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Review

Diffusible signals and interspecies communication in bacteria

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Many bacteria use cell–cell communication mediated by diffusible signal molecules to monitor their population density or confinement to niches and to modulate their behaviour in response to these aspects of their environment. Work on signalling systems within individual species has formed a platform for studies of interspecies interactions that can occur within polymicrobial communities in nature. In addition to signalling between organisms that synthesize the same or related signal molecules, it is becoming evident that bacteria can sense signal molecules that they do not synthesize, thereby eavesdropping on signalling by other organisms in their immediate environment. Furthermore, molecules such as antibiotics that are considered not to be signals for the producing species can have effects on gene expression in other bacteria that indicate a signalling function. Interspecies signalling can lead to alteration in factors contributing to the virulence or persistence of bacterial pathogens as well as influencing the development of beneficial microbial communities. Here we review our current understanding of interspecies signalling in bacteria and the signals involved, what is known of the underlying signal transduction mechanisms and their influences on bacterial behaviour.

Introduction

A major mechanism of cell–cell communication in bacteria involves the synthesis, release and detection of molecules called diffusible signal molecules (Waters & Bassler, 2005). Bacteria can utilize such systems to monitor their population density (the process of quorum sensing) and/or their confinement in particular environmental niches and to activate in consequence specific population-wide alterations in gene expression and bacterial behaviour. Cell–cell signalling thus allows a colony or group of organisms to behave in a co-ordinated fashion to regulate processes contributing to virulence, antibiotic production, biofilm formation and other developmental programmes. The signal molecules are often referred to as autoinducers, a term coined to reflect their activity in influencing the behaviour of the producing organism.

In nature bacteria are more likely to grow in polymicrobial communities than in monoculture. Interactions between the community members are required for community development and maintenance and can involve interspecies signalling mediated by the same molecules as used in intraspecies signalling. In addition to signal exchange between partners that utilize the same or related signal molecules, bacteria can also 'eavesdrop' on the communication of other organisms, modulating their behaviour in response to cell–cell signals that they do not synthesize. An emerging theme in the area of interspecies signalling is the involvement of antibiotics, which have not been considered to be intraspecies signals. At low concentrations (such as may occur in natural environments), some antibiotics have effects on bacterial behaviour and gene transcription that are distinct from those known or proposed to contribute to increased antibiotic tolerance, suggesting a role in signalling. Here we survey the current understanding of interspecies signalling in bacteria. We begin with a brief overview of intraspecies (autoinducer) signals and the criteria used to define them. We then discuss the roles of these molecules in interspecies signalling before going on to address the role of antibiotics as signals.

Abbreviations: AI-2, -3, autoinducer-2, -3; AIP, autoinducing peptide; DF, diffusible factor; DKP, diketopiperazine; DPD, 4,5-dihydroxy-2,3-pentanedione; DSF, diffusible signal factor; *N*-AHL, *N*-acylhomoserine lactone.

Molecules involved in intraspecies signalling

The autoinducer signal molecules produced by bacteria are structurally diverse (Fig. 1). Many Gram-negative bacteria use *N*-acylhomoserine lactones as signals, although other fatty acid derivatives such as 3-hydroxypalmitic acid methyl ester and *cis*-unsaturated fatty acids are also found. In contrast, many Gram-positive bacteria use amino acids or modified peptides as signal molecules. Fatty acid derivatives are however found as signal molecules in Gram-positive bacteria (for example the γ-butyrolactones of *Streptomyces* spp.) whereas cyclic dipeptides are found as signals in Gram-negative organisms (Fig. 1d, f). Both Gram-positive and Gram-negative bacteria use isomers of

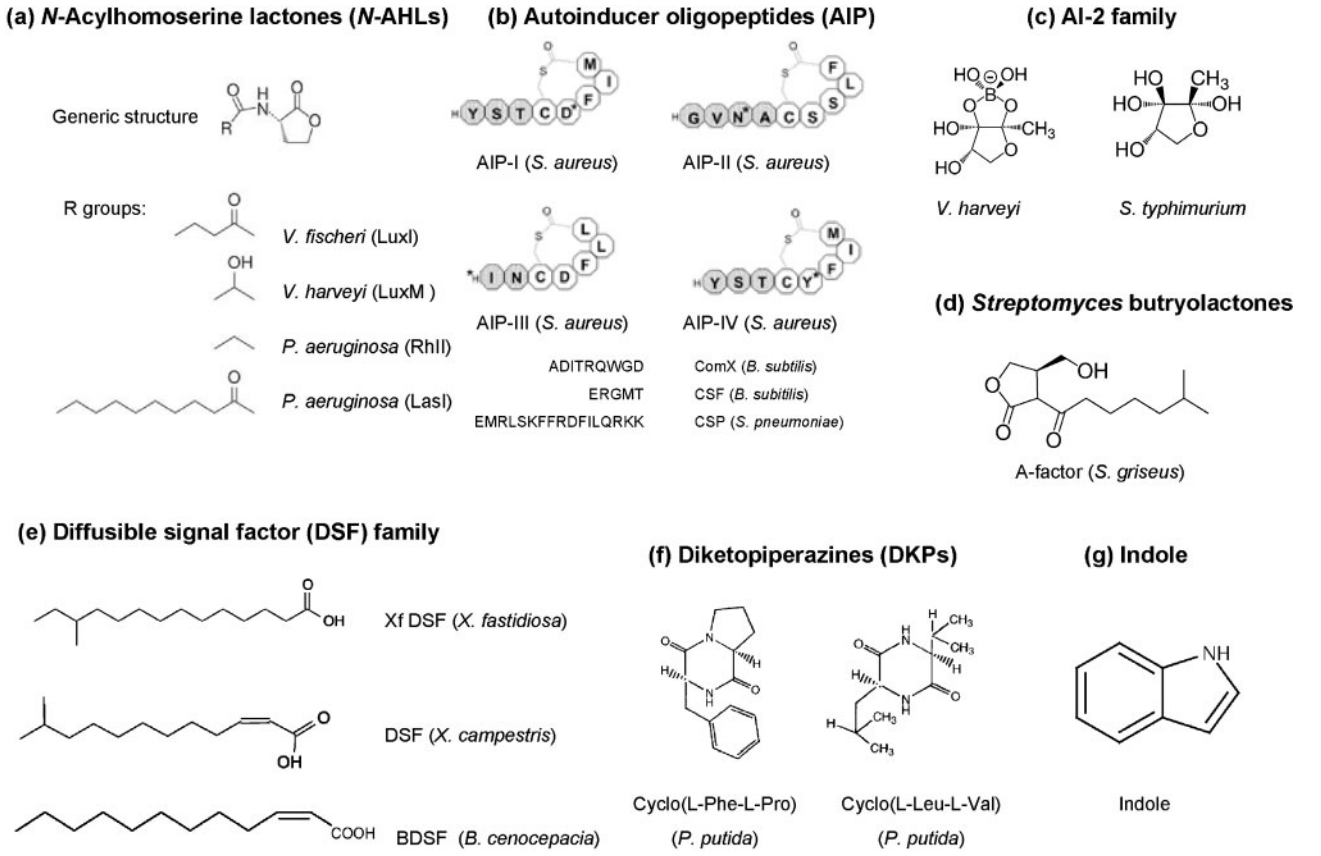

Fig. 1. Chemical molecules used for signalling in the bacterial world are structurally diverse. For details see text.

methyl-2,3,3,4-tetrahydroxytetrahydrofuran (the AI-2 autoinducer) as signals (Fig. 1). Signal molecules belonging to further structural classes such as indole and its derivatives, quinolones and (*S*)-3-hydroxytridecan-4-one have also been described (Diggle *et al.*, 2006; Higgins *et al.*, 2007; Lee *et al.*, 2007b). The molecular bases of the synthesis and perception of a number of these molecules and details of the signal transduction pathways have now been determined. As we will only briefly address these mechanisms, the reader is directed to several excellent and comprehensive reviews of this area (Whitehead *et al.*, 2001; Lyon & Novick, 2004; Waters & Bassler, 2005; Konaklieva & Plotkin, 2006).

Winzer *et al.* (2002) proposed four criteria to define signalling molecules involved in intraspecies communication. The first three address specific issues of the production (during specific stages of growth, under certain physiological conditions, or in response to changes in the environment), perception (the signal accumulates extracellularly and is recognized by a specific receptor) and cellular sensitivity (accumulation of the signal generates a concerted response, once a critical threshold concentration has been reached). The final and most important criterion is that the cellular response to the signal extends beyond physiological changes required to metabolize or detoxify it. By these criteria, the molecules described above can be considered intraspecies cell–cell signals in at least one organism, although molecules such as antibiotics would not qualify. As we will discuss, an extension of these considerations to interspecies signalling suggests that a number of molecules that would not be regarded as intraspecies cell–cell signals would nevertheless be eligible as interspecies signals.

Intraspecies signals with a role in cross-species communication

N-Acyl-L-homoserine lactones

The most intensively investigated signal molecules in Gram-negative bacteria are the *N*-acyl-L-homoserine lactones (*N*-AHLs). Two distinct mechanisms of signalling mediated by *N*-AHLs have been described. In most Gram-negative bacteria, the signal is generated by an *N*-AHL synthase of the LuxI family of proteins, and is perceived by an *N*-AHL receptor protein belonging to the LuxR family of transcriptional regulators. The *N*-AHL autoinducers bind to their cognate LuxR-type proteins only on reaching a critical threshold concentration. Autoinducer binding controls the transcriptional activity of the LuxR protein in regulating the expression of target genes, which can include

luxI. This establishes a positive feedback loop for *N*-AHL synthesis, although it must be noted that positive feedback is not a universal feature of *N*-AHL-mediated quorum-sensing systems. In some Gram-negative bacteria such as *Vibrio* spp., *N*-AHL synthesis is directed by a LuxM synthase (unrelated to LuxI) and perception of the signal involves a cytoplasmic membrane-associated sensor kinase. To date, *N*-AHL-dependent quorum-sensing circuits have been identified in a wide range of Gram-negative bacteria, where they regulate various functions including bioluminescence, plasmid conjugal transfer, biofilm formation, motility, antibiotic biosynthesis, and the production of virulence factors in plant and animal pathogens (Eberl, 1999).

N-AHLs vary in length, oxidation and saturation of the acyl chain (Fig. 1a). Signalling specificity arises because LuxR proteins can only bind particular *N*-AHLs and LuxI proteins only synthesize *N*-AHLs with a limited number of different acyl chains. For example, *Pseudomonas aeruginosa* contains two pairs of LuxR/LuxI homologues; LasI synthesizes *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (oxoC12-HSL), which is detected by LasR (Pearson *et al.*,1994, 1995), and RhlI synthesizes *N*-butanoyl-L-homoserine lactone (C4-HSL), which is detected by RhlR (Pearson *et al.*, 1995, 1997) (Fig. 1a).The occurrence of similar LuxIR systems in two species indicates the potential for interspecies signalling. Bacteria such as *Burkholderia cepacia* which have RhlR homologues are able to perceive and respond to *N*-AHLs produced by *Ps. aeruginosa* (Riedel *et al.*, 2001).

Bacterial species of the genera *Escherichia*, *Salmonella* and *Klebsiella* are intriguing in that they have a LuxR homologue, SdiA, but they do not contain a LuxI homologue or any other enzyme family that can synthesize *N*-AHLs (Ahmer, 2004). The function of SdiA is best understood in *Salmonella enterica* serovar Typhimurium, where the protein detects *N*-AHLs produced by other bacterial genera (Michael *et al.*, 2001). Upon *N*-AHL binding, SdiA activates two *Salmonella*-specific loci, the *rck* (resistance to complement killing) operon, which is carried on the *Salmonella* virulence plasmid, and *srgE* (*sdiA*-regulated gene), which is carried in the chromosome but is of unknown function (Ahmer *et al.*, 1998). The *rck* operon includes six genes, three of unknown function and three that play a role in adhesion to extracellular matrix and/or host cells and resistance to complement killing (Ahmer, 2004).

The function of SdiA in *Escherichia coli* and *Klebsiella* spp. is currently unclear. SdiA overexpression in *E. coli* O157 : H7 causes negative regulation of virulence factors (Kanamaru *et al.*, 2000) whereas in *E. coli* K-12 it results in a large pleiotropic response that includes inhibition of expression of genes determining chemotaxis and motility, repression of *tnaA*, which encodes an enzyme involved in indole synthesis, and induction of indole export via AcrEF. The genes/proteins affected by *sdiA* overexpression have however never been demonstrated to respond significantly to *sdiA* expressed from its natural position in the chromosome. Nevertheless the *sdiA* in *E. coli* is functional as it is required for *N*-AHL-induced expression of the heterologous *Salmonella srgE* gene (Ahmer, 2004). An involvement in indole synthesis and export is intriguing since this molecule has been shown to influence biofilm formation in *E. coli* in an SdiA-dependent fashion, leading to the suggestion that it is an interspecies signal. We will address this issue at a later point. Recent work has demonstrated that the refolding of recombinant SdiA of *E. coli* is activated in the presence of three different *N*-AHLs (C8-HSL, 3-oxo-C8-HSL and C6-HSL) (Yao *et al.*, 2006). This is indicative of the binding specificity of SdiA; upon overexpression many LuxR proteins remain insoluble unless the cognate *N*-AHLs are supplied in the medium.

Not all orphan LuxR family-type regulators may be involved in *N*-AHL binding. Two such proteins (OryR and XccR) have been recently described in *Xanthomonas oryzae* pv. *oryzae* and *Xanthomonas campestris* pv. *campestris* (*Xcc*), plant pathogens which do not synthesize *N*-AHLs (Ferluga *et al.*, 2007; Zhang *et al.*, 2007). Available evidence suggests that these proteins are not activated by *N*-AHLs, but by plant-derived components. OryR, which is required for full virulence to rice, regulates synthesis of two secreted proteins: a cell-wall-degrading cellobiosidase and a 20 kDa protein of unknown function (Ferluga *et al.*, 2007). Similarly XccR acts to positively regulate *pip*, encoding a proline iminopeptidase that is indispensable for full virulence of *Xcc* to cabbage (Zhang *et al.*, 2007). These findings suggest that some LuxR proteins are responsive to molecules other than *N*-AHLs, which has implications for bacterial interspecies signalling. However, the nature of the activating plant components is unknown and it remains a possibility that they are structural mimics of *N*-AHLs, which have been detected in plants.

AI-2, a signal molecule common to Gram-positive and Gram-negative bacteria

The only cell–cell signalling system identified to date that is shared by Gram-positive and Gram-negative bacteria is mediated by autoinducer-2 (AI-2) (Schauder & Bassler, 2001). The role of AI-2 as an intraspecies signal was revealed through studies of the control of bioluminescence in the marine bacterium *Vibrio harveyi* (Bassler *et al.*, 1997). This body of work established that biosynthesis of AI-2 requires the enzyme LuxS, whereas perception of AI-2 in *V. harveyi* requires the periplasmic AI-2-binding protein LuxP and the sensor kinase LuxQ. LuxPQ is one of three signal transduction systems that converge to control bioluminescence. The structure of the *V. harveyi* AI-2 molecule was determined as a boron diester of (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*S*-THMF) during establishment of the X-ray crystal structure of the LuxP to which AI-2 was bound (Chen *et al.*, 2002). In contrast, AI-2 from *Salmonella typhimurium* (*S. enterica*

serovar Typhimurium) has been characterized through binding to the distinct periplasmic protein LsrB as the enantiomeric *R*-THMF, from which borate is absent (Miller *et al.*, 2004; Xavier *et al.*, 2007) (Fig. 1c).

R- and *S*-THMF are derived by non-enzymic cyclization of 4,5-dihydroxy-2,3-pentanedione (DPD), the direct product of LuxS action. This reaction is one step in a pathway that serves to regenerate *S*-adenosylmethionine (SAM). When SAM is used as a methyl donor it is converted to *S*-adenosylhomocysteine (SAH), which is toxic to cells and must be eliminated. The enzyme Pfs converts SAH to *S*-ribosylhomocysteine (SRH), and then LuxS converts SRH to DPD and homocysteine, which is a precursor for methionine. This metabolic function of LuxS complicates the interpretation of experiments in which the role of AI-2 as intraspecies signal molecule is assessed through *luxS* mutation since phenotypes and/or changes in gene expression resulting from disruption of *luxS* could be metabolic in nature (Winzer *et al.*, 2002; Rezzonico & Duffy, 2007). Furthermore, it is possible that the extracellular occurrence of DPD/AI-2 reflects the excretion of a metabolic by-product. In *Salmonella*, AI-2 is generated during exponential growth and is then removed from the culture during stationary phase (Surette & Bassler, 1999). Addition of AI-2 to some bacteria leads to alterations only in genes potentially involved in uptake and catabolism of the molecule. Consequently by the criteria proposed by Winzer *et al.* (2002), AI-2 could not be considered an intraspecies signal in these organisms. In contrast, a signalling role for AI-2 is clear not only in *Vibrio* spp. but also in an expanding range of Gram-negative and Gram-positive bacteria, where the molecule acts to control diverse functions such as virulence factor production, cell motility, bacterial conjugation and biofilm formation (DeLisa *et al.*, 2001; Fong *et al.*, 2001; Stevenson & Babb, 2002; Xavier & Bassler, 2003; Lyon & Novick, 2004; Miller & Stevenson, 2004; Pei & Zhu, 2004).

The widespread nature of LuxS and AI-2 production amongst bacterial species has led to the proposal that AI-2 has a function in interspecies communication (Schauder & Bassler, 2001). Importantly, several observations have extended this concept through the demonstration that bacteria that cannot synthesize AI-2 can nevertheless respond to the molecule (Duan *et al.*, 2003; Rickard *et al.*, 2006). *Pseudomonas aeruginosa* does not have a *luxS* gene and therefore does not produce AI-2. Nevertheless this pathogen can detect AI-2 produced by bacteria within the oropharyngeal flora with consequent effects on virulence gene expression (Duan *et al.*, 2003). Co-culture of the human oral commensal bacteria *Actinomyces naeslundii* T14V and *Streptococcus oralis* 34 in flowing saliva promotes mutualistic and abundant biofilm growth (Fig. 2a). These effects are not seen in co-culture of *A. naeslundii* T14V with a *luxS* mutant of *Strep. oralis* 34, but are restored by addition of DPD at concentrations as low as 0.08 nM (Fig. 2a), a level that is two orders of magnitude lower than the detection limit of the *V. harveyi* AI-2 assay (Rickard *et al.*, 2006).

The molecular basis for AI-2 signal transduction in organisms outside the genus *Vibrio* is poorly understood and there are many outstanding questions. AI-2 binding proteins related to LuxP have only been found in *Vibrio* spp. (Sun *et al.*, 2004), although homologues of LsrB from *S. typhimurium* occur more widely. LsrB functions in concert with other Lsr proteins in the binding, uptake and metabolism of the AI-2 signal (Taga *et al.*, 2001, 2003). LsrA, LsrC and LsrD form an ABC transporter complex, homologous to the ribose transporter. It is unclear therefore whether this uptake system has a signalling function or if its role is restricted to catabolism of pentoses. Would elimination (by mutation) of the catabolism of AI-2 reveal a hitherto cryptic signalling system(s)? A related issue is whether AI-2 signalling in species other than *Vibrio* always requires a periplasmic binding protein component. Conceivably the signal could bind directly to the sensory input domain of a two-component sensor kinase.

AI-3/epinephrine/norepinephrine signalling

AI-3 is a bacterial cell–cell signal of unknown structure that activates transcription of virulence genes and controls virulence in enterohaemorrhagic *E. coli* O157 : H7. This bacterial signal-response system was originally defined through a role in interkingdom signalling as it is required for bacterial responses to the eukaryotic hormones epinephrine and norepinephrine. Perception of epinephrine/norepinephrine and AI-3 activates expression of genes of the LEE (locus of enterocyte effacement) pathogenicity island and of the flagella regulon (Sperandio *et al.*, 2000, 2002a, 2003). Activation of the flagella regulon by epinephrine and AI-3 requires the sensor kinase QseC and the response regulator QseB (Sperandio *et al.*, 2002b). A second two-component system, QseEF, which may also sense A1-3, epinephrine and norepinephrine, is essential for expression of LEE genes and for attaching and effacing lesion formation (Reading *et al.*, 2007).

AI-3 is produced by the combined microbial intestinal flora from healthy individuals, by commensal *E. coli*, *Klebsiella pneumoniae* and *Enterobacter cloacae* and by a range of pathogens including enteropathogenic *E. coli* strains from serogroups O26 : H11 and O111ac : H9, *Shigella* spp. and *Salmonella* spp. (Sircili *et al.*, 2004; Walters *et al.*, 2006). Furthermore, proteins related to the Qse components of the signalling cascades are present in a number of bacterial species. These findings suggest that AI-3 may be involved in interspecies signalling among intestinal bacteria as well as its role in interkingdom signalling via hormones as seen in *E. coli* (Hughes & Sperandio, 2008). Although the chemical structure of AI-3 has yet to be determined, preliminary analysis suggests that this signal is an aromatic compound and does not contain a sugar skeleton like AI-2 (J. R. Falck & V. Sperandio, unpublished data; discussed in Reading & Sperandio, 2006).

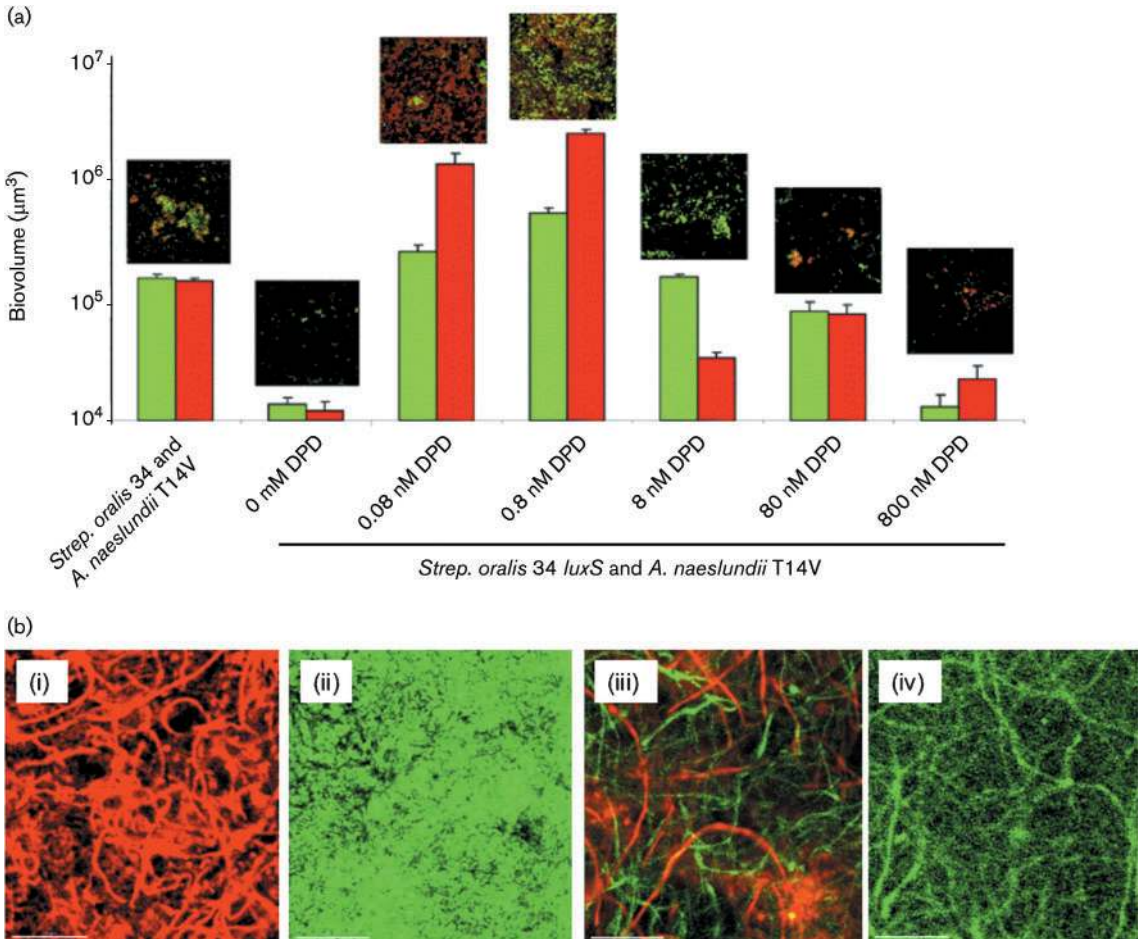

Fig. 2. Interspecies interactions through diffusible signal molecules can influence biofilm formation. (a) AI-2 is required for mutualistic biofilm formation between the oral bacteria *Streptococcus oralis* 34 and *Actinomyces naeslundii* T14V grown in saliva. This mutualistic interdigitated biofilm growth depends upon the production of AI-2 by *Strep. oralis* 34; mutation of the *luxS* gene in *Strep. oralis* abrogates the effect, which can be restored by addition of DPD (the AI-2 precursor) at very low concentration. Confocal images and measurements of biovolume of biofilms for *Strep. oralis* 34 strains (green bars) and *A. naeslundii* T14V (red bars) were made after 22 h growth at 37 °C in flow cells (data from Rickard *et al.*, 2006 with permission). (b) DSF from *Stenotrophomonas maltophilia* influences biofilm architecture of *Pseudomonas aeruginosa* PAO1, which does not produce the signal. Images are of 4-day-old biofilms in flow cells in FABL medium. Key: (i) *Ps. aeruginosa* PAO1; (ii) *Sten. maltophilia* K279a; (iii) Mixed culture of *Ps. aeruginosa* PAO1 and *Sten. maltophilia* K279a; (iv) *Ps. aeruginosa* PAO1 with 50 μM exogenous DSF. For these experiments, *Ps. aeruginosa* was tagged with mini-Tn7gfp (green fluorescence); *Sten. maltophilia* was visualized with Syto62 (red fluorescence). Scale bars, 20 μm.

Autoinducing peptides

Many cell–cell signalling systems in Gram-positive bacteria use modified peptides as signals to regulate functions such as virulence (*agr* system in staphylococci – Ji *et al.*, 1995; Peng *et al.*, 1988; and *fsr* system in enterococci – Clewell *et al.*, 2002; Haas *et al.*, 2002), competence (*com* system in bacilli – Hamoen *et al.*, 2003) and pneumococci – Tomasz, 1965; Havarstein *et al.*, 1995), and bacteriocin production (*pin* and *ssp* systems in lactic acid bacteria) (Fig. 1b). Most autoinducing peptide (AIP) signals are generated by cleavage from larger precursor peptides, and subsequent modifications that include substitution with isoprenyl groups and formation of lactone and thiolactone rings and lanthionines (Mayville *et al.*, 1999; Nakayama *et al.*, 2001; Ansaldi *et al.*, 2002). Signal release from the cell requires dedicated oligopeptide exporters, whereas signal perception is mediated by sensor histidine kinases located in the cytoplasmic membrane. Many Gram-positive bacteria communicate with multiple peptides in combination with other types of quorum-sensing signals.

The specificity of signalling has been well studied for the *agr* (accessory gene regulator) system in *Staphylococcus aureus* (Jarraud *et al.*, 2000; Dufour *et al.*, 2002), and the competence systems of *Bacillus subtilis* (Tortosa *et al.*,

2001) and *Streptococcus* spp. (Havarstein *et al.*, 1995; Whatmore *et al.*, 1999). In some cases the signalling peptide can be recognized not only by its cognate species but also by different strains of the same or related species. This interspecies signalling can exert either an inductive or an inhibitory effect on target gene expression in different organisms. For example, four AIP subgroups have been described in *Staph. aureus* isolates (Fig. 1b), while non-*Staph. aureus* AIPs have been detected in *Staph. epidermidis* and *Staph. lugdunensis* (Otto *et al.*, 2001; Dufour *et al.*, 2002). AIPs produced by one *Staph. aureus* strain inhibit expression of *agr* target genes in some of the other strains. Cross-inhibition between *Staph. aureus* strains and *Staph. epidermidis* or *Staph. lugdunensis* can also occur (Otto *et al.*, 2001; Dufour *et al.*, 2002). Cross-induction but not cross inhibition was observed for the ComX peptides from *Bacillus mojavensis* and *B. subtilis* strains (Tortosa *et al.*, 2001), the ComC peptides in *Streptococcus pneumoniae* isolates (Whatmore *et al.*, 1999) and the SalA lantibiotic peptides in *Streptococcus salivarius* and *Streptococcus pyogenes* (Upton *et al.*, 2001).

Although AIP-like signalling molecules have yet to be described in Gram-negative bacteria, there are at least two examples where the occurrence of peptide signals has been suggested. *Providencia stuartii*, a Gram-negative bacterium responsible for nosocomial and opportunistic infections in humans, produces an unknown quorum-sensing molecule that has a number of properties, including sensitivity to peptidases, which are consistent with those of a small peptide. This signal regulates cellular functions that include peptidoglycan acetylation, methionine transport and cysteine biosynthesis. The production of the signal depends upon the AarA protein, a member of the rhomboid family of intramembrane serine proteases (Rather & Orosz, 1994; Rather *et al.*, 1997). Related protein sequences are widespread, occurring in Gram-positive and Gram-negative bacteria as well as archaeal species (Gallio *et al.*, 2002). Functional analysis using a *Pr. stuartii aarA* mutant as a biosensor was used to demonstrate that proteins from diverse bacteria including *Ps. aeruginosa, B. subtilis* and *Strep. pyogenes* exhibited rhomboid activity to generate a signal recognized by *Pr. stuartii*. AarA acts in regulation of the Tat pathway of protein export, through cleavage of an N-terminal heptapeptide extension of TatA (Stevenson *et al.*, 2007). Several lines of evidence suggest that this peptide is however not the signal molecule. The role of the Tat pathway may be in transport of an enzyme that is involved in signal production or of the signal itself.

The second example of a proposed peptide signal molecule in Gram-negative bacteria comes from the plant pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). *Xoo* strains producing the extracellular AvrXa21 elicitor trigger host defence responses in rice lines carrying the Xa21 resistance gene. Although the *Xoo* molecule has not yet been isolated, it is established that the activity is dependent on eight *rax* genes that provide clues to its regulation, secretion and structure (Lee *et al.*, 2006, 2008). Extracellular AvrXa21 activity depends upon the RaxRH two-component system, the RaxABC type I secretion system and RaxPQST, which are required for activation and transfer of sulphate. Furthermore, AvrXa21 activity is produced in a cell-density-dependent manner. These properties have led to the suggestion that AvrXa21 is a secreted peptide that acts as a quorum-sensing molecule. Expression of the *raxSTAB* operon from *Xoo* in a related species, *Xanthomonas campestris* pv. *campestris*, confers AvrXa21 activity. This suggests that the core AvrXa21 molecule is conserved (Lee *et al.*, 2006), which may be important in the context of interspecies signalling within xanthomonads.

Diketopiperazines

Diketopiperazines (DKPs), also known as cyclic dipeptides, were originally extracted from culture supernatants of *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Citrobacter freundii* and *Enterobacter agglomerans* and have been shown to influence *N*-AHL-dependent quorum sensing in diverse fashions (Holden *et al.*, 1999, 2000). Representative structures are shown in Fig. 1(f). Cyclo(l-Pro-l-Met) produced by *E. coli* stimulates the swarming motility of a *swrI* mutant of *Proteus mirabilis* as effectively as C4-HSL (Holden *et al.*, 2000). In contrast, cyclo(l-Pro-l-Tyr) and other DKPs antagonize the quorum-sensing-regulated swarming of *Serratia liquefaciens* at a significantly lower concentration than those required to induce an *E. coli N*-AHL biosensor (Holden *et al.*, 1999). DKPs may mimic the action of *N*-AHLs by interacting with LuxR proteins at, or near, the *N*-AHL binding site (Holden *et al.*, 1999; Degrassi *et al.*, 2002). It has also been demonstrated that DKPs influence the transcription of specific stationary-phase-regulated genes in *E. coli* (Holden *et al.*, 1999). In some cases however the concentrations of DKPs required to see effects in bacteria are considerably higher than the levels of *N*-AHL required to activate the particular system under study (Lazazzera & Grossman, 1998). DKPs also have biological and pharmacological effects on cells of higher organisms (Prasad, 1995), suggesting a potential role in communication with plant and animal cells.

DSF (diffusible signal factor)

The synthesis of virulence factors in the plant pathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*) is controlled by cell–cell signalling mediated by the diffusible signal factor DSF (Barber *et al.*, 1997), which has been characterized as the unsaturated fatty acid *cis*-11-methyl-2-dodecenoic acid (Wang *et al.*, 2004; Fig. 1e). Synthesis and perception of the DSF signal require products of the *rpf* gene cluster. The synthesis of DSF is dependent on RpfF, which has some amino acid sequence similarity to enoyl-CoA hydratases, whereas the two-component system comprising the sensor kinase RpfC and regulator RpfG is implicated in DSF perception (Barber *et al.*, 1997; Slater *et al.*, 2000; Dow *et al.*, 2003; Ryan *et al.*, 2006). Homologues of Rpf proteins occur widely in xanthomonads, including

Xylella fastidiosa and other *Xanthomonas* spp. (which are plant pathogens) and *Stenotrophomonas maltophilia*, some strains of which are nosocomial pathogens. The *rpf*/DSF system controls diverse functions in these bacteria, including virulence, virulence factor synthesis, aggregative behaviour and biofilm formation (Newman *et al.*, 2004; Fouhy *et al.*, 2007; Huang & Wong, 2007a; Chatterjee *et al.*, 2008).

DSF activity from *Sten. maltophilia* strain WR-C has been shown to reside in a group of eight structurally related fatty acids that include *cis*-11-methyl-2-dodecenoic acid (the *Xcc* signal) and seven structural derivatives; two of these are saturated fatty acids whereas the others are unsaturated fatty acids with double bonds at position 2. These fatty acids vary in chain length from 12 to 14 carbons and in the position of the branched methyl group (Huang & Wong, 2007a). The molecule 12-methyltetradecanoic acid (Fig. 1e) has been identified in culture supernatants of *Xylella fastidiosa* as the putative DSF signal (Colnaghi Simionato *et al.*, 2007). The conservation of Rpf proteins and relatedness of DSF structures from different bacteria indicate that cross-species signalling between xanthomonads may well occur in nature, particularly since many of these organisms are associated with plants (Wang *et al.*, 2004; Colnaghi Simionato *et al.*, 2007; Huang & Wong, 2007b).

The findings from two recent reports have extended the scope of DSF-mediated interspecies signalling beyond the xanthomonads (Boon *et al.*, 2008; Ryan *et al.*, 2008). The first report concerns the characterization of a signal molecule related to DSF from *Burkholderia cenocepacia*. Culture supernatants of *B. cenocepacia* contain a compound with DSF-like activity, able to restore the biofilm and extracellular polysaccharide production phenotypes of an *rpfF* mutant of *Xcc* (Boon *et al.*, 2008). This signal molecule (BDSF) was identified by mass spectrometry and NMR analysis as *cis*-2-decenoic acid (Fig. 1e), which differs from DSF in the absence of the branched methyl moiety (Boon *et al.*, 2008). Synthesis of BDSF is dependent on an *rpfF* homologue found in *B. cenocepacia*. In the second report, Ryan and colleagues describe the influence of DSF on the behaviour of *Pseudomonas aeruginosa*, an organism that does not carry an *rpf* gene cluster and does not encode any protein that is highly related to RpfF. When grown in co-culture with *Sten. maltophilia*, *Ps. aeruginosa* develops biofilms with a filamentous architecture, different from the flat undifferentiated architecture seen with *Ps. aeruginosa* grown alone (Fig. 2b). These effects depend upon the presence of an intact *rpfF* gene in *Sten. maltophilia* and can be mimicked by addition of *cis*-11-methyl-2-dodecenoic acid to *Ps. aeruginosa* (Fig. 2b). DSF perception in *Ps. aeruginosa* depends on PA1396, a sensor kinase that has an input domain similar to that of RpfC, which is implicated in DSF perception in *Xcc* and leads to increased expression of stress-tolerance genes. Homologues of PA1396 occur in a number of other pseudomonads as well as unrelated bacteria (Ryan *et al.*, 2008). Taken together, these findings indicate a potential involvement of DSF or related molecules in interspecies communication involving non-xanthomonads such as *Ps. aeruginosa* and *B. cenocepacia*, which are major opportunistic human pathogens.

DF (diffusible factor)

Xcc synthesizes a second signal molecule called DF, which has a partially overlapping regulatory function with DSF (Chun *et al.*, 1997; Poplawsky & Chun, 1998, 1999; Poplawsky *et al.*, 1998, 2005). The DF signal molecule in *Xcc* strain B-24 regulates the production of both yellow pigments (xanthomonadins) and extracellular polysaccharide (EPS) (Poplawsky *et al.*, 1998) and is critical for epiphytic colonization and infection of the host plant (Poplawsky & Chun, 1998). Synthesis of DF depends upon the *pigB* locus and specifically *xanB2* (*XCC4014*), whose predicted amino acid sequence shows moderate similarity to putative pteridine-dependent dioxygenases from *Streptomyces* spp., but no homology to known regulatory genes (Poplawsky *et al.*, 2005). DF has been tentatively identified by mass spectrometry as a butyrolactone (Chun *et al.*, 1997). Butyrolactone signal molecules have been studied extensively in *Streptomyces* spp., where they control morphological differentiation and secondary metabolite production via quorum sensing (Horinouchi, 1999; Chater & Horinouchi, 2003), and recently the first gene for the biosynthesis of a *Streptomyces* butyrolactone signal was cloned (Shikura *et al.*, 2002; Kato *et al.*, 2007). Several *Streptomyces* strains are able to restore production of xanthomonadin and extracellular polysaccharide when streaked adjacent to an *Xcc xanB2* mutant (Poplawsky *et al.*, 2005). Determination of the structure of DF and elucidation of the mechanism(s) of DF perception should build upon these intriguing observations and help to establish whether a common strategy for the use and perception of butyrolactones as signalling molecules exists in *Xanthomonas* and *Streptomyces*.

Indole

Production of indole (Fig. 1g) and derivatives is widespread among plant and soil-associated bacteria (Morris, 1995; Patten & Glick, 1996, 2002; Theunis *et al.*, 2004) as well as some human and plant pathogens (Verstrepen *et al.*, 2004; Domergue *et al.*, 2005; Lee *et al.*, 2007a). Indole is generated through the degradation of tryptophan by tryptophanase, the product of the *tnaA* gene, and can reach levels up to 340 μM in stationary-phase cultures of *E. coli*. The role of indole as a potential signal molecule in *E. coli* was first revealed through analysis of factors required for the induction of the *astD*, *tnaB* and *gabT* genes by *E. coli* conditioned medium (Baca-DeLancey *et al.*, 1999; Wang *et al.*, 2001). Although indole itself acted as an inducer, conditioned medium from a *tnaA* mutant was still able to induce these genes, albeit to a lower extent than the wild-type. These findings suggested the occurrence of further, as-yet-unidentified, signals. The nature of the target genes (*tnaB* encodes a tryptophan permease, *astD*

and *gabT* encode enzymes involved in amino acid catabolism) has led to suggestions that indole should not be considered a cell–cell signal since it only induced genes involved in its own uptake or in catabolism.

More recent studies show a broader influence of indole on bacterial behaviour. Indole has been shown to regulate expression of several multidrug exporter genes in *E. coli*, via both BaeSR and CpxAR two-component signal transduction pathways and independently via the GadX transcriptional activator (Hirakawa *et al.*, 2005). Whether these systems directly sense indole is as yet unknown. An unknown metabolite of tryptophanase, derived from enteropathogenic *E. coli* (EPEC) or from commensal non-pathogenic strains, appears to directly or indirectly regulate toxin production within EPEC and to regulate the virulence in a nematode model (Anyanful *et al.*, 2005). Wood and colleagues recently showed that indole inhibits biofilm formation in *E. coli* K-12; mutation of either of two *E. coli* genes, *yliH* and *yceP*, which leads to lower intracellular indole concentrations, causes a dramatic increase in biofilm formation that can be reversed by addition of extracellular indole (Domka *et al.*, 2006). Intriguingly, these effects of exogenous indole are exerted through SdiA (Lee *et al.*, 2007a), the LuxR homologue of *E. coli* that responds to exogenous *N*-AHLs. SdiA has been shown to repress *tnaA*, as well as to induce indole export via AcrEF (Yao *et al.*, 2006). Oxidized derivatives of indole have diverse effects on biofilm formation in enterohaemorrhagic *E. coli* (Lee *et al.*, 2007b). Whereas indole and its hydroxylated derivatives 7-hydroxyindole and 5-hydroxyindole inhibit biofilm formation, 2-hydroxyindole has no effect and isatin (indole 2,3-dione) increases biofilm formation (Fig. 3).

What about the role of indole in interspecies communication? Indole positively influences the biofilm formation of *Pseudomonas fluorescens* and *Ps. aeruginosa*, even though these pseudomonads do not produce this signal (Lee *et al.*, 2007b). Furthermore, indole influences many of the quorum-sensing phenotypes and virulence factor production in *Ps. aeruginosa* (T. K. Wood, personal communication).

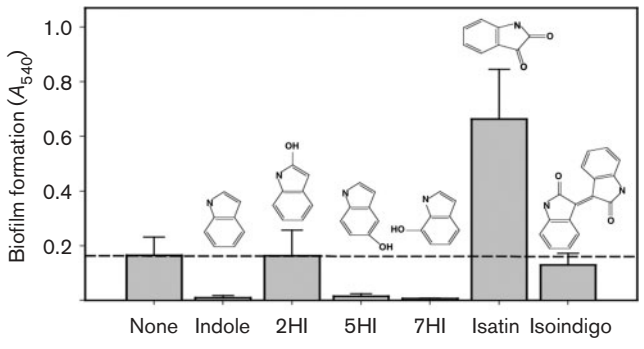

Fig. 3. Effects of indole, different hydroxyindoles and oxidized indole derivatives on formation of biofilms by enterohaemorrhagic *E. coli* measured in 96-well plates. Indole, 2-hydroxyindole (2HI), 5-hydroxyindole (5HI) and 7-hydroxyindole (7HI) were used at 1000 μM, whereas isatin and isoindigo were used at 250 μM. Biofilm formation was estimated by crystal violet staining (reproduced from Lee *et al.*, 2007a, with permission).

Antibiotics as interspecies signals

Antibiotics are naturally occurring organic molecules of low molecular mass (<3000 Da) that have been isolated by virtue of their ability to inhibit (or kill) living organisms; in most cases they act by binding to specific cellular targets. It is estimated that some of the biosynthetic pathways for antibiotics such as erythromycin and streptomycin are 500 million years old and abundantly distributed across the globe, and as a consequence the exposure to many bacteria is enormous (Baltz, 2007). An emerging notion is that antibiotics are not solely bacterial weapons but at subinhibitory concentrations can act as interspecies signalling molecules that may regulate the homeostasis of microbial communities (Davies, 1990, 2007; Davies *et al.*, 2006; Seshasayee *et al.*, 2006; Yim *et al.*, 2006, 2007).

The use of libraries of promoter-*lux* fusion constructions and transcriptome profiling has shown that most antibiotics demonstrate typical hormetic responses; at subinhibitory concentrations these compounds modulate the transcription of some 5–10 % of bacterial genes in the cell, often inducing 10- to 100-fold up- or downregulatory responses, with very limited effects on growth (Goh *et al.*, 2002; Tsui *et al.*, 2004; Brazas & Hancock, 2005; Lin *et al.*, 2005; Linares *et al.*, 2006). Since the promoters affected at subinhibitory concentrations depend to a large extent on the nature of the antibiotic class being used, it seems likely that in each case only transcripts associated with particular metabolic networks are affected. Nevertheless a small number of promoters do show specific patterns of activation for different antibiotics within the same class (Tsui *et al.*, 2004).

Subinhibitory antibiotic concentrations can increase expression of genes encoding bacterial determinants that influence interaction with host cells (Linares *et al.*, 2006; Marr *et al.*, 2007) and can induce biofilm formation (Hoffman *et al.*, 2005) (Fig. 4a). *Ps. aeruginosa* and *E. coli* respond to subinhibitory concentrations of aminoglycosides by forming antibiotic-resistant biofilms (Hoffman *et al.*, 2005). This is perhaps one strategy used by Gram-negative bacteria to counter antibiotic production by Gram-positive soil bacteria such as the streptomycetes. Tobramycin has a broad influence on gene expression in *Ps. aeruginosa*, which includes an upregulation of the gene encoding RsmA, a post-transcriptional regulator of the synthesis of virulence factors (Linares *et al.*, 2006; Lucchetti-Miganeh *et al.*, 2008). Antibiotics at subinhibitory levels do not always have a positive influence on biofilm formation; the semi-synthetic macrolide compound azithromycin decreases biofilm formation by

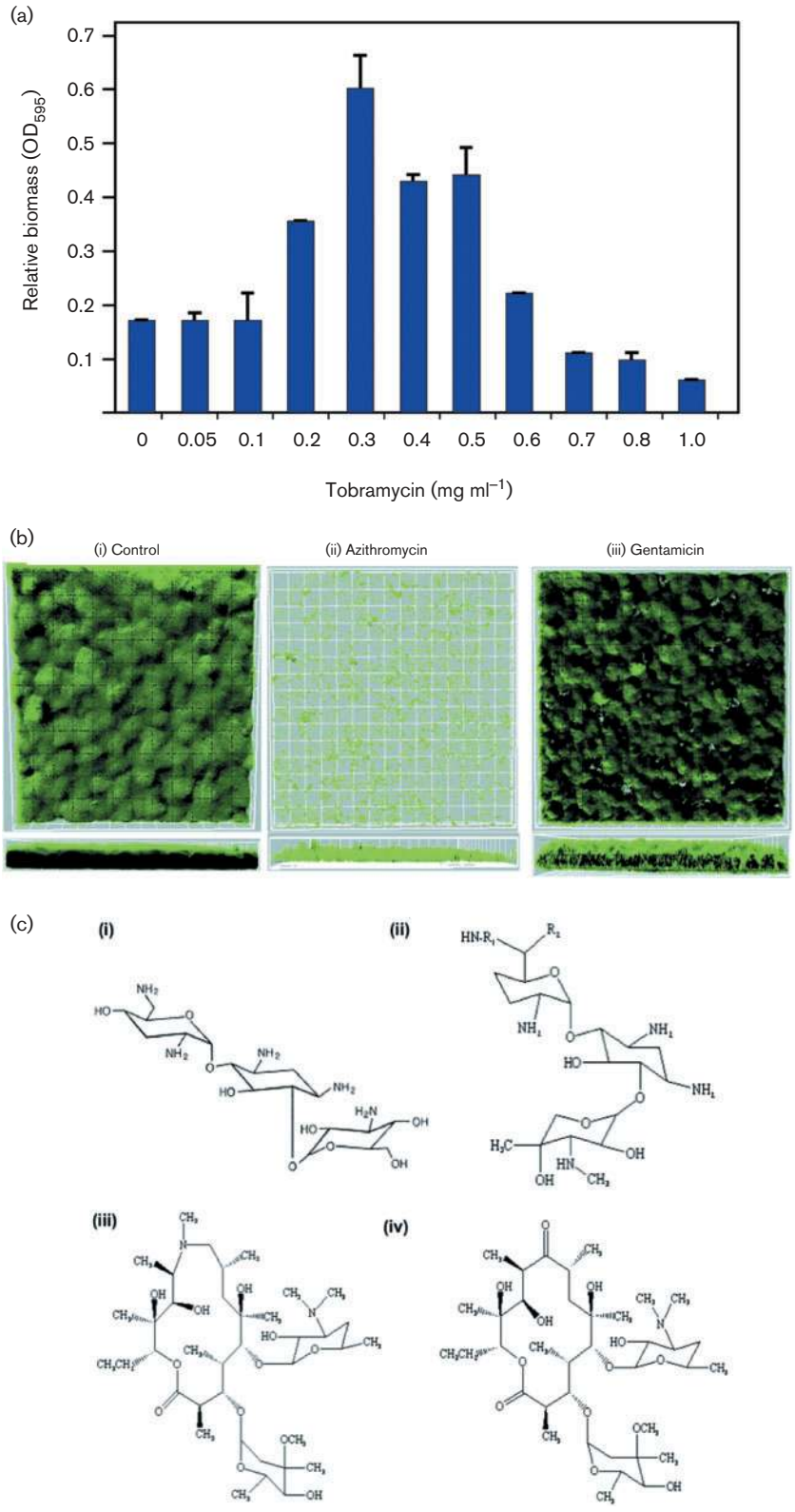

Fig. 4. Antibiotics at subinhibitory concentrations influence bacterial biofilm formation. (a) Tobramycin, an aminoglycoside produced by *Streptomyces tenebrarius*, promotes biofilm formation by *Pseudomonas aeruginosa* in plastic microtitre plates. This effect, which is optimal for *Ps. aeruginosa* at 0.3 μg ml^{-1}, is also induced by other aminoglycosides and in *E. coli* (data from Hoffman *et al.*, 2005; reproduced by permission from Macmillan Publishers Ltd [*Nature*] © 2005). (b) Azithromycin (a semi-synthetic macrolide antibiotic related to the natural product erythromycin) inhibits biofilm formation and decreases established biofilms of non-typable *Haemophilus influenzae*. Other antibiotics such as gentamicin have little or no effect. Images were captured using confocal laser scanning microscopy and are reproduced from Starner *et al.* (2008) with permission. (c) Structures of the aminoglycoside antibiotics tobramycin (i) and gentamicin [R_1, R_2=CH_3 or H] (ii) and the macrolide antibiotics azithromycin (iii) and erythromycin (iv).

Haemophilus influenzae (Starner *et al.*, 2008; Fig. 4c). Intriguingly, other antibiotics with a similar mechanism of antimicrobial action to azithromycin such as erythromycin (which is of highly related structure) and gentamicin have little or no effect on biofilm formation in *H. influenzae* (Starner *et al.*, 2008; Fig. 4b, c).

A key element among the criteria to define an intraspecies signal is that its effects on the producing organism should not be restricted to responses involved in signal metabolism or detoxification (Winzer *et al.*, 2002). If we use the same criteria to define an interspecies signal, antibiotics certainly qualify; they are extracellular components, produced at certain growth phases and whose effects on prokaryotic cells are not restricted to those that may contribute to antibiotic resistance (such as biofilm formation) (Linares *et al.*, 2006; Marr *et al.*, 2007). Antibiotics bind to specific cellular targets to exert their antimicrobial action, although it is not evident that all of the responses to subinhibitory concentrations are exerted through binding to the same targets. In *Ps. aeruginosa*, the biofilm response to subinhibitory concentrations of tobramycin requires Arr – a regulator that alters cyclic di-GMP levels. It is not known if tobramycin binds to Arr, which is membrane-associated, or if responses to other antibiotics involve other cyclic di-GMP signalling proteins.

Concluding remarks

An increasing research effort is being made to translate our knowledge of cell–cell interactions within species to understanding of interactions between bacteria in the polymicrobial communities that are characteristic of both natural environments and engineered environments such as microbial consortia used in wastewater treatment and bioremediation. In the clinical context, there is an increasing appreciation of the potentially important role for interspecies interactions in influencing bacterial virulence and response to therapy (Duan *et al.*, 2003; Ahmer, 2004; Hoffman *et al.*, 2005; Rickard *et al.*, 2006). The ability of certain pathogens to eavesdrop on signalling via molecules such as AI-2 and *N*-AHLs may allow these organisms to detect that they are in an environment of high bacterial density, such as may be found within a eukaryotic host, and consequently to activate expression of factors contributing to virulence and increased resistance against host defences. Interspecies interactions are however not restricted to those involving intraspecies signal molecules such as AI-2 and *N*-AHLs and can also involve, for example, antibiotics at subinhibitory concentrations. This may also be highly relevant in the clinical context. Although we have not addressed it here, interactions within bacterial communities can also involve enzymic degradation or modification of signals, which may manipulate the behaviour of the producing organism or of other organisms in a consortium. A number of such mechanisms have been described thus far and include hydrolysis of *N*-AHLs, phosphorylation of AI-2, oxidation of indole and degradation of DSF (Jensen *et al.*, 1995; Dong *et al.*, 2000, 2001; Labbate *et al.*, 2004; Roche *et al.*, 2004; Xavier & Bassler, 2005; Newman *et al.*, 2008).

The next few years offer the prospect of a substantial expansion of knowledge of bacterial interspecies communication, which will be provided both through an enhanced understanding of intraspecies signalling and through the further development of model systems of dual and multiple cultures to study bacterial behaviour within biofilms. We might expect to see the determination of the structures of intraspecies signals such as AI-3, DF and AvrXa21, the examination of roles of these and newly described signals such as 4-quinolones, including HHQ and PQS (reviewed by Diggle *et al.*, 2006), and CAI-1 (Higgins *et al.*, 2007) in interspecies signalling, and a deeper understanding of the mechanisms of perception of interspecies signals, e.g. AI-2 by *Ps. aeruginosa* and antibiotics by many bacteria. We also anticipate an expansion of the studies on the influence of eukaryotic microbes and eukaryotic hosts in development and maintenance of polymicrobial communities. Such further research efforts are warranted by our current (albeit limited) appreciation of the importance of interspecies communication and microbial community structure to plant and animal health.

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