‘Bac’ to the future: bioengineering lantibiotics for designer purposes

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Abstract

Bacteriocins are bacterially produced peptides or proteins that inhibit the growth of other bacterial strains. They can have a broad (effective against multiple genera) or narrow (effective against specific species) spectrum of activity. The diversity of bacteriocins found in Nature, in terms of both spectrum of activity and physiochemical properties, offers the possibility of multiple applications in the food and pharmaceutical industries. However, traditional screening strategies may not provide a sufficient range of natural molecules with specifically desired properties. Research suggests that bioengineering of existing bacteriocins has the potential to address this issue, extending the application of natural bacteriocins for use in novel settings and against different targets. In the present paper, we discuss the successful implementation of bioengineering strategies to alter and even improve the functional characteristics of a bacteriocin, using the prototypical lantibiotic nisin as an example. Additionally, we describe the recent use of the nisin-modification machinery in vivo to enhance the properties of medically significant peptides.

Introduction

Perhaps the most fascinating bacteriocins are the lanthionine-containing antibiotics or ‘lantibiotics’ [1]. Lantibiotics are ribosomally synthesized antimicrobial peptides produced by Gram-positive bacteria that are characterized by the presence of lanthionine and/or methyllethanionic residues, introduced by a series of enzyme-mediated post-translational modifications of a linear inactive precursor peptide [2–4] (Figure 1A). Other unusual post-translationally modified amino acids can also be present, the most frequent being dehydroalanine and dehydrobutyryl. When it comes to such post-translationally modified peptides, Nature has evolved countless permutations on the same central theme: dehydrate, cyclize and decorate [5,6]. The resulting highly modified products display diverse activities representative of the unique roles that they fulfill for each producer.

The continued discovery of novel lantibiotics is revealing the extent of potential applications for their use in human health, veterinary science and food preservation. Although a plethora of inhibitors has been isolated, they obviously evolved to confer a selective advantage on the producer in a particular niche and are not optimized for use outside that environment. For example, although lantibiotics have high heat- and protease-resistance, a major drawback for certain applications is the sensitivity of many to neutral or basic pHs. As such, attention has focused on the bioengineering of these peptides to work in multiple environments and to expand the activity profile of the compounds. The present review focuses on the efforts to redesign nisin, the first discovered and most widely used lantibiotic, for an array of applications. Furthermore, we describe recent research on using the nisin biosynthetic machinery to synthesize designer peptides in vivo.

Nisin, the prototypical lantibiotic

Nisin was discovered in 1928 (the same year as the discovery of penicillin) by Rogers and Whittier [7] and is the best studied of all lantibiotics. It exhibits a broad spectrum of inhibition against Gram-positive bacteria, including many food-borne pathogens. Nisin has a long record of safe use in food, with no significant resistance observed in routine practice, and is one of only a few bacteriocins to have been applied commercially [8]. Nisin was added to the positive list of food additives by the EU (European Union) as additive E234 (EEC Commission Directive 83/463/EEC, 1983) and has been approved by the WHO (World Health Organization, 1969) and the U.S. FDA (Food and Drug Administration, 1988) for use as a food preservative.

A PubMed search using the term ‘nisin’ recovers more than 1400 scientific papers and much is known about its structure, mode of action, autoregulation and immunity mechanisms [3]. Mature nisin is a pentacyclic cationic peptide (Figure 1B) that has two amphipathic structured domains [9]. The N-terminal domain, containing three (β-methyl)lanthionine rings (A, B and C) is linked to the C-terminal intertwined rings (D and E) by a flexible ‘hinge’ region consisting of three amino acids (Asn20–Met21–Lys22). The mode of action of nisin has been the focus of many elegant studies and our current understanding is that rings A and B form a ‘cage’ that facilitates binding of the pyrophosphate moiety of lipid II, thus interfering with cell wall synthesis [10]. This binding allows the C-terminal segment, via the hinge

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Figure 1 | Biosynthesis of nisin
(A) Formation of rings A and B only (see B) is shown for clarity. Serine and threonine residues of the unmodified core peptide (with N-terminal leader peptide attached; not shown) are dehydrated by NisB, forming dehydroalanine (DHA) and dehydrobutyrine (DHB) respectively. These modified residues can react with intramolecular cysteine residues, facilitated by NisC, generating the eponymous lanthionine (in the case of dehydroalanine) or β-methyl-lanthionine (in the case of dehydrobutyrine; ABU, aminobutyric acid). NisT then exports the modified core peptide with the leader peptide still attached. The extracellular serine protease NisP cleaves off the leader peptide, whereupon mature bioactive nisin is released. (B) The structure of mature nisin A with (β-methyl)lanthionine rings labelled A–E and hinge region boxed.

Bioengineering nisin for specific purposes
Despite the relative success of nisin in food applications, the lantibiotics can be largely considered an under-exploited class of natural products, especially when it comes to use in the medical arena [16–18]. Nisin, for example, has potential as an important therapeutic agent given its non-cytotoxic nature [19] and its potency against many clinically relevant bacteria, including multidrug-resistant pathogens such as MRSA (methicillin-resistant Staphylococcus aureus) and penicillin-resistant Streptococcus pneumoniae [20–23]. Unfortunately, despite this, the widespread use of nisin as a therapeutic agent has yet to be realized, in part due to the low solubility/stability of nisin at physiological pH hindering its systemic use. In fact, apart from the food industry, nisin has only found limited application in the veterinary field [24,25] and has only in the last few years been investigated as a mastitis treatment in humans [26]. For nisin, and the lantibiotic family as a whole, to realize their full therapeutic potential, it is imperative that the pharmacological properties of these molecules are optimized for the use in a variety of settings. Moreover, as antibiotic resistance is relentlessly increasing, the ability to direct the activity of nisin towards Gram-negative bacteria or to increase its potency against those Gram-positive bacteria that have a high level of innate resistance to native nisin would be useful. With this in mind, it seems that with the advent of lantibiotic bioengineering, many of these difficulties can potentially be overcome.

The gene-encoded and ribosomally synthesized nature of lantibiotics, coupled with the relatively relaxed substrate specificity of the modification enzymes, makes them ideal candidates for the implementation of bioengineering strategies [5,16,27]. Commonly used strategies can involve in vivo expression of a modified structural gene in the original producer strain, either in cis by replacing the original structural gene or in trans by complementing an inactivated copy of the structural gene. Such approaches are limited to producing novel variants that are compatible with the native biosynthetic, transport and immunity machinery.

Six natural variants of nisin have been identified to date. These are nisin A [28], Z [29], Q [30] and F [31], which are produced by Lactococcus lactis, and U and U2, which are produced by Streptococcus uberis [32], although the description of nisin U and U2 as true nisin variants is contentious [33]. This level of diversity suggests that the molecule is amenable to certain substitutions at particular residues and that it may be possible to modulate the functional characteristics of the peptide. Indeed, Nature has provided us with an excellent example in nisin Z, which differs from nisin A by having an asparagine rather than a histidine residue at position 27. Although these variants have similar activity spectra [34], they differ in that nisin Z exhibits a higher rate of diffusion [35] but is less soluble at low pH [36].

In order to explore this possibility for change throughout the nisin molecule, a variety of PCR-based approaches have been used, including site-directed, random and saturation mutagenesis have been used. In the first instance, this
fundamental analysis allows us to generate a blueprint of which residues are essential (for production and/or activity) and which are variable, thus greatly enhancing our understanding of the structure–activity relationships of this complex peptide. Secondly, it allows us to screen for nisin derivatives with improved functional properties, such as widened active pH range, better solubility, improved stability and enhanced potency against specific targets. Mutations introduced by bioengineering that have a positive impact on the biological activity or physicochemical properties of nisin are discussed below and summarized in Figure 2.

**Modulation of biological activity of nisin**

The first promising bioengineered nisin derivatives were enhanced against non-pathogenic targets. Initially, a M17Q/G18T nisin Z double mutant was identified that was twice as active as nisin Z against *Micrococcus flavus* [37]. Subsequently, a nisin Z T2S derivative, where the introduced serine residue was modified resulting in a Dha (dehydroalanine) residue at the mutated position, displayed a 2-fold increase in activity against *M. flavus* and *Streptococcus thermophilus* [11,38]. More recently, two ring A mutants (where ISL (Ile-Ser-Leu) at positions 4–6 is converted into KSI (Lys-Ser-Ile) or KFI (Lys-Phe-Ile)) were found to possess superior antimicrobial activity against some non-pathogenic strains, in addition to showing an enhanced capacity to inhibit the outgrowth of spores of *Bacillus subtilis*, compared with nisin A [39].

Nisin cannot inhibit Gram-negative bacteria in the absence of other stress factors such as chelating agents. Significantly, the first bioengineered derivatives of nisin that were enhanced with respect to activity against pathogenic bacteria, namely nisin Z N20K and M21K, were active against *Shigella*, *Pseudomonas* and *Salmonella* species [40]. These derivatives showed that the spectrum of antimicrobial activity of nisin can be extended by bioengineering to include Gram-negative bacteria. Subsequently, the identification of nisin A K22T proved that bioengineering can also yield derivatives that are more active against Gram-positive pathogens [41]. This derivative was discovered during the large-scale screening of
banks of bioengineered derivatives produced using a random mutagenesis approach. In the light of this breakthrough, the same study targeted the hinge region residues for site-directed and site-saturation mutagenesis, resulting in the identification of additional derivatives, most notably N20P, M21V and K22S, with enhanced specific activity against numerous Gram-positive pathogens. Nisin M21V, since renamed nisin V, is of particular note due to its enhanced antimicrobial activity against a wide range of targets, including numerous medically significant pathogens [41,42]. The superior activity of nisin V was subsequently validated in a food model against Listeria monocytogenes, a noteworthy outcome in view of the high natural resistance of L. monocytogenes to nisin [42].

Furthermore, these studies emphasize the benefits of bioengineering nisin to yield derivatives with greater antimicrobial activity against specific problematic microbes. Collectively, these results demonstrate that bioengineering can both alter the strain-specificity of nisin and improve its activity against target cells.

**Modulation of physicochemical properties of nisin**

One major avenue that has been explored in nisin bioengineering is the alteration of the overall charge of nisin in an attempt to modulate the solubility of the peptide. The introduction of a lysine residue at specific locations in nisin Z (N27K and H31K) caused the altered peptides to display an increase in solubility at neutral pH, while maintaining an equivalent antimicrobial activity and spectrum [36]. Another notable example of charge state modulation to obtain more soluble nisin derivatives comes from the aforementioned N20K and M21K mutants [40]. In addition to achieving anti-Gram-negative activity, the N20K and M21K variants were much more soluble, especially at alkaline pHs where wild-type nisin solubility is particularly poor. Fortunately, these enhanced mutants were also afforded greater stability at higher temperatures (a desirable characteristic given that nisin may have to endure heat treatment during food processing) and at neutral/alkaline pHs (which might allow the use of these derivatives in different foods to those low pH foods in which nisin is currently applied) than the wild-type peptide.

In a recent study, numerous nisin A derivatives were identified that were improved in another important physicochemical property, namely the ability to diffuse through complex polymers [43]. In the case of two derivatives [containing SVA (Ser-Val-Ala) and NAK (Asn-Ala-Lys) within the hinge region], it was found that this enhanced trait enabled the peptides to outperform nisin A when it came to control of L. monocytogenes in commercially produced chocolate milk containing carrageenan as a stabilizer.

**Modulation of properties of non-lantibiotic peptides by the nisin-modification machinery**

The nisin-modification machinery can be used to install dehydrated amino acids and lanthionine rings in small peptides that are fused to the nisin leader sequence [44]. This promiscuity has been exploited in order to successfully incorporate modified amino acids, not only into other lantibiotic peptides [45], but also into normally non-lantibiotic peptides [46]. A notable example involved the application of the nisin-modification machinery to effectively dehydrate, secrete and, in some cases, bring about ring formation, within derivatives of a variety of medically valuable peptides including angiotensin [47]. Significantly, the introduction of a thioether ring structure in some angiotensin peptides made them more resistant to proteolytic breakdown. Furthermore, one cyclized analogue showed enhanced biological activity (e.g. in lowering blood pressure) compared with its linear counterpart [48]. A similar approach significantly improved the proteolytic stability of luteinizing hormone release hormone [49], thus enhancing its therapeutic potential.

**Conclusions**

We can only speculate as to the role of bacteriocins in complex ecological niches. Perhaps their primary role is one of signalling between related strains, or perhaps they provide a competitive advantage in times of scarcity or in times of plenty. It is perhaps unnecessary to place too much emphasis on ‘why’ bacteriocins are produced, but simply accept that the wide distribution and great diversity of bacteriocin operons in sequenced genomes confirms an evolutionary advantage for their production.

Undoubtedly, the search for novel lantibiotics will continue to yield insights into the variety of structures and functions of these molecules, but their application is often restricted by their intrinsic properties. Although there has been a major focus on the production of nisin derivatives with improved specific activity in recent years, the implementation of bioengineering has also been fruitful with regard to the generation and identification of nisin derivatives with improved physicochemical properties. Although the mechanisms underlying the enhancement of these variants have yet to be elucidated, the fact that lantibiotic properties can be improved is an exciting development. In the near future, it should prove possible to apply the knowledge gained from such research in the implementation of rational mutagenesis strategies with a view to creating ‘designer’ nisin derivatives that are tailor-made for specific purposes [50]. Additionally, with further study, such derivatives should shed light on the biological activities that they are disrupting.

An improved understanding of the substrate tolerance of the biosynthetic enzymes involved in nisin production has yielded another promising avenue of research; the expanding of bioengineering studies to unnatural scaffolds in order to design completely novel modified peptides with potentially new biological activities and utilities. Furthermore, by facilitating the introduction of modified amino acids into both lantibiotic and non-lantibiotic peptides in vivo, the nisin-modification machinery represents an innovative tool to modulate and, thereby potentially improve, the properties of bioactive peptides.
With ongoing research, it is becoming apparent that the chemical space filled by the lantibiotic natural product family is continually expanding. Nature has thus provided us with a multitude of new possibilities.

Note added in proof (received 28 September 2012)

A recent publication [51] reports numerous derivatives of nisin A, varying at position 29, that display superior antimicrobial activity relative to nisin A against a range of Gram-positive pathogens. Significantly, a number of these derivatives are also enhanced against a range of Gram-negative pathogens, representing the first time that nisin has been improved against both Gram-positive and Gram-negative bacteria simultaneously.

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