Application of anti-listerial bacteriocins: monitoring enterocin expression by multiplex relative reverse transcription–PCR

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Abstract

Listeriosis is a deadly food-borne disease, and its incidence may be limited through the biotechnological exploitation of a number of anti-listerial biocontrol agents. The most widely used of these agents are bacteriocins and the Class II enterocins are characterized by their activity against *Listeria*. Enterocins are primarily produced by enterococci, particularly *Enterococcus faecium* and many strains have been described, often encoding multiple bacteriocins. The use of these strains in food will require that they are free of virulence functions and that they exhibit a high level expression of anti-listerial enterocins in fermentation conditions. Multiplex relative RT (reverse transcription)–PCR is a technique that is useful in the discovery of advantageous expression characteristics among enterocin-producing strains. It allows the levels of individual enterocin gene expression to be monitored and determination of how expression is altered under different growth conditions.

Listeriosis

Listeriosis is a serious bacterial infection most commonly caused by the ingestion of food contaminated with *Listeria monocytogenes* or one of a group of related species [1]. The disease affects mainly certain high-risk groups including, immunocompromised individuals, pregnant women, newborns and the elderly. It causes abortion, septicemia or infection of the CNS (central nervous system) and has been associated with self-limiting acute gastroenteritis [2]. The disease is a major public health issue in many parts of the world. In the U.K. between 2001 and 2009, there was an average of 192 cases per year. There was concern that this was nearly double that in the previous decade [3], although the situation did improve with 156 cases in 2010 [4]. In the U.S.A., there are an estimated 1600 cases each year and 250 deaths [5]. Mortality rates between 23 and 44% have occurred in various outbreaks [6]. Such worrying statistics have prompted many searches for novel agents active against *Listeria* over the last two decades.

Sources of anti-listerial agents

Owing to the ability of *L. monocytogenes* to survive the usual means of food preservation (refrigeration, low pH and high salt [7]), other approaches to inhibition have been sought. Plant derived extracts from a number of species limit growth or kill *Listeria*. In particular, several essential oils have been found to be effective [8–10].

The renewed interest in bacteriophages as antimicrobial agents, including the use of lytic enzymes from phages, has included the possibility of controlling *Listeria* by that means [11]. The use of drug molecules other than antibiotics, either alone [12] or in synergy with antibiotics [13], has also been tried as a potential therapeutic route. But, by far, the most thoroughly investigated agents have been the bacteriocins.

Bacteriocins

Bacteriocins are peptides synthesized on bacterial ribosomes that mediate antibiotic activity against other bacteria. They play a key role in the interactions between bacteria in natural populations [14]. Whereas the colicins of Gram-negative bacteria have found their niche as model systems for structural biology and mechanistic studies [15], the Gram-positive bacteriocins have enjoyed greater practical application for many years, particularly in food safety [16]. The diversity of bacteriocins being discovered in the lactic acid bacteria led to the proposal of a classification scheme [17] which, with modifications, still describes three major classes.

Class I, the lantibiotics, are small (<5 kDa) peptides characterized by the presence of unusual lanthionine and methyl-lanthionine amino acid residues formed by post-translational modification of dehydrated dehydroalanine and dehydrobutyrine amino acids. The best-known representative of this class is the familiar bacteriocin nisin. Some 37 years after its first reported use in cheese against clostridial spoilage in 1951 [18] and 35 years after its first appearance as a product, under the name Nisaplin®, by Aplin and Barret Ltd, in 1953 [19], nisin A received a GRAS (generally recognized as safe) status by the U.S. FDA (Food and Drug Administration)
Figure 1 | Batch fermentation of *E. faecium* LCW57

A typical fall in pH (■) is seen with an increase in biomass (‘OD600nm’, ●) and the phenotypic expression of anti-listerial activity (arbitrary units, ×10⁻³, ▲).

Figure 2 | Multiplex relative RT–PCR for enterocin A

Plots of average normalized fluorescence intensity against number of PCR cycles. Exponential curves fitted to the triplicate data show good correlation. (A) Comparison between 4 h (●) and 6 h (■). (B) Comparison between 6 h (●) and 8 h (■).
in 1988. The structure, biosynthesis and mode of action of lantibiotics have been reviewed extensively in [20–23].

Class II bacteriocins are a diverse group of small (<10 kDa) unmodified peptides, with characteristic activity against *L. monocytogenes*. Commonly heat-stable peptides with high glycine content, they are also cationic or amphiphilic and can be active against a broad range of Gram-positive bacteria [24].

Class III bacteriocins are large (>30 kDa) heat-labile proteins or enzymes that act in a different way from other bacteriocins. Most are produced by lactobacilli [25].

**Enterocins**

Enterocins produced by *Enterococcus* species are mainly class II bacteriocins distinguished by activity against *Listeria* spp. There are numerous examples of where a search for novel anti-listerial agents in natural food products has ended with the isolation of *Enterococcus faecium* strains harbouring multiple enterocins [26]. The production of multiple enterocins is a desirable trait as they may express activity under different conditions [27]. Such enterococci are often natural components of the flora of many foods such as artesanal cheeses where they contribute to the organoleptic properties [28]. In contrast, enterococci may also be pathogenic, and specific recommendations have been made regarding the safety of strains intended for human consumption [29], for example as protective cultures in fermentation.

Using the principle that the best place to search for antilisterial organisms was where *Listeria* occurs naturally, we isolated 17 strains with this property from raw sheep milk samples used for feta cheese production. All of the strains proved to be *E. faecium* harbouring genes for enterocins A and B, and either enterocin P or L50. They also lacked key virulence genes, so can be considered safe [30]. One of these strains, LCW57, has been used for molecular analysis of enterocin gene expression.

**Enterocin expression**

Usually, the detection of enterocin expression and the titration of activity have been done indirectly with a bioassay of the cell-free supernatant against the target micro-organism [31]. This phenotypic approach is potentially misleading and not only because of influencing factors such as the stability of the enterocin in the extracellular medium and variation in the interaction with target cells. Where multiple enterocins are produced, it is not known which is having the effect. It is now common to use qualitative PCR to detect and

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**Figure 3** | Multiplex relative RT-PCR for enterocin B

Plots of average normalized fluorescence intensity against number of PCR cycles. Exponential curves fitted to the triplicate data show good correlation. (A) Comparison between 4 h (●) and 6 h (■). (B) Comparison between 6 h (●) and 8 h (■).
identify enterocin genes [32] and RT (reverse transcription)–PCR has been used to detect enterocin gene transcription [33]. However, we examined the timing of enterocin gene expression using strain LCW57.

Batch culture of LCW57 followed the typical lactic acid bacterial pattern and anti-listerial activity appeared to climb between 4 and 6 h and then reach a plateau (Figure 1). The multiplex relative RT–PCR method of Spencer and Christensen [34] was used to measure the individual transcription of enterocin genes present in this strain. Although qualitative PCR had revealed the presence of the entA, entB and entP genes, initial amplification of cDNA showed that only entA and entB were expressed, and expression was detectable to the end of the batch at 12 h. The production of cDNA from these genes at the key time points of 4, 6 and 8 h of fermentation was compared with that from the 165 rRNA housekeeping genes. The RNA levels were indirectly measured by the level of fluorescence of specific PCR amplicons, generated from the cDNAs, after electrophoresis and staining. Predetermination of the minimum number of cycles required to detect cDNA from the housekeeping gene and the enterocin genes ensured that the PCRs were in exponential amplification. The fluorescence values for the enterocin product were normalized against those of the 16S rRNA product. The fluorescence values for the enterocin product were normalized against those of the 16S rRNA product. The number of PCR cycles required for the normalized enterocin signal to cross a threshold (in the classical real-time PCR manner) enables the determination of the difference in expression between the time points in the fermentation. It was revealed that entA expression declined 1.7-fold between 4 and 6 h and a further 3.7-fold between 6 and 8 h (Figure 2). entB expression fell 1.5-fold between 4 and 6 h and 1.52-fold between 6 and 8 h (Figure 3). The implication is that this strain, early in the growth phase, invests considerable energetic resources in the production of enterocins. In the natural mixed environmental population, this would select a competitive advantage. The observed stability of the enterocins (Figure 1) allows the diversion of this energy to other functions as resources dwindle with the decrease in fermentable substrates.

Conclusions

With the reluctance of some countries to use genetically modified bacteria in foodstuffs, there is likely to be a competitive advantage in the use of naturally occurring isolates. Specific PCRs can reveal the presence of multiple bacteriocin genes. Multiplex relative RT–PCR allows subtle differences in the expression of such genes to be monitored. This can be applied to real fermentation and aid in the optimization of conditions antagonistic to the survival of Listeria. The technique is relatively laborious, but has certain advantages: only the desired PCR product is quantified and not any PCR artefacts, it avoids the need for a qPCR (quantitative PCR) instrument, and no expensive labelled probe molecules are required to ensure specificity. For example, strain LCW57 enterocins A and B were shown to be expressed early in batch culture, which is an advantage, in that they could inhibit Listeria from an early stage. The latter decline in expression could be seen as a disadvantage. Alteration of the control of expression in this strain could lead to even higher levels of anti-listerial activity.

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References


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