

Newcastle University e-prints

Date deposited: 2010

Version of file: For Peer Review

Peer Review Status: Unknown

Citation for published item:

Landini P, Antoniani D, Burgess JG, Nijland R. [Molecular mechanisms of compounds affecting bacterial biofilm formation and dispersal](#). Applied Microbiology and Biotechnology 2010. ,86 3 813-823.

Further information on publisher website:

<http://www.springerlink.com/>

Publishers copyright statement:

This paper originally was published by Springer Verlag, 2010 and can be accessed (with permissions) from the URL below:

<http://dx.doi.org/10.1007/s00253-010-2468-8>

Always use the definitive version when citing.

Use Policy:

The full-text may be used and/or reproduced and given to third parties in any format or medium, without prior permission or charge, for personal research or study, educational, or not for profit purposes provided that:

- A full bibliographic reference is made to the original source
- A link is made to the metadata record in Newcastle E-prints
- The full text is not changed in any way.

The full-text must not be sold in any format or medium without the formal permission of the copyright holders.

Robinson Library, University of Newcastle upon Tyne, Newcastle upon Tyne.

NE1 7RU.

Tel. 0191 222 6000



Manuscript for review

Molecular mechanisms of compounds affecting bacterial biofilm formation and dispersal.

Journal:	<i>Applied Microbiology and Biotechnology</i>
Manuscript ID:	AMB-09-19216.R1
Manuscript Category:	Mini-Review
Date Submitted by the Author:	
Complete List of Authors:	Landini, Paolo; University of Milan, Dept. Biomolecular Sciences and Biotechnology Antoniani, Davide; University of Milan, Department of Biomolecular Sciences and Biotechnology Burgess, J. Grant; The Dove Marine Laboratory, School of Marine Science and Technology Nijland, Reindert; The Dove Marine Laboratory, School of Marine Science and Technology
Keyword:	Biofilm formation and dispersal, quorum sensing, c-di-GMP, target-directed screening, structure-directed screening, antimicrobial drugs



1
2
3 **Molecular mechanisms of compounds affecting bacterial biofilm formation and dispersal.**
4
5
6
7
8

9
10 Paolo Landini^{1*}, Davide Antoniani¹, J. Grant Burgess² and Reindert Nijland²
11

12
13 ¹Department of Biomolecular Sciences and Biotechnology
14

15 Università degli Studi di Milano
16

17 Via Celoria 26
18

19 20133 Milan
20

21 Italy
22

23
24 ² The Dove Marine Laboratory
25

26
27 School of Marine Science and Technology
28

29 Newcastle University, NE30 4PZ
30

31 United Kingdom
32
33
34
35

36 * corresponding author:
37

38 Tel. +39-02-50315028
39

40 Fax: +39-02-50315044
41

42 paolo.landini@unimi.it
43
44
45
46
47

48 Running title: Inhibitors of bacterial biofilms
49

50
51 Keywords: Biofilm formation and dispersal, quorum sensing, c-di-GMP, target-directed screening,
52
53 structure-directed screening, antimicrobial drugs
54
55
56
57
58
59
60

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).

1
2
3 Biofilm and planktonic cells differ significantly in their physiology, gene expression pattern
4 and even morphology. Bacteria growing in biofilms are less sensitive to treatments with
5 antimicrobial agents compared to planktonic cells (Costerton et al. 1995; Anderl et al. 2000; Ceri et
6 al. 2001). Although the molecular mechanisms of tolerance to antibiotics are not yet fully
7 understood, it has been proposed that the extracellular matrix can affect penetration of antibiotics
8 into bacterial cells. In addition, a dormant metabolic state of a fraction of biofilm cells would also
9 contribute to their decreased antibiotic sensitivity (reviewed in Lewis 2008). Interestingly, exposure
10 to subinhibitory concentrations of antibiotics can itself act as an environmental signal triggering
11 biofilm formation (Hoffman et al. 2005; Anderson and O' Toole 2008; Nucleo et al. 2009). Since
12 pathogenic bacteria are normally exposed to subinhibitory concentrations of antibiotics during
13 antimicrobial therapy in patients (Odenholt 2001), biofilm formation can therefore be further
14 increased by antibiotic treatment, posing a significant problem for the eradication of bacterial
15 infections.
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32

33
34
35 In addition to providing tolerance to antibiotic treatment, biofilms also play an important
36 role in virulence of many pathogenic bacteria. For instance, in *Pseudomonas aeruginosa*, many
37 virulence factors are expressed during biofilm formation (Wagner et al., 2004; Wagner et al., 2007).
38 In contrast, in other bacteria, such as *Staphylococcus aureus*, exotoxins and other virulence factors
39 are downregulated during biofilm growth (Kong et al., 2006). However, negative regulation of
40 virulence factors in bacterial biofilms can also be employed as a strategy for host infection by
41 pathogenic bacteria: indeed, biofilm growth results in high numbers of non-virulent biofilm
42 dwelling bacteria. When biofilms eventually disperse in a co-ordinated fashion, a large number of
43 planktonic bacteria, that quickly become virulent, are released simultaneously (Smith and Iglewski
44 2003; Tamayo et al. 2007; Karatan and Watnick 2009). These observations, and the fact that
45 bacterial resistance is undermining the efficacy of currently used antibiotics, indicate that there is a
46 strong need for novel approaches to target pathogenic bacteria growing in biofilms. Therefore, the
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 cellular processes of biofilm formation, maintenance and dispersal are important targets for the
4
5 discovery of novel chemical inhibitors. These inhibitors may be used either alone or in combination
6
7 with conventional antimicrobial agents in anti-infective therapies. To achieve this goal it is clear
8
9 that a full understanding of the basic biology of these processes is required to drive forward such
10
11 technologies.
12
13
14
15
16
17
18

19 **Target based screening**

20
21
22 A basic strategy for the discovery of biofilm inhibitors is the direct screening of chemical
23
24 compounds in biofilm formation assays (Junker and Clardy 2007; Richards et al. 2008; Rivardo et
25
26 al. 2009). However, such a direct approach also selects for non-specific biofilm inhibitors such as
27
28 detergents or biosurfactants which are not therapeutically useful. Although these classes of
29
30 molecules can display significant anti-biofilm activity under laboratory conditions, they often show
31
32 limited activity, or lack of selective toxicity towards bacteria, if used *in vivo*. In recent years, the
33
34 improvement in our understanding of the cellular processes controlling bacterial biofilms has
35
36 allowed the development of target-oriented approaches for the discovery of biofilm inhibitors.
37
38 Development of target-based screening constitutes a rational and effective strategy for discovery of
39
40 biofilm inhibitors. Characterization of quorum sensing as an important regulatory mechanism in
41
42 biofilm formation, and thus as a potential target for antimicrobials (Smith and Iglewski 2003;
43
44 Njoroge and Sperandio 2009), has led to the development of screening strategies for quorum
45
46 sensing inhibitors. In turn, identification of biofilm inhibitors through a target-based approach has
47
48 contributed to the elucidation of cellular processes controlling bacterial biofilms (Figure 1). The
49
50 discovery that several compounds with anti-biofilm activity (*e.g.*, halogenated furanones) are
51
52 quorum sensing inhibitors (Hentzer et al. 2002; Manefield et al. 2002; Bjarnsholt et al. 2005;
53
54 Persson et al. 2005; Rasmussen et al. 2005) confirmed the importance of this signaling system in
55
56 biofilm formation. More recently, the search for novel biofilm inhibitors has selected targets other
57
58
59
60

1
2
3 than quorum sensing, such as nucleotide biosynthesis (Attila et al. 2009, Ueda et al. 2009) and
4
5 production of the signal molecule cyclic di-GMP (c-di-GMP; Antoniani et al. 2010).
6
7
8
9

10 11 **Activity based screening for quorum sensing inhibitors**

12
13
14
15 Quorum sensing (QS) is a complex regulatory process dependent on bacterial cell density
16
17 (Miller and Bassler 2001; Karatan and Watnick 2009) and is typically involved in regulation of
18
19 genes involved in biofilm maturation and maintenance (Hammer and Bassler 2003; Marketon et al.
20
21 2003; Vuong et al. 2003; Ueda and Wood 2009). Indeed, since QS controlled regulatory pathways
22
23 are activated at high bacterial cell density, it is not surprising that QS is induced in biofilms, where
24
25 local cell concentrations can be more than 10-fold higher than planktonic cultures. In addition to its
26
27 role in biofilms, QS can control production of virulence factors in both Gram positive and Gram
28
29 negative pathogenic bacteria (Kong et al. 2006; Xu et al. 2006; Hegde et al. 2009). Thus, inhibitors
30
31 of QS, in addition to possessing antibiofilm activity, could also counteract bacterial pathogenicity.
32
33 During QS signal molecules, or autoinducers, are produced and secreted by the bacterial cells.
34
35 Autoinducer accumulation enables the cell to sense that a sufficient local concentration of bacteria
36
37 (a quorum) has been reached, in order to initiate concerted population responses, including biofilm
38
39 formation. Although regulation by QS is highly conserved in bacteria, its molecular mechanisms, as
40
41 well as the chemical nature of the autoinducers, differ significantly between Gram positive and
42
43 Gram negative bacteria (reviewed in Miller and Bassler 2001; Figure 2). In Gram negative bacteria,
44
45 autoinducers belong to the chemical class of the acyl-homoserine lactones (AHLs; Fuqua et al.
46
47 1996); additional species-specific QS systems make use of other autoinducers, such as
48
49 quinolonones in *P. aeruginosa* (McKnight et al. 2000), or the Diffusible Signal Factor (DSF), a
50
51 fatty acid (*cis*-11-methyl-dodecenoic acid) used as signal molecule by the plant pathogen
52
53 *Xanthomonas campestris* (Barber et al. 1997). AHL autoinducers are synthesized by enzymes of the
54
55 LuxI family and can bind transcription regulators of the LuxR family. AHL binding to LuxR
56
57
58
59
60

1
2
3 activates the transcription of QS-dependent genes. A scheme summarizing AHL-dependent QS in
4
5
6 *P. aeruginosa* is given in Figure 2A.
7

8
9 In contrast to Gram negative bacteria, the typical quorum sensing signal molecules in Gram
10
11 positive bacteria are short peptides (5-50 amino acids), synthesized by ribosomes and often
12
13 subjected to extensive post-translational modification (Miller and Bassler 2001; Li et al. 2002).
14
15 Binding of signalling peptides to sensor proteins in the cell membrane triggers a signal transduction
16
17 cascade which leads to phosphorylation of a response regulator and triggers QS-dependent gene
18
19 expression. A model of QS systems in Gram positive bacteria is the *agr* (accessory gene regulation)
20
21 system of *Staphylococcus aureus* (Figure 2B), where autoinducer-dependent phosphorylation of the
22
23 AgrA regulator, triggered by biofilm growth, leads to transcription activation of genes encoding
24
25 virulence factors (Novick et al. 1993; Balaban and Novick 1995). The different chemical nature of
26
27 signal molecules and of the molecular mechanisms involved in QS would suggest that QS inhibitors
28
29 can only be directed against either Gram positive or Gram negative bacteria. However, furanones,
30
31 an important class of inhibitors of QS in Gram negative bacteria, also show killing activity against
32
33 Gram positive bacteria and even Protozoa (Lönn-Stensrud et al. 2009; Zhu et al. 2009), suggesting
34
35 that they might target cellular processes other than QS. Indeed, exposure of the Gram positive
36
37 bacterium *Bacillus subtilis* to furanones triggers induction of stress response genes in a QS-
38
39 independent manner (Ren et al. 2004).
40
41
42
43
44
45
46

47
48 Additional, albeit indirect, evidence for the importance of QS systems based on AHLs in
49
50 various cellular processes of Gram negative bacteria is derived from the fact that both Gram
51
52 positive bacteria and eukaryotic (*e.g.* plant) cells can produce enzymes, such as lactonases and
53
54 acylases (Dong et al. 2002; Ozer et al. 2005; Park et al. 2005; Uroz and Heinonsalo 2008), able to
55
56 break down these signal molecules. These observations indicate that inhibition of AHL-mediated
57
58 cell-cell communication might confer an advantage in the competition with, or in the defence
59
60 against Gram negative bacterial infection. Search for natural products able to inhibit AHL

1
2
3 biosynthesis has led to the identification of halogenated furanones, produced by the marine alga
4
5 *Delisea pulchra* (Hentzer et al. 2002), and 4-nitro-pyridine-N-oxide (4-NPO) from garlic (*Allium*
6
7 *sativum*) cloves (Rasmussen et al 2005). These compounds have been identified using activity-
8
9 based screening in which expression of reporter genes under the control of QS-dependent promoters
10
11 was measured (Hentzer et al. 2002; Rasmussen et al. 2005). Further investigation of their
12
13 mechanism of action showed that furanones bind LasR (one of the regulatory proteins responding to
14
15 AHLs in *P. aeruginosa*) and act as competitive inhibitors of AHL binding. (Hentzer et al 2002).
16
17 Binding of furanones results in faster degradation of LasR, probably due to destabilization of its
18
19 conformation (Manefield et al. 2002), thus leading to complete inhibition of QS-dependent gene
20
21 regulation (Hentzer et al. 2003). Both furanones and 4-NPO inhibit biofilm formation while not
22
23 affecting cell growth, reduce *P. aeruginosa* virulence in experimental infection models and increase
24
25 its sensitivity to antibiotics (Hentzer et al. 2003). These results demonstrate the effectiveness of
26
27 using QS inhibitors in combination with antibiotics, in order to enhance their bactericidal effect.
28
29 Utilization of an antibiotic plus QS inhibitor combination therapy might also prevent the antibiotic-
30
31 dependent induction of biofilm formation observed in different pathogens (Hoffman et al. 2005;
32
33 Gotoh et al. 2008; Nucleo et al. 2009). Unfortunately, toxic and carcinogenic effects as well as poor
34
35 stability in aqueous solutions have greatly limited the utilization of halogenated furanones as
36
37 antimicrobials (Hentzer and Givskov 2003).
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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

1
2
3 mimicking chronic pneumonia in cystic fibrosis patients (Hoffmann et al. 2007). The molecular
4
5 mechanism of QS inhibition by macrolides has not yet been identified, but it seems likely that they
6
7 might only affect QS in an indirect fashion through interaction with their primary target, i.e. the
8
9 ribosome. An even partial inhibition of ribosome function can trigger synthesis of signal molecules
10
11 such as the alarmone ppGpp; this signal molecule is synthesized by the ribosome-associated RelA
12
13 and SpoT proteins in response to sudden stoppage in protein synthesis, which usually reflects
14
15 scarcity in the cellular amino acid pool (Svitil et al. 1993; Cashel et al. 1996). A role for ppGpp in
16
17 biofilm formation has been described in several reports (McLennan et al. 2008; Boehm et al. 2009),
18
19 although its precise function remains elusive so far. Since ppGpp affects the expression of a large
20
21 number of genes in the bacterial cell, it could be possible that macrolide-induced alterations in
22
23 intracellular ppGpp levels might affect expression of QS genes.
24
25
26
27
28
29
30
31
32

33 **Structure based screening for QS inhibitors**

34
35
36
37 In addition to activity-based assays, an alternative strategy for target-oriented discovery of
38
39 QS inhibitors is represented by structure-based screening of chemical compounds. This strategy
40
41 relies on the availability of a growing number of three-dimensional protein structures either
42
43 predicted by computational biology methods or characterized through biochemical structural
44
45 analysis. Using molecular modelling programs, it is possible to select potential inhibitors targeting
46
47 catalytic domains or key amino acid residues for protein activity using a virtual screening of small
48
49 molecules with known structures and chemical properties (Li et al. 2008; Kiran et al. 2008; Zeng et
50
51 al. 2008; Yang et al. 2009). This structure-based approach constitutes a primary virtual screening
52
53 followed by a secondary activity-based assay using reporter genes controlled by QS-dependent
54
55 promoters. Another important application of structure-based screening is provided by drug design,
56
57 which is not simply the virtual screening of pre-existing molecules, but the tailoring of new,
58
59
60

1
2
3 “custom made”, inhibitors based on the structure of a target protein. Proteins involved in QS of
4 Gram negative bacteria, in particular the LasR transcriptional regulator of *P. aeruginosa*, have been
5 used as a target in structure-based screening for biofilm inhibitors. This approach has led to the
6 identification of several compounds showing significant inhibition of QS in *P. aeruginosa* (Smith et
7 al. 2003; Müh et al. 2006; Geske et al. 2007; Amara et al. 2009); however, the number of inhibitors
8 displaying broad anti-biofilm activity remains low, possibly due to yet not identified resistance
9 mechanisms or to inability of QS inhibitors to reach their target in biofilms formed by clinical
10 isolates.
11
12
13
14
15
16
17
18
19
20
21

22 In Gram positive bacteria, QS directly regulates biofilm maintenance and dispersal, rather
23 than being a factor in its initial formation (Pratten et al. 2001; Yarwood et al. 2004). In addition, QS
24 systems of pathogenic Gram positive bacteria, such as the *agr* regulatory system of *S. aureus*, play a
25 fundamental role in regulation of virulence factors which contributes to pathogenicity of biofilm-
26 induced infections, and are therefore considered targets of great interest for antimicrobials able to
27 interfere with bacterial virulence (Recsei et al. 1986; Janzon and Arvidson 1990; Abdelnour et al.
28 1993; Kong et al. 2006; Abraham 2006).
29
30
31
32
33
34
35
36
37
38
39

40 An interesting mechanism which interferes with biofilm formation in *S. aureus* involves the
41 heptapeptide RIP. This peptide inhibits biofilm formation of *S. aureus in vivo* (Giacometti et al.
42 2003), possibly by blocking the *agr*-dependent QS system (Balaban et al. 2004). However, the *agr*
43 system might not be RIP primary target, since it has also been reported that inhibition of the *agr*
44 system increases biofilm formation (Vuong et al. 2003). Although the underlying biology remains
45 unclear, RIP appears to have an effect on biofilm formation, and as such, its structure is an
46 interesting subject for modelling studies aimed at the identification of other biofilm inhibitors.
47 Through structure-based virtual screening using RIP as a template, Kiran et al. (2008) identified
48 hamamelitannin, a tannic acid derivative from the bark of *Hamamelis virginiana* (witch hazel).
49 Interestingly, bark extracts of *H. virginiana* are used in natural medicine as astringent and possess
50
51
52
53
54
55
56
57
58
59
60

1
2
3 weak antibacterial activity (Iauk et al. 2003). Hamamelitannin displayed strong inhibition of QS in
4
5 *S. aureus* and other Gram positive bacteria. Similar to inhibitors of QS in Gram negative bacteria,
6
7 treatment with hamamelitannin does not result in any detectable growth inhibition of *S. aureus*, but
8
9 it effectively counteracts *S. aureus* infection in animal models (Kiran et al. 2008). This work
10
11 represents a clever variation of the structure-based screening approach in which the molecule used
12
13 for modelling studies was not the target of a desired inhibitor, but itself an inhibitor.
14
15
16
17
18
19
20
21

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

23
24
25 Over the last few years, it has become increasingly clear that modified nucleotides, such as
26
27 cyclic-di-guanosine monophosphate (c-di-GMP), play a pivotal role as signal molecules for biofilm
28
29 regulation (Figure 2A). Accumulation of c-di-GMP stimulates production of adhesion factors via a
30
31 variety of different mechanisms, *i.e.*, allosteric activation of protein activity, protein stabilization, or
32
33 regulation of gene expression at the transcriptional and translational levels (Kulasakara et al. 2006;
34
35 Weinhouse et al. 1997; Simm et al. 2004; Weber et al. 2006; Sudarsan et al. 2008). Intracellular
36
37 levels of c-di-GMP are determined by two classes of enzymes with opposite activities: diguanylate
38
39 cyclases (DGCs), which synthesize c-di-GMP, and c-di-GMP-phosphodiesterases (PDEs), that
40
41 hydrolyze it into the inactive di-guanylate phosphate (pGpG) form (reviewed in Tamayo et al.
42
43 2007). Genes involved in c-di-GMP biosynthesis and turnover are conserved in all *Eubacteria*,
44
45 while absent in animal species (Galperin 2004), thus suggesting that enzymes involved in c-di-GMP
46
47 biosynthesis might be an interesting target for antibiofilm agents. However, while genes encoding
48
49 DGCs and PDEs are present in remarkably high numbers in Gram negative bacteria, they are much
50
51 less abundant in Gram positives (Galperin 2004). Consistent with this large discrepancy, the role of
52
53 c-di-GMP in biofilm formation and maintenance has been well established in Gram negative
54
55 bacteria, while its importance in Gram positive bacteria remains questionable (Holland et al. 2008).
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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

1
2
3 abolishing “non essential” DNA synthesis, such as production of extracellular DNA. Indeed,
4
5 extracellular DNA is an essential component of the biofilm matrix in both Gram positive and Gram
6
7 negative bacteria (Allesen-Holm et al. 2006; Guiton et al. 2009) and treatment with DNase can
8
9 prevent biofilm formation (Whitchurch et al. 2002), a fact which suggests exploitable weaknesses in
10
11 the biofilm matrix. While in some instances eDNA originates from cell lysis (Ando et al. 2006,
12
13 Vilain et al. 2009), in *P. aeruginosa* and other pathogenic species eDNA release is mediated by
14
15 release of DNA-containing membrane vesicles in response to QS and possibly other cell signalling
16
17 mechanisms (Muto and Goto 1986; Kadurugamuwa and Beveridge 1995; Allesen-Holm et al.
18
19 2006), thus representing an important biofilm related cell process.
20
21
22
23
24
25
26
27
28

29 **Removal of bacterial biofilms by promoting their dispersal**

30
31
32 Biofilm dispersal can occur as a consequence of mechanical breakage of biofilms due to
33
34 flow or shear stresses. Often, however, dispersal is induced by the biofilm itself in response to
35
36 environmental cues, such as changes in nutrient availability (Sauer et al. 2004; Gjermansen et al.
37
38 2005), fluctuation in local oxygen concentrations (Thormann et al. 2005), or increase in nitric oxide
39
40 (Barraud et al. 2006). Biofilm dispersal is a naturally occurring process which may represent a
41
42 mechanism to escape starvation or other negative environmental conditions within a biofilm,
43
44 allowing bacterial cells the opportunity to migrate to a more favourable environment. In order to
45
46 promote their dispersal, biofilm cells need to produce enzymes able to degrade the EPS matrix that
47
48 surrounds them. To do this a wide variety of EPS-degrading enzymes are used. *P. aeruginosa*
49
50 secretes alginate lyase in (Boyd and Chakrabarty 1994), whereas the oral pathogen *Aggregatibacter*
51
52 *actinomycetemcomitans* (Kaplan et al. 2003) uses Dispersin B, a protein that specifically hydrolyzes
53
54 the glycosidic linkages of poly- β -1, 6-*N*-acetylglucosamine (PNAG), an EPS that functions as an
55
56 important biofilm determinant in both Gram negative and Gram positive microorganisms (Cramton
57
58
59
60

1
2
3 et al. 1999; Wang et al. 2004b). Cellular signals leading to biofilm dispersal are tightly
4 interconnected with regulatory signals controlling biofilm formation: for instance, in *S. aureus*,
5 production of extracellular serine proteases required for biofilm dispersal is controlled by the *agr*
6 QS system (Boles and Horswill 2008). Likewise, biofilm dispersal in *Xanthomonas campestris* can
7 be triggered by addition of Diffusible Signal Factor (DSF) that, as mentioned above, acts as a
8 diffusible QS signal (Dow et al. 2003; Wang et al. 2004a). DSF triggers expression of the *manA*
9 gene, encoding endo- β -1,4-mannanase, which results in EPS degradation and biofilm dispersal
10 (Dow et al. 2003). It has recently been reported that a monounsaturated fatty acid produced by *P.*
11 *aeruginosa*, *cis*-2-decenoic acid, can induce cell detachment from biofilms; interestingly, *cis*-2-
12 decenoic acid displays biofilm-dispersing effects on both Gram positive and Gram negative
13 bacteria, suggesting that monounsaturated fatty acids, unlike other autoinducers, might act as
14 “broad spectrum” signal molecules (Davies and Marques 2009). The enzyme lysine oxidase has
15 recently been implicated in the dispersal of biofilms in a number of Gram negative bacteria. This
16 enzyme has been shown to mediate cell death due to the production of hydrogen peroxide. Such cell
17 death is connected with the emergence of a phenotypically diverse dispersal population (Mai-
18 Prochnow et al. 2008). Thus the mechanisms by which dispersal is mediated are numerous,
19 complex and not fully characterised. One common theme is that dispersal causing compounds are
20 often active across the species barrier. For example, extracellular polysaccharides secreted by *P.*
21 *aeruginosa* exhibited dispersal activity against staphylococcal biofilms (Qin et al. 2009).
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47

48 Signal molecules which inhibit biofilm formation can also stimulate biofilm dispersal. This
49 is the case for c-di-GMP, which not only influences biofilm formation, but also affects the extent of
50 biofilm detachment (Morgan et al. 2006; Thormann et al. 2006). In *P. aeruginosa*, it has been
51 shown that treatment of biofilm-growing cells with toxic compounds, such as heavy metals, results
52 in detachment of biofilm cells, probably a defence response aimed at mobilizing bacterial cells. This
53 process requires the environmental sensor BdlA, which can trigger c-di-GMP degradation, in turn
54 resulting in the breakdown of the biofilm (Morgan et al. 2006). It was also observed that increased
55
56
57
58
59
60

1
2
3 levels of nitric oxide (NO) can induce dispersal (Barraud et al. 2006) through stimulation of c-di-
4
5 GMP phosphodiesterases activity (Barraud et al. 2009). Thus, inhibitors of c-di-GMP biosynthesis
6
7 might have the potential to promote dispersal of mature biofilm in addition to preventing its
8
9 formation.
10

11
12
13 As already mentioned, the enzyme Dispersin B, in addition to promoting self-dispersal in *A.*
14
15 *actinomycetemcomitans* biofilms by enzymatic degradation of the EPS poly-*N*-acetylglucosamine
16
17 (PNAG), can prevent biofilm formation and trigger biofilm detachment in any PNAG-producing
18
19 bacterial species. Exposure to Dispersin B in the presence of antibiotics (Donelli et al. 2007; Izano
20
21 et al. 2007) or disinfectants such as Triclosan results in synergistic biofilm removal and bacterial
22
23 killing (Darouiche et al. 2009). Unfortunately, combination of Dispersin B or other EPS-degrading
24
25 enzymes with antimicrobials can only find limited use for the treatment of biofilm-mediated
26
27 systemic infections, due to the immunogenic properties of bacterial enzymes. Induction of the
28
29 immune response in the host, with production of antibodies targeting EPS-degrading enzymes,
30
31 would prevent the enzymes from reaching their targets (*i.e.*, infection-causing biofilms) and block
32
33 their effects. Dispersin B in combination with Triclosan is now marketed in gel preparations for
34
35 treatment of wound and skin infections and for disinfection of medical devices, suggesting that
36
37 combinations of antimicrobials and EPS-degrading enzymes can represent a powerful tool for
38
39 biofilm eradication in these settings.
40
41
42
43
44
45
46
47
48
49
50

51 **Screening for biofilm dispersal compounds**

52

53
54 Screening for natural compounds that inhibit biofilm formation could be focussed on
55
56 organisms living in an environment where biofilms are common, such as the marine environment,
57
58 the most biologically diverse habitat on the planet. Because many marine creatures are not fouled,
59
60 they must have developed strategies against unwanted micro and macro fouling, either directly or

1
2
3 indirectly via symbiotic interactions with microorganisms. Indeed, marine algae, as well as marine
4
5 invertebrates such as sponges, nudibranchs and tunicates, are the source of a large number of
6
7 bioactive compounds, many of which probably function as a defence mechanism against predators
8
9 (Carté and Faulkner, 1986; Paul et al. 1990) and against colonization by bacterial pathogens (James
10
11 et al. 1996). A striking example of compounds which directly inhibit biofilms are the furanones,
12
13 initially isolated from the marine alga *Delisea pulchra* (Hentzer et al. 2002) a successful case study
14
15 which supports further investigation of marine organisms as a source of biofilm inhibitors.
16
17 Screening for natural products able to promote biofilm dispersal has led to the identification of
18
19 inhibitors of AHL-based QS, such as bromoageliferin and oroidin (Huigens et al. 2008; Richards et
20
21 al. 2008) produced by marine organisms, that, in addition to preventing biofilm formation, can
22
23 trigger biofilm detachment in Gram negative bacteria. Interestingly, a 2-aminobenzimidazole
24
25 derivative of oroidine is able to disperse biofilm in both Gram positive and Gram negative bacteria,
26
27 and its mode of action involves chelation of zinc ions (Rogers et al. 2009), suggesting that zinc
28
29 might play a role in stabilization of mature biofilms. Increasing evidence suggests that many of
30
31 these secondary metabolites are actually synthesized by microbial symbionts of marine organisms
32
33 (König et al. 2006; Burke et al. 2007). Thus, isolation and characterization of novel bacterial
34
35 species belonging to the microflora associated to marine organisms might constitute a promising
36
37 strategy for the identification of novel natural products with antimicrobial and anti-biofilm
38
39 activities.
40
41
42
43
44
45
46
47
48
49
50
51

52 **Concluding remarks**

53
54
55 Tolerance of bacterial biofilms to antibiotics can lead to failure of antibiotic therapies, thus
56
57 making inhibition and dispersal of biofilms an attractive therapeutic target. Although anti-biofilm
58
59 agents themselves might not kill the bacteria, they can make them more susceptible to conventional
60

1
2
3 antibiotics as well as to the action of the host immune system. The search for biofilm inhibitors
4
5 using either activity- or structure-based screening has led to the identification of a significant
6
7 number of biofilm inhibitors, including already marketed chemicals and compounds of potential
8
9 therapeutic use (Table 1). Implementation of novel target-oriented screening methods (*e.g.*, c-di-
10
11 GMP biosynthesis, biofilm dispersal) should yield an even greater number of promising biofilm
12
13 inhibitors that can be used directly or provide the starting material for drug development. In
14
15 addition, discovery of natural compounds with anti-biofilm activity and characterization of their
16
17 metabolic pathways can pave the way for functional meta-genomics-based studies of bacteria
18
19 producing bioactive compounds. A chemotherapeutic approach combining conventional antibiotics
20
21 and molecules with anti-biofilm activity could form the basis of future clinical protocols against
22
23 biofilm-mediated infections.
24
25
26
27
28
29
30
31
32
33
34
35
36

37 **Acknowledgments**

38
39
40 Research work in P.L.'s lab was supported by the Italian Foundation for Research on Cystic
41
42 Fibrosis (project FFC#9/2006, adopted by "*Gruppo Rocciatori di Belluno*") and by the CHEM-
43
44 PROFARMA-NET Research Program of the Italian Ministry for University and Research (Project
45
46 RBPR05NWWC_004). RN was funded by a fellowship from the European Community's Seventh
47
48 Framework Programme, under grant agreement PIEF-GA-2008-219592. JGB acknowledges
49
50 financial support from the Natural Environment Research Council (NERC) (Awards:
51
52 NER/T/S/2002/00586/2 and NE/G011206/1.)
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

References

Abdelnour A, Arvidson S, Bremell T, Rydén C, Tarkowski A (1993) The accessory gene regulator (*agr*) controls *Staphylococcus aureus* virulence in a murine arthritis model. *Infect Immun* 61:3879-3885.

Abraham WR (2006) Controlling biofilms of gram-positive pathogenic bacteria. *Curr Med Chem* 13:1509-1524.

Allesen-Holm M, Barken KB, Yang L, Klausen M, Webb JS, Kjelleberg S, Molin S, Givskov M, Tolker-Nielsen T (2006) A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Mol Microbiol* 59:1114-1128.

Amara N, Mashiach R, Amar D, Krief P, Spieser SA, Bottomley MJ, Aharoni A, Meijler MM (2009) Covalent inhibition of bacterial quorum sensing. *J Am Chem Soc* 131:10610-10619.

Anderl JN, Franklin MJ, Stewart PS (2000) Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob Agents Chemother* 44:1818-1824.

Anderson GG, O'Toole GA (2008) Innate and induced resistance mechanisms of bacterial biofilms. *Curr Top Microbiol Immunol* 322:85-105.

Ando T, Suzuki H, Nishimura S, Tanaka T, Hiraishi A, Kikuchi Y (2006) Characterization of extracellular RNAs produced by the marine photosynthetic bacterium *Rhodovulum sulfidophilum*. *J Biochem* 139:805-811.

Antoniani D, Bocci P, Maciag A, Raffaelli N, Landini P (2010) Monitoring of di-guanylate cyclase activity and of cyclic-di-GMP biosynthesis by whole-cell assays suitable for high-throughput screening of biofilm inhibitors. *Appl Microbiol Biotechnol* 85:1095-1104.

1
2
3 Attila C, Ueda A, Wood TK (2009) 5-Fluorouracil reduces biofilm formation in *Escherichia coli* K-
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Attila C, Ueda A, Wood TK (2009) 5-Fluorouracil reduces biofilm formation in *Escherichia coli* K-12 through global regulator AriR as an antivirulence compound *Appl Microbiol Biotechnol* 82:525-533.

Balaban N, Novick RP (1995) Autocrine regulation of toxin synthesis by *Staphylococcus aureus*. *Proc Natl Acad Sci U S A* 92:1619-1623.

Balaban N, Gov Y, Giacometti A, Cirioni O, Ghiselli R, Mocchegiani F, Orlando F, D'Amato G, Saba V, Scalise G, Bernes S, Mor A. (2004) A chimeric peptide composed of a dermaseptin derivative and an RNA III-inhibiting peptide prevents graft-associated infections by antibiotic-resistant staphylococci. *Antimicrob Agents Chemother* 48:2544-2550.

Barber CE, Tang JL, Feng JX, Pan MQ, Wilson TJ, Slater H, Dow JM, Williams P, Daniels MJ (1997) A novel regulatory system required for pathogenicity of *Xanthomonas campestris* is mediated by a small diffusible signal molecule *Mol Microbiol* 24:555-566.

Barraud N, Hassett DJ, Hwang SH, Rice SA, Kjelleberg S, Webb JS (2006) Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. *J Bacteriol* 188:7344-7353.

Barraud N, Schleheck D, Klebensberger J, Webb JS, Hassett DJ, Rice SA, Kjelleberg S (2009) Nitric oxide signaling in *Pseudomonas aeruginosa* biofilms mediates phosphodiesterase activity, decreased cyclic diguanosine-5'-monophosphate levels and enhanced dispersal. *J Bacteriol* 191:7333-7342.

Bjarnsholt T, Jensen PØ, Rasmussen TB, Christophersen L, Calum H, Hentzer, M, Hougen H, Rygaard J, Moser C, Eberl L, Høiby N, Givskov M (2005) Garlic blocks quorum sensing and promotes rapid clearing of pulmonary *Pseudomonas aeruginosa* infection. *Microbiology* 151:3873-3880.

1
2
3 Boehm A, Steiner S, Zaehring F, Casanova A, Hamburger F, Ritz D, Keck W, Ackermann M,
4 Schirmer T, Jenal U (2009) Second messenger signalling governs *Escherichia coli* biofilm
5 induction upon ribosomal stress. *Mol Microbiol* 72:1500-1516.
6
7

8
9
10
11 Boles BR, Horswill AR (2008) Agr-mediated dispersal of *Staphylococcus aureus* biofilms. *PLoS*
12 *Pathog* 4:e1000052.
13
14

15
16
17 Boyd A, Chakrabarty AM (1994) Role of alginate lyase in cell detachment of *Pseudomonas*
18 *aeruginosa*. *Appl Environ Microbiol* 60:2355-2359.
19
20

21
22
23 Burke C, Thomas T, Egan S, Kjelleberg S (2007) The use of functional genomics for the
24 identification of a gene cluster encoding for the biosynthesis of an antifungal tambjamine in the
25 marine bacterium *Pseudoalteromonas tunicata*. *Environ Microbiol* 9:814-818.
26
27

28
29
30
31 Cashel M, Gentry DR, Hernandez VJ, Vinella D (1996) The stringent response. In Neidhardt FC,
32 Curtiss R, Ingraham, JL, Lin ECC, Low KB, Magasanik B, Reznikoff W, Riley M, Schaechter M,
33 Umberger AE, (eds). *Escherichia coli* and *Salmonella typhimurium* cellular and molecular biology.
34 American Society of Microbiology, Washington DC, pp 1458–1496.
35
36
37

38
39
40
41 Carté B, Faulkner DJ (1986) Role of secondary metabolites in feeding associations between a
42 predatory nudibranch, two grazing nudibranchs and a bryozoan. *J Chem Ecol* 12:795–804.
43
44

45
46
47 Ceri H, Olson M, Morck D, Storey D, Read R, Buret A, Olson B (2001) The MBEC Assay System:
48 multiple equivalent biofilms for antibiotic and biocide susceptibility testing. *Methods Enzymol*
49 337:377-385.
50
51
52

53
54
55 Chan C, Paul R, Samoray D, Amiot NC, Giese B, Jenal U, Schirmer T (2004) Structural basis of
56 activity and allosteric control of diguanylate cyclase. *Proc Natl Acad Sci U S A* 101:17084-17089.
57
58
59
60

1
2
3 Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM (1995) Microbial
4
5 biofilms. *Annu Rev Microbiol* 49:711-745.
6
7

8
9 Cramton SE, Gerke C, Schnell NF, Nichols WW, Götz F (1999) The intercellular adhesion (*ica*)
10
11 locus is present in *Staphylococcus aureus* and is required for biofilm formation. *Infect Immun*
12
13 67:5427-5433.
14
15

16
17 Darouiche RO, Mansouri MD, Gawande PV, Madhyastha S (2009) Antimicrobial and antibiofilm
18
19 efficacy of triclosan and DispersinB combination. *J Antimicrob Chemother* 64:88-93.
20
21

22
23 Davies DG, Marques CN (2009) A fatty acid messenger is responsible for inducing dispersion in
24
25 microbial biofilms. *J Bacteriol* 191:1393-1403.
26
27

28
29 De N, Pirruccello M, Krasteva PV, Bae N, Raghavan RV, Sondermann H (2008) Phosphorylation-
30
31 independent regulation of the diguanylate cyclase WspR. *PLoS Biol* 6:e67.
32
33

34
35 Donelli G, Francolini I, Romoli D, Guaglianone E, Piozzi A, Rangunath C, Kaplan JB (2007)
36
37 Synergistic activity of dispersinB and cefamandole nafate in inhibition of staphylococcal biofilm
38
39 growth on polyurethanes. *Antimicrob Agents Chemother* 51:2733-2740.
40
41

42
43 Dong YH, Gusti AR, Zhang Q, Xu JL, Zhang LH (2002) Identification of quorum-quenching N-
44
45 acyl homoserine lactonases from *Bacillus* species. *Appl Environ Microbiol* 68:1754-1759.
46
47

48
49 Dow JM, Crossman L, Findlay K, He YQ, Feng JX Tang JL (2003) Biofilm dispersal in
50
51 *Xanthomonas campestris* is controlled by cell-cell signaling and is required for full virulence to
52
53 plants. *Proc Natl Acad Sci U S A* 100:10995-11000.
54
55

56
57 Fuqua C, Winans SC, Greenberg EP (1996) Census and consensus in bacterial ecosystems: the
58
59 LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annu Rev Microbiol* 50:727-751.
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Galperin MY (2004) Bacterial signal transduction network in a genomic perspective. *Environ Microbiol* 6: 552-567.

Geske GD, O'Neill JC, Miller DM, Mattmann ME, Blackwell HE (2007) Modulation of bacterial quorum sensing with synthetic ligands: systematic evaluation of N-acylated homoserine lactones in multiple species and new insights into their mechanisms of action. *J Am Chem Soc* 129:13613-13625.

Giacometti A, Cirioni O, Gov Y, Ghiselli R, Del Prete MS, Mocchegiani F, Saba V, Orlando F, Scalise G, Balaban N, Dell'Acqua G (2003) RNA III inhibiting peptide inhibits in vivo biofilm formation by drug-resistant *Staphylococcus aureus* *Antimicrob Agents Chemother* 47:1979-1983.

Gjermansen M, Ragas P, Sternberg C, Molin S, Tolker-Nielsen T (2005) Characterization of starvation-induced dispersion in *Pseudomonas putida* biofilms. *Environ Microbiol* 7:894-906.

Gotoh H, Zhang Y, Dallo SF, Hong S, Kasaraneni N, Weitaio T (2008) *Pseudomonas aeruginosa*, under DNA replication inhibition, tends to form biofilms via Arr. *Res Microbiol* 159:294-302.

Guiton PS, Hung CS, Kline KA, Roth R, Kau AL, Hayes E, Heuser J, Dodson KW, Caparon MG, Hultgren SJ (2009) Contribution of autolysin and Sortase a during *Enterococcus faecalis* DNA-dependent biofilm development. *Infect Immun* 77:3626-3638.

Hammer BK, Bassler BL (2003) Quorum sensing controls biofilm formation in *Vibrio cholerae*. *J Bacteriol* 185:101-104.

Hegde M, Wood TK, Jayaraman A (2009) The neuroendocrine hormone norepinephrine increases *Pseudomonas aeruginosa* PA14 virulence through the las quorum-sensing pathway. *Appl Microbiol Biotechnol* 84:763-776.

1
2
3 Hentzer M, Riedel K, Rasmussen TB, Heydorn A, Andersen JB, Parsek MR, Rice SA, Eberl L,
4
5 Molin S, Høiby N, Kjelleberg S, Givskov M (2002) Inhibition of quorum sensing in *Pseudomonas*
6
7 *aeruginosa* biofilm bacteria by a halogenated furanone compound. *Microbiology* 148:87-102.
8
9

10
11 Hentzer M, Givskov M (2003) Pharmacological inhibition of quorum sensing for the treatment of
12
13 chronic bacterial infections. *J Clin Invest* 112:1300-1307.
14
15

16
17 Hentzer M, Wu H, Andersen JB, Riedel K, Rasmussen TB, Bagge N, Kumar N, Schembri MA,
18
19 Song Z, Kristoffersen P, Manefield M, Costerton JW, Molin S, Eberl L, Steinberg P, Kjelleberg S,
20
21 Høiby N, Givskov M (2003) Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing
22
23 inhibitors. *EMBO J.* 22:3803-3815.
24
25

26
27 Hoffman LR, D'Argenio DA, MacCoss MJ, Zhang Z, Jones RA, Miller SI (2005) Aminoglycoside
28
29 antibiotics induce bacterial biofilm formation. *Nature* 436 :1171-1175.
30
31

32
33 Hoffmann N, Lee B, Hentzer M, Rasmussen TB, Song Z, Johansen HK, Givskov M, Høiby N
34
35 (2007) Azithromycin blocks quorum sensing and alginate polymer formation and increases the
36
37 sensitivity to serum and stationary-growth-phase killing of *Pseudomonas aeruginosa* and attenuates
38
39 chronic *P. aeruginosa* lung infection in *Cftr(-/-)* mice. *Antimicrob Agents Chemother.* 51:3677-
40
41 3687.
42
43

44
45 Holland LM, O'Donnell ST, Ryjenkov DA, Gomelsky L, Slater SR, Fey PD, Gomelsky M, O'Gara
46
47 JP (2008) A staphylococcal GGDEF domain protein regulates biofilm formation independently of
48
49 cyclic dimeric GMP. *J Bacteriol* 190:5178-5189.
50
51

52
53
54 Horswill AR, Stoodley P, Stewart PS, Parsek MR (2007) The effect of the chemical, biological, and
55
56 physical environment on quorum sensing in structured microbial communities. *Anal Bioanal Chem*
57
58 387:371-380.
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Huigens RW 3rd, Ma L, Gambino C, Moeller PD, Basso A, Cavanagh J, Wozniak DJ, Melander C (2008) Control of bacterial biofilms with marine alkaloid derivatives. *Mol Biosyst* 4:614-621.

Iauk L, Lo Bue AM, Milazzo I, Rapisarda A, Blandino G (2003) Antibacterial activity of medicinal plant extracts against periodontopathic bacteria *Phytother Res* 17:599-604.

Ichimiya T, Takeoka K, Hiramatsu K, Hirai K, Yamasaki T, Nasu M (1996) The influence of azithromycin on the biofilm formation of *Pseudomonas aeruginosa* in vitro. *Chemotherapy* 42:186-191.

Izano EA, Sadovskaya I, Vinogradov E, Mulks MH, Velliyagounder K, Ragunath C, Kher WB, Ramasubbu N, Jabbouri S, Perry MB, Kaplan JB (2007) Poly-N-acetylglucosamine mediates biofilm formation and antibiotic resistance in *Actinobacillus pleuropneumoniae*. *Microb Pathog* 43:1-9.

James SG, Holmström C, Kjelleberg S (1996) Purification and characterization of a novel antibacterial protein from the marine bacterium D2. *Appl Environ Microbiol* 62:2783-2788.

Janzon L, Arvidson S (1990) The role of the \square -lysin gene (*hld*) in the regulation of virulence genes by the accessory gene regulator (*agr*) in *Staphylococcus aureus*. *EMBO J* 9:1391-1399.

Junker LM, Clardy J (2007) High-throughput screens for small-molecule inhibitors of *Pseudomonas aeruginosa* biofilm development. *Antimicrob Agents Chemother* 51:3582-3590.

Kadurugamuwa JL, Beveridge TJ (1995) Virulence factors are released from *Pseudomonas aeruginosa* in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion. *J Bacteriol* 177:3998-4008.

1
2
3 Kaplan JB, Rangunath C, Ramasubbu N Fine DH (2003) Detachment of *Actinobacillus*
4 *actinomycetemcomitans* biofilm cells by an endogenous α -hexosaminidase activity. J Bacteriol
5
6 185:4693-4698.
7
8

9
10
11 Karatan E, Watnick PI (2009) Signals, regulatory networks, and materials that build and break
12 bacterial biofilms. Microbiol Mol Biol Rev 73:310-347.
13
14

15
16
17 Kiran MD, Adikesavan NV, Cirioni O, Giacometti A, Silvestri C, Scalise G, Ghiselli R, Saba V,
18
19 Orlando F, Shoham M, Balaban N (2008) Discovery of a quorum-sensing inhibitor of drug-resistant
20 staphylococcal infections by structure-based virtual screening. Mol Pharmacol 73:1578-1586.
21
22

23
24
25 Kong KF, Vuong C, Otto M (2006) *Staphylococcus* quorum sensing in biofilm formation and
26 infection. Int J Med Microbiol 296:133-139.
27
28

29
30
31 König GM, Kehraus S, Seibert SF, Abdel-Lateff A, Müller D (2006) Natural products from marine
32 organisms and their associated microbes. Chembiochem 7:229-238.
33
34

35
36
37 Kulasakara H, Lee V, Brencic A, Liberati N, Urbach J, Miyata S, Lee DG, Neely AN, Hyodo M,
38
39 Hayakawa Y, Asubel FM, Lory S (2006) Analysis of *Pseudomonas aeruginosa* diguanylate
40 cyclases and phosphodiesterases reveals a role for bis-(3'-5')-cyclic-GMP in virulence. Proc Natl
41 Acad Sci U S A 103:2839-2844.
42
43
44
45
46

47
48
49 Lewis K (2008) Multidrug tolerance of biofilms and persister cells. Curr Top Microbiol Immunol
50 322:107-131.
51

52
53
54 Li M, Ni N, Chou HT, Lu CD, Tai PC, Wang B (2008) Structure-based discovery and experimental
55 verification of novel AI-2 quorum sensing inhibitors against *Vibrio harveyi*. ChemMedChem
56 3:1242-1249.
57
58
59
60

1
2
3 Li YH, Tang N, Aspiras MB, Lau PC, Lee JH, Ellen RP, Cvitkovitch DG (2002) A quorum-sensing
4 signaling system essential for genetic competence in *Streptococcus mutans* is involved in biofilm
5 formation. *J Bacteriol* 184:2699-2708.
6
7

8
9
10
11 Lönn-Stensrud J, Landin MA, Benneche T, Petersen FC, Scheie AA (2009) Furanones, potential
12 agents for preventing *Staphylococcus epidermidis* biofilm infections? *J Antimicrob Chemother*
13 63:309-316
14
15

16
17
18
19 Mai-Prochnow A, Lucas-Elio P, Egan S, Thomas T, Webb JS, Sanchez-Amat A, Kjelleberg S
20 (2008) Hydrogen peroxide linked to lysine oxidase activity facilitates biofilm differentiation and
21 dispersal in several gram-negative bacteria. *J Bacteriol* 190:5493-5501.
22
23

24
25
26
27 Manefield M Rasmussen TB, Henzter M, Andersen JB, Steinberg P, Kjelleberg S, Givskov M
28 (2002) Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover.
29 *Microbiology* 118:1119-1127.
30
31

32
33
34
35 Marketon MM, Glenn SA, Eberhard A, González JE (2003) Quorum sensing controls
36 exopolysaccharide production in *Sinorhizobium meliloti* *J Bacteriol* 185:325-331.
37
38

39
40
41 McKnight SL, Iglewski BH, Pesci EC (2000) The *Pseudomonas* quinolone signal regulates *rhl*
42 quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* 182:2702-2708.
43
44

45
46
47 McLennan MK, Ringoir DD, Fridrich E, Svensson SL, Wells DH, Jarrell H, Szymanski CM,
48 Gaynor EC (2008) *Campylobacter jejuni* biofilms up-regulated in the absence of the stringent
49 response utilize a calcofluor white-reactive polysaccharide. *J Bacteriol* 190:1097-1107.
50
51

52
53
54
55 Miller MB, Bassler BL (2001) Quorum sensing in bacteria. *Annu Rev Microbiol* 55:165-199.
56
57

1
2
3 Mizukane R, Hirakata Y, Kaku M, Ishii Y, Furuya N, Ishida K, Koga H, Kohno S, Yamaguchi K
4
5 (1994) Comparative in vitro exoenzyme-suppressing activities of azithromycin and other macrolide
6
7 antibiotics against *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 38:528-533.
8
9

10
11 Morgan R, Kohn D, Hwang SH, Hasset DJ, Sauer K (2006) BdlA, a chemotaxis regulator essential
12
13 for biofilm dispersion in *Pseudomonas aeruginosa*. *J Bacteriol* 188:7335-7343.
14
15

16
17 Müh U, Schuster M, Heim R, Singh A, Olson ER, Greenberg EP (2006) Novel *Pseudomonas*
18
19 *aeruginosa* quorum-sensing inhibitors identified in an ultra-high-throughput screen. *Antimicrob*
20
21 *Agents Chemother* 50:3674-3679.
22
23

24
25 Muto Y, Goto S (1986) Transformation by extracellular DNA produced by *Pseudomonas*
26
27 *aeruginosa*. *Microbiol Immunol* 30:621-8.
28
29

30
31 Nalca Y, Jänsch L, Bredenbruch F, Geffers R, Buer J, Häussler S (2006) Quorum-sensing
32
33 antagonistic activities of azithromycin in *Pseudomonas aeruginosa* PAO1: a global approach.
34
35 *Antimicrob Agents Chemother* 50:1680-1688.
36
37

38
39 Njoroge J, Sperandio J (2009) Jamming bacterial communication: New approaches for the
40
41 treatment of infectious diseases. *EMBO Molec Med* 1:201-210.
42
43

44
45 Novick RP, Ross HF, Projan SJ, Kornblum J, Kreiswirth B, Moghazeh S (1993) Synthesis of
46
47 staphylococcal virulence factors is controlled by a regulatory RNA molecule. *EMBO J* 12:3967-
48
49 3975.
50
51

52
53 Nucleo E, Steffanoni L, Fugazza G, Migliavacca R, Giacobone E, Navarra A, Pagani L, Landini P
54
55 (2009) Growth in glucose-based medium and exposure to subinhibitory concentrations of imipenem
56
57 induce biofilm formation in a multidrug-resistant clinical isolate of *Acinetobacter baumannii*. *BMC*
58
59 *Microbiol* 9:270.
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Odenholt I (2001) Pharmacodynamic effects of subinhibitory antibiotic concentrations Int J Antimicrob Agents 17:1-8.

Ozer EA, Pezzulo A, Shih DM, Chun C, Furlong C, Lulis AJ, Greenberg EP, Zabner J (2005) Human and murine paraoxonase 1 are host modulators of *Pseudomonas aeruginosa* quorum-sensing FEMS Microbiol Lett 253:29-37

Park SY, Kang HO, Jang HS, Lee JK, Koo BT, Yum DY (2005) Identification of extracellular N-acylhomoserine lactone acylase from a *Streptomyces* sp. and its application to quorum quenching. Appl Environ Microbiol 71:2632-2641.

Paul VJ, Lindquist N, Fenical W (1990) Chemical defences of the tropical ascidian *Atapozoa*-sp. and its nudibranch predators *Nembrotha*-spp. Mar Ecol Prog Ser 59:109-118.

Persson T, Hansen TH, Rasmussen TB, Skindersø ME, Givskov M, Nielsen J (2005) Rational design and synthesis of new quorum-sensing inhibitors derived from acylated homoserine lactones and natural products from garlic. Org Biomol Chem 3:253-262.

Pratten J, Foster SJ, Chan PF, Wilson M, Nair SP (2001) *Staphylococcus aureus* accessory regulators: expression within biofilms and effect on adhesion. Microbes Infect 3:633-637.

Qin Z, Yang L, Qu D, Molin S, Tolker-Nielsen T (2009) *Pseudomonas aeruginosa* extracellular products inhibit staphylococcal growth, and disrupt established biofilms produced by *Staphylococcus epidermidis*. Microbiology 155:2148-2156.

Rasmussen TB, Skindersø ME, Bjarnsholt T, Phipps RK, Christensen KB, Jensen PO, Andersen JB, Koch B, Larsen TO, Hentzer M, Eberl L, Hoiby N, Givskov M (2005) Identity and effects of quorum-sensing inhibitors produced by *Penicillium* species. Microbiology 151:1325-1340.

- 1
2
3 Recsei P, Kreiswirth B, O'Reilly M, Schlievert P, Gruss A, Novick RP (1986) Regulation of
4 exoprotein gene expression in *Staphylococcus aureus* by agar. *Mol Gen Genet* 202:58-61.
5
6
7
8
9 Ren D, Bedzyk LA, Setlow P, England DF, Kjelleberg S, Thomas SM, Ye RW, Wood TK (2004).
10 Differential gene expression to investigate the effect of (5Z)-4-bromo- 5-(bromomethylene)-3-
11 butyl-2(5H)-furanone on *Bacillus subtilis*. *Appl Environ Microbiol* 70:4941-4949
12
13
14
15
16
17 Richards JJ, Ballard TE, Huigens RW 3rd, Melander C (2008) Synthesis and screening of an
18 oroidin library against *Pseudomonas aeruginosa* biofilms. *Chembiochem* 9:1267-1279.
19
20
21
22
23 Rivardo F, Turner RJ, Allegrone G, Ceri H, Martinotti MG (2009) Anti-adhesion activity of two
24 biosurfactants produced by *Bacillus* spp. prevents biofilm formation of human bacterial pathogens.
25
26
27
28
29
30
31 Rogers SA, Krayner M, Lindsey JS, Melander C (2009) Tandem dispersion and killing of bacteria
32 from a biofilm. *Org Biomol Chem* 7:603-6.
33
34
35
36
37 Sauer K, Cullen MC, Rickard AH, Zeef LA, Davies DG, Gilbert P (2004) Characterization of
38 nutrient-induced dispersion in *Pseudomonas aeruginosa* PAO1 biofilm. *J Bacteriol* 186:7312-26.
39
40
41
42
43 Simm R, Morr M, Kader A, Nimtiz M, Römling U (2004) GGDEF and EAL domains inversely
44 regulate cyclic di-GMP levels and transition from sessility to motility. *Mol Microbiol* 53:1123-
45
46
47
48
49
50
51 Smith KM, Bu Y, Suga H (2003) Induction and inhibition of *Pseudomonas aeruginosa* quorum
52 sensing by synthetic autoinducer analogs. *Chem Biol* 10:81-89.
53
54
55
56
57 Smith RS, Iglewski BH. (2003) *Pseudomonas aeruginosa* quorum sensing as a potential
58 antimicrobial target. *J Clin Invest* 112:1460-1465.
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Sudarsan N, Lee ER, Weinberg Z, Moy RH, Kim JN, Link KH, Breaker RR (2008) Riboswitches in eubacteria sense the second messenger cyclic di-GMP. *Science* 321:411-413

Svitil AL, Cashel M, Zyskind JW (1993) Guanosine tetraphosphate inhibits protein synthesis in vivo. A possible protective mechanism for starvation stress in *Escherichia coli*. *J Biol Chem* 268:2307-2311.

Tamayo R, Pratt JT, Camilli A (2007) Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. *Annu Rev Microbiol* 61:131-148.

Tateda K, Comte R, Pechere JC, Köhler T, Yamaguchi K, Van Delden C (2001) Azithromycin inhibits quorum sensing in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 45:1930-1933.

Thormann KM, Saville RM, Shukla S, Spormann AM (2005) Induction of rapid detachment in *Shewanella oneidensis* MR-1 biofilms. *J Bacteriol* 187:1014-21.

Thormann K, Duttler MS, Saville RM, Hyodo M, Shukla S, Hayakawa Y, Spormann AM (2006) Control of formation and cellular detachment from *Shewanella oneidensis* MR-1 biofilms by cyclic di-GMP. *J Bacteriol* 188:2681-91.

Ueda A, Attila C, Whiteley M, Wood TK (2009) Uracil influences quorum sensing and biofilm formation in *Pseudomonas aeruginosa* and fluorouracil is an antagonist. *Microbial Biotechnology* 2:62-74.

Ueda A, Wood TK (2009) Connecting quorum sensing, c-di-GMP, pel polysaccharide, and biofilm formation in *Pseudomonas aeruginosa* through tyrosine phosphatase TpbA (PA3885). *PLoS Pathog* 5:e1000483.

- 1
2
3 Uroz S, Heinonsalo J (2008) Degradation of N-acyl homoserine lactone quorum sensing signal
4 molecules by forest root-associated fungi. *FEMS Microbiol Ecol* 65:271-278.
5
6
7
8
9 Vilain S, Pretorius JM, Theron J, Brozel VS (2009). DNA as an adhesin: *Bacillus cereus* requires
10 extracellular DNA to form biofilms. *Appl Environ Microbiol* 75:2861-2868.
11
12
13
14
15 Vuong C, Gerke C, Somerville GA, Fischer ER, Otto M (2003) Quorum-sensing control of biofilm
16 factors in *Staphylococcus epidermidis*. *J Infect Dis* 188:706-718.
17
18
19
20
21 Wagner VE, Gillis RJ, Iglewski BH (2004) Transcriptome analysis of quorum-sensing regulation
22 and virulence factor expression in *Pseudomonas aeruginosa*. *Vaccine* 6;22 Suppl 1:S15-20.
23
24
25
26
27 Wagner VE, Li LL, Isabella VM, Iglewski BH (2007) Analysis of the hierarchy of quorum-sensing
28 regulation in *Pseudomonas aeruginosa*. *Anal Bioanal Chem* 387:469-479.
29
30
31
32
33 Wang LH, He Y, Gao Y, Wu JE, Dong YH, He C, Wang SX, Weng LX, Xu JL, Tay L, Fang RX,
34 Zhang LH (2004a) A bacterial cell-cell communication signal with cross-kingdom structural
35 analogues. *Mol Microbiol* 51:903-912.
36
37
38
39
40
41 Wang X, Preston JF 3rd, Romeo T (2004b) The *pgaABCD* locus of *Escherichia coli* promotes the
42 synthesis of a polysaccharide adhesin required for biofilm formation. *J Bacteriol* 186:2724-2734.
43
44
45
46
47 Weber H, Pesavento C, Possling A, Tischendorf G, Hengge R (2006) Cyclic-di-GMP-mediated
48 signalling within the sigma network of *Escherichia coli*. *Mol Microbiol* 62:1014-1034.
49
50
51
52
53 Weinhouse H, Sapir S, Amikam D, Shilo Y, Volman G, Ohana P, Benziman M (1997) c-di-GMP-
54 binding protein, a new factor regulating cellulose synthesis in *Acetobacter xylinum* *FEBS Lett*
55 416:207-211.
56
57
58
59
60
Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS (2002) Extracellular DNA required for
bacterial biofilm formation *Science* 295:1487.

1
2
3 Xu L, Li H, Vuong C, Vadyvaloo V, Wang J, Yao Y, Otto M, Gao Q (2006) Role of the *luxS*
4 quorum-sensing system in biofilm formation and virulence of *Staphylococcus epidermidis*. Infect
5
6 Immun 74:488-496.
7
8

9
10
11 Yang L, Rybtke MT, Jakobsen TH, Hentzer M, Bjarnsholt T, Givskov M, Tolker-Nielsen T (2009)
12 Computer-aided identification of recognized drugs as *Pseudomonas aeruginosa* quorum-sensing
13 inhibitors. Antimicrob Agents Chemother 53:2432-2443.
14
15
16

17
18
19 Yarwood JM, Bartels DJ, Volper EM, Greenberg EP (2004) Quorum sensing in *Staphylococcus*
20 *aureus* biofilms. J Bacteriol 186:1838-50.
21
22
23

24
25 Zeng Z, Qian L, Cao L, Tan H, Huang Y, Xue X, Shen Y, Zhou S (2008) Virtual screening for
26 novel quorum sensing inhibitors to eradicate biofilm formation of *Pseudomonas aeruginosa*. Appl
27 Microbiol Biotechnol 79:119-126.
28
29
30

31
32
33 Zhu H, Kumar A, Ozkan J, Bandara R, Ding A, Perera I, Steinberg P, Kumar N, Lao W, Griesser
34 SS, Britcher L, Griesser HJ, Willcox MD (2008) Fimbricide-coated antimicrobial lenses: their in
35 vitro and in vivo effects. Optom Vis Sci 85:292-300.
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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
<i>cis</i> -2-decenoic acid	signalling molecule	None	Activity-based screening	Davies and Marques 2009

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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).

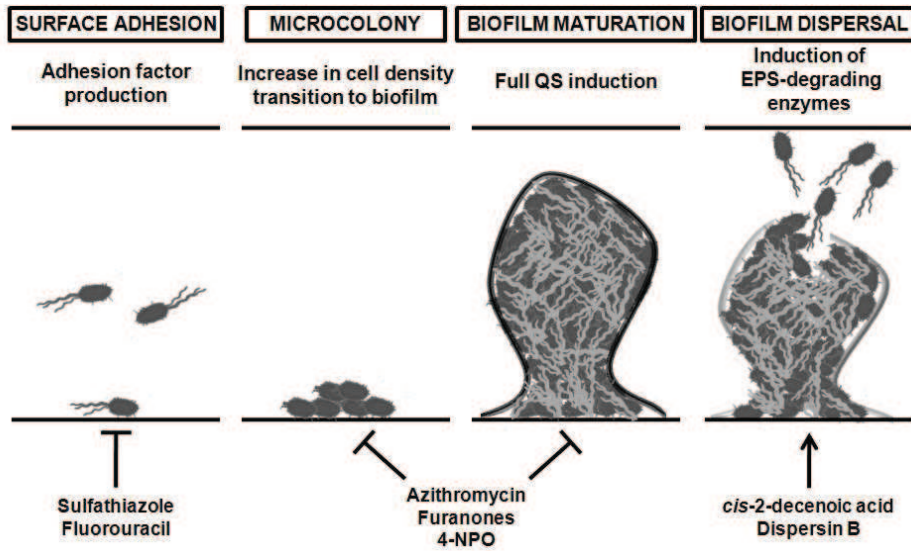
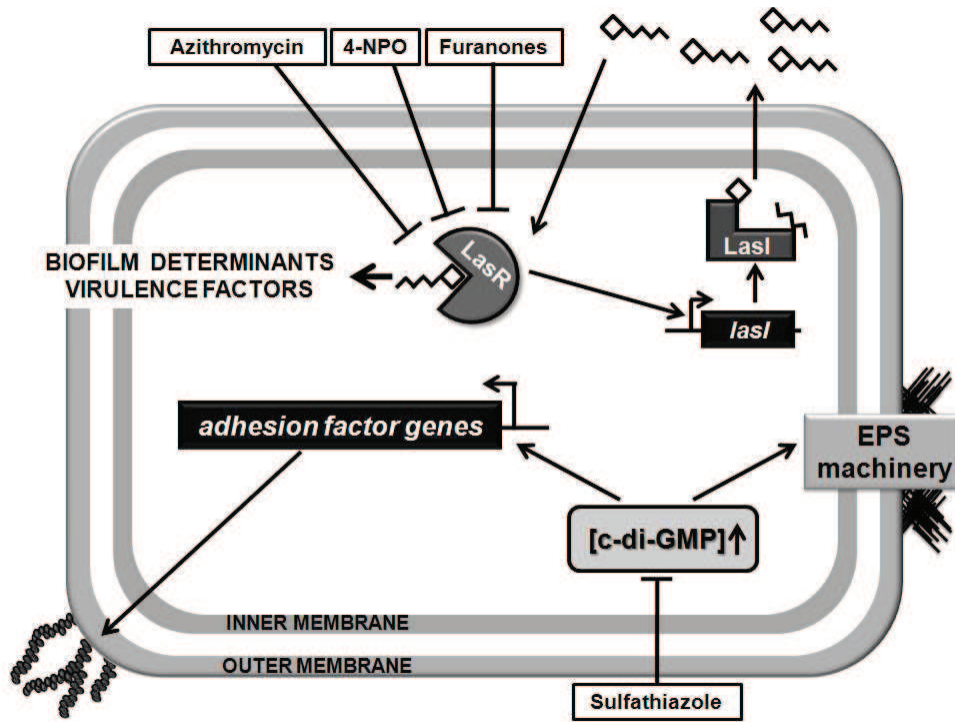


Fig. 1
346x197mm (72 x 72 DPI)

er Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



343x257mm (72 x 72 DPI)

Review

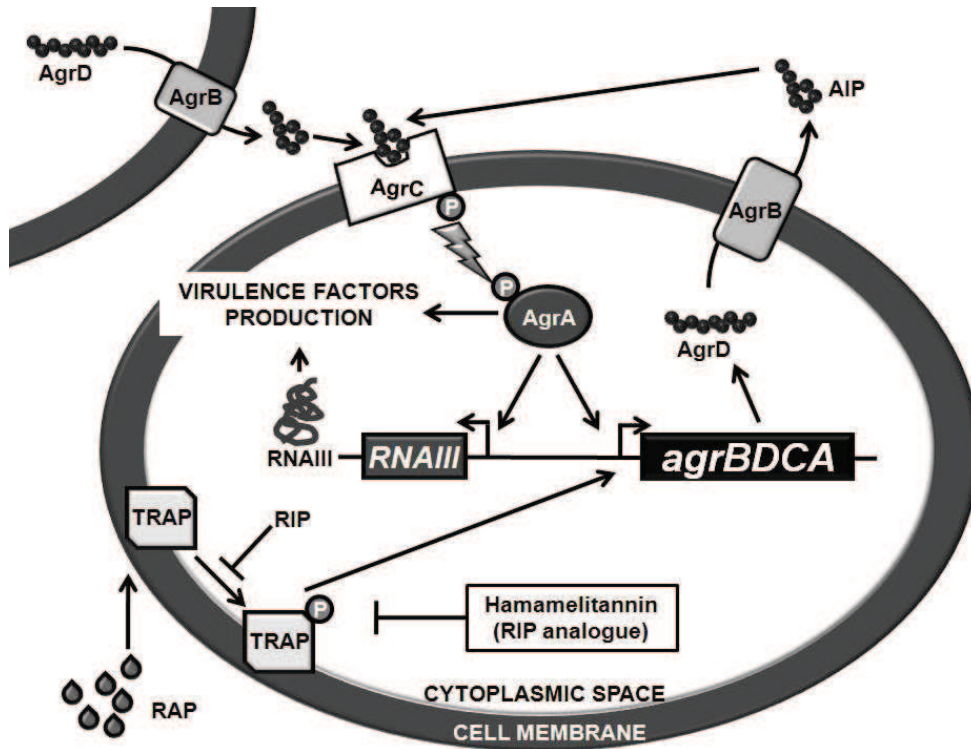


Fig. 2B
326x251mm (72 x 72 DPI)