

Antibiofilm Peptides: Potential as Broad-Spectrum Agents

 Daniel Pletzer, Robert E. W. Hancock

Centre for Microbial Diseases and Immunity Research, Department of Microbiology and Immunology, University of British Columbia, Vancouver, Canada

The treatment of bacterial diseases is facing twin threats, with increasing bacterial antibiotic resistance and relatively few novel compounds or strategies under development or entering the clinic. Bacteria frequently grow on surfaces as biofilm communities encased in a polymeric matrix. The biofilm mode of growth is associated with 65 to 80% of all clinical infections. It causes broad adaptive changes; biofilm bacteria are especially (10- to 1,000-fold) resistant to conventional antibiotics and to date no antimicrobials have been developed specifically to treat biofilms. Small synthetic peptides with broad-spectrum antibiofilm activity represent a novel approach to treat biofilm-related infections. Recent developments have provided evidence that these peptides can inhibit even developed biofilms, kill multiple bacterial species in biofilms (including the ESKAPE [*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species] pathogens), show strong synergy with several antibiotics, and act by targeting a universal stress response in bacteria. Thus, these peptides represent a promising alternative treatment to conventional antibiotics and work effectively in animal models of biofilm-associated infections.

Pathogenic bacteria are dynamically evolving organisms that are capable of causing infections in a large variety of hosts, including humans, animals, plants, and insects. The discovery and deployment of antibiotics had a massive impact on modern medicine, since many diseases caused by infectious bacteria (e.g., pneumonia) could be successfully treated. A large arsenal of different antibiotics, either from natural sources or produced by semisynthetic or synthetic means, has been developed, to help physicians treat bacterial infections. In a world without antibiotics, even minor injuries might lead to serious health problems, and procedures such as major surgeries, cytotoxic therapies, transplantations, etc., would be virtually impossible. Unfortunately, antibiotics are a victim of their own success, since their broad availability, low cost, and tremendous effectiveness have led to their overuse in hospitals and agricultural settings. This has created ideal conditions for the selection and spread of antimicrobial-resistant microorganisms. The most concerning microbes in hospitals are the so-called ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens. This global resistance threat, which has steadily increased over several decades, has led to less active or ineffective drugs (1). Recently, it was reported that a bacterial strain developed resistance toward polymyxin, a last-resort antibiotic for multidrug-resistant Gram-negative infections (2).

Antimicrobial agents are the first-line treatment for sepsis, a complex clinical syndrome involving an underlying infection in conjunction with a dysfunctional host immune response (i.e., systemic inflammatory response syndrome) (3). Given the 18 million cases of sepsis worldwide and the very high (~30%) rate of mortality, the 5 million deaths from sepsis (210,000 in the United States) reveal the enormous current impact of ineffective antibiotics combined with a lack of host-directed immune therapies for sepsis (4). Similarly, patients with a perturbed immune system (e.g., undergoing chemotherapy, immunosuppressive disease, major injuries, or burns) acquire infections that are often more difficult to treat. Another significant cause of microbial infections is biofilm-associated infections (5). Biofilms are structured aggregates of microorganisms embedded in a matrix composed of poly-

saccharides, extracellular DNA, proteins, and/or lipids (6). Biofilm-related infections typically involve colonization and strong attachment to various living (in chronic infections such as endocarditis, osteomyelitis, colonization of lungs [e.g., in cystic fibrosis or chronic obstructive pulmonary disease], dental caries, etc.) and nonliving (central venous and urinary catheters, stents, orthopedic prosthesis, mechanical heart valves, contact lenses, etc.) surfaces (7). Biofilm formation on surfaces can result in recalcitrant septic complications, and the only option for treating, e.g., an implant-related infection is to remove the device, treat the infection with antibiotics, and replace the implant (8). However, this procedure tremendously increases both treatment costs and patient trauma as well as increasing the risk of complications (9).

Biofilms are a growth adaptation to environmental stress that enables the organisms to resist stressors, including the host immune system and antibiotics, making them difficult to treat and eradicate (10, 11). If bacteria succeed growing and forming a biofilm within a host, the infection will become very difficult to treat and will further develop into a chronic biofilm-based infection (12). Bacteria growing in biofilms exhibit increased (adaptive) resistance to essentially all antibiotics, and while some antibiotics are used in such infections, with mixed success, to date not a single antibiotic has actually been developed for biofilm infections. Thus, while antibiotics are the single most successful medical intervention (13), we are urgently in need of alternative strategies to treat and prevent infections, especially biofilm infections.

Bacteria are rarely found as planktonic (free-swimming) organisms in nature but rather are found associated with surfaces or microbial mats in response to environmental stresses (14) and generally tend to be polymicrobial, often involving both bacteria

Accepted manuscript posted online 11 April 2016

Citation Pletzer D, Hancock REW. 2016. Antibiofilm peptides: potential as broad-spectrum agents. *J Bacteriol* 198:2572–2578. doi:10.1128/JB.00017-16.

Editor: G. A. O'Toole, Geisel School of Medicine at Dartmouth

Address correspondence to Robert E. W. Hancock, bob@hancocklab.com.

Copyright © 2016, American Society for Microbiology. All Rights Reserved.

and fungi. In the human body, most chronic infections are thought of as containing one prominent bacterium, although there are important exceptions, e.g., dental biofilms (known as plaque) that contain hundreds of species (15) or bacterial biofilms formed by multiple species in chronic nonhealing wounds (12). Biofilms undergo a staged development process that varies from organism to organism but generally follows at least five processes: surface association, gene expression changes leading to tight surface binding, microcolony formation, construction of a mature biofilm colony, and partial dissolution and dispersal of planktonic organisms that have the potential to nucleate new biofilms at distant locations. The processes are disparate but appear to involve various adhesins for initial attachment and formation of an intrabiofilm matrix (e.g., various polysaccharides, extracellular DNA, etc.) that may function in both tight binding and association of organisms in the biofilm. The basis for initiation of the biofilm development program is still unclear, but it appears to involve adherence to surfaces, which triggers regulatory responses, and stress or stress-like conditions, perhaps triggering the stringent stress response mediated through the nucleotide signal ppGpp. Other signals that have been described are cyclic dinucleotides and quorum sensing. Thus, biofilm formation is associated with significant lifestyle changes, and these provide both an opportunity and a substantial challenge when considering the medical aspects of biofilms. The opportunity is based on a series of unique events that can be considered potential targets for inhibiting biofilm bacteria, such as, e.g., interference with quorum-sensing pathways by inhibiting enzymatic degradation of produced signal molecules or by blocking signal reception/production (16). It has been demonstrated in *P. aeruginosa* that mutants defective in various quorum-sensing systems form flat, undifferentiated biofilms (17), suggesting that quorum-sensing signals act at the level of biofilm maturation in many cases, although the specific targeted signals vary from organism to organism.

The challenge, however, is that microbes growing in a biofilm demonstrate high resistance (10- to 1,000-fold compared to that for planktonic growth) toward almost all conventional antibiotics and antiseptics (18). Various factors have been proposed to account for this high resistance to antimicrobials, including the differential expression of many genes (some influencing antibiotic susceptibility), secretion of an extracellular matrix that might act to bind certain antibiotics, the trapping and consequent higher local concentration of antibiotic-degrading enzymes, and the low metabolic state at the base of the biofilm (19). Regardless of the cause, resistance is adaptive, since as biofilm-related resistant bacteria disperse from a biofilm, they revert to their planktonic susceptible phenotype (11). Susceptibility testing of recalcitrant biofilm cells is rarely performed in clinics or during the antibiotic discovery and development process, since conventional methods are not able to detect this type of resistance under laboratory conditions (19).

CONVENTIONAL COMBINATION THERAPY APPROACHES TO BIOFILM TREATMENT

Biofilm-related infections are difficult to treat in clinical environments, and to date no antimicrobial agent has been specifically developed to address this problem (19). Therefore, physicians often use a cocktail of various antibiotics, largely from distinct antibiotic classes (e.g., β -lactams paired with aminoglycosides or fluoroquinolones) (18). A potential benefit of having two anti-

microbial agents is a possible synergistic effect (i.e., an enhanced rate of killing of microbes) to more effectively treat bacterial infections such as those associated with ventilator-associated pneumonia or sepsis (20). However, the use of broad empirical coverage of two antibiotics from different classes raises the question of whether combined therapy is beneficial for the patient or whether it aids the development of multidrug-resistant bacteria.

Almost no data exist on synergistically treating organisms growing in biofilms. Although some potential combinations to overcome adaptive resistance of biofilms have been demonstrated (e.g., using a colistin-tobramycin combination for clearing *Pseudomonas*-related pulmonary biofilm infection) (21, 22), the molecular mechanisms driving the synergistic effects of two different antibiotic classes are poorly understood. For example, the aminoglycoside tobramycin and the macrolide clarithromycin show synergistic effects on clearing biofilms, although the individual antibiotics have only moderate effects (22). Conversely, combinations of tobramycin with other macrolides (such as azithromycin) show antagonistic effects, i.e., reduced effectiveness compared to that for individual usage (22). Thus, we need to gather more information about the mechanisms underlying synergistic treatment of biofilm infections.

A NOVEL APPROACH TO BIOFILM TREATMENT: ANTIBIOFILM PEPTIDES

In recent years, various approaches to treat bacterial biofilm infections (e.g., quorum-sensing antagonism, antibodies, antiadhesion strategies, bacteriophages, etc.) have been developed (23). One promising alternative is based on a new property of cationic, amphipathic peptides (24). These antibiofilm peptides are a distinct group of the antimicrobial/host defense peptides. Natural peptides range in size from 12 to 50 amino acids with a net charge of +2 to +9 (due to the presence of several Arg or Lys residues) and around 50% hydrophobic amino acids, and they have diverse amino acid sequences and structures (24). Interestingly, antimicrobial peptides developed for killing planktonic bacteria possess a broad-range activity and a low rate of inducing bacterial resistance (14), and they continue to be developed clinically. More than 2,600 peptides (25) have been identified in all forms of life. In higher organisms, they are part of the innate immune response and act as important host defense molecules. Their natural activities range from modulation of immunity and other host responses to direct bacterial killing (26).

Starting from the observation that the human cathelicidin LL-37 was able to inhibit *Pseudomonas* biofilm formation at 1/16 of its MIC for planktonic organisms (27, 28), it was subsequently demonstrated that antibiofilm peptides were a distinct subset of peptides with similar overall amino acid compositions but distinct structure-activity relationships compared to those of antimicrobial peptides (29). Generally speaking, it has been possible to generate novel antibiofilm peptides that (i) kill multiple species of bacteria in biofilms (with a minimal biofilm eradication concentration of less than 1 $\mu\text{g}/\text{ml}$), including major clinically relevant antibiotic-resistant Gram-negative and Gram-positive bacteria (Fig. 1A), (ii) work synergistically with antibiotics in multiple species (Fig. 1B), and (iii) are effective in animal models of biofilm infections such as, e.g., *P. aeruginosa* (30). Structure-activity relationship studies have confirmed no major overlap between antibiofilm and antimicrobial (versus planktonic bacteria) activities, and indeed organisms completely resistant to antibiotic peptides

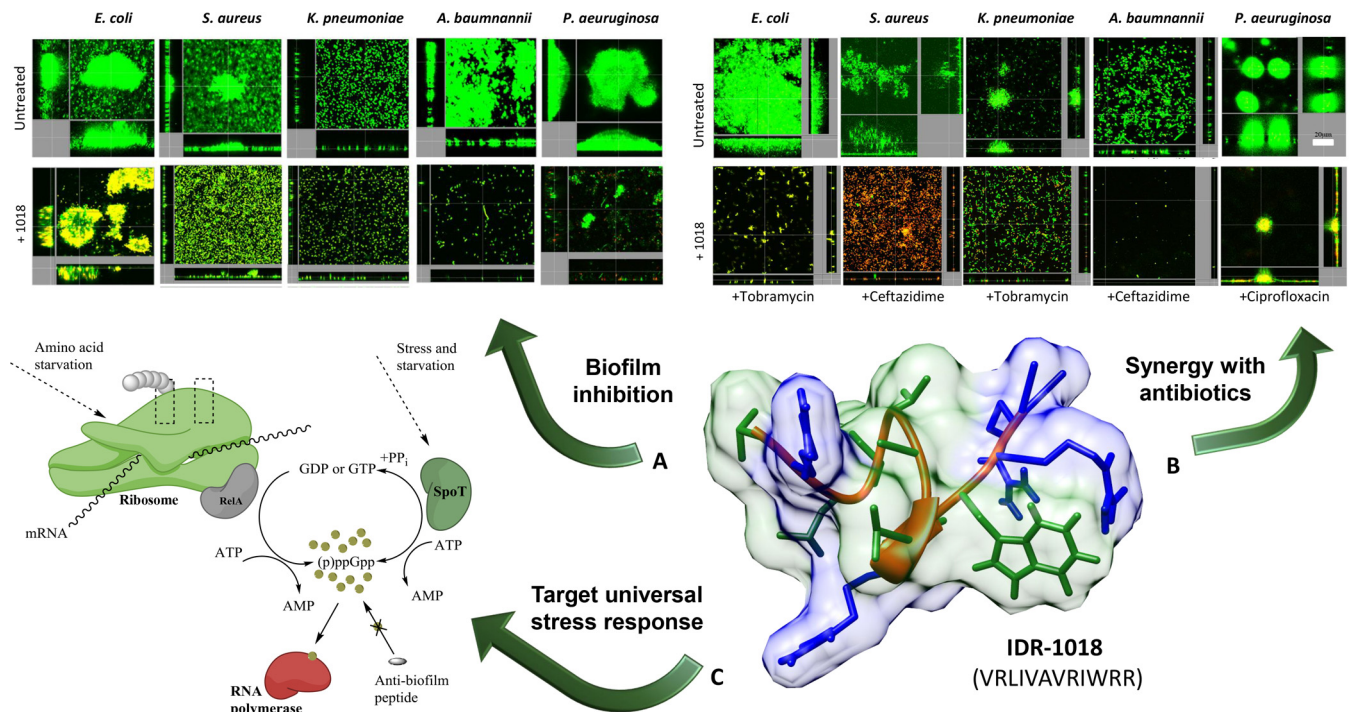


FIG 1 Overview of antibiofilm peptide activity based on the example of IDR-1018. The peptide structure was originally published by Wieczorek et al. (56), and the structural coordinate file was kindly provided by Suzana Straus. (Republished from reference 39.) (A) Antibiofilm peptides are able to inhibit developed biofilms and kill multiple species in biofilms, including the ESKAPE pathogens. (B) Antibiofilm peptides show strong synergy with several antibiotics, thereby completely eradicating preformed biofilms. (C) Antibiofilm peptides act by targeting a universal stress response in bacteria.

(e.g., *Burkholderia* spp.) can still be treated with antibiofilm peptides (29).

Intriguingly, LL-37 fits into the class of peptides termed host defense peptides, which can modulate the immune response to infection, thereby assisting the body in recognizing and clearing invading bacteria (31). Thus, LL-37 shows strong anti-inflammatory activity in dampening pathogen-induced proinflammatory cytokines as well as recruiting key effector cells responsible in responding to infections. LL-37 demonstrates a broad range of host-directed activities, including the upregulation of neutrophil responses, downregulation of proinflammatory cytokines and gamma interferon (IFN- γ) (31), increased angiogenesis (32), increased rat survival in a lethal sepsis model (33), and improved wound healing (34). As mentioned above, despite its weak antimicrobial activity, it inhibits and eradicates preformed bacterial biofilm formation at very low concentrations (27). Moreover, LL-37-derived peptides were able to eradicate existing biofilms of methicillin-resistant *S. aureus* (MRSA) in wounded human skin (35). Thus, this demonstrates a potential added benefit for antimicrobial infection, i.e., an ability to favorably modulate host immunity, especially suppressing inflammation, which often accompanies chronic biofilm-mediated infections.

BROAD SPECTRUM OF IMMUNOMODULATORY PEPTIDES WITH ANTIBIOFILM ACTIVITY

A major breakthrough was made when it was realized that peptides much smaller than LL-37 were able to effectively inhibit biofilms, including the nine-amino-acid peptide 1037. Indeed, a peptide originally selected as an optimized antibiofilm pep-

ptide, IDR-1018 (36), demonstrated very broad-spectrum activity against representative nosocomial pathogens, including *P. aeruginosa*, *Escherichia coli*, *A. baumannii*, *K. pneumoniae*, methicillin-resistant *S. aureus*, *Salmonella enterica* serovar Typhimurium, and *Burkholderia cenocepacia* (37). At 10 $\mu\text{g/ml}$, the peptide killed organisms even in preformed biofilms (Fig. 1A), while at 0.8 $\mu\text{g/ml}$, the peptide induced biofilm dispersal. Both flow cell biofilms and simple plastic adhesive biofilms could be inhibited.

It is important to highlight that increased dispersal of bacterial cells from biofilms might represent a potential danger in clinical settings, as dispersed cells may infect other organs or cause septic shock (38), indicating that it might be important to utilize such peptides in combination with antibiotics. In this regard, Refuvelle et al. (39) showed synergy between peptide 1018 and a variety of highly utilized clinical antibiotics (ceftazidime, ciprofloxacin, imipenem, and tobramycin) against several multi-drug-resistant ESKAPE pathogens (Fig. 1B). Due to the synergy between antibiofilm peptides and conventional antibiotics, a large drop in antibiotic MIC was observed with many combinations. Even short-term treatment with IDR-1018 and ciprofloxacin prevented biofilm formation and eradicated preexisting *P. aeruginosa* biofilms (39). Thus, these peptides represent a genuine antiresistance strategy for biofilm bacteria. Indeed, antibiofilm peptides were recently shown to reverse *K. pneumoniae* carbapenemase (KPC)-mediated resistance in *K. pneumoniae*, in that, e.g., 0.0625 $\mu\text{g/ml}$ meropenem combined with 0.125 $\mu\text{g/ml}$ DJK5 led to complete eradication of preexisting flow cell biofilms (40).

METHODS TO OPTIMIZE AND IMPROVE ANTIBIOFILM PEPTIDES

An easy, reliable, and relatively inexpensive method for screening large numbers of synthetic peptides is the SPOT synthesis (peptide array) technique (41). This procedure involves robotic synthesis of peptides on addressable spots, containing diglycine linkers, on a cellulose membrane by stepwise coupling of 9-fluorenylmethoxycarbonyl (Fmoc)-derivatized amino acids followed by deprotection and cleavage from the membrane (42). With this technique, hundreds of peptides can be synthesized in parallel and relatively inexpensively. Cherkasov et al. (43) used this approach to identify structure-activity relationships among the antimicrobial peptides, and this combined with neural network methods enabled computational predictions of the activity of virtual peptides based solely on the amino acid sequence.

Recently, Haney et al. (44) used a SPOT-synthesized peptide array on cellulose membranes to assess and improve both the antibiofilm and immunomodulatory (anti-inflammatory and chemokine induction) activities of hundreds of variants of the 12-mer peptides IDR-1002 and HH2 against *S. aureus* biofilms. They used single amino acid substitutions to exchange one of nine optimal amino acids, previously found in good antibiofilm peptides (29), into every position of these 12-mers. The information obtained about the biological activity of these peptides was combined and incorporated into the design of new peptides with more than one favorable substitution. This allowed the identification of a novel peptide, 2009, with an overall improved activity profile. However, they also pointed out that although individual improvement through substituting single amino acids leads to fine-tuning of a peptide, a combination of all possible improvements might lead to an unintended overall decrease in activity (e.g., decreasing hydrophobicity prevents membrane interaction).

In another approach, de la Fuente-Nunez et al. (30) designed and screened various protease-resistant D-enantiomeric peptides, including retro (D-amino acid sequence) and retro-inverso (reversed D-amino acid sequence) versions of peptides related to the known synthetic innate defense regulator (IDR) peptide 1018 that had demonstrated good broad-spectrum antibiofilm activity (37). It was found that D-amino acid versions of peptides tended to be more active; however, there was no obvious relationship between enantiomeric composition and activity, as might be expected if there was a specific “receptor” for these peptides in bacteria. Moreover, these peptides showed broad-spectrum activity, both preventing biofilm formation when added at the initial stages of biofilm formation and promoting bacterial dispersal and eradication when added after 2 days of biofilm growth. Like for peptide 1018, the best D-enantiomeric peptides showed synergy with selected conventional antibiotics in eradicating mature biofilms produced by various organisms (30).

Developing optimized antibiofilm peptides, while at the same time minimizing cytotoxicity, reducing proteolytic degradation, and promoting synergy with conventional antibiotics, is clearly an important step in fighting antibiotic-resistant biofilm infections (45, 46). One major challenge with the development of new antimicrobial peptides is still the high production costs of such peptides. Another important factor to consider is the importance of empirical testing of new compounds. While using an inexpensive high-throughput method such as biofilm detection using crystal violet might be suitable for screening of large peptide libraries, it

does not really mimic many types of biofilms, and it is necessary to experimentally confirm observed phenotypes using alternative methods such as flow cells. Possible approaches to reducing production costs are, e.g., shorter peptide analogs, recombinant peptide production, or improvement of pharmacokinetics to decrease the required dose (24). Additionally, computational modeling enables a strong understanding of structure-activity relationships among peptides. *In silico* approaches may drastically reduce time and costs by helping to identify, develop, and optimize novel peptides (e.g., with improved antimicrobial properties) (47).

PEPTIDES DESTROY COMPLEX ORAL BIOFILMS

Biofilms in nature and disease and especially in the oral cavity often involve consortia of different organisms. It was recently shown that peptide IDR-1018 is able to inhibit as well as destroy mixed-bacterial oral biofilms (48). Dental plaque is a biofilm-related oral infection caused by microbial communities comprised of hundreds of microorganisms exhibiting high antibiotic resistance (49). Thus, it was shown (48) that peptide 1018 was able to kill oral biofilms on hydroxyapatite (the primary mineral in tooth enamel) surfaces. Moreover, a combined treatment for only 1 to 3 min with IDR-1018 in combination with the traditional oral disinfectant chlorhexidine increased the antibiofilm activity, causing a significant reduction in biofilm volume and increased numbers of dead cells within the biofilms.

ANTIBIOFILM PEPTIDES TARGET A CELLULAR STRESS RESPONSE

Antimicrobial peptides have complex mechanisms of action, directly targeting either bacterial membranes, causing loss of cellular integrity and/or disruption of macromolecular synthesis dependent on a membrane-linked event (e.g., cell wall biosynthesis), or translocation through the membrane to reach an intracellular target (45). However, since antibiofilm peptides are distinguishable from antimicrobial peptides, they must have a unique mechanism of action that involves the biofilm lifestyle. Many events in biofilm development are highly variable between the different organisms that are targeted by antibiofilm peptides, including adhesion organelles, the matrix components, whether biofilms are structured or not, quorum-sensing mechanisms, and dispersal.

It is generally considered that starvation conditions promote biofilm formation and that bacteria living under starvation conditions show increased antibiotic tolerance (50). Antibiotic tolerance associated with nutrient limitation is a tightly controlled regulatory pathway. For example, activation of the stringent response leads to increased antibiotic tolerance during starvation in *P. aeruginosa* (50). Through activation of the stringent response, bacteria are able to cope with stressful environmental situations (including amino acid, carbon, nitrogen, iron, phosphorus, or lipid limitation, as well as oxidative and temperature stress). The stringent response involves the synthesis of the small signaling nucleotides (i.e., “alarmones”) guanosine pentaphosphate and the active moiety tetraphosphate (ppGpp), which serves as a secondary messenger molecule to coordinate the stress response by binding to and altering the specificity of RNA polymerase (Fig. 1C).

In most bacteria, the two homologous proteins RelA and SpoT are responsible for modulating intracellular concentrations of ppGpp (51). Interestingly, the stringent stress response is highly conserved among Gram-negative and Gram-positive bacteria, but

some bacteria (e.g., *S. aureus*) express only a single RelA/SpoT homolog, Rsh (30, 37, 51, 52). RelA is a monofunctional synthase able to generate pppGpp using GTP and ATP, which is subsequently dephosphorylated to ppGpp. SpoT or Rsh homologs have a bifunctional hydrolase-synthase activity and can therefore synthesize ppGpp and pppGpp (and hydrolyze it to GDP, GTP, or pyrophosphate) (Fig. 1C) (51).

The stringent response appears to play an important role in biofilm development, since mutants lacking both RelA and SpoT (or Rsh) are unable to form biofilms in various organisms (30, 37, 52). Activation of the stringent response affects transcription of hundreds of genes that collectively act to divert cellular energy away from cell division and into altered metabolism and stress-coping mechanisms. Currently, the specific role of (p)ppGpp in biofilm development is unknown, but it might be involved in initiating and/or perpetuating biofilm development and/or limiting dispersal (37).

The antibiofilm peptides 1018, DJK-5, and DJK-6 were all shown to act by binding to and triggering the degradation of ppGpp (30, 37). Direct interaction was demonstrated by the coprecipitation of IDR-1018 with ppGpp and by ³¹P nuclear magnetic resonance (NMR) line broadening (37). When added to cells from multiple different bacterial species treated with DL-serine hydroxamate (SHX) (a serine analog that inhibits seryl-tRNA synthetase) to trigger the stringent response, 1018 caused complete loss of ppGpp within 30 min as monitored using NMR spectrometry on intact cells and thin-layer chromatography of cellular contents. Furthermore, overexpression of the RelA synthase gene blocked peptide IDR-1018 action in *P. aeruginosa* and *S. aureus* strains, while genetically turning off ppGpp synthesis in 2-day-old *Pseudomonas* biofilms mimicked the action of 1018 on preformed biofilms.

PEPTIDES SHOW PROTECTION IN ANIMAL MODELS

Bacterial colonization on implant devices is a major threat to patients (8, 9) and antibiotic treatment an inevitable consequence, thereby increasing the risk of emerging multidrug-resistant bacteria. *In vivo* experiments in a murine model of *S. aureus* orthopedic implant biofilm infections showed that peptide IDR-1018 accelerates *S. aureus* clearance by decreasing the bacterial burden on implants, recruiting macrophages to the infection site, and preserving osseointegration (i.e., connection between the bone and implant) (53). Conversely, the D-enantiomeric protease-resistant peptides DJK-5 and DJK-6 were more active than peptide 1018 and its retro-inverso equivalent RI-1018 in showing protection against lethal *P. aeruginosa* biofilm infections in the nematode *Caenorhabditis elegans* and insect larvae of the moth *Galleria mellonella* (30).

Peptides have been tested for their ability to clear bacterial infections in various models, although in most cases the activity was attributed to immunomodulatory activity, increasing infection-resolving mechanisms such as induction of recruitment of phagocytic cells while suppressing inflammatory cytokines. Thus, activity has been demonstrated in animal infection models against *S. aureus*, *E. coli*, multidrug-resistant tuberculosis, and cerebral malaria as well as in models of wound healing and preterm birth (36, 54).

CONCLUSION

The treatment of bacterial infections caused by biofilm-producing microbes is currently a difficult and complex challenge but is important due to their major threat to human health. Biofilm-related infections are highly adaptively resistant to numerous antibiotics, and many infections of this type cannot be adequately treated with a single antimicrobial drug. Currently, there are no compounds available that specifically address biofilm infections, and the lack of appropriate clinical methods to determine the resistance profiles of clinical isolates under biofilm growth conditions is a major issue. The failure to treat chronic infections that are usually caused through biofilm recalcitrance highlights the urgent need for new strategies to fight these infections. High concentrations of antibiotics, which might inhibit or disperse biofilm growth, can be toxic to the human body, and even these are not guaranteed to successfully treat such infections. Hence, finding a treatment to block the distinct biofilm growth state represents a promising strategy. In particular, interrupting the complex regulatory systems involved in biofilm formation without selecting for resistant populations has the potential to lead the way in the future. Antibiofilm peptides have broad-spectrum activity and thus are exciting prospects. In addition, they demonstrate exciting synergy with conventional antibiotics. They appear to be less prone to cause resistance mechanisms (but can still engender resistance, as recently demonstrated for LL-37 [55]) than conventional antibiotics. Moreover, selective pressure on bacterial invaders can be minimized by utilizing them in combination with conventional antibiotics and/or by developing peptides with additional immunomodulatory properties. In summary, current research has shown that the combined treatment of antibiotics with antibiofilm peptides offers a very potent treatment of both biofilm and dispersed infection, thereby forming the basis for novel adjuvant therapies.

FUNDING INFORMATION

This work, including the efforts of Robert E. W. Hancock and Daniel Pletzer, was funded by HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID) (R33AI098701). This work, including the efforts of Robert E. W. Hancock and Daniel Pletzer, was funded by Gouvernement du Canada | Canadian Institutes of Health Research (CIHR) (MOP-123477).

D.P. received a Feodor Lynen postdoctoral fellowship from the Alexander von Humboldt Foundation, while R.E.W.H. holds a Canada Research Chair.

REFERENCES

1. WHO. 2014. Antimicrobial resistance: global report on surveillance. WHO, Geneva, Switzerland.
2. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu LF, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu JH, Shen J. 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* 16:161–168. [http://dx.doi.org/10.1016/S1473-3099\(15\)00424-7](http://dx.doi.org/10.1016/S1473-3099(15)00424-7).
3. Lyle NH, Pena OM, Boyd JH, Hancock REW. 2014. Barriers to the effective treatment of sepsis: antimicrobial agents, sepsis definitions, and host-directed therapies. *Ann N Y Acad Sci* 1323:101–114. <http://dx.doi.org/10.1111/nyas.12444>.
4. MacArthur RD, Miller M, Albertson T, Panacek E, Johnson D, Teoh L, Barchuk W. 2004. Adequacy of early empiric antibiotic treatment and survival in severe sepsis: experience from the MONARCS trial. *Clin Infect Dis* 38:284–288. <http://dx.doi.org/10.1086/379825>.
5. Davies D. 2003. Understanding biofilm resistance to antibacterial agents. *Nat Rev Drug Discov* 2:114–122. <http://dx.doi.org/10.1038/nrd1008>.

6. Sutherland IW. 2001. The biofilm matrix—an immobilized but dynamic microbial environment. *Trends Microbiol* 9:222–227. [http://dx.doi.org/10.1016/S0966-842X\(01\)02012-1](http://dx.doi.org/10.1016/S0966-842X(01)02012-1).
7. Kennedy P, Brammah S, Wills E. 2010. Burns, biofilm and a new appraisal of burn wound sepsis. *Burns* 36:49–56. <http://dx.doi.org/10.1016/j.burns.2009.02.017>.
8. Carmen JC, Roeder BL, Nelson JL, Ogilvie RL, Robison RA, Schaalje GB, Pitt WG. 2005. Treatment of biofilm infections on implants with low-frequency ultrasound and antibiotics. *Am J Infect Control* 33:78–82. <http://dx.doi.org/10.1016/j.ajic.2004.08.002>.
9. Romling U, Balsalobre C. 2012. Biofilm infections, their resilience to therapy and innovative treatment strategies. *J Intern Med* 272:541–561. <http://dx.doi.org/10.1111/joim.12004>.
10. Hoiby N, Ciofu O, Bjarnsholt T. 2010. *Pseudomonas aeruginosa* biofilms in cystic fibrosis. *Future Microbiol* 5:1663–1674. <http://dx.doi.org/10.2217/fmb.10.125>.
11. O'Toole G, Kaplan HB, Kolter R. 2000. Biofilm formation as microbial development. *Annu Rev Microbiol* 54:49–79. <http://dx.doi.org/10.1146/annurev.micro.54.1.49>.
12. Bjarnsholt T. 2013. The role of bacterial biofilms in chronic infections. *APMIS Suppl* <http://dx.doi.org/10.1111/apm.12099>.
13. Hancock REW. 2007. The end of an era? *Nat Rev Drug Discov* 6:28. <http://dx.doi.org/10.1038/nrd2223>.
14. Di Luca M, Maccari G, Nifosi R. 2014. Treatment of microbial biofilms in the post-antibiotic era: prophylactic and therapeutic use of antimicrobial peptides and their design by bioinformatics tools. *Pathog Dis* 70:257–270. <http://dx.doi.org/10.1111/2049-632X.12151>.
15. Karygianni L, Al-Ahmad A, Argyropoulou A, Hellwig E, Anderson AC, Skaltsounis AL. 2015. Natural antimicrobials and oral microorganisms: a systematic review on herbal interventions for the eradication of multispecies oral biofilms. *Front Microbiol* 6:1529. <http://dx.doi.org/10.3389/fmicb.2015.01529>.
16. Annous BA, Fratamico PM, Smith JL. 2009. Scientific status summary: quorum sensing in biofilms: why bacteria behave the way they do. *J Food Sci* 74:R24–R37. <http://dx.doi.org/10.1111/j.1750-3841.2008.01022.x>.
17. Rasamiravaka T, Labtani Q, Duez P, El Jaziri M. 2015. The formation of biofilms by *Pseudomonas aeruginosa*: a review of the natural and synthetic compounds interfering with control mechanisms. *Biomed Res Int* 2015:759348. <http://dx.doi.org/10.1155/2015/759348>.
18. Wu H, Moser C, Wang HZ, Hoiby N, Song ZJ. 2015. Strategies for combating bacterial biofilm infections. *Int J Oral Sci* 7:1–7. <http://dx.doi.org/10.1038/ijos.2014.65>.
19. Taylor PK, Yeung AT, Hancock REW. 2014. Antibiotic resistance in *Pseudomonas aeruginosa* biofilms: towards the development of novel anti-biofilm therapies. *J Biotechnol* 191:121–130. <http://dx.doi.org/10.1016/j.jbiotec.2014.09.003>.
20. Tamma PD, Cosgrove SE, Maragakis LL. 2012. Combination therapy for treatment of infections with gram-negative bacteria. *Clin Microbiol Rev* 25:450–470. <http://dx.doi.org/10.1128/CMR.05041-11>.
21. Herrmann G, Yang L, Wu H, Song Z, Wang H, Hoiby N, Ulrich M, Molin S, Riethmuller J, Doring G. 2010. Colistin-tobramycin combinations are superior to monotherapy concerning the killing of biofilm *Pseudomonas aeruginosa*. *J Infect Dis* 202:1585–1592. <http://dx.doi.org/10.1086/656788>.
22. Tre-Hardy M, Nagant C, El Manssouri N, Vanderbist F, Traore H, Vanechoutte M, Dehaye JP. 2010. Efficacy of the combination of tobramycin and a macrolide in an in vitro *Pseudomonas aeruginosa* mature biofilm model. *Antimicrob Agents Chemother* 54:4409–4415. <http://dx.doi.org/10.1128/AAC.00372-10>.
23. Lynch AS, Abbanat D. 2010. New antibiotic agents and approaches to treat biofilm-associated infections. *Expert Opin Ther Pat* 20:1373–1387. <http://dx.doi.org/10.1517/13543776.2010.505923>.
24. Hancock REW, Sahl HG. 2006. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat Biotechnol* 24:1551–1557. <http://dx.doi.org/10.1038/nbt1267>.
25. Wang G, Li X, Wang Z. 2016. APD3: the antimicrobial peptide database as a tool for research and education. 44:D1087–D1093. *Nucleic Acids Res* <http://dx.doi.org/10.1093/nar/gkv1278>.
26. Nijnik A, Hancock REW. 2009. Host defence peptides: antimicrobial and immunomodulatory activity and potential applications for tackling antibiotic-resistant infections. *Emerg Health Threats J* 2:e1. <http://dx.doi.org/10.13134/ehthj.09.001>.
27. Overhage J, Campisano A, Bains M, Torfs EC, Rehm BH, Hancock REW. 2008. Human host defense peptide LL-37 prevents bacterial biofilm formation. *Infect Immun* 76:4176–4182. <http://dx.doi.org/10.1128/IAI.00318-08>.
28. Dean SN, Bishop BM, van Hoek ML. 2011. Susceptibility of *Pseudomonas aeruginosa* biofilm to alpha-helical peptides: D-enantiomer of LL-37. *Front Microbiol* 2:128. <http://dx.doi.org/10.3389/fmicb.2011.00128>.
29. de la Fuente-Nunez C, Korolik V, Bains M, Nguyen U, Breidenstein EB, Horsman S, Lewenza S, Burrows L, Hancock REW. 2012. Inhibition of bacterial biofilm formation and swarming motility by a small synthetic cationic peptide. *Antimicrob Agents Chemother* 56:2696–2704. <http://dx.doi.org/10.1128/AAC.00064-12>.
30. de la Fuente-Nunez C, Reffuveille F, Mansour SC, Reckseidler-Zenteno SL, Hernandez D, Brackman G, Coenye T, Hancock REW. 2015. D-enantiomeric peptides that eradicate wild-type and multidrug-resistant biofilms and protect against lethal *Pseudomonas aeruginosa* infections. *Chem Biol* 22:196–205. <http://dx.doi.org/10.1016/j.chembiol.2015.01.002>.
31. Kahlenberg JM, Kaplan MJ. 2013. Little peptide, big effects: the role of LL-37 in inflammation and autoimmune disease. *J Immunol* 191:4895–4901. <http://dx.doi.org/10.4049/jimmunol.1302005>.
32. Koczulla R, von Degenfeld G, Kupatt C, Krotz F, Zahler S, Gloe T, Issbrucker K, Unterberger P, Zaiou M, Lebherz C, Karl A, Raake P, Pfosser A, Boekstegers P, Welsch U, Hiemstra PS, Vogelmeier C, Gallo RL, Clauss M, Bals R. 2003. An angiogenic role for the human peptide antibiotic LL-37/hCAP-18. *J Clin Invest* 111:1665–1672. <http://dx.doi.org/10.1172/JCI17545>.
33. Cirioni O, Giacometti A, Ghiselli R, Bergnach C, Orlando F, Silvestri C, Mocchegiani F, Licci A, Skerlavaj B, Rocchi M, Saba V, Zanetti M, Scalise G. 2006. LL-37 protects rats against lethal sepsis caused by gram-negative bacteria. *Antimicrob Agents Chemother* 50:1672–1679. <http://dx.doi.org/10.1128/AAC.50.5.1672-1679.2006>.
34. Shaykhiev R, Beisswenger C, Kandler K, Senske J, Puchner A, Damm T, Behr J, Bals R. 2005. Human endogenous antibiotic LL-37 stimulates airway epithelial cell proliferation and wound closure. *Am J Physiol Lung Cell Mol Physiol* 289:L842–L848. <http://dx.doi.org/10.1152/ajplung.00286.2004>.
35. Haisma EM, de Breij A, Chan H, van Dissel JT, Drijfhout JW, Hiemstra PS, El Ghalbzouri A, Nibbering PH. 2014. LL-37-derived peptides eradicate multidrug-resistant *Staphylococcus aureus* from thermally wounded human skin equivalents. *Antimicrob Agents Chemother* 58:4411–4419. <http://dx.doi.org/10.1128/AAC.02554-14>.
36. Mansour SC, de la Fuente-Nunez C, Hancock REW. 2015. Peptide IDR-1018: modulating the immune system and targeting bacterial biofilms to treat antibiotic-resistant bacterial infections. *J Pept Sci* 21:323–329. <http://dx.doi.org/10.1002/psc.2708>.
37. de la Fuente-Nunez C, Reffuveille F, Haney EF, Straus SK, Hancock REW. 2014. Broad-spectrum anti-biofilm peptide that targets a cellular stress response. *PLoS Pathog* 10:e1004152. <http://dx.doi.org/10.1371/journal.ppat.1004152>.
38. Hall-Stoodley L, Stoodley P. 2005. Biofilm formation and dispersal and the transmission of human pathogens. *Trends Microbiol* 13:7–10. <http://dx.doi.org/10.1016/j.tim.2004.11.004>.
39. Reffuveille F, de la Fuente-Nunez C, Mansour S, Hancock REW. 2014. A broad-spectrum antibiofilm peptide enhances antibiotic action against bacterial biofilms. *Antimicrob Agents Chemother* 58:5363–5371. <http://dx.doi.org/10.1128/AAC.03163-14>.
40. Ribeiro SM, de la Fuente-Nunez C, Baquir B, Faria-Junior C, Franco OL, Hancock REW. 2015. Antibiofilm peptides increase the susceptibility of carbapenemase-producing *Klebsiella pneumoniae* clinical isolates to beta-lactam antibiotics. *Antimicrob Agents Chemother* 59:3906–3912. <http://dx.doi.org/10.1128/AAC.00092-15>.
41. Frank R. 2002. The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports—principles and applications. *J Immunol Methods* 267:13–26.
42. Hilpert K, Winkler DF, Hancock REW. 2007. Peptide arrays on cellulose support: SPOT synthesis, a time and cost efficient method for synthesis of large numbers of peptides in a parallel and addressable fashion. *Nat Protoc* 2:1333–1349. <http://dx.doi.org/10.1038/nprot.2007.160>.
43. Cherkasov A, Hilpert K, Janssen H, Fjell CD, Waldbrook M, Mullaly SC, Volkmer R, Hancock REW. 2009. Use of artificial intelligence in the design of small peptide antibiotics effective against a broad spectrum of highly antibiotic-resistant superbugs. *ACS Chem Biol* 4:65–74. <http://dx.doi.org/10.1021/cb800240j>.
44. Haney EF, Mansour SC, Hilchie AL, de la Fuente-Nunez C, Hancock

- REW. 2015. High throughput screening methods for assessing antibiofilm and immunomodulatory activities of synthetic peptides. *Peptides* 71:276–285. <http://dx.doi.org/10.1016/j.peptides.2015.03.015>.
45. Fjell CD, Hiss JA, Hancock REW, Schneider G. 2012. Designing antimicrobial peptides: form follows function. *Nat Rev Drug Discov* 11:37–51. <http://dx.doi.org/10.1038/nrd3591>.
46. Maccari G, Di Luca M, Nifosi R, Cardarelli F, Signore G, Boccardi C, Bifone A. 2013. Antimicrobial peptides design by evolutionary multi-objective optimization. *PLoS Comput Biol* 9:e1003212. <http://dx.doi.org/10.1371/journal.pcbi.1003212>.
47. Jenssen H, Fjell CD, Cherkasov A, Hancock REW. 2008. QSAR modeling and computer-aided design of antimicrobial peptides. *J Pept Sci* 14: 110–104. <http://dx.doi.org/10.1002/psc.908>.
48. Wang Z, de la Fuente-Nunez C, Shen Y, Haapasalo M, Hancock REW. 2015. Treatment of oral multispecies biofilms by an anti-biofilm peptide. *PLoS One* 10:e0132512. <http://dx.doi.org/10.1371/journal.pone.0132512>.
49. Filoche S, Wong L, Sissons CH. 2010. Oral biofilms: emerging concepts in microbial ecology. *J Dent Res* 89:8–18. <http://dx.doi.org/10.1177/0022034509351812>.
50. Nguyen D, Joshi-Datar A, Lepine F, Bauerle E, Olakanmi O, Beer K, McKay G, Siehnel R, Schafhauser J, Wang Y, Britigan BE, Singh PK. 2011. Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. *Science* 334:982–986. <http://dx.doi.org/10.1126/science.1211037>.
51. Kalia D, Merey G, Nakayama S, Zheng Y, Zhou J, Luo Y, Guo M, Roembke BT, Sintim HO. 2013. Nucleotide, c-di-GMP, c-di-AMP, cGMP, cAMP, (p)ppGpp signaling in bacteria and implications in pathogenesis. *Chem Soc Rev* 42:305–341. <http://dx.doi.org/10.1039/C2CS35206K>.
52. Potrykus K, Cashel M. 2008. (p)ppGpp: still magical? *Annu Rev Microbiol* 62:35–51. <http://dx.doi.org/10.1146/annurev.micro.62.081307.162903>.
53. Choe H, Narayanan AS, Gandhi DA, Weinberg A, Marcus RE, Lee Z, Bonomo RA, Greenfield EM. 2015. Immunomodulatory peptide IDR-1018 decreases implant infection and preserves osseointegration. *Clin Orthop Relat Res* 473:2898–2907. <http://dx.doi.org/10.1007/s11999-015-4301-2>.
54. Mansour SC, Pena OM, Hancock REW. 2014. Host defense peptides: front-line immunomodulators. *Trends Immunol* 35:443–450. <http://dx.doi.org/10.1016/j.it.2014.07.004>.
55. Limoli DH, Rockel AB, Host KM, Jha A, Kopp BT, Hollis T, Wozniak DJ. 2014. Cationic antimicrobial peptides promote microbial mutagenesis and pathoadaptation in chronic infections. *PLoS Pathog* 10:e1004083. <http://dx.doi.org/10.1371/journal.ppat.1004083>.
56. Wiczorek M, Jenssen H, Kindrachuk J, Scott WR, Elliott M, Hilpert K, Cheng JT, Hancock REW, Straus SK. 2010. Structural studies of a peptide with immune modulating and direct antimicrobial activity. *Chem Biol* 17: 970–980. <http://dx.doi.org/10.1016/j.chembiol.2010.07.007>.