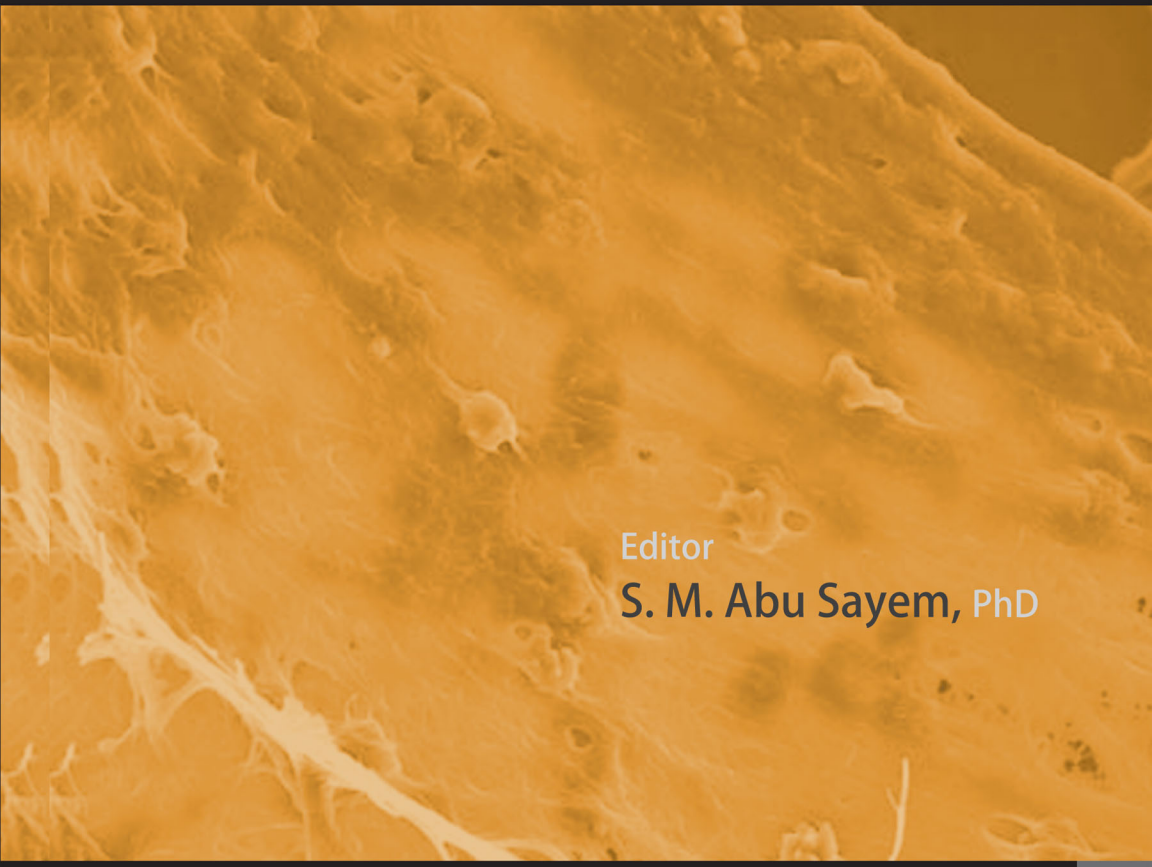
BIOFILM CONTROL and ANTIMICROBIAL AGENTS



Editor
S. M. Abu Sayem, PhD

Apple Academic Press

CRC Press
Taylor & Francis Group

BIOFILM CONTROL AND ANTIMICROBIAL AGENTS

This page intentionally left blank

BIOFILM CONTROL AND ANTIMICROBIAL AGENTS

Edited by

S. M. Abu Sayem, PhD

Apple Academic Press

TORONTO NEW JERSEY

CRC Press
Taylor & Francis Group
6000 Broken Sound Parkway NW, Suite 300
Boca Raton, FL 33487-2742

Apple Academic Press, Inc
3333 Mistwell Crescent
Oakville, ON L6L 0A2
Canada

Version Date: 20140128

International Standard Book Number-13: 978-1-4822-3949-2 (eBook - PDF)

ABOUT THE EDITOR

S. M. ABU SAYEM, PhD

Dr. S. M. Abu Sayem is an associate professor in the Department of Genetic Engineering and Biotechnology at Shahjalal University of Science and Technology, Sylhet, Bangladesh. He is a researcher and author, having published numerous peer-reviewed articles in the fields of structural biology, biofilms, and biotechnology.

This page intentionally left blank

CONTENTS

This page intentionally left blank

ACKNOWLEDGMENT AND HOW TO CITE

The chapters in this book were previously published in various places and in various formats. By bringing them together here in one place, we offer the reader a comprehensive perspective on recent investigations of biofilm control and antimicrobial agents. Each chapter is added to and enriched by being placed within the context of the larger investigative landscape.

We wish to thank the authors who made their research available for this book, whether by granting their permission individually or by releasing their research as open source articles. When citing information contained within this book, please do the authors the courtesy of attributing them by name, referring back to their original articles, using the credits provided at the beginning of each chapter.

This page intentionally left blank

LIST OF CONTRIBUTORS

Naif Abdullah Al-Dhabi
Department of Botany and Microbiology, Addiriyah Chair for Environmental Studies, College of Science, King Saud University, P.O. Box 2455, Riyadh, 11451, Saudi Arabia

David Andes
Department of Medicine, University of Wisconsin, Madison, Wisconsin, United States of America

Davide Antoniani
Department of Biosciences, University of Milan, Via Celoria 26, Milan, 20133, Italy

Chandrasekar Balachandran
Division of Microbiology, Entomology Research Institute, Loyola College, Chennai, 600 034, India

Sebastian Behrens
Geomicrobiology/Microbial Ecology Group, Centre for Applied Geosciences (ZAG), Eberhard-Karls-University Tübingen, Tübingen, Germany

Henrik Birkedal
Department of Chemistry, Faculty of Science and Technology, Aarhus University, Aarhus, Denmark

Federica Briani
Department of Biosciences, University of Milan, Via Celoria 26, Milan, 20133, Italy

J. Grant Burgess
Dove Marine Laboratory, School of Marine Science and Technology, Newcastle University, North Shields, United Kingdom

Thomas Carzaniga
Department of Biosciences, University of Milan, Via Celoria 26, Milan, 20133, Italy

S. Chusri
Faculty of Traditional Thai Medicine, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand and Natural Products Research Center, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand

Letizia Ciavatta
Institute of Biomolecular chemistry, National Center for Research, Naples, Italy

Lena Ciric
Department of Microbial Diseases, UCL Eastman Dental Institute, 256 Gray's Inn Road, London WC1X 8LD, UK

Angela Cordone
Department of Structural and Functional Biology, University of Naples Federico II, Naples, Italy

Leah E. Cowen
Department of Molecular Genetics, University of Toronto, Toronto, Ontario, Canada

Valentina Crocetta
Department of Biomedical Sciences, "G. d'Annunzio" University of Chieti, Via Vestini 31, 66100 Chieti, Italy and Center of Excellence on Aging, "G. d'Annunzio" University Foundation, Via Colle dell'Ara, 66100 Chieti, Italy

Maurilio De Felice
Department of Structural and Functional Biology, University of Naples Federico II, Naples, Italy

Gianni Dehò
Department of Biosciences, University of Milan, Via Celoria 26, Milan, 20133, Italy

Giovanni Di Bonaventura
Department of Biomedical Sciences, "G. d'Annunzio" University of Chieti, Via Vestini 31, 66100 Chieti, Italy and Center of Excellence on Aging, "G. d'Annunzio" University Foundation, Via Colle dell'Ara, 66100 Chieti, Italy

Giordano Dicuonzo
Center for Integrated Research, "Campus Biomedico" University, Via A. Del Portillo, 00128 Rome, Italy

Valentina Di Vincenzo
Department of Biomedical Sciences, "G. d'Annunzio" University of Chieti, Via Vestini 31, 66100 Chieti, Italy and Center of Excellence on Aging, "G. d'Annunzio" University Foundation, Via Colle dell'Ara, 66100 Chieti, Italy

Veeramuthu Duraipandiyan
Department of Botany and Microbiology, Addiriyah Chair for Environmental Studies, College of Science, King Saud University, P.O. Box 2455, Riyadh, 11451, Saudi Arabia

Hongjie Fan
Key Lab of Animal Bacteriology, Ministry of Agriculture, Nanjing Agricultural University, Nanjing, China

Nan Fang
State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing, China

Grzegorz Fila
Laboratory of Molecular Diagnostics, Department of Biotechnology, Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk, Kladki 24, 80-822 Gdansk, Poland

Ersilia Fiscarelli
"Bambino Gesù" Children's Hospital and Research Institute, Piazza Sant'Onofrio 4, 00165 Rome, Italy

He Gao
State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing, China and State Key Laboratory for Infectious Disease Prevention and Control, National Institute for Communicable Disease Control and Prevention, Chinese Centre for Disease Control and Prevention, Beijing, China

Renato Gennaro
Department of Life Sciences, University of Trieste, Via L. Giorgieri 1, 34127 Trieste, Italy

Sabine U. Gerbersdorf
Institute of Hydraulic Engineering, University Stuttgart, Stuttgart, Germany

Giovanni Gherardi
Center for Integrated Research, "Campus Biomedico" University, Via A. Del Portillo, 00128 Rome, Italy

Joana Graça
Centre of Biological Engineering, Institute for Biotechnology and Bioengineering (IBB), University of Minho, Campus de Gualtar, 4710–057 Braga, Portugal

Mariusz Grinholc
Laboratory of Molecular Diagnostics, Department of Biotechnology, Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk, Kladki 24, 80-822 Gdansk, Poland

Zhaobiao Guo
State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing, China

Michael J. Hall
School of Chemistry, Newcastle University, Newcastle upon Tyne, United Kingdom

Cédric Hubas
Département Milieux et Peuplements Aquatiques (DMPA), Muséum National d'Histoire Naturelle, UMR BOREA (Biologie des organismes et écosystèmes aquatiques) MNHN-CNRS-UPMC-IRD, Paris, France

Savarimuthu Ignacimuthu
Division of Microbiology, Entomology Research Institute, Loyola College, Chennai, 600 034, India

S. Jansrisewangwong
Faculty of Traditional Thai Medicine, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand

Inshad Ali Khan
Clinical Microbiology Division, Indian Institute of Integrative Medicine, Jammu, 180 001, India

Paolo Landini
Department of Biosciences, University of Milan, Via Celoria 26, Milan, 20133, Italy

S. Limsuwan
Faculty of Traditional Thai Medicine, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand and Natural Products Research Center, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand

Peter Lingström
Department of Cariology, Institute of Odontology at Sahlgrenska Academy, University of Gothenburg, 40530 Götegborg, Sweden

Guangjin Liu
Key Lab of Animal Bacteriology, Ministry of Agriculture, Nanjing Agricultural University, Nanjing, China

Hélder Lopes
Centre of Biological Engineering, Institute for Biotechnology and Bioengineering (IBB), University of Minho, Campus de Gualtar, 4710–057 Braga, Portugal

Susana Lopes
Centre of Biological Engineering, Institute for Biotechnology and Bioengineering (IBB), University of Minho, Campus de Gualtar, 4710–057 Braga, Portugal

Jose L. Lopez-Ribot
Department of Biology and South Texas Center for Emerging Infectious Diseases, University of Texas at San Antonio, Texas, United States of America

Helen V. Lubarsky
Sediment Ecology Research Group, Scottish Ocean Institute, School of Biology, University of St. Andrews, St. Andrews, Scotland, United Kingdom and Institute of Hydraulic Engineering, University Stuttgart, Stuttgart, Germany

Idalina Machado
Centre of Biological Engineering, Institute for Biotechnology and Bioengineering (IBB), University of Minho, Campus de Gualtar, 4710–057 Braga, Portugal

K. Maneenoon
Faculty of Traditional Thai Medicine, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand

Emiliano Manzo
Institute of Biomolecular chemistry, National Center for Research, Naples, Italy

Mario Mardirossian
Department of Life Sciences, University of Trieste, Via L. Giorgieri 1, 34127 Trieste, Italy

Rikke L. Meyer
The Interdisciplinary Nanoscience Center (iNANO), Faculty of Science and Technology, Aarhus University, Aarhus, Denmark and Department of Bioscience, Faculty of Science and Technology, Aarhus University, Aarhus, Denmark

S. Mukdee
Faculty of Traditional Thai Medicine, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand

Chinnasamy Muthukumar
Department of Botany and Microbiology, Addiriyah Chair for Environmental Studies, College of Science, King Saud University, P.O. Box 2455, Riyadh, 11451, Saudi Arabia

Joanna Nakonieczna
Laboratory of Molecular Diagnostics, Department of Biotechnology, Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk, Kladki 24, 80-822 Gdansk, Poland

Jeniel Nett
Department of Medicine, University of Wisconsin, Madison, Wisconsin, United States of America

Reindert Nijland
Dove Marine Laboratory, School of Marine Science and Technology, Newcastle University, North Shields, United Kingdom

Bente Nyvad
Department of Dentistry, Faculty of Health, Aarhus University, Aarhus, Denmark

Adele Papetti
Department of Drug Sciences, University of Pavia, Viale Taramelli 12, 27100 Pavia, Italy

David M. Paterson
Sediment Ecology Research Group, Scottish Ocean Institute, School of Biology, University of St. Andrews, St. Andrews, Scotland, United Kingdom

Maria O. Pereira
Centre of Biological Engineering, Institute for Biotechnology and Bioengineering (IBB), University of Minho, Campus de Gualtar, 4710–057 Braga, Portugal

Arianna Pompilio
Department of Biomedical Sciences, "G. d'Annunzio" University of Chieti, Via Vestini 31, 66100 Chieti, Italy and Center of Excellence on Aging, "G. d'Annunzio" University Foundation, Via Colle dell'Ara, 66100 Chieti, Italy

Stefano Pomponio
Department of Biomedical Sciences, "G. d'Annunzio" University of Chieti, Via Vestini 31, 66100 Chieti, Italy and Center of Excellence on Aging, "G. d'Annunzio" University Foundation, Via Colle dell'Ara, 66100 Chieti, Italy

Jonathan Pratten
Department of Microbial Diseases, UCL Eastman Dental Institute, 256 Gray's Inn Road, London WC1X 8LD, UK

Merete K. Raarup
Stereology and Electron Microscopy Research Laboratory and MIND Center, Aarhus University, Aarhus, Denmark

Michael Karunai Raj
Division of Microbiology, Entomology Research Institute, Loyola College, Chennai, 600 034, India and Research and Development Centre, Orchid Chemicals and Pharmaceuticals Ltd, Sozhanganallur, Chennai, 600119, India

Ranjith Rajendran
College of Medicine, Veterinary and Life Science, University of Glasgow, Glasgow, United Kingdom

Vikrant Singh Rajput
Clinical Microbiology Division, Indian Institute of Integrative Medicine, Jammu, 180 001, India

Gordon Ramage
College of Medicine, Veterinary and Life Science, University of Glasgow, Glasgow, United Kingdom

Francesco Ricciardi
Institute of Aquatic Ecology, University of Girona, Girona, Spain

Nicole Robbins
Department of Molecular Genetics, University of Toronto, Toronto, Ontario, Canada

S. M. Abu Sayem
Department of Structural and Functional Biology, University of Naples Federico II, Naples, Italy and Department of Genetic Engineering and Biotechnology, Shahjalal University of Science and Technology, Sylhet, Bangladesh

Sebastian Schlafer
The Interdisciplinary Nanoscience Center (iNANO), Faculty of Science and Technology, Aarhus University, Aarhus, Denmark, Department of Dentistry, Faculty of Health, Aarhus University, Aarhus, Denmark, and Department of Bioscience, Faculty of Science and Technology, Aarhus University, Aarhus, Denmark

Marco Scocchi
Department of Life Sciences, University of Trieste, Via L. Giorgieri 1, 34127 Trieste, Italy

Jing Shao
Key Lab of Animal Bacteriology, Ministry of Agriculture, Nanjing Agricultural University, Nanjing, China

Caterina Signoretto
Dipartimento di Patologia-Sezione di Microbiologia, Università di Verona, 37134 Verona, Italy

K. Sompetch
Faculty of Traditional Thai Medicine, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand

David Spratt
Department of Microbial Diseases, UCL Eastman Dental Institute, 256 Gray's Inn Road, London WC1X 8LD, UK

T. Srichai
Faculty of Traditional Thai Medicine, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand

Brigitte M. Städler
The Interdisciplinary Nanoscience Center (iNANO), Faculty of Science and Technology, Aarhus University, Aarhus, Denmark

Monica Stauder
DIPTERIS, University of Genoa, Corso Europa 26, 16132 Genoa, Italy

Fengjun Sun
State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing, China and Department of Pharmacy, Southwest Hospital, the Third Military Medical University, Chongqing, China

Duncan S. Sutherland
The Interdisciplinary Nanoscience Center (iNANO), Faculty of Science and Technology, Aarhus University, Aarhus, Denmark

Yafang Tan
State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing, China

Aleksandra Taraszkiewicz
Laboratory of Molecular Diagnostics, Department of Biotechnology, Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk, Kladki 24, 80-822 Gdansk, Poland

Hélène Tournu
Laboratory of Molecular Cell Biology, Institute of Botany and Microbiology, Katholieke Universiteit Leuven, Flanders, 3001 Leuven-Heverlee, Belgium and Department of Molecular Microbiology, VIB, Kasteelpark Arenberg 31, Flanders, 3001, Leuven-Heverlee, Belgium

Annabella Tramice
Institute of Biomolecular chemistry, National Center for Research, Naples, Italy

Anna Tymon
Department of Microbial Diseases, UCL Eastman Dental Institute, 256 Gray's Inn Road, London WC1X 8LD, UK

Priya Uppuluri
Department of Biology and South Texas Center for Emerging Infectious Diseases, University of Texas at San Antonio, Texas, United States of America

Patrick Van Dijck
Laboratory of Molecular Cell Biology, Institute of Botany and Microbiology, Katholieke Universiteit Leuven, Flanders, 3001 Leuven-Heverlee, Belgium and Department of Molecular Microbiology, VIB, Kasteelpark Arenberg 31, Flanders, 3001, Leuven-Heverlee, Belgium

Mario Varcamonti
Department of Structural and Functional Biology, University of Naples Federico II, Naples, Italy

S. P. Voravuthikunchai
Natural Products Research Center, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand and Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand

Li Wang
State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing, China

Yang Wang
College of Animal Science and Technology, Henan University of Science and Technology, Luoyang, China and Key Lab of Animal Bacteriology, Ministry of Agriculture, Nanjing Agricultural University, Nanjing, China

Peter L. Wejse
Arla Foods amba, Viby J., Denmark

Michael Wilson
Department of Microbial Diseases, UCL Eastman Dental Institute, 256 Gray's Inn Road, London WC1X 8LD, UK

Zongfu Wu
Key Lab of Animal Bacteriology, Ministry of Agriculture, Nanjing Agricultural University, Nanjing, China

Peiyuan Xia
Department of Pharmacy, Southwest Hospital, the Third Military Medical University, Chongqing, China

Ruifu Yang
State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing, China

Li Yi
Key Lab of Animal Bacteriology, Ministry of Agriculture, Nanjing Agricultural University, Nanjing, China

Anna Zanfardino
Department of Structural and Functional Biology, University of Naples Federico II, Naples, Italy

Egija Zaura
Department of Preventive Dentistry, Academic Centre for Dentistry Amsterdam (ACTA), Gustav Mahlerlaan 3004, 1081 LA Amsterdam, The Netherlands

Wei Zhang
Key Lab of Animal Bacteriology, Ministry of Agriculture, Nanjing Agricultural University, Nanjing, China

Yiquan Zhang
State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing, China

Dongsheng Zhou
State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing, China

INTRODUCTION

Biofilm is ubiqutoius; dental plaques, as well as the "gunk" that clogs drainage system, are examples of normal biofilm that we find in our day-to-day lives. Today, biofilm is considered the most prevalent mode of microorganism growth. Biofilm forms when planktonic bacteria adheres to surfaces and begins to excrete a slimy, glue-like substance that anchors them to all kinds of material—metals, paper, plastics, tissue, soil particles, food processing equipments, medical implant materials, and even artworks. Microbial biofilms on surfaces cost billions of dollars yearly in equipment damage, product contamination, energy losses, and medical infections; this is part of the reason why biofilm research is becoming so important.

Conventional methods for removing biofilm bacteria consist mainly of mechanical forces, such as scrubbing, heating, sonication, use of ultrasound, high pressure, and chemicals like ozone, hypochlorite, hypobromite, chloramines, tributylin, copper compounds, and antimicrobials such as antibiotics and disinfectants. However, mechanical, chemical, or antimicrobial approaches are often ineffective and are not able to successfully prevent or control the formation of unwanted biofilms without causing deleterious side effects. Mechanical forces are sometimes destructive towards the surface being treated and can be very expensive. On the other hand, the high dose of antimicrobials required to get rid of biofilm bacteria are environmentally undesirable, medically impractical, and sometimes pose serious health problems. In addition, repeated use of antimicrobial agents on biofilms can cause bacteria within the biofilm to develop an increased resistance to antimicrobial agents.

To discover novel, safe, and long-term solutions to the challenge imposed by biofilm, it is necessary to further understand the biofilm growth and detachment. Factors that lead to biofilm growth inhibition, biofilm disruption, or biofilm eradication are important for controlling biofilm. This book highlights some of the exciting research that has recently been done,

although it necessarily contains only a sample of all the recent insights that have been gained in this field.

Chapter 1, by Pompilio and colleagues, focuses on the use of biofilm in the treatment of cystic fibrosis. Treatment of cystic fibrosis-associated lung infections is hampered by the presence of multi-drug resistant pathogens, many of which are also strong biofilm producers. Antimicrobial peptides, essential components of innate immunity in humans and animals, exhibit relevant in vitro antimicrobial activity although they tend not to select for resistant strains. Three α-helical antimicrobial peptides, BMAP-27 and BMAP-28 of bovine origin, and the artificial P19(9/B) peptide were tested, comparatively to Tobramycin, for their in vitro antibacterial and anti-biofilm activity against 15 *Staphylococcus aureus*, 25 *Pseudomonas aeruginosa*, and 27 *Stenotrophomonas maltophilia* strains from cystic fibrosis patients. All assays were carried out in physical-chemical experimental conditions simulating a cystic fibrosis lung. All peptides showed a potent and rapid bactericidal activity against most *P. aeruginosa, S. maltophilia* and *S. aureusstrains* tested, at levels generally higher than those exhibited by Tobramycin and significantly reduced biofilm formation of all the bacterial species tested, although less effectively than Tobramycin did. On the contrary, the viability-reducing activity of antimicrobial peptides against preformed P. aeruginosa biofilms was comparable to and, in some cases, higher than that showed by Tobramycin. The activity shown by α-helical peptides against planktonic and biofilm cells makes them promising "lead compounds" for future development of novel drugs for therapeutic treatment of cystic fibrosis lung disease.

Nijland and colleagues explain in chapter 2 that microbial biofilms are composed of a hydrated matrix of biopolymers including polypeptides, polysaccharides and nucleic acids and act as a protective barrier and microenvironment for the inhabiting microbes. While studying marine biofilms, the authors observed that supernatant produced by a marine isolate of *Bacillus licheniformis* was capable of dispersing bacterial biofilms. They investigated the source of this activity and identified the active compound as an extracellular DNase (NucB). The authors have shown that this enzyme rapidly breaks up the biofilms of both Gram-positive and Gram-negative bacteria. They demonstrate that bacteria can use secreted nucleases as an elegant strategy to disperse established biofilms and to prevent

de novoformation of biofilms of competitors. DNA therefore plays an important dynamic role as a reversible structural adhesin within the biofilm.

Transition from planktonic cells to biofilm is mediated by production of adhesion factors, such as extracellular polysaccharides (EPS), and modulated by complex regulatory networks that, in addition to controlling production of adhesion factors, redirect bacterial cell metabolism to the biofilm mode. In chapter 3, Carzaniga and colleagues found that deletion of the pnp gene, encoding polynucleotide phosphorylase, an RNA processing enzyme and a component of the RNA degradosome, results in increased biofilm formation in *Escherichia coli*. This effect is particularly pronounced in the *E. coli* strain C-1a, in which deletion of the pnp gene leads to strong cell aggregation in liquid medium. Cell aggregation is dependent on the EPS poly-N-acetylglucosamine (PNAG), thus suggesting negative regulation of the PNAG biosynthetic operonpgaABCD by PNPase. Indeed, pgaABCD transcript levels are higher in the pnp mutant. Negative control of pgaABCD expression by PNPase takes place at mRNA stability level and involves the 5'-untranslated region of the pgaABCD transcript, which serves as a cis-element regulating pgaABCDtranscript stability and translatability. The authors' results demonstrate that PNPase is necessary to maintain bacterial cells in the planktonic mode through down-regulation of pgaABCD expression and PNAG production.

Wang and colleagues study *Streptococcus suis* (SS) in chapter 4: a zoonotic pathogen that causes severe disease symptoms in pigs and humans. Biofilms of SS bind to extracellular matrix proteins in both endothelial and epithelial cells and cause persistent infections. In this study, the differences in the protein expression profiles of SS grown either as planktonic cells or biofilms were identified using comparative proteomic analysis. The results revealed the existence of 13 proteins of varying amounts, among which six were upregulated and seven were downregulated in the *Streptococcus* biofilm compared with the planktonic controls. The convalescent serum from mini-pig, challenged with SS, was applied in a Western blot assay to visualize all proteins from the biofilm that were grown in vitro and separated by two-dimensional gel electrophoresis. A total of 10 immunoreactive protein spots corresponding to nine unique proteins were identified by MALDI-TOF/TOF-MS. Of these nine proteins, five (Manganese-dependent superoxide dismutase, UDP-N-acetylglucosamine

1-carboxyvinyltransferase, ornithine carbamoyltransferase, phosphoglycerate kinase, Hypothetical protein SSU05_0403) had no previously reported immunogenic properties in SS to our knowledge. The remaining four immunogenic proteins (glyceraldehyde-3-phosphate dehydrogenase, hemolysin, pyruvate dehydrogenase and DnaK) were identified under both planktonic and biofilm growth conditions. In conclusion, the protein expression pattern of SS, grown as biofilm, was different from the SS grown as planktonic cells. These five immunogenic proteins that were specific to SS biofilm cells may potentially be targeted as vaccine candidates to protect against SS biofilm infections. The four proteins common to both biofilm and planktonic cells can be targeted as vaccine candidates to protect against both biofilm and acute infections.

Secondary metabolites ranging from furanone to exo-polysaccharides have been suggested to have anti-biofilm activity in various recent studies. Among these, *Escherichia coli* group II capsular polysaccharides were shown to inhibit biofilm formation of a wide range of organisms and more recently marine *Vibrio sp*. were found to secrete complex exopolysaccharides having the potential for broad-spectrum biofilm inhibition and disruption. In chapter 5, Abu Sayem and colleageus report that a newly identified ca. 1800 kDa polysaccharide having simple monomeric units of α-D-galactopyranosyl-(1→2)-glycerol-phosphate exerts an anti-biofilm activity against a number of both pathogenic and non-pathogenic strains without bactericidal effects. This polysaccharide was extracted from a *Bacillus licheniformis* strain associated with the marine organism *Spongia officinalis*. The mechanism of action of this compound is most likely independent from quorum sensing, as its structure is unrelated to any of the so far known quorum sensing molecules. In their experiments the authors also found that treatment of abiotic surfaces with their polysaccharide reduced the initial adhesion and biofilm development of strains such as *Escherichia coli* PHL628 and Pseudomonas fluorescens. The polysaccharide isolated from sponge-associated *B. licheniformis* has several features that provide a tool for better exploration of novel anti-biofilm compounds. Inhibiting biofilm formation of a wide range of bacteria without affecting their growth appears to represent a special feature of the polysaccharide described in this report. Further research on such surface-active compounds might help developing new classes of anti-biofilm molecules with

broad spectrum activity and more in general will allow exploring of new functions for bacterial polysaccharides in the environment.

Combating dental biofilm formation is the most effective means for the prevention of caries, one of the most widespread human diseases. Among the chemical supplements to mechanical tooth cleaning procedures, non-bactericidal adjuncts that target the mechanisms of bacterial biofilm formation have gained increasing interest in recent years. Milk proteins, such as lactoferrin, have been shown to interfere with bacterial colonization of saliva-coated surfaces. Schlafer and colleagues study the effect of bovine milk osteopontin (OPN) in chapter 6, a highly phosphorylated whey glycoprotein, on a multispecies in vitro model of dental biofilm. While considerable research effort focuses on the interaction of OPN with mammalian cells, there are no data investigating the influence of OPN on bacterial biofilms. Biofilms consisting of *Streptococcus oralis, Actinomyces naeslundii, Streptococcus mitis, Streptococcus downei* and *Streptococcus sanguinis* were grown in a flow cell system that permitted in situ microscopic analysis. Crystal violet staining showed significantly less biofilm formation in the presence of OPN, as compared to biofilms grown without OPN or biofilms grown in the presence of caseinoglycomacropeptide, another phosphorylated milk protein. Confocal microscopy revealed that OPN bound to the surface of bacterial cells and reduced mechanical stability of the biofilms without affecting cell viability. The bacterial composition of the biofilms, determined by fluorescence in situ hybridization, changed considerably in the presence of OPN. In particular, colonization of S. mitis, the best biofilm former in the model, was reduced dramatically. OPN strongly reduces the amount of biofilm formed in a well-defined laboratory model of acidogenic dental biofilm. If a similar effect can be observed in vivo, OPN might serve as a valuable adjunct to mechanical tooth cleaning procedures.

In chapter 7, Lubarsky and colleagues argue that the accumulation of the widely-used antibacterial and antifungal compound triclosan (TCS) in freshwaters raises concerns about the impact of this harmful chemical on the biofilms that are the dominant life style of microorganisms in aquatic systems. However, investigations to-date rarely go beyond effects at the cellular, physiological or morphological level. The chapter focuses on bacterial biofilms addressing the possible chemical impairment of their

functionality, while also examining their substratum stabilization potential as one example of an important ecosystem service. The development of a bacterial assemblage of natural composition—isolated from sediments of the Eden Estuary (Scotland, UK)—on non-cohesive glass beads (<63 μm) and exposed to a range of triclosan concentrations (control, 2–100 μg L^{-1}) was monitored over time by Magnetic Particle Induction (MagPI). In parallel, bacterial cell numbers, division rate, community composition (DGGE) and EPS (extracellular polymeric substances: carbohydrates and proteins) secretion were determined. While the triclosan exposure did not prevent bacterial settlement, biofilm development was increasingly inhibited by increasing TCS levels. The surface binding capacity (MagPI) of the assemblages was positively correlated to the microbial secreted EPS matrix. The EPS concentrations and composition (quantity and quality) were closely linked to bacterial growth, which was affected by enhanced TCS exposure. Furthermore, TCS induced significant changes in bacterial community composition as well as a significant decrease in bacterial diversity. The impairment of the stabilization potential of bacterial biofilm under even low, environmentally relevant TCS levels is of concern since the resistance of sediments to erosive forces has large implications for the dynamics of sediments and associated pollutant dispersal. In addition, the surface adhesive capacity of the biofilm acts as a sensitive measure of ecosystem effects.

Machado and colleagues work aim to characterize endoscope biofilm-isolated (PAI) and reference strain *P. aeruginosa* (PA) adhesion, biofilm formation and sensitivity to antibiotics in chapter 8. The recovery ability of the biofilm-growing bacteria subjected to intermittent antibiotic pressure (ciprofloxacin (CIP) and gentamicin (GM)), as well as the development of resistance towards antibiotics and benzalkonium chloride (BC), were also determined. The capacity of both strains to develop biofilms was greatly impaired in the presence of CIP and GM. Sanitization was not complete allowing biofilm recovery after the intermittent cycles of antibiotic pressure. The environmental pressure exerted by CIP and GM did not develop *P. aeruginosa* resistance to antibiotics nor cross-resistance towards BC. However, data highlighted that none of the antimicrobials led to complete biofilm eradication, allowing the recovery of the remaining adhered population possibly due to the selection of persister cells. This

feature may lead to biofilm recalcitrance, reinforcement of bacterial attachment, and recolonization of other sites.

Development of biofilm is a key mechanism involved in *Staphylococcus epidermidis* virulence during device-associated infections. Chusri and colleagues aimed to investigate antibiofilm formation and mature biofilm eradication ability of ethanol and water extracts of Thai traditional herbal recipes including THR-SK004, THR-SK010, and THR-SK011 against *S. epidermidis* in chapter 9. A biofilm forming reference strain, *S. epidermidis* ATCC 35984 was employed as a model for searching anti-biofilm agents by MTT reduction assay. The results revealed that the ethanol extract of THR-SK004 (THR-SK004E) could inhibit the formation of *S. epidermidis* biofilm on polystyrene surfaces. Furthermore, treatments with the extract efficiently inhibit the biofilm formation of the pathogen on glass surfaces determined by scanning electron microscopy and crystal violet staining. In addition, THR-SK010 ethanol extract (THR-SK010E; 0.63–5 µg/mL) could decrease 30 to 40% of the biofilm development. Almost 90% of a 7-day-old staphylococcal biofilm was destroyed after treatment with THR-SK004E (250 and 500 µg/mL) and THR-SK010E (10 and 20 µg/mL) for 24 h. Therefore, the results clearly demonstrated THR-SK004E could prevent the staphylococcal biofilm development, whereas both THR-SK004E and THR-SK010E possessed remarkable eradication ability on the mature staphylococcal biofilm.

Gingivitis is a preventable disease characterised by inflammation of the gums due to the buildup of a microbial biofilm at the gingival margin. It is implicated as a precursor to periodontitis, a much more serious problem which includes associated bone loss. Unfortunately, due to poor oral hygiene among the general population, gingivitis is prevalent and results in high treatment costs. Consequently, the option of treating gingivitis using functional foods, which promote oral health, is an attractive one. Medicinal mushrooms, including shiitake, have long been known for their immune system boosting as well as antimicrobial effects; however, they have not been employed in the treatment of oral disease. In chapter 10, Ciric and colleagues explore the effectiveness of shiitake mushroom extract compared to that of the active component in the leading gingivitis mouthwash, containing chlorhexidine, in an artificial mouth model (constant depth film fermenter). The total bacterial numbers as well as numbers

of eight key taxa in the oral community were investigated over time using multiplex qPCR. The results indicated that shiitake mushroom extract lowered the numbers of some pathogenic taxa without affecting the taxa associated with health, unlike chlorhexidine which has a limited effect on all taxa.

Couroupita guianensis Aubl. (*Lecythidaceae*) is commonly called Ayahuma and the Cannonball tree. It is distributed in the tropical regions of northern South America and Southern Caribbean. It has several medicinal properties. It is used to treat hypertension, tumours, pain, inflammatory processes, cold, stomach ache, skin diseases, malaria, wounds and toothache. In chapter 11, Al-Dhabi and colleagues extracted the fruits of *Couroupita guianensis* with chloroform. Antimicrobial, antimycobacterial and antibiofilm forming activities of the chloroform extract were investigated. Quantitative estimation of Indirubin, one of the major constituent, was identified by HPLC. Chloroform extract showed good antimicrobial and antibiofilm forming activities; however it showed low antimycobacterial activity. The zones of inhibition by chloroform extract ranged from 0 to 26 mm. Chloroform extract showed effective antibiofilm activity against *Pseudomonas aeruginosa* starting from 2 mg/mL BIC, with 52% inhibition of biofilm formation. When the chloroform extract was subjected to HPLC-DAD analysis, along with Indirubin standard, in the same chromatographic conditions, the authors found that Indirubin was one of the major compounds in this plant (0.0918% dry weight basis). The chloroform extract showed good antimicrobial and antibiofilm properties. Chloroform extract can be evaluated further in drug development programmes.

Yersinia pestis synthesizes the attached biofilms in the *flea proventriculus*, which is important for the transmission of this pathogen by fleas. The hmsHFRS operons is responsible for the synthesis of exopolysaccharide (the major component of biofilm matrix), which is activated by the signaling molecule 3′, 5′-cyclic diguanylic acid (c-di-GMP) synthesized by the only two diguanylate cyclases HmsT, and YPO0449 (located in a putative operonYPO0450-0448). Sun and colleagues found in chpater 12 that the phenotypic assays indicated that the transcriptional regulator Fur inhibited the Y. pestisbiofilm production in vitro and on nematode. Two distinct Fur box-like sequences were predicted within the promoter-proximal region

of hmsT, suggesting that hmsT might be a direct Fur target. The subsequent primer extension, LacZ fusion, electrophoretic mobility shift, and DNase I footprinting assays disclosed that Fur specifically bound to the hmsT promoter-proximal region for repressing the hmsT transcription. In contrast, Fur had no regulatory effect on hmsHFRS and YPO0450-0448 at the transcriptional level. The detection of intracellular c-di-GMP levels revealed that Fur inhibited the c-di-GMP production. *Y. pestis* Fur inhibits the c-di-GMP production through directly repressing the transcription ofhmsT, and thus it acts as a repressor of biofilm formation. Since the relevant genetic contents for fur, hmsT, hmsHFRS, and YPO0450-0448 are extremely conserved between *Y. pestis* and typical *Y. pseudotuberculosis,* the above regulatory mechanisms can be applied to *Y. pseudotuberculosis.*

Fungal biofilms are a major cause of human mortality and are recalcitrant to most treatments due to intrinsic drug resistance. These complex communities of multiple cell types form on indwelling medical devices and their eradication often requires surgical removal of infected devices. In chapter 13, Robbins and colleagues implicate the molecular chaperone Hsp90 as a key regulator of biofilm dispersion and drug resistance. They previously established that in the leading human fungal pathogen, *Candida albicans*, Hsp90 enables the emergence and maintenance of drug resistance in planktonic conditions by stabilizing the protein phosphatase calcineurin and MAPK Mkc1. Hsp90 also regulates temperature-dependent *C. albicans* morphogenesis through repression of cAMP-PKA signalling. Here we demonstrate that genetic depletion of Hsp90 reduced *C. albicans* biofilm growth and maturation in vitro and impaired dispersal of biofilm cells. Further, compromising Hsp90 function in vitro abrogated resistance of *C. albicans* biofilms to the most widely deployed class of antifungal drugs, the azoles. Depletion of Hsp90 led to reduction of calcineurin and Mkc1 in planktonic but not biofilm conditions, suggesting that Hsp90 regulates drug resistance through different mechanisms in these distinct cellular states. Reduction of Hsp90 levels led to a marked decrease in matrix glucan levels, providing a compelling mechanism through which Hsp90 might regulate biofilm azole resistance. Impairment of Hsp90 function genetically or pharmacologically transformed fluconazole from ineffectual to highly effective in eradicating biofilms in a rat venous catheter infection model. Finally, inhibition of Hsp90 reduced resistance of biofilms of the

most lethal mould, *Aspergillus fumigatus*, to the newest class of antifungals to reach the clinic, the echinocandins. Thus, the authors establish a novel mechanism regulating biofilm drug resistance and dispersion and that targeting Hsp90 provides a much-needed strategy for improving clinical outcome in the treatment of biofilm infections.

Biofilms define mono- or multispecies communities embedded in a self-produced protective matrix, which is strongly attached to surfaces. They often are considered a general threat not only in industry but also in medicine. They constitute a permanent source of contamination, and they can disturb the proper usage of the material onto which they develop. Chapter 14, by Tournu and Van Dijck, relates to some of the most recent approaches that have been elaborated to eradicate *Candida* biofilms, based on the vast effort put in ever-improving models of biofilm formation in vitro and in vivo, including novel flow systems, high-throughput techniques and mucosal models. Mixed biofilms, sustaining antagonist or beneficial cooperation between species, and their interplay with the host immune system are also prevalent topics. Alternative strategies against biofilms include the lock therapy and immunotherapy approaches, and material coating and improvements. The host-biofilm interactions are also discussed, together with their potential applications in *Candida* biofilm elimination.

In the final chapter, chapter 15, Taraszkiewicz and colleagues review the recent literature concerning the efficiency of antimicrobial photodynamic inactivation toward various microbial species in planktonic and biofilm cultures. The review is mainly focused on biofilm-growing microrganisms because this form of growth poses a threat to chronically infected or immunocompromised patients and is difficult to eradicate from medical devices. We discuss the biofilm formation process and mechanisms of its increased resistance to various antimicrobials. We present, based on data in the literature, strategies for overcoming the problem of biofilm resistance. Factors that have potential for use in increasing the efficiency of the killing of biofilm-forming bacteria include plant extracts, enzymes that disturb the biofilm structure, and other nonenzymatic molecules. We propose combining antimicrobial photodynamic therapy with various antimicrobial and antibiofilm approaches to obtain a synergistic effect to permit efficient microbial growth control at low photosensitizer doses.

CHAPTER 1

POTENTIAL NOVEL THERAPEUTIC STRATEGIES IN CYSTIC FIBROSIS: ANTIMICROBIAL AND ANTI-BIOFILM ACTIVITY OF NATURAL AND DESIGNED α-HELICAL PEPTIDES AGAINST *Staphylococcus aureus*, *Pseudomonas aeruginosa*, AND *Stenotrophomonas maltophilia*

ARIANNA POMPILIO, VALENTINA CROCETTA, MARCO SCOCCH, STEFANO POMPONIO, VALENTINA DI VINCENZO, MARIO MARDIROSSIAN, GIOVANNI GHERARD, ERSILIA FISCARELLI, GIORDANO DICUONZO, RENATO GENNARO, and GIOVANNI DI BONAVENTURA

This chapter was originally published under the Creative Commons Attribution License. Pompilio A, Crocetta V, Scocch M, Pomponio S, Di Vincenzo V, Mardirossian M, Gherard G, Fiscarelli E, Dicuonzo G, Gennaro R, and Di Bonaventura G. Potential Novel Therapeutic Strategies in Cystic Fibrosis: Antimicrobial and Anti-Biofilm Activity of Natural and Designed α-Helical Peptides against Staphylococcus Aureus, Pseudomonas Aeruginosa*, and* Stenotrophomonas Maltophilia*.* BMC Microbiology ***12****, 145 (2012), doi:10.1186/1471-2180-12-145.*

1.1 BACKGROUND

Physicians treating patients with cystic fibrosis (CF) are increasingly faced with infections caused by multidrug-resistant strains. *Pseudomonas aeruginosa* and *Staphylococcus aureus* are the most common bacterial pathogens isolated from the CF respiratory tract where they cause persistent infections associated with a more rapid decline in lung function and survival [1,2]. In recent years, however, there has been an increasing number of reports on potentially emerging and challenging pathogens, probably due to improved laboratory detection strategies and to selective pressure exerted on bacterial populations by the antipseudomonal antibiotic therapy [2]. In this respect, both the overall prevalence and incidence of intrinsically antibiotic-resistant *Stenotrophomonas maltophilia* isolations from CF respiratory tract secretions have been recently reported [3-5].

Efforts to treat CF infections are also hampered by the high microbial adaptation to the CF pulmonary environment, resulting in an increased ability to form biofilms intrinsically resistant to therapeutically important antibiotics such as aminoglycosides, fluoroquinolones, and tetracycline [6-10].

Novel antimicrobial agents that could replace or complement current therapies are consequently needed to fight chronic infections in CF patients.

Antimicrobial peptides (AMPs) are naturally occurring molecules of the innate immune system that play an important role in the host defence of animals and plants [11-13]. Over the last years, natural AMPs have attracted considerable interest for the development of novel antibiotics for several reasons [14,15]: i) the broad activity spectrum, comprised multiply antibiotic-resistant bacteria; ii) the relative selectivity towards their targets (microbial membranes); iii) the rapid mechanism of action; and, above all, iv) the low frequency in selecting resistant strains. Although the antimicrobial activity of AMPs has been extensively reported in literature [13-17], only few studies have been reported with respect to CF pathogens [18-21].

Hence, in an attempt to evaluate the therapeutic potential of AMPs in the management of CF lung infections, for the first time in the present

study three cationic α-helical AMPs - two cathelicidins of bovine origin (BMAP-27, BMAP-28) and the artificial peptide P19(9/B) - were tested for their in vitro antibacterial effectiveness, as well as their in vitro anti-biofilm activity, against selected *S. aureus*, *P. aeruginosa*, and *S. maltophilia* strains collected from CF patients. The efficacy of the AMPs was compared to that of Tobramycin, selected as the antibiotic of choice used for chronic suppressive therapy in CF patients.

Since the conditions present in the CF patients' airway surface liquid could counteract the potency of antibiotics such as Tobramycin [22,23], in the present study all in vitro antimicrobial assays were carried out under experimental conditions simulating the physical-chemical properties observed in CF lung environment [24-26].

1.2 RESULTS

1.2.1 PHENOTYPIC FEATURES AND CLONAL RELATEDNESS OF CF STRAINS

A total of 9 out of 25 *P. aeruginosa* strains tested showed mucoid phenotype on MHA, while 3 exhibited SCV phenotype. Among 15 *S. aureus* isolates tested, 7 were methicillin-resistant.

PFGE analysis showed 8, 21, and 12 different pulsotypes among *S. aureus*, *S. maltophilia*, and *P. aeruginosa* isolates, respectively. Among *S. aureus* isolates, only the PFGE type 1 was shared by multiple strains, which comprised 8 isolates and 7 PFGE subtypes. Among *S. maltophilia* isolates, 2 multiple-strains PFGE types were observed: PFGE type 23 (5 isolates, 2 PFGE subtypes), and PFGE type 73 (2 isolates with identical PFGE profile). Among *P. aeruginosa* isolates, 5 multiple-strains PFGE types were observed: PFGE type 5 (6 isolates, 2 PFGE subtypes), PFGE type 1 (4 isolates with indistinguishable PFGE profile), PFGE types 9 and 11 (3 isolates each, with identical PFGE pattern), and PFGE type 8 (2 isolates, one PFGE subtype) (data not shown).

TABLE 1: In vitro activity of BMAP-27, BMAP-28, P19(9/B), and Tobramycin against *P. aeruginosa*, *S. maltophilia* and *S. aureus* CF strains

Bacterial strains (n)	Test agent:			
	BMAP-27	BMAP-28	P19(9/B)	TOBRAMYCIN
P. aeruginosa (25)				
MIC_{50}[a]	8	16	8	16
MIC_{90}[b]	16	32	32	>64
MIC_{range}	4-16	4–32	4–32	2->64
MBC_{50}[c]	8	16	16	32
MBC_{90}[d]	16	32	64	>64
MBC_{range}	4–16	4–64	4->64	2->64
MBC/MIC	1.3	1.2	1.9[e]	1.5[f]
S. maltophilia (27)				
MIC_{50}[a]	4	4	4	>64
MIC_{90}[b]	8	4	16	>64
MIC_{range}	4-8	2–8	4–32	4->64
MBC_{50}[c]	8	4	8	>64
MBC_{90}[d]	16	8	32	>64
MBC_{range}	4–32	2–16	4–64	8->64
MBC/MIC	1.9	1.3	1.7	1.3[g]
S. aureus (15)				
MIC_{50}[a]	64	8	64	>64
MIC_{90}[b]	>64	32	>64	>64
MIC_{range}	32->64	4–32	32->64	4->64
MBC_{50}[c]	>64	8	>64	>64
MBC_{90}[d]	>64	32	>64	>64
MBC_{range}	64->64	4–32	32->64	4->64
MBC/MIC	1.2[h]	1.2	1.2[i]	1.0[l]
Total (67)				
MIC_{50}[a]	8	4	8	>64
MIC_{90}[b]	>64	16	64	>64
MIC_{range}	4->64	2–32	4->64	2->64

TABLE 1: *Cont.*

Bacterial strains (n)	Test agent:			
	BMAP-27	BMAP-28	P19(9/B)	TOBRAMYCIN
MBC_{50} [c]	8	8	16	>64
MBC_{90} [d]	>64	16	>64	>64
MBC_{range}	4->64	2–64	4->64	2->64
MBC/MIC	1.5[m]	1.2	1.7[n]	1.4[o]

[a, b]MIC50 and MIC90: MIC (μg/ml) inhibiting 50 and 90% of the strains tested, respectively. [c, d]MBC50 and MBC90: MBC (μg/ml) eradicating 50 and 90% of the strains tested, respectively. Only isolates exhibiting in range MIC values were considered for killing quotient calculation (MBC/MIC): [e]n = 24; [f]n = 12; [g]n = 3; [h]n = 6; [i]n = 2; [m]n = 58; [n]n = 57;[o]n = 17.

1.2.2 IN VITRO ACTIVITY OF AMPS AND TOBRAMYCIN AGAINST PLANKTONIC CELLS: MIC, MBC

In order to determine the efficacy of AMPs, the antimicrobial activity was measured against 67 CF clinical isolates, and results are summarized in Table 1. Overall, BMAP-28 showed the widest activity spectrum among AMPs tested, as suggested by MIC90 and MBC90 values (16 μg/ml, for both), although all of them exhibited a species-specific activity. In fact, although AMPs showed comparable activity against *P. aeruginosa*, BMAP-28 was found to be more active than P19(9/B) against *S. maltophilia*, and resulted the best active AMP against *S. aureus* (MIC90: 32 μg/ml; MBC90: 32 μg/ml). Compared to AMPs, Tobramycin exhibited a lower activity (MIC90 and MBC90: >64 μg/ml) regardless of the species considered. Killing quotient values, calculated as MBC/MIC ratio, were <4 for all AMPs, as well as for Tobramycin, clearly suggesting a bactericidal activity. No differences in susceptibility levels to AMPs were found with regard to phenotype (mucoid, SCV, MRSA), pulsotype, or susceptibility to Tobramycin (data not shown).

TABLE 2: Antimicrobial activity of BMAP-27, BMAP-28, P19(9/B) and Tobramycin evaluated under different experimental conditions: "CF-like" (5% CO_2, pH 6.8, SCFM) and "standard CLSI-recommended" (aerobiosis, pH 7.2, CAMHB)

Bacterial strains	Susceptibility ($MIC_{CF\text{-}like}/MIC_{CLSI}$) to:			
	BMAP-27	BMAP-28	P19(9/B)	TOBRAMYCIN
P. aeruginosa				
Pa1	8/4	8/8	4/16	4/0.25
Pa5	8/4	16/16	8/8	16/2
Pa6	8/8	16/16	16/8	8/8
Pa9	8/4	16/16	16/8	64/1
Sm109	4/8	4/16	4/8	128/64
Sm126	8/16	8/32	4/32	256/64
Sm143	8/8	4/8	4/4	8/2
S. aureus				
Sa1	128/64	8/16	128/16	256/64
Sa3	64/64	4/32	64/16	256/16
Sa4	64/64	4/16	32/8	32/2
Sa7	64/16	4/16	64/8	256/2
Mean $MIC_{CF\text{-}like}/MIC_{CLSI}$	1.5	0.5	2.8	23.9
P. aeruginosa				
Pa1	8/8	8/16	16/32	4/1
Pa5	16/8	16/32	16/16	16/4
Pa6	16/8	16/16	16/32	8/8
Pa9	8/8	16/32	64/16	128/2
Sm109	8/16	8/16	8/8	256/128
Sm126	8/32	16/32	8/32	256/64
Sm143	16/8	8/8	4/4	8/8
Sa1	128/64	8/16	128/16	256/64
Sa3	64/64	4/32	64/16	256/32
Sa4	64/64	8/32	32/8	32/2
Sa7	64/ND[a]	8/16	64/8	256/4
Mean $MBC_{CF\text{-}like}/MBC_{CLSI}$	1.2	0.5	2.9	15.6

a ND, not determined.

MIC and MBC values obtained under CLSI-recommended or "CF-like" experimental conditions (see Materials and Methods section) are shown in Table 2. Comparative evaluation of these values showed that mean MICCF-like/MICCLSI and MBCCF-like/MBCCLSI values obtained for Tobramycin (23.9 and 15.6, respectively) were significantly higher than those observed for BMAP-27 (1.5 and 1.2, respectively; $p < 0.001$), BMAP-28 (0.5 and 0.5, respectively; $p < 0.001$), and P19(9/B) (2.8 and 2.9, respectively; $p < 0.001$), regardless of species tested, indicating a reduced antibiotic activity of Tobramycin in CF-like conditions.

1.2.3 BACTERICIDAL KINETICS

Time-killing results have been summarized in Figure 1. BMAP-27, BMAP-28, and P19(9/B) exerted a rapid bactericidal activity against *P. aeruginosa*, reducing the number of viable bacterial cells of at least 3 logs within 60 min of exposure. However, the bactericidal effect of BMAP-28 against *P. aeruginosa* was incomplete for two (Pa6 and Pa22) of the three strains tested, allowing bacterial regrowth after 24-h incubation, although at levels lower than those observed for untreated control. In parallel experiments, Tobramycin showed only a bacteriostatic effect against *P. aeruginosa*, causing no more than 1-log reduction in viable count after 24 h.

BMAP-27, BMAP-28 and P19(9/B) exerted bactericidal activity also against *S. maltophilia*, although with streaking strain-specific differences. Particularly, BMAP-28 exhibited only bacteriostatic effect against Sm192 strain, while P19(9/B) showed a rapid bactericidal effect against Sm138 strain, causing more than a 4-log reduction in viable count after 10 min-exposure. Tobramycin exhibited a late (after 24-h exposure) bactericidal effect only against Sm138 strain.

AMPs activity against *S. aureus* was significantly strain-specific, ranging from the rapid bactericidal activity of BMAP-28 against Sa10 strain, to the bacteriostatic effect of P19(9/B) and BMAP-28 against Sa4 strain. Tobramycin showed a bactericidal effect against all *S. aureus* strains tested, although allowing bacterial regrowth of Sa4 strain after 2-h exposure.

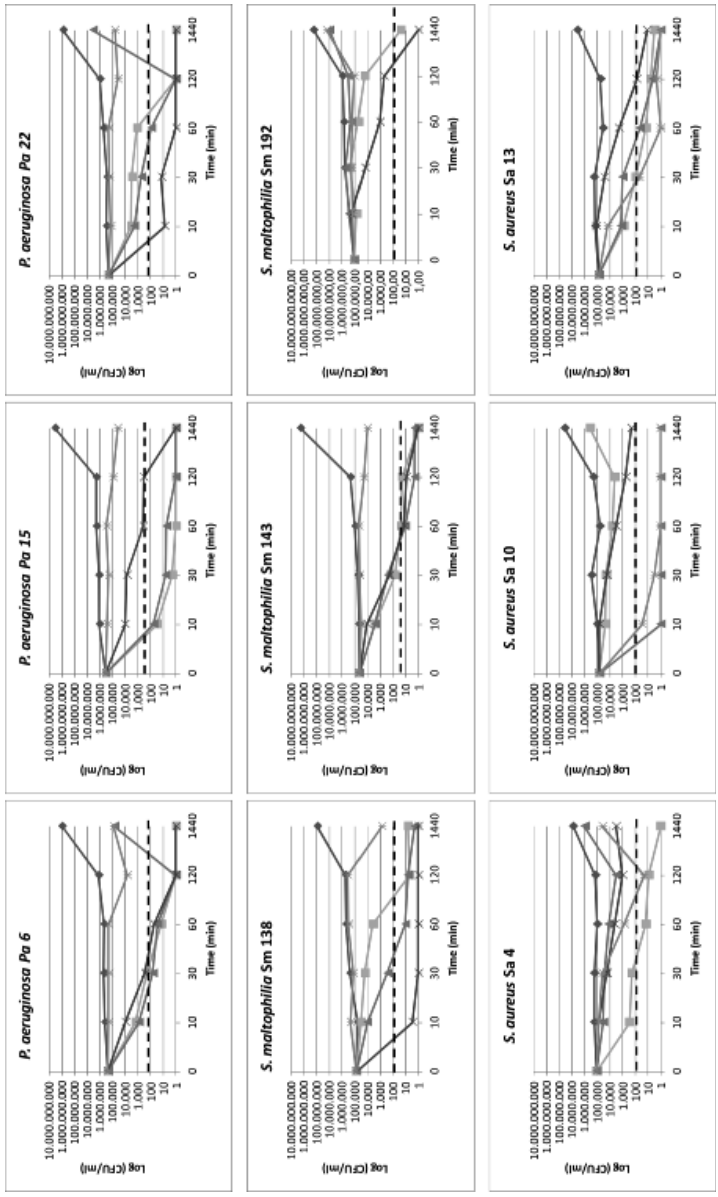


FIGURE 1: Time-killing kinetic of AMPs against CF strains. BMAP-27 (■), BMAP-28 (*), P19(9/B) (×), and Tobramycin () were tested at MIC value against representative *P. aeruginosa* (Pa6, Pa15, and Pa22), *S. maltophilia* (Sm138, Sm143, and Sm192), and *S. aureus* (Sa4, Sa10, and Sa13) CF strains. Controls (♦) were not exposed to drugs. Values are the mean of two independent experiments performed in triplicate. The dotted line indicates a 3-log reduction in viability.