REVIEW ARTICLE

Multi-species biofilms: how to avoid unfriendly neighbors

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Received 12 October 2011; revised 17 December 2011; accepted 22 December 2011. Final version published online 8 March 2012.

DOI: 10.1111/j.1574-6976.2012.00328.x

Editor: Dieter Haas

Keywords
biofilm formation; adhesion; bacterial interferences; mixed communities; biosurfactants; biofilm dispersion.

Abstract

Multi-species biofilm communities are environments in which complex but ill understood exchanges between bacteria occur. Although monospecies cultures are still widely used in the laboratory, new approaches have been undertaken to study interspecies interactions within mixed communities. This review describes our current understanding of competitive relationships involving nonbiocidal biosurfactants, enzymes, and metabolites produced by bacteria and other microorganisms. These molecules target all steps of biofilm formation, ranging from inhibition of initial adhesion to matrix degradation, jamming of cell–cell communications, and induction of biofilm dispersion. This review presents available data on nonbiocidal molecules and provides a new perspective on competitive interactions within biofilms that could lead to antibiofilm strategies of potential biomedical interest.

Introduction

In most environments, bacteria form multispecies communities and develop heterogeneous structures known as biofilms (Costerton *et al.*, 1987; Hall-Stoodley *et al.*, 2004). In contrast to liquid suspensions, the high cell density and reduced diffusion prevailing within biofilms provide opportunities for intense exchanges ranging from cooperation (for a detailed review of cooperative interactions see the accompanying paper by Elias and Banin appearing in this issue) to harsh competition (James *et al.*, 1995; Moons *et al.*, 2009). Such interactions can lead to physiological and regulatory alterations within biofilm bacteria, and this may eventually contribute to the selection of better adapted mutants. These interactions can influence the emergence and disappearance of species and therefore play an important role in the shaping of multispecies biofilm communities (Hibbing *et al.*, 2010; Dubey & Ben-Yehuda, 2011). Thus far, the studies of how bacteria relate to each other within these communities have often focused on antagonisms impairing fitness of bacterial competitors via, for instance, the production of toxins, scavenger molecules, and antimicrobials.

However, biofilm formation is a complex process involving multiple adhesion and dispersion events which, from initial surface contact to tri-dimensional maturation, can be shaped by microbial interactions that do not necessarily rely on growth-inhibiting molecules or processes (Fig. 1). Recently, the studies on mixed biofilm communities have shed light on a surprising diversity of nonbiocidal compounds targeting different stages of biofilm formation (Table 1). Although most of these compounds were first identified in monospecies cultures or studied in ecologically irrelevant experimental mixed species settings, they could be involved in biofilm population dynamics *in vivo*. This review describes how nonbiocidal molecules affect microbial interactions in biofilm environments and discusses their potential biological role and perspectives as alternative antibiofilm molecules of industrial and biomedical interest.

A cold welcome: Inhibition of initial adhesion

The first interactions between bacteria and surfaces are crucial for biofilm formation and, depending on the

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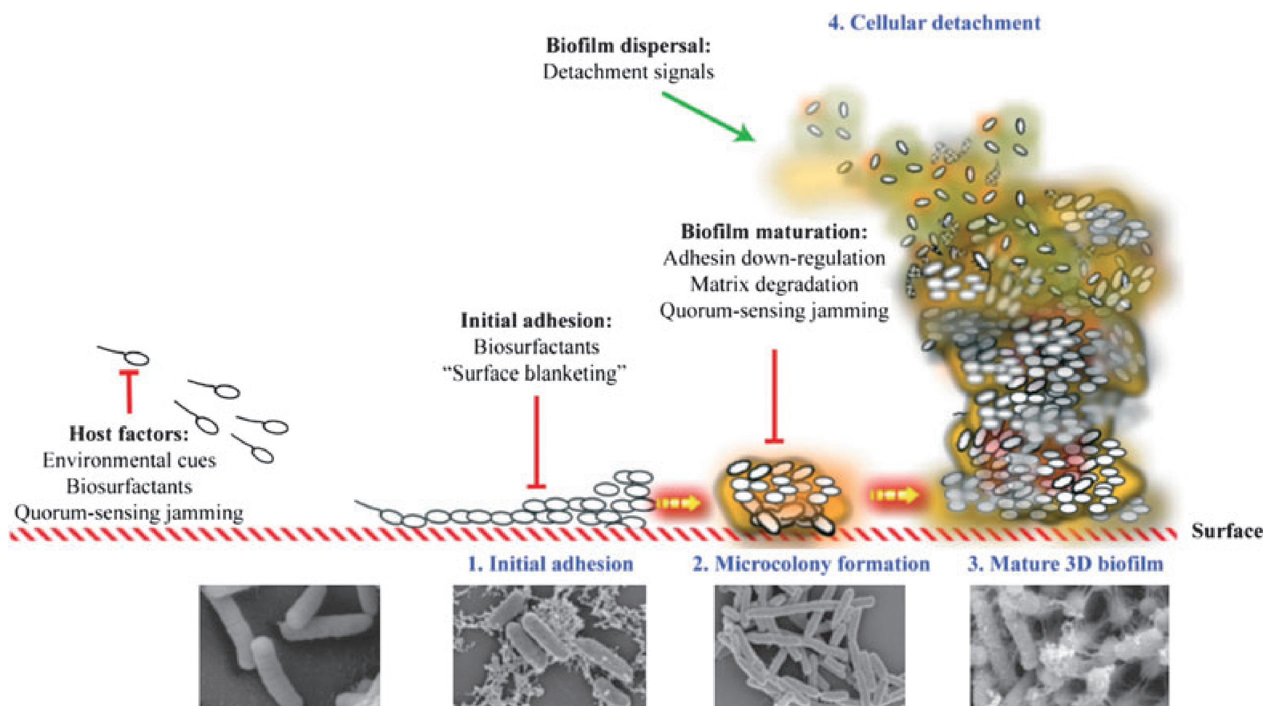

Fig. 1. Antibiofilm molecules act at several stages of the biofilm formation process. Biofilm formation is often described as a multistep process in which bacteria adhere to an abiotic or biotic surface, through surface charges and production of pili, fimbriae, and exopolysaccharides. After initial attachment, three-dimensional development starts with the building of microcolonies, in which different species already interact. The next step, biofilm maturation, is dependent on matrix production, which ensures cohesion and the three-dimensional structure of mature biofilms (Flemming & Wingender, 2010a). Scanning electron microscopy images representative of each steps are shown. The final step in biofilm formation is cellular detachment or dispersion, by which bacteria regain the planktonic lifestyle to colonize other surfaces. Microbial interferences can inhibit biofilm formation or enhance biofilm dispersion through different mechanisms and strategies at different stages of their development.

nature of the surface, can be driven by different mechanisms. Adhesion to abiotic surfaces, for instance, is often mediated by nonspecific events that primarily depend on cell-surface charge and hydrophobicity, the presence of extracellular polymers and organic conditioning film (Dunne, 2002). On the other hand, binding to biotic surfaces such as host tissues and mucosa epithelial cells can be mediated by specific receptors and influenced by host responses to bacterial colonization (Finlay & Falkow, 1989; Kline *et al.*, 2009). While environmental factors influence the initial steps of adhesion, bacterial activity *per se* has also been shown to alter the outcome of surface interactions through either production of antiadhesion molecules that modify surface physico-chemical properties, or composition of a physical bacterial barrier (surface 'blanketing') preventing surface contact with other competing bacteria.

Bacterial surface blanketing

One of the simplest strategies for avoiding initial colonization of competing strains is the rapid occupancy of all available adhesion sites, referred to as 'surface blanketing'. This strategy is illustrated in competition experiments between *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens* (An *et al.*, 2006). In a mixed species co-cultivation experimental model, *P. aeruginosa* rapidly spread through the surface via swarming and twitching motility, preventing *A. tumefaciens* adhesion. In contrast, a *P. aeruginosa flgK* motility-deficient mutant unable to spread quickly over a surface was no longer able to exclude *A. tumefaciens*, therefore allowing *A. tumefaciens* to form a mixed surface biofilm with *P. aeruginosa* (An *et al.*, 2006). Although this simple and intuitive strategy is often mentioned as a possible competition mechanism, the actual contribution of surface blanketing in interspecies interactions is currently not known.

Slippery surface: biosurfactant production

Bacteria have long been known to secrete biosurfactants altering surface properties such as wettability and charge (Neu, 1996; Banat *et al.*, 2010). The physiological roles of these surfactants, widespread among bacteria, are often unclear, but they generally weaken bacteria-surface and bacteria–bacteria interactions, therefore reducing the ability

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Table 1. Biofilm-inhibiting molecules produced by other bacteria. Different colors indicate successive stages of the biofilm life cycle

Susceptible strain	Produced by	Molecule	Step inhibited	Mechanism of action	Molecular basis	Reference
Broad spectrum	*Escherichia coli* UPEC	Group II capsule	Initial adhesion	Alteration of cell-surface and cell-to cell interactions	*kps* region	Valle *et al.* (2006)
Broad spectrum	*Lactobacillus acidophilus*	EPS	Initial adhesion	Downregulation of curli (*crl*, *csgA*, and *csgB*) and chemotaxis	–	Kim *et al.* (2009)
Streptococcus pyogenes	Several marine bacteria	–	Initial adhesion	Reduction of cell-surface hydrophobicity	–	Nithyanand *et al.* (2010)
Streptococcus pyogenes	*Bacillus horikoshii*	–	Initial adhesion	QS inhibition	–	Thenmozhi *et al.* (2009)
Vibrio spp.	*Streptomyces albus*		Initial adhesion	QS inhibition	–	You *et al.* (2007)
Broad spectrum	*Bacillus pumilus S6-15*	4-phenylbutanoic acid	Initial adhesion	Reduces hydrophobicity index and EPS production		Nithya *et al.* (2011)
Escherichia coli CFT073	*Bacillus subtilis*	Biosurfactant	Initial adhesion	–	–	Rivardo *et al.* (2009)
Broad spectrum	*Bacillus licheniformis*	α-D-galactopyranosyl-(1→2)-glycerol-phosphate	Initial adhesion	Independent of quorum sensing		Sayem *et al.* (2011)
Staphylococcus aureus	*Bacillus licheniformis*	Biosurfactant	Initial adhesion	–	–	Rivardo *et al.* (2009)
Streptococcus mutans	*Streptococcus gordonii*	Challisin	Initial adhesion	QS inhibition	*sgc*	Wang *et al.* (2011)
Streptococcus mutans	*Streptococcus salivarius*		Initial adhesion	CSP inactivation by *glrA*-dependent susceptibility		Tamura *et al.* (2009)
Gram-positive bacteria, yeast	*Streptococcus thermophilus A*	Biosurfactant	Initial adhesion	Reduction of cell-surface hydrophobicity	–	Rodrigues *et al.* (2006b)
Enterococcus faecalis	*Lactobacillus*	Surlactin	Initial adhesion	–	–	Velraeds *et al.* (1996)
Agrobacterium tumefaciens	*Pseudomonas aeruginosa*	Small diffusible molecule	Initial adhesion	–	–	Mowat *et al.* (2010)
Pseudomonas aeruginosa PAO1	*Bacillus* spp. SS4	Nonenzymatic	Initial adhesion	QS inhibition		Musthafa *et al.* (2011)
Gram-positive bacteria	*Escherichia coli* Ec300*Klebsiella pneumoniae* 342	Mannose-rich polysaccharide	Initial adhesion	Alteration of cell-surface interactions	*galF-his* region	Rendueles *et al.* (2011)
Broad spectrum	*Kingella kingae*	PAM galactan	Initial adhesion	–	*pamABCDE*	Bendaoud *et al.* (2011)
Streptococcus mutans	*Enterococcus faecium*	Protein	Initial adhesion	–	–	Kumada *et al.* (2009)
Salmonella enterica, Escherichia coli, Proteus mirabilis and Pseudomonas aeruginosa	*Bacillus subtilis*	Surfactin	Initial adhesion	Alteration of cell-surface interactions	sfp locus	Mireles *et al.* (2001)
Broad spectrum	*Streptococcus phocae* PI80	Biosurfactant	Initial adhesion	–	–	Kanmani *et al.* (2011)
Several marine bacteria	*Pseudoalteromonas* sp	–	–	–	–	Klein *et al.* (2011)

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Table 1. Continued

Susceptible strain	Produced by	Molecule	Step inhibited	Mechanism of action	Molecular basis	Reference
Staphylococcus epidermidis	*Pseudomonas aeruginosa*	Psl and Pel polysaccharide	Initial adhesion and biofilm detachment	–	*Psl* and *pel* *operons*	Qin *et al.* (2009)
Pseudomonas putida	*Pseudomonas aeruginosa*	2-heptyl-3-hydroxy-4-quinolone (PQS)	Initial adhesion and biofilm dispersion	Upregulation of swarming motility	*pqsABCDE*	Fernandez-Pinar *et al.* (2011)
Bacillus pumilus TiO1	*Serratia marcescens*	Glycolipid	Initial adhesion and biofilm detachment	Alteration of surface properties	–	Dusane *et al.* (2011)
Staphylococcus aureus	*Staphylococcus epidermidis*	Serine protease Esp	Initial adhesion, biofilm detachment	–	*esp*	Iwase *et al.* (2010)
Pseudomonas aeruginosa	Marine bacteria	–	Initial adhesion and biofilm detachment	QS inhibition and reduces cell-surface hydrophobicity	–	Nithya *et al.* (2010a, b)
Staphylococcus aureus, Staphylococcus epidermidis	*Lactobacillus acidophilus*	Biosurfactant	Initial adhesion, biofilm development and detachment	–		Walencka *et al.* (2008a)
Porphyromonas gingivalis	*Streptococcus intermedius*	Arginine deiminase	Irreversible attachment	Downregulation of two different fimbria (fimA and mfa1)	–	Christopher *et al.* (2010)
Broad spectrum	*Pseudomonas aeruginosa*	Quinolones (alkyl chain)	Biofilm maturation	Alteration of motility		Reen *et al.* (2011)
Streptococcus mutans	*Streptococcus salivarius*	Exo-β-D-fructosidase	Biofilm maturation	Sucrose digestion	fruA	Ogawa *et al.* (2011a)
Candida albicans	*Pseudomonas aeruginosa*	–	Biofilm maturation	Downregulation of biofilm-promoting genes, upregulation of biofilm-inhibiting genes, including YWP1	–	Holcombe *et al.* (2010)
Porphyromonas gingivalis	*Streptococcus crisatus*	Arginine deiminase	Biofilm maturation	Downregulation of long fimbria (*fimA)*	*arcA*	Wu & Xie (2010)
Broad spectrum	*Staphylococcus aureus*	Nuclease	Biofilm maturation	Degradation of nucleic acids	*Nuc1*	Tang *et al.* (2011)
Broad spectrum	*Bacillus licheniformis*	DNAse	Biofilm maturation and detachment	Nuclease activity, DNA degradation	*nucB*	Nijland *et al.* (2010)
Broad spectrum	*Bacillus subtilis* (potentially broad)	D-Aminoacids	Biofilm detachment	Detachment of amyloid fibers from cell wall	racemases	Kolodkin-Gal *et al.* (2010), Xu & Liu (2011)
Bordetella bronchiseptica	*Pseudomonas aeruginosa* PA01	Rhamnolipids	Biofilm detachment	–	*rhlAB*	Boles *et al.* (2005)
Streptococcus mutans	*Lactobacillus reuteri*	–	–	–	–	Soderling *et al.* (2011)

of bacteria and possibly other microorganisms to form and colonize biofilms (Rodrigues *et al.*, 2006b, c; Valle *et al.*, 2006; Walencka *et al.*, 2008b; Rivardo *et al.*, 2009; Jiang *et al.*, 2011; Rendueles *et al.*, 2011). For instance, the well-known surfactin, which is required for *Bacillus subtilis* swarming, also inhibits biofilm formation of different strains, including *Escherichia coli, Proteus mirabilis*, and *Salmonella enterica* (Mireles *et al.*, 2001). Similarly, *Pseudomonas* putisolvins, 12 amino acid lipopeptides linked to a hexanoic lipic chain, are active against other *Pseudomonas* strains (Kuiper *et al.*, 2004). Uropathogenic extraintestinal *E. coli*, on the other hand, were shown to prevent biofilm formation of a wide range of Gram-positive and Gram-negative bacteria because of the release of group 2 capsule, a high molecular weight polysaccharide encoded by the *kps* locus (Valle *et al.*, 2006; Whitfield, 2006). Group 2 capsule increases surface hydrophilicity and reduces bacterial adhesion by inhibiting cell-surface and cell-to-cell interactions in the developing biofilm (Fig. 2; Valle *et al.*, 2006). Recently, a 546-kDa exopolysaccharide (A101) isolated from a marine

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Vibrio was also shown to inhibit initial adhesion of both Gram-negative and Gram-positive bacteria (Fig. 3). In addition, the A101 polysaccharide also affected *P. aeruginosa* cell-to-cell interactions and induced biofilm dispersion of *P. aeruginosa,* but not of *Staphylococcus aureus* (Jiang *et al.*, 2011).

While bacterial adhesion may occasionally occur on bare surfaces, most bacterial adhesion events are likely to take place on surfaces already colonized by other microorganisms. Nonbiocidal tension-active molecules produced by adhering bacteria can prevent entry of incoming bacteria into already formed biofilms. For example, a natural *E. coli* isolate was shown to produce a mannose-rich polysaccharide that impairs *S. aureus* ability to adhere and colonize mature *E. coli* biofilm (Rendueles *et al.*, 2011). In the same study, up to 20% of the screened *E. coli* species produced antibiofilm compounds, suggesting that although colonization resistance could involve other mechanisms, widespread production of antibiofilm polysaccharides could significantly contribute to colonization resistance.

Sabotaging the new neighbors: Inhibition of biofilm maturation

After initial adhesion events, bacteria establish tight surface bonds and connections that enable characteristic biofilm three-dimensional growth and maturation (Fig. 1). This biofilm formation step can be impacted by several nonbiocidal bacterial activities.

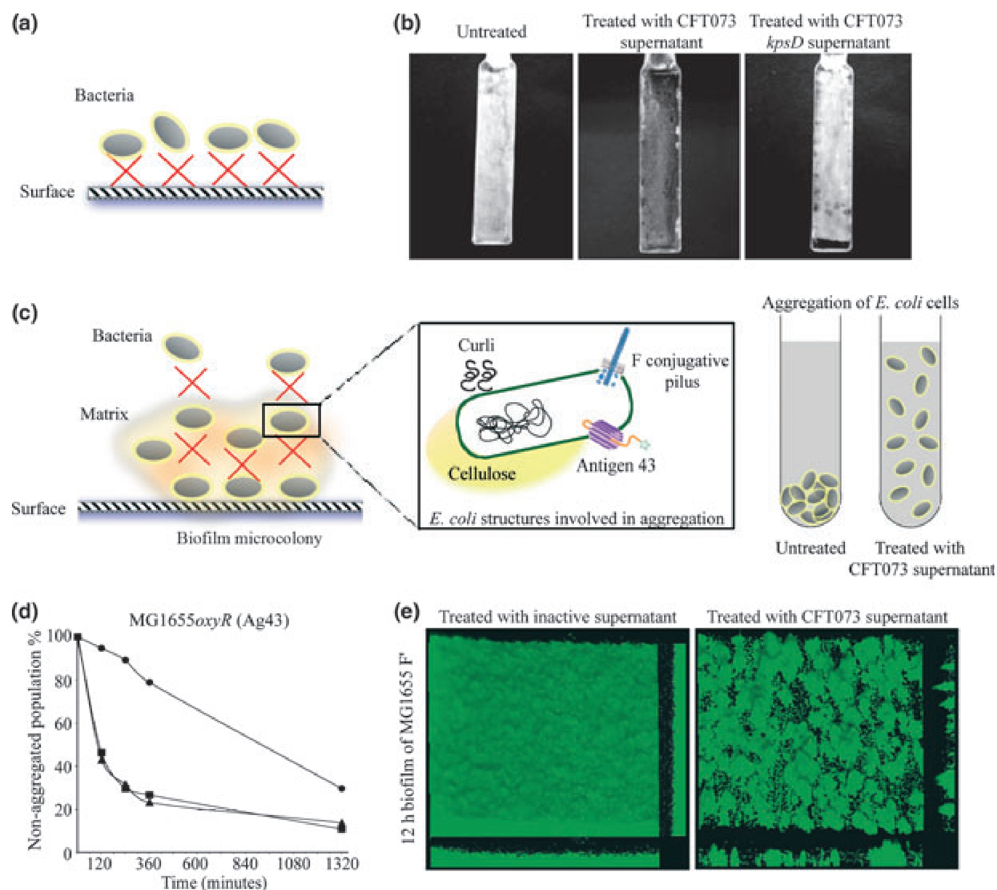

Fig. 2. Group 2 capsule alters cell-to-surface and cell-to-cell interactions. (a) Schematic representation of inhibitory cell-to-surface interactions. (b) Biofilm formation of *Escherichia coli* MG1655 F′ using untreated glass slides (control), glass slides treated with CFT073 supernatant (group 2 capsule) and glass slides treated with CFT073 Δ*kpsD* supernatant devoid of group 2 capsule. (c) Schematic representation of inhibitory cell-to-cell interactions. *Escherichia coli* possesses several extracellular structures that enable bacteria to interact among themselves, such as autotransporters (antigen 43), conjugative pili, curli, and polysaccharides such as cellulose. Expression of these factors generally leads to aggregation and clumping. (d) Autoaggregation assay with MG1655 ΔoxyR (Ag43 autotransporter adhesin overexpression); cells were diluted to $OD_{600\,nm}$ = 2 in 3 mL of M63B1 medium (triangles), treated either with CFT073 supernatant (circles) or Δ*kpsD* supernatant (squares). Adapted from Valle *et al.* (2006). (e) GFP-tagged MG1655 F inoculated in a flow cell and monitored by confocal microscopy. CFT073 or inactive supernatants were supplemented after 3 h of culture, and biofilms were grown for 12 h.

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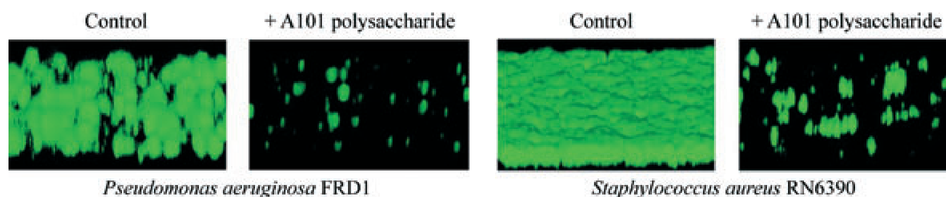

Fig. 3. Treatment of anti-biofilm molecules in *P. aeruginosa* and *S. aureus* biofilms. Flow cell images of *P. aeruginosa* FRD1 and *S. aureus* RN6390 without (control) and with 100 mg mL^{-1} A101 polysaccharide. *Pseudomonas aeruginosa* was cultured at 25 °C for 2 days and *S. aureus* was grown at 37 °C for 24 h. From Jiang *et al.* (2011).

Bonding inhibition: downregulating expression of competitor's adhesins

The studies of the oral ecosystem have provided valuable insight into several mechanisms leading to competitive inhibition of biofilm maturation at the transcriptional level. For instance, surface arginine deiminase ArcA of *Streptococcus cristatus* downregulates expression of *fimA,* which encodes the major subunit of *Porphyromonas gingivalis* long fimbriae and is required for irreversible attachment and further biofilm development (Xie *et al.*, 2000, 2007). A similar study reported that an ArcA homolog of *Streptococcus intermedius* also abolished biofilm formation, but not the growth rate of *P. gingivalis*, by downregulating expression of both short (*mfa1*) and long (*fimA*) fimbriae (Christopher *et al.*, 2010). While the exact mechanism behind this downregulation remains unclear, it has been shown that the regulatory role of ArcA is independent of ArcA deiminase activity (Xie *et al.*, 2007; Wu & Xie, 2010) and requires growth-phase-controlled release of ArcA into the extracellular medium by *S. intermedius* (Christopher *et al.*, 2010).

Matrix exopolysaccharides, besides being essential building blocks of most biofilms and protecting bacteria from desiccation, were recently reported to act as signaling molecules that induce gene expression changes in surrounding bacteria. Formation of biofilms by enterohemorrhagic *E. coli* (EHEC) was, for instance, strongly decreased in the presence of exopolysaccharides extracted from the probiotic bacterium *Lactobacillus acidophilus*. While EHEC growth rates and quorum sensing were not affected, transcription of genes for curli (*crl*, *csgA*, and *csgB*) and chemotaxis (*cheY*) was severely downregulated (Kim *et al.*, 2009). This suggested that *L. acidophilus* polysaccharides could interfere with expression of EHEC surface adhesins. The ability of *L. acidophilus* EPS to inhibit other Gram-positive and Gram-negative biofilms was also demonstrated in *Salmonella enteritidis, Salmonella typhimurium, Yersinia enterocolitica, P. aeruginosa*, and *Listeria monocytogenes* (Kim *et al.*, 2009).

Jamming communication of newcomers

Another hallmark of biofilm physiology is quorum sensing, a density- and dose-dependent communication system that coordinates gene expression at the community level (Bassler & Losick, 2006). While quorum sensing regulates a wide range of functions, controls many virulence traits, and plays an important role in bacterial biofilm formation, it is also involved in the development of mixed species populations (McNab *et al.*, 2003; An *et al.*, 2006). Following increasing interest in identification of molecules interfering with bacterial quorum sensing, it was early shown that bacteria themselves can impair, inhibit, and quench quorum sensing (Ji *et al.*, 1997; Dong *et al.*, 2001). For instance, the *agr* quorum sensing system involved in *S. aureus* virulence and colonization can be subjected to cross-inhibition by closely related strains (Ji *et al.*, 1997). Bacteria can also produce enzymes degrading some quorum sensing molecules, typically acylhomoserine lactones (AHLs), such as AHL lactonase, AHL acylases, and AHL oxidoreductases (Dong *et al.*, 2002; Dong & Zhang, 2005; Czajkowski & Jafra, 2009). Quorum sensing interferences also directly affect bacterial ability to form biofilm, as in the case of *Bacillus cereus* production of AiiA, an AHL lactonase that inhibits *Vibrio cholerae* biofilm formation (Augustine *et al.*, 2010), or bacterial extracts containing phenolic groups and aliphatic amines inhibiting biofilm formation by interfering with *P. aeruginosa* PAO1 quorum sensing (Nithya *et al.*, 2010b; Musthafa *et al.*, 2011).

The oral environment provides other examples of enzymes degrading bacterial communication signals. Two recent studies showed that the outcome of colonization by *Streptococcus mutans*, the primary etiologic agent of human dental caries, relies on successful interactions with other early dental colonizers such as, for instance, *Streptococcus gordonii.* However, *S. gordonii* secretes the serine protease challisin, which inactivates the *S. mutans* competence-stimulating peptide (CSP), a quorum sensing signaling molecule essential for biofilm formation, colonization, and subsequent plaque development (Senadheera

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& Cvitkovitch, 2008). In contrast, *Actinomyces naelundii*, another early colonizer of teeth, has weak overall protease activity that does not impair *S. mutans* in colonizing the shared niche, therefore indicating a role of challisin in preventing colonization by other *Streptococcus* spp. (Wang *et al.*, 2011).

Targeting the biofilm scaffold: matrix inhibition

As we have seen above, the biofilm matrix plays a key structural, defensive and sometimes regulatory role (Sutherland, 2001). It maintains bacterial cohesion, acts as a protective barrier and nutrient sink, and enables biofilm maturation (Flemming *et al.*, 2007; Flemming & Wingender, 2010). The biofilm matrix is therefore an ideal target for compromising the ability of other bacteria to establish and form biofilms (Otto, 2008; Jabbouri & Sadovskaya, 2010; Schillaci, 2011).

Degradation of polysaccharide components of the matrix

Major components of the matrix are polysaccharides (Flemming & Wingender, 2010), whose degradation could potentially prevent biofilm formation in mixed species context. Several enzymes degrading matrix polysaccharides have been identified. For instance, *Actinobacillus actinomycetemcomitans*, a predominant oral bacterium, produces dispersin B that degrades poly-*N*-acetylglucosamine (PNAG), a major polysaccharide component of many bacterial extracellular matrices (Kaplan *et al.*, 2003). This β-hexosaminidase, belonging to the glycosyl hydrolase family, is a matrix-degrading enzyme encoded by the *dspB* locus which can effectively interfere with and disperse pre-existing biofilms of *Staphylococcus epidermidis* by degrading its polysaccharide intercellular adhesin, as well as biofilms of other Gram-positive and Gram-negative bacteria (Kaplan *et al.*, 2004). Matrix-degrading enzymes have also been described for other bacteria, although their role in potential intra-biofilm competition is less clearly established, as opposed to self-destruction and biofilm dispersion (see section Forcing neighbors out: biofilm dispersion). For example, *P. aeruginosa* alginate lyase degrades alginate and *Methanosarcina mazei* disaggregatase reduces matrix polymers into trisaccharide units (Xun *et al.*, 1990; Boyd & Chakrabarty, 1994). Nevertheless, we cannot exclude that the primary role of such molecules is to control biofilm formation of producer themselves rather than antagonizing other species (see also section Avoiding neighbors: biofilm self-inhibition).

A recent study has shown that *Streptococcus salivarius*, a commensal bacterium colonizing the oral, tongue, and throat epithelia, produces a fructosyltransferase and an exo-beta-D-fructosidase (FruA) inhibiting matrix formation and hindering further biofilm development of other oral bacteria, including *S. mutans*. The inhibitory activity of FruA depends on sucrose concentration, because FruA is more active with increasing sucrose concentrations in *in vitro* (microtiter plates coated with hydroxyapatite and saliva) and *in vivo* models of *S. salivarius/S. mutans* mixed biofilm mimicking oral and teeth conditions (Ogawa *et al.*, 2011).

Degradation of nucleic acid component of the matrix

Nucleases such as DNase and RNase were shown to affect integrity of biofilms by degrading nucleic acid scaffold components of the extracellular matrix (Whitchurch *et al.*, 2002). Some bacteria release DNase into the medium and can inhibit biofilm formation of other DNA-dependent biofilm-forming strains. For example, the marine bacterium *Bacillus licheniformis* produces a broad spectrum DNase encoded by the *nucB* gene and is able to rapidly disperse (in 2 min) competing Gram-negative and Gram-positive biofilms and prevent *de novo* biofilm formation (Nijland *et al.*, 2010). Another recent study showed similar effects of the *S. aureus* nuclease Nuc1 upon the ability to form biofilms of several bacteria, including *P. aeruginosa, Actinobacillus pleuropneumoniae*, and *Haemophilus parasuis* (Tang *et al.*, 2011). In addition, there is much evidence that nucleases play a central role in shaping staphylococcal biofilm formation and architecture (Fredheim *et al.*, 2009; Mann *et al.*, 2009).

Degradation of protein components of the matrix

Nonbiocidal antibiofilm molecules can also target matrix-associated proteins. Proteins can either be thoroughly degraded or cut loose from bacterial cell walls by proteases. *Staphylococcus epidermidis*, a commensal bacterium from skin and nose epithelia, inhibits *S. aureus* biofilm formation through production of a serine protease, Esp, which degrades the *S. aureus* matrix without affecting its growth rate (Iwase *et al.*, 2010). Epidemiological studies showed that volunteer nasal cavities carrying Esp-secreting *S. epidermidis* were not colonized by *S. aureus*. Moreover, co-cultures of *S. aureus* with Esp for more than a year did not alter Esp efficiency of biofilm inhibition, indicating that no tolerance or resistance mechanisms arose over time. Interestingly, Esp also stimulates *in vivo* human beta defensin-2, which itself displays low bactericidal activity toward *S. aureus*. Hence, Esp production by *S. epidermidis*

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controls *S. aureus* biofilm formation in *in vitro* and *in vivo* contexts through different mechanisms; matrix degradation, inhibition of initial adhesion, and immune system stimulation (Iwase *et al.*, 2010).

Forcing neighbors out: biofilm dispersion

Dispersion is the final step in the life cycle of a biofilm and is considered a regulated process involving cell death, matrix-degrading enzymes, induction of cellular motility and potentially other environmentally triggered mechanisms (Boles *et al.*, 2005; Karatan & Watnick, 2009). Although some of the molecules involved in dispersion have a broad spectrum of activity against biofilms formed by other bacteria, dispersion has mostly been studied in monospecies cultures, and very few data are available on dispersion as a means of competing with other biofilm-forming bacteria in a mixed biofilm context.

The plant pathogen *Xanthomonas campestris* forms mannane-rich biofilms that clump plant vessels. *X. campestris* dissolves its own biofilms via production of a mannane-degrading enzyme, an endo-β-1,4-mannosidase regulated by *cis*-unsaturated fatty acid diffusible signal factors (DSFs; Ryan & Dow, 2011; Wang *et al.*, 2004). Two enzymes have been implicated in synthesis of DSF, RpfB, and RpfF, and a two-component regulatory system, RpfC–RpfG, that senses and transduces signals into the cells (Slater *et al.*, 2000). However, *X. campestris* DSF effects on other bacterial biofilms remain unknown. Following the description of *X. campestris* DSF, several other small fatty acids produced by other bacteria were characterized based on homology with the RpfF–RpfC genes of *X. campestris* implicated in cell-to-cell communication and antibiofilm activity through a signaling cascade involving histidine kinases (RpfC; Ryan & Dow, 2011). For instance, *cis*-2-decenoic acid produced by *P. aeruginosa* disperses *Klebsiella pneumoniae, E. coli, B. subtilis, S. aureus*, and even *Candida* biofilms, as shown by competition experiments (Davies & Marques, 2009). However, not all DSFs share the same mechanism of action or lead to similar phenotypes. For instance, DSF from *Stenotrophomonas maltophilia* does not disperse *P. aeruginosa* biofilms, but rather alters its biofilm architecture and induces formation of filamentous structures (Ryan *et al.*, 2008; Ryan & Dow, 2011). In addition, *N*-butanoyl-homoserine lactone from *Serratia marcescens* mediates its biofilm dispersion (Rice *et al.*, 2005), and *P. aeruginosa* rhamnolipids encoded by the *rhlAB* operon are involved in biofilm structure and dispersion (Boles *et al.*, 2005). Here again, however, there is still no evidence that these signals interfere with other biofilm-forming bacteria.

Another well-studied dispersion signal is nitric oxide (NO) produced by bacteria growing in the deep layers of biofilms under anaerobic conditions. Following microarray results that indicated that NO significantly downregulated adhesin synthesis in *P. aeruginosa* (Firoved *et al.*, 2004), it was shown that low (nanomolar) concentrations of NO control the ratio of biofilm versus planktonic cells and induce dispersion of various mono- and multispecies biofilms (Barraud *et al.*, 2009). Also in *P. aeruginosa*, NO induces swimming and swarming motility functions, leading to *P. aeruginosa* biofilm dispersion (Barraud *et al.*, 2006). In the presence of low concentrations of NO, the levels of intracellular c-di-GMP, a ubiquitous bacterial second messenger generally promoting biofilm formation (Hengge, 2009), were severely reduced because of upregulation of a phosphodiesterase, which degrades c-di-GMP (Barraud *et al.*, 2009).

D-amino acids produced by many bacteria at late stages of growth (Lam *et al.*, 2009) including stationary phase and biofilms were recently shown to disperse bacterial biofilms (Kolodkin-Gal *et al.*, 2010; Xu & Liu, 2011). In the specific case of *B. subtilis*, racemases encoded by *racX* and *ylmE* produce D-amino acids such as D-tyrosine, D-leucine, D-tryptophan, and D-methionine which substitute L-isoforms in the cell wall and inhibit TasA amyloid fiber anchorage (Kolodkin-Gal *et al.*, 2010; Romero *et al.*, 2011). Because tethering of TasA to the bacterial cell surface is an essential step in matrix-dependent biofilm maturation by *B. subtilis*, D-amino acid accumulation disrupts the *B. subtilis* biofilm. Although this is proposed to be a process by which bacteria can self-disperse their own biofilms, the fact that exogenous addition of D-amino acids also disassembles *S. aureus* and *P. aeruginosa* biofilms (Kolodkin-Gal *et al.*, 2010) suggests that D-amino acid production may also interfere with neighbors in the maturation of mixed biofilms. Different mechanisms of action for D-amino acids have been reported; for instance, D-amino acids inhibit accumulation of proteins in the *S. aureus* matrix and development of microcolonies (Hochbaum *et al.*, 2011), while D-tyrosine significantly reduces synthesis of auto-inducer 2 and extracellular polysaccharides (Xu & Liu, 2011).

Cross-kingdom antibiofilm behaviors

Evidence for nonbiocidal activities leading to limitation of biofilm development also exists across kingdoms (Lowery *et al.*, 2008). The best studied of these mild-mannered antagonistic interactions generally are fungi and bacteria (Hogan & Kolter, 2002; Hughes & Sperandio, 2008). For instance, in the case of *Candida albicans* and *P. aeruginosa,* two microorganisms that co-colonize the lungs of patients with cystic fibrosis or severe burn wounds,

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P. aeruginosa was shown to impair biofilm development and maturation of *C. albicans*. A transcriptome analysis of *Candida* genes in the presence of a *Pseudomonas* supernatant revealed downregulation of adhesion and biofilm formation genes and upregulation of *YWP1*, a protein known to inhibit biofilm formation (Holcombe *et al.*, 2010). Another group reported that *P. aeruginosa* can antagonize biofilm formed by other *Candida* species (Bandara *et al.*, 2010). Reciprocally, farnesol, produced by many fungi including *C. albicans*, has been shown to inhibit quinolone synthesis of *P. aeruginosa* and subsequently to downregulate quinolone-controlled genes such as those specifying pyocyanin, which is involved in *P. aeruginosa* virulence (Cugini *et al.*, 2007).

Fungi produce a wide range of secondary metabolites potentially involved in microbial interactions (Mathivanan *et al.*, 2008). Besides well-known antibiotics, fungi such as Ascomycotina produce zaragozic acids, which are competitive inhibitors of squalene synthase (Bergstrom *et al.*, 1993) and inhibit the formation of microdomains in bacterial membranes known as lipid rafts (Lopez & Kolter, 2010). Zaragozic acids have been recently shown to inhibit *B. subtilis* and *S. aureus* biofilms without affecting bacterial viability via inhibition of membrane lipid raft formation, where signaling and transport proteins involved in biofilm formation are clustered (Lopez & Kolter, 2010).

Another well-described cross-kingdom interaction is the use of molecular mimicry by *Delisea pulchra*, an Australian red alga. *D. pulchra* produces halogenated furanones (Givskov *et al.*,1996), which are similar to AHLs and inhibit quorum sensing of Gram-negative bacteria by reducing the AHL receptor half-life, thus altering AHL-dependent gene expression (Manefield *et al.*, 2002). Similarly, *Flustra foliacea*, a moss animal, produces an alkaloid reported to be an AHL antagonist (Peters *et al.*, 2003).

Many studies explored potential cross-talk between bacteria and their hosts (Hughes & Sperandio, 2008). The host innate response indeed possesses an arsenal of molecules against microbial pathogens, including antibiofilm compounds that efficiently reduce microbial surface colonization (Ardehali *et al.*, 2002, 2003; Hell *et al.*, 2009; Zinger-Yosovich *et al.*, 2010). For instance, PLUNC (palate, lung, nasal epithelium clone) is a protein secreted by epithelia in conducting airways as well as in several fluids including saliva, nasal, and tracheal fluids. This protein displays marked hydrophobicity and significantly reduces surface tension. At physiological concentrations, PLUNC inhibits *P. aeruginosa* biofilms in an *in vitro* model (Gakhar *et al.*, 2010). Similarly, numerous studies have described the antiadhesion role of bloodstream serum and albumin. Serum inhibits biofilm formation and enhances dispersion of *P. aeruginosa* by inducing twitching motility. These effects were demonstrated both *in vitro* and on *in situ* catheters, and it was suggested that the inhibitory activity is multifactorial rather than relying on a single serum component (Hammond *et al.*, 2008). In addition, human albumin also inhibits strong biofilm-forming *E. coli*, both in direct incubation or as pretreatment on a plastic surface. However, in the latter case, albumin-dependent iron chelation, and therefore growth limitation, may also be involved (Naves *et al.*, 2010). Other strategies, which involve iron as a regulatory element of bacterial lifestyle, can affect initiation of biofilm formation without affecting bacterial growth. For instance, lactoferrin is a protein naturally produced by humans which, at physiological concentrations, does not affect bacterial viability but reduces *P. aeruginosa* biofilms by chelating environmental iron (Singh *et al.*, 2002). Furthermore, lactoferrin was shown to induce twitching motility in *P. aeruginosa* and therefore to favor movement rather than sessile life within a biofilm (Singh, 2004). Motility induced by iron deficiency has been recently shown to be regulated by quorum sensing (Patriquin *et al.*, 2008).

More recently, it was reported that human nonspecific secretory immunoglobulin A (SIgA) was able to inhibit biofilm formation of *V. cholerae* without affecting the viability of the bacteria. *In vivo* studies have shown that IgA−/− mice are heavily colonized by *V. cholerae* compared with the wild type. Further experiments showed that the biofilm-inhibitory active element of SIgA is the mannose-rich secretory domain of SIgA. Consistently, mannose could also inhibit *V. cholerae* biofilm formation in a dose-dependent manner (Murthy *et al.*, 2011).

Biofilm-specific antiadhesion molecules?

Biofilms constitute an original lifestyle in which it has been estimated that up to 10% of the bacterial genome could be differentially regulated, compared to planktonic conditions (Whiteley *et al.*, 2001; Schembri *et al.*, 2003; Beloin *et al.*, 2004; Lazazzera, 2005). A few studies provide evidence that these changes in gene expression lead to production of biofilm-specific metabolites and polymers (Beloin *et al.*, 2004; Matz *et al.*, 2008; Valle *et al.*, 2008; Colvin *et al.*, 2011). Some of these biofilm-associated molecules display antagonist activities against other microorganisms in mixed species contexts. For example, accumulation of amino acid valine in biofilm formed by many Gram-negative bacteria inhibits the growth of several valine-sensitive *E. coli* natural isolates (Valle *et al.*, 2008). Similarly, *B. licheniformis* produces antimicrobial compounds against other *Bacillus* species when cultured as a biofilm, whereas biocidal activity is significantly reduced when grown in shaken cultures (Yan *et al.*, 2003).

While nonbiocidal antibiofilm molecules are not *stricto sensu* biofilm-specific, because traces can still be detected

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in planktonic conditions, such molecules appear to be strongly produced within a biofilm (Fig. 4a). For instance, genes involved in the synthesis and regulation of the Ec300p antibiofilm polysaccharide (e.g. *rfaH*) produced by a natural *E. coli* isolate (*E. coli* Ec300) are upregulated in late stationary phase and biofilms (Fig. 4b). Altogether, this leads to increased production of Ec300p within biofilms (Rendueles *et al.*, 2011). A linear polysaccharide (PAM galactan) is copiously produced within biofilms formed by the oral bacterium *Kingella kingae,* whereas yields obtained from batch cultures are significantly lower (Bendaoud *et al.*, 2011). While further studies of genes whose expression is cryptic under planktonic conditions may still uncover the existence of true biofilm-specific molecules (Ghigo, 2003; Korea *et al.*, 2010), high cell densities within biofilms have already revealed molecules which are poorly produced or not detected in batch cultures and which affect population dynamics in mixed bacterial communities (Bendaoud *et al.*, 2011; Rendueles *et al.*, 2011).

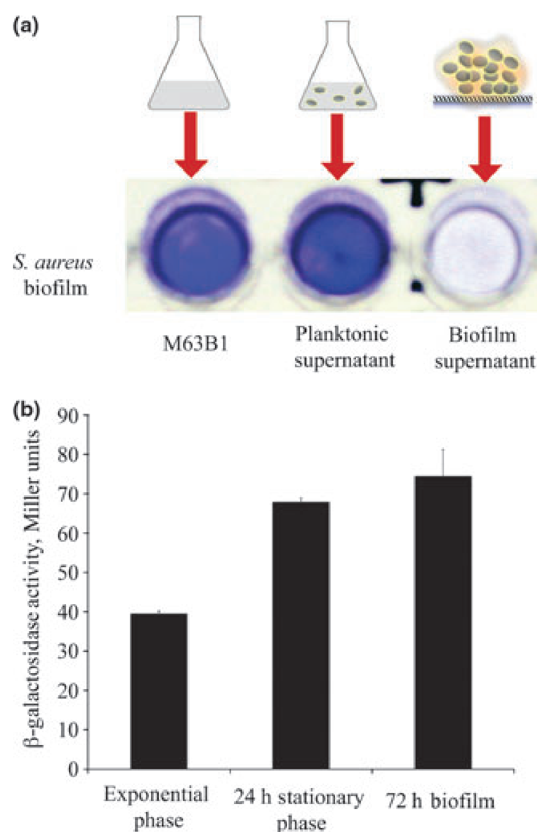

Fig. 4. Antiadhesion polysaccharide produced by *Escherichia coli* Ec300 is produced in higher quantities within biofilms. (a) *Staphylococcus aureus* biofilm inhibition upon the addition of planktonic or biofilm supernatant from *E. coli* Ec300. M63B1, control in which only M63B1 minimal medium was added. (b) Beta-galactosidase activity measurements of a *lacZ* transcriptional fusion in *rfaH*, the transcriptional regulator gene of *E. coli* Ec300 controlling antiadhesion polysaccharide, in exponential phase, late stationary phase (24 h) and biofilm (72 h). Adapted from Rendueles *et al.* (2011) and unpublished data.

Avoiding neighbors: biofilm self-inhibition

Many nonbiocidal antiadhesion molecules described in this review were first identified in monospecies biofilms, and their effects on biofilms formed by other bacteria were often studied only using purified compounds. The ecological role of these molecules has not always been analyzed in mixed biofilms, and their status of antiadhesion weapons interfering with competing neighbors may have been oversold. Indeed, considering that the ultimate strategy for bacteria to avoid interacting with other bacteria could be to inhibit their own ability to adhere to surfaces or to other bacteria in mixed biofilms, biofilm-inhibitory molecules may well serve other purposes. They may be involved in adhesion self-control so as to avoid the cost associated with building a biofilm. Alternatively, avoiding the formation of biofilm may reduce the fitness cost of sheltering spontaneous nonadhering scroungers that invade biofilms and benefit from the community goods without contributing to biofilm formation. Furthermore, far from being involved in intra-biofilm warfare, the net outcome of antiadhesion or dispersion molecules could be an increase in self-dispersion, enabling colonization of other niches or rescue of bacteria trapped in the nutrient- and oxygen-deprived matrix. The synthesis and release of the broad spectrum antibiofilm group 2 capsule by most extra-intestinal *E. coli* is an example in which a nonbiocidal antibiofilm molecule also has an effect upon the producing strain (Valle *et al.*, 2006). While *kps* mutants of uropathogenic *E. coli*, which are unable to synthesize the group 2 capsule, acquire the ability to form thick mature biofilms, wild-type strains are poor biofilm formers, and it is tempting to speculate that their resulting weak ability to mingle with an intestinal biofilm may be correlated with their frequent occurrence in the urogenital tract (Valle *et al.*, 2006).

This therefore raises the question of whether true interference molecules exist. One study reports that nonbiocidal interference molecules are inactive toward the producing strain such as *E. coli* Ec300, which is immune to its antiadhesion polysaccharide, but active against Gram-positive bacteria (Rendueles *et al.*, 2011). Future studies of mixed populations rather than monocultures should contribute to elucidating the ecological role of antibiofilm molecules.

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Lessons to be learned from bad-neighborliness

Although biofilms are ubiquitous and often beneficial, they are also harmful as industrial biofouling agents and as resilient infectious foyers of chronic infections in patients on medical devices (Costerton *et al.*, 1999; Donlan & Costerton, 2002; Parsek & Singh, 2003). This has led many studies to focus on identifying potential treatment of detrimental biofilms both in industrial and medical settings, notably related to catheter-associated biofilms (Francolini & Donelli, 2010; Donlan, 2011). In addition to new biocides and antimicrobial compounds, several alternative antibiofilm strategies have recently emerged. These approaches range from hydrophilic and nanoparticle coatings to more aggressive strategies such as bacteriophages and biofilm predation agents for grazing on problematic biofilm-forming, for instance, in drinking water facilities (Donlan, 2009; Sockett, 2009; Allaker, 2010).

Microbial interference compounds described in this review interfere with several aspects of adhesion and biofilm formation (Fig. 1) and might also be used for nonbiocidal biofilm control strategies (Fig. 5). Much effort has gone into chemical synthesis and screens for molecule-mimicking natural compounds. For instance, bicyclic 2-pyridone derivatives (or pilicides) have been identified in screening for inhibitors of assembly of type 1 pili (Pinkner *et al.*, 2006). They act as competitive inhibitors of chaperone-subunit association, an essential step in pili translocation to the bacterial surface. Similar molecules targeting other adhesion factors, such as curlicides, have also been reported to severely impair curli-dependent biofilm formation and pathogenesis (Pinkner *et al.*, 2006; Aberg & Almqvist, 2007; Cegelski *et al.*, 2009). Attenuation of virulence by acylated hydrazones of salicylaldehydes via inhibition of type III secretion in different strains of *Yersinia, Pseudomonas, E. coli*, and *Chlamydiae* has also been demonstrated (Aberg & Almqvist, 2007). Competitive inhibition for specific bacterial adhesion is a related strategy aimed at inhibiting fimbrial lectins using specific saccharidic ligands competing with cell-surface-exposed *bona fide* fimbriae ligands (Korea *et al.*, 2011). For instance, mannose-derived residues show high affinity for FimH and can subsequently inhibit adhesion (Klein *et al.*, 2010; Grabosch *et al.*, 2011). Other strategies pursue inhibition of synthases of second messengers involved in the biofilm formation process, such as diguanylate cyclases responsible for c-di-GMP formation (Antoniani *et al.*, 2010) or quorum sensing signals of multiresistant pathogens such as *S. aureus*, where the *agr* system is targeted by RNAIII-inhibiting peptides and their nonpeptide analog hamamelitannin (Kiran *et al.*, 2008); see Bjarnsholt *et al.* (2011) for review of other antiquorum sensing molecules.

Because initial adhesion is often seen as the first step in microbial pathogenesis (Finlay & Falkow, 1989), there is a strong interest in interference molecules hindering pathogen adhesion to mucosa or to indwelling medical devices as an alternative strategy to antibiotics (Reid *et al.*, 2001). In this context, biosurfactants such as glycolipids and

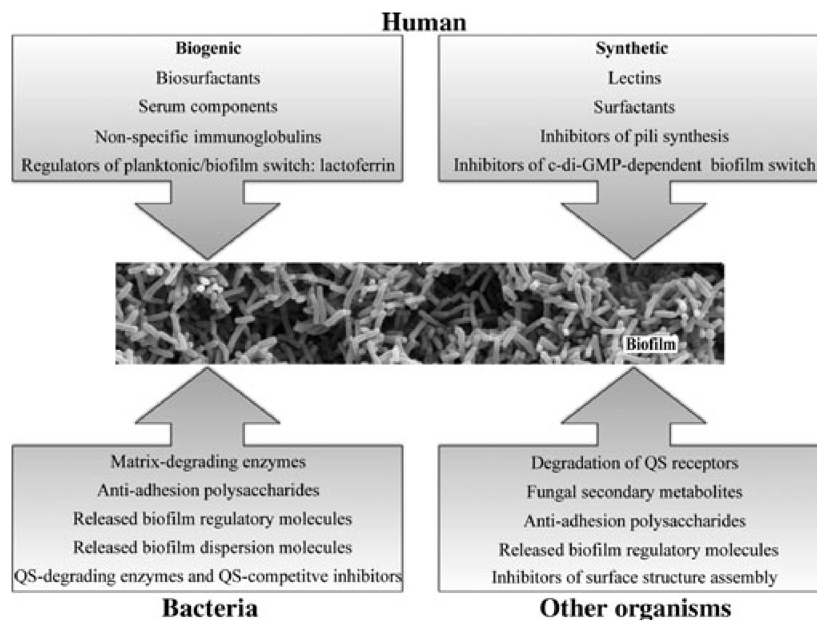

Fig. 5. Summary of nonbiocidal antibiofilm molecules described in this review and their mode of action.

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lipoproteins could play an important role in counteracting pathogen activity, as they exhibit low toxicity and high biodegradability effectiveness at different temperatures and pH (Rodrigues *et al.*, 2006a; Falagas & Makris, 2009; Zeraik & Nitschke, 2010).

Alternatively, instead of using purified antiadhesion compounds, whole (probiotic) commensals could be used for protecting a mammalian host via nonbiocidal competition with pathogens (Reid *et al.*, 2001; Kleerebezem & Vaughan, 2009; Quigley, 2010). Interactions between the commensal flora and incoming pathogens may have a positive effect on host health, as commensals act as physical barriers involved in resistance colonization and prevention of pathogen establishment. It has been shown that mice precolonized with several probiotic *E. coli*, including *E. coli* Nissle, are able to clear and avoid colonization of pathogenic *E. coli* O157:H7. Moreover, this barrier effect is not microbe-specific, as hosts precolonized with commensal *E. coli* strains can also lead to clearance of pathogenic *E. coli* (Leatham *et al.*, 2009). Co-incubation of *S. enterica* with aggregating and surface-blanketing *Lactobacillus kefir* strains significantly decreased *Salmonella*'s capacity to adhere to and invade Caco-2/TC-7 cells (Golowczyc *et al.*, 2007). In addition, *L. kefir* releases an unidentified compound that regulates virulence of *Salmonella,* as it significantly reduces induced microvillus disorganization (Golowczyc *et al.*, 2007). Commensal bacteria of the gut can also inhibit pathogen adhesion through induction of nonbiocidal host factors such as mucin production, which reduces the availability and accessibility of adhesion sites (Mack & Sherman, 1991). Co-incubation of lactobacilli with intestinal epithelial cells resulted in upregulation of MUC3 mucin production and correlated with reduced adhesion of enteropathogenic *E. coli* (Mack *et al.*, 2003).

Despite promises of nonbiocidal antibiofilm approaches (Fig. 5), no antibiofilm products are on the market yet. Although this might be attributed to high cost, low specificity and lack of financial interest on the part of pharmaceutical companies (Romero & Kolter, 2011), we should also consider potential drawbacks of certain antibiofilm approaches. Indeed, mixed communities often correspond to complex equilibria, the alteration of which could lead to drastic changes in population structure and composition, potentially leading to the emergence of opportunistic microorganisms or pathogens previously kept under control. Similarly, while the idea of dispersing mature biofilms formed by or hosting pathogens seems extremely tempting, massive bacterial release upon dispersion can have very serious drawbacks, including systemic infection and massive inflammatory responses, though these remain difficult to predict. Nevertheless, results from double-blind placebo-controlled studies are encouraging (Larsson *et al.*, 2008; Grandy *et al.*, 2010; Berggren *et al.*, 2011; Choi *et al.*, 2011; Davidson *et al.*, 2011). However, although attractive, these strategies will need to be carefully tested to determine their validity and health benefits.

Concluding remarks

In nature, bacteria interact with and influence each other in complex webs of multicellular behaviors. The studies of these interactions have shed light on the resources used by bacteria to thrive in mixed biofilm communities and have inspired us to design alternatives to antibiotics in the war against pathogenic microorganisms (Rasko & Sperandio, 2010). Targeting surface colonization rather than overall bacterial fitness is emerging as a promising approach, because nonbiocidal modification of pathogenic behavior causes milder evolutionary selective pressure and may therefore lead to the emergence of fewer resistant mutants and fewer toxicity issues. The effectiveness of antibiofilm approaches will be put to test in the coming years. Meanwhile, the hunt for antibiofilm molecules used alone or in combination with antibiotics and vaccines is under active investigation (Goldman *et al.*, 2006; Sanz *et al.*, 2007; Larsson *et al.*, 2008; Rowland *et al.*, 2010; Davidson *et al.*, 2011). It is clear, however, that no single molecule is likely to efficiently control biofilm formation in all types of contexts, underlining the need for a deeper understanding of antagonistic interactions in mixed bacterial populations.

Acknowledgements

We thank C. Beloin, C. Forestier, S. Bernier, M. Mourez, and L. Travier for helpful comments and critical reading of the manuscript. We thank J.B. Kaplan for scientific discussion. We are grateful to Brigitte Arbeille, Claude Lebos, and Gérard Prensier (LBCME, Faculté de Médecine de Tours) for their help in performing electronic microscopy. O.R. was supported by a fellowship from the Network of Excellence EuroPathogenomics; European Community Grant LSHB-CT-2005-512061.

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