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New trends in peptide-based anti-biofilm strategies: a review of recent achievements and bioinformatic approaches

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Antimicrobial peptides (AMPs) have a broad spectrum of activity and unspecific mechanisms of action. Therefore, they are seen as valid alternatives to overcome clinically relevant biofilms and reduce the chance of acquired resistance. This paper reviews AMPs and anti-biofilm AMP-based strategies and discusses ongoing and future work. Recent studies report successful AMP-based prophylactic and therapeutic strategies, several databases catalogue AMP information and analysis tools, and novel bioinformatics tools are supporting AMP discovery and design. However, most AMP studies are performed with planktonic cultures, and most studies on sessile cells test AMPs on growing rather than mature biofilms. Promising preliminary synergistic studies have to be consubstantiated and the study of functionalized coatings with AMPs must be further explored. Standardized operating protocols, to enforce the repeatability and reproducibility of AMP anti-biofilm tests, and automated means of screening and processing the ever-expanding literature are still missing.

Keywords: biofilm; antimicrobial peptide; therapeutic and prophylactic strategies; bioinformatics; anti-biofilm mechanisms of action

Introduction

Biofilms are complex, localized and fixed networks of microbial cells enclosed in a self-produced polymeric matrix, whose formation undergoes essentially four typical stages, namely (1) adherence of planktonic cells to the abiotic surface or tissue, (2) growth of the attached cells and production of the extracellular polymeric protective matrix (mainly proteins, polysaccharides and extracellular DNA resulting from autolysis (eDNA), (3) biofilm maturation involving the development of water channels and specialized zones, and (4) dispersion of cells and/or parts of the biofilm with subsequent colonization of other locations (Costerton et al. 1999; Donlan and Costerton 2002; Fey 2010; Høiby et al. 2010). The architecture of a mature biofilm is variable, ranging from flat homogenous layers of cells to highly organized cell clusters with a mushroom shaped structure containing water-filled channels (Wimpenny et al. 2000).

Even though there have been important research advances in the biofilm area, biofilm control is still an open and pivotal research field. Approximately 80% of human bacterial infections are caused by biofilms (Harro et al. 2010), mainly health care associated infections related to the implant of medical devices, eg urinary catheters, intravascular catheters and prosthetic heart valves. As such, microbial adhesion onto surfaces and the subsequent formation of biofilms are critical concerns for many biomedical applications (Donlan and Costerton 2002; Fey 2010). Indeed, the increasing resistance of biofilms to traditional antimicrobial treatments is considered the major cause of dissemination of antibiotic resistance in the nosocomial infections (Fey 2010; Spížek et al. 2010).

Biofilm resistance has been related to several factors (Hall-Stoodley and Stoodley 2009; Høiby et al. 2010; Corbin et al. 2011), such as (1) the higher frequency of mutation and horizontal gene transmission found in biofilms when compared to planktonic bacteria, which explains the rapid development of antibiotic resistance in biofilms; (2) the appearance of nutrient depleted zones throughout biofilms, due to oxygen and nutrient gradients, which cause bacteria to enter into a stationary phase-like dormancy and not be affected by antimicrobials; (3) the development of oxidative stress, caused by an imbalance between the formation of reactive oxygen species (ROS) and the antioxidant system, that increases mutability in biofilms; and (4) the delay of antibiotic penetration into the matrix of the biofilm, which contains polymers that bind to antibiotics and hinder their action, and antibiotic-degrading enzymes that deactivate them.

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The difficulty of successfully treating biofilm-associated infections and the increasing resistance of microbes to traditional treatments call for the discovery of compounds with novel mechanisms of action. Most of the antimicrobial products that have been developed are derivatives of already known compounds and target the same resistance mechanism, so their action can only be somewhat better. Now, research is drifting towards the discovery of non-traditional sources of antimicrobials, microbial genome sequencing focused on antibiotic gene expression, metagenomics, and investigation of new targets with respect to pathogenic bacteria. Within this scope, natural products, namely antimicrobial peptides (AMPs), stand out because they have a much higher hit rate in high-throughput screens than the combinational libraries of traditional antimicrobials. Moreover, natural products are usually much more complex than synthetic products and present scaffolds with viable and biologically validated starting points for the design of chemical libraries (Spížek et al. 2010).

The following sections describe AMPs and their application to biofilm prophylaxis and therapeutics. Emphasis is given to the main sources and categories of AMPs, their common anti-biofilm mechanisms of action, and customized bioinformatic approaches.

Sources of antimicrobial peptides

AMPs are short-length peptide antibiotics (between 15 and 30 amino acids), the majority of which are cationic, amphipathic, gene-encoded and directed to the cell membrane (Rossi et al. 2008; Melo et al. 2009; Splith and Neundorf 2011). AMPs are usually co-expressed in groups that act together (Lai and Gallo 2009), but despite their similarities, AMP sequences vary greatly. Usually, AMPs are classified as α-helical, β-sheeted, extended and looped (Melo et al. 2009) or ribosomally and non-ribosomally synthesized (Rossi et al. 2008).

AMPs come from a variety of sources and in many forms. Their widespread distribution throughout the animal and plant kingdoms suggests that AMPs play a fundamental role in the evolution of complex multicellular organisms. Despite their ancient lineage, AMPs have remained effective defensive weapons (Zasloff 2002). Indeed, it has been proposed that AMPs and AMP-directed resistance mechanisms have co-evolved, leading to a host-pathogen balance that has shaped the existing AMP portfolio (Peschel and Sahl 2006).

AMPs are part of the innate immune system of animals and plants, but can also be found in bacteria and fungi (Rossi et al. 2008; Splith and Neundorf 2011). Mammalian AMPs are generally expressed and easily induced in epithelial surfaces to repel assault by bacteria, viruses, fungi and parasites (Lai and Gallo 2009). AMPs have also been found in the glandular cells of amphibian skin, fish and most classes of invertebrates. In plants, AMPs help in the adaptation to stressful environmental conditions. For example, plant cells act as recognition sites against pathogen-derived metabolites (elicitors), leading to the accumulation of AMPs in the affected plant tissue (Kido et al. 2010). Additionally, bacteria and fungi produce AMPs as a defense mechanism and as a means of gaining competitive advantage against other microorganisms, sometimes of the same species (Sang and Blecha 2008).

Synthetic AMPs, produced by *de novo* synthesis or by modification of existing AMPs, emerged as an alternative to reduce production costs (Wimley and Hristova 2011). Recently, some reviews (Vooturi and Firestine 2010; Giuliani and Rinaldi 2011) have reported the engineering of AMP mimetics or peptidomimetics, ie non-peptide molecules aimed at retaining and improving the basic features of AMPs.

A resume of the sources and classifications of the various AMPs is available in Table 1.

Mechanisms of action of antimicrobial peptides

AMPs have been recognized as promising candidates for replacing classical antibiotics (Figure 1) due to their multiple mechanisms of action and low specificity in terms of molecular targets, which reduces the chance of acquired resistance (Zasloff 2002; Beckloff et al. 2007). Moreover, compared with conventional antimicrobials, which are generally active only against bacteria or fungi, AMPs exert activity against a broad spectrum of microorganisms, such as both Gram-negative and Gram-positive bacteria, including drug-resistant strains, parasites, enveloped viruses and even some cancer cells (Sang and Blecha 2008; Splith and Neundorf 2011; Wimley and Hristova 2011). AMPs are also cell specific and are able to distinguish host from non-host cells based on their charge (Beckloff et al. 2007). Besides their antimicrobial action, AMPs can also influence processes which support antimicrobial action, like cytokine release, chemotaxis, antigen presentation, angiogenesis and wound healing (Lai and Gallo 2009).

Conventional antibiotics usually act by inhibition of cell wall synthesis or DNA, RNA and protein synthesis (Sang and Blecha 2008; Chau 2010). Most AMPs permeabilize microbial membranes, inducing either a large-scale failure or small defects that dissipate the transmembrane potential, which results in cell death (Sang and Blecha 2008; Wimley and Hristova 2011). This mechanism of action does not depend on the recognition of chiral targets and,

Table 1. AMP sources and classes.

Source/type	Family	Structural features	Synthesis and localization	Spectrum of activity	Other characteristics
Mammals	Cathelicidins	• Gene encoded (Tomasinsig and Zanetti 2005) • 12–100 a.a. (Wang et al. 2011a) • Cathelin domain (highly conserved N-terminal region) (Bals and Wilson 2003; Tomasinsig and Zanetti 2005) • Peptides with little similarity besides their precursor protein (hCAP18) (Smet and Contreras 2005; Lai and Gallo 2009)	• Expressed in leukocytes (neutrophils, monocytes, NK cells, T and B cells), epithelial cells of the testis, skin, gastrointestinal and respiratory tracts (Bals and Wilson 2003; Smet and Contreras 2005)	• Bacteria • Fungi (Cirioni et al. 2006)	• Also produced by invertebrates, fish, birds, frogs and snakes (Cirioni et al. 2006; Lai and Gallo 2009)
	Defensins	• 3.5-6 kDa (Smet and Contreras 2005) • 6 conserved cysteine residues, forming 3 disulfide bridges (Smet and Contreras 2005) • 50α-defensins and 90β-defensins (Lai and Gallo 2009) • 4α neutrophil defensins (HNP-1 to 4), 2α enteric defensins (HD-5 and H-6) and 4β epithelial defensins (hβD-1 to 4) (http://peptidesaustralia.com/peptides-and-proteins-with-antimicrobial-activity/)	• Expressed in monocytes, macrophages, keratinocytes and epithelial cells of the respiratory, digestive, urinary and reproductive systems (Lai and Gallo 2009) • Stored in the neutrophils' granules and in Paneth cells (Lai and Gallo 2009)	• Bacteria • Fungi • Protozoans • Viruses (Cederlund et al. 2011)	• Antitumoral • Stimulate cell proliferation • Interferes with signal transduction pathways • Chemo-attract immune cells • Stimulate cytokine and adhesion molecules' expression
	Histatins	• 3-4 kDa • Histidine-rich (7 histidine residues) • Linear structure (Oppenheim et al. 1988; Smet and Contreras 2005) • Histatin 5: random coil structure in aqueous solvents and α-helix structure in non-aqueous solvents (Raj et al. 1998)	• Present in human saliva (Oppenheim et al. 1988; Smet and Contreras 2005) • Produced and secreted by the submandibular, sublingual and parotid glands (Smet and Contreras 2005)	• Bacteria • Fungi (Oppenheim et al. 1988; Smet and Contreras 2005)	• Inhibit inflammatory cytokine induction from human fibroblasts • Inhibit host and bacterial enzymes implicated in periodontal disease • Metallopeptide-like properties

(*continued*)

Table 1. (*Continued*).

Source/type	Family	Structural features	Synthesis and localization	Spectrum of activity	Other characteristics
Amphibians	Dermaseptins	• α-helical • Amphipathic • Undergo coil-to-helix transition upon binding to lipid bilayers • Signature pattern of a conserved Trp residue at position 3 and an AA(A/G) KAAL(G/N)A consensus motif in the midregion (Nicolas and El Amri 2009)	• Produced by *Phyllomedusa, Pachymedusa, Agalichnis* and *Hylomantis* frogs (Nicolas and El Amri 2009; Zhang et al. 2010) • Synthesized and stored in the granular glands of the skin (Nicolas and El Amri 2009)	• Bacteria • Yeast • Protozoa • Mollicutes • Enveloped viruses (Nicolas and El Amri 2009)	• Antimalarial (Nicolas and El Amri 2009)
	Phylloseptins	• 19–21 a.a. (1.7–2.1 kDa) • Highly conserved N-terminal region and C-terminal amidation • Amphiphilic (Leite et al. 2005; Zhang et al. 2010)	• Present in skin secretions of *Phyllomedusa* and *Hylomantis* frogs (Nicolas and El Amri 2009)	• Bacteria • Protozoans (Leite et al. 2005; Zhang et al. 2010)	–
	Magainins	• 23 a.a. (Zairi et al. 2009) • α-helical (Zairi et al. 2009) • Also known as PGS (peptide glycine serine) (Giovannini et al. 1987; Zairi et al. 2009)	• Present in the skin of the African clawed frog *Xenopus laevis* (Zairi et al. 2009; Duclohier 2010)	• Bacteria • Fungi • Viruses (Zairi et al. 2009; Duclohier 2010)	• Facilitate wound closure and reduce inflammation (Sai et al. 1995; Duclohier 2010) • Spermicidal (Reddy et al. 1996) • Antitumoral (Duclohier 2010) • Act as dimmers via the toroidal model (Duclohier 2010)
Crustaceans	Tachyplesins	• 17 a.a. • Unique arginine α-amide at the COOH terminal end (Nakamura et al. 1988) • Disulfide-stabilized β-sheet or β-hairpin conformation • 2 cross-strand disulfide bonds • 6 cationic residues (Doherty et al. 2006)	• Produced by horseshoe crabs' hemocytes (Nakamura et al. 1988; Minardi et al. 2007)	• Bacteria • Fungi • Viruses (Nakamura et al. 1988; Hancock and Lehrer 1998)	–

(*continued*)

Table 1. (*Continued*).

Source/type	Family	Structural features	Synthesis and localization	Spectrum of activity	Other characteristics
	Stylicins	• Anionic • Theoretical pI of 5 • 82 a.a. (8.9 kDa) • Proline-rich N-terminal region • C-terminal portion with 13 cysteine residues (Rolland et al. 2010; Rosa and Barracco 2010)	• Produced by the penaeid shrimp *Litopenaeus stylirostris* (Rolland et al. 2010)	Fungi (Rolland et al. 2010)	• Bind LPS (Rolland et al. 2010)
	Crustins	• 50 a.a. • 8 cysteine residues • Signal sequence at the N-terminus • Whey acidic protein (WAP) domain at the C-terminus • Tightly packed structure • 3 types: between the signal sequence and the WAP domain, type I crustins contain a cysteine-rich region, type II a glycine-rich and a cysteine-rich region and type III a proline-arginine-rich domain (Krusong et al. 2012)	• Produced by various crustaceans, including several shrimp species (Krusong et al. 2012)	• Bacteria (Krusong et al. 2012)	–
Plants	Thionins	• ~5 kDa • Cysteine-rich • Basic • 2 types: α/β-thionins and γ-thionins (or plant defensins) (Stec 2006)	• Produced by monocots (grains) and eudicots, including dicotyledonous plants (different species of mistletoe and *Pyrularia pubera*), and rosids (crambin) (Stec 2006)	• Bacteria • Fungi (Stec 2006)	–
	Cyclotides	• 30 a.a. • Amphipathic • Zero charge at neutral pH • Globular proteins • Head-to-tail cyclic backbone structure combined with a cystine knot • (Garcia and Camarero 2010; Smith et al. 2011)	• Produced by large plants (Garcia and Camarero 2010; Smith et al. 2011)	• Bacteria • Viruses • Insects (Garcia and Camarero 2010; Smith et al. 2011)	• Ultra-stable to thermal, chemical, and enzymatic degradation (Garcia and Camarero 2010; Smith et al. 2011)

(*continued*)

Table 1. (*Continued*).

Source/type	Family	Structural features	Synthesis and localization	Spectrum of activity	Other characteristics
AQ21	Lipid Transfer Proteins (LTPs)	• 2 families: LTP1 (90–95 a.a., 9 kDa) and LTP2 (70 a.a., 7 kDa), with different patterns of disulfide bridges (Kido et al. 2010)	• Produced by higher plants (Kader 1996)	• Fungi • Bacteria (Kader 1996; Wang et al. 2004)	• Transfer phospholipids between a donor and an acceptor membrane (Kader 1996; Kido et al. 2010)
Bacteria	Bacteriocins	• Ribossomally synthesized • Class I bacteriocins: small (18–39 a.a.); called lantibiotics because of their lanthionine or β-methyllanthionine residues, formed during post-translational modification • Class II bacteriocins: very diverse group not subject to extensive posttranslational modification • Class III bacteriolysins: large, heat-labile proteins; catalyze the hydrolysis of bacterial cell walls (Sang and Blecha 2008)	• Produced by different groups of bacteria and archaea (Gálvez et al. 2007; Sang and Blecha 2008)	• Bacteria of the same species (narrow spectrum) • Bacteria of other genera (broad spectrum) (Sang and Blecha 2008)	–
	Gramicidins	• Non-ribosomally synthesized • Prototypical channel formers • Gramicidins A, B, and C: linear penta-decapeptides; β-helix (6.3 a.a. per turn); alternating L- and D-a.a. composition except for position 2 (Gly) • Gramicidin S: backbone-cyclized cationic decapeptide; antiparallel β-sheet (Mogi and Kita 2009; Yala et al. 2011)	• Produced by the bacterium *Aneurinibacillus migulanus* (formerly known as *Bacillus brevis*) (Mogi and Kita 2009)	• Bacteria (Mogi and Kita 2009; Yala et al. 2011)	• Used to study organization, dynamics, and function of membrane-spanning channels (Yala et al. 2011) • Linear gramicidins enter lipid membranes forming a dimer channel that conducts a cation flow (Mogi and Kita 2009; Yala et al. 2011)

(*continued*)

Table 1. (*Continued*).

Source/type	Family	Structural features	Synthesis and localization	Spectrum of activity	Other characteristics
	Polymyxins	• Non-ribosomally synthesized • Pentabasic decapeptides • Contain a cyclo-heptapeptide ring with a C9 or C10 hydrophobic fatty acid chain through a α-amide linkage (Mogi and Kita 2009)	• Produced by Gram-positive bacterium *Bacillus polymyxa* (Mogi and Kita 2009)	• Gram-negative bacteria (Martti 2010)	• Polymyxins B and E (colistin) used in the last resort treatment of Gram-negative bacterial infections • Bind LPS (Martti 2010)
Fungi	Peptaibols	• 10–20 a.a. • Non-ribosomally synthesized • High proportion of genetically non-coded amino acids (α-aminoisobutyric acids (Aibs) or α-methylalanines and isovaline (Iva)) • C-terminal alcohol (Sang and Blecha 2008; Duclohier 2010)	• Produced by the hyphomycetous fungi of the *Trichoderma* genus (Duclohier 2010)	• Bacteria • Fungi (Sang and Blecha 2008)	–
Synthetic and Modified	Specifically (or selectively) targeted antimicrobial peptides (STAMPs)	• Targeting peptide domain fused to an antimicrobial, or 'killing', peptide domain, both functionally independent (Eckert et al. 2006a; He et al. 2010)	• Chemically synthesized with high-yield in vitro (Eckert et al. 2006a)	• Bacteria (Eckert et al. 2006a; He et al. 2010)	• Increased killing potency, selectivity, and kinetics (Eckert et al. 2006a)
	Synthetic antimicrobial peptidomimetics (SAMPs)	• Extremely short (Flemming et al. 2009)	• Developed through modifications of the cationic antimicrobial peptides (CAPs) (Flemming et al. 2009)	• Same as CAP used (Flemming et al. 2009)	• Improved pharmacokinetic properties (Flemming et al. 2009)
	Hydrophobic end-tagged AMPs	• Hydrophobic amino acid stretches at the C-terminal end (Malmsten et al. 2011)	• Developed through hydrophobic C-terminal tagging of AMPs (Malmsten et al. 2011)	• Bacteria (Malmsten et al. 2011)	• Improved bactericidal potency (Malmsten et al. 2011)
	Automatically designed peptide antibiotics (adepantins)	• Based on anuran AMPs (Juretić et al. 2009)	• *De novo* peptides, outputs of a Designer algorithm (Juretić et al. 2009)	• Gram-negative bacteria (Juretić et al. 2009)	• Designer algorithm uses an objective construction procedure, based on collected experimental data from anuran AMPs (Juretić et al. 2009)

(*continued*)

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Table 1. (*Continued*).

Source/type	Family	Structural features	Synthesis and localization	Spectrum of activity	Other characteristics
AMPs mimetics	Multimeric or Dendrimeric peptides	• Branched polymers with peptides attached centrally to a template or core matrix • Higher local concentration of bioactive units (Giuliani and Rinaldi 2011)	• *De novo* molecules with multiple peptide sequences added to an inner core usually of radially branched lysine residues (Bruschi et al. 2010; Giuliani and Rinaldi 2011)	• Bacteria (Bruschi et al. 2010; Giuliani and Rinaldi 2011) • Viruses (Giuliani and Rinaldi 2011)	• Resistant to peptidases and proteases • Increased activity (Giuliani and Rinaldi 2011)
	Peptoids or Amptoids	• Oligo N-substituted glycines • Isomers of AMPs with side chains attached to the backbone nitrogen rather than the α-carbon (Giuliani and Rinaldi 2011)	• *De novo* peptides constructed via a library of peptoid monomers tuned for hydrophobicity and side-chain charge (Chongsiriwatana et al. 2008; Giuliani and Rinaldi 2011)	• Bacteria • Fungi (Giuliani and Rinaldi 2011; Kapoor et al. 2011)	• Resistant to proteases • Reduced immunogenicity • Improved bioavailability • Highly tunable side-chain chemistry (Chongsiriwatana et al. 2008; Giuliani and Rinaldi 2011)
	Oligoacyllysines (OAKs)	• Composed of tandem repeats of acyllysines (Giuliani and Rinaldi 2011) • Hydrophobic and cationic (Giuliani and Rinaldi 2011)	• *De novo* peptides that mimic the primary structure and function of AMPs (Giuliani and Rinaldi 2011)	• Bacteria (Sarig et al. 2010; Giuliani and Rinaldi 2011)	• Does not form defined secondary structures due to optimal rotational freedom of the carbon atoms in an acyl chain • Increased vivo efficacy • Less toxic (Giuliani and Rinaldi 2011)
	Ceragenins	• Derivatives of bile acids with covalently attached amines • Cationic (Giuliani and Rinaldi 2011)	• *De novo* molecules whose design was inspired by the aminosterol squalamine (Giuliani and Rinaldi 2011)	• Bacteria (Savage et al. 2002; Giuliani and Rinaldi 2011)	• LPS and (lipoteichoic acid) LTA binding activity (Giuliani and Rinaldi 2011)
	DL-Amino acid diastereomers	• Do not retain α-helical structure of AMPs (Vooturi and Firestine 2010)	• *De novo* molecules obtained by incorporation of D-a.a. in the peptides (Vooturi and Firestine 2010)	• Bacteria (Vooturi and Firestine 2010)	• Increased activity • Resistant to proteases • Not cytotoxic on mammalian cells (Vooturi and Firestine 2010)

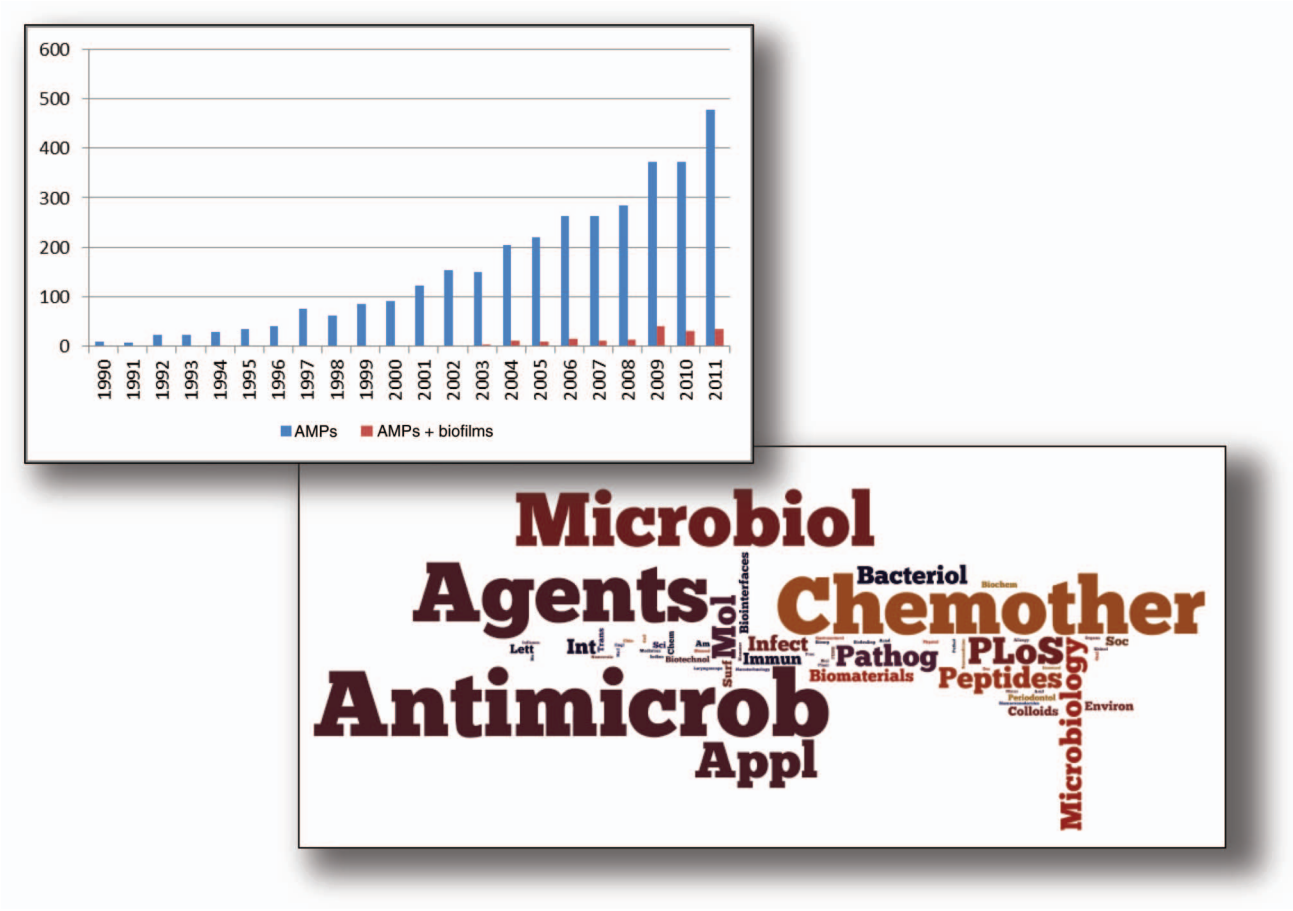

Figure 1. General statistics on the number of publications on AMPs (blue bars) and AMP applications to biofilms (red bars) in PubMed. The word cloud highlights research areas where these studies are most relevant.

therefore, all D-enantiomers are equally active, giving AMPs a broad action spectrum (Podda et al. 2006).

The mechanisms of action of AMPs are divided into pore and non-pore models (Wimley and Hristova 2011). Pore models account for the formation of membrane-spanning pores, namely the 'barrel stave pore model' (Rapaport and Shai 1991), in which AMPs interact to form a hydrophilic channel, and the 'toroidal pore model' (Ludtke et al. 1996), in which AMPs affect the curvature of the membrane. In turn, non-pore models comprise: the 'carpet model' (Gazit et al. 1996), which is the most cited model and accounts for the parallel deposition of AMPs on the membrane, causing global bilayer destabilization due to a detergent-like effect; the 'detergent model' (Ostolaza et al. 1993) that explains catastrophic collapse of the membrane using high concentrations of AMPs; the 'molecular shape models' (Bechinger and Lohner 2006), in which AMP-lipid interactions can be portrayed with phase diagrams; the 'lipid clustering model' (Epand and Epand 2009), in which AMPs induce lipid phase separation; the 'sinking raft model' (Pokorny et al. 2002), in which AMP activity is described in terms of binding, insertion and perturbation; and the 'interfacial activity model' (Rathinakumar and Wimley 2008), which is used to explain, predict and engineer the activity of AMPs. All the aforementioned models imply the need to reach a certain threshold concentration of AMPs in the membrane prior to disruption (Melo et al. 2009). Biomembrane interaction studies with model bacterial and mammalian lipid membranes are very important for determining the mechanisms of action of membrane active peptides (Gao et al. 2012).

Some AMPs act by alternative means, like binding to DNA, inhibiting cell wall, DNA, RNA and protein synthesis, and autolysin and inhibiting enzyme activity (Sang and Blecha 2008). The type of mechanism of action of AMPs can dictate their application fields. For example, it has been noticed that AMPs targeted at the

membrane are better suited for use in surface coatings than AMPs that act at an intracellular level (Bagheri et al. 2012).

More details about the mechanisms of action of AMPs are available in the reviews of Melo et al. (2009), Nguyen et al. (2011), Park et al. (2011), Splith and Neundorf (2011) and Wimley and Hristova (2011).

Anti-biofilm antimicrobial peptide strategies

Biofilm control can be achieved in three ways, namely (1) reduction of the planktonic population, (2) prevention of the initial adhesion of cells to the surface, and (3) removal of the established biofilm. Studies on biofilm-forming bacteria or yeasts in the planktonic state may open routes to the first strategy. However, *in vivo* application of the first strategy is quite complicated, given that the planktonic population in the body is widespread and identification of the presence of the planktonic biofilm-forming bacteria is difficult, so microbial adhesion must be prevented as the next step.

Conceptually, the easiest method for preventing microbial attachment is by pre-treating the surfaces. This can be achieved by impregnating the surface with an antimicrobial agent or using functionalized coatings that allow a localized antimicrobial delivery (Zilberman and Elsner 2008; Shukla et al. 2010; Yala et al. 2011). As reviewed by Glinel et al. (2012), several AMP-based coatings have been tested successfully.

When surface pre-treatments are not effective, biofilms may form. Strategies based on the administration of antimicrobials to the infected live tissue (antibiotic treatments) or the non-living surface (disinfectant treatments) must then be applied to kill the biofilm-growing microorganisms. A summary of the latest biofilm control studies using AMPs is depicted in Table 2.

Analysis of Table 2 shows that the AMPs tested on biofilms come from various natural sources, such as humans (AMP-IBP5; HBD3; LL-37; α-MSH), other mammals (BMAP-28; cathelicidin WAM1), amphibians (aurein 2.5; magainin I; phylloseptin-1), fish (chrysophsin-1; pleurocidin), arthropods (tachyplesin III), bacteria (gramicidin A; lacticin 3147; nisin) and plants (*Tn*-AFP1). Non-natural AMPs are classified into mimetics (peptoid 1; peptoid 1-C134mer; (RW)4D) and synthetic (F2,5,12W; KSL; PTP-7; Tet213; SAMPs Ltx5, Ltx9 and Ltx10; omiganan pentahydrochloride; STAMPs C16G2, M8G2, C16-33, M8-33 and G10KHc). A substantial part of the AMPs tested is synthetic, which means that improving the optimal performance of AMPs is nowadays becoming an important issue. Most of the microorganisms tested are bacteria, probably due to their ubiquity in Nature and their frequent association with infectious diseases and biofilms.

It is also noteworthy that a substantial number of biofilm-related studies, as seen in Table 2, cover mainly biofilm growth in the presence of AMPs, ie prophylactic strategies meant to prevent biofilm formation, rather than testing AMPs against pre-established biofilms, ie therapeutic strategies meant to treat existing biofilms. This suggests that prevention of biofilm formation is possibly the current favorite research strategy in the combat of nosocomial infections. However, more work must be done in order to evaluate the anti-biofilm efficacy of AMPs on mature biofilms. Some recent, successful studies on the development of AMP-mediated anti-biofilm prophylactic and therapeutic strategies can be found in Table 2.

One of the characteristics that appears to be linked to the anti-biofilm efficacy of some AMPs is their dual capacity to act both on the cytoplasmic membrane and on intracellular targets, once they have entered the cell. For example, it is thought that the synthetic AMP *meta*-phenylene ethynylene (mPE), based on magainin and active at nanomolar concentrations against biofilms of *Streptococcus mutans*, acts both as a membrane-active molecule, inhibiting lipopolysaccharides (LPSs), similar to magainin, and as an intracellular antibiotic by binding to DNA at equimolar ratios (Beckloff et al. 2007). Another example is pleurocidin, which is thought to inhibit nucleic acid and protein synthesis without damaging the cytoplasmic membrane of *Escherichia coli* at low concentrations (Patrzykat et al. 2002), but it is able to cause membrane leakage and pore-like channels at higher concentrations (Mason et al. 2006).

Some studies have also reported AMP activity against biofilms at lower concentrations than those required for killing planktonic cells. This is the case for the synthetic AMP NA-CATH:ATRA1-ATRA1 and the natural AMP LL-37, both from the cathelicidin family, that are effective against *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms, respectively. These AMPs are thought to act internally on the bacterial cells, affecting gene expression essential for the development of biofilms (Overhage et al. 2008; Dean et al. 2011). In *P. aeruginosa*, the AMP LL-37 also alters the expression of biofilm related genes, such as Type IV pili, rhamnolipid and Las quorum sensing systems at sub-antimicrobial levels, and genes associated with the assembly of flagella, involved in initial adherence during biofilm formation, were found to be down regulated (Overhage et al. 2008). LL-37 is also capable of inhibiting initial biofilm attachment (by 58–62%), suggesting that peptides of this kind may be interacting with bacterial adhesins as part of their

anti-biofilm mechanism (Dean et al. 2011). Another study (de la Fuente-Núñez et al. 2012) also showed that the AMP 1037 directly inhibits biofilms by reducing swimming and swarming motilities, stimulating twitching motility, and suppressing the expression of a variety of genes involved in biofilm formation in *P. aeruginosa* (eg PA2204).

Anti-adhesion may be one of the major anti-biofilm properties of AMPs, which allows them to be used as an effective pre-treatment strategy. The AMP nisin, which is known to interfere with cell wall synthesis and to form membrane pores (Peschel and Sahl 2006), retards biofilm formation without inhibiting the growth of *S. aureus* when immobilized in multi-walled carbon nanotubes (Qi et al. 2011). Another example is the cathelicidin-2 derived peptide, F2,5,12W, which suppresses *Staphylococcus epidermidis* biofilm formation at a concentration four times below the minimal inhibitory concentration (MIC), and this is reflected in decreased initial adhesion of the bacterial cells (Molhoek et al. 2011).

Anti-attachment capabilities may be related to binding of DNA also. DNA binding may facilitate the detachment or disruption of biofilm structures, since it has been reported that eDNA is involved in cell–cell attachment, having an important role in cell adhesion to surfaces and cell aggregation (Allesen-Holm et al. 2006; Das et al. 2010). This is the case for cationic AMPs and peptoids (Lobo et al. 2003; Otvos 2005; Hale and Hancock 2007). For example, the development of *P. aeruginosa* biofilms is disrupted by the enzyme DNase I (Whitchurch et al. 2002).

AMPs may also cause matrix disruption in biofilms. It is thought that the peptoid 1-C134mer, which has an hydrophobic tail and is active against *P. aeruginosa* biofilms, interacts strongly with and disrupt the hydrophobic matrix due to its surfactant like nature, facilitating deeper penetration (Kapoor et al. 2011).

Additionally, AMPs have some organism-specific features. For example, lactoferrin inhibits biofilm formation in *P. aeruginosa* due to its iron-chelating properties, increasing surface motility and causing the bacterial cells to wander around the surface, forming thin, flat biofilms (Singh et al. 2002). In turn, the inhibition of *Porphyromonas gingivalis* and *Prevotella intermedia* biofilms by lactoferrin is independent of the iron status of the protein. Lactoferrin may interact with the cell surface of these bacteria and interfere with their adherence (Wakabayashi et al. 2009), since this peptide is reported to interfere with the binding of *P. intermedia* to subepithelial matrix proteins, as well as fibroblasts and epithelial cells (Alugupalli et al. 1994; Alugupalli and Kalfas 1995).

Biofilm resistance to antimicrobial peptides

Although the development of resistance to AMPs is rare, some studies have reported this phenomenon. The general mechanisms of microbial resistance to AMPs, which are valid both for the planktonic and sessile states, include mutations that affect the structure and charge distribution of the cytoplasmatic membrane, modifications to the lipopolysaccharide structure of Gram-negative bacteria, and active pumping of the AMPs out of the cell (Altman et al. 2006). Specifically, it has been reported that Gram-negative bacteria have evolved mechanisms to remodel the composition of the outer membrane through modification of the LPS molecules (Miller et al. 2005), which impairs LPS-binding AMPs.

Biofilm structure is another factor correlated with biofilm resistance to AMPs. For instance, the increased survival of *E. coli* biofilms when treated with colistin is not related directly with biofilm forming ability, but rather to the organization of the biofilm (Folkesson et al. 2008). Also, there is some evidence that biofilm formation in *E. coli* induces tolerance to AMPs due to changes in intra-biofilm physiochemical gradients (Folkesson et al. 2008).

AMP activity over intracellular targets is countered by genetic mutations. Interestingly, in *S. aureus*, the regulatory system GraRS, which is involved in up-regulation of biofilm production, has been reported to mediate the resistance of the planktonic cells to AMPs (Herbert et al. 2007). In cystic fibrosis, where *P. aeruginosa* biofilms cause pneumonia, results show that colistin kills the stalk subpopulation (a deeper layer with low metabolic activity) preferentially, whereas the metabolically active cap-forming subpopulation in the upper layer becomes colistin resistant due to the up-regulation of the *pmr* and *mexAB-oprM* genes (Haagensen et al. 2007; Pamp et al. 2008).

Bioinformatic resources

Bioinformatic approaches are an invaluable asset to attain a thorough understanding of the activity of AMPs and thus exploit their potential as antimicrobial drugs. Current approaches address the screening and *in silico* modelling of novel AMPs as a means of accelerating the process of antimicrobial drug discovery and design, and the construction of information systems in support of the deposition, curation and comparison of AMP related data, as means of keeping track of the continuous discovery of new AMPs with diverse antimicrobial potential.

A schematic overview of existing tools and resources in support of AMP studies is presented in Figure 2, and details of the different areas of development and application are provided in the next subsections.

Table 2. Recent applications of AMPs in the control of biofilms.

		Minimal active concentration				
		Pre-treatment		Therapy		
AMPs	Microorganisms	B	P	B	P	Reference
1010cys	*Pseudomonas aeruginosa*	14.2 peptides nm^{-2} (on copolymer brush DMA/APMA) on Ti surfaces)		–		Gao et al. (2011a)
Aurein 2.5	*Bacillus subtilis* *Escherichia coli*	125 μM (MIC)	30 μM (MIC)	–		Dennison et al. (2009)
BMAP-28	*Staphylococcus aureus*	2 $\mu g\ ml^{-1}$ (MIC) 4 $\mu g\ ml^{-1}$ (MBC)		4 $\mu g\ ml^{-1}$ (MIC) 16 $\mu g\ ml^{-1}$ (MBC) 10 $\mu g\ ml^{-1}$ (CVC infection in rat)	–	Cirioni et al. (2006)
C16G2	*Streptococcus mutans*	–	3 μM	25 μM	–	Eckert et al. (2006b)
Chrysophsin-1	*Streptococcus mutans*	–	4 $\mu g\ ml^{-1}$ (MIC) 8 $\mu g\ ml^{-1}$ (MBC)	16 $\mu g\ ml^{-1}$	–	Wang et al. (2012)
F2,5,12W	*Staphylococcus epidermidis*	2.5 μM	10 μM	40 μM	–	Molhoek et al. (2011)
G10KHc	*Pseudomonas aeruginosa*	–	0.5 – 29 μM (MIC)	100 $\mu g\ ml^{-1}$	–	Eckert et al. (2006a)
Gramicidin A	*Listeria ivanovii* *Enterococcus faecalis* *Staphylococcus aureus* *Escherichia coli* *Candida albicans*	20 $\mu g\ ml^{-1}$ for 2h (cystamine self-assembled monolayers on gold surfaces)	–	–		Yala et al. (2011)
HBD3	*Staphylococcus aureus* *Candida albicans*	–	0.2 & 0.4 $\mu g\ m1^{-1}$ 25 & 50 $\mu g\ ml^{-1}$ (MIC & MBC)	0.2 $\mu g\ ml^{-1}$ 25 $\mu g\ ml^{-1}$ (on maxillofacial silicone elastomer disks)	–	(Shi et al. 2009)
KSL	*Streptococcus mutans*	62.5 $\mu g\ ml^{-1}$ (MIC)		0.25 mg ml^{-1}	–	(Liu et al. 2011)
Lacticin 3147	*Streptococcus mutans*	6.3 $\mu mol\ l^{-1}$	1.9–3.8 $\mu mol\ l^{-1}$ (MIC) 38 $\mu mol\ l^{-1}$ (MBC)	50 $\mu mol\ l^{-1}$	–	(Dobson et al. 2011)
LL-31	*Burkholderia pseudomallei*	–		20 ìM		(Kanthawong et al. 2012)
LL-37	*Burkholderia pseudomallei*	–		20 ìM		(Kanthawong et al. 2012)
	Staphylococcus aureus	10 $\mu g\ ml^{-1}$	1.27 $\mu g\ ml^{-1}$ (EC_{50})	–		(Dean et al. 2011)
	Candida albicans	20 $\mu g\ ml^{-1}$	–	–	20 $\mu g\ ml^{-1}$	(Tsai et al. 2011)
	Pseudomonas aeruginosa	16 $\mu g\ ml^{-1}$ (MIC)	64 $\mu g\ ml^{-1}$ (MIC)	4 $\mu g\ ml^{-1}$	–	(Overhage et al. 2008)
Ltx10	*Staphylococcus epidermidis* *Staphylococcus haemolyticus*	–	2 $\mu g\ ml^{-1}$ 4 $\mu g\ ml^{-1}$ (MIC)	50 $\mu g\ ml^{-1}$	–	(Flemming et al. 2009)
Ltx5	*Staphylococcus epidermidis* *Staphylococcus haemolyticus*	–	4 $\mu g\ ml^{-1}$ 8 $\mu g\ ml^{-1}$ (MIC)	50 $\mu g\ ml^{-1}$	–	(Flemming et al. 2009)
Ltx9	*Staphylococcus epidermidis* *Staphylococcus haemolyticus*	–	2 $\mu g\ ml^{-1}$ 4 $\mu g\ ml^{-1}$ (MIC)	50 $\mu g\ ml^{-1}$	–	(Flemming et al. 2009)
Magainin I	*Listeria ivanovii*	0.1 mg ml^{-1} (on SS-SC-Chi-Tere surfaces)	–	–		(Héquet et al. 2011)
	Listeria ivanovii *Bacillus cereus*	–		5% of a polymer brush		(Glinel et al. 2009)
Nisin	*Staphylococcus aureus*	1.25 ìg ml^{-1}	10 ìg ml^{-1}	–	–	(Qi et al. 2011)
	Listeria ivanovii	0.1 mg ml^{-1} (on SS-SC-Chi-Tere surfaces)	–	–		(Héquet et al. 2011)

(*continued*)

Table 2. (*Continued*).

AMPs	Microorganisms	Minimal active concentration: Pre-treatment B	Minimal active concentration: Pre-treatment P	Minimal active concentration: Therapy B	Minimal active concentration: Therapy P	Reference
Omiganan pentahydrochloride	MSSA MRSA MSSE MRSE	–	2–4 μg ml^{-1} 2–8 μg ml^{-1} 4 μg ml^{-1} 4 μg ml^{-1} (MIC)	1% omigan aqueous gel (on skin colonisation models)	–	(Rubinchik et al. 2009)
Peptoid 1 Peptoid 1-C134mer	*Pseudomonas aeruginosa*	12.5 ìM	12.5 ìM (MIC)	12.5 ìM	–	(Kapoor et al. 2011)
Phylloseptin-1	*Staphylococcus aureus*	5 μM (MBEC)	5 μM (MIC)	–		(Zhang et al. 2010)
Pleurocidin	*Streptococcus mutans* *Streptococcus sanguinis* *Streptococcus sobrinus* *Streptococcus gordonii* *Lactobacillus fermenti*	–	8–16 μg ml^{-1} (MIC) 16 μg ml^{-1} (MBC)	64 μg ml^{-1}	–	(Tao et al. 2011)
PTP-7	*Staphylococcus aureus*	4 ìM	2–4 ìM (MIC) 4–8 ìM (MBC)	20–40 ìM	–	(Kharidia and Liang 2011)
Tachyplesin III	*Pseudomonas aeruginosa*	4 & 32 μg ml^{-1} (MIC & MBC) 10 μg ml^{-1} (for 30 min. *in vivo* on ureteral stents)	2 & 4 μg ml^{-1} (MIC & MBC)	–		(Minardi et al. 2007)
Tet20	*Pseudomonas aeruginosa*	10.2 peptides nm^{-2} (on copolymer brush DMA/APMA) on Ti surface)		–		(Gao et al. 2011a)
Tet213	*Staphylococcus aureus* *Pseudomonas aeruginosa*	~9 mg cm^{-2}		–		(Kazemzadeh-Narbat et al. 2010)
	Pseudomonas aeruginosa	0.20 chains nm^{-2} (on copolymer brush DMA/APMA) on Ti surface)		–		(Gao et al. 2011b)
		14.5 peptides nm^{-2} (on copolymer brush DMA/APMA) on Ti surface)		–		(Gao et al. 2011a)
Tet26		10.5 peptides/nm^2 (on copolymer brush DMA/APMA) on Ti surface)		–		(Gao et al. 2011a)
Tn-AFP1	*Candida tropicalis*	32 μg ml^{-1} (MIC)		–		(Mandal et al. 2011)
α-MSH	MRSA	–		12 μM	3 μM	(Madhuri et al. 2009)
(RW)4D	*Escherichia coli*	40 μM		–		(Hou et al. 2009)

Note: MSSA = methicillin-sensitive *Staphylococcus aureus*; MRSA = methicillin-resistant *S. aureus*; MSSE = methicillin-sensitive *Staphylococcus epidermidis*; MRSE = methicillin-resistant *S. epidermidis*; CVC = central venous catheter; B = biofilm; P = planktonic; MIC = minimal inhibitory concentration; MBC = minimal bactericidal concentration; MBEC = minimal biofilm eradication concentration; EC_{50} = half maximal effective concentration; IC_{50} = 50% growth inhibitory concentration. Pre-treatment accounts for the growth of cells in the presence of the AMP and also for the surface treatment, when indicated. Therapy accounts for the application of AMPs to the pre-grown biofilms or cells.

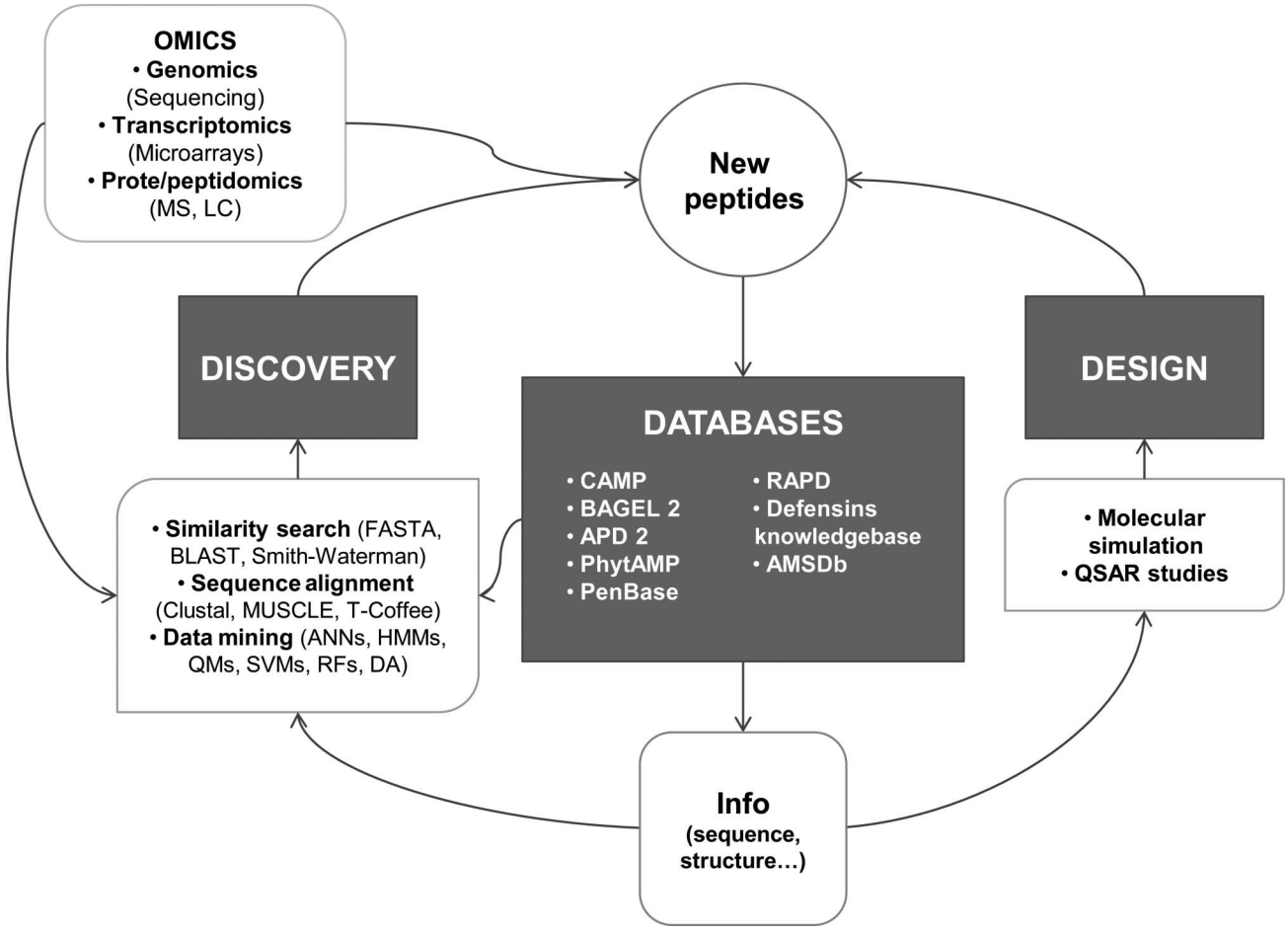

Figure 2. Bioinformatics resources for AMP studies. MS = mass spectrometry; LC = liquid chromatography; ANNs = artificial neural networks; HMMs = hidden Markov models; QMs = quantitative matrices; SVMs = support vector machines; RFs = random forests; DA = discriminant analysis; QSAR = quantitative structure–activity relationship.

Discovery and classification of AMPs

Several 'omics' platforms are addressing the development of computational strategies to identify distinct classes of AMPs (Hammami and Fliss 2010; Pestana-Calsa et al. 2010). Among them, there are consolidated tools for local and global alignments of DNA and protein sequences such as FASTA (Lipman and Pearson 1985), BLAST (Altschul et al. 1990), HMMER (Finn et al. 2011) and the Smith-Waterman algorithm (Smith and Waterman 1981), and data mining tools that apply large-scale computational analysis algorithms to the information saved in genomic and proteomic databases, as well as in published structure–activity data. Indeed, advanced algorithms based in Hidden Markov Models (HMMs) (Eddy 1998), Artificial Neural Networks (ANNs) (Hopfield 1988), Quantitative Matrices (QMs) (Brusic et al. 1998), Support Vector Machines (SVMs) (Zhang 2001), Random Forests (RFs) (Breiman 2001), Discriminant Analysis (DA) (Lachenbruch and Goldstein 1979), Minimum Redundancy Maximum Relevance (mRMR) (Peng et al. 2003) and Incremental Feature Selection (IFS) (Liu and Setiono 1998) have been successfully applied to AMP data mining.

Some authors have used predictive data mining to assess the antimicrobial potential of new peptides. For example the AMPer method (Fjell et al. 2007) recognizes individual classes of AMPs (such as defensins, cathelicidins and cecropins) and discovers novel AMP candidates based on HMMs fed on publicly available data; the BACTIBASE method (Hammami et al. 2007, 2010) uses HMMs to produce bacteriocin profiles for each known family and the sequence analysis tool HMMER to provide statistical descriptions of family consensus sequences in order to support sequence-based searches on the bacterial families producing bacteriocins; the AntiBP and AntiBP2 methods (Lata et al. 2007, 2010) predict antibacterial peptides applying ANN, QM and SVM models to the analysis of the N and C terminal residues

of proteins; the CAMP method (Thomas et al. 2010) uses RF, DA and SVM models to predict the antimicrobial activity of peptide sequences; the BAGEL2 method (de Jong et al. 2010) combines HMMs and simple decision rules in the prediction of bacteriocin sub-classes; the DAMPD method (Sundararajan et al. 2011) predicts AMPs based on SVMs that can classify peptides into one of 27 AMP families in catalogue; and the tool of Wang et al. (2011b) integrates protein BLAST (BLASTP) and a feature selection method based on mRMR and IFS models to select the optimal features for the prediction of AMPs *vs* non-AMPs. In addition, Juretić et al. (2009, 2011) have been addressing the correlation between the physical characteristics of natural AMPs and high selectivity to generate potential peptide antibiotics not homologous to any existing natural or synthetic AMPs. This has resulted in the creation of a structure-selectivity database (AMPad) of frog-derived, helical AMPs. The AMPA web application (http://tcoffee.crg.cat/apps/ampa) (Torrent et al. 2011) was constructed for assessing the antimicrobial domains of proteins, based on an antimicrobial propensity scale for each amino acid (related to the IC_{50} values for all amino acid replacements in the AMP bactenecin 2A) and identifying the regions (>12 amino acids in length) located below the threshold, which are considered putative antimicrobial domains. There is also a search method (Fernandes et al. 2009) for sequence similarity and physico-chemical properties followed by a fuzzy inference system in order to find AMPs that are more appropriate for certain target domains. Finally, the Antibp server (http://www.imtech.res.in/raghava/antibp/index.html) (Lata et al. 2007) predicts antibacterial peptides in protein sequences, with QM, ANN and SVM based methods using the binary patterns of peptide sequences.

A detailed review on designing AMPs is available (Fjell et al. 2012).

Genomics

In genomics, DNA sequencing and mapping constitute a high yield and an useful step for seeking AMP-coding genes (Pestana-Calsa et al. 2010; Pestana-Calsa and Calsa Jr. 2011). One of the most used methods for AMP discovery is the analysis of homologs of known genes by comparing new sequences with sequences of biochemically characterized AMPs (Hammami and Fliss 2010; Pestana-Calsa and Calsa Jr. 2011), since the sequence segments with high identity tend to share structure and function. For example: an *in silico* search based on HMMs (Silverstein et al. 2005) supported genome assignment of 317 defensin-like sequences in *Arabidopsis thaliana*; a novel cathelicidin-like AMP, designated cathelicidin-AM, was identified (Yan et al. 2011) by mining the genome of panda using BLAST and the sequence of a dog cathelicidin; and the systematic mining of the *Nasonia vitripennis* genome (Tian et al. 2010), using the tools BLASTP, translated BLAST nucleotide (TBLASTN) and ScanProsite (http://prosite.expasy.org/scanprosite/scanprosite-doc.html), has delivered a large and diverse peptidome AMP repertoire of defensins, abaecins, hymenoptaecins and tachystatins.

Transcriptomics and proteomics

Transcriptome and proteome profiling offer powerful approaches for studying antimicrobial inhibitory action (Hutter et al. 2004; Freiberg et al. 2005; Overton et al. 2011). It is possible to screen a large number of compounds for antimicrobials, and also to predict their mechanism of action by gene expression profiling (Hammami and Fliss 2010). In principle, the mRNA and protein profiles generated in response to the imposition of antimicrobial stress reflect modulation of particular cellular functions, and provide a signature of the type of stress imposed (Overton et al. 2011). For example: an integrated transcriptome and proteome profiling of drug exposed bacteria (Overton et al. 2011) revealed 22 novel virulence factors from methicillin-resistant *S. aureus* (MRSA) and novel information on killing mechanisms of the AMP ranalexin; a Super SAGE transcriptional analysis (Kido et al. 2010) investigated AMP candidates in Brazilian plants; and a comparative proteomics study (Kim et al. 2011) evaluated milk proteome profiles during acute and chronic phases of *S. aureus* intramammary infection, revealing unique host protein expression profiles depending on the infecting strain and the phase of infection, and implicating the protein CPP3 as a potential AMP.

Peptidomics

Peptidomics addresses the study of small polypeptides, eg by coupling bi- or multidimensional liquid chromatography to mass spectrometry (Pestana-Calsa et al. 2010). Thirty-four novel AMPs from skin secretions of *Rana nigrovittata* were identified by integrating genomics and peptidomics analysis (Ma et al. 2010). Also, a functional peptidomics approach (Wang et al. 2008), integrating peptidome and transcriptome studies of the defensive skin secretion of the frog *Agalychnis callidryas* has led to the identification of three new AMPs: DRP-AC4 (dermaseptin), ARP-AC1 (adenoregulin) and CRP-AC1 (caerin). More recently, a peptidomics-based approach (Osaki et al. 2011) allowed the identification of the AMP-IBP5, which exhibits

antimicrobial activity against Gram-negative and Gram-positive bacteria and fungi at concentrations similar or lower than those of cathelicidin and β-defensin-2. This was accomplished by applying tandem mass spectrometry (MS/MS) techniques to the characterization of a pool of naturally occurring peptides released by exocytosis from cells in culture.

Additionally, peptidomic approaches tackle the study of resistance mechanisms, in support of the development of more sustainable antimicrobial agents (Shen et al. 2010). For example, proteins regulating AMP resistance in *Vibrio parahaemolyticus* have been discovered using membrane subproteome analysis (Shen et al. 2010).

Design of antimicrobial peptides

Simulations and structural bioinformatic approaches can help to focus experimental work that, in turn, will eventually guide and verify simulations (Matyus et al. 2007). Classical molecular dynamics simulations have been proven useful in describing elements of the mechanism of action of AMPs, including the binding of peptides to the bilayer surface, the properties of surface-bound complexes, peptide insertion, structural modifications of lipid bilayers, and the structure of model pores constructed from AMPs and lipids (Matyus et al. 2007). For example, new synthetic cationic peptides with two LPS- and lipid A (LA)-binding sites, similar to AMPs such as protegrin 1, thanatin, and androctonin, were synthesized *de novo* based on molecular modeling simulation and quantitative structure-activity relationship (QSAR) analysis (Frecer et al. 2004).

QSAR analyses, which are used broadly in chemoinformatics to correlate the variation in measured biological activity with the variation in molecular structure, are now being used as a tool for antimicrobial drug discovery (Hammami and Fliss 2010). QSAR analyses have been applied successfully to the analysis of AMP data, resulting in a model that quantifies linear sequence patterns (Hilpert et al. 2006), contact energy between neighboring amino acids (Jenssen et al. 2008) and amphipathicity (Frecer et al. 2004). Also, QSAR analyses have been used in the production of lactoferricin, bactenecin, and protegrin derivatives (Hilpert et al. 2008), and the optimization of the activity of novispirin G10 (Taboureau et al. 2006; Blondelle and Lohner 2010). Moreover, a 3D-QSAR analysis of a series of AMPs (Bhonsle et al. 2007; Blondelle and Lohner 2010) revealed that for any particular AMP, cell selectivity and potency are controlled by the chemical composition of the target cell membrane, and a combined approach of atomic-resolution QSARs with ANNs (Cherkasov et al. 2008; Fjell et al. 2009; Blondelle and Lohner 2010), applied to a large data set of peptides containing high sequence diversity, has shown that the most potent peptides exhibit a high frequency of the amino acids tryptophan, arginine, lysine, isoleucine and leucine.

Information storage and search

A comprehensive database on AMPs with information on their activity would facilitate the study of peptide potential, enabling and promoting sequence-specificity and sequence-activity studies (Hammami et al. 2009; Thomas et al. 2010). Although many AMPs are now well characterized, much information is still missing or scattered over scientific literature, ie its collection and analysis is troublesome and implies time consuming manual curation.

Available and fully operational databases on AMPs are presented in Table 3. Most AMP databases specialize in one type of AMP family, like BAGEL2 (bacteriocins), PhytAMP (plant AMPs), Defensins Knowledgebase (defensins), BACTIBASE (bacteriocins), Peptaibol Database (peptaibols) and PenBase (penaidins). This is due to the diversity of AMPs that are being described and the need to accommodate the most extensive (or application-specific) subclasses into specialized databases. Bioinformatic tools accounting for similarity search (FASTA, BLAST, Smith-Waterman), sequence alignment (ClustalW, T-Coffee and MUSCLE), AMP prediction and physico-chemical profile analysis are present in the majority of the databases described, allowing users easy access to peptide/DNA sequence analysis.

Databases covering proteins and antimicrobials are also considered useful resources for AMP related studies. For example: UniProt (http://www.uniprot.org/), which is a central repository of protein sequence and function data; Protein Data Bank (http://www.pdb.org/), which covers the processing and distribution of 3D biological macromolecular structure data; CyBase (http://www.cybase.org.au/), which keeps cyclic protein sequences and structures; AMIC-BASE (http://www.reviewscience.com/), which documents the antimicrobial properties of natural compounds; EROP (Endogenous Regulatory Oligo-Peptide knowledgebase)-Moscow (http://erop.inbi.ras.ru/index.html) (Zamyatnin 1991; Zamyatnin et al. 2006), which is a curated oligopeptide (2-50 amino acid residues) sequence database; Norine (http://bioinfo.lifl.fr/norine/), which is a database for nonribosomal peptides with tools for their analysis; PepBank (http://pepbank.mgh.harvard.edu/), which is a database on biologically active peptides; PeptideDB database (http://www.peptides.be/), which assembles all naturally occurring signalling peptides from animal source, which are

Table 3. Contents and tools (similarity search, sequence alignment, prediction and others) of current AMP-related databases.

Database (URL and reference)	Contents	Tools			
		Similarity search	Sequence alignment	Prediction	Other
AMSDb http://www.bbcm.units.it/~tossi/amsdb.html	Gene encoded AMPs and proteins from animals and plants.	–	–	No	–
APD2 http://aps.unmc.edu/AP/main.php; Wang et al. (2009)	Categorizes peptides into families, sources, post-translational modifications, and binding targets.	–	–	Yes	Improvement of design peptides.
BACTIBASE http://bactibase.pfba-lab-tun.org/main.php; Hammami et al. (2007, 2010)	Bacteriocin natural AMPs.	BLAST, FASTA, Smith-Waterman	ClustalW, MUSCLE and T-Coffee	Yes (structure)	Physicochemical profile analysis; HMMER.
BAGEL2 http://bagel2.molgenrug.nl; de Jong et al. (2010)	Identifies putative bacteriocins.	BLAST(p or x)	–	Yes (ORFs prediction)	–
CAMP http://www.bicnirrh.res.in/antimicrobial; Thomas et al. (2010)	AMP sequences (experimentally validated and predicted), sources, activity (MIC values), reference literature, target and non-target organisms.	BLAST	–	Yes (sequences with antimicrobial activity based on SVMs, RFs, DA)	Physicochemical profile analysis.
DAMPD http://apps.sanbi.ac.za/dampd/; Seshadri Sundararajan et al. (2011)	Manually curated AMPs (update and replacement of the ANTIMIC database)	BLAST	ClustalW	Yes (signal peptide cleavage sites; sequence for antimicrobial activity based on SVMs)	HMMER; hydrophobicity calculator; graphical views; sub-database selection – UniProt, Swiss-Prot and TrEMBL
Defensins knowledgebase http://defensins.bii.a-star.edu.sg/; Seebah et al. (2007)	Sequence, structure and activity of defensins Related information on patents, grants, research laboratories and scientists, clinical studies and commercial entities.	BLAST	ClustalW	No	Analogs search.
MiniCOPE http://www.copewithcytokines.org/cope.cgi?key= Innate%20immunity%20defense%20peptides%20MiniCOPE%20Dictionary	Dictionary available from COPE (Cytokines & Cells Online Pathfinder Encyclopedia) for innate immunity defense peptides with antibiotic activities.	–	–	No	–
PenBase http://www.penbase.immunaqua.com; Gueguen et al. (2006)	Specialized database for the penaeidin family of AMPs, providing information about their properties, diversity and nomenclature.	BLAST	ClustalW	No	Database primers for subgroup determination.
Peptaibol database http://peptaibol.cryst.bbk.ac.uk/home.shtml; Whitmore and Wallace (2004)	Peptaibol related information from works published in scientific journals.	–	–	–	–
PhytAMP http://phytamp.pfba-lab-tun.org/main.php; Hammami et al. (2009)	Plant AMPs, including taxonomic, microbiological and physicochemical data.	BLAST, FASTA, Smith-Waterman	ClustalW, MUSCLE and T-Coffee	No	HMMER
RAPD http:/faculty.ist.unomaha.edu/chen/rapd/index.php; Li and Chen (2008)	Recombinantly-produced AMPs. Information about expression host, fusion strategy, release method and yield.	–	–	No	DNA translator; peptide calculator; DNA sequence convertor.
YADAMP http://www.yadamp.unisa.it/; Piotto et al. (2012)	Information on AMPs active against bacteria based on an extensive literature search.	–	–	–	–

derived by cleavage from prepropeptide precursor proteins; the Novel Antibiotics DataBase (http://www.nih.go.jp/~jun/NADB/search.html), which contains substances reported first in the Journal of Antibiotics; and the Amicbase Drugs-Online 2011 report (http://www.manetec-52.de/apps/amicbase_drugs-online/base.nsf), which provides antibiotic inhibitory data on licensed drugs in Europe, Japan and the USA.

Current challenges to AMP application

Despite all the aforementioned advantages, most AMP-based strategies present some limitations that must be overcome to achieve widespread implementation of such strategies in biotechnological and clinical settings.

AMP production and application

AMPs are difficult and expensive to obtain in large quantities (Beckloff et al. 2007), mainly due to the complex processes needed for their extraction, isolation and purification. While large amounts of AMPs may be applied to a localized external site (as topical agents), the use of AMPs for internal infections raises some problems: (1) their antimicrobial power is reduced in the presence of biological fluids, (2) inadequate safety margins and rapid renal clearance, (3) the non-specific effect of AMPs may compromise the eradication of a specific infectious agent, and (4) some AMPs display toxicity to the host cells and may induce unwanted pro-inflammatory responses (Baltzer and Brown 2011; Batoni et al. 2011). Thus, efforts are being focused on modifying or synthesizing *de novo* AMPs that are easier to produce and may overcome some of the limitations referred to above.

Prophylactic strategies

Although most anti-biofilm AMP studies have focused on the prevention of biofilm formation, little is known about some prophylaxis-oriented evaluations. For example, the immobilization or 'grafting' of AMPs into surfaces. This immobilization of AMPs, alone or combined, onto a biomaterial surface may circumvent some of the limitations of the peptides, such as a short half-life and cytotoxicity associated with higher concentrations of soluble peptides (Costa et al. 2011). The rationale, at this point, is to obtain functionalized surfaces with proven anti-adhesive and anti-biofilm traits to later be tested as potential harmless biomaterials. Several promising studies have already been made, namely with polymer brushes, which have the advantage of being robust and can be generated in most biomedical materials (Gao et al. 2011a, 2011b). Glinel et al. (2012) report on the existing findings in this field, but further studies are required in order to escalate findings onto a broader range of biomedical surfaces and devices.

Therapeutic strategies

Therapeutic studies are a pressing need as means of assessing the ability of AMPs to kill already existing biofilms. Indeed, it is common knowledge that mature biofilms are more difficult to eradicate, mainly due to the exopolymeric matrix surrounding the cells. Moreover, that mature biofilms are resistant to treatment may be due to their slow growth rate and low metabolic activity and thus, most antibiotics will not be useful because their primary target is metabolically active cells (Lewis 2001; Walters et al. 2003). AMPs have the ability to act on slow-growing or even non-growing bacteria because they act mainly by permeabilizing and/or forming pores within the cytoplasmic membrane. However, there is still little understanding about mechanisms of action of anti-biofilm AMPs.

Naturally, studies on the toxicity and the post-AMP effect (PAE) are the next steps. Toxicity studies on animal cell lines are fundamental for evaluating AMPs as possible therapeutic drugs to be administered to human patients. Likewise, the analysis of biofilm behavior in response to AMP administration cycles and the post-treatment existence of persister cells are very important to ensure the effectiveness and improvement of AMP strategies.

Synergism studies

Synergistic compounds are a relatively unexplored source of new pharmaceutical products. The combination of innovative and conventional antimicrobial compounds is being put into practice to improve both the prophylactic and therapeutic efficacy of some drugs and lower drug dosage, thus reducing toxic side effects (Ncube et al. 2008; Wei et al. 2011).

Natural synergic mechanisms are known in Nature, namely those involved in AMP action. This explains why AMPs require much lower physiological concentrations than those *in vitro* to be effective (Lai and Gallo 2009). There are already some examples of the positive outcomes of this type of approach in biofilms, although the number of studies is still far from what would be expected given the prospects of this line of research (Table 4).

Most synergism studies involving AMPs are centered on the planktonic state. Also, AMPs are tested in combination with other chemicals but rarely with other AMPs. Studies focus either on Gram-

positive and Gram-negative bacteria, more precisely into the biofilm-forming strains of *P. aeruginosa, S. aureus* and *S. mutans*. Due to their clinical profile, these strains are of obvious interest but studies should be extended to other biofilm-forming species to evaluate and take full advantage of the potential of AMPs.

Standardization of laboratory procedures

Current protocols for laboratory research lack standardization, and therefore validity, in some ways. Specifically, the lack of standard protocols hampers the quality of the information generated by researchers and makes the reproduction of results in different laboratories difficult. Studies often target the same scenario but the results are not comparable because they are performed using different methods of analysis, which result in diverse biological data and lead to varying conclusions (Jackson et al. 2001).

There are three basic statistical requirements which should be attained in order to establish a standard operating procedure (SOP): ruggedness, repeatability and reproducibility. Ruggedness is the resistance to change in the results produced by an analytical method when minor deviations are made from the experimental conditions described in the procedure. Repeatability accounts for random errors in the measurements and includes the contributions from any part of the procedure that varies within a run, including gravimetric and volumetric errors, heterogeneity of the materials being tested, and variation in the chemical treatment stages. Reproducibility relates to the ability of the procedure to be reproduced by others, namely in different laboratories. Between-laboratory variation arises from factors such as variations in calibration standards, differences between local interpretations of the protocol, changes in equipment or reagent source, and environmental factors, such as differences in the average climatic conditions (Thompson et al. 2002).

Currently, no SOP is available for AMP studies, but there are some biofilm and antibiotic SOPs. The SOPs proposed for biofilm studies are specific for: the growth of reproducible mixed biofilms of *P. aeruginosa* (ATCC#700829), *Pseudomonas fluorescens* (ATCC# 700830) and *Klebsiella pneumoniae* (ATCC#700831), analyzed in terms of structure and viable cell counts using a flat-plate, open channel reactor (Jackson et al. 2001); the growth of *P. aeruginosa* biofilm using the CDC biofilm reactor (CBR) system (Goeres et al. 2005); the assessment of resistance of *Staphylococcus aureus* biofilm cells to disinfectants (Luppens et al. 2002); and the optimized quantification of enterococci biofilms using microtitre-plates (Extremina et al. 2011). Also, as a first step towards the establishment of a SOP for methods of analysis, several authors have studied and compared methods in use on biofilm related research for evaluating biofilm disinfectant efficacy with different fluid dynamics (CDC, drip flow and static biofilm reactor systems) (Buckingham-Meyer et al. 2007), comparing methods for quantification of microbial biofilms grown in microtiter plates (crystal violet (CV), Syto9, fluorescein diacetate (FDA), resazurin, XTT and dimethyl methylene blue (DMMB) assays) (Peeters et al. 2008), and checking the validity of the harvesting and disaggregating steps (Hamilton et al. 2009).

The SOPs proposed for antibiotic studies address, among other things: the assessment of antibiotic resistance using a disc susceptibility test for various genera of microorganisms (Andrews 2001; Kronvall et al. 2003; Wikler et al. 2009a) and specifically, for aquaculture-organisms at international level (Huys et al. 2005) and infrequently isolated or fastidious bacteria (Jorgensen et al. 2005); broth dilution and disk diffusion antifungal susceptibility testing of yeasts (Rex et al. 2008, 2009); and dilution antimicrobial susceptibility tests for aerobic bacteria (Wikler et al. 2009b).

Data standardization and interchange

Understanding AMP related phenomena requires the integration of heterogeneous data sources. This implies the development and use of annotation standards such as controlled vocabularies and ontologies, ie a formal description of the concepts and relationships involved in AMP studies that facilitate both communication between researchers and the computational use of domain knowledge for multiple purposes (Consortium 2004).

These integration efforts have already succeeded in some research areas and various ontologies are being developed and available for use. One major example is the Gene Ontology (GO) project, which provides consistent descriptors for gene products, in different databases, and standardizes classifications for sequences and sequence features (Consortium 2004). Other examples follow, such as the Chemical Entities of Biological Interest (ChEBI), which is a freely available dictionary of molecular entities focused on 'small' chemical compounds (Degtyarenko et al. 2008), or the Plant Ontology Database, which aims to create, maintain and facilitate the use of a controlled vocabulary for plants, providing a semantic framework to make meaningful cross-species and database comparisons (Avraham et al. 2008). Some work has also been focused on antibiotics, namely the DebugIT Core Ontology, which focuses on patients, diseases,

Table 4. Synergism studies involving AMPs.

Tested compounds				
Compound 1 (AMPs)	Compound 2 (AMP or other)	Microorganisms	Culture state	Reference
6752	Lysostaphin	*Staphylococcus aureus*	Planktonic	Desbois and Coote (2011)
Bactenecin	Protegrin 1	*Escherichia coli*	Planktonic	Yan and Hancock (2001)
	Indolicidin			
	Protegrin 1	*Pseudomonas aeruginosa*	Planktonic	Yan and Hancock (2001)
Bacteriocin PsVP-10	Chlorhexidine	*Streptococcus mutans*	Planktonic	Lobos et al. (2009)
		Streptococcus sobrinus		
BMAP-28	Quinupristin/dalfopristin (Q/D)	*Staphylococcus aureus*	Both	Cirioni et al. (2006)
	Linezolid			
	Vancomycin			
Bovine lactoferrin	Lysostaphin	*Staphylococcus aureus*	Planktonic	Desbois and Coote (2011)
Cecropin	Rifampicin	*Pseudomonas aeruginosa*	Planktonic	Cirioni et al. (2008b)
Colistin	Lysostaphin	*Staphylococcus aureus*	Planktonic	Desbois and Coote (2011)
	Tobramycin	*Pseudomonas aeruginosa*	Biofilm/*in vivo*	Herrmann et al. (2010)
	Rifampicin	*Pseudomonas aeruginosa*	Planktonic/mouse pneumonia model	Aoki et al. (2009)
	Imipenem	*Pseudomonas aeruginosa*	Planktonic	Cirioni et al. (2007)
D-9-mer peptides	Tetracycline	*Pseudomonas aeruginosa*	Planktonic	Iwasaki et al. (2007)
	Piperacillin	*Staphylococcus aureus*		
	Chloramphenicol			
	Cefotaxime			
	Rifampicin			
Daptomycin	Lysostaphin	*Staphylococcus aureus*	Planktonic	Desbois and Coote (2011)
Dermaseptin S3(1-16)	Lysostaphin	*Staphylococcus aureus*	Planktonic	Desbois and Coote (2011)
G10KHc	Tobramycin	*Pseudomonas aeruginosa*	Planktonic	Eckert et al. (2006b)
Gaegurin 6 (GGN6)	Xylitol	*Streptococcus gordonii*	Planktonic	Kim et al. (2003)
GK14K4	Lysostaphin	*Staphylococcus aureus*	Planktonic	Desbois and Coote (2011)
Gomesin	Lysostaphin	*Staphylococcus aureus*	Planktonic	Desbois and Coote (2011)
hLF (1-11)	Lysostaphin	*Staphylococcus aureus*	Planktonic	Desbois and Coote (2011)
Indolicidin	Protegrin 1	*Pseudomonas aeruginosa*	Planktonic	Yan and Hancock (2001)
	Protegrin 1	*Escherichia coli*	Planktonic	Yan and Hancock (2001)
KR-20	Lysostaphin	*Staphylococcus aureus*	Planktonic	Desbois and Coote (2011)
Lactoferrin	Ciprofloxacin	*Porphyromonas gingivalis*	Biofilm	Wakabayashi et al. (2009)
	Clarithromycin			
	Minocycline			
LL-37	Protegrin 1	*Pseudomonas aeruginosa*	Planktonic	Yan and Hancock (2001)
	Protegrin 1	*Escherichia coli*	Planktonic	Yan and Hancock (2001)
	Protegrin 1	*Enterococcus faecalis*	Planktonic	Yan and Hancock (2001)
	Bactenecin			
Magainin 2	Rifampicin	*Pseudomonas aeruginosa*	Planktonic	Cirioni et al. (2008b)
	Lysostaphin	*Staphylococcus aureus*	Planktonic	Desbois and Coote (2011)
	PGLa	*Escherichia coli*	Planktonic	Westerhoff et al. (1995); Tremouilhac et al.(2006); Strandberg et al. (2009)

(*continued*)

Table 4. (*Continued*).

Tested compounds				
Compound 1 (AMPs)	Compound 2 (AMP or other)	Microorganisms	Culture state	Reference
Melanotropin-α	Lysostaphin	*Staphylococcus aureus*	Planktonic	Desbois and Coote (2011)
meta-phenylene ethynylene (mPE)	Chlorhexidine	*Streptococcus mutans*	Planktonic	Beckloff et al. (2007)
MUC7-12mer	Lysostaphin	*Staphylococcus aureus*	Planktonic	Desbois and Coote (2011)
Nigrocin-OG13	Lysostaphin	*Staphylococcus aureus*	Planktonic	Desbois and Coote (2011)
Nisin	Lysostaphin	*Staphylococcus aureus*	Planktonic	Desbois and Coote (2011)
	Sodium fluoride	*Staphylococcus aureus*	Both	Tong et al. (2011)
Odorranain-G1	Lysostaphin	*Staphylococcus aureus*	Planktonic	Desbois and Coote (2011)
Parasin-I	Lysostaphin	*Staphylococcus aureus*	Planktonic	Desbois and Coote (2011)
Pexiganan	Lysostaphin	*Staphylococcus aureus*	Planktonic	Desbois and Coote (2011)
Polymyxin B	Lysostaphin	*Staphylococcus aureus*	Planktonic	Desbois and Coote (2011)
PTP6	Chlorhexidine	*Streptococcus sobrinus*	Planktonic	Kim et al. (2003)
Ranalexin	Lysostaphin	*Staphylococcus aureus*	Planktonic/*in vivo*	Graham and Coote (2007); Desbois et al. (2010); Desbois and Coote (2011)
Tachyplesin III	Imipenem	*Pseudomonas aeruginosa*	Planktonic	Cirioni et al. (2007)
	G-CSF TZP	Mixed (primarily *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., Gram-positive enterococci and staphylococci, and anaerobic *Bacteroides* spp.)	*In vivo* (murine neutropenic intraabdominal infection)	Cirioni et al. (2008a)
	TZP	*Pseudomonas aeruginosa*	Biofilm/*in vivo* (rat model ureteral stent infection)	Minardi et al. (2007)

Note: Both = biofilm and planktonic.

pathogens, their analyses and medications, allowing a semantic integration of antibiotics resistance patterns (Schober et al. 2010). As for AMPs, no nomenclature is yet available, apart from generic protein ontology, such as the Protein Ontology (PRO) that provides a formal, logically-based classification of specific protein classes (Natale et al. 2011).

On a need basis, the curation teams of AMP databases are taking the lead in this subject: PenBase suggests a nomenclature for penaeidins, based on the genus and species of the source, its subgroup and an identification number (http://www.penbase.immunaqua.com/); APD2 proposes a nomenclature for AMPs based on properties, source and a combination of property and source data, and provides a glossary for terms and abbreviations for the entire database (http://aps.unmc.edu/AP/main.php).

Data screening and processing

As the volume of related literature increases, it becomes urgent to help researchers and database curators to keep up with new literature, especially to provide the means for the systematic screening and prioritization of literature of interest. Bioinformaticians/chemoinformaticians can benefit from this support as well, in particular to delineate new hypotheses to be modeled and mined.

Given the success reported in other biomedical domains, the integration of semi-automated mechanisms into AMP curation workflows would seem to be an ideal application for text mining. State-of-the-art recognizers can be used to tag organisms (Gerner et al. 2010), chemical entities (Jessop et al. 2011) and genes, proteins and other biological entities (Settles 2005). The vocabulary on AMPs, eg derived from the UniProt knowledge base (Magrane and Consortium 2011) and antibiotics lexicon derived from the ARDB database (Liu and Pop 2009) and the antibiotics list in Wikipedia (Wikipedia 2012), can support the pattern matching of AMP mentions and further development of suitable recognizers. Also, to start with, article screening and ranking could be based on statistical considerations, such as the number and diversity of unique concepts detected in the texts, weighted by the relevance associated with the different biological categories, and the degree of certainty associated with detection.

To date, the curation of AMP databases is exclusively manual and little has been reported on text mining developments (Jorge et al. 2012).

Conclusions

In the nosocomial scenario, infections are often caused by or associated with microbial adhesion and biofilm development. Compared to planktonic (non-attached) cells, bacteria growing in biofilms have a specific phenotype and demonstrate higher resistance to antimicrobial agents. Therefore, biofilms are difficult to kill and eradicate, contributing to surface persistent contamination and the prevelance of nosocomial pathogens.

The challenges posed to conventional strategies have led to the investigation of alternative prophylaxis and therapeutics, namely AMP-based strategies. These strategies take advantage of the non-specific mechanisms of action of AMPs and the wide availability of AMP-deriving sources. AMPs act mainly by destabilizing cellular membranes, which is a mechanism low in specificity and therefore impairs the development of resistance. Also, AMPs are spread throughout Nature, and can be synthesized or modified to improve their action and reduce production costs.

To date, most AMP studies have focused on planktonic cells. Nevertheless, since biofilms are involved in 80% of human bacterial infections (Harro et al. 2010), it is crucial to extend the studies on biofilm inhibition and AMP dispersion effects to already preformed biofilms and to developing biofilms. Also, synergism studies involving AMPs are needed to discover successful AMP-AMP or AMP-drug combinations for lowering the dosage and minimizing the side effects of antimicrobials.

Finally, it is noteworthy that the discovery and design of AMPs is being revolutionized by the emergence of novel bioinformatic tools and resources, mainly 'omics' experiments and predictive applications.

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