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REVIEW ARTICLE

Novel alternatives to antibiotics: bacteriophages, bacterial cell wall hydrolases, and antimicrobial peptides

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Summary

Extensive research has been conducted on the development of three groups of naturally occurring antimicrobials as novel alternatives to antibiotics: bacteriophages (phages), bacterial cell wall hydrolases (BCWH), and antimicrobial peptides (AMP). Phage therapies are highly efficient, highly specific, and relatively cost-effective. However, precautions have to be taken in the selection of phage candidates for therapeutic applications as some phages may encode toxins and others may, when integrated into host bacterial genome and converted to prophages in a lysogenic cycle, lead to bacterial immunity and altered virulence. BCWH are divided into three groups: lysozymes, autolysins, and virolysins. Among them, virolysins are the most promising candidates as they are highly specific and have the capability to rapidly lyse antibiotic-resistant bacteria on a generally species-specific basis. Finally, AMP are a family of natural proteins produced by eukaryotic and prokaryotic organisms or encoded by phages. AMP are of vast diversity in term of size, structure, mode of action, and specificity and have a high potential for clinical therapeutic applications.

Introduction

For more than half a century, the human society has been relying primarily on antibiotics to treat infectious diseases caused by pathogenic bacteria. However, the emergence of bacterial resistance to antibiotics following widespread clinical, veterinary, and animal agricultural usage has made antibiotics less and less effective (Teuber 2001; Heuer *et al.* 2006). We are now facing the threat of superbugs, i.e. pathogenic bacteria resistant to most or all available antibiotics. It was warned by the World Health Organization that those multiple antibiotic-resistant pathogens would very likely bring the world back to the pre-antibiotic era.

Till now, the pharmaceutical industry has been coping with this problem by modifying existing antibiotics and developing new ones. However, only three new classes of antibiotics (i.e. lipopeptides, oxazolidinones, and streptogramins) have entered the medicine market in the last four decades and all of them are for the treatment of gram-positive (G^+) bacterial infections. Besides, bacteria have proven to have the capability of developing and spreading antibiotic resistance promptly, making this strategy increasingly less effective. This clearly highlights the need for new antibacterial agents with fundamentally different modes of action than that of traditional antibiotics.

The enormous demand has triggered worldwide efforts in developing novel antibacterial alternatives. Bacteriophages (phages), bacterial cell wall hydrolases (BCWH), and antimicrobial peptides (AMP) are among the most promising candidates. In this review, we strive on providing a comprehensive picture outlining current knowledge regarding these antimicrobial alternatives, including their mode of action, specificity and safety, antimicrobial efficiency, and their advantages and disadvantages relevant to potential clinical applications.

Bacteriophages

General description of bacteriophage

Discovered independently by Frederick Twort and Félix d'Hérelle respectively in 1915 and 1917, phages are 'bacterium eaters' that kill bacterium by causing its lysis

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(bacteriolysis). Antibacterial properties of phages were assessed earlier on, but unfortunately the mechanism was not well defined, leading to trial failures. Moreover, the exquisite specificity of the phage treatment limited their use in the early days. Developed in the same period, antibiotics with a large spectrum of action and well-understood mechanisms became popular in western countries. Phage therapy was however extensively used in eastern European countries mainly in the former Soviet Union and Georgia. Because of the actual rise of antibiotic-resistant bacteria, a revitalization of interests on phage therapy has been observed in the last two decades in western countries (Brussow 2005; Skurnik and Strauch 2006).

It is estimated that there are about 10^{31} phages on earth and approximately 5100 have been identified and reported towards the end of last century (Ackermann 2001). These phages are classified into 13 families according to their morphological characteristics, type of nucleic acid, and presence or absence of envelope or lipid. It was noticed that 4950 out of the 5100 reported phages are 'tailed phages', which are composed of an icosahedral head and a tail. All those tailed phages have double-stranded DNA (dsDNA) as genome and are lytic phages that encode endolysins, also named virolysins (Young *et al.* 2000, Bernhardt *et al.* 2002). These phages are classified into three families according to the morphological features of the tail: *Myoviridae* (contractile tail), *Siphoviridae* (long noncontractile tail), and *Podoviridae* (extremely short tail). The rest of the phages, constituting only 4% of the total, are classified into 10 families. They are cubic, filamentous, or pleomorphic phages with dsDNA, single-stranded DNA (ssDNA), double-stranded RNA (dsRNA), or single-stranded RNA (ssRNA) genome (Ackermann 2001).

Phages can also be conventionally classified into two categories according to the strategies they use to escape their hosts: filamentous phages and lytic phages (Young *et al.* 2000; Bernhardt *et al.* 2002). Filamentous phages continuously extrude from their hosts without causing host lysis, whereas all other phages are lytic phages that encode gene products to compromise or destroy the bacterial cell wall, leading to bacteriolysis. Lytic phages can be further divided into two classes according to their bacteriolytic mechanisms: (i) the virolysin-holin system to hydrolyse bacterial cell wall and (ii) the single lytic factor to compromise the strength of the cell wall. Most known phages, including all tailed phages, are large lytic phages with dsDNA genome encoding virolysin-holin system. Virolysin is a muralytic enzyme that hydrolyses peptidoglycan in bacterial cell wall and holin is a small peptide that oligomerizes in the membrane to form disruptive membrane lesions, allowing virolysins to access the cell wall at a programmed time of the phage's life cycle (Ugorcakova and Bukovska 2003). The other lytic phages are small phages with ssDNA or ssRNA genome and encode a single lytic factor inducing bacteriolysis by inhibition of the cell wall biosynthesis or other mechanisms.

Mode of action and specificity of bacteriophages

The most common mode of action of phages is the bacteriolysis, which occurs naturally at the end of the phage lytic cycle, by disruption of the cell wall caused by the virolysin-holin system or the single lytic factor. It could also happen in the adsorption stage if a high multiplication of infection (MOI) is used, in which a substantially large number of phage particles attach to the same bacterial cell (Tarahovsky *et al.* 1994).

Another mode of action involves genetically modified phages, especially filamentous ones, which do not cause cell lysis and cannot be used directly for phage therapy. Hagens and Blasi (2003) and Hagens *et al.* (2004) have shown that *Pseudomonas aeruginosa* filamentous phage can be genetically modified by replacing the transportation gene (i.e. the gene involved in the extrusion of phage particles from the host bacterium) with a restriction enzyme gene so that the phages loses the ability to extrude from bacterial cells for its multiplication, but acquires the ability to digest the bacterial nucleic acid. They have demonstrated that this type of genetically modified filamentous phages could be used as effective anti-infection agents and have the benefit to reduce the release of membrane-associated endotoxins (lipopolysaccharide), leading to significantly higher rates of mice survival in comparison with therapies using lytic phages.

The specificity of phages comes from the first essential step of phage infection cycle: the attachment of phage tail to a specific receptor on the surface of the bacterial cell (adsorption). There seem to have been some controversies with regard to how specific a phage could really be. While some authors tend to think that phages are species-specific (Merril *et al.* 2003), it seems to be more accurate to say that phages typically attack bacteria on a strain-specific basis (Bradbury 2004). Nevertheless, there is no doubt that phages are much more specific than antibiotics. While this high specificity of phages made them less appealing in comparison with broad-spectrum antibiotics in the early days, it is now highly appreciated as a major merit because phage therapy would not affect the microbial flora of the hosts like more broad-spectrum antibiotics would normally do.

Antimicrobial efficacy of phage therapy

There is an accumulation of evidences that phages can be employed for clinical treatment or prevention of

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infectious diseases caused by both G^+ and gram-negative (G^-) bacteria (Bull *et al.* 2002; Stone 2002). After the works of Smith *et al.* (1987) on the treatment of *Escherichia coli* infections using experimental mice and calves, there have been many published reports on the successful efficacy of phages against experimental infections by G^- and G^+ bacteria, mostly in animal models. Moreover, phages were also shown to be effective for the elimination of food poisoning pathogens such as *Listeria monocytogenes*, *Campylobacter jejuni*, and *Salmonella* spp. (Greer 2005). On 18 August 2006, Food and Drug Administration (FDA) approved the use of phages for the treatment of ready-to-eat meat: a combination of six viruses was designed to be sprayed on ready-to-eat meat to eradicate strains of *L. monocytogenes* (Petty *et al.* 2007). There is no doubt that phages, after extensive studies and careful selection of phage candidates, will eventually become one of the most effective antibacterial alternatives.

Advantages and disadvantages of phage therapy

Matsuzaki *et al.* (2005) summarized the advantages of phage therapy over antibiotic therapy as follows: (i) it is effective against multidrug-resistant pathogenic bacteria; (ii) substitution of the normal microbial flora does not occur because the phages kills only the targeted pathogenic bacteria; (iii) it can respond quickly to the appearance of phage-resistant bacterial mutants because the frequency of phage mutation is significantly higher than that of bacteria; (iv) developing costs for a phage treatment is cheaper than that of new antibiotics; and (v) side-effects are very rare.

However, there are still some concerns such as: (i) rapid cell lysis of bacteria may result in the release of large amount of bacterial membrane-bound endotoxins; (ii) some phages may encode toxins; (iii) lack of pharmacokinetic data; (iv) neutralization of phages by the host immune system may lead to failure of phage therapy; (v) conversion of lytic phages to lysogenic phages (prophages) leads to bacterial immunity to attacks by the corresponding lytic phages and may also change the virulence of the bacteria.

Some of the aforementioned concerns have been successfully addressed by different approaches. To address the endotoxins release issue, Hagens *et al.* (2004) demonstrated, with the construction of a recombinant phage derived from *P. aeruginosa* filamentous phage (described before), that the rapid release of membrane-bound endotoxins was significantly reduced as well as the animal mortality rate.

Administration of phages may also induce immunological response in hosts. However, neutralization of phages by the host immune system may not be a significant obstacle towards clinical application of phage therapies *per se*. In fact, it was demonstrated that immunological response to phages takes seven administrations before to be significant (Merril *et al.* 1996), whereas there are evidences that single administration of phages are usually enough to eliminate sensitive pathogenic bacteria for effective infection treatment (Smith *et al.* 1987; Wang *et al.* 2006). However, there are also experimental data showing that, in some cases, single administration of single or cocktail phages may not be sufficient (Tanji *et al.* 2005). In these cases, developing long circulating phages that have adopted the capability to avoid the host immune system might be an effective countermeasure (Merril *et al.* 1996).

Conversion of lytic phages to lysogenic prophages occurs when phages enter the lysogenic cycle and insert their genome into the bacterial genome. Bacteria containing prophage are immunized against the attack by the corresponding lytic phages (Cheng *et al.* 1999). This is called the lysogenic immunity and leads to acquired resistance and this may also alter the virulence of the bacteria.

Evidences have shown that phages may carry harmful genes (Skurnik and Strauch 2006). When a prophage excises its genome from the bacterial genome to enter the replicative lytic cycle, some part of the bacterial genome can be up-taken and become part of the phage genome. Therefore, phages may carry toxins and virulence factors from one bacteria to another (Brussow *et al.* 2004). Phage-associated conversion of Tox^- *Streptococcus pyogenes* into Tox^+ bacteria was also confirmed *in vitro* and *in vivo* (Broudy and Fischetti 2003). Thorough studies should therefore be carried out before selecting a phage candidate for therapeutic applications to avoid carrying or transferring toxic genes.

Another possible obstacle to phage therapy is that bacteria may acquire resistance to phages via different mechanisms such as: (i) integrating a phage into its genome by entering the lysogenic cycle, which renders the bacterium lysogenic immunity (see earlier discussions); (ii) losing phage receptor from the bacterial surface; (iii) acquiring horizontally a restriction modification system that degrades the injected phage nucleic acid; and (iv) loss of a gene essential for phage replication or assembly (Skurnik and Strauch 2006). Nevertheless, induced resistance of bacteria to phages is so far not considered a significant concern because phages mutate at frequencies significantly higher than that of bacteria and there are abundant experimental evidences showing that the mutation of phages would be enough to maintain the efficacy of phage therapies. This implies continuous phage isolation and selection for efficient phage therapy, which is, however, largely easier, shorter, and cheaper than discovering new antibiotics.

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Despite the aforementioned concerns, which could be easily dealt with by properly selecting and characterizing phage candidates, phage therapy is highly efficient, highly specific, and relatively cost-effective. This therapy is effective on all bacteria and will be, without any doubt, one of the most promising antimicrobial alternatives to fight antibiotic-resistant bacterial pathogens.

Bacterial cell wall hydrolases

General description of BCWH

BCWH are enzymes that degrade peptidoglycan, the major component of the bacterial cell wall, and cause bacteriolysis. Different forms of BCWH can be used to treat infectious diseases, including purified native enzymes, denatured enzymes, partial digests (Masschalck and Michiels 2003), and recombinant proteins endogenously over-expressed in transgenic animals or plants for enhancement of host defense (Yazawa *et al.* 2006). Lysozymes denatured by heating or dithiothreitol reduction have been shown to have nonenzymatic bactericidal activities by causing membrane perturbation or activating the autolytic system of the bacteria (Masschalck and Michiels 2003). There are many sources of BCWH including animals, insects, plants, bacteria, and phages. Although enzymes from different sources may have similar structures and functionalities, they have important differences with significant impacts on their clinical applications. In this complex scenario, we propose to classify BCWH according to their sources into the following categories: (i) lysozymes, BCWH of eukaryotic origin and components of the innate defence system of their producers; (ii) autolysins, bacteria encoded by BCWH that have a variety of physiological functions in the bacterial life cycle; and (iii) virolysins, phage-encoded BCWH responsible for the lysis of bacterial cells and the release of phage particles at the end of a phage life cycle.

Lysozymes

Based on our classification, lysozymes are BCWH of eukaryotic origins, e.g. those produced by animals and plants. Lysozymes are important components of the innate defence system of their hosts and are known to exert antimicrobial activities against a broad spectrum of bacterial, fungal, and viral pathogens (Abdou *et al.* 2007). These enzymes, alone or in combination with other antimicrobials, have found wide applications as food preservatives and pharmaceuticals (Masschalck and Michiels 2003; Niyonsaba and Ogawa 2005). The distribution, activity and particularly the number of genes that encode lysozymes vary considerably among species. For instance, it has been reported that among mammals, humans and pigs contain a single lysozyme gene, whereas camels and mice contain two, and cows, sheep, and deer contain ten. In humans, lysozymes are present in various tissues including skin and secretions such as saliva, tears, urine, milk, respiratory, and cervical secretions (Masschalck and Michiels 2003).

Autolysins

Bacterium-encoded BCWH, autolysins are usually membrane-bound proteins that have BCWH activities, and each bacterial species contains one or more autolysins (Garcia and Dillard 2006). Numerous autolysins have been identified and have been shown to be involved in a variety of physiological cell functions such as cell wall biosynthesis, cell separation, cell adhesion, and virulence (Heilmann *et al.* 2005). They may also, as the name suggests, cause bacteriolysis and lead to the death of the host bacterium by various mechanisms (Garcia and Dillard 2006).

Virolysins

Virolysins are BCWH encoded by lytic dsDNA phages and produced in phage-infected bacterial cells towards the end of the phage lytic cycle. The name 'virolysin' was first adopted by Ralston in the 1950s (Ralston *et al.* 1955). However, virolysins are now most commonly referred as endolysins, and were sometimes called lysozymes, lysins, and lytic enzymes, which are too generic and confusing. To avoid this confusion, it is necessary to assign a unique name to each group of lytic enzymes and 'virolysin' is a good choice for the phage-encoded ones, reflecting their viral (phage) origin.

Virolysins are capable of degrading peptidoglycan when applied (as purified proteins) on bacterial cells, resulting in rapid lysis of the bacteria. They possess several important features including a narrow antibacterial spectrum and activity against bacteria regardless of their antibiotic sensitivity. It is also important to note that there are evidences that development of resistance by bacteria sensitive to a virolysin is unlikely. Furthermore, the results of preclinical studies indicate that, unlike phage therapy, therapeutic use of virolysins should not be prevented by immunogenicity, toxicity, or resistance issues (Borysowski *et al.* 2006).

Mode of action and specificity of BCWH

The primary bactericidal mechanism of BCWH is the lytic enzymatic activities of these enzymes, i.e. attacking specific sites in the peptidoglycan network, leading to peptidoglycan hydrolysis and consequently bacteriolysis (Masschalck and Michiels 2003). Bacterial cell wall is a unique structure comprised primarily of a peptidoglycan

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network (also called murein) embedded with other compounds and, in the case of G^- bacteria, surrounded by a lipid-rich outer membrane (Dmitriev *et al.* 2005; Bower and Rosenthal 2006). The hydrolysis of bacterial cell wall requires two distinct functions of BCWH: (i) binding to specific sites on the bacterial cell wall leading to the correct positioning of BCWH as the essential first step; and then (ii) cleaving specific peptidoglycan bonds. It is now clear that most BCWH achieve these two functions by two separate domains. For instance, LytA amidase, the major autolysin of *Streptococcus pneumonia*, has a molecular mass of *c.* 36 kDa and a two-domain modular structure (Garcia *et al.* 1986). The *C*-terminal domain is responsible for the attachment to teichoic or lipoteichoic acid residues on the surface of pneumococcal bacteria (Usobiaga *et al.* 1996). The *N*-domain, on the other hand, is responsible for the lytic activity against pneumococcal cell wall. This modular structure is shared by almost all known BCWH including lysozymes (Lopez and Garcia 2004; Niyonsaba and Ogawa 2005), autolysins (Mesnage and Fouet 2002), and virolysins (Low *et al.* 2005).

A secondary bactericidal mechanism of BCWH, the nonlytic mechanism, has also attracted the attention of the research community in the recent years (Ibrahim *et al.* 2001; Masschalck *et al.* 2002). The nonlytic mechanism is based on the cationic and amphiphilic properties of BCWH or its derived peptide to provoke membrane perturbation or to activate the autolytic system of bacteria. As will be discussed later, this nonlytic bactericidal mechanism is shared by other antimicrobial peptides.

The specificity of BCWH varies with their sources. While the specificity of autolysins as exogenous antimicrobial agents is not well documented, it has been well established that lysozymes are broad-spectrum lytic enzymes that have antibacterial activities against most G^+ bacteria and virolysins are narrow-spectrum enzymes that are functional only to specific bacterial species or sub-species.

Lysozymes are a group of broad-spectrum lytic enzymes effective against most G^+ bacteria, but nonfunctional for most G^- bacteria owing to the protection offered by the outer membrane of G^- bacteria. It is worth to emphasize that some important G^+ pathogenic bacteria are also resistant to lysozymes. Several resistance mechanisms that have been characterized include: (i) *O*-acetylation of the *N*-acetylmuramic acid (NAM) of peptidoglycan as reported for *Staphylococcus* (Bera *et al.* 2005, 2006); (ii) attachment of other polymers (e.g. polysaccharides) to the cell wall, as described also for *Staphylococcus* (Fedtke *et al.* 2004); (iii) D-aspartic acid incorporation as discovered in *Lactococcus lactis* (Veiga *et al.* 2006); and (iv) synthesis of lysozyme inhibitors to neutralize the enzymatic activity (Bukharin and Valyshev 2006).

On the other hand, virolysins are narrow-spectrum lytic enzymes that are functional to a specific group of G^+ bacteria and there is a huge variety of specificity within the various virolysins (Fischetti 2005). Interestingly, studies with different pathogenic bacteria have shown that repeated exposure of bacteria to sub-lethal doses of corresponding virolysins does not lead to the development of enzyme-resistant strains (Low *et al.* 2005). This observation was tentatively explained by the fact that choline, an essential structural component of G^+ cell wall (Fischer 2000), served as receptor for binding of virolysins to the bacterial cell wall (Lopez and Garcia 2004).

Although lysozymes and virolysins have very similar structures (the two-domain modular structure) and functionalities (hydrolysing peptidoglycan by attacking specific bonds), they are totally different in terms of specificity. This indicates that virolysins must have acquired some unique recognizing mechanisms that allow them to attack specific G^+ bacteria, including those resistant to lysozymes and other virolysins, while being inefficient on the rest of bacteria.

Antimicrobial efficacy of BCWH

Extensive studies on the antimicrobial efficacy of different groups of BCWH, mostly lysozymes (Niyonsaba and Ogawa 2005) and virolysins (Fischetti 2005), have been carried out in the last two decades.

Lysozymes were shown to have antimicrobial activities towards bacteria, fungi, and viruses (Reddy *et al.* 2004; Wang *et al.* 2005; Lee-Huang *et al.* 2005). They are mainly used in food preservation and processing, but also have applications in veterinary and human medicine. Nevertheless, as previously mentioned, a few virulent G^+ bacterial pathogens have acquired resistance to lysozymes (Bera *et al.* 2005; Abdou *et al.* 2007), making them less attractive as an alternative to antibiotics.

On the other hand, virolysins have the capacity of rapidly killing pathogenic G^+ bacteria on a generally species-specific basis, *in vitro* or *in vivo*. Since 2000, some virolysins have been demonstrated to be efficient and safe antimicrobials, and could potentially be used for the control of pathogens on mucous membranes or as biowarfare countermeasures for *Bacillus anthracis* (Schuch *et al.* 2002; Fischetti *et al.* 2006). Interestingly, virolysins are rapidly effective at low dosages in the order of milligrams or even micrograms per litre (Fischetti 2005; Fischetti *et al.* 2006). This rapid killing of sensitive bacteria by virolysins at relatively low dosage is not only important in the sense of therapy costs, but could be one way whereby the enzymes would avoid being neutralized by the immune response or causing severe allergic responses in hosts (Fischetti 2005).

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Although autolysins are in general less studied as alternative antimicrobials, the concept of activating the autolytic system of pathogenic bacteria using cationic peptides and other compounds has been investigated with encouraging results (Ginsburg 2001). However, no reports have been found regarding the clinical use of exogenous autolysins for the treatment of infectious diseases, except the suggestion that they might be used as vaccines because of their ability to provoke immunological responses against their corresponding bacteria (Lock *et al.* 1992).

Advantages and disadvantages of BCWH therapy

The most significant advantages of BCWH as potential antibacterial alternatives include: (i) they are efficient against antibiotic-resistant bacteria by using killing mechanisms totally different from that of the antibiotics; (ii) they are well understood and safe; and (iii) immunogenicity is not a concern to their effectiveness. The major disadvantage of BCWH as antibacterials is the absence of effect on most G^- bacteria as a result of the presence of the outer membrane. Moreover, some important G^+ pathogens are already resistant to lysozymes and data on the efficacy of autolysins are limited. It is encouraging however, that virolysins were shown to be an efficient antibacterial agent and repeated exposure to sub-lethal doses did not lead to the development of bacterial resistance. Consequently, virolysins appear to be the most promising antibacterial alternative among the three different types of BCWH. The only obstacle to their clinical application is probably the high production costs in comparison with that of phages and antibiotics, but future works should solve this issue.

Antimicrobial peptides

General description of AMP

AMP are another major group of promising novel alternatives to antibiotics based on their effectiveness, safety, and enormous diversity. This is a large family of naturally occurring peptides from diverse sources, having diverse structures and functionalities. We will present AMP according to their origins: eukaryotic AMP, bacteriocins, and phage-encoded AMP.

Eukaryotic AMP

Eukaryotic AMP are naturally occurring cationic peptides of molecular weight in the range of 1–5 kDa. They usually possess a net positive charge at physiological pH, allowing them to interact with anionic cytoplasmic membrane. They are highly amphipathic molecules with hydrophobic and hydrophilic moieties segregating into distinct patches on the molecular surface, which allow them to form pores in the cytoplasmic membrane, leading to enhanced permeability of the membrane and loss of cell content. More than 700 AMP produced by animals and plants have been identified to date (Straus and Hancock 2006). In humans, three distinct groups were characterized: defensins, cathelicidins, and histatins.

Defensins are a large family of eukaryotic AMP typically containing six cysteine residues that form three intramolecular disulfide bridges, resulting in a triple-stranded β-sheet structure. They have been isolated from organisms ranging from plants to invertebrates and mammals including humans. Defensins are believed to play an important role in the innate immune defense of organisms, protecting against invading pathogens by killing bacteria and other microbes (Bowdish *et al.* 2006). During the last two decades, a series of endogenous α- and β-human defensins were discovered including six α-defensins and four β-defensins (HBD) (Niyonsaba and Ogawa 2005; Pazgier *et al.* 2006). Among them, HBD-3 is particularly attractive for its strong antibacterial activity, relative salt-insensitiveness and low toxicity for the host (Batoni *et al.* 2006; Dhople *et al.* 2006). HBD-3 was found to exhibit a broad-spectrum antimicrobial activity against various G^- and G^+ bacteria as well as fungi and viruses at low micromolar concentrations (Weinberg *et al.* 2006).

Cathelicidins are a group of various mammalian cationic AMP with a common evolutionary origin of their genes but displaying a remarkable variety of sizes, sequences, and structures (Zanetti *et al.* 2002). All cathelicidins contain an *N*-terminal cathelin domain and a *C*-terminal cationic antimicrobial domain that becomes active after cleavage. Just as lysozymes and defensins, cathelicidins are important components of the innate defence system of their hosts. In addition to their diverse antimicrobial activities, cathelicidin can bind to and neutralize the effects of endotoxin, making them good candidates for therapeutic uses. So far, about 30 cathelicidins have been found in mammals. However, only one human cathelicidin, LL-37, has been identified (Durr *et al.* 2006). LL-37 is found throughout the body and has been shown to exhibit a broad spectrum of antimicrobial activity against a variety of bacterial, fungal, and viral pathogens (Nilsson *et al.* 1999). This unique human cathelicidin is generally known to have salt-insensitive microbiocidal activity (Turner *et al.* 1998). It appears to play a key role in repair of damaged tissue and wound closure, by promoting wound neovascularization and re-epithelialization of healing skin (Heilborn *et al.* 2003).

Histatins constitute a group of small histidine-rich, cationic multifunctional proteins present in the saliva of human and some nonhuman primates, with a molecular

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weight typically less than 5 kDa. Histatins are characterized by a high content of histidine, which accounts for about 25% of all amino acids (Shimada 2006). The most significant function of histatins may be their antifungal activity against *Candida albicans* and *Cryptococcus neoformans* (Tsai and Bobek 1998). The mechanism of histatins' action on *C. albicans* is not clear, but appears to be different from that of the azole-based antifungal drugs, which interrupt ergosterol synthesis. Histatins are promising antifungal therapeutic agents for the following reasons: (i) they have little or no toxicity; (ii) they possess high biocidal activities against azole-resistant fungal species and most of the fungal species tested; and (iii) their efficiency against *Candida* is similar to the azole-based antifungals (Tsai and Bobek 1998).

Bacteriocins

Bacteriocins are AMP produced by bacteria and have relatively narrow killing spectra. They are specific to bacterial strains closely related to the producing strain (Kirkup Jr 2006). According to Klaenhammer (1988), 99% of all bacteria may produce at least one bacteriocin, which provides the basis for an optimistic view that it is only a matter of directing enough research resources to this area to identify more bacteriocins for different bacteria. Bacteriocins are categorized based on several criteria, including producing strains, common resistance mechanisms, protein structure (unusual amino acids), and mode of action (Blinkova *et al.* 2003). We will briefly present some representative bacteriocins according to their origins, i.e. (i) colicins from G^- bacteria; (ii) lantibiotics from G^+ bacteria; (iii) halocins from archaeabacteria; and (iv) phage tail-like bacteriocin.

Colicins are the most well-studied G^- bacteriocins and were first discovered in 1925 by Gratia (2000). They are produced by and kill *E. coli* bacteria. The producing *E. coli* strain of a particular colicin has an immune gene that protects it against the colicin it produces, whereas sensitive *E. coli* strains do not carry the immune gene. Colicin production is mediated by the SOS regulon and is therefore produced under stress. It is worth to mention that colicins do not include all the bacteriocins produced by *E. coli*. Microcins, which are also produced by *E. coli*, have been treated as a separate class because of their significantly smaller molecular weight. To date, at least 11 colicins and 7 microcins have been identified (Gordon *et al.* 2005).

Lantibiotics, the most important G^+ bacteriocins, are produced by lactic acid bacteria (LAB), *Staphylococcus*, and many other G^+ bacteria. They characteristically contain unusual amino acids such as lantine, which are introduced to the structure by post-translation modifications and are essential for the biological activities of these bacteriocins. Over the 49 different lantibiotics identified towards the end of 2004 (Chatterjee *et al.* 2005), nisin is the most commercially important member of this family. Nisin is a type A lantibiotic that binds to lipid II, a precursor lipid component of the cell wall of sensitive bacteria and blocks the cell wall biosynthesis (Cheigh and Pyun 2005).

Our knowledge on bacteriocins from archaea is still quite limited at present and halocins, bacteriocins produced by the *Halobacteriaceae* family, are the most well-studied bacteriocins of archaea (Platas *et al.* 2002; Sun *et al.* 2005). Halocin production is recognized as a nearly universal feature of halobacteria. However, only a handful of halocins, including halocin H1, H4, H6, S8, and R1, have been purified and described in detail. The limited number of known halocins exhibit substantial diversity in size, ranging from proteins as large as 35 kDa (e.g. H4) to peptides as small as 3·6 kDa (e.g. S8). The halocin peptides, also named as microhalocins, are usually quite resistant to acid, base, and organic solvent, and their activities are usually unaffected by desalting or boiling (Li *et al.* 2003).

Phage tail-like bacteriocins are a special category of bacteriocins which are of large molecular weights, by opposition to most other AMP. Like other bacteriocins, they are produced by bacteria and are specific to bacterial species closely related to the producing stain. Several phage tail-like bacteriocins have been identified, including enterocoliticin produced by *Yersinia enterocolitica* (Strauch *et al.* 2003), serracin P by *Serratia plymithicum* (Jabrane *et al.* 2002), Carotovoricin Er by *Erwinia carotovora* (Nguyen *et al.* 1999), and xenorhabdicin by *Xenorhabdus nematophilus* (Thailer *et al.* 1995). They are genetically and morphologically similar to phage tails (Damasko *et al.* 2005; Sicard *et al.* 2005; Smarda and Benada 2005) and it is generally accepted that they have common ancestors with corresponding phage tails.

Phage-encoded AMP

There are two types of phage-encoded AMP: phage-encoded lytic factors and the phage tail complexes. To date, three different types of phage-encoded lytic genes have been isolated, all from phages with small ssDNA or ssRNA genomes. These lytic factors have similar functionality as that of the virolysin-holin system of large lytic phages, i.e. inducing bacteriolysis at a programmed time to allow the release of phage particles into the environment, however, through completely different mechanisms (i.e. the nonenzymatic mechanisms). The tree types of lytic factors can be represented by *E* in φX174 (Young and Young 1982; Mendel *et al.* 2006), *L* in MS2/GA classes of RNA phages (Remaut *et al.* 1982), and A_2 in the Qβ/SP classes (Model *et al.* 1979; Young *et al.* 2000).

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The *E* and *L* genes encode small membrane proteins while the A_2 maturation protein is not a distinct lysis protein of Qβ/SP phages but a protein that binds to the host sex pilus with a secondary role as a lytic factor. Protein *E*, the best-understood lytic factor among the three, is a specific inhibitor of the enzymatic reaction catalysed by the phospho-MurNAc-pentapeptide translocase (MraY), an integral membrane protein essential for peptidoglycan biosynthesis (Mendel *et al.* 2006). It was demonstrated that the peptidyl-prolyl isomerase of the host is essential for the bacteriolytic activity of *E* (Young *et al.* 2000) and that the *E*-mediated bacteriolysis requires continued host cell division, like penicillin. These observations suggest that protein *E* acts in a way very similar to the antibiotics and can be viewed as a 'protein antibiotic' (Bernhardt *et al.* 2002). The modes of action of the other lytic factors (e.g. *L* and A_2) have yet to be elucidated.

Another type of phage-encoded AMP is the phage tail complexes, which are large molecular assembly of peptide subunits responsible for recognition and attachment to specific receptors on the surface of sensitive bacterium (i.e. adsorption), penetrating the outer membrane if applicable (G^- bacteria), and local lysis of peptidoglycan on the site of attachment on the cell wall, and injection of phage genome into the host bacterium. The tail of bacteriophage T4 is probably the best-understood phage tail (Arisaka *et al.* 2003; Leiman *et al.* 2003; Miller *et al.* 2003; Rossmann *et al.* 2004). It has a large molecular weight, the ability to penetrate the outer membrane of *E. coli*, and the lytic activity for the hydrolysis of peptidoglycan. Although a number of phage tails have been expressed, no report has been found regarding the antimicrobial properties of purified phage tails. Nevertheless, the possibility of utilizing phage tail complexes as antimicrobial alternatives is real, especially for G^- bacteria, where we can take advantage of their outer membrane penetrating mechanisms.

Mode of action and specificity of AMP

AMP have a large variety of different bactericidal mechanisms. Although many of them still need to be elucidated, four of them are well understood and presented here. The first mechanism, thought to be the killing mechanism of the majority of eukaryotic AMP, is the formation of ion channels or pores across the cytoplasmic membrane of bacteria, which causes membrane perturbation, dissipation of the electrochemical gradient across the cell membrane, and loss of cell content (Li *et al.* 2005; Lohner and Blondelle 2005; Toke 2005). The second mechanism is the inhibition of cell wall biosynthesis. For example, several short members of the lipid II-targeting lantibiotics use an alternative bacteriolytic mechanism by removing the lipid II from the cell division site (or septum) to block cell wall synthesis (Breukink and De Kruijff 2006; Hasper *et al.* 2006); and the *E* protein, a phage-encoded lytic factor, inhibits the cell wall synthesis via the inhibition of the MraY activity as described before. The third mechanism will kill bacteria via the ribonuclease (RNase) or deoxyribonuclease (DNase) activities of some AMP. For example, colicin E9 kills sensitive *E. coli* cells by DNA degradation (i.e. the DNase activity) (Vankemmelbeke *et al.* 2005) and colicin E3 by catalysing a specific ribonucleolytic cleavage of 16S rRNA (i.e. the RNase activity) (Zarivach *et al.* 2002). The fourth mechanism is used by phage tail-like bacteriocins to kill bacteria through specific binding of bacteriocins to the bacterial receptor, which provokes dispolarization and perforation of the cytoplasmic membrane, inducing membrane perturbations (Damasko *et al.* 2005).

Regarding the specificity, bacteriocins, including phage tail-like bacteriocins, have a very high specificity (Nguyen *et al.* 1999; Fimland *et al.* 2005). They are usually toxic to bacterial strains closely related to the producing strains. Although no reports were found on the specificity of phage-encoded lytic factors, there is no doubt that their toxicities are limited to bacteria because their bacteriolytic activity relies on the inhibition of the biosynthesis of peptidoglycan, a unique component of the bacterial cell wall (Bernhardt *et al.* 2002). On the other hand, most eukaryotic AMP are broad-spectrum antimicrobials and many of them are toxic to both bacterial and eukaryotic cells (Asthana *et al.* 2004; Antonenko *et al.* 2005) whereas most of the bateriocins do not show toxicity to animals at their effective antimicrobial concentration (Kobayashi 2002). Not surprisingly, there is a correlation between their toxicity and specificity: nontoxic and narrow specificity for bacteriocins *vs* broad-spectrum and toxicity for eukaryotic AMP.

Antimicrobial efficacy of AMP

Extensive studies have been carried out on the antimicrobial efficacy of different families of AMP. AMP of prokaryotic origins, such as bacteriocins, have been demonstrated to have high efficacy in eliminating bacterial species closely related to the producing strains (Riley and Wertz 2002). For instance, local injections of staphylococcin A-1262a was used to treat 50 patients with a variety of staphylococcal lesions and a complete recovery was observed in 42 of them (Anisimov and Amoako 2006). The streptococcal bacteriocin, tomicide, was used for protection of white mice from staphylococcal infection and a single oral administration of the preparation of this bacteriocin immediately after infection protected one-third of the mice from local staphylococcal infection

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(Blinkova *et al.* 2003). Enterocoliticin, a phage-tail-like bacteriocin, has been successfully administered as an antimicrobial compound by oral route for the treatment of mice infected with *Y. enterocolitica* (Damasko *et al.* 2005). Nisin is so far the most commercially successful AMP: it has been used extensively as a food preservative worldwide without substantial development of bacterial resistance (Delves-Broughton 2005). It is active at low concentrations [minimum inhibitory concentration (MIC) within low nanomolar range] against many strains of G^+ bacteria, including drug-resistant strains and food-borne pathogens such as *Clostridium botulinum* and *L. monocytogenes*, which represents a multibillion dollar market. A comprehensive review on the antimicrobial efficacy of AMP has been published recently (Zaiou 2007), which summarizes the potent activities of AMP against a broad range of micro-organisms encompassing G^- and G^+ bacteria, fungus, parasites, and enveloped viruses, as well as the importance of AMP in inflammatory conditions including psoriasis, respiratory disorder, inflammatory lung disease, inflammatory bowel disease (IBD), rheumatoid arthritis, and atherosclerosis. Numerous natural and synthetic AMP have been used in clinical and preclinical trials, however with limited success, and none of them has obtained FDA approval for any topical or systemic medical applications, although a few of them appear to be quite promising (Marr *et al.* 2006; Zaiou 2007).

Advantages and disadvantages of AMP therapy

The properties that make eukaryotic AMP promising alternatives to antibiotics include: (i) broad-spectrum activity (antibacterial, antiviral, antifungal); (ii) rapid and potent bactericidal activity; (iii) low level of induced resistance (Gordon *et al.* 2005); and (iv) vast variety of AMP in terms of structures and antimicrobial functionalities, representing a tremendous potential. However, as pointed out before, so far no natural or modified AMP have FDA approval for medical applications. Marr *et al.* (2006) summarized the major obstacles for the therapeutic application of eukaryotic AMP as follows: (i) lack of higher efficiency than existing standard method of care; (ii) toxicity; and (iii) high costs of drug development and manufacturing. By opposition, prokaryotic AMP are safer because of their narrow spectra and seem to be efficient *in vivo*. Even if they are not significantly more efficient than current treatment and are more costly to produce, these negative criteria about AMP should be considered as minor points and the urgent need of efficient treatments to protect the population from the threat of the widespread antibiotic-resistant bacterial pathogens should be the new priority.

Conclusions

A vast number of potential antimicrobial alternatives including phages, BCWH, and AMP are under investigation to overcome the growing issue of bacterial resistance to antibiotics. Phages, when properly selected, offer the most cost-effective alternative. They are highly specific, effective, and safe for the treatment and prevention of infectious diseases. They were proved to be efficient in bacterial elimination and recently accepted for food treatment to counter food contamination during storage. Among the BCWH, virolysins are the most favourable choice because of their high specificity and their capacity to rapidly eliminate pathogenic bacteria that have acquired resistance to lysozymes. They represent a powerful alternative to antibiotics, and the relatively high production cost is probably the only obstacle to the commercial production and clinical application for efficient bacterial treatment, which should be resolved in future development. AMP are a promising family of natural antimicrobials with vast diversity in terms of sources, structures, functionalities, antimicrobial spectra, and modes of action. Despite of the lack of commercial success, it is reasonable to be optimistic about the development of natural and/or synthetic AMP for the treatment of certain infections. Future studies should turn these antimicrobial alternatives into practical clinical substitutes to antibiotics and prove their anticipated efficacy, safety, and affordability.

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