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Mutacins and their Potential Use in Food Preservation

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ABSTRACT

Mutacins are proteinaceous antibacterial substances produced by *Streptococcus mutans*, an indigenous bacterial inhabitant of the oral cavity. The metabolism of *S. mutans* is similar to that of lactic acid bacteria (LAB) used in fermented food. Actually, only a few well-studied mutacins have been described. Mutacins B-Ny266, B-JH1140, I, III, and K8 are linear lantibiotics. Mutacins II and H-29B are globular lantibiotics. Mutacins GS-5/Smb and BHT-Aαβ are dipeptide lantibiotics. Mutacins N and BHT-B are non-lantibiotic peptides while mutacin IV is a non-lantibiotic dipeptide. Some of these mutacins are active against most Gram-positive foodborne pathogens. Nisin is actually the only lantibiotic bacteriocin used as a food additive and pediocin-like bacteriocins are considered to be next in line if more antibacterial proteins are to be approved in the future. However, nisin- and pediocin-resistant mutants appear relatively easily while resistant mutants against mutacins B-JH1140 and B-Ny266 could not be obtained. Mutacins thus have potential for controlling foodborne pathogens and spoilage bacteria. New methods for producing and purifying these small peptides will contribute towards developing food grade antimicrobials for use in food products. More research is needed on the applications of bacteriocins in food systems.

Keywords: bacteriocin, biopreservative, biofilm inhibition, food additive, GRAS, hurdle technology, lantibiotic, peptide purification, *Streptococcus mutans*

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INTRODUCTION

The increasing concerns of consumers about the possible adverse health effects from the presence of chemical additives in food associated with the increasing demand for minimally processed food and the need for the industry to preserve the shelf life and safety of food products have stimulated research interest in finding new natural effective food preservatives.

The history of food preservation is associated with the presence of lactic acid bacteria (LAB) in foodstuff. Today LABs are recognised as producers of a plethora of metabolites involved in preservation properties of a large variety of fermented food products. Among these metabolites are bacteriocins (Chen and Hoover 2003; Gálvez *et al*. 2007).

Bacteriocins are ribosomally synthesised proteinaceous antibacterial substances produced by bacteria (Jack *et al*. 1995; Cotter *et al*. 2005a) with activity directed either against related species (narrow spectrum) or across genera (broad spectrum). They can thus provide a defence mecha-

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nism for the producing strain as they kill other competitive bacteria. Discrepancies persist between theories as to whether bacteriocins serve as defence mechanisms or attack weapons (Dykes and Hasting 1997; Riley and Wertz 2002; Gordon *et al*. 2006). Nevertheless, bacteriocins are able to kill sensitive bacteria. Work on bacteriocins has also been driven by the need to face the fast and continuous increase and widespread bacterial resistance to conventional antibiotics (Berger-Bächi 2002; Sharma *et al*. 2005). This public health problem impels the development and rapid use of new types of antibacterial substances. Bacteriocins and lantibiotics represent candidates with great potential against pathogen infections (Pag and Sahl 2002; Joerger 2003; Chatterjee *et al*. 2005; Cotter *et al*. 2005b; Gillor *et al*. 2005). Similarly in the food industry, the need for more natural food biopreservatives has greatly stimulated research on LAB bacteriocins (Ross *et al*. 1999; O'Sullivan *et al*. 2002; Chen and Hoover 2003; Cotter *et al*. 2005a; Deegan *et al*. 2006; Gálvez *et al*. 2007). Particular cautions need to be taken before using new LAB strains in food, and genomics can help by providing new insights into LAB activities and their safety (Konings *et al*. 2000).

The bacteriocins produced by *Streptococcus mutans*, an indigenous oral bacterium, were first studied by Kelstrup and Gibbons (1969) and termed mutacins by Hamada and Ooshima (1975a). Although there are many reports showing that *S. mutans* produces inhibitory substances, only a few such inhibitors have been isolated and characterised as mutacins. The diversity of bacteriocin production by *S. mutans* has been recently highlighted, as they produce 40% of the listed streptococcal antimicrobial peptides (Nes *et al*. 2006). Historically, interest in mutacins was based on their usefulness as possible anti-caries agents and the role they play in the colonization of the oral cavity (Hamada and Ooshima 1975a, 1975b; Weerkamp *et al*. 1977). They were also used as an epidemiological fingerprinting tool and in determining the distribution of mutacin-producing *S. mutans* strains (Rogers 1976a; Groonroos *et al*. 1998).

Despite the fact that *S. mutans* is physiologically a LAB (Adjic *et al*. 2002) and is part of the normal human microbiota (Hamada and Slade 1980; Caufield *et al*. 2007), it is also considered a pathogen (Mitchell 2003; Banas 2004). In fact, *S. mutans* should be considered as a pathogen whose virulence is only linked to diet and environment (van Palenstein Helderman *et al*. 1996). Moreover, *S. mutans* does not seem to be as detrimental or as dangerous as some enterococci found in food products (Franz *et al*. 2003).

In their diversity, bacteriocins produced by *S. mutans* present potential for use as food biopreservatives. Even though *S. mutans* is considered as a pathogen by the majority of the research community, potentially safe biotechnical application of mutacins may be envisioned with heterologous production by generally recognised as safe hosts or by the addition of cell-free partially purified preparations in foodstuffs.

STREPTOCOCCUS MUTANS

S. mutans was first described by J. K. Clarke in 1924 after he isolated it from a carious lesion. Later, serological, protein profiles, cell wall structures and gross DNA composition studies confirmed that there was considerable variation amongst the large number of isolates identified as *S. mutans*. Based on these studies, *S. mutans* isolates were sub-divided into a number of distinct species some of which were of animal and some of human origin (Coykendall 1989). Thus the "mutans streptococci" were born and the name *S. mutans* was retained to describe the more common of the two main human strains, the other being *Streptococcus sobrinus*. The retention of the name *S. mutans* has lead to some confusion but was necessary to comply with the rules governing scientific nomenclature. *S. mutans* inhabit the oral cavity of virtually everybody (Hamada and Slade 1980). *S. mutans* is considered the main microbial aetiological agent of dental caries. However, the high genotypic and phenotypic variation existing within the species influences its cariogenic potential (Balakrishnan *et al*. 2002; Napimoga *et al*. 2004; Saxena *et al*. 2005; Guo *et al*. 2006; Waterhouse and Rusell 2006; Waterhouse *et al*. 2007). Sucrose is converted by glucosyltransferase enzymes to produce a sticky, extracellular, dextran-based polysaccharide that allows *S. mutans* to adhere to each other and to the teeth, forming dental plaque. Other primary virulence mechanisms include glucan binding proteins and its combined acidogenicity and acid resistance (Mitchell 2003; Ferretti and McShan 2006).

CLASSIFICATION OF MUTACINS

Many classifications, essentially based on their molecular structure and mechanism of action, have been proposed for bacteriocins (Klaenhammer 1993; Nes *et al*. 1996). With the advancement in bacteriocin studies, a recent classification was proposed by Cotter *et al*. (2005a), which groups bacteriocins into two main classes. Heng and Tagg (2006) proposed a modification to group bacteriocins into four distinct classes in accordance with Cotter *et al*. (2006) that we will adopt here (**Table 1**).

Mutacins were grouped in the past mainly by physical, biochemical, genetic, and phenotypic characteristics such as the inhibitory spectra, resistance to solvents, heat, proteolytic enzymes, and molecular mass (Kelstrup and Gibbons 1969; Bondi *et al*. 1991; Caufield *et al*. 1985; Tagg and Banister 1979; Morency *et al*. 1995, 2001; Bekal-Si Ali *et al*. 2002; Waterhouse and Russell 2006). These classifications were preliminary and only the purification and sequencing of the peptides will reveal the real nature of these mutacins.

Chikindas *et al*. (1997) have reviewed some of the mutacins that were well characterised before 1997. Since then, more biochemical and genetic information has been acquired on mutacins and four types have been described: lan-

Table 1 Classification of bacteriocins produced by Gram-positive bacteria (adapted from Cotter *et al*. 2005a, 2006; Heng and Tagg 2006)

	Class I-lantibiotics	**Class II**	**Class III**	**Class IV**
Definition	Post-translationally modified peptides containing lanthionines and/or unsaturated amino acids	Non-lanthionine containing, heat-stable peptides	Large heat-labile proteins. Mostly the "colicin-like" bacteriocins	Cyclic peptides whose N and C termini are covalently linked
Specific Molecular Weight	< 5 kDa	< 10 kDa	> 10 kDa	No specification
Subgroups	**Type Ia:** Elongated peptides with a net positive charge *Subtype AI*: Nisin-like *Subtype AII*: SA-FF22-like **Type Ib:** Globular peptides with a net negative charge or no charge **Type Ic:** Multi-component bacteriocins	**Type IIa:** Pediocin-like bacteriocins that show antilisterial activity with a N-terminal sequence: YGNGVXC **Type IIb:** Miscellaneous bacteriocins **Type IIc:** Multi-component bacteriocins	**Type IIIa:** Bacteriolytic **Type IIIb:** Non-lytic Not much is known about this group	None
Examples	Nisin, lacticin 481, mersacidin, lacticin 3147, **mutacins B-Ny266, B-JH1140, H-29B, J-T8 (II), I, III, Smb and BHT-A**	Pediocin PA1, thermophilin 13, aureocin A53, **mutacins F-59.1, IV, V, N and BHT-B**	Lysostaphin, helveticin J	Enterocin AS-48

Table 2 Activity of known mutacins against spoilage and foodborne pathogenic bacteria.

Mutacin	Producing strain	Bacteriocin Class	Similarity with other well described bacteriocins	Activity spectra	References
I	*S. mutans* CH43, UA140	Class Ia-lantibiotic	Epidermin	Similar to mutacin B-Ny266	Schnell *et al*. 1988; Mota-Meira *et al*. 2000; Qi *et al*. 2000a, 2000b, 2001
II or J-T8	*S. mutans* T8, UA96, 17A2	Class Ia-lantibiotic	Lacticin 481 group (variacin, macedocin among others, see Dufour *et al*. 2006)	*Clostridium* spp. (*sporogenes*[a], *tyrobutyricum*, *perfringens*), *Bacillus* spp. (*subtilis*, *cereus*), *Listeria monocytogenes*, *Staphylococcus aureus*, various spoilage LAB	Parrot *et al*. 1989; Novak *et al*. 1993; Pridmore *et al*. 1996; Krull *et al*. 2000; Balakrishnan *et al*. 2002; Dufour *et al*. 2006; van den Berghe *et al*. 2006
III	*S. mutans* UA787	Class Ia-lantibiotic	Epidermin	Similar to mutacin B-Ny266	Schnell *et al*. 1988; Qi *et al*. 1999; Mota-Meira *et al*. 2000; Qi *et al*. 2000b
IV[b] NlmA NlmB	*S. mutans* UA140, UA159, GS-5, K34-1	Class IIb or class IIc	Thermophilin 13	Similar to thermophilin 13 (*Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Listeria*, *Bacillus* spp., and *Clostridium* spp.)	Marciset *et al*. 1997; Qi *et al*. 2001; Hale *et al*. 2005b; Yonezawa and Kuramitsu 2005
V NlmC	*S. mutans* UA159	Supposed Class IIb	Not known	Non-streptococcal species, *Lactococcus lactis*	Hale *et al*. 2005a, 2005b; van der Ploeg 2005
1140	*S. mutans* JH1140	Class Ia-lantibiotic	Epidermin	Similar to mutacin B-Ny266	Schnell *et al*. 1988; Hillman *et al*. 1998; Mota-Meira *et al*. 2000
B-Ny266	*S. mutans* Ny266	Class Ia-lantibiotic	Epidermin	*L. monocytogenes*, *Campylobacter jejuni*, *B. cereus*, *B. subtilis*, *C. perfringens*, *C. sporogenes*[a], *C. tyrobutyricum*, *S. aureus*, various spoilage LAB	Schnell *et al*. 1988; Mota-Meira *et al*. 1997, 2000
N	*S. mutans* N	Class IIb	Partial similarity with mutacin I	Close to that of mutacin I by deferred antagonism tests. *Streptococcus pyogenes*, oral streptococci	Balakrishnan *et al*. 2000; Hale *et al*. 2004
Smb[c] SmbA SmbB	*S. mutans* GS-5, BM71, K34-1	Class Ic-lantibiotic	Lacticin 3147, Plantaricin W	Similar to lacticin 3147 and plantaricin W (*L. monocytogenes*, *S. aureus*), various spoilage LAB	Morgan *et al*. 1999, 2000; Holo *et al*. 2001; Hyink *et al*. 2005; Yonezawa and Kuramitsu 2005; Petersen *et al*. 2006
BHT-A[c] BHT-Aα BHT-Aβ					
BHT-B	*S. mutans* K34-1, GS-5, *S. rattus* 67-3, FA1, GF71, IB, LG-1	Class IIb	Aureocin A53	Similar to aureocin A53 (*E. faecium*, *S. aureus*)	Netz *et al*. 2002a, 2002b; Hyink *et al*. 2005
H-29B	*S. mutans* 29B	Class Ia-lantibiotic	Lacticin 481 group	Similar to mutacin II	Dufour *et al*. 2006; Nicolas *et al*. 2006
K8[c] MukA1 MukA2 MukA3	*S. mutans* K8	Class Ia-lantibiotic	Lacticin 481 group	Similar to macedocin and variacin (*L. monocytogenes*, *S. aureus*, *B. cereus*, *B. subtilis*, *C. botulinum*, *C. tyrobutyricum*)	Pridmore *et al*. 1996; Dufour *et al*. 2006; van den Berghe *et al*. 2006; Robson *et al*. 2007

[a] *C. sporogenes* belongs to group I (proteolytic) *Clostridium* spp. with the proteolytic strains of the neurotoxin-producing pathogen *Clostridium botulinum*
[b] The first characterisation of mutacin IV included two peptides designated NlmA and NlmB (class IIc bacteriocin) (Qi *et al*. 2001). However, Hale *et al*. (2005b) showed that disruption of the *nlmB* gene has no impact on the activity of mutacin IV, placing mutacin IV in class IIb
[c] Multi-component mutacin

tibiotic monopeptides (bacteriocin class I type A or type B), lantibiotic dipeptides (bacteriocin Class I type C), non-lantibiotic monopeptides (bacteriocin Class IIc), and non-lantibiotic dipeptides (bacteriocin Class IIb). The major characteristics of the new mutacins are summarised in **Table 2**.

ACTIVITY SPECTRA OF MUTACINS

Following the characterisation of many mutacins, their biotechnological potential was evaluated by determining their spectrum of activity (**Table 2**). Mota-Meira *et al*. (2000) and Morency *et al*. (2001) have demonstrated that numerous mutacinogenic strains, as for other lantibiotic-producing strains, inhibited the growth of many foodborne pathogenic bacteria such as *Listeria monocytogenes*, *Bacillus cereus*, *Clostridium perfringens*, *Staphylococcus aureus* and *Campylobacter jejuni* and numerous food spoilage bacilli and clostridia as well as LABs. Mutacins can also inhibit many streptococci and enterococci, including a considerable number of clinical multiresistant strains (Mota-Meira *et al*. 2000, 2005). The mutacins tested were also active against some Gram-negative pathogens such as *Neisseria gonorrhoeae*, *Helicobacter pylori* and to a lesser extent against a hyperpermeable *Escherichia coli* strain (Mota-Meira *et al*. 2000, 2005). Mutacin B-Ny266 is active against many nisin A-resistant strains (*L. monocytogenes* Scott A, *Pediococcus acidilactici*), oxacillin-resistant strains (*N. gonorrhoeae*, *Enterococcus faecalis*, *S. aureus*, *and S. epidermidis*) and vancomycin-resistant strains (*N. gonorrhoeae*, *E. faecalis*) (Mota-Meira *et al*. 2000). Mutacin Smb was active against 11 species of streptococci, as well as *E. faecalis* and *S. aureus* (Petersen *et al*. 2006). The lantibiotic mutacins I, II and III are active against most Gram-positive bacteria tested whereas the non lantibiotic mutacins IV and mutacin N have a narrower activity spectrum limited to the *S. sanguinis* and *S. mitis* species, which are the main competitors of *S. mutans* in the oral microbial community (Qi *et al*. 2000b, 2001; Kreth *et al*. 2005), while mutacin V seems mainly to target non-streptococcal species (Hale *et al*. 2005a).

Minimum inhibitory concentrations (MICs) have been

determined for some mutacins. Mota-Meira *et al*. (2000) compared MICs of mutacin B-Ny266 to the lantibiotic nisin A, and the antibiotics vancomycin and oxacillin. The lantibiotics were found to be as active as these antibiotics against most of the strains tested (actinobacilli, bacilli, clostridia, corynebacteria, enterococci, listeria, mycobacteria, neisseria, staphylococci, streptococci, *H. pylori*, and *C. jejuni*). Mutacin B-Ny266 was even active against strains resistant to nisin A, oxacillin and vancomycin. Its wide spectrum of activity in the nanomolar range makes mutacin B-Ny266 an excellent candidate for antibiotherapy or to be used as biopreservative in the food industry. Mutacins I and III were shown to be more efficient than nisin against methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VRE) and *S. epidermidis*, showing MICs lower than 10 μg/mL (Qi *et al*. 1999, 2000b). Mutacins I, II, III and IV are very active against group A streptococci and penicillin-resistant *Streptococcus pneumoniae* with MICs lower than 1 μg/mL (Qi *et al*. 2000b).

MODE OF ACTION OF MUTACINS

Although four types of mutacin have been described (monopeptide lantibiotic, dipeptide lantibiotic, monopeptide non-lantibiotic, and dipeptide non-lantibiotic), only the mode of action of the monopeptide lantibiotic mutacin II has been experimentally studied. Mutacin II was grouped into the type AII lantibiotics by biochemical tests (Krull *et al*. 2000). Generally, lantibiotics of type AII (Jung 1991; Pag and Sahl 2002) were found to kill sensitive cells by forming pores that perturb the cellular membrane. Mutacin II was shown rather to inhibit energy metabolism of sensitive cells by depolarizing, in a transient manner, the electrical transmembrane potential, the transmembrane pH gradient and by partially inhibiting amino acid transport (Chikindas *et al*. 1995). This observed mode of action of mutacin II is related to that of the type B lantibiotics. However, mutacin II shares partial sequence similarities with lantibiotics of type A, now described as the lacticin 481 lantibiotic group (Dufour *et al*. 2006). Twomey *et al*. (2002) observed that the N-terminus of the lacticin 481 group and the C-terminus of the mersacidin group (type B) share the motif GXXXTX (T/S)XEC, which includes several residues involved in the bridging of lacticin 481 (van den Hooven *et al*. 1996) and mutacin II (Krull *et al*. 2000). Actagardin and mersacidin also share with mutacin II a Glu-Cys motif which is proposed to be responsible for the inhibition of murein biosynthesis (Zimmermann and Jung 1997; Sahl and Bierbaum 1998). Lantibiotics are known to possess a dual activity against sensitive cells. First they act by disrupting the cellular membrane by forming pores leading to dissipation of membrane potential and efflux of small metabolites. Second, they block peptidoglycan synthesis (Bauer and Dicks 2005). These two functions are ensured by their link to the peptideglycan precursor lipid II serving as docking or target molecule (Brötz *et al*. 1998; Breukink *et al*. 1999; Wiedemann *et al*. 2001). For mutacin B-Ny266, binding to membrane via lipid II has been proposed as a model for its mechanism of action (Hsu *et al*. 2004). Also, an alternative mechanism of action has been described for lipid II-targeted lantibiotics that are too short to form a pore across the bilayer membrane (such as mutacins from group B) but maintain their antibacterial activity. The lantibiotic activity was shown to result in removal of lipid II from the septum, which inhibits peptidoglycan synthesis and causes cell death (Hasper *et al*. 2006). The mode of action of other mutacins was preliminarily deduced from computational analysis using sequence comparison with antimicrobial peptides with known function and modes of action. A membrane-spanning domain was found in the N-terminal region of the peptide sequence of mutacin N by comparison to the transmembrane domain VIII of the *E. coli* PTS enzyme IIc and also observed in the C-terminus of the peptide (Balakrishnan *et al*. 2000). A membrane disruptive mechanism similar to that of aureocin A53 was predicted for mutacin BHT-B, which has similarities with this tryptophan-rich and amphipathic bacteriocin (Netz *et al*. 2002a; Hyink *et al*. 2005). Similarly, the mode of action of SmbAB and BHT-Aαβ peptides were predicted based on sequence comparison with the two-component lacticin 3147αβ. The proposed mechanism is that the α peptide binds to the docking molecule lipid II and then recruits the β peptide, which forms pores in the membrane of the target cell (Hyink *et al*. 2005; Morgan *et al*. 2005; Yonezawa and Kuramitsu 2005; Wiedemann *et al*. 2006).

MUTACIN RESISTANCE

Many bacteriocin resistance mechanisms have been reported among usually sensitive bacteria, associated with a different phenotypic and genetic traits (Ennahar *et al*. 2000; Ramnath *et al*. 2000; Dalet *et al*. 2001; Héchard *et al*. 2001; Gravesen *et al*. 2002; Vadyvoloo *et al*. 2002; Ramnath *et al*. 2004; Vadyvoloo *et al*. 2004a, 2004b; Xue *et al*. 2005; Kramer *et al*. 2006). While resistant mutant strains were easily obtained for nisin (Mazzotta *et al*. 1997; Crandall and Montville 1998), no mutacin-resistant mutants have been reported in the literature (Dagry 1996; Smith *et al*. 2003).

For lantibiotic resistance, only nisin resistance has been investigated in detail. Nisin-resistant variants of many organisms have been isolated after exposure to the lantibiotic (Mazzotta and Montville 1997; Gravesen *et al*. 2002 for *L. monocytogenes*; Mazzotta *et al*. 1997 for *Clostridium botulinum*; Garde *et al*. 2004 for *Streptococcus thermophilus*; Mantovani and Russell 2001 for *Streptococcus bovis*; Peschel *et al*. 1999 for *S. aureus*). Nisin resistance has been related to alterations in the cell wall (Garde *et al*. 2004), membrane fatty acid and/or phospholipid composition (Mazzotta and Montville 1997), and the amount of lipoteichoic acids in the membrane composition (Peschel *et al*. 1999; Mantovani and Russell 2001). A nisin resistant profile is acquired through alterations in the expression of genes that are involved in cell wall and cytoplasmic membrane biosynthesis (Kramer *et al*. 2006). According to Kramer *et al*. (2006) four major mechanisms of acquired resistance have been defined (i) preventing nisin from reaching the cytoplasmic membrane, (ii) reducing the acidity of the extracellular medium, thereby stimulating the binding of nisin to the cell wall, (iii) preventing the insertion of nisin into the membrane, and (iv) possibly transporting nisin across the membrane or extruding nisin out of the membrane.

Class IIa bacteriocin resistance has been reported for normally sensitive bacteria (Ennahar *et al*. 2000). In *Listeria* species, low resistance levels (two- to four-fold) to class IIa bacteriocins are caused by alterations in membrane lipid composition (Crandall and Montville 1998; Vadyvaloo *et al*. 2002, 2004a). An increase in positive charges in the membrane decreases the sensitivity to the class IIa bacteriocins (Vadyvaloo *et al*. 2004b). High resistance levels (1000-fold) in *L. monocytogenes* and *E. faecalis* result primarily from the loss of a mannose permease component (Ramnath *et al*. 2000; Dalet *et al*. 2001; Héchard *et al*. 2001; Gravesen *et al*. 2002). High resistance levels in *L. monocytogenes* are also caused by the loss of a regulating transcription factor (Robichon *et al*. 1997; Dalet *et al*. 2001), which positively regulates the expression of the mannose permease. Diep *et al*. (2007) identified a similar mechanism between target cell recognition and immunity self-protection for class II bacteriocins involving the mannose phosphotransferase components IIC and IID as the common target. In fact, the mannose transporter complex seems to act as an open door for bacteriocin invasion including microcins produced by Gram-negative bacteria, which harbour some similarities in structure with lantibiotics from Gram-positive bacteria (Jack and Jung 2000; Bieler *et al*. 2006).

ARE MUTACINS SUITABLE FOR USE IN THE FOOD INDUSTRY?

Limitations to produce and use mutacin in food systems

Recently, Gálvez and co-workers (2007) reviewed the most important factors influencing the efficacy of bacteriocins in food systems. In this chapter, virtual pathogenicity of *S. mutans*, interactions of bacteriocins with food matrices and factors related to the obtaining of pure mutacins will be discussed. Also, conceivable solutions based on intrinsic properties and test procedures concerning bacteriocin applications in food are proposed for mutacins.

Pathogenicity

The major objection to the use of *S. mutans* and/or its products in the food industry is that *S. mutans* is recognised as a "pathogen". Nobody wants to put *S. mutans* in food to increase risk of dental caries, do they? However, is *S. mutans* really a "pathogen"? *S. mutans* is considered as the major cause of dental caries, but what is *S. mutans* really guilty of?

S. mutans produces acid from sugars, but so do all the LAB used in milk fermentation processes. It produces water-soluble exopolysaccharides, which is often a desired trait in the food industry (Tettelin 2004) but also water-insoluble glucans, which promote adherence to the cells and are referred to as unwanted exopolysaccharides in food (de Vuyst and Degeest 1999). *S. mutans* is considered a "pathogen" because production of soluble and insoluble glucan by extracellular and cell-associated glycosyltransferase promotes accumulation of *S. mutans* cells on the surface of the teeth while carbohydrate metabolism leads to production of lactic acid destroying the tooth enamel (Mitchell 2003). However, who is the real culprit? Is it *S. mutans*, or the refined sugar, particularly sucrose, in the diet? In fact, *S. mutans* should not be considered as a pathogen to be afraid of, but as one whose only light virulence is linked to the diet (Rolla 1989; van Palenstein Helderman *et al*. 1996).

S. mutans is an indigenous inhabitant of the oral cavity. It was found to be present in almost every human (Hamada and Slade 1980) and apart from leading to dental caries, it does not cause any damage to its host under normal conditions. Moreover, *S. mutans* is not the only cariogenic species, as dental caries occurs in its absence (Kleinberg 2003). *S. mutans* has rarely been shown to be transmitted horizontally among individuals (Berkowitz 2003). Even people living in close proximity do not share the same *S. mutans* strains (Alaluusua 1991; Klein *et al*. 2004). Transmission of *S. mutans* was demonstrated to occur only from mother to infants during a very narrow period (Caufield *et al*. 1993). Thus, *S. mutans* is not usually contagious. However, *S. mutans* may be associated with endocarditis (Moreillon and Que 2004; Nomura *et al*. 2006). This appears only after oral surgery when *S. mutans* gets access to the blood circulation and ends up colonising the cardiac valves. Won't some polysaccharide-producing LAB bacteria do the same if they get access to the blood circulation?

No gene encoding toxins have been detected in the *S. mutans* genome and few virulence traits are reported for *S. mutans* compared to other pathogenic streptococcal species (Ajdic *et al*. 2002; Mitchell 2003). Probable virulence factors identified in *S. mutans* are adhesins allowing adhesion of cells to exracellular matrix and initiation of biofilm formation, glucan-producing and glucan-binding exoenzymes, mainly involved in extracellular sucrose metabolism and ensuring aggregation of cells, several proteases and peptidases to provide amino acid nutrients, and other membrane and extracellular proteins which could stimulate the immune system (Ajdic *et al*. 2002; Mitchell 2003). In fact, inactive relics of pathogenesis-related genes are also found in the genome of the food-related LAB species *Streptococcus thermophilus* (Tettelin 2004; Mora *et al*. 2005).

Interaction with food material

One limitation to the use of bacteriocins in food is their interaction with food constituents, which can affect their activity (Aasen *et al*. 2003). Food structure, food composition (nutrients, ingredients, additives), buffering capacity of food products and their associated physicochemical factors such as pH, temperature, water activity (a_w), atmosphere (O_2, CO_2), redox potential, and microbial load can influence *in situ* bacteriocin activity in food products (Gálvez *et al*. 2007). All these obstacles should be studied before mutacins can be used as food preservatives. It has been observed that the growth of some sensitive strains in the presence of sucrose can abolish their sensitivity to some mutacins (Hamada and Ooshima 1975b; Rogers 1976b) and that a high sucrose diet reduced the activity of mutacin C3603 *in vivo* (Ikeda *et al*. 1985). However, our results (Morency and Lavoie 1991) and those of others (Delisle 1975; Weerkamp *et al*. 1977; Hamada *et al*. 1986; Willcox and Drucker 1988) indicate that this is not always the case. Each mutacin being a different substance, their activity in food will have to be assessed individually.

Production and purification

The difficulties in producing mutacins or in obtaining good mutacin yield in liquid media are well known (Parrot *et al*. 1989; Nicolas *et al*. 2004) and few mutacins have been purified from liquid cultures (Novak *et al*. 1993; Mota-Meira *et al*. 1997; Nicolas *et al*. 2006). Bacteriocin production is influenced by several environmental factors, such as pH, temperature, concentration of nitrogen and carbohydrate sources, and the presence of essential elements (vitamins, oligo-elements) (Parente and Ricciardi 1999). An inexpensive medium based on whey permeate was found for mutacin production making commercial production possible (Nicolas *et al*. 2004).

The major limiting factor, which is obtaining pure bacteriocin, should not be an insurmountable problem. General methods of bacteriocin purification are based on their biochemical properties: cationic and amphiphilic peptides. The general process for bacteriocin purification often involves a straight-forward four-step process (Berjeaud and Cenatiempo 2004). The methods most frequently used for isolation, concentration and purification include salt precipitation of bacteriocin from culture supernatant, cationic exchange chromatography and reverse-phase high performance liquid chromatography (Saavedra *et al*. 2004). Large scale food grade purification of bacteriocin still requires improvement (Guyonnet *et al*. 2000; Uteng *et al*. 2002), as the majority of processes involve the use of toxic organic solvents. Use of food grade mutacins could also be enabled by applying food grade purification procedures developed by Coventry *et al*. (1996) and Wan *et al*. (1996), which use diatomite calcium silicate or other ingestible silica compounds to purify different bacteriocins to homogeneity. This purification process involved both electrostatic and hydrophobic interactions of bacteriocins with food grade adsorbing resins. The use of food grade emulsifier under limited concentrations for human consumption to desorb bacteriocins is also possible (Daeschel 1993; Coventry *et al*. 1996; Janes *et al*. 1998). We previously described a technique of purification using hydrochloric acid instead of trifluoroacetic acid (Gaussier *et al*. 2002). Methanol could also be replaced by ethanol in the elution process. Another possible avenue to explore would be to use shrimp exoskeleton (mainly made of chitin) as a solid phase in the chromatography process (Casal *et al*. 2006). However, although chitin is closely related structurally to chitosan (poly-D-glucosamine), which shows no toxicity to mammals, and is approved by FDA as a food additive, it was recently shown to induce the accumulation in tissue of IL-4-expressing innate immune cells, including eosinophils and basophils responsible for allergic reactions, when given to mice (Reese *et al*. 2007). Potential food grade alternatives to these processes also include immuno-

affinity chromatography (Suarez *et al*. 1997).

Heterologous production of mutacins in GRAS bacteria

Heterologous expression systems for bacteriocins production may offer advantages over the original producer, such as allowing control of bacteriocin gene expression and achieving higher production levels by selected food grade LAB. Heterologous expression may also contribute to the production of hybrid bacteriocins with improved properties and effectiveness in food systems (Rodrigez *et al*. 2002; Ingham and Moore 2007). Construction of multibacteriocinogenic strains is also possible in order to contravene bacteriocin resistance development. With the advances in knowledge acquired on the genetics of mutacin production, we can envision that some cocktails of mutacins targeting a broader spectrum of pathogens could be produced by one food grade LAB strain. Research efforts are still necessary to improve the production levels of heterologous expression of bacteriocin-lantibiotic in genetically modified GRAS (Generally Recognised As Safe) organisms.

Stability

The biochemical properties of lantibiotics make them resistant to a wide range of temperatures (up to 100°C for 15-30 min and some up to 121°C for 15 min) and pH (2 to 12) (Delves-Broughton *et al*. 1996; Lawton *et al*. 2007). The high stability of lantibiotics is explained by their thioether bridges which lock the molecule into the biologically active conformation as well as the presence of D-amino acids that protect them against the activity of certain proteases (Ryan *et al*. 1999). However, from our mutacin producing strains, all mutacins were sensitive to pronase E, proving their digestibility, and a few demonstrated reduced activity after heat treatment (80°C, 1 h) suggesting their potential use in pasteurized food products (Parrot *et al*. 1990; Morency *et al*. 1995; Nicolas *et al*. 2006). Mutacins H-29B and B-Ny266 also showed resistance to autoclaving (121°C, 15 min) (Nicolas *et al*. 2006).

APPLICATIONS

Biopreservatives

Can pure mutacin be used as a food biopreservative? Bacteriocins produced by *S. mutans* have interesting properties suitable to use as food biopreservative. Similar to bacteriocins produced by LAB, mutacins are inactivated by enzymes from the gastrointestinal tract, they are heat and pH-tolerant, they have a bactericidal effect against many foodborne pathogenic and spoilage bacteria. However, unlike LAB bacteriocins, no mutacin-resistant mutants have been reported in the literature. Other bacteriocins produced by GRAS streptococcal species are currently intentionally or unintentionally used either in the food and feed industries or in various probiotic treatments and are seriously considered for a number of other applications (Kirkup 2006; Diez-Gonzalez 2007). By their homology with well-characterized bacteriocins, some mutacins could find application in food products.

Mutacin II (H-29B) and mutacin K8 are related to the well-described lacticin 481 and to other lantibiotics of the lacticin 481 group (Dufour *et al*. 2006). Many studies have reported the effectiveness of this type of lantibiotic in food products (O'Sullivan *et al*. 2003; Ávila *et al*. 2006; Dufour *et al*. 2006). For example, proposed applications are variacin powder preparation to inhibit *B. cereus* growth in chilled dairy products (O'Mahony *et al*. 2001), warnericin RB4 against spoilage bacteria in acidic fruit juice-based drinks (Minamikawa *et al*. 2005), macedocin-producing strains as bioprotective cultures in cheese making (van den Berghe *et al*. 2006). Mutacins Smb and BHT-A are related to the well-studied lacticin 3147, which has demonstrated its potential as a food preservative by inhibiting many pathogens and spoilage bacteria such as *L. monocytogenes*, *B. cereus* and *S. aureus* (Morgan *et al*. 1999, 2000; Scannell *et al*. 2000; Morgan *et al*. 2001; Cotter *et al*. 2005a).

Peptide A from mutacin IV shows homology with peptide A (ThmA) of thermophilin 13, a bacteriocin that is naturally found in yoghurt. Furthermore, the *S. mutans* genome appears to code for additional ThmA-like peptides (loci SMU.613 and SMU.616 in the *S. mutans* genome, GenBank accession number AE014133). Thermophilin 13 is a dipeptide non-lantibiotic bacteriocin able to inhibit growth of different species of *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Listeria*, *Bacillus* spp., and *Clostridium* spp. It even prevents outgrowth of spores of *B. cereus* and *Clostridium botulinum* (Marciset *et al*. 1997). Thermophilin 13 was shown to act by dissipating the membrane potential and the pH gradient in liposomes by forming pores and no component from the membrane sensitive strains (e.g. lipid or proteinaceous receptor) seems to be required for its activity (Marciset *et al*. 1997).

Mutacin B-Ny266 is basically related to the lantibiotic nisin, which is the only purified bacteriocin approved for use as a food additive. It has been used for more than thirty years against foodborne and spoilage bacteria in several food systems such as low-acid canned foods and dairy products (Delves-Broughton *et al*. 1996; Chen and Hoover 2003). Toxicology of pure nisin has been tested and found harmless for human consumption (Delves-Broughton *et al*. 1996). Mutacin B-Ny266 shows no haemolytic activity against blood erythrocytes and no apparent deleterious effect in a mouse model (Mota-Meira *et al*. 1997, 2005). As for nisin, mutacin B-Ny266 has a broad activity spectrum against important foodborne and food spoilage Gram-positive bacteria and its activity is enhanced at acidic pH (Mota-meira *et al*. 2000; Nicolas *et al*. 2004). Furthermore, mutacin B-Ny266 remained active against bacterial strains that are resistant to nisin A (Mota-Meira *et al*. 2000), placing this mutacin as a good candidate to be used when nisin resistant mutants are present in a food system.

As certain food spoilage and foodborne pathogen strains can be naturally resistant or develop resistance to one particular bacteriocin (Ennahar *et al*. 2000), combinations of bacteriocins represent valuable strategies to eliminate the propagation of resistant bacterial contaminants in food products (Hanlin *et al*. 1993; Bouttefroy and Milliere 2000; Vignolo *et al*. 2000).

Cheese ripening

Enzymatic coagulation, draining, and ripening ensure transformation of milk into cheese. Ripening represents a critical step in the maturation of cheese and development of cheese flavour and texture. Many complex biochemical reactions of proteolysis, lipolysis and glycolysis take place during the maturation period. These reactions are generally performed by starter cultures and their enzymes. To reduce the cost of cheese production, different methods have been developed to accelerate the cheese ripening step, such as the use of recombinant enzymes or microencapsulated enzymes (Azarnia *et al*. 2006). Mutacins could be used to increase the rate of starter culture lysis and enhance the release of intracellular enzymes such as aminopeptidase during cheese ripening, as has been proposed for other bacteriocins (Ryan *et al*. 1996; Morgan *et al*. 1997; Ross *et al*. 1999; O'Sullivan *et al*. 2003; Ávila *et al*. 2006). The oral cavity naturally contains mutacin-producing strains that show broad spectra inhibitory activity not only against pathogenic and food spoilage microorganisms, but also against starter and non-starter LAB. Under the proper conditions, pure mutacins could be used to accelerate cell lysis during cheese ripening (**Table 3**).

Biofilm inhibition

Biofilm formation is an emerging problem in sanitation and

Table 3 Summary of proposed applications for some mutacins.

APPLICATIONS
Addition of purified or semi-purified mutacins as food additive/biopreservative
-Inhibition of nonstarter LAB proliferation in cheese making
-Inactivation of pathogenic bacteria in cheese (*L. monocytogenes*, *S. aureus*)
-Inhibition of *C. tyrobutyricum* (late-blowing defect in cheese)
-Inactivation of *L. monocytogenes* in ready-to-eat foods (e.g. hot dog sausage)
-Inhibition of spoilage bacteria in processed foods (e.g. *Bacillus* spp.)
-Inhibition of spoilage bacteria in acidic fruit-juice-based drinks
-Inhibition of spoilage bacteria in beer fermentation processes
Immobilized mutacins in food contact surfaces
-Equipment and surfaces in the food plant to avoid bacterial biofilm formation
-Food packaging, e.g. in meat packaging for inhibition of pathogenic (*L. monocytogenes*) and food spoilage bacteria
Adjunct in raw milk products
Acceleration of cheese ripening
In combination with other bacteriocins to eliminate the propagation of resistant bacterial contaminants in food products
In combination with other barriers in hurdle technology
-in MAP[a] and VP[b] to inhibit the pathogenic (*L. monocytogenes*) and spoilage bacteria (LAB e.g. *Lactobacillus* spp., clostridia)
-low pH, low NaCl concentration, moderate temperatures, high hydrostatic pressure or pulsed electric fields

[a] MAP: modified atmosphere packing
[b] VP: vacuum packing

safety of the food environment (reviewed by Kumar and Anand 1998; Lyndsay and von Holy 2006). Biofilms are complex communities of microorganisms irreversibly attached to a substratum, interface, or to each other, producing an extracellular polymeric matrix (Costerton 1995; Kumar and Anand 1998; O'Toole and Ghannoum 2004). Cells in biofilms are often more resistant to treatment with antimicrobial compounds, such as disinfectants, than planktonic cells (Costerton *et al*. 1995). Cells of pathogenic or spoilage microorganisms may survive cleaning and disinfection, detach and contaminate the food products resulting in post-processing contamination (Kumar and Anand 1998). Undesirable biofilms and their decomposition products on surfaces may lead to reduced heat transfer, increased resistance to flow, and corrosion of food processing equipment (Kumar and Anand 1998). Biofilms in the food industry can be eliminated by physical and chemical methods. In addition, the use of antimicrobial compounds such as bacteriocins adsorbed onto surfaces has been proposed to inhibit biofilm formation (Kumar and Anand 1998). Daeschel *et al*. (1992) and Bower *et al*. (1995) showed that nisin adsorbed to food contact surfaces lowered the incidence of surface contamination by *L. monocytogenes*. Ming *et al*. (1997) proposed the use of bacteriocins on food packaging materials for the biocontrol of *L. monocytogenes* on meats. Ercolini *et al*. (2006) used polythene films coated with a solution of bacteriocin 32Y from *Lactobacillus curvatus* to package frankfurters artificially contaminated with *L. monocytogenes*. The antimicrobial package was effective in inhibiting the growth and survival of the pathogen on the surface of the food product during storage. Joerger (2007) reviewed the effectiveness of films to which antimicrobials, such as nisin, food-grade acids and salts, chitosan, plant extracts, and enzymes such as lysozyme and lactoperoxi-dase, were incorporated for food applications. He pointed out that antimicrobial films still face limitations and suggested that they be used as part of a hurdle strategy to provide safe foods. Mutacins such as mutacin B-Ny266 having specific or broad activity spectra against several foodborne bacteria could find similar applications (**Table 2** and **Table 3**).

Combination of mutacins with other hurdle technologies

The concept of hurdle technology implies the activity of various antimicrobial factors to which foodborne microorganisms are exposed to reduce or control their population in food products. Many hurdles have been developed to enhance the control of foodborne and spoilage microorganisms found in food products (Leistner 2000). Recently, bacteriocins have gained great interest as part of one hurdle since they can be easily combined with other selected preservation technologies without losing their antibacterial activity. Combinations of bacteriocins with chemical substances, heat treatment, modified atmosphere packaging, pulsed electric field, high hydrostatic pressure, irradiation and pulsed magnetic fields have often been studied, showing a synergistic effect in inhibitory activity against target microorganisms (reviewed by Gálvez *et al*. 2007). Indeed, by their particular physical and chemical properties, bacteriocins can be applied to food and can resist treatments such as temperature, addition of chemicals, high pressure processing or pulsed electric fields. The combination of different kinds of hurdle technologies avoids development of resistant bacteria. It also widens the inhibitory activity spectra of the antimicrobial factors targeting Gram-positive and Gram-negative spoilage bacteria thus improving the preservation of foodstuff (Chen and Hoover 2003; Gálvez *et al*. 2007). The counterpart of this kind of process is often the reduction of the organoleptic properties of food products. Bacteriocin efficiency in food matrices depends also on several food-related factors to which one particular bacteriocin molecule could be less susceptible. Impacts of such food-related factors on mutacin activity are yet unknown and remain to be studied.

CONCLUDING REMARKS

Over the last five years, advances in bacteriocin research have been spectacular, contributing to the better understanding of the molecular mechanism of action and structure-function relationships. Some crucial studies have clarified the enzymatic activity responsible for the modification reactions in the biosynthesis of lantibiotics given the possibility of synthesizing dehydrated and unsaturated residues *in vitro* (Xie *et al*. 2004; Li *et al*. 2006). A more thorough understanding of the mechanisms involved in bacteriocin immunity is emerging (Cotter *et al*. 2005a). However, effective application of bacteriocins in food will be restricted until comprehensive studies of their activity, solubility, stability in food products and more importantly, their toxicity are carried out. While a plethora of bacteriocins have gained interest in food applications, to date only nisin is legally approved for use in the food industry (Delves-Broughton *et al*. 1996; Chen and Hoover 2003). Pediocin, a class IIa bacteriocin and lacticin 3147 (class Ic) exhibiting broad activity spectra, are prototypes of new potentially applicable bacteriocins with properties more specific than nisin for application in particular food products. Mutacins, in their diversity, possess the general properties of these bacteriocins. They are active against various Gram-positive and Gram-negative bacteriocin-resistant foodborne pathogens and

spoilage bacteria (Mota-Meira *et al*. 2000, 2005). Due to the cautions over the use of enterococci and streptococci in the food industry, heterologous production of mutacins constitutes an attractive mean of exploiting their broad antibacterial activity. Finally, application of bacteriocins in food should not be seen as the cure to food spoilage and contamination, but rather as a valuable tool contributing to the hurdle concept for food preservation and food safety.

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