Resistance to bacteriocins produced by Gram-positive bacteria

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Bacteriocins are prokaryotic proteins or peptides with antimicrobial activity. Most of them exhibit a broad spectrum of activity, inhibiting micro-organisms belonging to different genera and species, including many bacterial pathogens which cause human, animal or plant infections. Therefore, these substances have potential biotechnological applications in either food preservation or prevention and control of bacterial infectious diseases. However, there is concern that continuous exposure of bacteria to bacteriocins may select cells resistant to them, as observed for conventional antimicrobials. Based on the models already investigated, bacteriocin resistance may be either innate or acquired and seems to be a complex phenomenon, arising at different frequencies (generally from $10^{-9}$ to $10^{-2}$) and by different mechanisms, even amongst strains of the same bacterial species. In the present review, we discuss the prevalence, development and molecular mechanisms involved in resistance to bacteriocins produced by Gram-positive bacteria. These mechanisms generally involve changes in the bacterial cell envelope, which result in (i) reduction or loss of bacteriocin binding or insertion, (ii) bacteriocin sequestering, (iii) bacteriocin efflux pumping (export) and (iv) bacteriocin degradation, amongst others. Strategies that can be used to overcome this resistance are also addressed.

Introduction

Bacteriocins are prokaryotic antimicrobial proteins or antimicrobial peptides. As many of these substances reported in the literature have a broad spectrum of action (Heng et al., 2007), exhibiting antagonistic activity against several pathogens, they have potential biotechnological applications (Bastos & Ceotto, 2011; Cotter et al., 2013). Due to the risks derived from the presence of food-borne pathogens or spoilage bacteria in food, and also from the chemical preservatives used in food to control undesirable micro-organisms, the interest in the discovery of new biopreservatives has increased recently (Bastos & Ceotto, 2011). Due to the low availability of new drugs that could be used to control drug-resistant pathogens, bacteriocins have become an important option (Cotter et al., 2013). Therefore, many bacteriocins have been investigated with respect to their potential use in promoting human, plant and animal health, and in enhancing food safety and quality.

Gram-positive bacteria are the major source of bacteriocins examined for biotechnological applications (Bastos & Ceotto, 2011; Cotter et al., 2005, 2013). These substances are ribosomally synthesized, and are generally cationic and hydrophobic. Different schemes of classification have been proposed for bacteriocins produced by these micro-organisms. In this review, we adopt a classification (Table 1) which is an update of the classification proposed by Bastos et al. (2009), where bacteriocins are divided into four major classes, combining the propositions of Heng et al. (2007), Nes et al. (2007), Bierbaum & Sahl (2009), Nissen-Meyer et al. (2009) and van Belkum et al. (2011), with modifications. Most classes have been subdivided into types or subclasses. Class I bacteriocins, the so-called lantibiotics, are small, heat-stable peptides that contain modified amino acids formed by post-translational modifications (Bierbaum & Sahl, 2009). Class II bacteriocins are small, heat-stable peptides with no modified amino acids (Nissen-Meyer et al., 2009). Heat-labile antimicrobial proteins are included in class III (Heng et al., 2007), whereas class IV is composed of heat-stable cyclic peptides (van Belkum et al., 2011).
As many bacteriocins exhibit activity at nanomolar concentrations against the target micro-organisms, are bactericidal against drug-resistant bacteria and are non-cytotoxic to eukaryotic cells, biotechnological applications have been proposed for most of them (Bastos et al., 2009, 2010; Bastos & Ceotto, 2011; Cotter et al., 2005, 2013). Examples are given in Table 2.

Mechanisms involved in bacteriocin action

The mechanisms involved in the inhibitory activity of bacteriocins produced by Gram-positive bacteria towards target cells have been shown to be diverse. As most peptide bacteriocins are cationic, interaction between these molecules and anionic components of the bacterial cell surface is considered to play an essential role in the initial stages of bacteriocin activity (Jack et al., 1995). In a general manner, these bacteriocins are presumed to cause cell death through membrane permeabilization and pore formation, due to their cationic and amphipathic characteristics. Some bacteriocins are able to impair cell wall formation, thus compromising cell envelope strength (Bastos et al., 2010; Bierbaum & Sahl, 2009).

Class I bacteriocins (lantibiotics) may possess two modes of action, both involving lipid II as a docking molecule. Lipid II is present in the cytoplasmic membrane and plays a central role in building the cell wall through the transport of peptidoglycan monomers from inside out (Breukink et al., 1999). Some type AI lantibiotics, such as nisin, interact with lipid II and then with the membrane, producing a poration complex at this site. Binding of nisin to lipid II also inhibits cell wall synthesis by blocking the lipid II cycle (Breukink et al., 2003; Brotz et al., 1998; van Heusden et al., 2002). With regard to types AII and IB lantibiotics, and to lactococcin 972 (a subclass IIc bacteriocin), their antimicrobial action is due only to the

Table 1. Classification proposed for bacteriocins produced by Gram-positive bacteria

<table>
<thead>
<tr>
<th>Classification</th>
<th>Relevant features</th>
<th>Type/subclass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I (lantibiotics)</td>
<td>Small, linear peptides (&lt;5 kDa) with modified amino acids</td>
<td>IA (linear)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IB (globular)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IC (two components)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ID (reduced antimicrobial activity)</td>
</tr>
<tr>
<td>Class II</td>
<td>Small, linear peptides (&lt;10 kDa) without modified amino acids</td>
<td>IIA (pediocin-like)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IIB (two components)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IIC (leaderless peptide)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IID (formed by specific degradation of larger proteins)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IIE (more than two components)</td>
</tr>
<tr>
<td>Class III</td>
<td>Large, heat-labile proteins (&gt;25 kDa)</td>
<td>IIIA (bacteriolysins)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IIIB (non-lytic)</td>
</tr>
<tr>
<td>Class IV</td>
<td>Small, cyclic peptides (&lt;8 kDa)</td>
<td>–</td>
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</tbody>
</table>

*This subclass encompasses a miscellaneous collection of bacteriocins with distinct features.

Table 2. Examples of biotechnological applications of bacteriocins produced by Gram-positive bacteria

<table>
<thead>
<tr>
<th>Bacteriocin (type/subclass)</th>
<th>Producer micro-organism</th>
<th>Potential application(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nisin (IA)</td>
<td>Lactococcus lactis</td>
<td>Food biopreservative and bovine mastitis prevention</td>
<td>Cotter et al. (2005)</td>
</tr>
<tr>
<td>Nukacin 3299 (IA)</td>
<td>Staphylococcus simulans</td>
<td>Prevention of streptococcal bovine mastitis</td>
<td>Ceotto et al. (2010)</td>
</tr>
<tr>
<td>Mutacin 1140 (IA)</td>
<td>Streptococcus mutans</td>
<td>Prevention of dental caries</td>
<td>Hillman et al. (2007)</td>
</tr>
<tr>
<td>Hyicin 3682 (IA)</td>
<td>Staphylococcus hyicus</td>
<td>Control of phytopathogens</td>
<td>Fagundes (2014)</td>
</tr>
<tr>
<td>Lacticin 3147 (IC)</td>
<td>Lactococcus lactis</td>
<td>Bovine mastitis prevention</td>
<td>Ryan et al. (1999)</td>
</tr>
<tr>
<td>Pediocin PA-1 (IIa)</td>
<td>Pediococcus acidilactici</td>
<td>Food biopreservative</td>
<td>Cotter et al. (2005)</td>
</tr>
<tr>
<td>Aureocin A53 (IId)</td>
<td>Staphylococcus aureus</td>
<td>Prevention and control of bovine mastitis</td>
<td>Coelho et al. (2007)</td>
</tr>
<tr>
<td>Lysostaphin (IIIa)</td>
<td>S. simulans subsp. staphylyoticus</td>
<td>Prevention and control of human and animal infections caused by S. aureus</td>
<td>Bastos et al. (2010)</td>
</tr>
<tr>
<td>Enterocin AS-48 (IV)</td>
<td>Enterococcus faecalis</td>
<td>Food biopreservative</td>
<td>Gálvez et al. (2007)</td>
</tr>
</tbody>
</table>
inhibition of cell wall synthesis (Bierbaum & Sahl, 2009; Islam et al., 2012; Martínez et al., 2000). Finally, the two-component lantibiotics (type IC) initiate their action with the interaction of peptide A1 (also called α) and the cytoplasmic membrane, resembling the type A1 mode of action. Lipid II is then targeted, blocking the transport of the peptidoglycan monomers, whilst the A1 peptide suffers a conformational change, exposing the A2 (also called β) peptide-binding site, thus creating the pore formation complex (Wiedemann et al., 2006).

The best understood mechanism of action of class II bacteriocins is exemplified by subclass IIa – the pediocin-like bacteriocins. These bacteriocins use the mannose–phosphotransferase system (Man-PTS) as a docking molecule through which they exert their activity, leading to permeabilization of the cell membrane, disruption of the proton motive force and depletion of the ATP pool, which, consequently, results in arrest of all cellular biosynthesis (Nissen-Meyer et al., 2009; Ramnath et al., 2000, 2004). Diep et al. (2007) also showed that some subclass IIC bacteriocins employ a similar mechanism to kill the target cells. The mode of action of all subclass IIb bacteriocins investigated to date also involves membrane permeabilization to a variety of small molecules (Nissen-Meyer et al., 2009). Aureocin A53 is a subclass IId bacteriocin which rapidly dissipates the membrane potential and simultaneously stops biosynthesis of DNA, polysaccharides and proteins in the target cells. The bacteriolytic activity of aureocin A53 is derived from extensive cell lysis via generalized membrane destruction (Netz et al., 2002).

Lysostaphin is the most studied member of subclass IIIa and its lytic action is due to glycylglycine endopeptidase activity, specifically against the cell wall of some staphylococcal species (Schindler & Schuhardt, 1965). Lysostaphin cleaves the cross-bridges of the peptidoglycan, which are composed of five glycine residues (Browder et al., 1965).

Finally, class IV bacteriocins, similar to most bacteriocins from classes I and II, permeabilize the membrane of the susceptible cells, causing dissipation of the membrane potential, leakage of ions and cell death (van Belkum et al., 2011).

Bacteriocin immunity

Bacteriocin-producer strains are often protected from the activity of their own products by immunity systems, whose encoding genes are generally found in the bacteriocin gene clusters (Bierbaum & Sahl, 2009; Heng et al., 2007; Nissen-Meyer et al., 2009; van Belkum et al., 2011). Consequently, a bacteriocin-producing strain is generally not sensitive to the action of its cognate bacteriocin. Additionally, related bacteriocins may exhibit cross-immunity, as observed for some subclass IIa (Fimland et al., 2002) and subclass IIb (Oppegård et al., 2010) bacteriocins, and for the lantibiotics PepS and epicidin 280 (Heidrich et al., 1998), amongst others. However, for at least some subclass IIB bacteriocins, the cross-immunity is target cell dependent, which suggests that its function is dependent on a cellular component, probably the bacteriocin receptor (Oppegård et al., 2010).

The mechanisms involved in immunity are still poorly understood for most bacteriocins. In many systems, bacteriocin producers rely on a single small protein for self-protection. These proteins have been detected either anchored to the membrane surface (Dubois et al., 2009; Heidrich et al., 1998) or embedded in the membrane (Bierbaum & Sahl, 2009; Coelho et al., 2014). Some are largely exported, remaining trapped at the membrane and within the cell wall compartment (Hoffmann et al., 2004). The roles proposed for these proteins include blocking the insertion of the bacteriocin into the membrane or protection of a dedicated target, shielding it from the bacteriocin (Dubois et al., 2009).

For some class II bacteriocins, the immunity protein forms a strong complex with the bacteriocin bound to its receptor, preventing cells from being killed (Diep et al., 2007). In the examples already described, the receptors are components of the Man-PTS, which is generally composed of three proteins: enzyme I (EI), histidine phosphocarrier protein (HPr) and enzyme II (EII). EI and HPr are cytoplasmic proteins involved in the transfer of a phosphate group to EII (Postma et al., 1993). EII consists of four subunits: IIA, IIB, IIC and IID. Subunits IIA and IIB are located in the cytoplasm, whereas subunits IIC and IID form a membrane-bound complex through which the sugar molecules enter the cell. Diep et al. (2007) demonstrated that lactococcin A uses the subunits IIC and IID as a receptor on the target cells and that the immunity protein (LciA) binds to the bacteriocin–receptor complex to confer immunity to producer cells. A similar mechanism of immunity has also been shown for subclass IIa bacteriocins (Diep et al., 2007).

For some class II bacteriocins, immunity relies on the activity of putative membrane-bound metallopeptidases which belong to the Abi protein family (Kjos et al., 2010a). As these proteins are proteases, Kjos et al. (2010a) postulated that Abi-mediated immunity arises by either direct degradation of the bacteriocin or modification of a receptor protein.

Another immunity mechanism reported for some class II bacteriocins depends on the activity of multi-drug transporter proteins, which participate in bacteriocin immunity by removing bacteriocin that enters the cytoplasmic membrane from the outside (Gajic et al., 2003).

For some lantibiotics (Bierbaum & Sahl, 2009) and aureocin A53 (Nascimento et al., 2012), immunity results from the combined action of a cognate immunity protein, which binds to bacteriocin molecules on the bacterial cell membrane, and a multi-component ATP-binding cassette (ABC) transporter, which removes the bacteriocin from cells. Proteins involved in immunity to cyclic bacteriocins are probably membrane-associated, but the role of a
cognate immunity protein is also complemented by ABC transporters, required for full immunity to cyclic peptides (van Belkum et al., 2011).

Lysostaphin producers do not have specific immunity genes. However, the peptidoglycan synthesized by the lysostaphin-producer strains is resistant to the hydrolytic activity of the bacteriocin, due to the incorporation of serine residues into the third and fifth positions of the pentapeptide cell wall cross-bridges (Ehlert et al., 2000; Thumm & Götz, 1997). Lysostaphin is unable to hydrolyse glycolcerine and serylglycine peptide bonds. The product encoded by the lif (lysostaphin immunity factor) gene is required for such incorporation, changing the lysostaphin target in the producer cells (Ehlert et al., 2000; Thumm & Götz, 1997).

**Bacteriocin resistance development**

Once a new antimicrobial agent is described, and proven to be safe and effective against pathogens, it is crucial to evaluate the potential risks of resistance development upon prolonged exposure to it ( Cotter et al., 2013). The frequency at which susceptible organisms can develop resistance to a given bacteriocin is, therefore, a very important issue to consider when bacteriocin-based biocatalysis strategies are proposed. Bacteriocins have not been extensively used in the clinical setting ( Cotter et al., 2013). Nisin has long been used as a food biopreservative, but nisin resistance has not been reported amongst food-spoilage micro-organisms in the food industry ( Blake et al., 2011). Therefore, our understanding of the potential for bacteriocin resistance development has been revealed primarily from experiments performed under laboratory conditions ( Cotter et al., 2013).

Bacteriocin resistance is often not defined clearly in the literature ( Katla et al., 2003). Enhanced nisin resistance in *Listeria monocytogenes* is generally defined as <10-fold increase in MIC ( Gravesen et al., 2004). If the mutant is able to grow in the presence of the highest bacteriocin concentration tested, it is generally considered a high-level resistant mutant. In a study published by Blake et al. (2011), mutants displaying an eightfold or less decrease in nisin susceptibility were considered to exhibit low resistance, whilst those exhibiting a 32-fold increase in nisin MIC were considered to be highly resistant. However, for subclass IIa bacteriocins, mutants were considered to be highly resistant to a given bacteriocin when (i) they were able to grow on agar plates containing ≥1600 arbitrary units (AU) bacteriocin ml−1 ( Rasch & Knochel, 1998), (ii) the MICs/IC50 were 1000 times higher than those for the parental strains ( Gravesen et al., 2002b; Katla et al., 2003) or (iii) the IC50 was ≥1 μg ml−1 ( Tessema et al., 2009). When mutants were inhibited by <1600 AU bacteriocin ml−1 ( Rasch & Knochel, 1998) or when the IC50 was <1 μg ml−1 ( Tessema et al., 2009), they were characterized as having enhanced tolerance or low-level resistance. As can be seen, there is no consensus amongst investigators in relation to what is considered high-, moderate- or low-level resistance to bacteriocins.

One critical factor in susceptibility tests is the bacteriocin solution. In some studies, purified preparations [given in μg or international units (IU)] were used, whereas in others the fermentates (given in AU) of the producer organisms were employed. Therefore, we agree with Katla et al. (2003) who pointed out that it is generally difficult to compare studies on susceptibility to bacteriocins due to differences in experimental approaches and terminology.

Several methods have been used to determine differences in bacterial susceptibility to bacteriocins ( Katla et al., 2003), including plating of saturated cultures on agar media containing either a single dose or different amounts of the bacteriocins. However, the most sensitive method is the microtitre plate assay, employing bacteriocin solutions of known concentrations ( Katla et al., 2003). After incubation at the appropriate conditions, bacterial growth is monitored either visually or by optical density.

Bacterial antimicrobial peptides generally act fast, which drastically reduces the possibility of resistance development in their presence in a population containing only susceptible target cells ( Cotter et al., 2005). However, mutations resulting in deresistance to bacteriocins may arise in the absence of bacteriocins, being selected in their presence. Bacteriocin resistance may develop on solid media, and in food and animal model systems ( Blake et al., 2011; Climo et al., 2001; Graversen et al., 2002b; Rasch & Knochel, 1998; Wan et al., 1997). As, to best of our knowledge, there has not been a review published on this subject, we highlight the main findings concerning the resistance to bacteriocins produced by Gram-positive bacteria in the present publication.

According to Collins et al. (2012), the mechanisms involved in bacteriocin resistance can be divided into two groups: acquired resistance (that developed by a formerly susceptible strain) and innate resistance (that intrinsically found in particular genera or species). Several genetic loci have been linked with either innate or acquired resistance. Their involvement in bacteriocin resistance has generally been shown by mutations generated by gene knockouts or deletions, complementation or overexpression analysis. A given gene is considered to be involved in innate resistance when mutations in the gene lead to bacteriocin sensitivity, whereas it is considered to be associated with acquired resistance when mutations in the gene lead to bacteriocin resistance. The cell alterations responsible for resistance and resulting from some of these mutations have not yet been elucidated. Nevertheless, descriptions of the genes will be included in the present review as their roles in bacteriocin resistance deserve further investigation and could attract the attention of investigators, as this knowledge is important for optimization of bacteriocin use ( Tessema et al., 2011).

The bacteriocins most studied with regard to the development of resistance are nisin, lacticin 3147, pediocin-like bacteriocins and lysostaphin. Both nisin and lacticin 3147 are produced by strains of *Lactococcus lactis*. Nisin A is the most studied lantibiotic, and it has already
found applications as a biopreservative in a broad range of foods worldwide and in the prevention of bovine mastitis (Cotter et al., 2005). Lactocin 3147 is a two-peptide lantibiotic which is bactericidal against many pathogens. Its use as a food preservative has been proposed to control food-spoilage bacteria as well as pathogens (Suda et al., 2012). It has also been used in teat seals for prevention of intramammary infections in dry cows (Suda et al., 2012). Pediocin-like bacteriocins are subclass IIa peptides produced by strains of Pediococcus spp. and other lactic acid bacteria, which exhibit a strong anti-listerial activity (Nissen-Meyer et al., 2009). Listeria monocytogenes is a food-borne pathogen which causes listeriosis – an infection that can be fatal (Freitag et al., 2009). Pediocin PA-1/AcH is also approved for use in food (Bastos & Ceotto, 2011). Lysostaphin is an enzyme produced by Staphylococcus simulans subsp. staphyloyticus, which exhibits bacteriolytic activity towards staphylococci, including Staphylococcus aureus. It is the most investigated class III bacteriocin in relation to clinical applications (Bastos et al., 2010).

Fig. 1 summarizes some mechanisms involved in resistance to bacteriocins, which will be further explained in the text. However, readers interested in a more detailed description of the experiments performed and the systems investigated in each case are referred to the original publications.

Innate resistance

Innate susceptibility to bacteriocins may vary greatly amongst different bacterial strains (Katla et al., 2003). However, few studies have attempted to compare a large number of strains. In the study performed by Rasch & Knochel (1998), using 381 strains of L. monocytogenes, none of the strains was considered naturally nisin resistant and only two strains (0.52 %) were characterized as exhibiting enhanced nisin tolerance, being able to grow on agar plates containing 500 IU nisin ml\(^{-1}\). However, 20 strains (5.2 %) were considered resistant to pediocin PA-1, growing on 1600 AU ml\(^{-1}\), and 34 strains (8.9 %) were characterized as exhibiting enhanced tolerance to pediocin, growing on 400 AU ml\(^{-1}\). When 200 strains of L. monocytogenes were tested by Katla et al. (2003) for susceptibility to nisin and three subclass IIa bacteriocins (pediocin PA-1, sakacin P and sakacin A), a 60–360-fold difference in sensitivity was observed. The susceptibility differences measured in the microtiter plates assays were comparable to those observed for the same strains in food model systems (Katla et al., 2002).

Innate bacteriocin resistance may result from different mechanisms. The main mechanisms are summarized in Fig. 2.

Resistance due to immunity mimicry

Immune mimicry is a mechanism which contributes to protection specifically towards bacteriocins. In such cases, non-bacteriocin-producing strains carry genes which encode functional homologues of bacteriocin immunity systems, also designated ‘orphan immunity genes’. This phenomenon has been described for lantibiotics (Draper et al., 2009) and class II bacteriocins (Finland et al., 2002). It has been shown that heterologous expression of these orphan immunity genes confers protection against the cognate bacteriocin (Draper et al., 2012).

Resistance due to bacteriocin degradation

This resistance mechanism also seems to be specific for bacteriocins. Some nisin-resistant strains of Bacillus spp. produce an enzyme, nisinase, which degrades nisin (Jarvis, 1967). Nisinase was shown to be produced by strains of Bacillus cereus and Paenibacillus (formerly Bacillus) polymyxa during sporulation. Nisinase breaks the C-terminal lanthionine ring (Jarvis & Farr, 1971) and is also active against subtilin, but not against bacitracin, polymyxin and gramicidin (Jarvis, 1967). This absence of cross-resistance towards antibiotics is quite relevant. If bacteriocin-resistant mutants become simultaneously resistant to antibiotics, such findings may create concern with regard to clinical bacteriocin applications.

Nisin degradation has also been observed in some non-nisin-producing La. lactis strains. This degradation is catalysed by the nisin resistance protein NSR protease localized on the cell membrane, which removes the nisin C-terminal tail (Sun et al., 2009).

Butcher & Helmann (2006) proposed that the proteolytic activity of YqeZ (a predicted membrane-integrated protease), encoded by the \(\sigma^W\)-regulated yqeZyqfAB operon, could provide protection against the lantibiotic sublancin 168 in bacilli.

Resistance associated with growth conditions

Innate bacteriocin resistance seems to depend on various factors. Listeria monocytogenes 412 stationary-phase cells exhibited higher resistance to either nisin (300 IU ml\(^{-1}\)) or pediocin PA-1 (320 AU ml\(^{-1}\)) than exponential-phase cells (Jydegaard et al., 2000). However, no effect of growth phase was observed for a high pediocin concentration (2560 AU ml\(^{-1}\)). Tolerance to pediocin PA-1 was also observed in osmotically stressed cultures (6.5 % NaCl) and in cold-stressed cells (5 °C for 60–80 min) (Jydegaard et al., 2000). The former results were probably explained by changes in electrostatic interactions between the bacteriocin and the cell surface in the presence of high ion concentrations. Additionally, cell morphology may change due to increased osmolarity of the growth medium, leading to changes in the cell envelope (Jydegaard et al., 2000).

Nisin resistance of L. monocytogenes has also been associated with acid stress response (Begley et al., 2010; van Schaik et al., 1999). The glutamate decarboxylase system is considered the most important system employed by this species to withstand low pH stress (Begley et al., 2010). In this system, glutamate undergoes decarboxylation to
\(\gamma\)-aminobutyrate and carbon dioxide. In this reaction, catalysed by GadD1, GadD2 and GadD3, ATP is formed, contributing to ATP pools within the cell. \(\gamma\)-Aminobutyrate is then transported by the antiporters GadT1 and GadT2 (Begley et al., 2010). Begley et al. (2010) investigated a complete set of gad deletion mutants of \textit{L. monocytogenes} LO28 and found that only the \(\Delta\text{gadD1}\) mutant was susceptible to nisin, exhibiting a 10^2-fold reduction survival in the presence of 300 \(\mu\)g nisin ml\(^{-1}\). They also observed a 40 \% reduction in the intracellular ATP levels found in the \(\Delta\text{gadD1}\) mutant. Based on these data, Begley et al. (2010) proposed that, as nisin activity ultimately leads to release of ATP and cell death, GadD1 may restore the intracellular ATP pools, leading to nisin resistance. Similar results were observed for \(\text{gadB}\) mutants of \textit{La. lactis} (Begley et al., 2010).

Fig. 1. Some mechanisms and genes involved in bacterial resistance to bacteriocins produced by Gram-positive bacteria. Bac, bacteriocin; \textit{brg}, bacteriocin resistance gene; CM, cytoplasmic membrane; Crm, chromosome; CW, cell wall; gad, glutamate decarboxylase; \textit{imm}, immunity; LTA, lipoteichoic acid; PBP, penicillin-binding protein; \textit{sig}, sigma factor; TA, teichoic acid; 2/3CS, two/three-component signal transduction system.
Resistance due to changes in the bacterial cell envelope

Many Gram-positive bacteria have teichoic acids (TAs) or lipoteichoic acids (LTAs) as major constituents of the cell wall (Fischer, 1988). These molecules extend through the peptidoglycan to the outer cell surface and their backbone is highly charged by deprotonized phosphate groups. However, backbone esterification with D-alanine reduces the cell wall net negative charge by the introduction of basic amino groups (Fischer, 1988). An example of a system contributing to innate bacteriocin resistance in Gram-positive bacteria is the \textit{dlt} operon, which codes for proteins required for the incorporation of \textit{D}-alanine to TAs and LTAs (Peschel \textit{et al.}, 1999). Mutations in the \textit{dltA} gene result in increased sensitivity to bacteriocins due to a defective \textit{D}-alanine incorporation to TAs or LTAs. Consequently, the mutant cells carry an increased negative surface charge, resulting in increased interaction of bacteriocins with the bacterial surface and, therefore, in cell inhibition (Peschel \textit{et al.}, 1999).

The involvement of the \textit{dlt} operon in innate resistance to bacteriocins and other cationic antimicrobial peptides (CAMPs) is probably a common occurrence amongst Gram-positive bacteria, and it has been experimentally proven for \textit{S. aureus} (Peschel \textit{et al.}, 1999), \textit{Clostridium difficile} (McBride & Sonenshein, 2011a) and \textit{Bacillus cereus} (Abi Khattar \textit{et al.}, 2009), amongst other species. Moreover, the expression of the \textit{dltDABC} genes was shown to increase when \textit{Clostridium difficile} WT cells were grown in the presence of bacteriocins (McBride & Sonenshein, 2011a).

A functional MprF protein is required for the biosynthesis of lysylphosphatidylglycerols (Peschel \textit{et al.}, 2001), whose presence in the bacterial cytoplasmic membrane also reduces the net negative charge of the cell envelope, contributing to bacteriocin resistance. An alteration in the cell envelope charge due to defective incorporation of lysine to membrane phospholipids is also responsible for the increased sensitivity to bacteriocins and other CAMPs exhibited by \textit{mprF} gene mutants (Peschel \textit{et al.}, 2001; Samant \textit{et al.}, 2009; Thedieck \textit{et al.}, 2006).

The response regulator VirR, which is part of the two-component signal transduction system (2CS) VirRS, positively controls the expression of both \textit{dltA} and \textit{mprF} in \textit{L. monocytogenes} (Mandin \textit{et al.}, 2005). In \textit{S. aureus}, the role of VirRS in the regulation of the \textit{dlt} operon and the \textit{mprF} gene is played by the 2CS GraRS (Falord \textit{et al.}, 2011). Therefore, as expected, inactivation of VirR or GraR results in enhanced bacterial susceptibility to bacteriocins and other CAMPs (Falord \textit{et al.}, 2011; Kawada-Matsuo \textit{et al.}, 2013a; Thedieck \textit{et al.}, 2006).

Alternative sigma factors, which control cell envelope structure and charge, may also play a role in innate resistance.
resistance, as reported by Kingston et al. (2013) for Bacillus subtilis. In this micro-organism, the sigma factors $\sigma^M$ and $\sigma^X$ contribute to resistance against lantibiotics, as mutants lacking $\sigma^M$ and $\sigma^X$ exhibit an increased susceptibility to nisin, mesacidin, gallidermin and subtilin. $\sigma^M$ contributes to resistance through expression of ItaSa, which encodes a stress-activated LTA synthase, whereas $\sigma^X$ activates the synthesis of phosphatidylethanolamine, which decreases the net negative charge of the cell envelope (Cao & Helmann, 2004) and the expression of the dlt operon (Cao & Helmann, 2004; Kingston et al., 2013).

The sigB gene is an important mediator of the bacterial stress response and is also involved in the resistance of L. monocytogenes to bacteriocins. In the studies performed by Begley et al. (2006), a mutation of sigB of L. monocytogenes resulted in a reduced tolerance to nisin (300 $\mu$g ml$^{-1}$) and lactacin 3147 (100 AU ml$^{-1}$) in broth survival experiments, suggesting the role of SigB in innate resistance. A total of 55 genes, including those encoding putative efflux pumps, penicillin-binding proteins (PBPs), autolysins and proteins involved in the modification of the bacterial cell envelope, were shown to be positively regulated by SigB using microarray analysis. However, the exact role of SigB in L. monocytogenes bacteriocin resistance has not yet been determined, although Begley et al. (2006) postulated that SigB may control membrane charge or lipid composition, as alteration of these characteristics affects bacteriocin binding or insertion. SigB may also regulate transporters involved in bacteriocin efflux. However, contradictory results were reported by Palmer et al. (2009), who showed that a $\Delta$sigB mutant of L. monocytogenes was more resistant to nisin in broth survival assays, suggesting a role for SigB in acquired resistance. Such discordance may, however, be explained by differences in the experiments performed, such as assay procedures, bacteriocin concentrations and growth phase of L. monocytogenes (Laursen et al., 2014).

Kawada-Matsu et al. (2013b), studying the 2CS NsrRS found in Streptococcus mutans, observed that mutants affected in the nrsr genes exhibited an increased susceptibility to nisin. NsrRS regulates the expression of NsrX, which is predicted to be a membrane-bound protein. The results of a nisin-binding assay showed that a $\Delta$nsrX mutant exhibiting increased sensitivity to nisin could only bind significantly to nisin when complemented with a functional nrsr gene. Based on these results, the authors proposed that NsrX, or an unidentified factor modified through NsrX, binds to nisin and inhibits its binding to lipid II, preventing nisin activity.

Several other 2CSs have been implicated in innate bacteriocin resistance. These are encoded by an operon which codes for a response regulator and a histidine kinase (HK). This operon lies upstream of a second operon which encodes a putative ABC transporter, generally composed of two separate domains: an ATP-binding domain and a permease domain (Blake et al., 2011). The ABC transporter is regulated by the 2CS and is believed to participate in bacteriocin resistance by mediating its export (Blake et al., 2011). Examples of 2CSs with such a role in innate resistance include graRS/vraFG (Kawada-Matsu et al., 2013a) and braRS (also called nsaRS or bcrRS/vraDE (Hiron et al., 2011; Kawada-Matsu et al., 2013a), both found in S. aureus, and lcrRS/lctFEG (in this case, a three-component ABC transporter), in Streptococcus mutans (Kawada-Matsu et al., 2013b). The BraRS system was shown to be specific for nisin and nukacin ISK-1. Conversely, GraRS confers broad-spectrum resistance against CAMPs (Kawada-Matsu et al., 2013b).

Another example of a transporter involved in innate bacteriocin resistance is AnrAB, described in L. monocytogenes (Collins et al., 2010a). This transporter was also shown to provide protection against bacitracin and $\beta$-lactam antibiotics (Collins et al., 2010a).

Other genetic loci involved in innate resistance

Collins et al. (2010b) described a locus, lmo1967, required for innate nisin resistance in L. monocytogenes. This locus is a homologue of the tellurite resistance gene tela, and its role in innate nisin resistance was shown by analysing different types of mutant. All mutants became fourfold more susceptible to the lantibiotics nisin and gallidermin, and also to cefuroxime, cefotaxime and bacitracin. This was the first report associating a tela gene with innate bacteriocin resistance, although no mechanism has yet been proposed to explain the resistance phenotype.

Acquired resistance

There is substantial variation in the frequency of spontaneous mutations resulting in bacteriocin resistance, depending on the micro-organism, the bacteriocin and the strain tested, and the assay employed, including the bacteria/bacteriocin ratio and environmental conditions (Blake et al., 2011; Gravesen et al., 2002a; Mazzotta et al., 1997; Ming & Daeschel, 1993). The mechanisms involved in acquired resistance are also quite diverse (Fig. 3).

Resistance to lantibiotics

The frequency of spontaneous mutations resulting in nisin resistance varies from $<10^{-9}$ to $10^{-2}$ in L. monocytogenes (de Martinis et al., 1997; Gravesen et al., 2002a; Ming & Daeschel, 1993) and from $10^{-8}$ to $10^{-2}$ in other Gram-positive organisms (Mazzotta et al., 1997). Strains with moderate resistance (an eightfold increase in MIC) are isolated more frequently (Blake et al., 2011; Gravesen et al., 2002a). However, mutants exhibiting a higher-level resistance (a 32-fold increase in MIC) could be selected by plating mutants with moderate resistance onto media containing higher nisin concentrations (Blake et al., 2011). The development of nisin resistance could be reduced by increasing the nisin concentration (Gravesen et al., 2002a).
The frequency of nisin (100 IU ml\(^{-1}\)) resistance development in \(L.\) monocytogenes strain ScottA was found to drop (at least 100-fold) when the strain was grown at 10 °C and in the presence of decreasing pH (pH 5.5 and 6.0) and decreasing NaCl concentration (2.0 and 0.5%) (de Martinis et al., 1997). Growth on NaCl (6.5%) at 30 °C also reduced the frequency of nisin (500 IU ml\(^{-1}\)) resistance development (<5×10\(^{-8}\)) (Gravesen et al., 2002a). Nisin resistance seems to have little impact on bacterial fitness, and resistance seems to be stable in most mutants in the absence of selection (Blake et al., 2011; Gravesen et al., 2002a; Mantovani & Russell, 2001).

An interesting phenomenon described by Blake et al. (2011) was the isolation of a small percentage (5%) of nisin-resistant mutants of \(S.\) aureus (with a fourfold increase in MIC) which formed small-colony variants on solid medium. These mutants displayed a 32-fold increase in gentamicin susceptibility and were menadione auxotrophs.

Mutants spontaneously resistant to lacticin 3147 do not arise at high levels of bacteriocin or at high frequencies (generally <10\(^{-7}\)) (Coakley et al., 1997; Guinane et al., 2006; Ryan et al., 1998), and the few mutants isolated were only able to withstand low levels of bacteriocin (100 AU ml\(^{-1}\)) (Guinane et al., 2006). However, mutants resistant to moderate levels of lacticin 3147 (up to 600 AU ml\(^{-1}\)) could be selected by repeated exposure to bacteriocin (Guinane et al., 2006). The resistance phenotype was stable.

Guinane et al. (2006) also investigated the antibiotic sensitivity of lacticin 3147 spontaneously resistant mutants and reported that no change in susceptibility was observed for the majority of antibiotics tested. The only exceptions were enhanced neomycin resistance and increased cephalosporin sensitivity exhibited by some mutants. Based on these results, it can be concluded that lacticin 3147 resistance is not expected to be of concern if this bacteriocin is used commercially. Moreover, if resistance develops, conventional antimicrobials could be employed in the clinical setting for bacterial control (Guinane et al., 2006).

The mechanisms responsible for acquired resistance to lantibiotics vary amongst strains and species. In many micro-organisms investigated, the resistance phenotype was associated with cell wall alterations, such as abnormal cell wall synthesis and autolysin inhibition (Maisnier-Patin & Richard, 1996), the presence of higher amounts of modified LTAs (Kramer et al., 2008; Mantovani & Russell, 2001) and increased thickness at the septum (Kramer et al., 2008) or content of rhamnose (Xuanyuan et al., 2010). Changes in cytoplasmic membrane composition may also render a bacterium resistant to lantibiotics. A nisin-resistant

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**Fig. 3.** Examples of mechanisms involved in acquired bacteriocin resistance.
variant of *L. monocytogenes* Scott A, isolated by exposure to increasing concentrations of nisin, was shown to produce less diphosphatidylglycerol and more phosphatidylglycerol than the parental strain (Verheul et al., 1997). As nisin penetrates more deeply into lipid monolayers of diphostatidylglycerol than those of other lipids, including phosphatidylglycerol, the resistance exhibited by the mutant was attributed to a reduction in the diphosphatidylglycerol content of the cytoplasmic membrane (Verheul et al., 1997). Crandall & Montville (1998) also reported that nisin resistance in *L. monocytogenes* ATCC 700302 involved alterations in cellular membrane composition, such as a lower ratio of C₁₅: C₁₇ fatty acids and the presence of more phosphatidylethanolamine and less phosphatidylglycerol and cardiolipin. All these changes may result in a less fluid cytoplasmic membrane and increased rigidity, which may prevent nisin from inserting into its target. Moreover, the decrease in phosphatidylglycerol content and, therefore, in the net negative charge of the lipid bilayer may also hamper nisin’s ability to bind to and interact with the membrane (Crandall & Montville, 1998).

Regulators have also been shown to contribute to acquired resistance. Cotter et al. (2002) reported that the LisRK 2CS is involved in listerial susceptibility to nisin. A ΔlisK mutant (lacking the HK sensor component) of strain LO28 displayed enhanced resistance to nisin, manifested by a significantly shorter lag phase in the presence of 300 μg nisin ml⁻¹. The expression of some genes, including *lmo1021* which encodes another HK (subsequently named LiaS), was significantly reduced in the mutant. The LisRK 2CS regulates the LiaFSR₄m 3CS, which in turn regulates the listerial response to environmental stresses (Fritsch et al., 2011). The LiaFSR₄m 3CS is induced in the presence of antibiotics and CAMPs which target the cell wall or the membrane (Fritsch et al., 2011; Mascher et al., 2004). Expression of a functional LiaFSR₄m seems to result in extensive remodelling of the protein composition of the cytoplasmic membrane (Fritsch et al., 2011) and in a decreased transcription of *lmo2229*/*pbb2229*, which codes for a protein similar to PBPs (named PBP2229) (Collins et al., 2012), rendering the cell more susceptible to nisin.

A ΔliaS mutant exhibited an increased tolerance to nisin (a twofold increase in MIC), but an increased susceptibility to cephalosporins and disinfectants (Collins et al., 2012). This link between nisin resistance and sensitivity to antimicrobials acting on the cell wall had been observed previously by others (Crandall & Montville, 1998; Gravesen et al., 2001). The nisin resistance of the double-mutant ΔliaS ΔlisK was only marginally greater than that of the ΔliaS mutant. However, *lmo2229*/*pbb2229* transcription increased in the ΔliaS mutant and, even more intensely, in the ΔliaS ΔlisK double mutant. Mutation of *lmo2229* in the ΔliaS ΔlisK mutant resulted in a large increase in nisin susceptibility (a 32-fold reduction in MIC) (Collins et al., 2012), suggesting that PBP2229 plays an important role in the nisin-resistant phenotype of this mutant. Collins et al. (2012) then proposed that, as PBPs catalyze the incorporation of the disaccharide–pentapeptide moiety of lipid II by the growing peptidoglycan chain (Gravesen et al., 2004), PBP2229 may contribute strongly to nisin resistance by shielding lipid II.

In a nisin-resistant mutant of *Clostridium difficile*, exhibiting a fourfold increase in MIC, the resistance phenotype was attributed to an increased expression of a three-component ABC transporter encoded by the operon *cprABC* and probably involved in peptide export (McBride & Sonenshein, 2011b). This operon is regulated by a putative orphan HK (*cprK*). This mutant was also resistant to gallidermin (McBride & Sonenshein, 2011b).

### Resistance to class II bacteriocins

To date, only cells resistant to subclass IIA bacteriocins, lactococcin A and lactococcin 972 (both belonging to subclass IIc) have been investigated. For class II bacteriocins, resistance has been found to be either spontaneous (Katla et al., 2003; Rasch & Knochel, 1998) or induced by exposure. Resistance frequencies from 10⁻⁹ to 10⁻³ have been reported for subclass IIA bacteriocins (Dykes & Hastings, 1998; Gravesen et al., 2002a; Rekhif et al., 1994; Tessema et al., 2009; Vadyvaloo et al., 2002) and from 10⁻⁷ to 10⁻⁴ for lactococcin A (Kjos et al., 2011). Environmental stress in the form of low temperature (10 °C), reduced pH (5.5) or the presence of NaCl (6.5 %) did not influence the frequency of pediocin PA-1 resistance (Gravesen et al., 2002a). The resistance developed proved to be stable in some mutants (Gravesen et al., 2002a; Kjos et al., 2011; Rekhif et al., 1994; Tessema et al., 2011) but unstable in others (Dykes & Hastings, 1998; Tessema et al., 2011).

Different mechanisms are associated with resistance development to class II bacteriocins and the level of resistance depends on the mechanism involved.

In many cases, resistance to subclass IIA bacteriocins and lactococcin A involves their receptors (encoded by the *mpt/ptn* operon) and downregulation of their expression (Gravesen et al., 2002b; Kjos et al., 2011; Opsata et al., 2010; Rammath et al., 2000, 2004). High-level resistance of Gram-positive bacteria to subclass IIA bacteriocins has been reported to result from either loss or reduction of expression of Man-PTS, either in naturally resistant isolates or in induced resistant mutants (Gravesen et al., 2002b; Kjos et al., 2011; Opsata et al., 2010; Tessema et al., 2011). In many instances, the mutants no longer synthesized the MptA subunit from Man-PTS (Gravesen et al., 2002b) or its synthesis was found to be reduced (Tessema et al., 2009). Reduced transcription of the *mptC* or *mptD* gene was observed in mutants of *Enterococcus faecalis* (Opsata et al., 2010) or *L. monocytogenes* (Kjos et al., 2011). Site-directed mutagenesis in the extracellular loop of MptC, the main determinant for specific targeting by subclass IIA bacteriocins (Kjos et al., 2010b), was performed and two mutations caused a significant reduction in receptor activity for eight subclass IIA bacteriocins, resulting in
bacteriocin resistance. The point mutation Q88F caused MIC values to increase six- to 500-fold, depending on the bacteriocin tested and the G89H mutation totally disrupted the receptor function for all tested bacteriocins (Kjos et al., 2010b). In-frame deletions in the mptD gene also led to resistance of *L. monocytogenes* to mesentericin Y105 (Dalet et al., 2001). Moreover, the introduction of the *L. monocytogenes* mptC gene or the *L. lactis* ptnABCD rendered a resistant strain of *L. lactis* sensitive to subclass Ila bacteriocins (Ramnath et al., 2004) or to lactococcin A (Kjos et al., 2011), respectively.

In other studies performed with *L. monocytogenes* (Robichon et al., 1997) and *Enterococcus faecalis* (Dalet et al., 2000), resistance to subclass Ila bacteriocins was linked to the regulatory gene *rpoN*. *rpoN* encodes the alternative sigma factor σ^IIa^ (SigL). SigL is involved in activation of the *mptA* / *BCD* operon, which codes for EIIAB, EIIC and EIID in *L. monocytogenes* and *Enterococcus faecalis* (Héchard et al., 2001), in conjunction with ManR (the transcriptional activator for σ^IIa^) (Dalet et al., 2001). Mutations in *rpoN* resulted in loss of mpt expression, which in turn led to mesentericin Y105 resistance in both micro-organisms (Dalet et al., 2000; Héchard et al., 2001). Mutations in ManR of *L. monocytogenes* (Dalet et al., 2001; Gravesen et al., 2002b) or in its homologue of *Enterococcus faecalis* (Opsata et al., 2010) also rendered the cells resistant to subclass Ila bacteriocins. As both regulatory proteins RpoN and ManR are required for active transcription of the genes encoding the subclass Ila bacteriocin receptor, the bacteria become resistant if the receptor is not synthesized (Diep et al., 2007). The molecular switch that turns off or downregulates Man-PTS expression in resistant cells is reportedly a stable phenomenon (Gravesen et al., 2002b; Kjos et al., 2011; Rekhif et al., 1994).

Opsata et al. (2010) performed transcriptome analyses in order to compare *Enterococcus faecalis* cells sensitive and resistant (with mutation in the mpt operon) to pediocin PA-1. About 200 genes exhibited a significantly altered transcription in the mutant cells. Most of these genes encoded functions involved in energy metabolism and transport. Some genes were downregulated (e.g. genes involved in glycolysis), whereas others were upregulated (e.g. genes involved in transportation and degradation of secondary sugar sources). The metabolism was shifted from lactic acid fermentation to mixed fermentation in the mutant. Similar findings have also been reported in resistant mutants of *L. monocytogenes* (Naghmouchi et al., 2007; Vadyvaloo et al., 2004). As discussed by Vadyvaloo et al. (2004), the shift in metabolism and the subsequent change in the end products will profoundly influence both the organoleptic properties and spoilage potential of the food product in which pediocin is used as a biopreservative. Moreover, these changes generally exhibit fitness costs, such as reduced growth rate in broth (Gravesen et al., 2002a; Vadyvaloo et al., 2004), but this fitness cost was not observed when the pediocin PA-1-resistant mutants were grown in a meat model at 5 °C (Gravesen et al., 2002a).

A strain with a high resistance to subclass Ila bacteriocins also displayed a reduced ability to grow on 0.4 % glucose (Man-PTS dependent), but grew normally on cellobiose (Man-PTS independent) relative to the growth of parental cells (Kjos et al., 2011). Similar results were reported for lactococcin A-resistant mutants, although in this case the alternative sugar used was galactose (Man-PTS independent) instead of cellobiose (Kjos et al., 2011).

Although the downregulation of Man-PTS expression is the main resistance mechanism associated with high-level resistance to various class II bacteriocins, there are other mechanisms which normally occur in mutants with low resistance. In the latter, normal or high Man-PTS gene expression is generally observed (Kjos et al., 2011). Although the exact nature of these mechanisms is still unknown, there is evidence that suggests the involvement of changes in the cell envelope, such as: (i) phospholipids with increased ratios of unsaturated to saturated fatty acids and short- to long-acyl chain species (Vadyvaloo et al., 2002), which result in an increased membrane fluidity; (ii) increased ratios of saturated fatty acids, leading to a decrease in membrane fluidity (Limonet et al., 2002); (iii) a decrease in the cell wall negative charge by an increase in D-alanine content in TAs (Vadyvaloo et al., 2004); (iv) a higher content of peptidoglycan with tripeptide side-chains and a reduced content of pentapeptide side-chains, leading to a profound change in the peptidoglycan structure (Roces et al., 2012a); and (v) efflux of the bacteriocin by the twin-arginine translocase system (Tessema et al., 2011).

Recently, Laursen et al. (2014) reported that *L. monocytogenes* stress caused by exposure to a single sublethal pediocin concentration resulted in increased expression of LisRK, and SigB- and SigL-dependent genes, implicating these regulators in acquired subclass Ila bacteriocin resistance. These authors proposed that, in the presence of the bacteriocin: (i) the LisRK system senses the cell envelope damage caused by pediocin and initiates a response which results in cell envelope alterations in an attempt to maintain its integrity; (ii) SigB-dependent genes participate in a more general defence mechanism, whose role in bacteriocin sensitivity has not yet been elucidated, and (iii) SigL-dependent genes (involved in protein synthesis, nutrient transport and motility) participate in a phase of cell reconstruction. Consequently, decreased bacteriocin susceptibility is observed.

High induction of Llmg2447 was observed in a lactococcin 972-resistant mutant of *L. lactis*. Llmg2447 is predicted to encode a putative anti-sigma factor (Roces et al., 2012b). An elevated expression of this gene led to high lactococcin 972 resistance (a 400-fold decrease in susceptibility) in *L. lactis*, with no cross-resistance to nisin, bacitracin, penicillin G and vancomycin (Roces et al., 2012b). However, the way in which Llmg2447 protects *L. lactis* from lactococcin 972 remains to be elucidated (Roces et al., 2012b).
Resistance to class III bacteriocins

Lysostaphin is the only class III bacteriocin investigated in relation to resistance development. Resistance to lysostaphin (a five- to 15-fold increase in MIC) occurred both in vitro (at frequencies of $<10^{-9}$ to $10^{-1}$) and in a rabbit model of experimental endocarditis (at a frequency of $10^{-6}$) (Climo et al., 2001; Kusuma & Kokai-Kun, 2005) following exposure to low doses of lysostaphin (0.25–0.50 MIC). This resistance has generally been associated with mutations in femAB and results in an alteration of the peptidoglycan cross-bridges, in which one or more serine residues replace glycine (Gargis et al., 2010; Strandén et al., 1997). Moreover, this resistance mechanism is generally accompanied by increased $\beta$-lactam susceptibility (Climo et al., 2001; Guignard et al., 2005; Kiri et al., 2002; Rohrer & Berger-Bäch, 2003) and by loss of fitness in the mutants (Kusuma et al., 2007). Therefore, Kusuma et al. (2007) recommended the inclusion of $\beta$-lactam antibiotics in all therapeutic trials with lysostaphin to both suppress resistance and promote synergy.

Resistance to cyclic bacteriocins

To date, at least 12 cyclic bacteriocins have been described (Potter et al., 2014; van Belkum et al., 2011). Enterocin AS-48, a bacteriocin produced by Enterococcus faecalis and the first reported to be cyclic, is the prototype of this group and is the best characterized class IV bacteriocin (van Belkum et al., 2011).

With regard to resistance mechanisms against cyclic bacteriocins, it is not surprising that not many studies have been undertaken, and that the only models investigated are enterocin AS-48, which has potential application as a food preservative (Gálvez et al., 2007), and garvicin ML.

A mutant of L. monocytogenes with higher tolerance to enterocin AS-48 (MIC 0.5 µg ml$^{-1}$) was studied by Mendoza et al. (1999). The resistance phenotype was, however, unstable as it was lost after 11 steps of subcultivation of the mutant without enterocin AS-48. Cell envelope changes were implicated in the resistance, such as a higher proportion of branched fatty acids as well as a higher C$_{15}$:C$_{17}$ ratio. The cell wall thickness was also increased, although it seemed to be less dense.

Later, Grande Burgos et al. (2009) investigated the effect of enterocin AS-48 against vegetative cells of Bacillus cereus by transcriptome analysis. Expression of 24 genes changed significantly after exposure to a subinhibitory bacteriocin concentration (0.5 µg ml$^{-1}$). Most genes ($n=20$) were upregulated and encoded membrane-associated or secreted proteins. The genes which are part of an operon that encodes a PadR-type transcriptional regulator and a hypothetical membrane protein were found to be the most upregulated. The upregulation was only observed in the presence of enterocin AS-48, but not of nisin. Bacillus subtilis 168 lacks that operon, and is sensitive to enterocin AS-48 and nisin. However, when the gene encoding the membrane protein was introduced and expressed in this strain, the transformant exhibited increased resistance to enterocin AS-48, but not to nisin (Grande Burgos et al., 2009). Therefore, this membrane protein is involved in the resistance mechanism of Bacillus cereus cells against enterocin AS-48 by a hitherto unknown mechanism.

In relation to garvicin ML, a bacteriocin produced by Lactococcus garvieae DCC43, Gabrielsen et al. (2012) characterized various spontaneous mutants of La. lactis IL1403 with lower susceptibility to garvicin ML (with a six- to 11-fold decreased sensitivity). These mutants lost the ability to metabolize starch and maltose, and carried a 13.5 kb chromosomal deletion. The malEFG operon, which encodes a maltose ABC transporter, was included in the deleted region. When the mutants were complemented with these three genes, they recovered the susceptibility to garvicin ML. Higher expression levels of the operon resulted in higher bacteriocin sensitivity, suggesting an important role of the maltose ABC transporter in the antagonistic activity of garvicin ML, potentially as a target receptor for the bacteriocin (Gabrielsen et al., 2012).

Cross-resistance amongst bacteriocins

As the combined use of bacteriocins, especially of those with different mechanisms of action, may reduce (Gravesen et al., 2002a) or prevent (Coelho et al., 2007; Vignolo et al., 2000) the development of bacteriocin resistance, it is critical to determine whether cross-resistance will occur following the spontaneous development of resistance to one of the two types of bacteriocins (Gravesen et al., 2004). In this respect, contradictory reports are found in the literature. No cross-resistance between nisin and pediocin PA-1 was found by Rasch & Knöchel (1998) in L. monocytogenes strains which were shown to exhibit naturally enhanced tolerance to either nisin or pediocin PA-1. Similar results have been reported by others. Rekhif et al. (1994) reported that mutants of L. monocytogenes resistant to one of three class II bacteriocins tested [mesenterocin 52B (IIc), curvaticin 13 (IIa) and plantaricin C19 (IIa)] exhibited some degree of cross-resistance to the other two, but not to nisin. Cross-resistance between lactocin 3147, nisin and lactocin 481 (all lantibiotics) was reported by van Schaik et al. (1999) and by Guinane et al. (2006), but cross-resistance to the class II bacteriocin lactococcin ABM was not observed (Guinane et al., 2006). In one case, resistance to a subclass IIa bacteriocin was reported to confer increased susceptibility to nisin (Boutetfroy & Millière, 2000). Conversely, cross-resistance between bacteriocins belonging to different classes has also been observed. Crandall & Montville (1998) reported that nisin resistance in L. monocytogenes conferred cross-resistance to pediocin PA-1. In another study, a strain of L. monocytogenes displayed resistance to nisin, pediocin PA-1 and leucocin S – bacteriocins belonging to at least two distinct classes (Bruno & Montville, 1993). Gravesen et al.
(2004) found a degree of cross-resistance between nisin and subclass IIa bacteriocins (pediocin PA-1 and leucocin A). They also observed complete cross-resistance amongst subclass IIa bacteriocins (pediocin PA-1, leucocin A and carnobacteriocin B2). Indeed, cross-resistance between different subclass IIa bacteriocins has been reported frequently (Dykes & Hastings, 1998; Gravesen et al., 2002a, b; Ramnath et al., 2004; Rasch & Knochel, 1998).

**Strategies to overcome bacteriocin resistance**

Resistance to a given bacteriocin arises by different mechanisms, even amongst strains of the same bacterial species. Moreover, similar resistance mechanisms are shared by bacteriocins belonging to different classes. From the examples described above, it can be concluded that the bacterial cell envelope plays an important role in both innate and acquired resistance. However, the development of bacteriocin resistance does not hamper the intended use of a given bacteriocin as there are strategies that can be used to prevent or overcome this resistance.

In food technology, the risk of developing bacteriocin resistance may be reduced by the judicious use of a multiple-hurdle preservation strategy, combining several methods to preserve food (Bastos & Ceotto, 2011; Cotter et al., 2005). Kaur et al. (2013) showed that *L. monocytogenes* mutants resistant to nisin, pediocin 34 and enterocin FH99 did not become resistant to low pH, sodium chloride, potassium sorbate or sodium nitrite. Resistant variants were equally or more sensitive than the WT strain. Therefore, according to these authors, bacteriocin resistance should not affect the use of hurdle preservation technologies employing bacteriocins to improve food safety. However, other studies have shown that salt stress at low temperature could provide cross-protection to *L. monocytogenes* against nisin (Bergholz et al., 2013). Thus, it is advisable to take into consideration the potential for cross-protective effects between different hurdle technologies used for food preservation.

Another approach to avoid the emergence of bacteriocin-resistant bacteria may be the combined use of bacteriocins with different mechanisms of action, which may additionally allow the use of lower bacteriocin doses (Bastos & Ceotto, 2011). Such an approach can be illustrated by the combination of nisin and curvaticin 13 (subclass IIa) to control *L. monocytogenes* (Bouillefroy & Millière, 2000). The bactericidal activity against this pathogen resulting from this combination was higher than that of either bacteriocin alone. Similar results were observed by Kaur et al. (2013), who combined nisin, pediocin 34 and enterocin FH99 (both subclass IIa bacteriocins) to control *L. monocytogenes*. Moreover, the combination of nisin (500 IU ml⁻¹) and pediocin PA-1 (1720 AU ml⁻¹) reduced the frequency of resistance development (a 10²–10³ reduction, depending on the strain tested) in *L. monocytogenes* (Gravesen et al., 2002a).

As resistance to a given bacteriocin may extend to other bacteriocins, especially of the same subclass, it is advisable that combinations should be tested with bacteriocins belonging to different classes or, at least, subclasses. Even so, preliminary tests aiming to investigate the development of cross-resistance should be performed before combining distinct bacteriocins, so that the emergence of resistance can be minimized. Macwana & Muriana (2012) published a study in which they proposed a method, based on mode of action, to classify bacteriocins into functional groups that could be used for identifying the most adequate combination of bacteriocins for use in biocontrol.

Complementary use of conventional drugs and bacteriocins may be another potential approach to prevent the emergence of resistant micro-organisms (Coelho et al., 2007; Cotter et al., 2013). Most traditional antimicrobials generally act as enzyme inhibitors. Most bacteriocins, however, target the membranes of sensitive cells leading to cell death. Therefore, development of cross-resistance to antimicrobials with different modes of action is expected to occur less frequently (Coelho et al., 2007).

**Conclusions**

The studies reported in the literature suggest that bacteriocin resistance may develop during bacteriocin exploitation for biotechnological applications. Most bacteriocin resistance does not result in great fitness costs. If resistance makes the cells sick, the bacteriocin-sensitive cells are expected to exhibit a higher growth rate and predominate in the culture or in the foods, and the mutants will be outnumbered and presumably eliminated. Therefore, such sick mutants would not have any impact on microbial control by bacteriocins. As pointed out by Gravesen et al. (2002a), the frequency of development of resistance to bacteriocins cannot be predicted, as it will depend on the specific strain and growth conditions. The main proposed application for most bacteriocins is food preservation. Although experiments performed in vitro are relevant to measure important parameters, such as an assessment of the ease with which resistance arises, the impact that resistance exerts upon bacterial fitness and the stability of the resistance in the absence of selection (Blake et al., 2011), more studies on bacteriocin resistance development in food model systems are required in order to measure its frequency and assess its impact on microbial control. Food systems are heterogeneous in composition, and their intrinsic factors may affect bacteriocin activity and the development of bacteriocin resistance (Bastos & Ceotto, 2011). According to Gravesen et al. (2002a), the presence of gradients of food components may create local areas which may favour the development of, presumably, low-level bacteriocin resistance. As in many cases these resistant mutants can be stable, it is conceivable that the occurrence of further mutations in these mutants may result in the emergence of high-level bacteriocin resistance (Gravesen et al., 2002a). However, such assumptions deserve investigation.
Finally, considering the importance of bacteriocins in preventing and controlling bacterial infections, investigation on the functions involved in bacteriocin resistance should also continue, as this knowledge could facilitate the development of strategies to increase bacterial susceptibility to bacteriocins (Begley et al., 2006; Collins et al., 2012). However, we would like to point out, as already proposed by Katla et al. (2003), the urgent need for guidelines for the classification of different micro-organisms according to their susceptibilities to various bacteriocins, which would not only facilitate the comparison of different studies, but also help to identify new lines of investigation on this topic.

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Bacteriocin resistance mechanisms


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Studies on prolysostaphin processing


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