Redefining the Persistent Infection in Root Canals: Possible Role of Biofilm Communities

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Abstract

Current concepts suggest that persisting infections subsequent to endodontic therapy are caused by one or two bacterial species that are “too robust” to be eliminated by conventional treatment measures. As a consequence, numerous studies are exploring the characteristics of these “most” resistant organisms to define an effective treatment strategy to eradicate them from root canals. By taking an ecological perspective, the main objective of this review is to present evidence that the nature of persisting endodontic infections depends not on the robustness of the organisms in the infected site, but on their capability of adapting their physiology to the new environmental conditions set by the treatment. Changes in the environment, such as an increase in pH by calcium hydroxide or the effect of antimicrobials, are capable of triggering genetic cascades that modify the physiological characteristics of bacterial cells. Surface adherence by bacteria to form biofilms is a good example of bacterial adaptation and one that is pertinent to endodontic infections. Increasing information is now available on the existence of polymicrobial biofilm communities on root canal walls, coupled with new data showing that the adaptive mechanisms of bacteria in these biofilms are significantly augmented for increased survival. This ecological view on the persisting infection problem in endodontics suggests that the action of individual species in persisting endodontic infections is secondary when compared to the adaptive changes of a polymicrobial biofilm community undergoing physiological and genetic changes in response to changes in the root canal environment. (J Endod 2007; 33:652–662)

Key Words

Bacterial adaptation, Enterococcus faecalis, microbial ecology, pathogens, physiological changes

A traditional concept that explains infectious processes occurring in humans suggests that diseases are produced as the result of the aggressive invasion of harmful microorganisms, which battle with the human host’s defenses, triggering mechanisms that release antibodies and immune cells. The impact of such an approach generates a predisposition to search for those “most dangerous” microorganisms that can cause/trigger the most severe damage to the host. In line with this view, infectious processes of the oral cavity were proposed to be caused by a relatively small number of organisms from the diverse collection of species found in the human mouth (1). In caries, for example, the frequent isolation of Streptococcus mutans from carious lesions (2–4) generated a considerable number of studies to explore the ex vivo features of this bacterium. Research findings showing the significant acid-tolerant capabilities of S. mutans defined this organism as “the” agent responsible for initial enamel and dentin demineralization. Similarly, in periodontal disease, the frequent recovery of proteolytic microorganisms from deep periodontal pockets, such as Porphyromonas gingivalis, increased the attention of periodontists to these bacteria because they were considered key etiological agents of the disease (5, 6). The main disadvantage with this traditional view of the infectious process, especially in oral infections, is that the determination of true cause-and-effect relationships is not always possible. Consequently, the predominance of certain microorganisms at a given site may be the result of the disease itself rather than that of the initiating agent (7). Recently, the “ecological plaque hypothesis” (8–15) has improved on these classic infectious concepts to explain the etiology of caries and periodontal disease. This hypothesis suggests that the organisms associated with the disease may also be present at sound sites, but at levels too low to represent a clinical threat. In other words, disease is produced as the result of changes in the local environmental conditions that will shift the balance of the resident flora.

Root canal infections have a different nature than that of caries or periodontitis because they become established in originally sterile compartments of the oral cavity. In many cases, this led to the concept that the etiology of root canal infections involves only a single pathogen. For example, the predominance of certain proteolytic black-pigmented anaerobic organisms in cultures from infected root canals associated with acute symptoms suggested that these organisms are foremost etiological agents in such cases (14, 15). Recently, the frequent recovery of Enterococcus faecalis in root canals associated with persistent infections brought about an intense research interest in this bacterium. E. faecalis has become the ideal organism to test different irrigants, medicaments, and antiseptic solutions used in endodontics ex vivo, with findings that revealed its innate resistance capacity (16–18). This extensive interest in E. faecalis, perhaps driven by its ability to grow under almost any laboratory condition (19), resulted in the concept that the organism is the sole etiological agent for chronic endodontic infections. Consequently, the focus on E. faecalis resulted in much less information on the existence of other organisms in such infections that may possess similar tolerating characteristics to E. faecalis and that would shed light on the existence of a polymicrobial persisting community. Thus, it is not surprising that ecological parameters in root canal infections are not often discussed.

From an ecological perspective, the root canal can be considered a highly controlled environment with a limited number of niches. Although niches are composed by a variety of environmental factors that limit the growth of one species relative to others (20), the main limiting factors in root canal niches that influence bacterial colonization are, for instance, oxygen and nutrient availability (21). After root canal treatment, other
limiting factors become involved, such as pH and the short-/long-term effects of the antibacterial medicaments applied. The limiting factors for three different niches in root canals are depicted in Fig. 1, from which it can be reasonably assumed that bacterial survival in such controlled environments, especially after root canal treatment, is based on the capacity of organisms to adapt to the existing conditions.

Although traditional views suggest that the organisms surviving root canal treatment are a selected group of the “most robust” organisms, the application of ecological parameters indicates that bacterial survival after root canal treatment will depend not on the robustness of the organisms, but on how good an adaptor the organism is to the new limiting factors in their corresponding niches. Furthermore, as in every natural microenvironment, the adaptive capabilities of individual organisms are exponentially augmented when growing in biofilm communities. As proposed in this review, data now exist (Fig. 2) that provide an argument for the inclusion of the biofilm concept in the etiology of persisting endodontic infections. The foundation for this ecological approach to endodontic infections suggests that the most dangerous “pathogen” is not an individual species, but a polymicrobial entity that undergoes physiological and genetic changes triggered by changes in the root canal environment.

Pathogens and Virulence

The accepted definitions of microbial pathogen and microbial virulence were formulated largely from the study of infections caused by a single etiological agent, although both represent combinations of highly complex parameters. As a result, classifications of microbial pathogens place the primary responsibility for causing a determined disease at all times on the microorganism [for a review see Casadevall and Pirofski (22)]. Likewise, the virulence of a pathogen is generally defined as the degree of pathogenicity or ability of the organism to cause disease measured by an experimental procedure (23).

The theory of the single pathogen as the etiological agent of a specific disease is derived from Robert Koch’s Postulates, which are based on the work accomplished by Koch and his coworkers on diseases, such as, tuberculosis, diphtheria, anthrax, and cholera, that were determined to be caused by specific microbial entities at a time when the most prominent medical and scientific communities denied their importance [see Kaufmann and Schaible (24)]. By current standards, however, there are very few microorganisms to which the term pathogen can be applied invariably (25). A common example is group A streptococci, which are etiological agents of acute rheumatic fever, rheumatic heart disease, post-streptococcal glomerulonephritis, and invasive infections causing at least 517,000 deaths each year (26). Not included in such a traditional “pathogenic” categorization are the various microorganisms that we encounter on a daily basis coexisting peacefully with humans and from which a small number of them may be capable of a pathogenic phase. For example, Escherichia coli is ubiquitous, asymptptomatically colonizing the human intestines and is widely distributed in the environment, yet, after experiencing specific genetic variations, this organism can cause epidemic dysentery and neonatal meningitis, among other diseases (27). Another example is Listeria monocytogenes, which is well adapted as a saprophyte for peaceful survival in soil and decaying vegetation, but it has also another phase where it acts as an intracellular invader capable of causing serious infections in humans (28–30). Current research suggests that the ability of these opportunistic organisms to switch from the harmless to the pathogenic state appears to occur in response to environmental changes that are mediated through complex regulatory pathways, which reversibly modulate the expression of virulence factors. The advent of bacterial genomic studies has significantly increased our understanding of the pathogenic state of many microbes with the on/off virulence switch, in fact, constituting a valuable marker of individual microbial pathogens (31–33).

Oral Pathogens

Historically, much of the earlier research into dental caries and the various periodontal diseases was focused on correlating a single specific organism with the disease to satisfy Koch’s Postulates. We now know that not all diseases are the result of the action of a single organism and this is particularly true of the oral cavity where all of the microbial diseases associated with tissue destruction involve more than one type of organism and are, therefore, “mixed” infections (34). This polymicrobial nature of oral disease has its basis in the characterization of the organisms present in dental plaque and their potential roles in dental caries, gingivitis, and periodontitis (35–37). Early data (38) recognized the association of mutants streptococci, including Streptococcus mutans and S. sobrinus, with the initial phase of human dental caries because their acidogenic and aciduric properties permitted them to create a low-pH environment in dental plaque after the ingestion of sugars. In addition, lactobacilli and certain acid tolerant non-mutans streptococci can now be considered virulent with respect to dental caries (39, 40). In periodontal disease, the use of animal models suggested that Actinomyces naeslundii was involved in the destructive alveolar bone loss characteristic of advanced periodontitis (41), whereas evidence was also presented in the 1970s that black-pigmented organisms, such as Porphyromonas gingivalis, were directly involved in periodontitis (36).

Marsh (7) proposed the “ecological plaque hypothesis” to explain changes in the ecology of dental plaque that lead to the development of caries or periodontal disease. This hypothesis constitutes a dynamic model in which plaque-mediated diseases are the consequence of imbalances in the resident microflora resulting from an enrichment within the microbial community of the above mentioned “oral pathogens” (9–11, 13). In caries, for example, potentially cariogenic bacteria may be found naturally in dental plaque, but at neutral pH and with a conventional low-sugar diet, the levels of such potentially cariogenic bacteria are clinically insignificant. If the intake of fermentable carbohydrates increases, the low pH provoked in plaque favors the proliferation of acidogenic and aciduric bacteria, such as mutants streptococci and lactobacilli, which promote enamel demineralization.
Figure 2. Preparation of a tooth apex associated with chronic infection as observed with scanning electron microscope. Overview in (a) at ×40 magnification shows the main root canal obturated with gutta-percha (arrow). Magnifications of ×150 in (b) and ×300 in (c) show a gap between the gutta-percha and the root canal wall, a section is marked in (c) for higher magnification. Images (d), (e), and (f) show the marked section at magnifications of ×2,500, ×7,000, and ×10,000, respectively. Accumulations of bacterial cells adhered in the gap between the gutta-percha and the root canal wall are observed. The specimen was provided by Dr. Olle Heningsson, Department of Endodontics, Malmö University, and the images were obtained with a Hitachi electron microscope.
that this study was focused primarily in proving the occurrence of failure–associated root canals (45). As in other related works (46 – 49), PCR methodology seems to be exclusively directed to find only E. faecalis, ignoring the rest of the flora present that may be as important as E. faecalis in provoking the treatment failures.

On the other hand, recent investigations have confirmed the polymicrobial nature of root canal infections (50, 51). In a study with monkeys (50), different combinations of bacteria were experimentally inoculated in root canals and periapical lesions were induced. The teeth were treated endodontically and followed-up radiographically and histologically for 2 to 2.5 years. In the root canals with bacteria present when the root filling was removed, 30 of the 31 canals had persisting periapical lesions. Importantly, more of these nonhealed lesions were associated with various combinations of bacterial strains, that is, mixed infections, than single strains. Previously, the same research group (52) also found that when an “eight-strain collection” of species, derived from one infected root canal, was re-inoculated in equal proportions into other monkey teeth, species such as Bacteroides oralis (now Prevotella oralis) dominated in mixed infections and showed a more potent capacity for tissue destruction. Furthermore, B. oralis could not be re-isolated from inoculated root canals after the experimental period when inoculated as a pure culture. In another study using the tissue cage model implanted subcutaneously in the backs of rabbits, the same collection of eight bacterial strains from monkey root canals were inoculated in different combinations and individual species. The combination of B. oralis, Fusobacterium necrophorum, Peptostreptococcus anaerobius, and Streptococcus milleri was the most predominant and induced higher titers of circulating antibodies than that obtained with individual inoculations, such as E. faecalis (53).

Even if we accept the polymicrobial nature of root canal infections, one of the major problems in understanding endodontic infections is that we still extrapolate between individual organisms growing in liquid (planktonic) cultures and the in vivo situation. A significant literature now exists demonstrating that the physiology of a bacterium in planktonic culture is profoundly different from that of the same organism growing on a surface in a biofilm (see review by Costerton et al. (54)). For instance, planktonic bacteria are more sensitive to antimicrobial agents because of their ease of diffusion within the bulk fluid, whereas biofilm bacteria are notably resistant to these agents (55–59). In this context, the study of biofilms in root canal infections has included biofilms formed by mixed cultures of anaerobic bacteria in extracted teeth (60, 61) or by pure cultures of E. faecalis (62, 63). Biofilms of five root canal isolates have also been used to test the antimicrobial efficacy of endodontic irrigants, such as sodium hypochlorite (NaOCl) (2.25%), 0.2% chlorhexidine, 10% povidone iodine, and 5 ppm colloidial silver, with NaOCl shown to be the most effective agent of this group (64). In addition, our research group tested the alkaline tolerance of species isolated from chronically infected root canals and found that E. faecalis and other Gram-positive organisms, such as Lactobacillus paracasei, Olsenella uli, or Streptococcus gordontii, shared similarly high alkaline-tolerant capabilities when growing in planktonic conditions. S. anginosus, S. oris, and F. nucleatum, on the other hand, were greatly affected by the alkaline stress (see Fig. 3) (65). Of importance, however, was the observation that this difference in alkaline tolerance was not apparent when the strains were tested in biofilms because all seven strains showed a similar high tolerance to alkaline pH (Fig. 3). These findings not only show the capacity of root canal bacteria other than E. faecalis to adapt to alkaline stress, but also provide further evidence that bacteria in surface-adhered biofilm consortia are more resistant to environmental stress than when grown in liquid culture.

**Endodontic Pathogens**

Currently, there is no substantial evidence indicating that certain microorganisms of the microbial flora in root canal infections are more virulent than others. With this in mind, Sundqvist and Figdor (42) stated that a proper definition for endodontic pathogens should include every organism capable of inducing the tissue destruction in apical periodontitis. In reality, however, the majority of endodontic-microbiology studies refer to the endodontic pathogen as the bacterium isolated from a symptomatic-associate root canal that grows in the laboratory in a specific media. By this approach, the most frequently recovered species will assume the role of major endodontic pathogen. In persistent root canal infections, for example, the frequent occurrence of monocolonies of E. faecalis has raised suspicion that this bacterium may be the sole organism persisting in the root canals. Considering that mono-infections rarely if ever occur in nature, it is possible that the apparent pure cultures of E. faecalis could be the result of sampling and culturing techniques that favor it over other organisms at the site that were either in low numbers or were physiologically inactive or dormant (see later). For instance, in a commonly cited study (43), from the total 100 root-filled teeth with apical periodontitis sampled E. faecalis was reported as the most frequently recovered organism (32%), although in 32% of the cases with persistent lesion no microbe could be isolated. In yet nine root-filled teeth without periapical lesion that showed bacterial growth, the organism was found in one case. In a similar study, 25 root-filled teeth requiring retreatment were sampled and E. faecalis was found in 14 of those 20 teeth with bacterial growth (44). However, it would seem that this study was focused primarily in proving the occurrence of E. faecalis in root-filled teeth rather than in exploring the microbial flora in persisting infections. Similarly, in a recent study using a sophisticated nested PCR technique, the target bacterium E. faecalis was found in 41 of 50 (82%) untreated root canals and 38 of 50 (76%) treatment-failure–associated root canals (45). As in other related works (46 – 49), PCR methodology seems to be exclusively directed to find only E. faecalis, ignoring the rest of the flora present that may be as important as E. faecalis in provoking the treatment failures.

**Figure 3.** Fluorescence micrographs using Live/Dead fluorescence staining for bacterial viability. Cells stained fluorescent green represent viable cells, whereas cells stained fluorescent red are nonviable or damaged. In the first column, images show planktonic cells of three root canal strains at neutral media (pH 7). The middle column shows planktonic cells after exposure to pH 10.5 for 4 hours, and the right column shows biofilm cells exposed to alkaline challenge (pH 10.5) for 4 hours. Bars, 2 μm. Images are published with permission of Blackwell Publishing. International Endodontic Journal, Chávez de Paz et al. (65).

**Physiological Status of Bacteria**

The previous discussion relative to the adaptation and survival of oral bacteria under environmental stress indicates the importance
of the physiological state of bacteria with respect to the potential level of activity in disease processes. However, the exact description of the status of a microorganism can be complex given the numerous terms used to describe different physiological states, such as, dead, moribund, starved, dormant, resting, quiescent, viable-but-not-culturable, injured, sublethally damaged, inhibited, resuscitable, living, active, and vital (66). Many of these terms are used conceptually and do not reflect the actual knowledge of the state of the organism in question (67). That this gap in information exists is apparent from recent bacterial genomic sequencing data that indicate how little we still know about bacterial physiology. This can be seen in studies with Haemophilus influenzae, which has 756 genes of unknown function in its genome out of a total of 1,743 genes (68).

Viability of bacteria is conventionally defined as the capacity of cells to perform all cell functions necessary for survival under given conditions (66). The simplest method we have used to assess bacterial viability is the plate count method, where the number of viable cells approximates the number of colony-forming units. Bacteria, therefore, have been classified into two physiological groups: those that can and those that cannot readily be grown to detectable levels in vitro (67). In root canal infections, we have cultivated microbial strains from root canal samples in the laboratory, using growth media that contained a specific substrate, and then identified the cultivated bacteria at the physiological, biochemical, and, more recently, at the molecular level. The metabolic properties of these bacterial isolates were then used to infer the potential roles of these and related microorganisms in the environment. Under some circumstances, however, such methods may underestimate the number of viable bacteria for a variety of reasons, such as cases where slightly damaged organisms are present (69), the laboratory growth media used are deficient for one or more essential nutrients required for the growth of some bacteria in the sample (70), or viable cells are present that have lost their ability to form colonies (71). Furthermore, if the bacteria exist in a biofilm they may assume a status of low-metabolic activity similar to stationary-phase planktonic growth for the majority of time (72, 73). The bacteria in such low active states may be undetectable by regular culture techniques.

Currently, a variety of methods have been developed to assess the physiological status of bacterial cells, including metabolic activity, the integrity of the cell, or the presence of nucleic acids (Fig. 4). Perhaps the most useful are fluorescent probes that target different cellular functions, such as membrane potential, enzyme activity, nucleic acids, and membrane integrity [see review by Joux and Lebaron (74)]. Based on the detection of the amount of RNA in bacterial cells, fluorescence in situ hybridization (FISH) has been applied to identify and, to an extent, determine the physiological state of different species in their natural environments (75). One weakness of FISH is the generation of false negative results with bacteria possessing low physiological activity because they exhibit low amounts of RNA, resulting in low signal intensity (76). In endodontics, FISH has been used to visualize and identify bacteria from periapical lesions of asymptomatic root-filled teeth (77). Alternative fluorescent probes to test bacterial viability are the Live/Dead BacLight kit and the tetrazolium salts 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT) and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). The Live/Dead kit tests the integrity of the cell membrane by applying two nucleic acid stains, SYTO-9 and propidium iodide (PI), which can simultaneously detect dead/injured (fluorescent red by stain with PI) and intact cells (fluorescent green by staining with SYTO-9) (78, 79). This fluorescent probe has been used to assess the viability of root canal strains ex vivo (65) and to determine the autoaggregation and coaggregation of bacteria isolated from teeth with acute endodontic infections (80). Furthermore, the outcome of such a procedure can be seen in Fig. 5 with a histological preparation of a root apex specimen obtained after surgical removal of a tooth associated with persistent radiographic signs and symptoms. The filamentous organisms observed in the histological images (Fig. 5a and b), are also observed under the fluorescent microscope, where their viability can be assessed (cells stained fluorescent green in Fig. 5c and d). The tetrazolium salts INT and CTC are often used as markers of bacterial respiratory activity, as well as viability [for a review see Creach et al. (81)]. With these relatively simple methods, a good correlation between the number of INT/CTC-positive cells and the CFU count can be obtained.

In conclusion, the physiological state of bacteria in root canals, whether they are viable, dormant, injured, or in another state of their life cycle, is a crucial marker that measures their involvement in periapical inflammation rather than only the identification of species from DNA fragments.

Mechanisms of Adaptation

There are a number of different mechanisms used by bacteria that permit them to adapt to the environment. Biofilm formation (55), physiological modification (82), stress response (83), and the creation of subpopulations of cells (84) are among some of the adaptive mechanisms used by bacteria along with various mechanisms involving the exchange of genetic material between bacteria (85). The exploration of these mechanisms can aid us in understanding the bacterial survival under the limiting environments, such as that found in the root canal. One of the most relevant features of adaptation for oral bacteria is the adhesion to surfaces that leads to the formation of plaque biofilms, which serves not only to aid in their retention in the oral cavity, but also results in increased survival (86). Interestingly, this ability to form complex biofilm communities is not lost when oral organisms colonize other sites in the human body. For example, species from viridians oral streptococci, such as Streptococcus oralis and S. gordonii, have been found to form plaque biofilms on the endocardium and valve leaflets and are thus considered major etiological agents in endocarditis (87, 88). Similarly, oral microorganisms are able to colonize root canals by adhering to the dentine walls as shown in microphotographs of a tooth apex associated with a chronic infection taken with a scanning electron microscope (Fig. 6). Aggregations of microorganisms can be seen adhering to the inner walls of an accessory canal under high magnification, thus demonstrating the retention of these biofilm communities even after root canal treatment.

Biofilms

Given that surface-associated microbial communities are the main form of colonization and retention by oral bacteria in the mouth, it is not unreasonable to assume that biofilms also form in root canals having the
same properties as the parent communities colonizing the enamel and cementum surfaces (see Figs. 2 and 6). Biofilms form when planktonic bacteria in a natural liquid phase are deposited on a surface containing an organic conditioning polymeric matrix or “conditioning film” (see Fig. 7). In this dynamic process many other organisms co-adhere to the surface and grow with some cells detaching from the biofilm over time (89–92). Biofilm formation in root canals, as hypothesized by Svensäter and Bergenholtz (93), is probably initiated at some time after the first invasion of the pulp chamber by planktonic oral organisms after some tissue breakdown. At this point, the inflammatory lesion frontage that moves successively toward the apex will provide the fluid vehicle for the invading planktonic organisms so these can multiply and continue attaching to the root canal walls. Interestingly, bacteria have been observed to detach from inner root canal surfaces and occasionally mass in the inflammatory lesion per se (94). This observation could explain how the inflammatory lesion front serves as a fluid source for bacterial biofilm detachment and colonization of other inaccessible sites in the root canal. Thus, when biofilms are formed on surfaces located beyond the reach of mechanical removal and the effects of antimicrobials, host-derived proteins from remaining necrotic tissues and bacterially produced adhesive substances will provide the proper prerequisites for the survival of microbes.

Biofilms in root canals have been confirmed by examinations of extracted teeth with periapical lesions. For example, when sections were viewed by transmission electron microscopy, dense aggregates of cocci and rods embedded in an extracellular matrix were observed along the walls (95), whereas studies using scanning electron microscopy showed microcolonies of cocci, rods, and filaments on root canal walls (93, 96, 97). Such root canal biofilms can be seen in Figs. 2 and 6.

Introducing this biofilm concept to endodontic microbiology is a major step forward in our understanding of root canal infections, especially those of the persistent kind, because microorganisms growing in biofilms are better protected from adverse environmental changes and other antimicrobial agents (56, 98). Apart from the physical protection provided by the extracellular matrix [see review by Branda et al. (99)], additional protection is afforded by physiological changes initiated by the bacteria after their adhesion to the surface (55, 100, 101). These phenotypic changes by the biofilm bacteria usually result in increased resistance to antimicrobial agents, in some cases up to 1,000-fold greater than that of the same microorganisms living planktonically (56, 57). Evidence already exists showing that biofilms of oral bacteria are more resistant to chlorhexidine, amine fluoride, amoxycillin, doxycycline, and metronidazole than planktonic cells (58, 59). As mentioned previously, our group found that the viability of susceptible root canal strains to alkaline stress in planktonic cultures was considerably increased when these strains were exposed to the same alkaline stress in biofilms (65). The increased resistance of the strains in biofilms compared to planktonic cultures of the same organism thus raises questions...
Figure 6. Preparation of a tooth apex associated with chronic infection as observed with scanning electron microscope (also depicted in Figure 2). Overview in (a) at ×80 magnification shows an accessory root canal (arrow). Magnification of ×400 in (b) shows a wall of the accessory canal with evidence of a microbial biofilm, a section is marked for higher magnification. Images (c) and (d) show the marked section at a magnification of ×3,000. Accumulations of bacterial cells adhered to the root canal wall are observed. The specimen was provided by Dr. Olle Henningsson, Department of Endodontics, Malmö University, and the images were obtained with a Hitachi electron microscope.

Figure 7. Stages of biofilm formation [modified from Svensäter and Bergenholtz (93)].
as to the validation of studies using exclusively liquid-grown cultures in laboratory tests.

During the various stages of biofilm development, as well as throughout the various sections of the biofilm, cells are in different physiological states. Cells at the base of the film, for example, may be dead or lysing, whereas those near the surface may be actively growing; however, even with such physiological diversity, it can be argued that the majority of time cells in biofilms are in a status equivalent to cells in the stationary phase of growth (72, 73, 102). From the perspective of the persisting root canal flora, however, one might imagine that such “stationary-phase” cells might maintain a low but sufficient metabolic activity to provoke periapical inflammation. Thus, from the metabolic perspective, they will not be “dead” but would, theoretically, be able to contribute to the persistence of inflammation.

Morphological Changes

Adaptation of cells to environmental shift in some cases may trigger phenotypic changes in cell morphology. Figure 8 illustrates the morphologic changes undertaken by a root canal strain of Lactobacillus paracasei grown in biofilms exposed to a commercial rinsing solution of sodium fluoride at a concentration of 0.2%. Column A shows phase contrast and Live/Dead images of the 1-day-old biofilms cells of L. paracasei growing in optimal conditions in peptone yeast glucose media (PYG) at 37°C. In Column B, images of biofilms of L. paracasei, exposed to a 0.2% sodium fluoride solution for 1 day and then reactivated in PYG at 37°C for 1 day, show changes in the cellular morphology of L. paracasei. Similar changes were observed with the root canal strain of Streptococcus anginosus. The explanation for this phenomenon is scant; however, such changes are consistent with stress responses triggering changes while the cells are dividing. For example, colonies of an antibiotic-resistant strain of Pseudomonas aeruginosa were found to have different morphologies when exposed to kanamycin with the agent resulting in the formation of smaller and rougher colonies than wild-type cells. However, when this P. aeruginosa strain was grown on antibiotic-free media for 5 days, colonies appeared smooth and large just as their wild-type. The authors suggested that these phenotypic variations observed in the resistant variants were transient (82). With the advent of tools such as confocal laser scanning microscopy, numerous structural proteins have been shown to be involved in bacterial morphological alterations. Some of these proteins have a good molecular similarity to tubulin and are thus capable of forming scaffolding-like filamentous assemblies in bacteria (103, 104). For instance, the structural proteins, crescentin and MreB, were recently implicated as scaffolds responsible for maintaining shape within Caulobacter crescentus (105, 106). In any case, it is clear that environmental stress results in phenotypic changes to bacterial cells and such modifications constitute important markers of bacterial adaptation.

Proteomics

A variety of environmental changes can trigger bacterial adaptation resulting in the coordinated induction of stress proteins or “heat-shock” proteins. Such proteins have been well characterized for diverse oral species, such as S. mutans and S. oralis among others, as generated when exposed to both heat and acid stress (83, 107–109). Intra cellular expression of different proteins was also reported for E. faecalis upon alkaline stress (110, 111). These specific bacterial adaptive pathways also include the expression of cytosolic proteins that are released outside the cell as shown by the release of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from S. gordonii after a change in pH from 6.5 to 7.5 (112). Furthermore, in a recent study, we observed that selected cytosolic proteins were as well induced and released to the external media in cultures of root canal isolates exposed to sublethal alkaline stress (65). The proteins Dnak, Hpr, and fructose-1,6-bisphosphate aldolase (FBA) were released extracellularly at high levels by root canal strains of S. anginosus, S. oralis, and S. gordonii. Interestingly, these copious protein excretions were more intense with planktonic cultures than with biofilms, and thus such sublethal effects of a stress may be altered when an organism assumes growth in a biofilm.

Formation of Subpopulations

Bacterial adaptation also includes the creation of subpopulations of cells at the population level. This generation of diversity within a population is achieved either by genetic pathways involving constitutive or transient mutators and contingency loci, or by modifications of cellular phenotypes (113, 114). Groups of cells have been found to persist
after exposure to lethal doses of antibiotics and new growing populations appear in the culture (115, 116). These persister cells (1) may represent cells in some protected part of their cell cycle, (2) are capable of rapid adaptation, (3) are in a dormant state, or (4) are unable to initiate programmed cell death in response to the stimulus (84). Thus, such persisters cells represent a recalcitrant subpopulation that will not die and are capable of initiating a new population with normal susceptibility once the antibacterial effect has been dissipated (117–119). To date, these cells have been reported to occur only after the exposure of a bacterial population to high doses of a single antimicrobial agent, which triggered the appearance of persister cells exhibiting multiple drug resistance (120, 121). The frequency of persister occurrence and the mechanism(s) involved in their appearance is unclear, although one hypothesis with *E. coli* suggests that persister cells are regulated by the expression of chromosomal toxin–antitoxin genes (122). In this case, the operon HipA seems to be responsible for tolerance to ciprofloxacin and mitomycin C in stationary-phase planktonic cells and *E. coli* biofilms (122). It was also previously proposed that the expression of toxins drives bacteria reversibly into the slow growing, multiple drug-resistant phenotypes by "shutting down" antibiotic targets (84). In the context of root canal bacteria, the formation of such persisting populations that are capable of surviving imposed endodontic treatment measures, as the increase of alkaline levels resulting from application of calcium hydroxide (65), would explain how organisms are able to survive and remain in the environment until the effects of noxious stimuli have dissipated.

**Concluding Remarks and Directions for Future Research**

The survival of bacteria in root canals after treatment is based on the capability of the individual organisms to adapt to the environment within the consortium. Therefore, the study of the adaptive mechanisms used by organisms to survive in such a highly controlled environment, with limiting nutrients plus the effects of the antibacterial medicaments, is important to our understanding of persisting root canal infections. The ability of the organisms in such infections to form biofilms can be seen as the most important adaptive mechanism used by bacteria to survive the environmental changes resulting from the treatment protocol. Thus, from the realization that all oral microorganisms are capable of forming a biofilm and that such surface-associated communities exist in root canals, it is possible to apply the "biofilm concept" to clinical treatment; that is, efforts should not be directed to specific individual organisms, but to a group of well-adapted organisms undoubtedly possessing increased resistance to a variety of antimicrobial agents.

We should obtain much better understanding of the characteristics and properties of bacterial biofilms in root canals and the degree to which such microbial communities enhance survival from environmental changes. A glance at how a biofilm consortium of root canal strains cope with alkaline stress is shown in Chávez de Paz (123). Prospectively, the application of molecular biology will be invaluable to identifying, for instance, biofilm-expressed genes by root canal colonizers, and permit the identification of genes involved in adaptation signaling. From this knowledge we should come to identify key adaptive events occurring in the root canal microflora while organisms are exposed to root canal treatment measures. This path of research should aid in finding mechanisms that can block such processes.

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