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Antimicrobial antagonists against food pathogens; a bacteriocin perspective

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ABSTRACT

Efforts are continuing to find novel bacteriocins with enhanced specificity and potency. Traditional plating techniques are still being used for bacteriocin screening studies, however, the availability of ever more bacterial genome sequences and the use of *in silico* gene mining tools have revealed novel bacteriocin gene clusters that would otherwise have been overlooked. Furthermore, synthetic biology and bioengineering-based approaches are allowing scientists to harness existing and novel bacteriocin gene clusters through expression in different hosts and by enhancing functionalities. The same principles apply to bacteriocin producing probiotic cultures and their application to control pathogens in the gut. We can expect that the recent developments on bacteriocins from Lactic Acid Bacteria (LAB) described here will contribute greatly to increased commercialisation of bacteriocins in food systems.

INTRODUCTION

Consumer awareness of the effect of diet on health has led to a demand for minimally processed foods in which chemical preservatives are replaced by more natural alternatives. Traditionally foods were preserved by (LAB), natural constituents of fermented foods, which confer their preservative effects by the production of lactic acid, hydrogen peroxide and small peptides known as bacteriocins. Bacteriocins are active against a number of genera (broad spectrum) or particular species (narrow spectrum) [1-3] and are very diverse, varying in size, structure and specificity. The fact that many bacteriocins are produced by food-grade LAB and possess potent antimicrobial activity means that they are ideally suited to controlling food spoilage and pathogenic bacteria [4-6]. Bacteriocins can be broadly divided into two classes: class I, of which the lantibiotics (post-translationally modified peptides containing unusual amino acids) are the best-known example and class II, containing unmodified peptides [7]. Their mode of action is likely driven by the primary structure of the bacteriocin with membrane permeabilisation being a very common theme. The producing culture is protected by the production of specific immunity proteins and the low levels of resistance detected so far makes them desirable alternatives to antibiotics. [6]. Their main advantage over chemical preservatives is their ability to preserve without affecting the sensory qualities of the food while adhering to the demand for natural preservatives. The ideal bacteriocin should be potent at low concentrations, active against a range of spoilage and pathogenic organisms, innocuous to the host and economical to produce [8]. These antimicrobials can be introduced into a food through incorporation of the bacteriocin-producing strain into the food product (most commonly in fermented foods), the generation and use of a bacteriocin-containing fermentate or as a more concentrated bacteriocin-containing food preservative. Currently only two bacteriocins are being used commercially as food preservatives: nisin produced by *Lactococcus lactis,* (marketed as Nisaplin and under other brand names), has been used commercially for 50 years [9] and carnocyclin A (marketed as Micocin) a circular bacteriocin produced by *Carnobacterium maltaromaticum* UAL307 is an approved biopreservative in the US and Canada developed to inhibit *Listeria monocytogenes* in ready-to-eat meat (RTE) products [10]. This review focuses predominantly on bacteriocins as antimicrobial antagonists and efforts to develop them as viable food biopreservatives. (See Figure 1)

THE CONTINUING SEARCH FOR NOVEL BACTERIOCINS

A primary focus of bacteriocin research is identifying novel bacteriocins and bacteriocin-producing strains for specific applications. The general consensus is that the bacteriocin/bacteriocin-producer that is best suited to controlling a problematic spoilage/pathogenic microorganism will often be one that is found in the same environmental niche. This is based on the expectation that bacteriocins provide an advantage to competitors fighting for scarce resources in a particular environment. A prime example relates to *Weissella hellenica* QU 13, isolated from a barrel in which Japanese pickles are fermented, which was found to produce two leaderless bacteriocins, weissellicin V, homologous to the class IId Enterocin L50A and L50B, and weisselicin M. In the latter case, it is notable that this novel broad spectrum class IId antimicrobial is effective against *Bacillus coagulans*, a known contaminant of pickle fermentations. Thus, strain QU 13 is a good example of a fermentation-associated isolate which has the potential to be employed to control an undesirable microbial contaminant [11]. *Lactococcus garvieae* is a pathogen affecting farmed and fresh fish from marine and freshwaters and is also considered an emerging zoonotic pathogen. Garvicin A, a novel class IIb bacteriocin produced by the human isolate *L. garvieae* 21881, inhibits other *L. garvieae* stains and has potential to treat or prevent *L. garvieae* infections. More specifically, it is suggested that the purified bacteriocin in combination with probiotic LAB would be useful in the fight against *L. garvieae* infections [12]. Another *L. garvieae* strain, a fermented pork sausage isolate *L. garvieae* BCC 43578, produces garvieacin Q, a novel class IId bacteriocin active against other *L. garvieae* and *L. monocyto genes* [13]. The ability to control *L. monocyto genes* is a particularly highly sought-after trait and it is thus notable that enterocin W, a two component lantibiotic produced by *Enterococcus faecalis* NKR-4-1 isolated from *pla-ra* Thai fermented fish [14], exhibits activity against this pathogen. Given that *Staphylococcus aureus* is also a major concern for the food industry, it is interesting that bactofencin A, a cationic disulphide bond-containing bacteriocin similar to eukaryotic defensins, is active against *S. aureus*. In addition to the unusual nature of this bacteriocin, it is notable that its producer, the porcine isolate *Lactobacillus salivarius* DPC6502, does not contain a classical immunity-like gene, but instead encodes a *dltB* homologue that confers resistance [15]. While the examples provided above relate to strains that produce a single bacteriocin, it should be noted that the production of multiple bacteriocins by a single strain can be advantageous as the various bacteriocins are likely to have different modes of action, thereby extending the spectrum of inhibition and reducing the likelihood of development of resistance. The genome of *Enterococcus faecium* NKR-5-3, isolated from *pla-ra* Thai fermented fish, encodes 5 enterocins, NKR-5-3 A, B, C, D and Z and produces at least four of them, that is NKR-5-3 A, B, C and D. Enterocin NKR-5-3C was confirmed to be a class IIa bacteriocin which exhibits potent antilisterial activity. The other bacteriocins are proposed to represent different classes but further investigations are required to establish this definitively [16,17].

THE PARTICULAR EXPANSION IN NUMBERS OF CIRCULAR BACTERIOCINS

Although previously regarded as being rare, the discovery of circular bacteriocins has become more common in recent years. This is notable as these bacteriocins are thought by some to have the potential to form the next generation of biopreservatives as a consequence of their stability and activity. Indeed, gassericin A, garvicin ML, lactocyclin Q and leucocyclin Q produced by LAB inhibit a range of Gram-positive bacteria including food spoilage bacteria and food pathogens [18]. The remarkable stability and activity of these bacteriocins is attributed to their head to tail cyclisation which confers the bacteriocins with increased protease and heat resistance [19,20]. Garvicin ML is a recently discovered circular bacteriocin produced by *L. garvieae* DCC43 isolated from a Mallard duck which inhibits *L. garvieae* [21]. Leucocyclin Q, produced by a Japanese pickle isolate *Leuconostoc mesenteroides* TK41401, is particularly active against *B. coagulans* which, as noted above, is a major pickle food spoilage organism [22]. Studies relating to the mode of action of these, and indeed other, bacteriocins continue to also attract attention. Notably, in this regard, Liu *et al* [23] recently noted that sublethal doses of carnocyclin A induced an adaptation response in *L. monocytogenes* 08-5923 by affecting genes responsible for cell wall biosynthesis and metabolic function maintenance.

NEW STUDIES RELATING TO THE USE OF BACTERIOCINS AS PART OF A HURDLE APPROACH TO PRESERVATION

Bacteriocins can become more effective biopreservatives when used in combination with other antimicrobial hurdles such as organic acids, chelating agents or essential oils. These additive or synergistic phenomena act by reducing the levels of bacteriocin required for target inhibition and, in some instances, can even extend the spectrum of inhibition of bacteriocins to include Gram-negative microorganisms [2]. *Cronobacter sakazakii* DPC6445 is an opportunistic Gram-negative pathogen associated with powdered infant milk formula (PIF) which has been associated with meningitis, septicaemia and necrotizing enterocolitis in premature and immunocompromised babies. Producing PIF that could be reconstituted at 40-50°C without risk of *C. sakazakii* infection is of interest to the food industry. Significantly, it has recently been established that nisin or lacticin 3147 when combined with the lactoperoxidase system inhibited *C. sakazakii* outgrowth for 8 hours, thereby providing an excellent example of a combinatory approach to improving the safety of PIF [24].

It has also been frequently demonstrated that using bacteriocins in combination with chelators such as ethylenediaminetetraacetic acid (EDTA) can expand the antimicrobial spectrum of a bacteriocin. Indeed, although carnocyclin A is not effective against *Escherichia coli*, *Pseudomonas aeroginosa* or *Salmonella* Typhimurium when tested alone, it can inhibit *E. coli* and *P. aeruginosa* when combined with 40 mM EDTA. Anti-*E. coli* and *S.* Typhimurium activity could be improved even further when nisin, rather than carnocyclin A, was combined with 40 mM EDTA [10].

Bacteriocins can also be utilised by applying them to a food surface. Due to lower concentrations being sufficient for efficacy in these circumstances, production costs are reduced. The use of immobilised bacteriocins, such as nisin, as components of antimicrobial packaging has been the focus of increasing levels of research, though it is important to appreciate that understanding the mode of action of specific bacteriocins is important to ensuring further progress in the area. In one instance nisin was absorbed on both hydrophobic and hydrophilic food films and the effectiveness of the active surface against *L. monocytogenes, Bacillus cereus* and *S. aureus* was compared. It was established that the hydrophilic surfaces were more bioactive and absorbed higher quantities of nisin than the hydrophobic surfaces and that *S. aureus* was most sensitive to the nisin functionalised films [25]. Class IIb lactocin 705 and the pediocin like class IIa lactocin CL705 also possess potential in this regard. These *Lactobacillus curvatus* CRL705-produced bacteriocins are active against spoilage LAB and *Listeria* and have been incorporated into wheat gluten films to assess their ability to inhibit *L. monocyto genes* in meat products. The bacteriocin-containing gluten film, made at pilot scale, retained antimicrobial activity for 50 days which, importantly, is the shelf life of RTE meat products such as cooked sausages [26]. More specifically, the film reduced *L. monocyto genes* levels in Wiener sausages at day 45 by 2.5 log cycles relative to controls [27]. In addition to food surfaces, the surfaces of equipment can also serve as a site for the contamination of food by food spoilers and pathogens such as *L. monocytogenes*. Many such bacteria can colonise surfaces such as stainless steel and form biofilms. Biocides are routinely used to clean processing equipment but biofilms can be particularly difficult to remove. It has recently been established that combining sub-inhibitory concentrations of the class IIc enterocin AS-48 with concentrations of biocides 4-10 fold lower than their MICs inhibited the growth of planktonic (non-biofilm) *L. monocyto genes*. Unsurprisingly, higher concentrations of both bacteriocin and biocide were required to inhibit sessile cells though synergy was still observed [28]. Proteomic analysis of the exposure of *L. monocyto genes* to enterocin AS-48 revealed that planktonic and sessile cells respond differently upon exposure to the bacteriocin. Planktonic cells may compensate for changes in cytoplasmic permeability by reinforcing carbohydrate transport and metabolism while sessile cells shift carbohydrate metabolism and reinforce protein synthesis. Both cells states also exhibit a differing response to stress [29].

BACTERIOCIN ENGINEERING

Bacteriocins are ribosomally encoded and therefore are amenable to genetic manipulation through engineering, which is defined as modifying the amino acid sequence of a protein to change its structure and function [30]. Bioengineering (engineering inside the cell) and the use of synthetic biology-based (*in vitro* engineering) approaches have contributed significantly to our understanding of the roles specific amino acids play in structure and activity and resulted in the production of bacteriocins which have extended bioactivity against selected pathogens [31]. The structure-activity relationship of nisin has been extensively studied through bioengineering and this has enabled researchers to design variants with enhanced activity against specific targets. Nisin S29G, with enhanced activity against *S. aureus* SA113, was found by screening a bank of nisin A variants following site directed mutagenesis specifically targeted against this residue. This resulted in the generation of a number of variants with improved activity against both Gram-positive and Gram-negative pathogens. Indeed, this is the first instance upon which bioengineering of a bacteriocin has led to enhanced activity of this kind [32]. Saturation mutagenesis at another location in nisin, lysine 12, resulted in the finding that a K12A derivative displays increased specific activity against food pathogens such as *B. cereus*, *S. aureus* and *S. agalactieae* but not against *L. monocytogenes* [33]. Another region of the nisin peptide, the three amino acid 'hinge' region, is particularly amenable to change and bioengineering of this region has had beneficial consequences [34]. Indeed, Rouse *et al*. [35] created a bank of hinge mutants and found that nisin peptides containing hinges consisting of SVA or NAK (rather than the original NMK) displayed an enhanced ability to diffuse through complex polymers, a trait which enabled the variants to outcompete nisin A controlling *L. monocyto genes* in commercially produced chocolate milk containing the stabiliser carrageenan. Furthermore, Healy *et al.* [36] used site directed mutagenesis of the hinge region to create a novel bank of nisin derivatives and found that AAK, NAI and SLS had enhanced activity towards some microorganisms. On the basis of the observation that the incorporation of small, chiral amino acids at this location generally has positive consequences, AAA-containing and SAA-containing 'hinge' derivatives were designed, created and ultimately became the first example of enhanced nisin derivatives to be generated through rational design.

In the case of another lantibiotic, actagardine A, saturation mutagenesis was employed to engineer each amino acid, with the exception of those involved in bridge formation, in turn through using saturation mutagenesis. Through this approach it was established that the V15F variant demonstrates enhanced activity against *Clostridium difficile*, *E. faecium* and *E. faecalis* [37]. The ribosomal nature of bacteriocins also allows for more dramatic changes. To highlight this point, the anti-Gram-negative microcin V was combined, through asymmetrical PCR, with the anti-Gram-positive enterocin 35 to generate the chimeric bacteriocin Ent35-MccV which is active against both Gram-positive and Gram-negative pathogens and thus could be of value to the food or pharmaceutical industries [38]. Finally, it is now possible to bioengineer circular peptides by introducing a covalent bond between the N and C termini using advances in molecular biology and protein engineering techniques [30]. Theoretically these techniques could allow the generation of more stable bacteriocins with extended applications that could be employed by the food industry. Synthetic biology, considered complementary to bioengineering, is another promising area that provides insights into structure-stability relationships and the mechanism of action of bacteriocins [39,40]. In one instance, Solid Phase Peptide Synthesis (SPPS) has been used to synthesise, and modify, lantibiotics such as lacticin 481. Using this approach, the role of lanthionine and methyllanthionine residues was investigated by replacing them with diastereoisomers. In this case it was established that activity was lost, suggesting that the 3D structures were modified [41]. Synthetic biology also inspired Kong *et al* [42] to clone the nisin biosynthesis pathway from *Lactococcus lactis* K9 into a plasmid and express it in a nisin deficient strain. They also overexpressed nisin A using constitutive promoters and further optimised yield by integrating the structural peptide determinant *nisA*, overexpression cassettes and the recombinant pathway into a single circuit enabling the strain to produce 6 fold higher levels of nisin. This could potentially reduce the cost of nisin production for the food industry and also provides a means via which novel bacteriocin clusters identified through genome mining (see below) could be harnessed. Further efforts to increase bacteriocin yield have led to the use of synthetic genes encoding bacteriocins being cloned into and expressed in yeasts. A synthetic gene designed using adapted codon usage from the amino acid sequence of enterocin A from *E. faecium* T136 was cloned into *Pichia pastoris* X-33EAS and production levels increased 21.4 fold and antimicrobial activity against a number of listeria strains increased 4-603 fold when compared to the natural producer [43].

GENOME MINING

In the past bacteriocin-producing strains have been identified primarily on the basis of culture-based approaches. However, traditional plating techniques will reveal bacteriocin producing cultures only if the culture produces the bacteriocin under the conditions used for laboratory growth and only if it is effective against the target organism chosen for the overlay. Recently there has been a move to supplement traditional mining techniques with exploring the genomes of microorganisms from under-exploited environments which could be a reservoir of novel bacteriocins. The number of genome sequences being deposited in public databases is continually increasing as a consequence of significant developments in next generation sequencing technologies. This information is often freely available through online databases and provides an opportunity for screening a wide number of microorganisms to identify those which have the potential to produce bacteriocins [44,45]. This is seen as the dawn of a new era in which *in silico* and bioengineering based approaches can complement, and potentially supersede, culture based methods [45]. Despite this potential, finding bacteriocin genomes can be a challenge due to the small size of the structural peptides and diversity of their operons. BAGEL 3 is a fast genome mining tool that can identify putative bacteriocins based on conserved domains in structural, biosynthetic, transport and immunity genes [46]. In addition the BACTIBASE database is a manually curated repository of bacteriocin sequences that can also be helpful. [47]. Mass spectrometry is also being used more often in the quest for novel bacteriocins. Natural Product Peptidogenomics is a mass spectrometry based genome mining approach that connects chemotypes with biosynthetic gene clusters, the objective being to match a series of mass shifts from MS^n spectrum of a putative bacteriocin to the genes responsible for production [48].

Zendo and co-workers [49,50] developed a rapid screening method using electrospray ionisation liquid chromatography/mass spectrometry (ESI/LC/MS) coupled with statistical analysis of antimicrobial spectra to accelerate the discovery of novel bacteriocins isolated from various sources. An example of a novel lantibiotic that has recently been discovered using a genome mining and PCR approach is the broad spectrum cerecidin A1 and cerecidin A7 from *B. cereus* strain As 1.1846 isolated from spoiled soya milk. The *cer* locus differs from other class II lantibiotics in that it contains seven tandem precursor *cerA* genes and the cerecidins are notably active against multidrug resistant *S. aureus* (MDRSA) and vancomycin resistant *E. faecalis* (VRE) [51].

PROBIOTICS

Finally, over the last few years there has been growing evidence that bacteriocin production confers a number of advantages on probiotic strains. It is proposed that the ability to produce bacteriocins may help a strain to establish itself in a new niche, inhibit competitors and pathogens, alter the composition of the microbiota and even modulate the host immune system [52]. A recent study of the gut microbiota of elderly Irish subjects revealed *Enterococcus* strains with anti-listerial activity, which merit closer attention with a view to investigating their use as probiotic strains. In addition, a *Lactobacillus gasseri* strain producing gassericin T was isolated during the same screening programme [53]. Notably, *Lb. gasseri* bacteriocins are very active against Gram-positive pathogens and have potential as food preservatives due to their heat stability and pH stability. *Lb. gasseri* have been evaluated as probiotics and these investigations have also highlighted its tolerance of low pH environments, resistance to bile salts, ability to adhere to the host epithelium modulate the innate and adaptive immune system [54]. There have also been a number of recent studies that have highlighted the impact of the Abp118 bacteriocin by *Lactobacillus salivarius* UCC118 on the overall composition of the gut microbiota and on the host epithelium [55-57]. Finally, a study of LAB associated with fish for human consumption showed that bacteriocin activity against fish pathogens is a widespread probiotic property. Indeed LAB active against lactococcosis were common among LAB isolated from edible fish, further supporting the theory that the best place to find antimicrobials against a specific pathogen is in the niche the pathogen proliferates [58].

CONCLUSION

In conclusion, there is a continued drive to find novel bacteriocins that can control food pathogens more effectively. Novel LAB bacteriocins continue to be discovered and the use of LAB that produce multiple bacteriocins is receiving renewed attention. These screening programmes are being aided by the use of genome mining and mass spectrometry to find and characterise new bacteriocins while new engineering based approaches are being used in parallel to improve previously identified bacteriocins for particular applications /targets. There is great potential to carry out investigations that would assess the impact of bacteriocins on an entire food microbial consortia as has been done previously to assess the impact of bacteriocins on gut microbial populations [56, 59].

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