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Molecular mechanisms of compounds affecting bacterial biofilm formation and dispersal.

Paolo Landini[1]*, Davide Antoniani[1], J. Grant Burgess[2] and Reindert Nijland[2]

[1]Department of Biomolecular Sciences and Biotechnology

Università degli Studi di Milano

Via Celoria 26

20133 Milan

Italy

[2] The Dove Marine Laboratory

School of Marine Science and Technology

Newcastle University, NE30 4PZ

United Kingdom

* corresponding author:

Tel. +39-02-50315028

Fax: +39-02-50315044

paolo.landini@unimi.it

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Abstract

Bacteria can switch between planktonic forms (single cells) and biofilms, *i.e.*, bacterial communities growing on solid surfaces and embedded in a matrix of extracellular polymeric substance. Biofilm formation by pathogenic bacteria often results in lower susceptibility to antibiotic treatments and in development of chronic infections; thus, biofilm formation can be considered an important virulence factor. In recent years, much attention has been directed towards understanding the biology of biofilms and towards searching for inhibitors of biofilm development and of biofilm-related cellular processes. In this report, we review selected examples of target-based screening for anti-biofilm agents: we focus on inhibitors of quorum sensing, possibly the most characterized target for molecules with anti-biofilm activity, and on compounds interfering with the metabolism of the signal molecule cyclic di-GMP metabolism and on inhibitors of DNA and nucleotide biosynthesis, which represent a novel and promising class of biofilm inhibitors. Finally, we discuss the activation of biofilm dispersal as a novel mode of action for anti-biofilm compounds.

Introduction

Bacteria are able to switch between two different "lifestyles": single cells (planktonic mode) and biofilms. A biofilm is defined as a sessile microbial community characterized by adhesion to a solid surface and by production of a matrix, which surrounds the bacterial cells and include extracellular polysaccharides (EPS), proteins and DNA. Transition from planktonic cells to biofilm is regulated by a variety of environmental and physiological cues, such as bacterial cell density, nutrient availability and cellular stress. A detailed discussion of biofilm-related cellular processes and of their molecular mechanisms goes beyond the aim of this mini-review: extensive descriptions of the biology of biofilm development can be found in excellent reviews devoted to this subject (Miller and Bassler, 2001; Tamayo et al., 2007; Karatan and Watnick, 2009).

Biofilm and planktonic cells differ significantly in their physiology, gene expression pattern and even morphology. Bacteria growing in biofilms are less sensitive to treatments with antimicrobial agents compared to planktonic cells (Costerton et al. 1995; Anderl et al. 2000; Ceri et al. 2001). Although the molecular mechanisms of tolerance to antibiotics are not yet fully understood, it has been proposed that the extracellular matrix can affect penetration of antibiotics into bacterial cells. In addition, a dormant metabolic state of a fraction of biofilm cells would also contribute to their decreased antibiotic sensitivity (reviewed in Lewis 2008). Interestingly, exposure to subinhibitory concentrations of antibiotics can itself act as an environmental signal triggering biofilm formation (Hoffman et al. 2005; Anderson and O' Toole 2008; Nucleo et al. 2009). Since pathogenic bacteria are normally exposed to subinhibitory concentrations of antibiotics during antimicrobial therapy in patients (Odenholt 2001), biofilm formation can therefore be further increased by antibiotic treatment, posing a significant problem for the eradication of bacterial infections.

In addition to providing tolerance to antibiotic treatment, biofilms also play an important role in virulence of many pathogenic bacteria. For instance, in *Pseudomonas aeruginosa*, many virulence factors are expressed during biofilm formation (Wagner et al., 2004; Wagner et al., 2007). In contrast, in other bacteria, such as *Staphylococcus aureus*, exotoxins and other virulence factors are downregulated during biofilm growth (Kong et al., 2006). However, negative regulation of virulence factors in bacterial biofilms can also be employed as a strategy for host infection by pathogenic bacteria: indeed, biofilm growth results in high numbers of non-virulent biofilm dwelling bacteria. When biofilms eventually disperse in a co-ordinated fashion, a large number of planktonic bacteria, that quickly become virulent, are released simultaneously (Smith and Iglewski 2003; Tamayo et al. 2007; Karatan and Watnick 2009). These observations, and the fact that bacterial resistance is undermining the efficacy of currently used antibiotics, indicate that there is a strong need for novel approaches to target pathogenic bacteria growing in biofilms. Therefore, the

cellular processes of biofilm formation, maintenance and dispersal are important targets for the discovery of novel chemical inhibitors. These inhibitors may be used either alone or in combination with conventional antimicrobial agents in anti-infective therapies. To achieve this goal it is clear that a full understanding of the basic biology of these processes is required to drive forward such technologies.

Target based screening

A basic strategy for the discovery of biofilm inhibitors is the direct screening of chemical compounds in biofilm formation assays (Junker and Clardy 2007; Richards et al. 2008; Rivardo et al. 2009). However, such a direct approach also selects for non-specific biofilm inhibitors such as detergents or biosurfactants which are not therapeutically useful. Although these classes of molecules can display significant anti-biofilm activity under laboratory conditions, they often show limited activity, or lack of selective toxicity towards bacteria, if used *in vivo*. In recent years, the improvement in our understanding of the cellular processes controlling bacterial biofilms has allowed the development of target-oriented approaches for the discovery of biofilm inhibitors. Development of target-based screening constitutes a rational and effective strategy for discovery of biofilm inhibitors. Characterization of quorum sensing as an important regulatory mechanism in biofilm formation, and thus as a potential target for antimicrobials (Smith and Iglewski 2003; Njoroge and Sperandio 2009), has led to the development of screening strategies for quorum sensing inhibitors. In turn, identification of biofilm inhibitors through a target-based approach has contributed to the elucidation of cellular processes controlling bacterial biofilms (Figure 1). The discovery that several compounds with anti-biofilm activity (*e.g.*, halogenated furanones) are quorum sensing inhibitors (Hentzer et al. 2002; Manefield et al. 2002; Bjarnsholt et al. 2005; Persson et al. 2005; Rasmussen et al. 2005) confirmed the importance of this signaling system in biofilm formation. More recently, the search for novel biofilm inhibitors has selected targets other

than quorum sensing, such as nucleotide biosynthesis (Attila et al. 2009, Ueda et al. 2009) and production of the signal molecule cyclic di-GMP (c-di-GMP; Antoniani et al. 2010).

Activity based screening for quorum sensing inhibitors

Quorum sensing (QS) is a complex regulatory process dependent on bacterial cell density (Miller and Bassler 2001; Karatan and Watnick 2009) and is typically involved in regulation of genes involved in biofilm maturation and maintenance (Hammer and Bassler 2003; Marketon et al. 2003; Vuong et al. 2003; Ueda and Wood 2009). Indeed, since QS controlled regulatory pathways are activated at high bacterial cell density, it is not surprising that QS is induced in biofilms, where local cell concentrations can be more than 10-fold higher than planktonic cultures. In addition to its role in biofilms, QS can control production of virulence factors in both Gram positive and Gram negative pathogenic bacteria (Kong et al. 2006; Xu et al. 2006; Hegde et al. 2009). Thus, inhibitors of QS, in addition to possessing antibiofilm activity, could also counteract bacterial pathogenicity. During QS signal molecules, or autoinducers, are produced and secreted by the bacterial cells. Autoinducer accumulation enables the cell to sense that a sufficient local concentration of bacteria (a quorum) has been reached, in order to initiate concerted population responses, including biofilm formation. Although regulation by QS is highly conserved in bacteria, its molecular mechanisms, as well as the chemical nature of the autoinducers, differ significantly between Gram positive and Gram negative bacteria (reviewed in Miller and Bassler 2001; Figure 2). In Gram negative bacteria, autoinducers belong to the chemical class of the acyl-homoserine lactones (AHLs; Fuqua et al. 1996); additional species-specific QS systems make use of other autoinducers, such as quinolonones in *P. aeruginosa* (McKnight et al. 2000), or the Diffusible Signal Factor (DSF), a fatty acid (*cis*-11-methyl-dodecenoic acid) used as signal molecule by the plant pathogen *Xanthomonas campestris* (Barber et al. 1997). AHL autoinducers are synthesized by enzymes of the LuxI family and can bind transcription regulators of the LuxR family. AHL binding to LuxR

activates the transcription of QS-dependent genes. A scheme summarizing AHL-dependent QS in *P. aeruginosa* is given in Figure 2A.

In contrast to Gram negative bacteria, the typical quorum sensing signal molecules in Gram positive bacteria are short peptides (5-50 amino acids), synthesized by ribosomes and often subjected to extensive post-translational modification (Miller and Bassler 2001; Li et al. 2002). Binding of signalling peptides to sensor proteins in the cell membrane triggers a signal transduction cascade which leads to phosphorylation of a response regulator and triggers QS-dependent gene expression. A model of QS systems in Gram positive bacteria is the *agr* (accessory gene regulation) system of *Staphylococcus aureus* (Figure 2B), where autoinducer-dependent phosphorylation of the AgrA regulator, triggered by biofilm growth, leads to transcription activation of genes encoding virulence factors (Novick et al. 1993; Balaban and Novick 1995). The different chemical nature of signal molecules and of the molecular mechanisms involved in QS would suggest that QS inhibitors can only be directed against either Gram positive or Gram negative bacteria. However, furanones, an important class of inhibitors of QS in Gram negative bacteria, also show killing activity against Gram positive bacteria and even Protozoa (Lönn-Stensrud et al. 2009; Zhu et al. 2009), suggesting that they might target cellular processes other than QS. Indeed, exposure of the Gram positive bacterium *Bacillus subtilis* to furanones triggers induction of stress response genes in a QS-independent manner (Ren et al. 2004).

Additional, albeit indirect, evidence for the importance of QS systems based on AHLs in various cellular processes of Gram negative bacteria is derived from the fact that both Gram positive bacteria and eukaryotic (*e.g.* plant) cells can produce enzymes, such as lactonases and acylases (Dong et al. 2002; Ozer et al. 2005; Park et al. 2005; Uroz and Heinonsalo 2008), able to break down these signal molecules. These observations indicate that inhibition of AHL-mediated cell-cell communication might confer an advantage in the competition with, or in the defence against Gram negative bacterial infection. Search for natural products able to inhibit AHL

biosynthesis has led to the identification of halogenated furanones, produced by the marine alga *Delisea pulchra* (Hentzer et al. 2002), and 4-nitro-pyridine-N-oxide (4-NPO) from garlic (*Allium sativum*) cloves (Rasmussen et al 2005). These compounds have been identified using activity-based screening in which expression of reporter genes under the control of QS-dependent promoters was measured (Hentzer et al. 2002; Rasmussen et al. 2005). Further investigation of their mechanism of action showed that furanones bind LasR (one of the regulatory proteins responding to AHLs in *P. aeruginosa*) and act as competitive inhibitors of AHL binding. (Hentzer et al 2002). Binding of furanones results in faster degradation of LasR, probably due to destabilization of its conformation (Manefield et al. 2002), thus leading to complete inhibition of QS-dependent gene regulation (Hentzer et al. 2003). Both furanones and 4-NPO inhibit biofilm formation while not affecting cell growth, reduce *P. aeruginosa* virulence in experimental infection models and increase its sensitivity to antibiotics (Hentzer et al. 2003). These results demonstrate the effectiveness of using QS inhibitors in combination with antibiotics, in order to enhance their bactericidal effect. Utilization of an antibiotic plus QS inhibitor combination therapy might also prevent the antibiotic-dependent induction of biofilm formation observed in different pathogens (Hoffman et al. 2005; Gotoh et al. 2008; Nucleo et al. 2009). Unfortunately, toxic and carcinogenic effects as well as poor stability in aqueous solutions have greatly limited the utilization of halogenated furanones as antimicrobials (Hentzer and Givskov 2003).

An interesting case of molecules combining antibiotic and anti-biofilm activities is the macrolide antibiotics, in particular azithromycin. This antibiotic shows very poor antimicrobial activity against *P. aeruginosa* and other Gram negative bacteria, in particular clinical isolates (Hoffmann et al. 2007). However, azithromycin interferes with *P. aeruginosa* biofilm formation (Mizukane et al. 1994; Ichimiya et al. 1996) by blocking AHL-mediated QS (Tateda et al. 2001; Nalca et al. 2006). Treatment with azithromycin can attenuate chronic *P. aeruginosa* lung infection and significantly reduce bacterial load in the lungs of *Cftr*$^{-/-}$ mice, an animal infection model

mimicking chronic pneumonia in cystic fibrosis patients (Hoffmann et al. 2007). The molecular mechanism of QS inhibition by macrolides has not yet been identified, but it seems likely that they might only affect QS in an indirect fashion through interaction with their primary target, i.e. the ribosome. An even partial inhibition of ribosome function can trigger synthesis of signal molecules such as the alarmone ppGpp; this signal molecule is synthesized by the ribosome-associated RelA and SpoT proteins in response to sudden stoppage in protein synthesis, which usually reflects scarcity in the cellular amino acid pool (Svitil et al. 1993; Cashel et al. 1996). A role for ppGpp in biofilm formation has been described in several reports (McLennan et al. 2008; Boehm et al. 2009), although its precise function remains elusive so far. Since ppGpp affects the expression of a large number of genes in the bacterial cell, it could be possible that macrolide-induced alterations in intracellular ppGpp levels might affect expression of QS genes.

Structure based screening for QS inhibitors

In addition to activity-based assays, an alternative strategy for target-oriented discovery of QS inhibitors is represented by structure-based screening of chemical compounds. This strategy relies on the availability of a growing number of three-dimensional protein structures either predicted by computational biology methods or characterized through biochemical structural analysis. Using molecular modelling programs, it is possible to select potential inhibitors targeting catalytic domains or key amino acid residues for protein activity using a virtual screening of small molecules with known structures and chemical properties (Li et al. 2008; Kiran et al. 2008; Zeng et al. 2008; Yang et al. 2009). This structure-based approach constitutes a primary virtual screening followed by a secondary activity-based assay using reporter genes controlled by QS-dependent promoters. Another important application of structure-based screening is provided by drug design, which is not simply the virtual screening of pre-existing molecules, but the tailoring of new,

"custom made", inhibitors based on the structure of a target protein. Proteins involved in QS of Gram negative bacteria, in particular the LasR transcriptional regulator of *P. aeruginosa*, have been used as a target in structure-based screening for biofilm inhibitors. This approach has led to the identification of several compounds showing significant inhibition of QS in *P. aeruginosa* (Smith et al. 2003; Müh et al. 2006; Geske et al. 2007; Amara et al. 2009); however, the number of inhibitors displaying broad anti-biofilm activity remains low, possibly due to yet not identified resistance mechanisms or to inability of QS inhibitors to reach their target in biofilms formed by clinical isolates.

In Gram positive bacteria, QS directly regulates biofilm maintenance and dispersal, rather than being a factor in its initial formation (Pratten et al. 2001; Yarwood et al. 2004). In addition, QS systems of pathogenic Gram positive bacteria, such as the *agr* regulatory system of *S. aureus*, play a fundamental role in regulation of virulence factors which contributes to pathogenicity of biofilm-induced infections, and are therefore considered targets of great interest for antimicrobials able to interfere with bacterial virulence (Recsei et al. 1986; Janzon and Arvidson 1990; Abdelnour et al. 1993; Kong et al. 2006; Abraham 2006).

An interesting mechanism which interferes with biofilm formation in *S. aureus* involves the heptapeptide RIP. This peptide inhibits biofilm formation of *S. aureus in vivo* (Giacometti et al. 2003), possibly by blocking the *agr*-dependent QS system (Balaban et al. 2004). However, the *agr* system might not be RIP primary target, since it has also been reported that inhibition of the *agr* system increases biofilm formation (Vuong et al. 2003). Although the underlying biology remains unclear, RIP appears to have an effect on biofilm formation, and as such, its structure is an interesting subject for modelling studies aimed at the identification of other biofilm inhibitors. Through structure-based virtual screening using RIP as a template, Kiran et al. (2008) identified hamamelitannin, a tannic acid derivative from the bark of *Hamamelis virginiana* (witch hazel). Interestingly, bark extracts of *H. virginiana* are used in natural medicine as astringent and possess

weak antibacterial activity (Iauk et al. 2003). Hamamelitannin displayed strong inhibition of QS in *S. aureus* and other Gram positive bacteria. Similar to inhibitors of QS in Gram negative bacteria, treatment with hamamelitannin does not result in any detectable growth inhibition of *S. aureus*, but it effectively counteracts *S. aureus* infection in animal models (Kiran et al. 2008). This work represents a clever variation of the structure-based screening approach in which the molecule used for modelling studies was not the target of a desired inhibitor, but itself an inhibitor.

Inhibitors of nucleotide biosynthesis and DNA replication as anti-biofilm agents

Over the last few years, it has become increasingly clear that modified nucleotides, such as cyclic-di-guanosine monophosphate (c-di-GMP), play a pivotal role as signal molecules for biofilm regulation (Figure 2A). Accumulation of c-di-GMP stimulates production of adhesion factors via a variety of different mechanisms, *i.e.*, allosteric activation of protein activity, protein stabilization, or regulation of gene expression at the transcriptional and translational levels (Kulasakara et al. 2006; Weinhouse et al. 1997; Simm et al. 2004; Weber et al. 2006; Sudarsan et al. 2008). Intracellular levels of c-di-GMP are determined by two classes of enzymes with opposite activities: diguanylate cyclases (DGCs), which synthesize c-di-GMP, and c-di-GMP-phosphodiesterases (PDEs), that hydrolyze it into the inactive di-guanylate phosphate (pGpG) form (reviewed in Tamayo et al. 2007). Genes involved in c-di-GMP biosynthesis and turnover are conserved in all *Eubacteria*, while absent in animal species (Galperin 2004), thus suggesting that enzymes involved in c-di-GMP biosynthesis might be an interesting target for antibiofilm agents. However, while genes encoding DGCs and PDEs are present in remarkably high numbers in Gram negative bacteria, they are much less abundant in Gram positives (Galperin 2004). Consistent with this large discrepancy, the role of c-di-GMP in biofilm formation and maintenance has been well established in Gram negative bacteria, while its importance in Gram positive bacteria remains questionable (Holland et al. 2008).

Thus, as observed for quorum sensing inhibitors, it appears that promising targets for biofilm control might follow a strict divide between Gram positive and Gram negative bacteria.

Thanks to our knowledge of cellular processes controlled by c-di-GMP-related enzymes and to the availability of structural data on at least two different DGCs (PleD from *Caulobacter crescentus* and WspR from *P. aeruginosa* (Chan et al. 2004; De et al. 2008), target-oriented screening for DGC inhibitors can be performed using either structure-based or activity-based approaches. Recently, we have described screening assays for inhibitors of c-di-GMP biosynthesis that rely on monitoring of the production of curli and cellulose, two important adhesion factors in *E. coli*. Screening of a commercially available chemical library using these assays has demonstrated that sulfathiazole, a known antimicrobial, can inhibit c-di-GMP biosynthesis and prevent biofilm formation at subinhibitory concentrations (Antoniani et al. 2010). It is possible that reduction of intracellular c-di-GMP levels by sulfathiazole depends on inhibition of tetrahydrofolate biosynthesis, in turn affecting thymidine intracellular pools and DNA synthesis, rather than being mediated by direct binding to DGCs. It has recently been reported that fluorouracil, which blocks DNA replication through inhibition of nucleotide biosynthesis, can prevent biofilm formation at concentrations not affecting planktonic cell growth (Attila et al. 2009; Ueda et al. 2009). This demonstrates that nucleotide biosynthesis inhibitors also show anti-biofilm activity and suggest that a decrease in cellular nucleotide pools negatively affects biofilm formation. Consistent with this finding, surface adhesion is impaired by mutations in genes responsible for nucleotide biosynthesis (Ueda et al. 2009). Inhibition of nucleotide biosynthesis might block production of modified nucleotides acting as signal molecules for biofilm formation, such as c-di-GMP, and stimulate their degradation and recycling in nucleotide triphosphate biosynthesis for DNA and RNA production. Another possibility might be that an even partial inhibition of nucleotide biosynthesis, such as observed at sulfathiazole or fluorouracil concentrations not affecting bacterial growth, might result in shortage of deoxyribonucleotides for DNA replication. The bacterial cell may then react by

abolishing "non essential" DNA synthesis, such as production of extracellular DNA. Indeed, extracellular DNA is an essential component of the biofilm matrix in both Gram positive and Gram negative bacteria (Allesen-Holm et al. 2006; Guiton et al. 2009) and treatment with DNase can prevent biofilm formation (Whitchurch et al. 2002), a fact which suggests exploitable weaknesses in the biofilm matrix. While in some instances eDNA originates from cell lysis (Ando et al. 2006, Vilain et al. 2009), in *P. aeruginosa* and other pathogenic species eDNA release is mediated by release of DNA-containing membrane vesicles in response to QS and possibly other cell signalling mechanisms (Muto and Goto 1986; Kadurugamuwa and Beveridge 1995; Allesen-Holm et al. 2006), thus representing an important biofilm related cell process.

Removal of bacterial biofilms by promoting their dispersal

Biofilm dispersal can occur as a consequence of mechanical breakage of biofilms due to flow or shear stresses. Often, however, dispersal is induced by the biofilm itself in response to environmental cues, such as changes in nutrient availability (Sauer et al. 2004; Gjermansen et al. 2005), fluctuation in local oxygen concentrations (Thormann et al. 2005), or increase in nitric oxide (Barraud et al. 2006). Biofilm dispersal is a naturally occurring process which may represent a mechanism to escape starvation or other negative environmental conditions within a biofilm, allowing bacterial cells the opportunity to migrate to a more favourable environment. In order to promote their dispersal, biofilm cells need to produce enzymes able to degrade the EPS matrix that surrounds them. To do this a wide variety of EPS-degrading enzymes are used. *P. aeruginosa* secretes alginate lyase in (Boyd and Chakrabarty 1994), whereas the oral pathogen *Aggregatibacter actinomycetemcomitans* (Kaplan et al. 2003) uses Dispersin B, a protein that specifically hydrolyzes the glycosidic linkages of poly-β-1, 6-*N*-acetylglucosamine (PNAG), an EPS that functions as an important biofilm determinant in both Gram negative and Gram positive microorganisms (Cramton

et al. 1999; Wang et al. 2004b). Cellular signals leading to biofilm dispersal are tightly interconnected with regulatory signals controlling biofilm formation: for instance, in *S. aureus*, production of extracellular serine proteases required for biofilm dispersal is controlled by the *agr* QS system (Boles and Horswill 2008). Likewise, biofilm dispersal in *Xanthomonas campestris* can be triggered by addition of Diffusible Signal Factor (DSF) that, as mentioned above, acts as a diffusible QS signal (Dow et al. 2003; Wang et al. 2004a). DSF triggers expression of the *manA* gene, encoding endo-β-1,4-mannanase, which results in EPS degradation and biofilm dispersal (Dow et al. 2003). It has recently been reported that a monounsaturated fatty acid produced by *P. aeruginosa*, *cis*-2-decenoic acid, can induce cell detachment from biofilms; interestingly, *cis*-2-decenoic acid displays biofilm-dispersing effects on both Gram positive and Gram negative bacteria, suggesting that monounsaturated fatty acids, unlike other autoinducers, might act as "broad spectrum" signal molecules (Davies and Marques 2009). The enzyme lysine oxidase has recently been implicated in the dispersal of biofilms in a number of Gram negative bacteria. This enzyme has been shown to mediate cell death due to the production of hydrogen peroxide. Such cell death is connected with the emergence of a phenotypically diverse dispersal population (Mai-Prochnow et al. 2008). Thus the mechanisms by which dispersal is mediated are numerous, complex and not fully characterised. One common theme is that dispersal causing compounds are often active across the species barrier. For example, extracellular polysaccharides secreted by *P aeruginosa* exhibited dispersal activity against staphylococcal biofilms (Qin et al. 2009).

Signal molecules which inhibit biofilm formation can also stimulate biofilm dispersal. This is the case for c-di-GMP, which not only influences biofilm formation, but also affects the extent of biofilm detachment (Morgan et al. 2006; Thormann et al. 2006). In *P. aeruginosa*, it has been shown that treatment of biofilm-growing cells with toxic compounds, such as heavy metals, results in detachment of biofilm cells, probably a defence response aimed at mobilizing bacterial cells. This process requires the environmental sensor BdlA, which can trigger c-di-GMP degradation, in turn resulting in the breakdown of the biofilm (Morgan et al. 2006). It was also observed that increased

levels of nitric oxide (NO) can induce dispersal (Barraud et al. 2006) through stimulation of c-di-GMP phosphodiesterases activity (Barraud et al. 2009). Thus, inhibitors of c-di-GMP biosynthesis might have the potential to promote dispersal of mature biofilm in addition to preventing its formation.

As already mentioned, the enzyme Dispersin B, in addition to promoting self-dispersal in *A. actinomycetemcomitans* biofilms by enzymatic degradation of the EPS poly-*N*-acetylglucosamine (PNAG), can prevent biofilm formation and trigger biofilm detachment in any PNAG-producing bacterial species. Exposure to Dispersin B in the presence of antibiotics (Donelli et al. 2007; Izano et al. 2007) or disinfectants such as Triclosan results in synergistic biofilm removal and bacterial killing (Darouiche et al. 2009). Unfortunately, combination of Dispersin B or other EPS-degrading enzymes with antimicrobials can only find limited use for the treatment of biofilm-mediated systemic infections, due to the immunogenic properties of bacterial enzymes. Induction of the immune response in the host, with production of antibodies targeting EPS-degrading enzymes, would prevent the enzymes from reaching their targets (*i.e.*, infection-causing biofilms) and block their effects. Dispersin B in combination with Triclosan is now marketed in gel preparations for treatment of wound and skin infections and for disinfection of medical devices, suggesting that combinations of antimicrobials and EPS-degrading enzymes can represent a powerful tool for biofilm eradication in these settings.

Screening for biofilm dispersal compounds

Screening for natural compounds that inhibit biofilm formation could be focussed on organisms living in an environment where biofilms are common, such as the marine environment, the most biologically diverse habitat on the planet. Because many marine creatures are not fouled, they must have developed strategies against unwanted micro and macro fouling, either directly or

indirectly via symbiotic interactions with microorganisms. Indeed, marine algae, as well as marine invertebrates such as sponges, nudibranchs and tunicates, are the source of a large number of bioactive compounds, many of which probably function as a defence mechanism against predators (Carté and Faulkner, 1986; Paul et al. 1990) and against colonization by bacterial pathogens (James et al. 1996). A striking example of compounds which directly inhibit biofilms are the furanones, initially isolated from the marine alga *Delisea pulchra* (Hentzer et al. 2002) a successful case study which supports further investigation of marine organisms as a source of biofilm inhibitors. Screening for natural products able to promote biofilm dispersal has led to the identification of inhibitors of AHL-based QS, such as bromoageliferin and oroidin (Huigens et al. 2008; Richards et al. 2008) produced by marine organisms, that, in addition to preventing biofilm formation, can trigger biofilm detachment in Gram negative bacteria. Interestingly, a 2-aminobenzimidazole derivative of oroidine is able to disperse biofilm in both Gram positive and Gram negative bacteria, and its mode of action involves chelation of zinc ions (Rogers et al. 2009), suggesting that zinc might play a role in stabilization of mature biofilms. Increasing evidence suggests that many of these secondary metabolites are actually synthesized by microbial symbionts of marine organisms (König et al. 2006; Burke et al. 2007). Thus, isolation and characterization of novel bacterial species belonging to the microflora associated to marine organisms might constitute a promising strategy for the identification of novel natural products with antimicrobial and anti-biofilm activities.

Concluding remarks

Tolerance of bacterial biofilms to antibiotics can lead to failure of antibiotic therapies, thus making inhibition and dispersal of biofilms an attractive therapeutic target. Although anti-biofilm agents themselves might not kill the bacteria, they can make them more susceptible to conventional

antibiotics as well as to the action of the host immune system. The search for biofilm inhibitors using either activity- or structure-based screening has led to the identification of a significant number of biofilm inhibitors, including already marketed chemicals and compounds of potential therapeutic use (Table 1). Implementation of novel target-oriented screening methods (*e.g.*, c-di-GMP biosynthesis, biofilm dispersal) should yield an even greater number of promising biofilm inhibitors that can be used directly or provide the starting material for drug development. In addition, discovery of natural compounds with anti-biofilm activity and characterization of their metabolic pathways can pave the way for functional meta-genomics-based studies of bacteria producing bioactive compounds. A chemotherapeutic approach combining conventional antibiotics and molecules with anti-biofilm activity could form the basis of future clinical protocols against biofilm-mediated infections.

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Table 1. List of selected biofilm inhibitors.

Compound	**Mechanism of biofilm inhibition**	**Other reported biological effects**	**Identification**	**References**
Furanones and structural analogues	AHL binding by LasR protein	Antimicrobial activity on Gram positive bacteria	Activity-based screening	Hentzer et al. 2002; Müh et al. 2006
Azithromycin	Inhibition of LasR-dependent gene expression	Protein synthesis inhibitor	Evaluation of antimicrobial activity	Nalca et al. 2006; Hoffmann et al. 2007
4-NPO	Inhibition of LasR-dependent gene expression	None	Activity-based screening	Rasmussen et al. 2005
Hamamelitannin	RIP analogue (RNAIII inhibitor)	None	Structure-based virtual screening	Kiran et al. 2008
Sulfathiazole	Inhibition of c-di-GMP biosynthesis	Inhibition of tetrahydrofolate biosynthesis	Activity-based screening	Antoniani et al. 2010
Fluorouracil	Inhibition of AriR biofilm regulatory protein	Inhibition of nucleotide biosynthesis	Activity-based screening	Attila et al. 2009
Dispersin B	Enzymatic degradation of biofilm matrix	None	Genetic screening for mutants in biofilm formation	Kaplan et al. 2003
DNase I	Enzymatic degradation of biofilm matrix	Degradation of DNA	Target-oriented direct testing	Whitchurch et al. 2002
cis-2-decenoic acid	signalling molecule	None	Activity-based screening	Davies and Marques 2009

Figure legends

Figure 1. Schematic representation of biofilm development and transition from and to the planktonic lifestyle. The main events linked to the different stages of biofilm development are indicated. Examples of chemical compounds affecting biofilm-related cell processes are shown. Inhibition is represented by the blunt arrow; stimulatory effects are represented by a pointed arrow. See text for further details. (Abbreviations: QS= quorum sensing; EPS= extracellular polysaccharides)

Figure 2. Summary of regulatory processes controlling biofilm formation, maintenance and dispersal in the Gram negative bacterium *P. aeruginosa* (Figure 2A) and in the Gram positive bacterium *S. aureus* (Figure 2B). Figure 2A: acyl-homoserine lactone autoinducers (AHLs; represented by the diamond and the squiggly line) can diffuse through the cell membranes. AHLs accumulation and binding to the LasR protein trigger activation of biofilm- and virulence-related genes (above in the figure). Production of adhesion factors can be controlled by intracellular accumulation of c-di-GMP, which can act as allosteric activator of EPS biosynthesis or as co-factor in gene expression regulation (below in the figure). Inhibitors of the two regulatory processes are shown. Figure 2B: the AgrD oligopeptide (the QS autoinducer) is synthesized as a linear peptide modified and exported by the AgrB protein. Its accumulation leads to interaction with the AgrC sensor protein, which phosphorylates the AgrA response regulator, leading to transcription activation of virulence-related genes. Full activation of the QS system requires autophosphorylation of TRAP, a sensor protein which responds to RAP, another oligopeptide autoinducer. Autophosphorylation of TRAP is inhibited by the RIP protein and can be blocked by the QS inhibitor Hamamelitannin. Figure 2B was adapted from (Horswill et al. 2007).

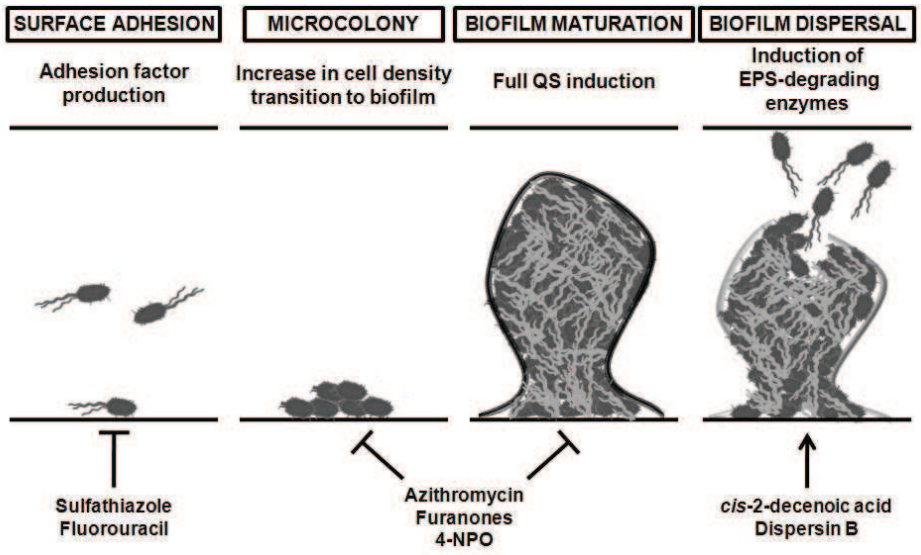
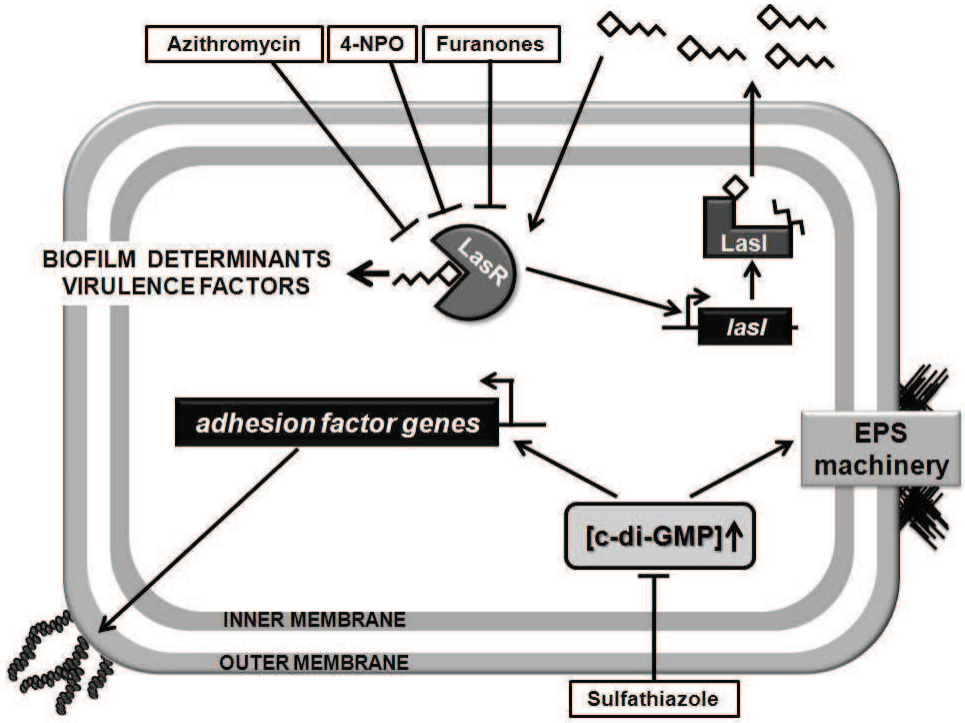


Fig. 1
346x197mm (72 x 72 DPI)



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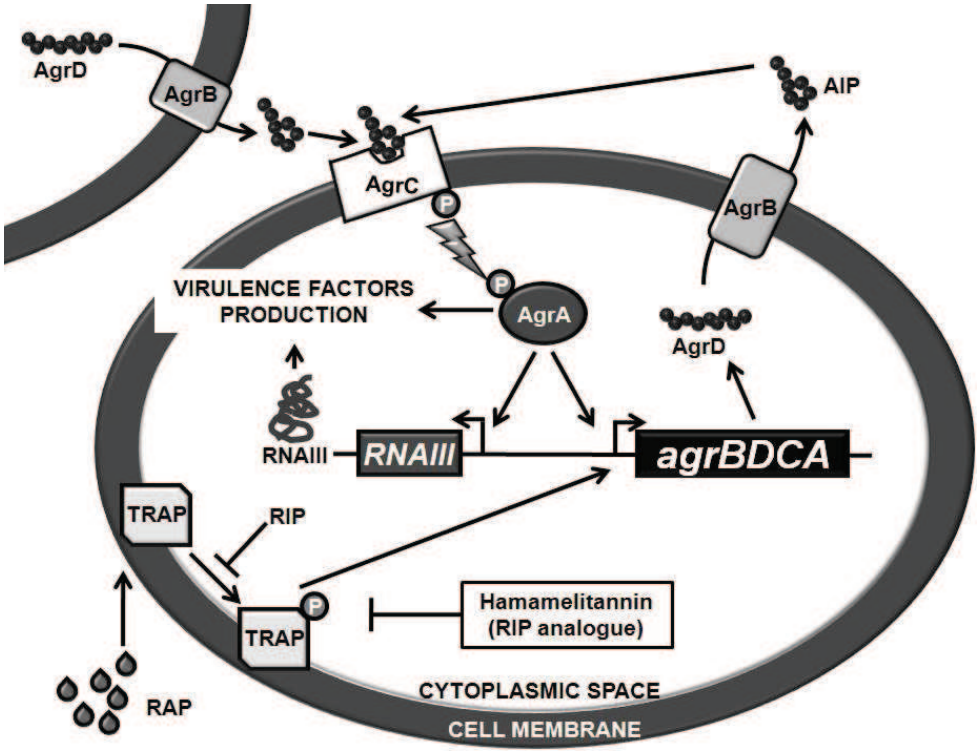

Fig. 2B
326x251mm (72 x 72 DPI)