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Biofilm infections, their resilience to therapy and innovative treatment strategies

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Abstract

Biofilm formation of microorganisms causes persistent tissue and foreign body infections resistant to treatment with antimicrobial agents. Up to 80% of human bacterial infections are biofilm associated; such infections are most frequently caused by *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and Enterobacteria such as *Escherichia coli*. The accurate diagnosis of biofilm infections is often difficult, which prevents the appropriate choice of treatment. As biofilm infections significantly contribute to patient morbidity and substantial healthcare costs, novel strategies to treat these infections are urgently required. Nucleotide second messengers, c-di-GMP, (p)ppGpp and potentially c-di-AMP, are major regulators of biofilm formation and associated antibiotic tolerance. Consequently, different components of these signalling networks might be appropriate targets for antibiofilm therapy in combination with antibiotic treatment strategies. In addition, cyclic di-nucleotides are microbial-associated molecular patterns with an almost universal presence. Their conserved structures sensed by the eukaryotic host have a widespread effect on the immune system. Thus, cyclic di-nucleotides are also potential immunotherapeutic agents to treat antibiotic-resistant bacterial infections.

Introduction

Microbial communities, commonly termed biofilms, are the most ancient multicellular life forms on earth as evidenced by billion-year-old fossils. The preference of natural microbial communities for a sessile lifestyle was observed almost 80 years ago [1]. However, it was not until the 1980s that it was recognized that biofilm formation plays a pathogenic role during the infection process [2]. Today, the almost ubiquitous involvement of biofilm formation during chronic and to some extent acute infection has been realized [3]. Thus, biofilm formation of microbes leads to persistent infections resistant to conventional antimicrobial treatment and is today a major cause of treatment failure.

Increased knowledge about the molecular mechanisms of biofilm formation is important for the development and analysis of *in vivo* biofilm models and to establish innovative treatment strategies for biofilm infections. Such knowledge has accumulated in recent years leading to the recognition that despite some specific variations, there are common structural and regulatory mechanisms involved in bacterial biofilm formation.

Here, we describe the basis of treatment resistance of biofilm infections and discuss the global role of nucleotide second messenger signalling pathways in the regulation of biofilm formation and biofilm-related characteristics, such as antibiotic resistance and formation of persister cells. Next, the different approaches to interfere with these signalling pathways to develop novel antibiofilm strategies are presented. The role of cyclic di-nucleotide second messengers in inhibition of biofilm formation as extracellular signalling molecules as well as their role in the communication of microbes with the host resulting in immune stimulation is also discussed. Finally, we consider how these characteristics of cyclic di-nucleotide second messengers can be used to develop complementary approaches to treat biofilm-associated infections.

The problem: biofilm-related infections are refractory to antimicrobial treatment

According to the National Institutes of Health, up to 80% of human bacterial infections involve biofilm-associated microorganisms. Common human diseases such as dental caries and periodontitis are caused by biofilm-forming bacteria. Biofilm formation has been implicated in persistent tissue infections such as chronic wound infection, chronic otitis media, chronic osteomyelitis, chronic rhinosinositis, recurrent urinary tract infection, endocarditis and cystic fibrosis-associated lung

infection 3. Biofilm-forming bacteria are also associated with chronic inflammatory diseases such as Crohn's disease 4. In addition, recent experimental evidence indicates a role of biofilm formation in acute infections 5, 6. As the adherence of microorganisms to tissue is part of the process of acute infection, the impact of biofilm formation in infection might in fact be underestimated. There is also a high incidence of biofilm formation on artificial devices such as catheters, stents, orthopaedic implants, contact lenses and implantable electronic devices 3, 7. Examples of *in vitro* and *in vivo* biofilm formation are shown in Fig. 1.

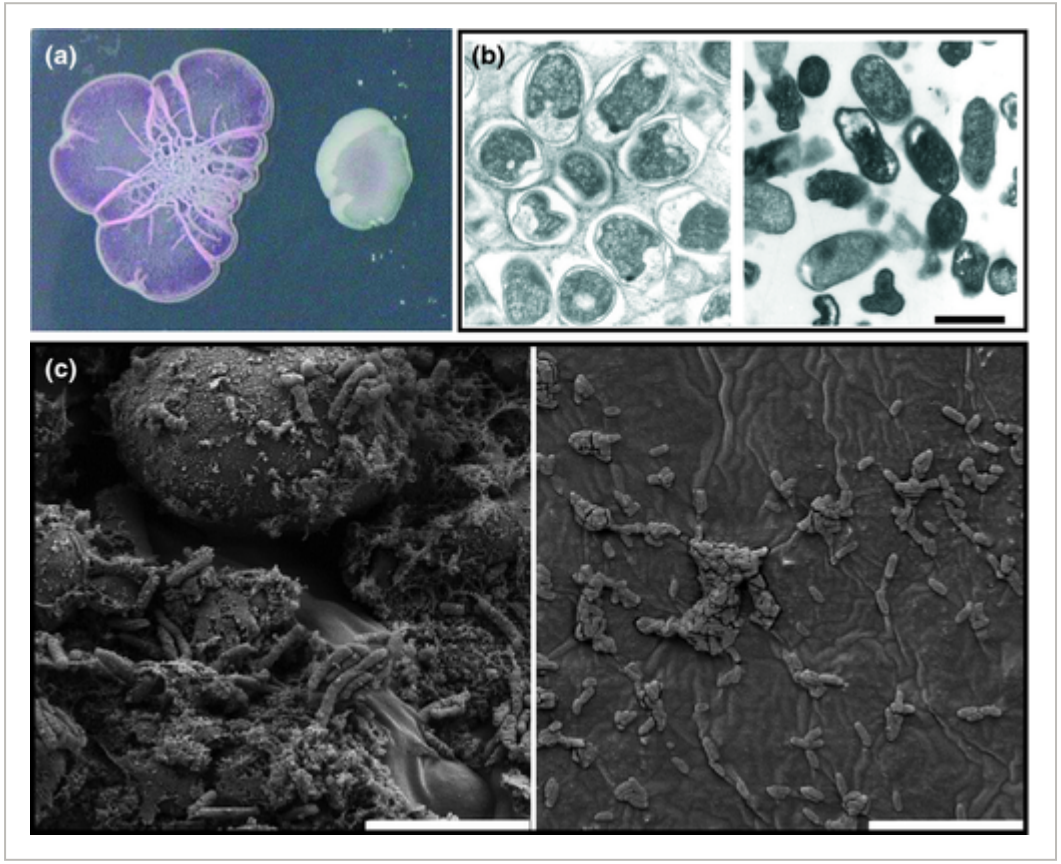

Figure 1

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Examples of bacterial biofilm formation. (a) Biofilm formation of *Salmonella* typhimurium clinical isolates grown on an agar plate. Left, biofilm-forming colony; right, colony without biofilm. Biofilm colonies by *Escherichia* coli and Pseudomonas spp. have a similar appearance due to the production of extracellular matrix components which bind the dye Congo red. (b) Electron microscopic analysis of biofilm formation. Left, biofilm-forming S. typhimurium are surrounded by an extracellular matrix; right, non-biofilm-forming S. typhimurium cells. Pictures taken by Manfred Rohde, Helmholtz Center for Infection Research, Braunschweig, Germany. (c) Biofilm formation on an urinary catheter. Left, catheter biofilm of a patient with urinary tract infection caused by *E. coli*; *right, in vitro catheter biofilm by the same E. coli isolate. Pictures taken by Heinrich Lünsdorf, Helmholtz Center for Infection Research*, *Brauschweig, Germany. Reproduced from [*158*] with permission of the publisher Springer Science+Business Media*.

Bacteria frequently involved in biofilm-associated infections include the Gram-positive pathogens *Staphylococcus epidermidis*, *Staphylococcus aureus* and Streptococcus species and Gram-negative *Pseudomonas aeruginosa* and Enterobacteriaceae such as *Escherichia coli* (Table 1). Biofilm infections challenge the 'one disease, one infectious agent' paradigm; more than one bacterial species can cause a biofilm infection at the same site, and these infections are often polymicrobial, with interaction between microbes increasing persistence. Consequently, personalized antimicrobial treatment strategies are likely to emerge.

Table 1. Major pathogens involved in biofilm-associated disease

Bacterial species	Biofilm infection	References
Escherichia coli	Acute and recurrent urinary tract infection, catheter-associated urinary tract infection, biliary tract infection	3, 6, 7
Pseudomonas aeruginosa	Cystic fibrosis lung infection, chronic wound infection, catheter-associated urinary tract infection, chronic rhinosinusitis, chronic otitis media, contact lens-related keratitis	3, 44
Staphylococcus aureus	Chronic osteomyelitis, chronic rhinosinusitis, endocarditis, chronic otitis media, orthopaedic implants	3, 7
Staphylococcus epidermidis	Central venous catheter, orthopaedic implants, chronic osteomyelitis	3, 7
Streptococcus pneumoniae	Colonization of nasopharynx, chronic rhinosinositis, chronic otitis media, chronic obstructive pulmonary disease	10, 12

Bacterial species	Biofilm infection	References
Streptococcus pyogenes	Colonization of oral cavity and nasopharynx, recurrent tonsilitis	3

Biofilm infections are chronic with a low-grade immune response and thus contribute to patient morbidity. Substantial healthcare costs are caused by biofilm infections due to their high frequency, their resistance to antibiotic treatment and the need to remove the infected foreign body to cure the infection 7, 8. In addition, biofilms contribute to the emergence and spread of antimicrobial resistance 7. From a wider perspective, biofilm formation favours the colonization of the human host by potential pathogens, as well as the transmission and persistence of these pathogens in the environment 9, 10.

Biofilm infections are difficult to diagnose, as conventional culture methods often fail to reliably detect the infectious agent(s), which prevents the use of adequate treatment strategies. A number of criteria, first proposed by Parsek and Singh 11, have been developed to support accurate diagnosis of biofilm infections 8, 11-13. These criteria include (i) the presence of a localized infection with aggregated bacteria at the infection site, (ii) resistance to antibiotic treatment and (iii) ongoing ineffective host immune responses. To overcome the problem of poor diagnosis, reliable and accurate detection of biofilm pathogens is supported by improved sample preparation and the use of advanced molecular and visualization techniques. Of note, there is an urgent need to improve early diagnosis of biofilm infections to enhance the efficacy of therapy. The development of early diagnostic techniques is especially valuable for individuals at high risk of developing biofilm-associated infections including patients with orthopaedic implants who are affected by *S. aureus* bacteraemia 14.

Biofilm characteristics

What makes biofilm infections so difficult to treat? Biofilm formation by microbes is a developmental process (Fig. 2) 15. Initially a (motile) cell approaches a surface, and the bacterium adheres reversibly to the surface. In the next step, irreversible attachment occurs with the development of microcolonies that produce an extracellular matrix (Fig. 1b). The subsequent development of the mature three-dimensional biofilm architecture includes regulated motility. Upon biofilm dispersal, the cells undergo controlled lysis and escape from the microbial community. In the human host, bacteria form a biofilm on a biotic (e.g. an epithelial cell lining) or an abiotic (foreign body) surface. The abiotic surface is usually coated with proteins or other host

molecules forming a conditioning film that alters the adhesion capabilities of microbes. In addition, biofilm formation can even occur without a surface. This is the case, for example, in cystic fibrosis-related lung infection where *P. aeruginosa* forms dense matrix-enclosed cell aggregates in the viscous mucus that are not attached to the epithelial cell lining. In addition, host cells can become an integral part of the biofilm and host components can be incorporated in the biofilm matrix.

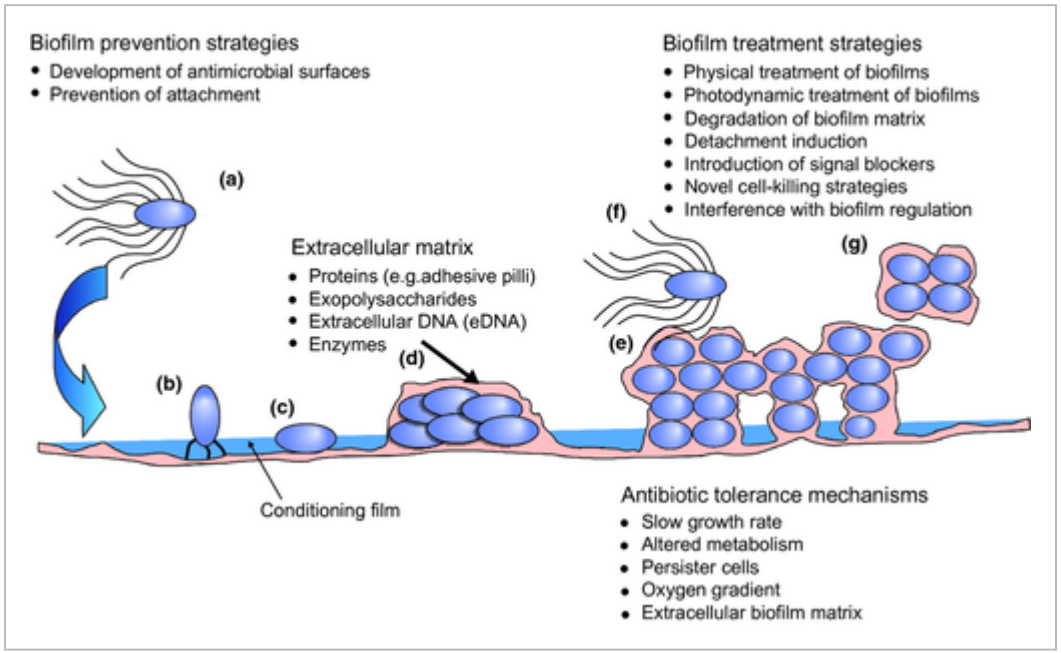

Figure 2

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Biofilm formation is a developmental process. The different stages of biofilm formation include the free-swimming cell (a), reversible attachment to the surface (b), irreversible attachment to the surface (c), formation of microcolonies through cell division and extracellular matrix production (d) and formation of a mature three-dimensional biofilm architecture (e). Cells can actively disintegrate from the biofilm (f) or passively be shed through mechanical disruption (g).

Mature biofilms are highly resistant not only to the action of the innate and adaptive immune defence systems, but also to the action of antimicrobial agents and disinfectants. There are several possible mechanisms underlying this phenotypic resistance (Fig. 2), which may depend on the type of antibiotic treatment and the organism: slow rate of growth in the biofilm, altered

metabolism, titration and inactivation of antimicrobial agents by the extracellular matrix and the presence of an existing oxygen gradient that prevents the action of some antibiotics 7, 12. In addition, biofilms contain a large subpopulation of so-called persister cells, that is, dormant cells, which survive antimicrobial treatment 16 and adapt to a slow growth rate through the emergence of small colony variants 7. A limited diffusion of antimicrobials into biofilms has been suggested, but in most instances, no direct evidence has been provided 17, 18.

The challenge: defining suitable biofilm models

In vitro biofilm models are indispensable to elucidate the molecular mechanisms of biofilm formation. They have also been highly valuable for determining the role of biofilm formation in the infectious process. However, the results of *in vitro* investigation of biofilm formation in clinical isolates have not been entirely consistent with the findings from *in vivo* studies 19. This might be due to the poor correlation between *in vitro* and *in vivo* biofilm formation, the undefined role of biofilm formation in the infection process or insufficient knowledge about the role of biofilms in health and disease settings. For example, almost all *P. aeruginosa* strains, irrespectively of their origin, form biofilms to a large extent in *in vitro* models. However, alginate, a major biofilm matrix component that supports persistence of *P. aeruginosa* in the cystic fibrosis lung, plays a minor role in biofilm formation *in vitro*. In addition, correlation between biofilm formation and the ability to cause invasive disease could not be demonstrated for clinical isolates of *Streptococcus pneumoniae*. As biofilm formation has been experimentally shown to negatively affect acute invasive disease of *S. pneumoniae* and *Streptococcus pyogenes* 20, 21, biofilm formation might initially aid colonization of the human host by Streptococcus species 10. Also, the intercellular adhesion (ica) polysaccharide was considered the major determinant mediating biofilm formation and consequently virulence in the nosocomial pathogen *S. epidermidis*; this was supported by data that invasive strains carry the *ica* locus more frequently than commensal strains 19. The higher incidence of *ica* occurrence in strains recovered from infections can be related in part to the fact that it inhibits colonization of the healthy human skin; however, individuals who have contact with the healthcare system are more frequently colonized with *ica*-positive strains. Thus, *ica*-negative strains can still be pathogenic harbouring alternative factors that contribute to biofilm formation in defined clinical settings.

Novel approaches to prevent biofilm formation

Novel approaches to prevent biofilm formation and to treat infections by biofilm-forming bacteria are currently in development 22. Antiadhesive surfaces with altered physical, chemical and topographical properties that prevent adhesion and thereby

biofilm formation are also being sought 23. Other strategies to prevent biofilm formation have focused on compounds that inhibit the production of functional bacterial adhesins 24. Approaches to aid the dissolution of already established biofilms include physical treatment of the biofilm, photodynamic therapy, targeting of the biofilm matrix for degradation, delivery of signal blockers, interference with biofilm regulation, induction of biofilm detachment and development of cytotoxic strategies to treat biofilm-forming bacteria 22, 25, 26. Although numerous *in vitro* studies have demonstrated effective antibiofilm treatment, only a few *in vivo* (preclinical) or clinical studies have demonstrated improved treatment of biofilm infections (Table 2). For example, interference with homoserine-lactone (HSL) quorum sensing (QS) signalling has been successfully used in combination with antibiotics to treat *P. aeruginosa* biofilms *in vivo* 27. HSL signalling, however, negatively controls biofilm formation in pathogenic bacteria other than *P. aeruginosa* 28. It is generally agreed that effective treatment of biofilms requires a combination therapy of an antibiofilm compound with an effective antibiotic, but no antibiofilm therapies are in current clinical use.

Table 2. Preclinical and clinical antibiofilm treatment strategies

Treatment	Rationale	Biofilm-associated infection model	Effect	Reference
QS inhibitor[a] + tobramycin	QS inhibitors affect biofilm architecture, enhancing susceptibility to antibiotics and disinfectants, and expression of virulence factors	Mouse intraperitoneal foreign body infection model; *Pseudomonas aeruginosa*	Significant reduction of colony-forming unit counts when compared to monotherapy with either compound	27

Treatment	Rationale	Biofilm-associated infection model	Effect	Reference
garlic extract, ajoene	QS inhibitors (e.g. ajoene)	associated lung infection model; *P. aeruginosa*		
Mannitol + gentamicin	Metabolites feeding in the upper steps of the glycolysis pathway create NADH, which contributes to the	Mouse urinary catheter infection	Reduction of biofilm viability by $10^{1.5}$ versus no effect of	101

[a] Quorum-sensing (QS) inhibitors: furanone C-30, ajoene and horseradish juice extract.

[b] Garlic extract had no statistically significant effect in a clinical trial of cystic fibrosis patients infected with *P. aeruginosa* 163.

There are several challenges to be met in the development of novel antibiofilm therapies. Screening for effective antibiofilm compounds requires models relevant to the clinical situation 29. Although *in vitro* investigation of biofilm formation has made significant progress in the last decade, the *in vivo* molecular mechanisms remain poorly understood 12. In addition, the complexity of biofilm formation makes it difficult to develop a compound that will affect this process in more than one species. However, conserved extracellular matrix components and regulatory mechanisms of biofilm formation have been discovered 30, 31. Almost ubiquitous regulatory mechanisms of biofilm formation in many Gram-positive and Gram-negative pathogens include second messenger signalling by nucleotides. These signalling systems, which are described below, might therefore provide targets for the development of antibiofilm compounds and/or the treatment of biofilm infections through their signalling and immunostimulatory properties.

The c-di-GMP signalling network

In recent years, the second messenger cyclic dimeric guanosine monophosphate (c-di-GMP) has evolved as a key activator of biofilm formation in bacteria from all branches of the phylogenetic tree 32. Originally, c-di-GMP was discovered as an allosteric regulator of cellulose synthase in the fruit-degrading bacterium *Gluconacetobacter xylinus* about 20 years ago 33. c-di-GMP is synthesized by so-called GGDEF domain proteins and degraded by the unrelated EAL and HD-GYP domain proteins (Fig. 3) 34.

The c-di-GMP signalling network is the most complex secondary signalling system discovered in bacteria with more than 100 c-di-GMP-metabolizing proteins in some species. However, the network can vary significantly in complexity and c-di-GMP signalling is completely absent in some bacteria. This signalling network is especially prominent in γ-proteobacteria, including the major human pathogens *P. aeruginosa*, *Salmonella typhimurium*, *E. coli* and *Vibrio cholerae*, which possess numerous c-di-GMP-metabolizing proteins; but also Gram-positive Clostridia and Mycobacteria species can harbour the c-di-GMP signalling pathway.

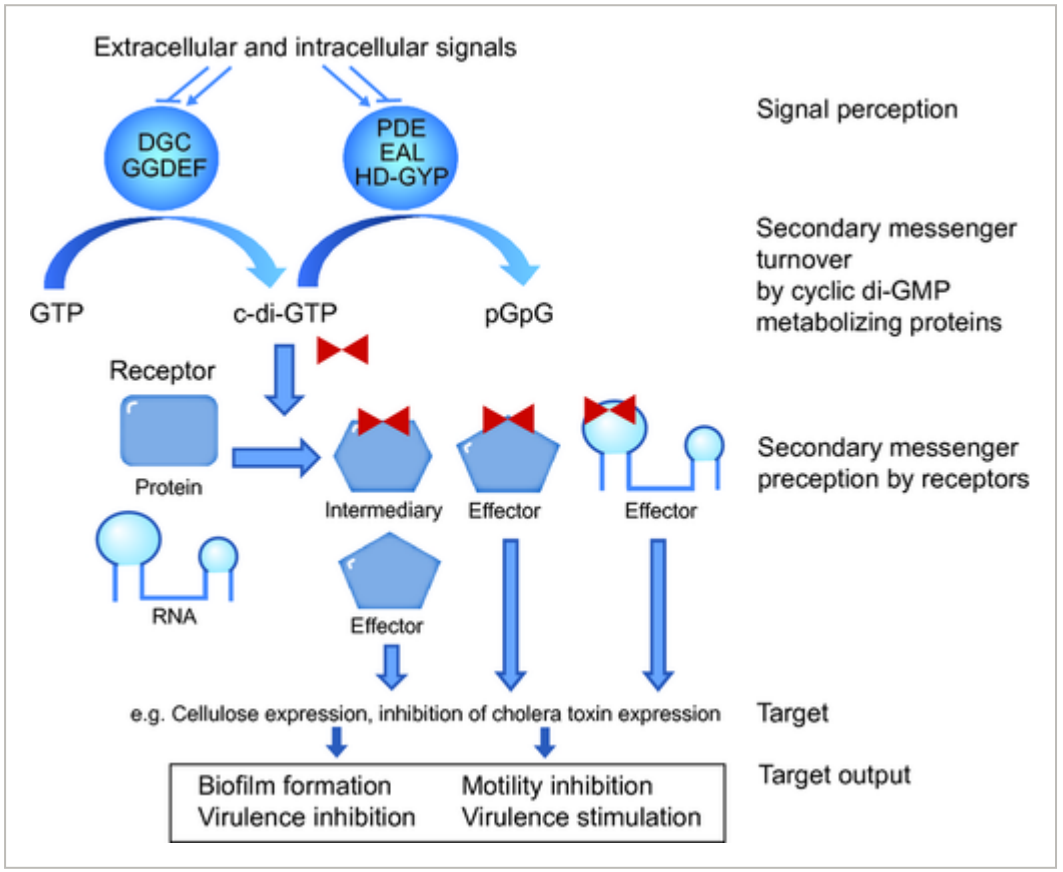

Figure 3

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Basic principles of a nucleotide second messenger signalling pathway with c-di-GMP as an example. An extra- or intracellular signal is sensed by a receptor or directly by sensory domains of the c-di-GMP-metabolizing proteins. As a consequence of signal perception, alteration of the activity of the enzymes involved in c-di-GMP turnover will lead to a temporal and/or spatial alteration of the concentration of c-di-GMP. Second messenger concentrations are sensed by c-di-GMP-binding proteins (receptors) or RNA aptamers. C-di-GMP receptors can function as intermediaries that transmit the signal either by protein–protein interactions to effector proteins or by RNA–RNA interactions to a downstream functional platform. Effector proteins, which can be itself receptors can function as transcription factors or may have enzymatic activity. The physiological consequences of c-di-GMP interactions with cellular receptors include alterations in major essential cellular functions such as replication, gene expression, RNA turnover, translation, post-translational enzymatic activity and protein functionality and degradation. The overall biological target output is stimulation of biofilm formation with activation of the biogenesis of adhesive extracellular matrix components, restriction of motility, alteration of protein secretion and other functions. Alternative global target outputs include inhibition of motility and inhibition or stimulation of virulence.

c-di-GMP signalling has a wide range of effects on bacterial physiology, from the regulation of antibiotic production in *Streptomyces coelicolor* to secretion of toxins in human pathogens such as *V. cholerae* 30, 35, 36. The most prominent role of c-di-GMP signalling is, however, the regulation of bacterial behaviour with the activation of biofilm formation and suppression of motility 37, 38. This lifestyle regulation by c-di-GMP is conserved in all bacterial species that have been investigated. Consequently, as biofilm formation is a hallmark of persistent infections, c-di-GMP signalling is likely to play a significant role in the regulation of processes associated with persistence. The role of c-di-GMP signalling in acute infections is less clear, but emerging evidence indicates that tight regulation is required for a full acute virulence phenotype 35.

The activation of biofilm formation by c-di-GMP signalling includes the regulation of several individual physiological processes. The expression of extracellular matrix components such as exopolysaccharides, adhesive pili, surface-anchored adhesins, secretion systems and extracellular DNA, and cell death and motility which cumulatively lead to a mature three-dimensional biofilm structure are regulated by c-di-GMP signalling 32. Cellular metabolism, nutrient usage and cell cyclic progression are also occasionally regulated by c-di-GMP.

Regulation of biofilm-associated processes occurs on the transcriptional, post-transcriptional and post-translational levels upon c-di-GMP binding to a protein or RNA receptor (Fig. 3) 39-41. Protein receptors include effectors that change their output activity upon c-di-GMP binding. For example, diverse transcriptional regulators activate biofilm-related genes following c-di-GMP binding. Other c-di-GMP receptors are enzymes such as ribonucleases and glycosyltransferases. PilZ domain proteins and

degenerated GGDEF and EAL domain proteins are intermediaries that undergo a conformational change upon c-di-GMP binding and act on an effector through protein–protein interactions.

The c-di-GMP pool available for binding to a specific receptor is frequently regulated by more than one c-di-GMP-metabolizing protein 42, 43. Thereby, the activities of c-di-GMP-metabolizing proteins are tightly regulated from the level of gene expression to allosteric feedback inhibition in response to intra- and extracellular conditions 32. c-di-GMP-metabolizing proteins can be regulated in concert by global transcriptional factors, such as the stress sigma factor RpoS and at the post-transcriptional level by the RNA-binding protein CsrA which regulates carbon metabolism. Whereas RpoS expression is required for biofilm formation and leads to an overall rise in c-di-GMP levels, CsrA inhibits biofilm formation and downregulates the c-di-GMP levels in *E. coli*.

c-di-GMP-metabolizing proteins are multidomain proteins consisting of N-terminal signalling domain(s) and C-terminal c-di-GMP-metabolizing domains 30. Phosphotransfer within two-component signalling cascades is a common signal that couples c-di-GMP turnover activity with extracellular signals sensed by membrane-standing histidine kinases. Oxygen, nitrogen oxide and light are directly sensed by the sensor domains of c-di-GMP-metabolizing proteins. Furthermore, c-di-GMP synthesizing activity is allosterically regulated by binding of c-di-GMP to an inhibitory site in the GGDEF domain. Signals that cumulatively downregulate c-di-GMP concentrations in the cell can be used as potential therapies for biofilm-associated infections (see below). Bacterial biofilm formation is a cause of persistent infection 3. A role for c-di-GMP is implied by the fact that c-di-GMP-regulated components such as the exopolysaccharide alginate contribute to persistence in *P. aeruginosa* infection of the cystic fibrosis lung 44. Indeed, small colony variants, highly adhesive antibiotic-resistant variants of *P. aeruginosa*, which emerge after long-term colonization of the cystic fibrosis lung, have elevated c-di-GMP levels 45, 46. Nevertheless, using a chinchilla model of otitis media, overexpression of c-di-GMP has recently been shown to increase the persistence of the infection 47. Of note, the regulation of biofilm components by c-di-GMP-metabolizing protein *in vivo* and *in vitro* can be significantly different 48.

As cells in biofilms show resistance to antimicrobial treatment and c-di-GMP targets are involved in resistance to antimicrobial agents and disinfectants, it is likely that the c-di-GMP signalling network contributes to antimicrobial resistance. However, only one c-di-GMP-metabolizing protein, the phosphodiesterase PvrR, has been demonstrated to control the switch between antimicrobial-susceptible and -resistant forms of *P. aeruginosa* 49.

c-di-GMP signalling also contributes to the acute infection process. The formation of bacterial aggregates (morulae) that resembles biofilm formation by the obligate intracellular pathogens *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* in white blood cells is dependent on c-di-GMP signalling 5, 50. This signalling is also required for pathogenicity of *P. aeruginosa* in a murine model of thermal injury 51. Frequently, however, c-di-GMP signalling inhibits the acute virulence phenotype. Loss of virulence has been attributed to the inhibition of flagella-related functions, suppression of toxin secretion and/or the deregulated expression of biofilm extracellular matrix components 52-54. In these instances, the c-di-GMP signalling system might also provide a potential target for interference with the process of acute infection.

The c-di-AMP signalling network

Recently it was discovered serendipitously, whilst determining its crystal structure, that the second messenger molecule cyclic dimeric adenosine monophosphate (c-di-AMP) is associated with the DNA integrity scanning protein DisA from *Bacillus subtilis* 55. This finding immediately led to the suggestion that c-di-AMP might be yet another bacterial second messenger 56.

Is c-di-AMP signalling as widespread as other second messenger pathways? The identification of the basic components of c-di-AMP turnover, di-adenylate cyclase activity in the DAC domain of the DNA integrity scanning protein DisA 55 and physiologically relevant c-di-AMP phosphodiesterase activity in a subgroup domain of the DHH family 57 provides a clue to the phylogenetic distribution of the c-di-AMP signalling pathway. It is interesting that c-di-AMP signalling seems to have a fundamentally different phylogenetic distribution compared with c-di-GMP signalling. For example, c-di-AMP-metabolizing proteins are not found in most branches of the proteobacteria; they are absent in important Gram-negative pathogens such as *P. aeruginosa* and *E. coli*. However, c-di-AMP signalling components can be found in major Gram-positive pathogens such as Staphylococci, Enterococci and Clostridia, as well as in *Treponema pallidum* and *Borrelia burgdorferi*. In addition, the c-di-AMP pathway is present in intracellular pathogens such as Chlamydia and Mycoplasma spp. It is worth noting that the c-di-AMP signalling network appears to be far less complex than the c-di-GMP system.

Although the physiological functions of c-di-AMP signalling have not been extensively studied, emerging data indicate that this pathway could be a potential target for antimicrobial and antibiofilm therapy. First, cumulative evidence suggests that c-di-AMP signalling is essential as deletion of di-adenylate cyclase activity was found to be deleterious in a number of investigated pathogens and *B. subtilis* 58-61. Second, a general role of c-di-AMP signalling in the increased resistance to various stress factors such as acid and oxidative stress has been suggested in Gram-positive bacteria 57, 62, 63. In *S. aureus*, c-di-AMP has a role in

resistance to extreme cell wall stress 64. A mutant of the c-di-AMP phosphodiesterase (i.e. mutation in the *gdpP* gene) suppressed the lethal effect caused by the absence of lipotechoic acid in the cell wall, enhanced the cross-linking of the peptidoglycan and altered the expression of autolysin. Third, elevated c-di-AMP levels are associated with increased resistance to some cell wall-active antimicrobials in *S. aureus* and *B. subtilis* [64, 65]. Fourth, c-di-AMP is secreted by virulent *Listeria monocytogenes* to activate a cytosolic surveillance pathway (CSP) in host immune cells such as macrophages, which stimulates type 1 interferon production 66. Balanced c-di-AMP production is likely to have a role in virulence as it is attenuated by deregulation of c-di-AMP delivery 67. Finally, there are indications that biofilm formation is stimulated by c-di-AMP signalling 64.

In summary, c-di-AMP seems to have an essential role in intracellular pathogens and plays a role in antibiotic and stress resistance, virulence and biofilm formation. In addition, as c-di-AMP is required for efficient sporulation [68, 69], it might also be a target for prevention of sporulation in food-contaminating pathogens such as *Bacillus cereus* and Clostridia spp. In future studies, c-di-AMP receptors need to be identified and the targets of c-di-AMP signalling elucidated.

The (p)ppGpp signalling network

The term (p)ppGpp is used for the two modified nucleotides ppGpp and pppGpp, which are tetra- and penta-phosphate guanosines. These compounds were initially described in *E. coli* as mediators of the control of ribosomal content in response to amino acid availability, the so-called stringent response 70. Today, (p)ppGpp is considered a global stress response regulator that responds to additional environmental inputs including phosphorus, fatty acid and iron starvation. In *E. coli* and most γ-proteobacteria, (p)ppGpp turnover depends on the activity of two enzymes: RelA, a (p)ppGpp synthetase and SpoT, a bifunctional enzyme with synthetase and hydrolase activities. The mechanisms by which *E. coli* senses amino acid and fatty acid starvation by RelA and SpoT, respectively, have been demonstrated 70. In the absence of certain amino acids, RelA is activated after interaction with stalled ribosomes. During fatty acid starvation, the acyl carrier protein interacts with SpoT promoting (p)ppGpp synthesis under conditions of limited fatty acid synthesis. However, these mechanisms of sensing nutrient starvation may not be universal amongst bacteria [71, 72]. Moreover, non-homologous enzymes are involved in (p)ppGpp turnover in different bacteria. Although the majority of bacteria encode for a RelA/SpoT homologue (RSH), several Gram-positive species and *V. cholerae* also code for small (p)ppGpp synthetases and/or hydrolases unrelated to RSH. The described diversity in (p)ppGpp turnover enzymes suggests substantial complexity of the (p)ppGpp-mediated sensing of environmental changes amongst bacteria 73; however, this requires further investigation.

(p)ppGpp acts via the following mechanisms. (i) (p)ppGpp directly affects transcription by interacting with RNA polymerase (RNAP), thereby stimulating or repressing expression of the specific promoter 74. (ii) Interaction of RNAP with (p)ppGpp decreases the affinity for RpoD (major sigma subunit during exponential growth), promoting interaction with other sigma factors such as RpoS (stationary phase sigma subunit). This mechanism is known as sigma factor competition. (iii) It seems that (p)ppGpp binds to proteins other than RNAP and thereby affects translation, replication and RNA turnover 75. (iv) In addition, there is crosstalk between (p)ppGpp and other second messengers, such as c-di-GMP, c-di-AMP and cAMP 76.

Alteration in the level of (p)ppGpp above a certain threshold leads to deviation of resources from active growth to promote rapid adaptation to stress and survival under harsh conditions 77, 78. This reprogramming of the cell reduces basic cellular functions, such as protein synthesis, cell division and cell wall synthesis, and induces protective responses against oxidative and osmotic stress. Moreover, (p)ppGpp can coordinate the regulation of cellular processes such as sporulation, competence, antibiotic production and cell-to-cell communication 79. The relevance of (p)ppGpp in the control of virulence has been extensively studied in different bacterial pathogens 80. *In vivo* and *in vitro* experiments have shown that the (p)ppGpp signalling pathway coordinates the expression of virulence factors required at different steps during both acute and persistent infectious processes; (p)ppGpp contributes to initial adherence to host tissues during infection, host cell invasion and intracellular survival. Moreover, *in vivo* experiments have highlighted the pivotal role of (p)ppGpp in pathogenesis.

(p)ppGpp is also involved in the control of biofilm formation and maturation by important Gram-positive and Gram-negative pathogens including *Listeria monocytogenes*, *Streptococcus mutans*, *Enterococcus faecalis*, *V. cholerae* and uropathogenic *E. coli* 81-85. In the majority of studies, a clear reduction in biofilm formation is detected in the absence of (p)ppGpp, consistent with a role in protection against environmental stress. Direct regulation of biofilm-promoting type 1 fimbriation by (p)ppGpp has been described for uropathogenic *E. coli* strains 85. In other bacteria, (p)ppGpp acts indirectly by altering the levels of central regulators that control the expression of biofilm-promoting genes 84. In *S. mutans*, inactivation of *relA* causes a significant reduction in biofilm formation, presumably by deregulation of the *luxS* gene involved in quorum sensing 82. In *E. coli*, (p)ppGpp interferes with the CsrA regulatory pathway that negatively controls biofilm formation by inducing expression of non-coding regulatory RNAs that inhibit the function of CsrA 75.

(p)ppGpp also contributes to antimicrobial drug resistance 86. The mechanisms behind the (p)ppGpp-mediated resistance to microcin J25 and penicillins in *E. coli* and to vancomycin in *Enterococcus faecalis* have been investigated 87, 88. Moreover, it is well known that nutrient starvation, the signal that increases (p)ppGpp levels and a common condition in biofilm

subpopulations, causes an increased tolerance to antibiotic exposure. The conventional explanation of this observation has been that passive mechanisms such as slow growth and a low level of metabolic activity in senescent cells would promote antibiotic resistance, as most of the antibiotic targets are barely active. Recently, however, an active response to starvation that causes an increase in tolerance to antibiotic exposure mediated by (p)ppGpp has been shown in *P. aeruginosa* 89. After nutrient depletion, (p)ppGpp promotes a decrease in the synthesis of pro-oxidant molecules such as hydroxy-2-alkyl quinolones and a concomitant increase in the expression of oxidative stress-protecting enzymes such as catalase and superoxide dismutase. Of note, during antibiotic exposure in a murine biofilm model, the stringent response mutant was more efficiently eradicated than the wild type. Thus, (p)ppGpp signalling is ubiquitous amongst pathogens and has a central role in promoting biofilm formation, drug resistance, stress survival and virulence.

The cAMP signalling network

The first of the second messengers to be described was cAMP. It is ubiquitously present amongst different life forms. In prokaryotic cells, the cAMP signal is commonly relayed via cAMP receptor protein (CRP)-like transcription factors.

In *E. coli*, the cAMP–CRP complex is a metabolic sensor that regulates gene expression in response to carbohydrate availability (commonly known as catabolite repression) and the energy status of the cell. In addition, cAMP plays a pivotal role in the fine-tuning of a diverse set of cellular processes, including pathogenicity. cAMP deficiency causes attenuation of virulence in different pathogens, via regulation of the expression of toxins, adhesins and secretion systems associated with virulence 90. It is intriguing that the *M. tuberculosis* genome contains at least fifteen adenylate cyclase homologues, suggesting that cAMP signalling has both a relevant and complex role in the biology of this major pathogen 91.

The involvement of cAMP in the modulation of biofilm formation has not been extensively studied. The available data, however, point to a negative role of cAMP in biofilm formation through repression of the *cdgA* di-guanylate cyclase in *V. cholerae* and transcriptional repression of the biofilm-promoting factor type 1 fimbriae in uropathogenic *E. coli* strains and *Serratia marcescens* [92, 93]. The potential use of cAMP signalling as a target to prevent biofilm formation warrants further investigation.

Antibiotic resistance phenotypes related to nucleotide second messenger signalling and biofilm formation

Several antibiotic resistance phenotypes affect biofilm formation and/or nucleotide second messenger signalling, or vice versa. However, these inter-relationships have not been fully explored.

Persister cells

A minor fraction of a bacterial population consists of persister cells, which are highly drug tolerant 16. There are high levels of persister cells in recalcitrant chronic bacterial infections such as *P. aeruginosa* infection of the lung in patients with cystic fibrosis and possibly tuberculosis infection 16. These cells are particularly abundant in biofilms. The mechanism of dormancy of persister cells is not fully understood, but may be due to the expression of toxin–antitoxin (TA) systems 94. Shifting the TA balance through degradation of the antitoxin activates the toxin and induces a bacteriostatic-like effect leading to dormancy. TA systems are also intimately involved in the regulation of biofilm formation and affect the c-di-GMP network 95. In *E. coli*, the antitoxin MqsA, which is also a transcriptional regulator, represses expression of the biofilm regulatory cascade including the stress sigma factor *rpoS* and the biofilm regulator *csgD*, which reduces cellular levels of c-di-GMP 96. In addition, the level of persister cells is reduced in a (p)ppGpp-deficient strain. It has been suggested that the toxin HipA might elicit an increase in (p)ppGpp level causing a concomitant increase in antibiotic tolerance 97. According to the proposed model, inhibition of translation and expression of the cell stasis-associated HipA toxin will induce the activation of ribosome-associated (p)ppGpp synthetases 98, 99.

Toxin–antitoxin systems are highly redundant in bacterial genomes, as *E. coli* and *M. tuberculosis* possess at least 37 and 88 TA systems, respectively. Consequently, global mechanisms of TA system regulation and persister formation 94, 100 are potential targets for successful elimination of persister cells. Further studies will reveal whether the c-di-GMP and (p)ppGpp signalling pathways have a global impact on the regulation of persister cell formation and TA systems and may thus provide alternative targets for persister cell elimination. Reversion of dormancy by stimulation of metabolism is another approach to target the antibiotic tolerance of persister cells. It has recently been demonstrated in a biofilm-associated animal infection model that this approach can enable aminoglycoside-specific killing of persister cells (Table 2) 101.

Antioxidant defence

A common cytotoxic mechanism of bactericidal antibiotics, independently of their specific molecular target, is the induction of oxidative damage as a result of hydroxyl radical production 102. Furthermore, bacteria have general strategies to counteract the deleterious effects of antimicrobial agents such as endogenous H_2S production which protects against exposure to a wide

range of antibiotics in both Gram-positive and Gram-negative bacteria 103. H_2S acts as an antioxidant by chelating iron, which prevents the Fenton reaction (a hallmark of antibiotic-induced oxidative stress) and consequently suppresses DNA damage. Moreover, H_2S stimulates catalase and superoxide dismutase activities in the cell. This H_2S-mediated protection against antibiotics is reminiscent of the molecular mechanism of (p)ppGpp-mediated antibiotic tolerance induced by nutrient starvation as discussed above 89. The production of H_2S is ubiquitous amongst bacteria, although different metabolic pathways have been described. To promote bacterial killing by blocking H_2S-mediated antibiotic resistance in biofilms will therefore require the inhibition of diverse H_2S-producing enzymes as this molecule, which easily diffuses through biological membranes, might otherwise exert a protective effect on the bacterial community.

Antibiotic–biofilm interaction

In addition to a growth inhibiting effect, antibiotics are important as signalling molecules. Exposure of bacteria to a subminimum inhibitory concentration (MIC) of different classes of antibiotics with diverse cellular targets can globally affect gene expression regulating not only biofilm formation, but also stress response, virulence and motility 104, 105. Importantly, sub-MIC antibiotic levels can affect biofilm formation positively and negatively which potentially determines treatment outcome. For example, the beneficial effect of low-dose chemotherapy with the macrolide antibiotic azithromycin for the treatment of lung infection with *P. aeruginosa* might be partially due to its inhibition of biofilm formation 106.

Antibiotic exposure can stimulate biofilm formation. For example, the commonly used aminoglycoside tobramycin induced biofilm formation of *P. aeruginosa* and *E. coli* at sub-MIC levels. It is interesting that this effect is mediated directly or indirectly through the c-di-GMP signalling system 107. In *S. epidermidis*, expression of the *icaADBC* operon, encoding the biofilm matrix component poly-*N*-acetylglucosamine (PNAG), is induced after exposure to a wide variety of antibiotics. In addition, antibiotic-mediated induction of biofilm formation is observed in *ica*-negative strains. Similarly, in *E. coli*, antibiotics targeting translation induce biofilm formation by upregulation of PNAG synthesis 76. Biofilm activation occurs through (p)ppGpp signalling, which acts synergistically with c-di-GMP. The mechanisms by which antibiotic exposure affects biofilm formation are targets to improve the efficacy of antimicrobial therapy.

Another aspect of antibiotic–biofilm interaction is the alteration of the mechanism of biofilm formation upon acquisition of antibiotic resistance. For example, methicillin-resistant *S. aureus* isolates acquire PNAG-independent biofilm formation, which is

related to c-di-AMP signalling 108. Expression of β-lactamases type A and D, but not other types, decreased biofilm formation in *P. aeruginosa* and *E. coli*, most probably through interference with normal cell wall turnover 109.

Approaches to interfering with second messenger signalling pathways

There are several rational approaches to interfere with second messenger signalling pathways that alter the activities of the second messenger metabolizing network: manipulation of metabolizing activities, interference with second messenger perception and direct inactivation of the second messenger molecules. Most experimental studies have targeted the c-di-GMP signalling network; however, the same principles of interference can be applied to the manipulation of other second messenger pathways.

Manipulation of enzymatic activities

Intrinsic expression of c-di-GMP-specific phosphodiesterases prevents biofilm formation and/or leads to biofilm dispersal which can be mimicked by ectopic expression of certain phosphodiesterases in biofilm-forming bacteria such as *S. typhimurium*, *E. coli*, *P. aeruginosa* and *Clostridium difficile* 37, 110-113. Similarly, the general stimulatory effect of (p)ppGpp on biofilm formation has been demonstrated in a wide range of pathogens either by blocking or by promoting ectopic expression of (p)ppGpp synthetases 81, 83-85. These examples show that in principle, biofilm formation can be effectively prevented by manipulation of nucleotide second messenger metabolizing activities, either by inhibition of the synthesizing activity or by stimulation of phosphodiesterase activity, or both, which cumulatively leads to reduction in the second messenger concentration. Phage therapy, which had already been used successfully in the pre-antibiotic era to treat bacterial infections, has recently been proven to be effective in treating biofilm infections (Table 2). It is possible that delivery of nucleotide second messenger degrading proteins can be combined with phage therapy to increase efficacy against biofilm infections 114.

Signals that target second messenger pathways

External signals that either inhibit nucleotide synthases and/or activate phosphodiesterases can be used to control biofilm formation. It has been demonstrated that NO, which is also intrinsically produced by prokaryotes, inhibits biofilm formation and/or triggers biofilm dispersal in a variety of microorganisms when applied exogenously at levels below the MIC 115 and is thus an example of a global biofilm inhibitor. At the molecular level, reduction in biofilm formation by the freely diffusible NO occurs through direct or indirect interference with the c-di-GMP or c-di-AMP-metabolizing components, which ultimately leads

to a decrease in second messenger concentration 116, 117. In *P. aeruginosa*, exposure to NO stimulates c-di-GMP-specific phosphodiesterase activity that promotes biofilm dispersal by a pathway which also responds to other dispersion signals 112, 118, 119. In *E. coli*, NO directly binds to the PAS sensory domain of a phosphodiesterase, which stimulates cyclic di-nucleotide degrading activity and eventually leads to biofilm dispersal 120, 121. The level of NO must be regulated as high concentrations were found to induce biofilm formation, probably as part of a protective response 122. Of interest, biofilm upregulation is also mediated by the c-di-GMP signalling network 123. Very high concentrations of NO subsequently exhibit a bactericidal effect.

Other commonly used dispersion signals that can aid the dissolution of medically important biofilms have been identified in studies of biofilm dispersion 28. In addition, cell death and lysis, which precede the dispersal of biofilm microcolonies, are phenotypes that can be targeted in c-di-GMP-mediated biofilm eradication approaches.

Interference with signal perception

Although c-di-GMP and other nucleotides are in principle freely diffusible molecules, data suggest that they are localized by receptor binding (Fig. 3). Thus, the sequestration of c-di-GMP by high-affinity receptors removes the nucleotide from the general signalling system and promotes biofilm dispersal [124, 125]. Consequently, overexpression of c-di-GMP receptors mimics the phenotype of phosphodiesterase expression. To decrease c-di-GMP levels in the cells by sequestration through receptors could be an effective method to prevent biofilm formation or to dissolve existing biofilms.

Synthetic chemistry

In addition to the genetic approaches to control second messenger pathways by manipulating the expression of turnover proteins and receptors, synthetic chemistry approaches can be used to interfere with the signalling function. In the most obvious approach, synthetic analogues of second messengers can be designed to interfere with the synthesis, degradation or receptor perception of the messenger. A (p)ppGpp analogue has been described which effectively inhibits RelA synthase activity of Gram-positive and Gram-negative bacteria *in vitro* 126. In the obligate intracellular pathogens *A. phagocytophilum* and *E. chaffeensis*, 2′-*O*-(tert-butyldimethylsilyl) (TBDMS)-c-di-GMP, a hydrophobic analogue of c-di-GMP, has been shown to specifically interfere with the biofilm phenotype required for growth in host cells 5, 50, 127. *A. phagocytophilum* and *E. chaffeensis* harbour only one di-guanylate cyclase making the target choice straightforward. In general, however, it is not clear whether targeting of a single turnover protein or all components of the c-di-GMP metabolic network is the most useful strategy.

c-di-GMP-binding sites in proteins are diverse and bind different conformations of the nucleotide or even dimeric c-di-GMP aggregates 32. This diversity of binding sites allows the development of class-specific c-di-GMP pathway inhibitors. It has been demonstrated experimentally that a non-hydrolysable analogue of c-di-GMP is conformationally locked and shows binding specificity for a phosphodiesterase, but not for a di-guanylate cyclase and a c-di-GMP receptor 128. Selective inhibition of phosphodiesterases will prevent virulence and/or promote biofilm formation.

It will not be trivial to design synthetic analogues of nucleotide second messenger molecules or small molecules that globally interfere with components of these signalling pathways due to substantial sequence diversity, for example in GGDEF domain di-guanylate cyclases. As an alternative approach, it has been suggested that the signalling molecule could be targeted directly by small molecules, which interact with c-di-GMP, to form biologically inactive higher-order complexes 129. Due to highly altered structures, these complexes are not expected to bind to c-di-GMP-binding sites and thus remain biologically inactive. Future work will show whether these aromatic intercalators, previously shown to bind to DNA, indeed have the potential to function as biofilm inhibitors *in vivo* 129, 130. Formation of higher-order aggregates has not been reported for the other nucleotide second messengers, c-di-AMP and (p)ppGpp.

On the other hand, global screens have detected biofilm inhibitory compounds that interfere with nucleotide secondary signalling pathways. For example, a molecule with *S. mutans* antibiofilm activity, described after screening of a library of 506 compounds, causes significant downregulation of *relA*, highlighting the role of (p)ppGpp in biofilm formation 131.

Inherent microbial components that inhibit biofilm formation

Clear understanding of the mechanisms of disaggregation to liberate planktonic cells from the biofilm (Fig. 2) and competitive inhibitory mechanisms in multispecies biofilms provides new strategies to design efficient antibiofilm drugs 28, 132.

Studies of the mechanisms of biofilm disaggregation have demonstrated that mature biofilms of *B. subtilis* secrete several non-proteinaceous cellular factors that not only induce disaggregation of existing biofilms but also prevent biofilm formation. The first cellular factor identified was a mixture of D-amino acids (D-Tyr, D-Leu, D-Met and D-Trp), which causes the mislocation of crucial components of the extracellular matrix by substitution of the D-Ala present in the peptide side chain of the peptidoglycan 106, 133. As D-amino acids are produced by many bacteria and also prevent biofilm formation in *S. aureus* and *P. aeruginosa*, they have emerged as global antibiofilm compounds. Another biofilm-disassembly factor of *B. subtilis* is

norspermidine; this polyamine interacts with exopolysaccharides, which are major component of the extracellular matrix. The disassembling activity of norspermidine relies on a basic motif of three methylene groups flanked by two positively charged amino acids 134. Synthetically produced compounds carrying this motif were found to be active as biofilm disassembling agents. It is remarkable that norspermidine inhibits biofilm formation in both major Gram-positive and Gram-negative pathogens. Although the composition of the extracellular matrix varies between species, these findings suggest that approaches based on D-amino acids and norspermidine might be exploited as general antibiofilm strategies.

Many bacteria secrete factors that competitively inhibit biofilm formation in other species. For example, although certain polysaccharides are part of the extracellular matrix of biofilms (Fig. 2), the group II capsular polysaccharide produced by certain strains of *E. coli* was shown to prevent biofilm formation 132. Broad spectrum activity was demonstrated as biofilm formation was not only prevented in *E. coli* strains, but also in *P. aeruginosa*, *S. aureus* and other pathogens. Recently, additional antibiofilm polysaccharides have been discovered 135.

The presence of the commensal flora prevents the intrusion of pathogens. Recently, it was demonstrated that commensal strains of *S. epidermidis* that secrete the EspA protease prevent biofilm formation and nasal colonization by *S. aureus* by a unique, non-bactericidal mechanism (Table 2) 136. This finding might lead to novel mechanisms of interference as the nasal cavity is the primary reservoir of *S. aureus* and a major risk factor for transmission and infection.

Application of extracellular c-di-GMP

In addition to its role as an intracellular second messenger, extracellular c-di-GMP has been reported to paradoxically act as a biofilm inhibitor. Exposure to c-di-GMP of different clinically relevant *S. aureus* strains including a human methicillin-resistant *S. aureus* isolate inhibited not only biofilm formation but also cell-to-cell adhesion and bacterial adherence to human epithelial cells 137. In a mouse model of mastitis, bacterial load was reduced upon co-administration of c-di-GMP with bacteria 138. It is noteworthy that *S. aureus* does not possess a functional c-di-GMP signalling pathway. A membrane-standing receptor that senses extracellular c-di-GMP has not been identified. In addition, the broader significance of this finding remains to be demonstrated as the repressive effect of extracellular c-di-GMP on biofilm formation remains restricted to *S. aureus*.

Immunostimulation using cyclic di-nucleotides: a complementary strategy to inhibit biofilm formation?

Microorganisms that cause biofilm-associated infections and cancer cells have evolved analogous redundant strategies to resist drug treatment and to effectively evade the immune system. Immunotherapy has been successful as part of cancer treatment 139, and this therapeutic approach has also been considered for biofilm-related infections. In particular, active or passive immunization strategies based on proteins or polysaccharides specifically expressed by biofilm cells have been developed 140.

The cyclic di-nucleotides c-di-GMP and c-di-AMP are microbial-associated molecular patterns as they are present in bacteria, but not in higher eukaryotes. The almost exclusive bacterial presence makes them suitable for interkingdom communication (i.e. between microorganisms and their hosts) and as targets of immunosurveillance. Indeed, their conserved structures are sensed by both mouse and human cells with widespread effects on the innate and adaptive immune responses 141-143. For example, in dendritic cells exposed to c-di-GMP *in vitro*, maturation was induced and chemokine production and receptor expression were increased 141. These cells have a central role in bridging the innate and adaptive immune responses. Consistent with *in vitro* effects, the production of specific antibodies is enhanced by *in vivo* application of c-di-GMP alone or in combination with an antigen 141, 144.

Subsequent studies have shown that the receptors for cyclic di-nucleotides are located inside host cells. Cyclic-di-AMP is secreted into the cytoplasm by the intracellular bacterium *L. monocytogenes*, which causes the activation of the CSP 66. A robust type 1 interferon response is also triggered by *Legionella pneumophila* overexpressing a di-guanylate cyclase in host macrophages or cytosol-delivered c-di-GMP alone 145, 146. Cyclic di-nucleotides are therefore prominent non-viral CSP-activating ligands. Recently, STING, an essential signalling adaptor, which links cytosolic detection of DNA to downstream events, was identified as a c-di-GMP receptor 147. Considering the broad effects of cyclic di-nucleotides on functions of the immune system, it is expected that additional detection and signalling mechanisms by the host for immunosurveillance of cyclic di-nucleotide second messengers will soon be uncovered 145. Although the biological consequences are different, cytosolic delivery of cyclic di-nucleotides resembles the delivery of cAMP by intracellular *M. tuberculosis* 90.

The immunomodulatory functions described above in combination with the non-toxicity and stability of cyclic di-nucleotides support their use as immunotherapeutics. Indeed, c-di-GMP, c-di-AMP and cyclic dimeric inosine monophosphate, which is not a naturally occurring nucleotide, have been shown to function successfully as an adjuvant in vaccination strategies 148, 149, leading to the clearance of microbial infections 150-152. Although cyclic di-nucleotides have not been used as adjuvants in combination with biofilm-related antigens, their higher efficacy to mount an immune response compared to established adjuvants makes them safe candidate molecules for active or passive immunization strategies against biofilm infections. In

addition, as cyclic di-nucleotides *per se* are highly immunomodulatory, local administration might aid the clearance of these infections through immunostimulation in combination with complementary antibiofilm therapies.

Future perspectives

Biofilm infections are resistant to conventional antimicrobial treatment and require the development of innovative combination therapies for successful eradication. Candidate targets for the development of antimicrobial treatment strategies include bacterial nucleotide second messenger signalling systems, which integrate and amplify changes in the intra- and extracellular environment, mediate resistance to environmental stress and monitor the nutrient status leading to adaptation of the bacterial cell (Fig. 4).

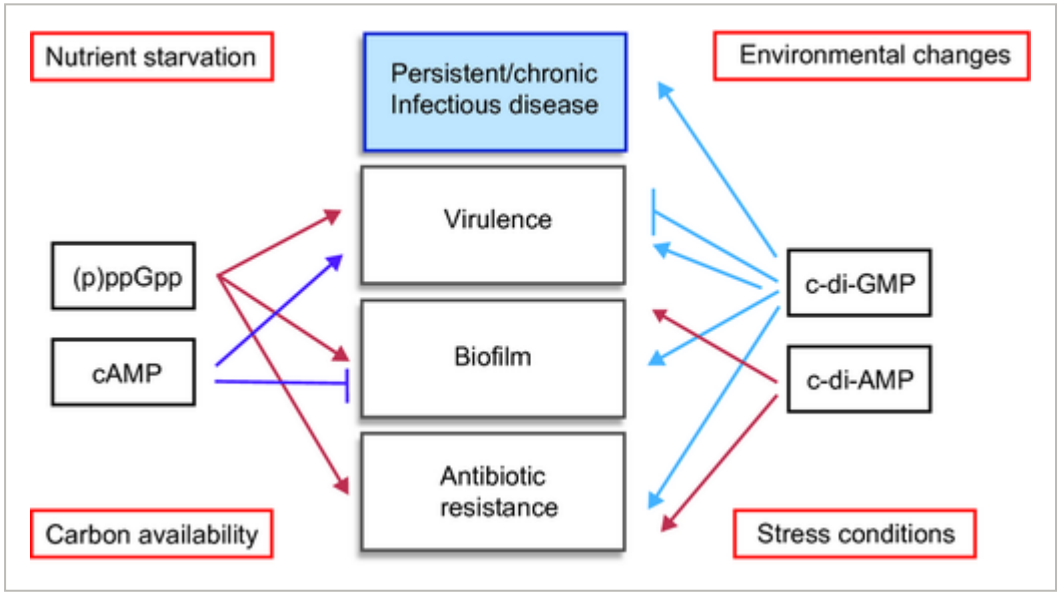

Figure 4

Open in figure viewer | PowerPoint

The second messenger systems in bacteria: major stimulatory signals and their effect on biofilm formation, virulence, antibiotic tolerance and persistent infections.

Thus, c-di-GMP is a major regulator of the switch between the sessile (biofilm) and motile bacterial lifestyle and between the acute and chronic infection state. c-di-AMP has emerged as a global stress response regulator that modulates the cell wall and affects biofilm formation and antibiotic resistance. (p)ppGpp plays a major role in the adaptation of bacteria to a broad range of environmental stress factors and coordinates cellular processes such as virulence, biofilm formation and drug resistance. A common feature of these nucleotide second messengers is their promotion of resistance to antimicrobial agents through effects on biofilm formation, but also independent of such effects. Consequently, nucleotide second messenger signalling systems are suitable targets for antibiofilm and/or antibacterial interference. Because of the absence of cyclic di-nucleotides in higher eukaryotes combined with their broad effects on the host immune response and cell growth, cyclic di-nucleotides are likely to prove effective as therapeutic agents.

The regulation of biofilm formation by nucleotide second messengers is complex. Despite considerable progress, few molecular components of these signalling systems have been identified. Increased knowledge regarding nucleotide receptors, the targets of the nucleotide signals, target output and other molecular aspects of these signalling systems in major pathogens will help to identify more precisely targets for the development of novel antibiofilm and antimicrobial therapies. In addition, the relationship between persister cell formation and nucleotide second messenger signalling is worth investigating in more detail and may lead to the discovery of novel global mechanisms to treat this subpopulation of survivors of antimicrobial therapies.

As regulation of biofilm components by nucleotide second messenger signalling systems might differ *in vitro* and *in vivo*, the precise role of individual elements and their targets in *in vivo* infection needs to be defined. *In vivo* models of biofilm formation in combination with expression technology may shed light on this regulation [153].

Biofilm populations consist of heterogeneous cell types, which are bistable with respect to nucleotide second messenger signalling pathways [154] and respond differently to antibiotic treatment [155]. Therefore, considering the rapid emergence of resistance, more than one antibiofilm/antimicrobial agent might be required to effectively treat biofilm infections.

Furthermore, the wide impact of nucleotide signalling systems on bacterial physiology makes them ideal tools to engineer bacteria with defined and controllable secretory, adhesive and multicellular properties, for example for the design of strains with improved antibiotic production or novel probiotics.

Because of the pronounced effect of cyclic di-nucleotides on the host immune system, these molecules are ideal candidate therapeutic agents for the treatment of biofilm infections. To tailor bacteria to accurately and efficiently deliver cyclic di-nucleotides for therapeutic purposes will require increased knowledge of the expression of cyclic di-nucleotide-metabolizing proteins by bacteria in the host and inside host cells and the delivery mechanisms of these nucleotides for presentation to the antibacterial surveillance systems of the host. As c-di-GMP has also been reported to affect host cell growth, cyclic di-nucleotides might even be applied for cancer treatment [156], [157].

In summary, global regulators of bacterial physiology have emerged not only with important roles in biofilm formation, virulence and antibiotic resistance, but also as immunostimulatory molecules with adjuvant function. All these characteristics can be taken into consideration for the development of treatment strategies for bacterial infections resistant to conventional antimicrobial agents.

Conflict of interest statement

No conflicts of interest to declare.

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