sucrose.

CONCLUSIONS: Tonsilloliths exhibit biofilm structure and the formation of chemical gradients through physiological activity. Although tonsillectomy is an option for treating cryptic infections, understanding the morphology and biofilm characteristics of tonsilloliths may stimulate scientists to use limited or targeted remedies in the future.

Biofilm formation is increasingly being recognized as a virulence factor for infections associated with the pharynx and upper airway.[1] Biofilms are assemblages of bacteria that are attached to surfaces (in the case of otolaryngological infections, epithelial and mucosal surfaces). Biofilms have been identified on middle ear epithelium in pediatric patients suffering with otitis media,[2] on adenoid and tonsil tissue,[3,4] and in the sinuses of patients suffering from rhinosinusitis.[5]

Biofilms are most commonly distinguished from bacterial colonization by the presence of an extracellular matrix enveloping the bacteria and structural features such as cell clusters, towers, and evidence of stratification suggesting a developmental process of a bacterial community, rather than the casual attachment of single cells. Scanning electron microscopy has been most commonly used to identify biofilms associated with clinical specimens. Confocal microscopy has the advantage that specific fluorescent staining can be used to identify chemical components (ie, proteins, nucleic acids, carbohydrates), bacteria species (by fluorescent in situ hybridization, FISH), and viability on fully hydrated specimens.[2,4]

Bacteria in biofilms are difficult to kill with antibiotics and, because they are localized, can also act as a nidus for acute episodes.[6] These characteristics are increasingly associated with the high recurrence rate and chronicity associated with many otolaryngological infections.[1] Another characteristic feature of biofilms is the development of chemical gradients due to consumption of nutrients (such as oxygen) and the production of metabolic end products such as lactic acid, which in turn creates microniches within the biofilm, allowing the coexistence of many different types of bacteria. For example, the consumption of oxygen by aerobic bacteria at the top of dental biofilms creates underlying anaerobic regions that can then be occupied by anaerobic pathogens.[7-9]

Tonsilloliths are another feature of the pharynx that appears to have characteristics in common with biofilms, particularly with dental biofilms. Tonsilloliths act as a localized concentration of aerobic and anaerobic bacteria, they calcify over time, progressing from soft gels to hard "stones," and they are often associated with inflammation (ie, in gingivitis and tonsillar inflammation).[10,11] Tonsilloliths form within tonsillar crypts and are associated with halitosis, thought to be caused by the release of volatile sulfur compounds pro-

duced from sulfur metabolism of anaerobic bacteria.[11,12] Tonsilloliths are also associated with chronic cryptic tonsillitis, which is the most common indication for adult tonsillectomy.

It was the goal of the present study to determine whether tonsilloliths could be considered biofilms by using conventional thin section histology on 16 patients, with more detailed analysis on a subset of multiple tonsilloliths from both tonsils of two patients by using confocal microscopy and dissolved oxygen (DO), pH, and nitrous oxide (N_2O) microelectrodes for respiration, acid fermentation and denitrification, respectively. Further, we wished to assess the influence of fluoride ion (F^-) on the reduction of acidic regions in the tonsillolith. Fluoride is a common ingredient of dentifrices used for caries control, and we have recently shown that sodium fluoride (NaF) reduced sucrose-stimulated pH drop in dental biofilms.[7]

MATERIALS AND METHODS

Collection of Tonsilloliths

After obtaining institutional review board approval and informed consent, tonsilloliths were collected randomly from 16 young adult patients undergoing elective tonsillectomy for chronic tonsillitis. None of these patients had any acute infections or antibiotic therapy in the preceding 3 months. First, prior to tonsillectomy, tonsilloliths alone were extracted by blunt pressure or they were dissected and removed in atraumatic technique in situ within tonsil crypts, stored in physiological saline, and shipped under sterile conditions to the laboratories for microbiological biofilm studies (Allegheny-Singer Research Institute, Pittsburgh) for whole-organ histopathological examination (St Lukes Roosevelt Hospital, New York).

Thin Section Histology

Tonsil specimens were oriented and sectioned in a manner to best demonstrate the crypts and tonsilloliths. The specimens were thinly sectioned, stained with hematoxylin-eosin (H&E), and examined with light microscope by a senior surgical pathologist.

Confocal Microscopy

Tonsilloliths from two patients were selected for more in-depth analysis by confocal microscopy. Individual tonsilloliths were mounted by placing them on a thin layer of cooled molten agar (15%) in a 35-mm Petri plate. The tonsilloliths were gently placed onto the agar with tweezers, avoiding contact with the intended viewing region, but they were not submerged. The specimens were stained according to manufacturer's instructions by pipetting approximately 50 μL of BacLight Live Dead kit (Invitrogen, Carlsbad, CA) onto each tonsillolith. After 30 minutes of incubation at room temperature in the dark, the petri plate was flooded with Hanks balanced salt solution with calcium chloride and magnesium chloride, but without phenol red (cat# 14025, Invitrogen). The tonsilloliths were observed with a Leica DM RXE microscope attached to a TCS SP2 AOBS confocal system (Leica Microsystems, Exton, PA); a ×63 long working distance water immersion objective was used, thus ensuring that the specimens were never dehydrated.

Microelectrode Studies

Individual tonsilloliths collected from two patients were mounted in cooled molten agar as described in previous text. Diluted saliva (10% in 1% phosphate buffer solution, PBS) was flowed over the tonsilloliths at a flow rate of 0.5 mL/min with the use of a peristaltic pump (Ismatec; Cole Parmer Instrument Co., Vernon Hills, IL). The unstimulated saliva was collected on ice and pooled from six young healthy volunteers of both sexes, with good oral hygiene and no detectable oral malodor by organoleptic methods. The saliva was used within 3 hours of collection, diluted in 1 percent PBS, and then spun down at 3500 rpm for 25 minutes. The supernatant was decanted. We diluted the saliva to lower the viscosity so that we could pump the fluid across the tonsilloliths for microelectrode measurements at a flow rate of 0.5 mL/min. The chamber was positioned on a 37°C heater. Because we were interested in determining whether denitrification (an anaerobic respiration) was occurring in the tonsilloliths, we measured the nitrate concentration in the undiluted saliva with a nitrate microelectrode.[8] The microelectrode was calibrated with the use of potassium nitrate (KNO_3) between 0 and 500 μM. The concentration of nitrate in the undiluted saliva was 763 μM. Because the measured saliva nitrate from our volunteers fell close to the median found in a clinical study of 209 people,[13] we decided to use it as a representative physiological concentration.

Thereafter, KNO_3 was added to the diluted saliva to make up to 763 μM NO_3^- ion. For the measurement of physiological activity in response to sugar, a pulse of sucrose (2.5% or 10%) was added to the medium. Physiological activity in the tonsilloliths was measured with either a 1) dissolved oxygen, 2) pH, or 3) N_2O microelectrode.

Microelectrodes were prepared and calibrated as previously described[8] with measurements performed by using a semiautomated setup. Concentration measurements were made at various depths above, and into, the tonsilloliths. The location of the surface of the tonsillolith was determined by observation with a dissecting microscope and designated as depth 0. The development of an oxygen profile with anaerobic regions within the tonsilloliths was assumed to be indicative of aerobic respiration; the production of acid was indicative of acid fermentation; and the production of N_2O was indicative of denitrification.

Conversion Rates at Different Depths in the Tonsilloliths

For calculation of the local conversion rates within the biofilm, a stepwise algorithm was used as described thor-

oughly by Gieseke et al.[9] To determine where acidification was occurring within the tonsillolith, we calculated rate data in terms of the relative H^+ ion production or consumption from the pH data, because the actual proton concentration is a function of buffering. First, we converted pH to H^+ concentration using $[H^+] = 10^{-pH}$ (mol 1^{-1}). We then expressed the production rate at different depths in the tonsilloliths and under the various conditions as relative values; those values were expressed as a percentage of the maximum rate.

RESULTS

Histology

Examination of the tonsil specimens stained with H&E under low magnification demonstrated tonsil crypts and surrounding benign lymphoid hyperplasia (Fig 1). Within the crypts tonsilloliths of various sizes were documented in early or mature stages of their development. A layer of fibrinous debris and inflammatory cells was identified between the tonsillolith and the crypt epithelium. Squamous epithelium of the tonsil surface was noted to extend toward the lining of the tonsil crypt. At the depth of the tonsillar crypt, some areas of mucosal changes from squamous epithelium to so-called lymphoepithelium were noted. These benign-appearing lymphoid cells overran (and obscured) the nonkeratinizing squamous epithelium; this type of epithelium can be seen lining the inflamed cryptic areas adjacent to tonsilloliths. Free mature tonsilloliths appeared as a round amorphous mass with a basophilic appearance. In the early stages of tonsillolith formation the biofilm was found to be adherent to the crypt epithelium. At high magnification the tonsilloliths were composed of a dense matrix and large quantities of microbial colonies composed of rods and cocci.
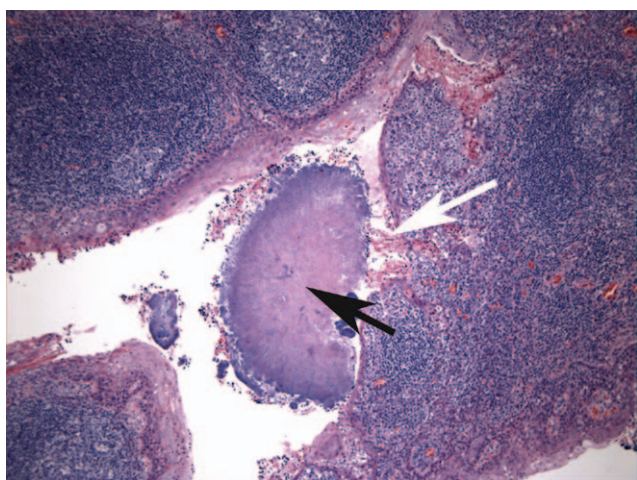

Figure 1   Tonsillolith (*black arrow*) within tonsillar crypt lined by squamous epithelium with reactive lymphoid hyperplasia. Fibrinous debris and inflammatory cells (*white arrow*).

Confocal Microscopy

The tonsilloliths were composed of densely packed bacteria including rods, cocci, and filamentous bacteria held together within an extracellular matrix (Fig 2A). In some places there was evidence of stratification, with fusiform rod-shaped bacteria concentrated on the outside and cocci on the inside layer (Fig 2B). The fusiform rods had a distinctive morphology consistent with that of *Fusobacterium nucleatum*. The composition of the matrix was undetermined because the reflected imaging technique was nonspecific. We also observed corncob structures consisting of cocci surrounding filamentous bacteria (Fig 3). Corncob structures are a common feature of dental biofilms and have been shown to consist of various bacteria including *F. nucleatum* (filamentous gram-negative rods) and *Streptococcus sanguis* (gram-positive cocci).[14]

Microelectrode Studies

The tonsilloliths consumed oxygen when exposed to unsupplemented 10 percent saliva, so that at 0.6 mm depth the DO was reduced to approximately 30 percent of air saturation (Fig 4A). The absence of N_2O production suggested that there was no denitrification (Fig 4B). When the 10 percent saliva was supplemented with sucrose, aerobic respiration, denitrification, and acid fermentation were stimulated. The oxygen concentration within the tonsillolith was further reduced to approximately 10 percent of air saturation (Fig 4A). The pH dropped from 7.2 to a minimum of approximately 6.3 at a depth of 0.2 mm with 2.5 percent sucrose and to 5.7 with 10 percent sucrose (Fig 4C). When NaF was added to the 10 percent saliva/10 percent sucrose medium, the pH increased, suggesting that the fluoride was inhibiting acid fermentation. Aerobic respiration was maximal in the surface layer down to a depth of 125 µm; denitrification was greatest between 50 µm and 200 µm; and acid fermentation occurred predominantly at depths greater than 100 µm.

DISCUSSION

The thin histology sections and confocal imaging revealed that the tonsilloliths contained a high density of bacteria, a diversity of cell morphologies showing that they were polymicrobial in nature, and that they were stratified. The fusiform morphology and corncob structures suggested that *F. nucleatum* was present and the images were similar to those of dental biofilms.[15] In future studies FISH might be employed to confirm the identification of *F. nucleatum*. Tsuneishi et al[11] had detected *F. nucleatum* in tonsilloliths by 16S ribosomal bacterial DNA sequencing and noted filaments in scanning electron micrographs. *F. nucleatum* is an obligate anaerobe that can produce volatile sulfur compounds associated with bad breath.[16] Noting that the filaments were in the interior of the tonsillolith, these authors hypothesized that this was because the interior was presum-
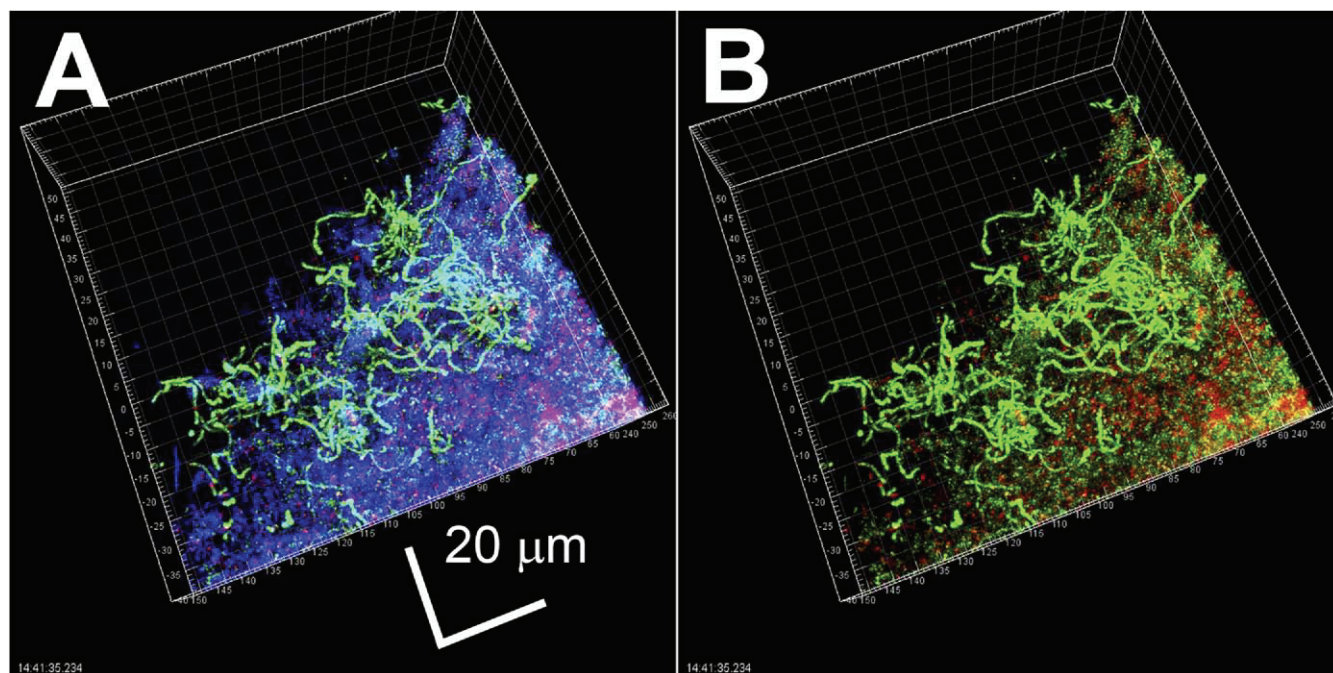
Figure 2   Confocal microscopy of tonsillolith. (**A**) Live (*green*) and dead (*red*) bacteria within matrix (*blue*). (**B**) Subtracting the matrix shows live filamentous bacteria on the surface, with rods and cocci deeper.

ably anaerobic and therefore a favorable environment for anaerobes. In the present study we observed the fusiform bacteria on the surface of the tonsillolith.

The microelectrode data showed that the bacteria in the tonsilloliths were metabolically active and that oxygen consumption in the top layers depleted oxygen deeper in the tonsillolith. The addition of sucrose stimulated oxy-

Figure 3   Corncob structures (*white arrow*) of green cocci surround red filamentous bacteria. Cocci predominantly surround the corncobs.

gen consumption and acid fermentation in a similar manner to that seen in dental biofilms.[7] However, it was interesting to note that even at a depth of 600 μm the dissolved oxygen concentration was still greater than 10 percent of saturation. In biofilms containing aerobic bacteria, oxygen is usually almost fully consumed at depths of greater than 100 μm. The higher concentrations that we measured in the present study might be explained by either a relatively low population of active aerobic bacteria or the presence of channels through the tonsillolith, which might have transported aerated medium; however, we did not note an extensive network of channels during confocal examination. The microelectrode profiles indicated that there was a stratification of physiological activity with aerobic respiration at the top of the tonsillolith, acid fermentation deeper in the tonsillolith, and an overlapping denitrifying zone in between. The acidified nitrite decomposes to nitric oxide (NO), which then has a bactericidal effect.[17] To defend against the highly reactive NO, which is also produced by human phagocytes, some bacteria possess enzymes with NO reductase activity, which reduces NO to N_2O.[18] It is therefore possible that the observed N_2O production we measured in the acidic region of the tonsillolith might not be attributable to just denitrification for energy, but may also be attributable to a NO detoxification defense strategy by competing bacteria within the tonsillolith. The microelectrode data were useful in demonstrating that the tonsillolith bacteria were capable of forming chemical gradients and that there was metabolic stratification (a characteristic of biofilms); however, the ex situ condition, removed from host immunity and creating an altered surrounding environment, will almost certainly have
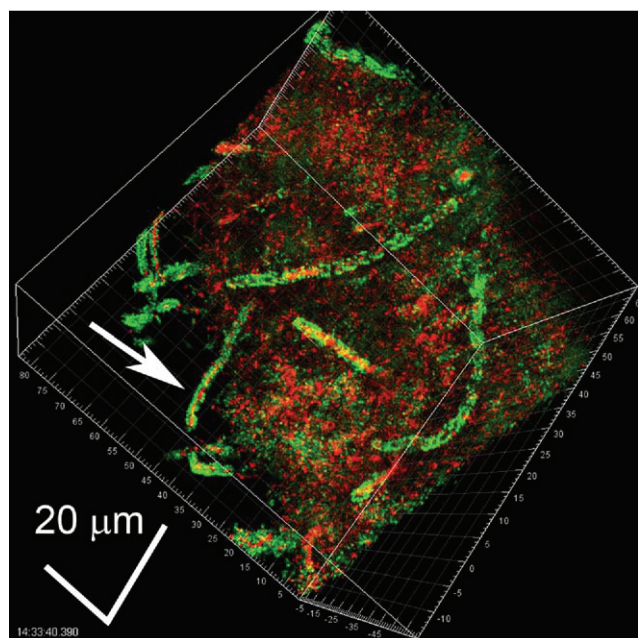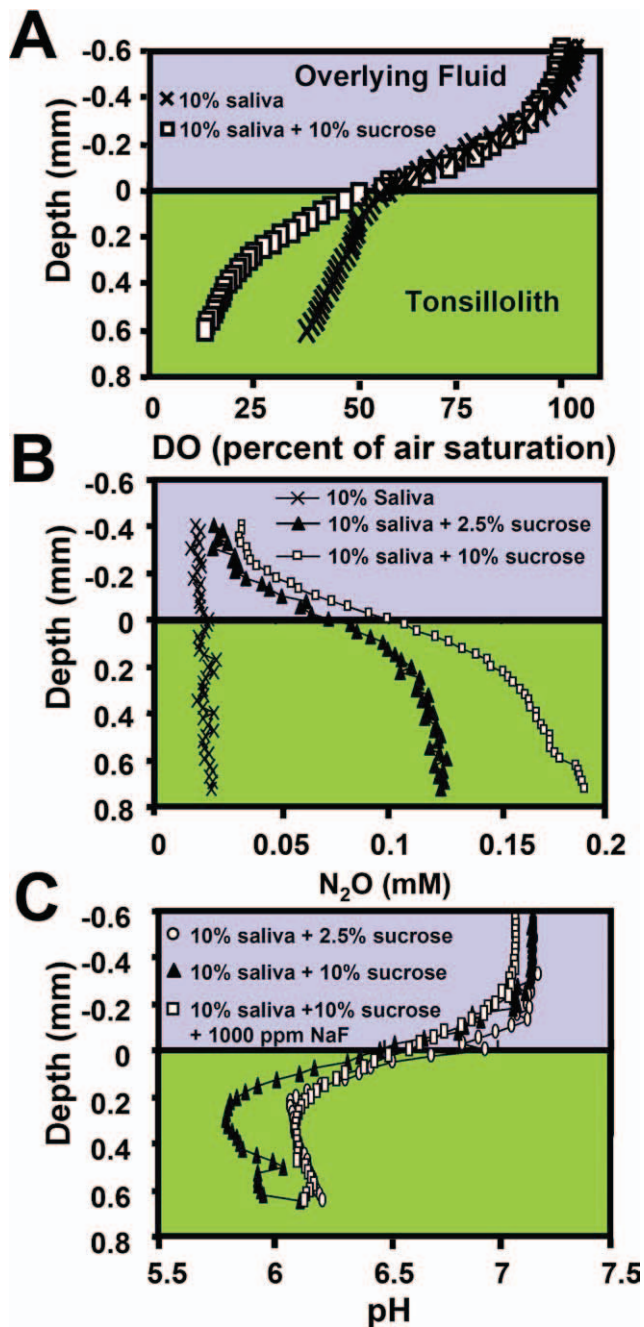
Figure 4 Metabolic activity of bacteria. (**A**) Oxygen consumption; (**B**) N$_2$O production (denitrification); (**C**) pH. Sucrose caused the pH to drop; NaF inhibited acid fermentation, raising pH.

a significant impact on activity, so absolute values of conversion rates and concentrations should not be taken as indicative of the in vivo condition. The purpose here was not to suggest we could extrapolate our data to determine exact conversion rates or concentrations in vivo, but to use microelectrodes to demonstrate that the tonsilloliths were capable of metabolic activity consistent with biofilms, thus supporting our conclusions from histological analysis.

Finally, we added fluoride to assess the influence on acid fermentation. Fluoride reversed the pH drop caused by a pulse of sucrose from 5.7 at a depth of 200 μm to the

original 6.2 in the presence of 10 percent saliva alone. This response was similar to that observed in dental biofilms; taken together our data suggest that mechanical, optical,[19] and/or chemical control strategies developed to control dental biofilms might be considered for potential treatments for tonsilloliths.

Finally, it should be noted that although microelectrodes have been used for environmental biofilms and for ex vivo dental biofilms grown on coupons,[20] to our knowledge the present study is the first to employ microelectrodes to measure chemical gradients in biofilms grown on a mucosal surface.

CONCLUSIONS

Tonsilloliths are microbial biofilms that exhibit structural heterogeneity, and the physiological activity of these biofilms results in the formation of chemical gradients and microniches. Currently, complete or intracapsular tonsillectomy is considered an option for treating chronic cryptic infections in adults; however, understanding the morphology and biofilm characteristics of tonsilloliths may stimulate clinicians to conduct randomized controlled trials of adequate sample size to assess the efficacy of using less invasive methods for treating chronic cryptic tonsillitis in the future.

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Paul Stoodley, confocal microscopy, microelectrodes, editing; **Dirk DeBeer,** microelectrodes; **Mark Longwell,** microelectrodes; **Laura Nistico,** microbiology, confocal microscopy; **Luanne Hall-Stoodley,** micro-

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