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Strategies to improve the bacteriocin protection provided by lactic acid bacteria

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Abstract

Lactic acid bacteria (LAB) produce a wide variety of antimicrobial peptides (bacteriocins) which contribute to the safety and preservation of fermented foods. This review discusses strategies that have or could be employed to further enhance the commercial application of bacteriocins and/or bacteriocin-producing LAB for food use.

Introduction

Bacteriocin production is a desirable trait among LAB from the perspective of controlling microbial populations in fermented foods in order to extend product shelf-life and safety. Bacteriocins produced by LAB are a diverse group of ribosomally-synthesized antimicrobial peptides which may be divided into two main groups i.e. class I peptides, which contain post-translational modifications, and class II, or unmodified, peptides [1]. Broad spectrum bacteriocins, such as nisin (class I), inhibit Gram positive food-borne pathogens and spoilage microbes and, when combined with additional hurdles, Gram negative targets [2]. Narrow spectrum bacteriocins can also be of value, for example, lactococcin A (class II) has a lytic effect on sensitive lactococci which, through the release of key enzymes, can accelerate cheese ripening and enhance the development of important organoleptic properties [3]. Bacteriocins may be introduced into a food *via in situ* production by bacterial starter or adjunct strains in fermented foods, by the addition of purified or semi-purified preparations (e.g. nisin containing powders such as Nisaplin) or as an ingredient based on a fermentate of a bacteriocin producing strain (such as ALTA2431 which contains pediocin PA1). However, the commercial application of specific bacteriocins can be hindered by low or inconsistent production levels, high production costs, a non-ideal antimicrobial spectrum and potency, the risk of the emergence of resistance and the poor/lack of growth of some producing strains in particular foods. This review discusses some of the strategies developed to overcome such limiting factors.

Influence of growth parameters

Many studies have been dedicated to optimising bacteriocin production by manipulating growth media composition, temperature or pH [4-6]. Investigations of

alternative carbon, nitrogen and mineral sources have successfully led to increased bacteriocin yields or more cost effective production [7-9]. Another strategy has been the inclusion of additional stimuli. In the case of *Lactobacillus plantarum* NC8, a starter strain used in Spanish-style green olive fermentations, and *Leuconostoc citreum* GJ7, a kimchi isolate, this occurs through the addition of specific adjunct strains that induce bacteriocin-production [10,11].

Use of conjugation to transfer a bacteriocin producing phenotype

Conjugation provides a natural mechanism by which genes can be transferred from one LAB to another while maintaining the food grade status of the recipient strain. As many bacteriocins are plasmid encoded, this approach has been widely exploited to disseminate bacteriocin-producing phenotypes. This is exemplified by the generation of over 30 food grade *Lactococcus* strains that produce the broad spectrum two-component class I bacteriocin lacticin 3147 through the transfer of pMRC01, a conjugative plasmid which contains the corresponding genetic determinants [12]. Although the presence of pMRC01 may impose an additional metabolic burden on LAB, which can increase cell permeability and autolysis, it does not impact on the acidification capacity of the strain [13]. In addition to the transfer of the bacteriocin producing phenotype itself, bacteriocin production (and the associated bacteriocin immunity phenotype) can also serve as a food grade selectable marker when transferring additional, plasmid-linked, industrially relevant traits. Indeed, while conjugal transfer of pMRC01 to the lacticin 481 producing host *Lactococcus lactis* DPC5552 generated a co-producing transconjugant which exhibited synergistic activity [14], both bacteriocins have served as effective food grade selectable markers for the transfer of bacteriophage resistance phenotypes to important commercial dairy

starter strains, thereby reducing phage sensitivity [15,16]. It should also be noted that the conjugal transfer of plasmids encoding other traits, such as lactose utilisation and protease activity, can facilitate increased bacteriocin production by adapting the producing strain for better growth in food environments. Indeed, Garcia-Parra et al, reported a 40-fold increase in nisin production by one such transconjugant in milk [17]. The genetic determinants for nisin are also located on a conjugative transposon enabling its food-grade transfer to other LAB [18], and the nisin resistance phenotype has also been widely exploited as a selection marker for food grade improvement of starter strains [19,20].

Strategies have also been adapted to mobilize bacteriocin-encoding non-conjugative plasmids for the improvement of starter strains in a food grade manner [21,22]. Such plasmids, unable to mediate their own transfer, require the sequence of the origin of transfer (*oriT*) and *mobA* gene, while the genes encoding other conjugal functions are supplied *in trans* from a conjugative plasmid or sex factor. *L. lactis* IFPL359 transconjugants generated in this way to harbour the lacticin 3147-encoding non-conjugative plasmid pBaC105 successfully accelerated proteolysis and development of sensory characteristics of semi-hard goat cheese [21].

Aside from the ‘donor’ strain from which these plasmids and transposons are being mobilised, it should also be noted the genetic composition of recipient strains can ultimately influence the success of the conjugative approach. Indeed, *L. lactis* subsp *lactis* IL1403 is frequently selected for studies of lactococcin A (LcnA; class II)-type bacteriocins as this strain contains chromosomal analogues of the genes involved in LcnA secretion and maturation (i.e. *lcnC* and *lcnD*). Indeed, the extent of bacteriocin production by this strain following the conjugal transfer of pS140, a plasmid harbouring the genetic determinants for a lactococcin A-like bacteriocin, was greater

than that of other lactococcal transconjugants, presumably as a consequence of the additional copies of *lcnCD* already present [23].

Subcloning and expression of bacteriocin genes or gene clusters

Subcloning and expression of bacteriocin genes and gene clusters has also been applied as a means of conferring a bacteriocin positive phenotype on LAB strains or to facilitate over-production in a strain that is already a natural bacteriocin producer. Indeed, a particularly effective strategy employed for the overproduction of various class I bacteriocins has been the introduction of additional copies of biosynthesis-associated genes to an existing bacteriocin-producing host. This has led to greater yields of nisin [24,25] and of the individual lacticin 3147 component peptides, Ltnα and Ltnβ, as well as improved yields of bioengineered lacticin 3147 variants [26]. Such studies have also established that the introduction of additional copies of immunity (self-protection) genes can be important to overcome self-toxicity-associated limitations when overproducing these peptides.

The heterologous production of class II bacteriocins by LAB is dependant on several factors such as the host strain, the expression and secretion systems employed, plasmid stability and copy number and the presence of the cognate bacteriocin immunity genes. While expression systems employing constitutive promoters and inducible promoters (such as the nisin-inducible promoter, (P_{nisA}, of the NIsin Controlled gene Expression (NICE) system [27]) have both been highly exploited, inducible systems have in general been more successful. Regardless of promoter, the strategies involved have varied from cloning of the entire, intact bacteriocin biosynthetic gene cluster [28,29] to the creation of gene fusions (to facilitate efficient bacteriocin transport) through the exploitation of bacteriocin leader or secretion

signals [30-37]. Yeast based platforms have also shown considerable promise. These may be useful for the large-scale production of bacteriocins or for yeast based fermentations [38-41].

Although these recombination techniques can facilitate increased levels of bacteriocin production and activity, and the construction of improved multi-bacteriocin producing strains, they remain genetically modified organisms (GMO) which may limit their application in the wider Food Industry.

Bioengineering of bacteriocin peptides

There have been a number of instances in which bioengineering of bacteriocin structural genes (through manipulation of the gene in a natural producer or in a strain which produces the bacteriocin heterologously) has been employed with a view to expanding or altering the associated antimicrobial spectrum. This strategy initially evolved from a desire to gain a better appreciation of the importance of specific residues or domains within these peptides, i.e. to assess the negative consequences of mutating these regions. However, this approach has evolved such that strains with greater antimicrobial potency have resulted which can potentially provide for the better control of spoilage or pathogenic microbes.

Bioengineering-based strategies were first applied to LAB producers of class I bacteriocins, with the targeting of nisin [42-44] being of greatest relevance to this review. Subsequent, manipulations of nisin were crucial with respect to elucidating the mechanism of action of the peptide [45-48]. From these, and more recent studies, some bioengineered nisins are notable by virtue of possessing enhanced antimicrobial activity against at least one, albeit non-pathogenic, Gram positive target [49-52]. Other bioengineered derivatives of nisin have been identified which more effectively

158 inhibit one or more pathogenic targets. The majority of these are derivatives in which
159 residues within a central, 3-amino acid, stretch known as the ‘hinge’ region have been
160 altered. Here, examples include nisins N20K and M21K [53], nisin M21V [54-56],
161 nisin K22T (figure 1), [55] and nisin N20P [54]. Recently, nisin peptides in which
162 serine 29 has been altered have drawn attention by virtue of exhibiting enhanced
163 activity against both Gram positive and Gram negative pathogens [57]. It should also
164 be noted that there have also been instances in which the nisin structural gene has
165 been altered to facilitate the production of other natural variants of nisin (nisin Z, F
166 and Q; [58]) or in a manner that has resulted in peptides which exhibit enhanced
167 diffusion through complex matrices [59]. Other class I bacteriocin producing LAB,
168 i.e. the producers of lacticin 3147 and lacticin 481, have also been the focus of
169 bioengineering-based strategies. In these cases, the outcomes have been of greater
170 importance from a fundamental science, rather than applied, perspective [60-66], with
171 only one example of a partial enhancement having been described to date [64]. Aside
172 from the lantibiotics, as derivatives of the unmodified class II bacteriocins can be
173 generated both synthetically [67,68] or through heterologous expression [69-77] with
174 relative ease, there are relatively few examples of instances in which LAB producers
175 of the class II bacteriocins have themselves been engineered. However, the potential
176 exists to reconstitute production of some of the more interesting derivatives in the
177 original host strain should the need or desire arise.
178 It is important to note that while all bioengineering based strategies are valid if the
179 aim is to create bacteriocins for fundamental analyses or applications by the
180 pharmaceutical industry, the application of bioengineered bacteriocin peptides as food
181 preservatives is a bigger obstacle in some jurisdictions. Indeed, many of the strategies
182 employed to produce the engineered bacteriocins described above involve approaches

that result in the producer needing to be described as a GMO. However, alternatives exist. Indeed, self cloning of non-pathogenic microorganisms is not considered to lead to a GMO as long as containment of the organism is guaranteed (directive 90/219/EC). Accordingly, the temporary introduction of plasmids, the deletion of specific DNA sequences, or introduction of DNA from another micro-organism belonging to the same species fall within the definition of self-cloning. Thus, subtle alterations to bacteriocin structural genes (such as the changing of single codons) made using food grade strategies [78] fall outside the remit of the Contained Use legislation and therefore are not regulated as GMOs.

Conclusions

There are various methods available to improve the bacteriocin-mediated protection provided by food grade LAB. While genetic manipulation by recombinant and bioengineering based approaches offer great promise, only strains which have been modified through non-recombinant approaches can be directly added to food. In addition to the further improvement of existing strains, advances with respect to high throughput screening strategies are likely to result in the identification of novel antimicrobials with considerable potential for food applications. Few naturally occurring multi-bacteriocin producing LAB have been identified [79-83]. However, ongoing developments in traditional microbiological, mass spectrometric, molecular and bioinformatic screening techniques [84,85] has led to the isolation [86-94] and characterisation of several novel bacteriocins [95-98] reported this year alone which may find applications in food. Regardless of the strain and bacteriocin in question, it is fair to say that the application of bacteriocin producing LAB, alone or in combination with additional antimicrobial hurdles [2], continues to be a relatively

underutilised strategy that, through various enhancements such as those described here, could be more widely applied by the food industry.

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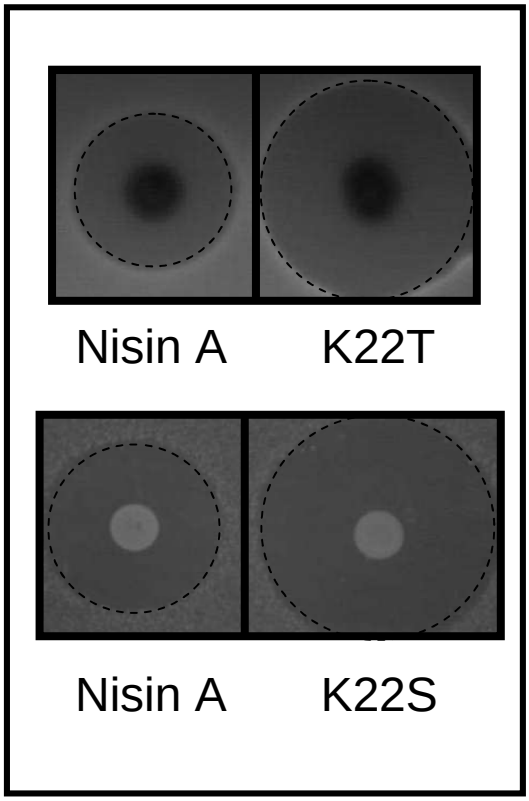

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569 **Figure 1.** Enhanced activity of bioengineered *L. lactis* producing nisin derivatives

570 mutated within the 'hinge' region, K22T and K22S, against *Streptococcus agalactiae*

571 ATCC13813 as compared with the respective nisin A producing controls (as adapted

572 from Field et al., (2008)).