Bettina Basrani
Editor

Endodontic Irrigation

Chemical Disinfection of the Root Canal System

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This book is dedicated:

To my father, Enrique, for leaving his fingerprints of endodontic passion in my life
To my mother, Clarita, and mother-in-law, Enid, for being my dearest and most unconditional fans
To my husband, Howard, for helping me, every day, in becoming a better person
To my children, Jonathan and Daniel, for teaching me what life is really about
To my coworkers, Shimon, Cal, Anil, Andres, Gevik, and Pavel, for being my second family
Finally, to my students for making me a better teacher

Foreword

Apical periodontitis is an infectious disease related to the presence of microorganisms in the root canal system of teeth. Its treatment therefore must be directed at eliminating or, at the very least, reducing the infecting microbiota, to levels that allow healing to occur. Advances in microbiology have identified the nature and complexity of the infecting microbiota and the ability of some of its members to collectively survive under the harshest of conditions. The treatment of apical periodontitis has historically been based upon two pillars, the mechanical removal of necrotic tissue and microorganisms from the root canal system and the irrigation of the root canal system with chemical agents, to supplement removal of tissue and microorganisms from areas of the system that were mechanically prepared, as well as address the presence of tissue and microorganisms at sites in the system that mechanical preparation could not reach. Research has shown that despite the nature and design of the instruments used in the mechanical preparation of the system, significant reduction in the concentrations of tissue and microorganisms in complex root canal systems can only be achieved when irrigation of the system is an integral part of the treatment undertaken. Over the years, different irrigants have been used in endodontic treatment, but only one, sodium hypochlorite, has proven itself to be consistently effective. Its effectiveness is a product of its concentration and the manner in which it is introduced into the root canal system. Because of the toxic nature of sodium hypochlorite, both of these factors pose a potential risk to the patient if tissues surrounding the tooth are inadvertently exposed to the agent during use.

In this textbook, Dr. Basrani, a noted authority in root canal irrigation, has recruited a panel of prominent authors to discuss the merits, limitations, and safety of the various sodium hypochlorite delivery systems currently being used in endodontic treatment. Some attention is also paid to the influence that mechanical root canal preparation has in impeding or promoting their therapeutic effect. With an eye to the future, Dr. Basrani has also included chapters concerned with evolving technologies in the field of supplemental root canal disinfection, technologies that have shown promise in avoiding the potential risks associated with sodium hypochlorite use, while achieving and, in some instances, exceeding sodium hypochlorite's effectiveness in tissue and microbial reduction.

In view of the importance of irrigation of the root canal system in its broadest form, to the outcome of endodontic treatment, this textbook is a must-read for all clinicians who include endodontics as an integral part of their dental practice.

Toronto, ON, Canada Calvin D. Torneck, DDS, MS, FRCD(C)

Preface

When I was invited by Springer International Publishing to edit a book in irrigation, I felt like a dream came true. I have been working on endodontic irrigation for close to 20 years. While doing my PhD at Maimonides University in Buenos Aires, Argentina, I was invited work with a periodontist, Dr. Piovano, and microbiologist, Dr. Marcantoni, who became my initial mentors. After a couple of meetings together, we recognized how much periodontics and endodontics have in common: (a) similar etiological factor of the diseases (bacterial-/biofilm-related causes), (b) similar treatments (both disciplines mechanically clean the tooth surface either with curettes or endodontic files), and (c) both chemically disinfect the surface (medicaments and irrigants). However, the big difference is that, as endodontists, we seal the canal as tridimensionally as possible, while in periodontal treatment this step is difficult to achieve.

When we recognized the similarity in the procedure, we started to analyze the medicaments that periodontal therapy applied, and chlorhexidine (CHX) was the "new" topical drug at that time. We wondered: if CHX is used for periodontics, why not for endodontics? This is how my irrigation pathway began in 1995, and that path opened to new amazing and unexpected routes. I was able to complete my PhD and published in vitro papers on the use of CHX as an intracanal medicament and other papers on the mixture of CHX with calcium hydroxide with my new supervisors Dr. Tjadehane and Dr. Canete. Finally, this motivation and interest in irrigation research brought me to Canada to continue this line of investigation with the research group at the University of Toronto, under the wise guidance of Dr. Shimon Friedman and Dr. Calvin Torneck and the inquisitive minds of the residents who went through our program. Today, the disinfection research is reaching for new horizons with the leading research of Dr. Anil Kishen and his lab. I am so proud of being part of such a prestigious group of researchers and remarkable group of human beings.

Chemical disinfection of the root canal system is now the bread and butter of modern endodontic therapy. Even though we have new and sophisticated file systems in the market, the key to endodontic success is based on chemical disinfection. This book is intended to convey the most recent challenges and advances in cleaning the root canal. We start by analyzing the main etiological factors of apical periodontitis in Chapter 1, and Dr. Luis Chaves de Paz explains the importance of the biofilms in causing endodontic diseases. In Chapter 2 Dr. Marco A. Versiani, Jesus D. Pécora, and Manoel D. Sousa-Neto,

with distinctive studies on microCT, explain dental anatomy in great detail. In Chapter 3 on irrigation dynamics was written by Dr. Christos Boutsioukis and Lucas W.M. van der Sluis explained in detail why the irrigants do not reach the apical part of the canal and what we can do to improve irrigation dynamics. For the more academic-oriented readers, we have Chapter 4 Drs. Shen Y, Gao Y, Lin J, Ma J, Wang Z, and Haapasalo M described different methods on studying irrigation. In Chapter 5, Dr. Gevik Malkhassian and I put together the most common irrigant solutions used in endodontics along with the pros and cons of their use. Chapter 6 Dr Gary Glassman describes accidents and mishaps during irrigation. We then have Dr Jorge Vera in Chapter 7 describing how patency file may (or may not) affect irrigation efficacy Chapters 8 to 14 are dedicated to each irrigation technique written by experts in each of these fields: Dr. Pierre Matchou for manual dynamic technique, Drs. Gary Glassman and Karine Charara for apical negative pressure, Dr. John Nusstein for sonic and ultrasonics, Drs. Zvi Metzger and Anda Kfir for SAF, Drs. Amir Azarpahazoo and Zahed Mohammadi for ozone, Dr. David Jaramillo for PIPS, and Dr. Anil Kishen and Anie Shersta for photo activation disinfection. Two chapters are dedicated to inter-appointment therapy, with Dr. Zahed Mohammadi and Dr. Paul Abbott (Chap. 15) describing the use of antibiotics in endodontics and Professor José F. Siqueira Jr and Isabela N. Rôças describing the details on intracanal medications (Chap. 16).

Two chapters are dedicated to modern and current points of interest, Chap. 17 on irrigation in the era of re-treatment written by Dr. Rodrigo Sanches Cunha and Dr. Carlos Eduardo da Silveira Bueno and Chap.18 on irrigation in the era of revascularization by Dr. Anibal R. Diogenes and Nikita B. Ruparel.

The vision of this book would never have been possible without the dedication and hard work of this astounding team of scientists with such different backgrounds but with the same enthusiasm for endodontic disinfection. The collaborators of this textbook are bringing their expertise and knowledge from Brazil, Iran, Peru, Mexico, Canada, Australia, USA, Israel, France, Greece, and Holland. To all of them, to my coauthors, thank you!

Toronto, ON, Canada Bettina Basrani

Acknowledgments

I would like to start by thanking Springer International Publishing for giving me the wonderful opportunity of editing a textbook on chemical disinfection of the root canal system. I appreciate the trust, patience, and knowledge they demonstrated throughout the whole process. I also want to thank Dean Haas, Faculty of Dentistry, University of Toronto, for granting me the 6-month sabbatical to focus on this project, and I have a deep appreciation to the whole endodontic department of the faculty of dentistry for their motivation and constant support. Special thanks to Warrena Wilkinson for editing some of the chapters and Dr. Calvin Torneck for the thoughtful writing of the preface.

Gratitude goes to the collaborators of this book. It was a great pleasure to invite you to participate in this project, and your motivated and enthusiastic responses were always encouraging. Thanks for your expertise and dedication.

Finally, I want to recognize my family. I have to start by thanking my father, Professor Emeritus Dr. Enrique Basrani, for showing me what a life of an endodontist looks like. He lived in Buenos Aires, Argentina, and divided his time between academics and clinical practice, while he wrote six textbooks in endodontics, finishing his last one on his death bed. He never stopped working. I should say: he never stopped doing what he loved. Now, as I follow in his steps, dividing my own time between academics and clinical practice, and feel him guiding me in spirit in all that I do. Secondly, I want to thank my mother, Clarita, and mother-in-law, Enid Alter for listening and understanding when sometimes I think that life is overpowering. My brother Dr. Damian Basrani and his family always have a special place in my heart. Howard, my beloved and precious husband, thanks for being there for me, always. Without your presence in my life, I would not be able to be the person that I am today. And to my beautiful children, Jonathan and Daniel, for being as enthusiastic as I am in everything they do.

I want to conclude by thanking all my students, from the undergraduate to graduate program and participants in lectures and workshops. You are the ones who make us better teachers, the ones who challenge us, who inspire us to give our best, and the ones who I also dedicate this book to.

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Microbial Biofilms in Endodontics

1

Luis E. Chávez de Paz

Abstract

Microorganisms colonizing different sites in humans have been found to grow predominantly in complex structures known as biofilms. Biofilms are dynamic systems with attributes of both primordial multicellular organisms and represent a protected mode of growth that allows cells to survive. The initial stage of biofilm formation includes the attachment of bacteria to the substratum. Bacterial growth and division then leads to the colonization of the surrounding area and the maturation of the biofilm. The environment in a biofilm is not homogeneous; the bacteria in multispecies biofilms are not randomly distributed, but rather are organized to best meet their requirements. The implications of this mode of microbial growth in the context of endodontic infections are discussed in this chapter. Although there is an initial understanding on the mechanisms of biofilm formation in root canals and its associated resistance to clinical antimicrobial regimens, this topic is still under investigation. A greater understanding of biofilm processes should lead to novel, effective control strategies for endodontic biofilm control and a resulting improvement in patient management.

Introduction

In nature, bacteria are able to live either as independent free-floating cells (planktonic state) or as members of organized surface-attached microbial communities called biofilms. Biofilms are composed of microorganisms that are embedded in a self-produced extracellular matrix which bind cells together [17, 18, 30]. Biofilms have major clinical relevance as they provide bacteria with protective environments against stresses, immune responses, antibacterial agents, and antibiotics [31, 33]. After several decades of intense research, it is now well established that biofilm formation is a developmental process that begins when a cell attaches to a surface and it is strictly regulated in response to environmental conditions [33].

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One of the most relevant features of oral bacteria is their intrinsic ability to continuously form complex biofilm communities, also known as dental plaque. Oral biofilm formation serves not only to aid in retention of bacteria in the oral cavity, but also results in their increased survival [34, 35]. In root canals of teeth, biofilms have been confirmed by examinations of extracted teeth with periapical lesions [71]. 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 [61], while studies using scanning electron microscopy have shown microcolonies of cocci, rods, and filaments on root canal walls [59, 74, 83]. The biofilm mode of growth contributes to resistance to host defenses, and within the biofilm, there are formed subpopulations of cells that are phenotypically highly resistant to antibiotics and biocides [13, 16, 24, 46]. Although there is no generally agreed upon mechanism to account for this broad resistance to antimicrobials, the extent of the problem in endodontics is considerable.

Formation of Microbial Biofilms

Formation of a bacterial biofilm is a developmental process that begins when a cell attaches to a surface. The formation of microbial biofilms includes several steps that can be divided in two main parts: (a) the initial interactions of cells with the substrate and (b) growth and development of the biofilm (see Figs. 1.1 and 1.2).

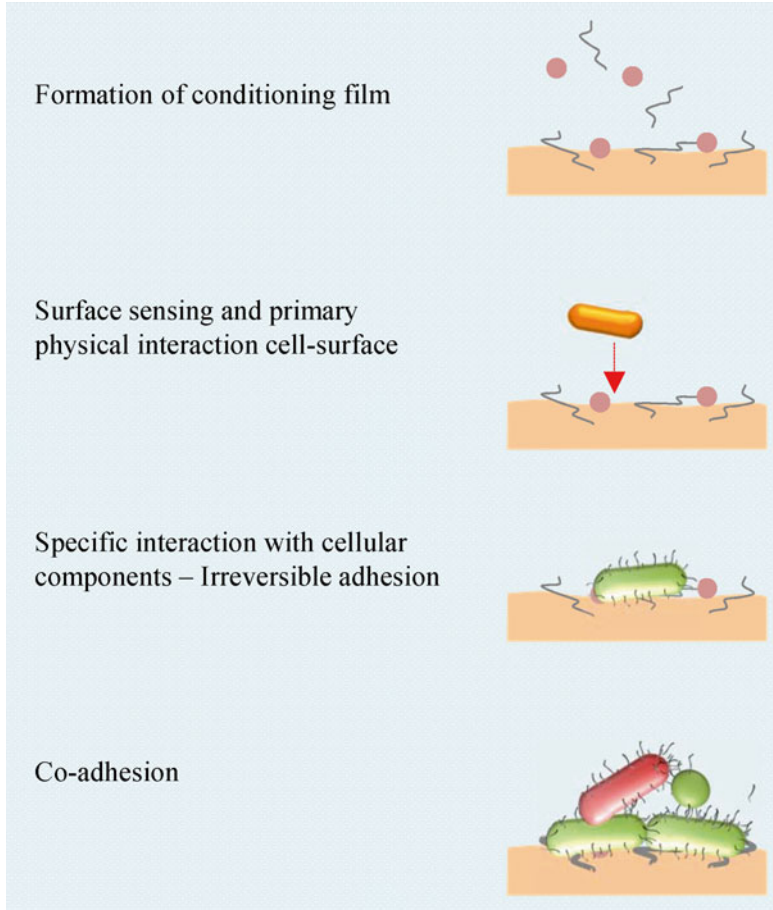

Fig. 1.1 Initial stages of biofilm formation. Schematic outlining the general approaches of initial cellular interaction of planktonic cells with coated substrates. In the initial phase, a "clean" surface is coated with environmental elements. At the second stage, a planktonic cell that approaches the coated surface initiates adhesion by adjusting a number of regulatory mechanisms known as surface sensing. In the following stage, irreversible adhesion occurs by association of specific cell components such as pili, flagella, exopolymers, etc. Lastly, co-adhesion with other organisms is achieved by specific interspecies interactive mechanisms

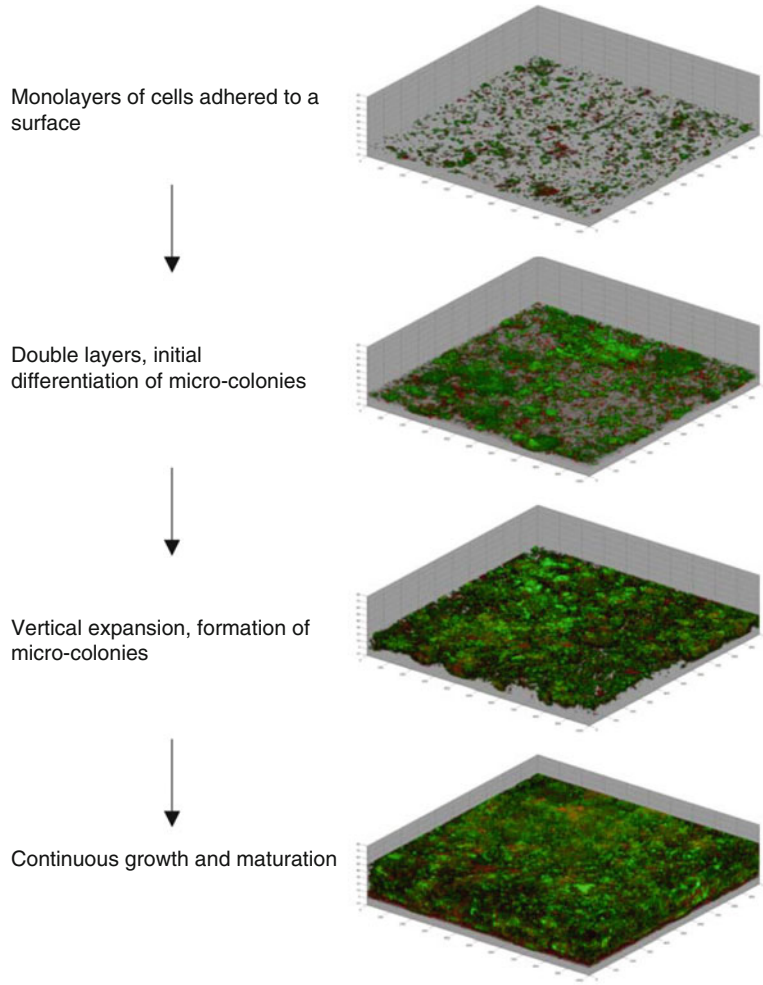

Fig. 1.2 Biofilm growth and maturation. Image sections showing reconstructed three-dimensional biofilm images at a magnification of ×100. Biofilms were stained with LIVE/DEAD stain, resulting in live and dead bacteria appearing as green or red, respectively. 3D images show confocal images of biofilm formation by oral bacteria at 1, 3, 5, and 7 days of growth, respectively. *Upper image* shows the first stage of biofilm growth at day 1; second and third images show subsequent stages of biofilm formation at day 3 and 5, respectively. *Bottom image* shows the fourth stage of biofilm formation at day 7. Damaged organisms appear red and undamaged organisms appear *green*

Biofilms initiate formation when a free-floating cell (cell in planktonic state) is deposited on a substratum coated with an organic conditioning polymeric matrix or "conditioning film" (Fig. 1.1). Conditioning films are composed by constituents of the local environment like water, salt ions, albumin, or fibronectin. When the first bacterial cells arrive, there is a weak and reversible contact between the cell and the conditioning film resulting from physical interactions such as Brownian motion, gravitation, diffusion, or electrostatic interactions [21]. Specific interactions with bacterial surface structures such as flagella and pilus are also important in the initial formation of a biofilm. The next step is when the adhesion of the cell to the substrate becomes irreversible. This is partly due to surface appendages overcoming the repulsive forces between the two surfaces and also helped by the sticky exopolymers secreted by the cells. These hydrophilic exopolymers have a complex and dynamic structure [22].

As depicted in Fig. 1.2, the second part of the formation of a biofilm comprises its growth and development. Development of a biofilm occurs as a result of adherent cells replicating and by additional cells adhering to the biofilm [37]. This is an overall dynamic process where many microorganisms co-adhere to one another and interact in the now active communities. Consequently during growth some cells will be detaching from the biofilm over time [6, 8, 28, 47].

Biofilms Developed in Root Canals

As 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 [10]. Microorganisms have been found to colonize by adhering to dentine walls in all the extension of the root canals. These aggregations of microorganisms have been observed adhered to the inner walls of complex apex anatomies and accessory canals [61, 71]. When these biofilm communities 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.

In 2004, Svensäter and Bergenholtz [83] proposed a hypothesis for biofilm formation in root canals. Biofilm formation in root canals is probably initiated just after the first invasion of the pulp chamber by oral organisms following the pulp tissue inflammatory breakdown. The inflammatory lesion frontage will then move successively towards the apex providing the fluid vehicle for the invading 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 [61, 71]. This observation could explain how the inflammatory lesion front serves as a fluid source for bacterial biofilm detachment and colonization of other remote sites in the root canal.

Resistance to Antimicrobials

Biofilm bacteria usually have an increased resistance to antimicrobial agents, in some cases up to 1,000-fold greater than that of the same microorganisms living in liquid suspension [27, 38]. Biofilms formed by oral bacteria are more resistant to chlorhexidine, amine fluoride, amoxicillin, doxycycline, and metronidazole than planktonic cells [46, 75]. Therefore, it is reasonable to assume that biofilms formed in root canals will also share the same resistant properties as oral bacteria, a fact that will affect the overall prognosis of root canal treatments. The high resistance capacity of biofilm communities from root canal bacteria was shown in a series of experiments that tested the resistance of biofilms formed by bacteria isolated from infected root canals to alkaline stress [12]. In this study, the viability of susceptible root canal strains in planktonic cultures was found to be considerably increased when the same strains were exposed to the same alkaline stress in biofilms.

The reasons for the increased resistance of bacteria when forming a biofilm are believed to be multiple, and currently, there is no generally agreed upon specific mechanism(s). It would seem that resistance is dependent in multiple factors such as the substrate, microenvironment, and age of the biofilm [80, 81]. There are, however, a number of known mechanisms that account for this broad resistance and can be divided in two main groups: (a) physical and (b) acquired. The physical protection is mainly related to the impaired penetration of antibiotics through the biofilm matrix. As it is illustrated in Fig. 1.3, acquired resistance is divided into three subcategories: differentiation of cells with low metabolic activity, differentiation of cells that actively respond to stress, and differentiation of cells with a very high persistent phenotype.

Physical Barrier to the Penetration of Antimicrobials in Biofilms

The main barrier that will hinder the penetration of antibiotics into the biofilm is the extracellular matrix [7, 26]. The extracellular matrix is the backbone of the biofilm and it is very complex in its composition, wide ranging between polysaccharides, proteins, nucleic acids, and lipids. The extracellular polymeric substances (EPS) provide not only physical and adhesive stability to the biofilm, but they also form the scaffold for the three-dimensional architecture that interconnects and organizes cells in biofilms [26].

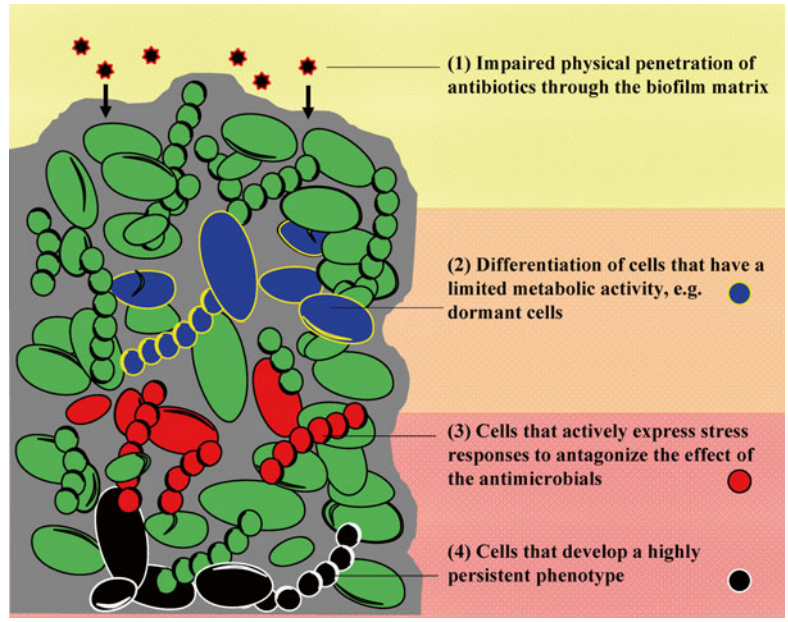

Fig. 1.3 Mechanisms of resistance by biofilm bacteria. The illustration depicts different mechanisms of resistance by biofilm bacteria. Slow or incomplete penetration of antimicrobials through the matrix (*1*). Concentration gradients of metabolites and waste will form zones where subpopulations of bacteria are differentiated. These subpopulations have different antimicrobial resistance capacities depending on their metabolic activity (dormant cells labeled blue) (*2*) or if they develop an active stress response mechanism (*red cells*) (*3*). Finally, a subpopulation of persister cells may also develop (*black cells*) (*4*)

Table 1.1 Novel biofilm matrix components recently found and under current research

Biofilm matrix component	Biofilm-forming species	Reference
Exopolysaccharide	*Bacillus subtilis (NCIB3610)*	[7]
Poly-gamma-DL-glutamic acid	*B. subtilis (RO-FF-1)*	[79]
Poly-N-acetyl glucosamine (PNAG)	*S. aureus*	[66]
Amyloid fibers of the protein TasA	*B. subtilis*	[72]
Protein BapL	*L. monocytogenes*	[39]
BAP proteins	*S. aureus*	[87]
Extracellular protein, MabA	*Lactobacillus rhamnosus*	[88]
Extracellular DNA (eDNA)	*Bacillus cereus*, *S. aureus, and L. monocytogenes*	[55, 70, 91]

Critical to matrix function is the distribution of the varied molecular-complex components that influences the developmental, homeostatic, and defensive processes in biofilms. Because of the marked diversity of EPS – inclusive of glycoproteins, proteoglycans, and insoluble hydrophobic polymers, among other components depending on the species involved – it is not surprising that this slimy substance delays considerably the diffusion of antimicrobials [81]. For example, it has been directly observed a profound retardation in the delivery of a penicillin antibiotic from penetrating a biofilm formed by a betalactamase-positive bacterium [3].

Due to the physical protection provided by the biofilm matrix, intense research is ongoing that aim to target the identification of novel matrix components. This novel research on matrix components will provide evidence for the identification and application of matrix-degrading enzymes that may prevent formation and/or activate dispersal of biofilms [45]. Some examples of novel biofilm matrix components that are currently studied are listed in Table 1.1.

State of Nutrient Deprivation and Dormancy

It has been observed that 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, while those near the surface may be actively growing [19, 80].

However, the majority of time cells in biofilms are in a dormant state that is equivalent to cells in the stationary phase of growth [64, 65]. In particular this dormant state is hypothesized to be common in biofilms that are formed in microenvironments where nutrients are scarce, such as treated root canals of teeth [14]. This dormant physiological state related to the general stress response and associated survival responses may offer an explanation for the resistance of biofilm cells to antimicrobials.

Bacteria under the stress of nutrient deprivation have developed efficient adaptive regulatory mechanisms to modify their metabolic balance away from biosynthesis and reproduction [40, 73]. One such mechanism involves the stringent response, a global bacterial response to nutritional stress that is mediated by the accumulation of the alarmones guanosine tetraphosphate and guanosine pentaphosphate, collectively known as (p) ppGpp [25, 68, 85]. For example, (p)ppGpp plays an important role for low-nutrient survival of *E. faecalis*, an organism that is known to withstand prolonged periods of starvation and remain viable in root-filled teeth for at least 12 months [58, 67]. Furthermore, the alarmone system (p)ppGpp has also a profound effect on the ability of *E. faecalis* to form, develop, and maintain stable biofilms [15]. These improved understanding of the alarmone mechanisms underlying biofilm formation and survival by *E. faecalis* may facilitate the identification of pathways that could be targeted to control persistent infections by this organism.

From the perspective of the persisting root canal flora, it is reasonable to assume that such dormant cells might "wake up" at some point in time and resume their metabolic activity to provoke periapical inflammation. Thus, from the metabolic perspective, the reactivation of dormant cells will render biofilm bacteria able to contribute to the persistence of inflammation. For example, a recent case report of a tooth that was adequately treated and showed no signs of disease revealed recurrent disease after 12 years. Histopathologic and histobacteriologic analyses showed a heavy dentinal tubule infection surrounding the area of a lateral canal providing evidence on the persistence of an intraradicular infection caused by bacteria possibly located in dentinal tubules [90].

The above hypothesis on the reactivation of biofilm cells was tested in a recent study [14]. Biofilm cultures of oral isolates of *Streptococcus anginosus* and *Lactobacillus salivarius* were forced to enter a state of dormancy by exposing them to nutrient deprivation for 24 h in buffer. After the starvation period the number of metabolically active cells decreased dramatically to zero and their cell membrane integrity was kept intact. Biofilm cells were then exposed to a "reactivation period" with fresh nutrients, but even after 96 h, the cultures were dominated by undamaged cells that were metabolically inactive. This phenomenon was not observed for cells in a planktonic state that were rapidly reactivated after 2 h. The data produced by this study showed that biofilm cells exhibit a slow physiological response and, unlike cells in planktonic culture, do not reactivate in short time periods even under optimal conditions. This observation highlights the difference in physiology between the biofilm and planktonic cultures and also confirms the slower physiological response of biofilm cells [53, 54], a mechanism that may account as a strategy of biofilm bacteria to resist stressful conditions.

Formation of Phenotypically Different Subpopulations

Bacteria within biofilms differ in their phenotype, depending on the spatial location of the cells within the community [81, 96]. There is now consistent evidence that has proven the presence of subpopulations of cells within biofilms that significantly differ in their antibiotic susceptibility [32, 41]. This phenomenon is correlated with differences in chemical concentration gradients that create unique microenvironments within biofilm communities. Simultaneously, adaptive variability allows the cells to respond to their local environmental conditions [69, 97]. Numerous studies have investigated the creation of these phenotypically different subpopulations and their mechanisms including genetic alterations, mutations, genetic recombination, and stochastic gene expression. For example, Weiser et al. described two distinct phenotypic variants in *S. pneumoniae* that switched between a pheno-

type with the ability to adhere and coexist among eukaryotic cells and a phenotype that was less capable to adhere but was better adapted to evade the host immune response during inflammation or invasive infection [94]. Of interest is the fact that both phenotypes of *S. pneumoniae* differed in their production of capsular polysaccharide having the inflammation-resistant phenotype an increased production of up to two to six times more capsular polysaccharide. These differences were accentuated by changes in the environmental concentration of oxygen; decreased oxygen levels correlated with an increase in capsular polysaccharide expression.

Interestingly, the formation of subpopulation in biofilms, where physiological differences are in play, has been demonstrated to occur in multispecies biofilms by root canal bacteria [11]. This was shown using four root canal bacterial isolates that, when cocultured, reacted concurrently to the absence of glucose in the culture medium. Although the overall cell viability of the four-species community was not affected by the lack of glucose, there was a significant variation in the 3D structure of the biofilms. In addition, patterns of physiologic adaptation by members of the community to the glucose-deprived medium were observed. The metabolic activity was concentrated in the upper levels of the biofilms, while at lower levels the metabolism of cells was considerably decreased. Subpopulations of species with high glycolytic demands, streptococcus, and lactobacilli were found predominating in the upper levels of the biofilms. This distinct spatial organization in biofilms grown in the lack of glucose shows a clear reorganization of the community in order to satisfy their members' metabolic pathways in order to enable the long-term persistence of the community. This result lends support to the hypothesis that the reorganization of subpopulations of cells in multispecies biofilms is also important for survival to stress factors from the environment [76].

Bacterial Cells That Persist

Groups of cells have been found to persist following exposure to lethal doses of antibiotics and new growing populations appear in the culture [48, 49]. These persister cells (a) may represent cells in some protected part of their cell cycle, (b) are capable of rapid adaptation, (c) are in a dormant state, or (d) are unable to initiate programmed cell death in response to the stimulus [49]. Thus, such persister 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. To date, these cells have only been reported to occur 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 [51]. The frequency of persister occurrence and the mechanism(s) involved in their appearance are unclear, although one hypothesis with *Escherichia coli* suggests that persister cells are regulated by the expression of chromosomal toxin–antitoxin genes [42]. 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 [42]. It has also been proposed that the expression of toxins drives bacteria reversibly into the slow-growing, multiple drug-tolerant phenotypes by "shutting down" antibiotic targets [50]. In the context of root canal bacteria, the formation of such persisting populations that are capable of surviving imposed endodontic treatment measures, as rise of the alkaline levels due to application of calcium hydroxide [12], would explain how organisms are able to survive and remain in the environment until the effects of noxious stimuli have dissipated.

Methods to Study Bacteria in Biofilms

The previous discussion relative to the capacity of biofilm bacteria to resist exposure to antimicrobials indicates the importance of studying the physiological state of bacteria with respect to their potential level of activity in the disease processes. However, the exact description of the status of a microorganism can be complex especially in chronic infections such as apical periodontitis. Currently, a variety of microscopic in situ methods have been developed to identify subpopula-