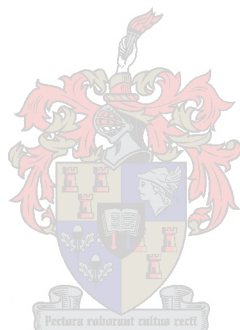Cloning of the *gfp* (green fluorescent protein) gene downstream of the *ldh* promoter in a bacteriocin-sensitive strain of *Lactobacillus sakei* to serve as a reporter strain in bacteriocin studies

by

Petronella F. Liss

Thesis in partial fulfilment of the requirements for the degree of Masters of Science at the University of Stellenbosch.

Supervisor: Prof. L.M.T. Dicks

December 2003

Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: Date:

Summary

Lactobacillus plantarum 285, isolated from sorghum beer, produces bacteriocin 285, which displays activity against several food spoilage organisms. For future application of bacteriocin 285 in the food industry, it was important to characterize the peptide and identify the genes encoding its production. The effect of bacteriocin 285 on sensitive cells was determined through the use of an indicator (sensitive) organism, *Lactobacillus sakei* DSM 20017. The indicator strain was genetically modified to express GFP (green fluorescent protein), with the aim of quantifying the antibacterial activity of bacteriocin 285 as a function of GFP fluorescence.

Bacteriocin 285 proved to be identical to plantaricin 423 produced by *L. plantarum* 423. Plantaricin 423 is a class IIa bacteriocin and displays antimicrobial activity towards a broad spectrum of bacteria, including several food spoilage organisms. The sensitivity of *L. sakei* DSM 20017 towards antibacterial peptides produced by *Lactobacillus curvatus* DF38, *L. plantarum* 285, *Lactobacillus casei* LHS and *Lactobacillus salivarius* 241 is not limited to the growth stage of the organism. Cells remained sensitive to all four of these bacteriocins, from lag phase to late exponential growth. To inhibit growth of up to 90% of the cells of *L. sakei* DSM 20017, 1 AU/ml bacteriocin 285 (7 ng/ml) of partially purified bacteriocin 285 was required. However, to kill all viable cells of *L. sakei* DSM 20017, 16 AU/ml (110 ng/ml) of partially purified bacteriocin 285 was required.

The gfp_{uv} gene, encoding GFP_{uv}, was cloned downstream of the *ldh* promoter and successfully expressed in *L. sakei* DSM 20017. However, GFP_{uv} fluorescence could not be used as a direct method to quantify the antimicrobial activity of bacteriocin 285, since cells of strain DSM 20017 remained fluorescent for prolonged periods after treatment with lethal concentrations of the bacteriocin. The non-viability of the cells was confirmed with epifluorescence microscopy and a LIVE/DEAD® *Baclight*™ Bacterial Viability Probe. Cells that were stained with the viability probe indicated that the majority of untreated *L. sakei* DSM 20017 cells were viable. However, treatment of strain DSM 20017 with 16 AU/ml bacteriocin 285 rendered all visible cells non-viable.

Opsomming

Lactobacillus plantarum 285 wat uit sorgumbier geïsoleer is, produseer bakteriosien 285. Die bakteriosien toon aktiwiteit teen verskeie organismes wat voedselbederf veroorsaak. Vir toekomstige aanwending van bakteriosien 285 in die voedselindustrie was dit belangrik om die peptied te karakteriseer en die gene wat vir die produksie daarvan kodeer, te identifiseer. Die effek van bakteriosien 285 op sensitiewe selle is bepaal deur die gebruik van 'n indikator (sensitiewe)-organisme, *Lactobacillus sakei* DSM 20017. Die indikator-organisme is geneties verander om die GFP (groen fluoreserende proteïen) uit te druk. Die doel was om die antibakteriese aktiwiteit van bakteriosien 285 te kwantifiseer as 'n funksie van GFP fluorisensie.

Bakteriosien 285 is identies aan plantarisien 423 wat deur *L. plantarum* 423 produseer word. Plantarisien 423 is 'n klas IIa bakteriosien en vertoon antimikrobiese aktiwiteit teenoor 'n wye verskeidenheid bakterieë, insluitende verskeie organismes wat voedsel bederf. Die sensitiwiteit van *L. sakei* DSM 20017 teenoor antibakteriese peptiede wat deur *Lactobacillus curvatus* DF38, *L. plantarum* 285, *Lactobacillus casei* LHS en *Lactobacillus salivarius* 241 geproduseer word, word nie beïnvloed deur die groeifase van die organisme nie. Selle het sensitief gebly teenoor al vier die bakteriosiene van sloer- tot laat eksponensiële groei. Om groei van tot 90% van *L. sakei* DSM 20017 selle te inhibeer, word 1 AU/ml (7 ng/ml) gedeeltelik gesuiwerde bakteriosien 285 benodig. Om alle lewensvatbare *L. sakei* DSM 20017 selle te dood, word 16 AU/ml (110 ng/ml) gedeeltelik gesuiwerde bakteriosien 285 benodig.

Die *gfp$_{uv}$*-geen, wat GFP$_{uv}$ kodeer is stroomaf van die *ldh*-promoter gekloneer en suksesvol in *L. sakei* DSM 20017 uitgedruk. GFP$_{uv}$ fluoresensie kon nie as direkte metode gebruik word om die antimikrobiese aktiwiteit van bakteriosien 285 te bepaal nie, aangesien die selle van *L. sakei* DSM 20017 fluoreserend gebly het lank na behandeling met dodelike konsentrasies van die bakteriosien. Die lewensvatbaarheid van die selle is bevestig deur epifluoresensie-mikroskopie en 'n LIVE/DEAD® *Baclight*™ bakteriese lewensvatbaarheidspeiler. Selle van *L. sakei* DSM 20017 wat deur die peiler gekleur is, het gewys dat die meeste selle wat nie deur bakteriosien 285 behandel was nie, lewensvatbaar was. Behandeling van *L. sakei* DSM 20017 met 16 AU/ml bakteriosien 285 het al die sigbare selle gedood.

Acknowledgements

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INTRODUCTION

INTRODUCTION

Lactic acid bacteria play an important role in the fermentation of various foods and beverages. Most of the species characterized thus far do not form toxins and are in general considered to be non-pathogenic (de Vuyst and Vandamme, 1994). The preservative role of lactic acid bacteria in fermented foods and their production of organoleptic compounds have been well documented. Most of the antimicrobial substances such as lactic acid, acetic acid, hydrogen peroxide and bacteriocins (antimicrobial peptides) are produced *in situ* by starter cultures, rendering the product less perishable. These natural inhibitors could replace unwanted chemical additives such as sulfur dioxide, benzoic acid, sorbic acid, nitrate and nitrite, or reduce the concentration needed. Although sulfur dioxide, sodium benzoate and sorbic acid are considered to be safe food preservatives, the general tendency is to avoid chemical additives. In the United States of America, 0.05% of the population are sulfite-sensitive (Lester, 1995).

Bacteriocins are small, ribosomally synthesized antimicrobial peptides (Héchard and Sahl, 2002) with no characteristic flavour or taste. Most bacteriocins produced by lactic acid bacteria exhibit activities towards a broad range of Gram-positive species, but not Gram-negative bacteria, molds or yeast (De Vuyst and Vandamme, 1994). Bacteriocins produced by lactic acid bacteria are subdivided into three distinct classes, namely class I, class II and class III. Class I bacteriocins undergo posttranslational modifications, whereas bacteriocins of class II undergo no posttranslational modifications. Class II bacteriocins are subgrouped in groups IIa to IIc. Bacteriocins grouped in class IIa are characterized by the presence of YGNV and CXXXXCXV sequence motifs in their N-terminal sections. Class IIa bacteriocins strongly inhibit growth of *Listeria* spp. Since *Listeria* is a food-borne pathogen, class IIa bacteriocins have potential as biopreservatives in food and animal feed (Ennahar *et al.*, 2000).

The mode of action of many bacteriocins have been described. In most cases, the destruction of the target or sensitive cells are expressed as a decrease in viability (bacteriostatic) or lowering of cell numbers (bactericidal). Viable cell counts and optical density readings of cultures are often not sensitive enough to detect small changes in the target cell brought about by bacteriocins.

Bacteriocin 285, which is produced by *Lactobacillus plantarum*, has an inhibition spectrum similar to plantaricin 423, a broad-spectrum bacteriocin produced by *L. plantarum* (Van Reenen and Dicks, 1996). For future application of bacteriocin 285 in the food industry, it was important to characterize the peptide, identify the genes encoding its production, and study its mode of action.

The effect of bacteriocin 285 on sensitive cells was studied by using an indicator sensitive strain of *Lactobacillus sakei* (DSM 20017). *Lactobacillus sakei* DSM 20017 was chosen as indicator organism, since it is sensitive to several bacteriocins. Furthermore, the sequence of

the *ldh* gene of *L. sakei* has been published (Van den Berg, et al., 1995). Strain 20017 was genetically modified to express GFP (green fluorescent protein). The inhibition of *L. sakei* DSM 20017 would then, theoretically, result in a decrease in GFP fluorescence. A similar approach was taken by Simon *et al.* (2001). In the latter case, the authors used luciferase luminescence to quantify the effect of bacteriocins on a number of bacteria. A decrease in luminescence was observed when sensitive cells were treated with the bacteriocins. Use of GFP as a reporter has several advantages. GFP fluorescence is relatively easily detectable, requires no addition of exogenous substrate or energy source, the cells need no processing and individual cells can be monitored (Bloemberg *et al.*, 1997). A reporter strain containing the *gfp* gene could be useful as a tool for rapid identification and quantification of bacteriocin activities by using fluorescence microscopy, fluorimetry or flow cytometry.

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LITERATURE REVIEWS

MINI REVIEW
LACTIC ACID BACTERIA: METABOLISM, BACTERIOCINS AND EXPRESSION SYSTEMS

Lactic acid bacteria belong to the family Lactobacteriaceae. They are Gram-positive, catalase negative, oxidase negative, non-sporulating microaerophilic and acid-tolerant with lactate as the main fermentation product from carbohydrates (Sneath *et al.*, 1986). They are dependent on carbohydrates for their energy supply and are mostly non-motile. Lactic acid bacteria can be either cocci (*Lactococcus, Vagococcus, Leuconostoc, Oenococcus, Pediococcus, Melissococcus, Aerococcus, Tetragenococcus, Streptococcus* and *Enterococcus*) or rods, e.g. *Lactobacillus, Weissella* and *Carnobacterium* (Schlegel and Schmidt, 1986).

The Lactobacteriaceae do not contain haemins (catalase, cytochromes), nevertheless they are able to generate ATP in the presence of oxygen through substrate-level phosphorylation. Lactic acid bacteria require a number of vitamins, aminoacids, purines and pyrimidines as growth factors, rendering these bacteria well adapted for growth on milk and other media that are rich in nutrients. Unlike most other bacteria, lactic acid bacteria can utilise lactose. This ability is, however, shared with a number of bacteria from the intestine (Martin, 1996).

Lactic acid bacteria are usually found in milk and environments where milk is produced and processed (*Streptococcus diacetilactis, Lactobacillus casei, Lactobacillus fermentum, Lactobacillus lactis, Lactobacillus bulgaricus* and *Lactobacillus helviticus*), in the intestinal tracts and mucous membranes of mammals (*Streptococcus salivarius, Streptococcus pyogenes, Streptococcus bovis, Streptococcus pneumoniae, Lactobacillus acidophilus* and *Bifidobacterium* spp.), and in intact and rotting plants (*Lactobacillus brevis, Lactobacillus plantarum, Lactobacillus delbrueckii, L. fermentum, Streptococcus lactis* and *Leuconostoc mesenteroides*) (Schlegel and Schmidt, 1986).

Since lactic acid bacteria produce large quantities of lactic acid, they easily become dominant under appropriate environmental conditions. This makes it easy to isolate "pure cultures" from products like sour milk and sauerkraut (Schlegel and Schmidt, 1986). Examples of fermented food products are pickles, sauerkraut, sausages, yoghurt, cheese, tofu, and soy sauce. Other industrial advantages of lactic acid bacteria are that they grow rapidly, they can secrete proteins and they can express homologous and heterologous proteins. An increasing number of laboratories have started to study the genetics of lactic acid bacteria (de Vuyst and Vandamme, 1994). These genetic studies have been valuable in

the analysis of industrially important traits and their improvement by genetic, metabolic and protein engineering.

1. Metabolism of lactic acid bacteria

Lactobacteriaceae are classified into two groups according to their ability to ferment glucose to lactate or to additional products (Mayrhuber *et al.*, 1999). Homofermentative lactic acid bacteria produce 90% pure lactate. They metabolise glucose via the glycolytic pathway. Heterofermentative lactic acid bacteria lack the important enzymes of the glycolytic pathway (aldolase and triose-phosphate isomerase) and metabolise glucose by the pentose-phosphate pathway to yield lactate, ethanol, and carbon dioxide (Schlegel and Schmidt, 1986).

Fermentation is defined as an energy-yielding process in which molecules within the catabolic pathway serve both as electron donors and acceptors (Schlegel and Schmidt, 1986). The substrate is metabolized without the involvement of an exogenous oxidising agent. During fermentation of hexoses, the sugars are transported into the cells and are phosphorylated. A high-energy phosphate bond is required to activate the sugar. In most bacteria, the transport of free sugar and phosphorylation is done via an ATP-dependent glucokinase. In some species the phosphoenolpyruvate-sugar phosphotransferase system is used (Schlegel and Schmidt, 1986).

The first step of fermentation is glycolysis that occurs in the cytoplasm in the absence of oxygen. Glycolysis is used by all obligatory homofermentative and facultatively heterofermentative lactic acid bacteria. During glycolysis fructose-1,6-diphosphate is formed from fructose-6-phosphate. A fructose-1,6-diphosphate aldolase splits the fructose-1,6-diphosphate into dihydroxyacetonephosphate and glycelaldehyde-3-phosphate. Through substrate-level phosphorylation at two sites, glyceraldehyde-3-phosphate (and dihydroxyacetonephosphate through glyceraldehyde-3-phosphate) is converted to pyruvate with the net yield of 2 ATP. Under conditions of excess sugar and a limited concentration of dissolved oxygen, pyruvate is reduced to lactate by a NAD^+-dependent lactate dehydrogenase, thereby reoxidising the NADH that was formed during glycolysis. The stereospecificity of the lactate dehydrogenase and the presence of lactate racemase (catalyses the conversion from L- to D-lactate) determines whether D(-)-, L(+)-, or DL-lactate is formed (Schlegel and Schmidt, 1986).

In heterofermentative lactic acid bacteria, the initial degradation of glucose is via the pentose phosphate pathway. The pathway is characterized by initial dehydrogenation steps with the

formation of 6-phospho-gluconate, which becomes decarboxylated. The ribulose-5-phosphate which is formed, is converted to xylulose-5-phosphate by an epimerase. The next reaction (thiamine pyrophosphate-dependent) is catalysed by phosphoketolase and cleaves the molecule into glyceraldehyde-3-phosphate and acetylphosphate. Glyceraldehyde-3-phosphate is metabolized in the same way as for the glycolytic pathway and results in the formation of lactic acid. When no other electron acceptor is available, acetyl phosphate is reduced to ethanol via acetyl CoA and acetaldehyde. Heterolactic fermentation yields one mole each of lactic acid, ethanol and CO_2 (Schlegel and Schmidt, 1986)

Lactic acid is an organic hydroxy acid with uses in the chemical, industrial and food processes. Lactic acid formed in fermentation processes is normally a racemic mixture (DL forms) (Martin, 1996). Since humans have only the L-lactate dehydrogenase enzyme, only the L(+) isomer of lactate can be assimilated (Soccol et al., 1994), making the L(+) isomer most important for the food industry. Half of the lactic acid produced worldwide, is manufactured by fermentation processes and is employed in the food industry as an acidulant (Ward, 1989).

Considering that the lactate dehydrogenase enzyme plays such a vital role in the conversion of pyruvate to lactate, it has been studied in depth (Clarke et al., 1994; Malleret et al.,1998). Malleret et al. (1998) determined the sequence of the L-lactate dehydrogenase gene of L. sakei. Lactobacillus sakei produces both L- and D-lactate. The production of L-lactate results from the conversion of pyruvate to L-(+) by L-lactate dehydrogenase. Part of the L-lactate is consumed and an equal amount of D-lactate is produced by lactate racemase that transforms L-lactate to D-lactate. The presence of lactate racemase in L. sakei is considered to be a characteristic trait of the species (Sneath et al., 1986). In several lactic acid bacteria, the D-lactate dehydrogenase is responsible for the production of D-lactate. Genes sharing sequence similarities with D-lactate dehydrogenase have been reported (Delcour et al., 1993; Bernard et al., 1994).

2. Bacteriocins

Bacteriocins were discovered in 1925 when E. coli V was shown to produce an antimicrobial compound active against E. coli φ (Gratia, 1925). The antimicrobial substances produced by E. coli were named colicins and are active against other Enterobacteriaceae. Both Gram-positive and Gram-negative bacteria produce bacteriocins that are plasmid-encoded, proteinaceous compounds and are active mainly against closely related strains or species (Tagg et al., 1976). It has, however, become evident that bacteriocins produced by lactic acid

bacteria do not display bactericidal activity only towards species that are closely related to them (Klaenhammer, 1993).

2.1. Classes of bacteriocins

The genetic organization, structure and mode of action of bacteriocins has only recently been determined. Although heterogenous, bacteriocins produced by lactic acid bacteria are subdivided into three distinct classes, based on genetic and biochemical resemblances (Nes *et al.*, 1996) and their primary structure (Héchard and Sahl, 2002). Some bacteriocins are secreted via ABC (ATP-binding cassette) transporter systems and others by means of a sec-dependent pathway. Class I bacteriocins are modified peptides. Lantibiotics (class I) are small, heat-stable, lanthionine-containing bacteriocins. Class I bacteriocins undergo extensive post-translational modification before secretion (Nes *et al.*, 1996).

Class II bacteriocins are unmodified peptides that are small, heat-stable (100°C to 121°C), non-lanthionine containing, membrane active peptides. Class II bacteriocins consist of a large heterologous group produced by different species of the lactic acid bacteria (Klaenhammer, 1993). Regardless of this heterogeneity, all class II bacteriocins display a very conserved N-terminal leader peptide and a characteristic double-glycine-type ($Gly^{-2} Gly^{-1}$ Xaa) proteolytic processing site. The organization of the operon structures encoding these bacteriocins reflects the conserved mechanism of secretion and processing. The genetic determinants involved in the production of several class II bacteriocins have been genetically studied in detail (Holo *et al.*, 1991; Marugg *et al.*, 1992; Axelsson and Holck, 1995). Class II bacteriocins can be divided into three groups: Class IIa are the *Listeria*-active bacteriocins with a Tyr-Gly-Asn-Gly-Val (YGNGV) consensus sequence in the N-terminal side of the peptide. Class IIb includes bacteriocins that consists of two peptides. Both these peptides are necessary for full activity. Class IIc includes all bacteriocins that are heat stable, small and are non-modified and translated with *sec*-dependent leaders (Klaenhammer, 1993).

Class III bacteriocins are poorly understood and have not been studied extensively. These bacteriocins are large heat-labile proteins larger than 30kDa (Nes *et al.*, 1996).

2.2. Antibacterial activity of bacteriocins

Some bacteriocins are membrane-active peptides that destroy the integrity of the cytoplasmic membrane through the formation of channels. This causes a change in membrane permeability that leads to the leakage of low molecular mass metabolites or dissipation of the proton motive force, causing inhibition of energy production and biosynthesis of proteins or nucleic acids (De Vuyst and Vandamme, 1994). Differences

between narrow or broad host-range bacteriocins seem to be correlated with a specific receptor being needed for activity. Some of the class II bacteriocins require a specific receptor molecule for adsorption. Nisin, a class IA lantibiotic, on the other hand, does not require a membrane receptor, but requires an energized membrane, which appears to be dependent on the phospholipid composition of the membrane (Sahl, 1991).

Some bacteriocins, such as leucocin A (Hastings and Stiles, 1991) and lactocin 27 (Upreti and Hinsdill, 1975) act bacteriostatically. However, the designation of a lethal versus a static effect depend on aspects of the assay system, including the buffer or the broth, the purity of the inhibitor, the indicator species and the cell density used (De Vuyst and Vandamme, 1994).

It may be usual for bacteriocin-producing strains to produce more than one bacteriocin. *Enterococcus faecium* CTC 492 produces enterocin A (class IIa bacteriocin) and enterocin B. These bacteriocins have different inhibitory spectra (Eijsink *et al.*, 1998). Enterocins L50A and L50B, produced by *E. faecium* L50, can be active individually, but behave synergistically when acting together (Cintas *et al.*, 1998). Some bacteriocins, such as lactococcins G/G (Nissen-Meyer *et al.*, 1992), lactococcins M/N (Van Belkum *et al.*, 1991 a), and plantaricins EF and JK (Diep *et al.*, 1996), require the complementary action of two different peptides to achieve biological activity.

2.3. Class IIa bacteriocins and their mode of action

Class IIa bacteriocins are the major subgroup of bacteriocins because of their variation, activities and potential applications (Ennahar *et al.*, 2000). Class IIa bacteriocins are the most extensively studied group of bacteriocins. Their strong inhibitory effects towards the foodborne pathogen *Listeria monocytogenes* facilitates their use in a variety of foods as biopreservatives. Interestingly, all class IIa bacteriocins are produced by lactic acid bacteria associated with food (Ennahar *et al.*, 2000).

Bacteriocin production was initially thought to have been solely plasmid-encoded (Sablon *et al.*, 2000). However, genes encoding several class IIa bacteriocins are located on the chromosome as shown for enterocin A from *E. faecium* (Aymerich *et al.*, 1996), divercin V41 produced by *Carnobacterium divergens* V41 (Métivier *et al.*, 1998) and sakacin P from *L.sakei* Lb674 (Hühne *et al.*, 1996). The structural gene for class IIa bacteriocins and its surrounding regions reveal one to three operon-like structures, which may be divergently transcribed and are involved in the production and extracellular translocation, the immunity of

the producers and, in several cases, the regulation of bacteriocin synthesis (Nes *et al.*, 1996).

Class IIa bacteriocins show a remarkable conservation of gene arrangement (Ennahar *et al.*, 2000). The bacteriocin structural gene encodes an inactive form of the bacteriocin, called prepeptide, that contains a leader sequence with two glycine residues at its C-terminus. These glycine residues may serve as a recognition signal for a *sec*-independent ABC transporter (Klaenhammer, 1993; Nes *et al.* 1996). Operons of most class IIa bacteriocins posses at least two genes encoding proteins that are homologous to ABC-transporters and their accessory proteins, which are required for the externalization of the bacteriocins (Ennahar *et al.*, 2000). The bacteriocin structural gene always precedes an immunity gene, with which it is cotranscribed. For bacteriocins that are transcriptionally regulated, additional open reading frames exist in the proximity of the structural gene. These open reading frames are always in the same order and form a putative three-component signal-transduction autoregulatory cassette that encodes a possible induction factor, a histidine kinase and a response regulator (Ennahar *et al.*, 2000).

Class IIa bacteriocins, like other low molecular weight bacteriocins, are formed as ribosomally synthesized pre-peptides containing a N-terminal leader sequence. The leader sequence is cleaved at a specific processing site as the peptide is exported to the outside of the cell (Håvarstein *et al.*, 1994). The leader peptide is removed by the same protein that is associated with bacteriocin transport (Håvarstein *et al.*, 1994; Nes *et al.*, 1996). The leader peptides of class IIa bacteriocins vary in length of 18 to 27 amino acid residues. The C-terminus contain two glycine residues at positions –2 and –1 relative to the processing site for cleavage, which are believed to serve as signal peptides for the processing and secretion of class IIa bacteriocins by a transport system that involves an ATP-type translocator and an accessory protein. The two glycine residues may function as a recognition signal for this *sec*-independent transporter system (Klaenhammer, 1993; Håvarstein *et al.*, 1994 and Nes *et al.*, 1996).

Consensus elements other than the two glycine residues are also present. Leaders with the same size show particular high sequence similarities. Subsequently, when the charges and hydrophobicity of the individual amino acid residues within the double glycine leaders of class IIa bacteriocins are carefully thought about, it seems that these characteristics have been conserved consistently in the corresponding positions. There exist a significant degree of similarity of the hydropathic profiles of these residues. The residues at positions -4, -7, -12 and -15 are hydrophobic, whereas the residues at position -5, -6 and -11 are hydrophilic

(Ennahar *et al.*, 2000). The similarities between double glycine leaders indicates that the corresponding ABC-transporters and associated proteins are also similar, which may allow the heterologous expression of a class IIa bacteriocin using a secretion machinery of another bacteriocin (Ennahar *et al.*, 2000).

Only nanomolar quantities of class IIa bacteriocins are needed to be inhibitory towards sensitive organisms. Class II bacteriocins mainly induce membrane permeabilisation and leakage of molecules. They show a narrow inhibition spectra, limited mostly to species or strains, which are related to producers. Class IIa bacteriocin activity is directed mainly against low G+C Gram-positive bacteria such as *Listeria*, *Enterococcus* and *Clostridium* (Hechard and Sahl, 2002).

The most extensively studied class IIa bacteriocins are pediocin PA-1 and other identical bacteriocins produced by *Pediococcus acidilactici*. Pediocin AcH-treated cells leak UV-absorbing materials and potassium ions (Buhnia *et al.*, 1991). Pediocin PA-1 is identical to pediocin AcH (Klaenhammer, 1993) and shares sequence similarities with several other antilisterial bacteriocins, such as sakacin A and P and Leucocin A. Pediocin-like peptides are active against a broad range of Gram-positive bacteria, including *L. monocytogenes*. The function of the consensus sequence in the N-terminal region of the peptides is unknown. Mature pediocin PA-1 is cationic and highly hydrophobic. In sensitive cells, pediocin PA-1 acts on the cytoplasmic membrane by dissipating ion gradients and inhibiting transport of amino acids. Chikindas *et al.* (1993) proposed a possible mode of action: Pediocin PA-1 dissipates the membrane potential of treated *Pediococcus pentosaceus* cells and amino acids and other low molecular weight compounds are released. Pediocin PA-1 needs a specific receptor at the cell surface and forms hydrophilic pores in the cytoplasmatic membrane of a sensitive cell and thereby modifies the permeability of the membranes in a voltage-independent manner.

Data from Chen *et al.* (1997) indicated that pediocin PA-1 induced the release of compounds from liposomes in a concentration-dependent manner. This data suggests that no target protein is needed for bacteriocin activity. It was also shown that electrostatic interactions are responsible for binding to liposomes and not the YGNGV consensus motif. However, in the study by Chikindas *et al.* (1993), pediocin PA-1 hardly induced any leakage from liposomes. The authors suggested that a protein receptor may be required for bacteriocin activity.

A study of Fimland *et al.* (1998) also suggested the presence of a specific receptor on the cell surface of sensitive cells. They showed that a 15-mer peptide fragment, that constituted

residue 20 to 34 from pediocin PA-1, inhibited the activity of pediocin PA-1 and other subclass IIa bacteriocins, which suggested that the 15-mer peptide fragment interferes specifically with the interacting bacteriocins and target cells.

Abee (1995) proposed a model for the mode of action of class IIa bacteriocins. First, a proteinaceous receptor is involved in binding of the bacteriocins. Second, a proton motive force-independent insertion of the bacteriocin into the cytoplasmic membrane follows. The third step is the aggregation of monomers in the membrane that result in pore formation.

2.4. Pore formation by nisin, a class I bacteriocin

Ramseier first reported the mode of action of nisin in 1960. He observed that nisin-treated bacteria leaked UV-absorbing cellular material, which was the result of a detergent-like membrane disruption (Ramseier, 1960). Nisin-treated cells are killed rapidly. There is an instant depolarisation of the cell membrane, cellular material leakage and biosynthetic processes are halted (Ojcius and Young, 1991).

Liposomes made from membrane lipids are not affected by nisin (Chikindas et al.,1993). Nisin and epidermin-related bacteriocins use a docking molecule, lipid II, for specific binding to bacterial membranes to form effective, targeted pores in the cell membrane of the sensitive organism (Abee, 1995). At the same time, cell wall biosynthesis is suppressed through trapping the precursor in the cell membrane.

Nisin inhibits peptidoglycan biosynthesis by binding to the membrane-bound precursor undecaprenylpyrophosphoryl-MurNAc(pentapeptide)-GlcNAc, also known as lipid II (Linnet and Strominger, 1973). The N-terminal part of nisin is essential for binding to the membrane-bound cell wall precursor lipid II, resulting in inhibition of peptidoglycan synthesis. The C-terminal part of nisin is important for initial binding to the anionic cell wall polymer and therefore, for in vivo antimicrobial activity.

Nisin also has the ability to form non-targeted pores (Klein and Entian, 1994). Nisin can activate cell wall hydrolyzing enzymes and can induce autolysis as in the case of staphylococci. The peptide releases an N-acetylmuramoyl-L-alanine amidase and an N-acetylglucosamidase, which are both cell wall hydrolyzing enzymes. These hydrolyzing enzymes are strongly cationic proteins that bind to the cell wall via electrostatic interactions with the negatively charged teichoic-, teichuronic-, and lipoteichoic acids. The cationic peptides displace enzymes from the cell wall intrinsic inhibitors by a cation exchange-like

process that results in an apparent enzyme activation and cell lysis (van Belkum *et al.*, 1991 b).

The antibiotic activity of nisin is based on a variety of activities which may be used in different combinations for individual sensitivities of different bacterial species. The concentration of nisin and those bacteriocins related to nisin required to kill bacteria is in the nanomolar range. This is achieved through combining two killing mechanisms: inhibition of peptidoglycan synthesis and through pore formation (van Belkum *et al.*, 1991b).

Driessen *et al.* (1995) suggested that pore formation by positively charged lantibiotics, such as nisin, involves the local perturbation of the bilayer structure and a *delta psi* - dependent reorientation of these molecules from surface-band into a membrane-inserted configuration. The primary target for nisin activity is the cytoplasmic membrane of the sensitive bacterial cell. The initial association of these cationic peptides with the membrane may be partly charge dependent.

A membrane potential that is negative inside is needed before a *trans*-membrane orientation is adopted (Abee, 1995). Various parameters, such as the phospholipid composition of the membrane and the pH may influence the threshold potential. At acidic pH values, nisin A and Z are more active and could permeabilize membranes at very low and even at completely absent membrane potentials. Nisin A forms pores with diameters from 0.2 to 1.2 nm in black lipid membranes that allow passage of hydrophilic solutes with molecular masses of up to 0.5 kDa. Nisin A and Z have been shown to induce leakage of ATP from sensitive cells.

Nisin and other lantibiotics function through the barrel-stave mechanism that has been proposed for cytolytic pore-forming proteins (Ojcius and Young, 1991). This mechanism involves three steps. First, the binding of the bactericidal peptide molecules to the membrane. Second, a membrane potential (inside negative) – dependent insertion into the cell membrane. The third step is the aggregation of molecules that results in the formation of a water-filled pore.

2.5. Immunity and resistance towards bacteriocins

Three observable traits can award non-sensitivity to bacteriocins (Klaenhammer, 1993), namely (i) immunity that is genetically linked with bacteriocin production and employs the most powerful level of non-sensitivity, (ii) resistance can occur as the introduction of spontaneous mutants following selection on the bacteriocin; and (iii) resistance obtained from a gene that is not genetically linked with bacteriocin production.

For all class II bacteriocins that were studied genetically until now, a protein conferring immunity to the producer organism was encoded in the 3' end of the bacteriocin operon, for example lactococcin G (Nes *et al.*, 1996), pediocin PA-1 (Marugg *et al.*, 1992), plantaricin A (Diep *et al.*, 1996) and sakacin A (Axelsson and Holck, 1995). These immunity proteins have a high pI (Diep *et al.*, 1996). Immunity proteins associated with two-peptide bacteriocins consist of 110 to 154 amino acids that contain several transmembrane domains (van Belkum *et al.*, 1991b; Fremaux *et al.*, 1993; Nes *et al.*, 1996), while immunity proteins of the one-peptide bacteriocins are generally smaller and consist of 51 to 113 residues. These proteins contain one, two, or no putative transmembrane helices (van Belkum *et al.*, 1991b; Marugg *et al.*, 1992; Axelsson and Holck, 1995). A new class of immunity proteins was reported that consist of 247 to 257 residues spanning the cytoplasmic membrane seven times (Diep *et al.*, 1996). Based on these findings, immunity proteins exert their effect at the cytoplasmic membrane.

The efficacy of bacteriocins as antimicrobial agents is compromised by the emergence of organisms that acquired resistance to bacteriocins. Strains resistant to nisin have been described for several different bacteria such as *L. monocytogenes* (Ming and Daeschel, 1995) and *Clostridium botulinum* (Mazotta *et al.*, 1997).

Comprehending resistance to nisin is important, since it is the only bacteriocin allowed by the World Health Organization (WHO) in food (Chihib *et al.*, 1999). Understanding the mechanism of resistance may assist in avoiding resistance. It seems that modifications of the cytoplasmic membrane components, such as higher phase transition temperature which corresponds to a higher percentage of straight chain as well as lower percentage of branched fatty acids, are associated with the nisin resistance phenotype (Chihib *et al.*, 1999).

Chihib *et al.* (1999) investigated the involvement of the cell envelope in nisin resistance of the Gram-negative organism *Pectinatus frisingensis*. The nisin-resistant strain showed a significantly different fatty acid composition compared to the wild-type strain. The differences in the two membranes suggested the involvement of membrane fluidity in preventing nisin's bactericidal effect.

Gravesen *et al.* (2002) investigated the development of resistance in *L. monocytogenes* to the two major bacteriocins pediocin PA-1 and nisin A. The frequency of resistance development and the associated fitness costs varied considerably between strains. The influence of environmental stress on resistance development was specific to each

bacteriocin. The frequencies of bacteriocin resistance development to the class IIa bacteriocin, pediocin PA-1, have been observed to be ca. 10^{-6} for the 20 strains that were tested. They found two strains that showed pediocin resistance frequencies of ca. 10^{-4}. These strains displayed only intermediate sensitivity to pediocin PA-1. Pediocin resistance have been shown to be stable in all spontaneous mutations. Osmotic pressure (6.5% NaCl), low temperature (10°C) and low pH (5.5) do not influence the development of resistance to pediocin. Resistant mutants had a growth rate reduction of 44% compared to the maximum specific growth rate of the wild-type strain. The high stability of acquired mutations could imply that secondary mutations would arise that could improve the fitness cost, as observed previously in antibiotic-resistant mutants. *Listeria monocytogenes* strains showed resistance frequencies of 10^{-7} to 10^{-2} to nisin. Strains that showed the highest frequency of resistance were intermediately sensitive to nisin. The environmental factors that did not influence pediocin resistance, reduced nisin resistance to lower than 5×10^{-8}. Nisin resistant mutants have lower growth rate reductions. The bacteriocin-resistant mutants were not more sensitive to stress, as the growth rates were not lowered by environmental stress (5°C, 6.5% NaCl or pH 5.5). The development of simultaneous resistance to pediocin and nisin in a single strain at a frequency of 10^{-6} emphasises the importance of maintaining a low contamination level. It was proposed that the resistance of *L. monocytogenes* to all class IIa bacteriocins will arise at a frequency of ca. 10^{-6}. The number of listerial cells can, therefore, be a determining factor of the effectiveness of bacteriocins.

Resistance to bacteriocins is not necessarily linked to resistance to antibiotics. *Pectinatus frisingensis* strains with acquired resistance to nisin, were not resistant to the antibiotic polymyxin B (Chihib *et al.*, 1999). It was assumed that nisin and polymyxin B probably act by different ways on *P. frisingensis* cells.

3. Expression systems

In the last decade, considerable advances have been made in the genetic studies of lactic acid bacteria. A great number of genetic techniques, transformation protocols, and sophisticated vectors, integration and amplification systems have been developed (de Vos *et al.*, 1997). Additionally, the genome from *Lactococcus lactis* has been sequenced and numerous projects are underway to sequence other lactic acid bacteria (de Vos, 1999). For further scientific and industrial development of lactic acid bacteria, it is indispensable that novel genes and new combinations of genes be expressed. For this reason, various constitutive and inducible gene expression systems and protein targeting systems have been developed (de Vos, 1999).

3.1. Constitutive expression systems

Transcription initiation receives interest because of its important role in the efficiency and control of gene expression. In *L. lactis*, the *rpoD* gene that encodes the major sigma factor have been characterized (de Vos, 1999). The precise contacts of the *L. lactis* sigma factor with the lactococcal promoter have, however, not been determined. A typical lactococcal promoter includes the conserved −35 and −10 hexamers as found in *E. coli*. Unlike the *E. coli* promoter, however, lactococcal promoters contain a TG dinucleotide at position −15 and includes a region immediately upstream from the −35 region that is AT rich (de Vos *et al.*, 1997). Based on the sequence of eleven unidentified promoters and twenty presumed promoters, Pouwels and Leer (1993) concluded that a typical *Lactobacillus* promoter shows significant similarity to promoters from *E. coli* and *Bacillus subtilis*. They further analysed the sequences of seventy published genes and indicated that the region encompassing the translation start codon AUG also shows extensive similarity to that of *E. coli* and *B. subtilis*.

Many *Lactobacillus* genes are expressed in *E. coli* indicating that their expression signals are similar enough to be recognised in other bacteria. Likewise, the DNA-dependent RNA polymerase from *L. acidophilus* transcribed some, but not all, *E. coli* promoters *in vitro* (Natori *et al.*, 1988). The lactobacilli are also diverse in their percentage GC content (*L. acidophilus* 32-37% against *Lactobacillus fermentum* 53%) and these differences will result in varied regulatory sequences and bias codon usage that can significantly affect gene expression (Pouwels and Leer, 1993). Pouwels and Leunissen (1994) compared the level of gene expression for amino acids encoded by two codon sets and suggested that highly expressed genes have a preference for C or G in the third position, thereby promoting tRNA availability in an A/T rich organism. It is also interesting that near the transcription and translational starts of *Lactobacillus* sequences (about 70 were studied), a high A/T content occurs in the 5' region of the genes, specifically the first five codons following the initiation codon. This correlation is held for species with high (*Lactobacillus helveticus*) or low (*Lactobacillus delbrueckii* subsp. *bulgaricus*) percent contents.

Nilsson and Johansen (1994) cloned and sequenced a tRNA operon (*trnA*) from *L. lactis* consisting of seven tRNA genes and a 5S rRNA gene. They aligned the promoters and examined similarities between them. A consensus sequence of promoters preceding rRNA operons and tRNA operons from *Lactococcus* species including a previously undescribed conserved sequence (AGTTATTC) was revealed. A conserved sequence (GTACTGG) at +1, which includes the transcription initiation site, was also found.

Three different procedures have been followed to isolate lactic acid bacterial promoters (de Vos, 1999):

i)   Screening of plasmid and transposon vectors that carry promoterless reporter genes can aid in the identification of new promoters. Waterfield *et al.*(1995) discovered 10 promoters with different efficiencies by exploiting the promoterless *lux* gene.

ii)   Identification of promoters from genes that have been studied, especially from house-keeping genes which have been used to identify several strong and constitutive promoters.

iii)   The construction and screening of synthetic promoters that were obtained from the consensus *L. lactis* promoter in which the sequences of the spacer regions were randomized have led to the identification of strong, constitutive promoters.

3.2. Controlled expression systems

Regulated gene expression systems for lactic acid bacteria are essential in industrial fermentations, since controllable overproduction of proteins at high levels at any desired moment is made possible (Kuipers *et al.*, 1997). When the level of expression is correlated with the amount of inducer added, regulated gene expression allows studies of the effects of varying intracellular levels of protein production on metabolic pathways.

In lactic acid bacteria, a number of genes and operons have been identified that are inducible and respond to environmental factors (Kok, 1996). The lactose operon, the operon for nisin generation and genes for the metabolism of xylose in *Lactobacliius pentosus* were studied because of their importance in the food industry. Consequently, these genes were found to be regulatable.

Several studies have been conducted on regulated gene expression, mostly in *L. lactis* (Table 1).

Table 1. Inducible expression systems for *Lactococcus lactis*

Inducible expression systems for *Lactococcus lactis* (Kuipers *et al.*, 1997)			
Inducible element	Inducing factor	Expressed gene(s)	Ratio induced: uninduced
LacA or *lacR* promoter	Lactose	*cat*-86, *luxAB*	< 10
LacA/T7 promoter	Lactose	Gene for TTFC	< 20
DnaJ promoter	High temperature	*amyS*	< 4
SodA promoter	Aeration	*lacZ*	2
PrtP or *prtM* promoter	Absence of peptides	*gusA*	< 8
Repressor/operator φrlt	Mitomycin	*lacZ*	70
PA170 promoter	Low pH/temperature	*lacZ*	50 – 100
TrpE promoter	Absence of tryptophan	*lacZ*	100
φ31 promoter and ori	φ31 infection	*lacZ*	> 1000
NisA or *nisF* promoter	Nisin	*gusA, pepN, lytHA,nox, estA, pstH, pepO, ccpA*	> 1000

Of the systems listed in Table 1, only the *nisA/nisF*-promoter system will be discussed.

The nisin-controlled expression system (NICE) is a highly versatile, strongly controlled food-grade process for controlled expression of proteins (de Vos, 1999). This system is based on signal transduction by the two-component regulatory system that consists of the response-regulator protein NisR and the sensor histidine-kinase NisK present in the nisin gene cluster of *L. lactis* (Kuipers *et al.*, 1997).

This regulatory system is one of the well-known peptide pheromone-dependent quorum sensing systems that are present in a variety of Gram-positive bacteria, where a secreted peptide functions as the input signal for a specific sensor component of a two-component signal transduction system (Kleerebezem *et al.*, 1997). The NICE system has been used successfully in a variety of bacteria, including *Bacillus, Enterococcus, Lactococcus, Lactobacillus* and *Streptococcus* spp.

Nisin is a post-transcriptionally modified antibacterial peptide that is produced by several strains of *L. lactis* and is used in the food industry. Extracellular nisin performs as the signal for transcriptional activation of the bacteria's own structural, immunity and several other

biosynthetic genes. The expression system is based on plasmids that contain genes under the control of the *nisA* promoter fragments (Kuipers *et al.*, 1997). These plasmids are introduced into strains that contain the NisR and NisK proteins, but cannot produce nisin.

The NisR and NisK proteins are required for signal production and will be expressed at nearly undetectable levels. Upon addition of nisin to growth medium of cells during logarithmic growth, transcription of the genes on the plasmid, that are under *nisA* control, is activated. The level of expression is proportional to the amount nisin added. It was also found that the *nisF* promoter, which controls expression of the *nisFEG* genes, can be activated by nisin in a similar way to the *nisA* promoter (Kuipers *et al.*, 1997).

The *nisFEG* genes are involved in immunity to nisin. An advantage of nisin as an inducer molecule is that it has a history of safe use in food products and is therefore considered to be food-grade (Kuipers *et al.*, 1997).

The NICE system is easy to use and is inexpensive, as only 0.05 - 5 microgram nisin per litre or less than 0.1% of nisin-producing *Lactococcus lactis* is needed for induction of cultures. These concentrations of nisin are low enough that other starter culture bacteria will not suffer any damage. The system can be used in lactic acid bacteria other than *Lactococcus lactis* when the *nisRK* genes are introduced on a plasmid and another plasmid is introduced with the *nisA* promoter and the genes of interest. The NICE system can be used to produce lethal proteins, as the expression is extremely tightly controlled and the protein of interest cannot be detected in the uninduced state. The NICE system has a further advantage of high protein production of up to 60% of the total intracellular protein, which can be important for industrial enzyme production (Kuipers *et al.*, 1997).

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MINI REVIEW

METHODS USED TO DETECT MICROBIAL CELL NUMBERS, WITH THE EMPHASIS ON GREEN FLUORESCENT PROTEIN (GFP) AS A REPORTER SYSTEM

Various methods to detect bacteria have been established. Some methods aim at establishing the presence and/or identity of bacteria (PCR, FISH), whereas other methods are used to establish the number of bacteria in a sample (e.g. plate counts, fluorimetry, flow cytometry). Some of these methods, including their advantages and disadvantages, will be discussed in this mini review.

The polymerase chain reaction (PCR) is a powerful technique that allows detection of minute quantities of DNA or RNA. In the medical field, various infectious agents can be detected with high specificity and sensitivity, including bacteria, parasites, rickettsia and viruses (Thomson and Bertram, 2001). Several PCR protocols, differing from each other in the choice of genomic targets and primers, have been used to detect *Helicobacter pylori* infections (Kabir, 2001). Substances in faeces that inhibit PCR can be removed by pre-PCR methods such as biochemical separation by column chromatography and isolation *of H. pylori* with immunomagnetic beads. These techniques yield results with a high degree of sensitivity and specificity (Kabir, 2001). PCR can be used to detect coliform bacteria by means of signal amplification. DNA sequence coding for the *lacZ* (beta-galactosidase) gene and the *uidA* (beta-D glucuronidase) gene have been used to detect total coliforms and *E. coli*, respectively (Rompre *et al.*, 2001). However, quantification with PCR is still lacking in precision and requires extensive laboratory work.

Sequencing of the 16S rRNA gene does not only serve as an important tool for determining phylogenetic relationships between bacteria, but also for bacterial detection and identification. Sequence identification is useful for slow-growing, unusual and fastidious bacteria and bacteria that are poorly differentiated by conventional methods (Patel, 2001).

The fluorescent *in situ* hybridization (FISH) technique involves the use of oligonucleotide probes to detect complementary sequences inside specific cells. Oligonucleotide probes designed specifically for regions of the 16S rRNA of bacteria can be used to detect and determine the concentration of the bacteria within a mixed culture (Rompre *et al.*, 2001).

MALDI-TOF (matrix-assisted laser desorption/ionisation-time of flight) mass spectrometry is generally used to characterize large biomolecules. The versatility and rapidity of MALDI-TOF

analysis led to the development of MALDI-TOF methods that included bacteria. These methods comprise the rapid characterization of bacteria (unprocessed cells) at genus, species and strain level (Lay, 2001). MALDI-TOF methods have also been developed to analyse bacterial RNA and DNA, to detect recombinant proteins and to characterise targeted or unknown proteins (Lay, 2001).

Immunoassays are based on antibodies detecting and binding to antigens from specific bacteria. Kabir (2001) described an immunoassay to detect the presence of *H. pylori* in faeces. However, the method that was described detected live and dead bacteria, which could lead to false positive results.

Flow cytometry (FCM) is a rapid and sensitive technique that can determine cell numbers and measure various physiological characteristics of individual cells (such as relative size) which are suspended in liquid medium by using appropriate fluorescent probes. The labelled cells are illuminated by a 488 or 635 nm laser and the fluorescence intensity or light scatter (cell size and complexity) is recorded. Flow cytometry can detect microorganisms rapidly in low concentrations (Shapiro, 2001). In a study by Maksimow *et al.* (2002), *E. coli* cells producing a red fluorescent protein of *Discosoma* sp. (drFP583 DsRed) were investigated with flow cytometry using an excitation of 488 nm. They also determined whether GFP and red fluorescent protein of *Discosoma* sp. (drFP583) could be detected simultaneously from a single bacterial cell. Their results showed that enhanced GFP and drFP583 proteins can be detected simultaneously from single bacteria with a standard flow cytometer with simple optical configuration.

Confocal microscopy allows the three-dimensional observation of bacteria without physically sectioning the bacteria. Samples can be observed without extensive preparation processes. The interaction of bacteria with each other and their environment can be studied *in situ* over a period of time. Takeuchi and Frank (2002) reported that the fate of microbial populations in food systems depends on processing, distribution and storage conditions, as well as decontamination procedures that are applied to inactivate and remove them. They found confocal microscopy suitable to determine the physiological status of microorganisms without disrupting their physical relationship with a food system, which is useful for determining the means by which microorganisms survive decontamination treatments.

Bacteria present in a sample can be cultured on plates and the number of viable bacteria determined. It is, however, often necessary to obtain numbers of specific bacteria in a sample. Medium that selectively promotes the growth of specific bacteria, whereas other

bacteria are suppressed (Roy, 2001) have been developed to culture and enumerate specific bacteria. MRS medium supplemented with neomycin, paromomycin, nalidixic acid and lithium chloride is an example of such a selective medium. The medium is recommended for selective enumeration of bifidobacteria in dairy products.

Adenosine triphosphate (ATP) bioluminescence can be used to detect microbial contamination within 27 hours (Jimenez, 2001). Samples are subjected to lysis to release microbial ATP and light production is quantitated. The method is rapid, reliable, and cost effective. However, to maintain the detection time within 27 hours, different enrichment broths are required for neutralization of antimicrobial ingredients in finished products and to provide specific nutrients for growth optimization.

Green fluorescent protein

Green fluorescent protein, GFP, is a spontaneously fluorescent protein isolated from coelenterates. The role of GFP in the jellyfish *Aequorea victoria* is to transduce the blue chemiluminescence of another protein, aequorin, to green light (Morin and Hastings, 1971). Wild-type GFP is a 27-kDa monomer consisting of 238 amino acids that has the unique characteristic of emitting green light when excited with UV-light with the wavelength of 360 nm to 400 nm or blue light with a wavelength of 440 nm to 480 nm. GFP is the only well-characterized example of a protein that displays strong, visible fluorescence without any additional substrates or co-factors (Heim and Tsien, 1996). It maintains this fluorescence even after prolonged incubation in strong denaturing agents such as 6 M guanidine HCl, 8 M urea or 1% sodium dodecyl sulfate (SDS) (Bokman and Ward, 1981). GFP has a broad range of pH stability, retaining conformation from pH 5.5 to 12.0, it is non-toxic and is extremely thermostable, surviving temperatures up to 65°C. The fact that GFP requires no addition of substrates or co-factors to fluoresce and its ability to be expressed in a variety of organisms has made it a valuable universal reporter gene.

GFP can tolerate N- and C-terminal fusion to a broad variety of proteins, therefore, making it suitable as a protein tag (Olsen *et al.*, 1995). The demonstration by Chalfie *et al.* (1994) that GFP can be expressed as a functional transgene have opened many ways to investigate cell, developmental and molecular biology. Fluorescent GFP has been expressed in bacteria (Chalfie *et al.*, 1994), yeast (Kahana *et al.*, 1995), slime mold (Moores *et al.*, 1996), plants (Casper and Holt, 1996), Drosophila (Wang and Hazelrigg, 1994), zebra fish (Amsterdam *et al.*, 1996) and mammalian cells (Ludin *et al.*, 1996).

1. GFP as a reporter in fungi

The fungus *Aspergillus flavus* grows in corn kernels, where it secretes carcinogenic aflatoxin. To monitor the growth of the fungus in corn, GFP was used as a marker (Wanglei *et al.*, 1999). The GFP gene was fused to the promoter of the coding region of *A. flavus aflR*. The transformants expressed green fluorescence that was detectable under a standard laboratory UV light without a microscope or filters. *A. flavus* that express GFP can be a useful tool in screening corn genotypes for resistance to aflatoxin accumulation and can increase the efficiency of screening procedures for corn samples (Wanglei *et al.*, 1999).

Siedenberg *et al.* (1999) reported that wild-type GFP expressed in *Aspergillus niger* does not fluoresce, neither is the full translation product detectable. However, a mutant form of GFP (Ser65Thr-GFP) formed a fluorescent protein that was sufficient to study the glucoamylase promoter.

2. GFP in mice

The use of GFP as marker is not limited to single-cell organisms. GFP has also been used in higher eukaryotes. Takada *et al.* (1997) developed a method to selectively produce transgenic mice using GFP as marker. This solved the problem of low efficiency of transgenic animal production by microinjection, which has been a serious obstacle in the production of transgenic livestock. Using this method, they obtained eight fetuses and four live-born mice derived from 55 GFP-positive blastocysts. GFP expression was also observed in bovine blastocysts (Takada *et al.*, 1997). This suggests that this method could contribute to the efficient production of transgenic livestock.

3. GFP in plants

A cereal transformation vector, containing the strong constitutive rice actin promoter *act*-1, a multiple cloning site and the *nos* terminator, was constructed (Ahlandsberg *et al.*, 1999). Fusion of a plant-optimized *gfp* gene to *act*-1 in pN1473 resulted in the vector pN1473GFP. To assess the suitability of pN1473 and GFP as a reporter system in barley transformation, two barley cultivars (Baronesse and Golden Promise) were transformed by microprojectile bombardment. Transient GFP expression in transformed embryogenic callus material was detected by fluorescence microscopy less than 12 hours after transformation. The presence of the *gfp* gene in callus and regenerated plantlets was confirmed by PCR amplification and DNA gel-blot analysis.

The use of transgenic crops has generated concerns about transgene movement to unintended hosts and the associated ecological consequences. A solution to these potential

problems is to monitor the presence and expression of an agronomically important gene by linking it to a marker gene, such as *gfp*, encoding the green fluorescent protein. Harper *et al.* (1999) pursued this concept to characterize the performance of three different *gfp* genes in transgenic tobacco.

4. GFP in bacteria

4.1. Detection of mutants

Green fluorescent protein has been used in a broad variety of applications. Cariello *et al.* (1998) were first to report the use of green fluorescent protein for frameshift mutation detection. A plasmid-based bacterial system was constructed where mutated cells fluoresced and non-mutated cells did not fluoresce. This was made possible by a +1 DNA frameshift mutation in the coding region of GFP. The assay was used to detect reversion and forward frameshift mutations caused by various substances, such as ICR-191 and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG).

4.2. Bacterial survival studies

Fratamico *et al.* (1997) constructed recombinant *E. coli* O157:H7 strains that contained easily identifiable and stable markers. These strains could be used as positive controls in microbial assays. They could also be useful in studies monitoring bacterial survival and the behaviour of *E. coli* O157:H7 in food and in a food processing environment. The *A. victoria* green fluorescent protein gene on plasmid vector pGFP was introduced into strains of *E. coli* O157:H7. The *E. coli* strains which contained pGFP were indistinguishable from their parent strains in biochemical and immunological assays and in multiplex PCR. At 37°C, all recombinant strains maintained the vectors and expressed the green fluorescent protein when grown with and without antibiotic selection. Strains of *E. coli* O157:H7 which emitted bright green light when excited with UV light were used in studies examining the survival of the organism in apple cider and in orange juice. In apple cider the organism declined to undetectable levels in 24 days at refrigeration temperature, while in orange juice the strains survived with only small decreases in numbers during the 24-day sampling period (Fratamico *et al.*, 1997).

4.3. Behaviour of bacteria under different conditions

Beef has been implicated in the largest outbreaks of *E. coli* O157:H7 in the United States (Ajjarapu and Shelef, 1999). One difficulty presented in studies regarding the fate of this pathogen, was that it had to be detected at levels considerably lower than the levels of the competing microorganisms. A GFP-expressing *E. coli* strain (O157:H7) was used to monitor the behaviour of the pathogen in ground beef mince stored aerobically from freshness to

spoilage at 2 and 10°C. In addition, effects of sodium salts of lactate, diacetate and buffered citrate and combinations of sodium lactate and di-acetate were evaluated using GFP-expressing *E. coli* O157:H7. Green fluorescence emitted by the GFP-recombinant *E. coli* strain was stable throughout the study. Fluorescence was intense, also in the presence of high numbers of other microorganisms (Ajjarapu and Shelef, 1999).

Bloemberg *et al.* (1997) utilised plasmid-borne GFP in *Pseudomonas* spp. to observe individual cells in a population in experimental models and in natural environments. GFP has also been used to monitor the survival of genetically engineered organisms by plating to examine culturable cells and by microscopy to examine the total number of genetically engineered organisms (Leff and Leff, 1996). Therefore, even unculturable, genetically engineered organisms were monitored and their abundance could be determined by microscopic analysis of samples.

4.4. Bacterial responses to stresses

Hyung *et al.* (1999) constructed three stress probe plasmids which used GFP as an noninvasive reporter to elucidate cellular stress responses in cells. Three heat shock stress protein promoter elements were fused to a mutated *gfp* gene, *gfp*$_{uv}$. Chemical and physical stress caused recombinant *E. coli* HB101 cells to express GFP$_{uv}$, which was detected as green fluorescence.

4.5. Examination of gene expression

Freitag and Jacobs (1999) examined *Listeria monocytogenes* intracellular gene expression by using GFP. The ActA protein of *L. monocytogenes* is an essential virulence factor that is required for intracellular bacterial motility and spread from cell to cell. The *plcB* gene, which is cotranscribed with *actA*, encodes a broad-specificity phospholipase C that contributes to lysis of host cell vacuoles and cell-to-cell spread. A fusion between the *actA-plcB* genes and the GFP gene was used to examine in detail patterns of *actA/plcB* expression within infected tissue culture cells. Results emphasized the ability of *L. monocytogenes* to sense the different host cell compartment environments encountered during the course of infection and to regulate virulence gene expression in response.

4.6. GFP as a reporter for protein localization

GFP was used by Feilmeier *et al.* (2000) as a reporter for protein localization in *E. coli*. Gene fusions between the carboxy-terminal end of *malE* (maltose binding protein, MBP) and a *gfp* gene optimized for fluorescence in bacteria (GFP$_{uv}$) were obtained. Fluorescence was only detected where the MBP signal sequence was deleted. Through cell fractionation studies,

the MBP-GFP hybrid protein was localized to the cytoplasm. Further results obtained suggested that GFP could serve as a reporter for genetic analysis of bacterial protein export and of protein folding.

4.7. GFP as a marker in biofilms

Skillman *et al.* (1998) used GFP to examine the interactions between pairs of enteric bacterial species and their effects on subsequent biofilm development over a period of 24 hours. *Enterobacter agglomerans*, *Klebsiella pneumoniae* and *Serratia marcescens* were isolated from industrial biofilms. A plasmid encoding GFP from *A. victoria* was transformed into strains of *E. agglomerans* and *E. coli* ATCC 11229. The development of dual-species biofims, containing one fluorescent and one non-fluorescent partner, was examined by GFP fluorescence. Furthermore, it was possible to study the adhesion of the strains to other cells or cell constituents or the invasion into pre-existing biofilms. Cooperation between *E. agglomerans*/GFP and *K. pneumoniae* resulted in a 54% and a 23% increase in biofilm formation, respectively, when compared with single-species biofilms. *E. coli*/GFP and *S. marcescens* stably co-existed in biofilms but did not affect the growth of each other. The other bacterial partnerships examined were competitive, with the end result that one species dominated the biofilm. In this application of GFP, a convenient technique was provided to study mixed-species biofilm communities where unique interactions between species determine the true properties of the resultant biofilms (Skillman *et al.*, 1998).

4.8. Mutants of GFP

GFP has the advantage as a reporter, that it forms a fluorophore without the addition of external agents, provided the dissolved oxygen levels are higher than 0.025 p.p.m. (Hansen et al., 2001). However, wild-type GFP has two main disadvantages (Scholz *et al.*, 2000). At temperatures above 25°C large amounts of insoluble GFP is formed, as protein folding at these temperatures is insufficient. Another problem is the relatively low intrinsic fluorescence of GFP. These disadvantages have been abolished by creating GFP mutants.

GFP$_{uv}$ has three mutations (F99S, M153T and V163A). This mutant displays an increased folding efficiency at 37°C. Cells expressing GFP$_{uv}$ are 16 times brighter than cells expressing wild-type GFP. The problem with low intrinsic fluorescence was solved by the creation of the mutant GFPmut1 that has two mutations in its chromophore (F64L and S65T). This mutant displays a 35-fold increase in fluorescence compared to wild-type GFP. However, only 20% of GFPmut1 is folded correctly at 37°C (Scholz *et al.*, 2000).

The intensity of the fluorescence signal accurately reflects the GFP protein concentration (Misteli and Spector, 1997; Scholz et al., 2000) and thus a reporter of gene expression. Scholz et al. (2000) constructed an expression system based on the tetA promoter fused with a gfp+ gene to compare GFP as a reporter with β-galactosidase. It was demonstrated that the fluorescence of GFP+ can be determined as accurately as β-galactosidase activity to quantify gene expression in vivo. The luxAB genes are also accurate reporters of gene expression, but can activate or repress transcription from certain promoters. The gfp+ gene have not been shown to influence the activity of promoters. Under constant promoter expression, GFP fluorescence intensity can be used quantitavely to determine cell numbers. The relationship between fluorescence intensity of GFP and absorbance (OD_{600}) from GFP-expressing cells is linear up to an OD (600 nm) of 5 for diluted E. coli DH5α cells.

5. GFP in lactic acid bacteria

Lactic acid bacteria constitute a family of safe Gram-positive bacteria that are well known for their use in industrial food fermentations and for their probiotic properties (Morteau and Rambaud, 1993). During the last fifteen years, a variety of molecular biology tools have been developed for lactic acid bacteria, mainly to isolate functional expression target signals. These reporter genes included the chloroamphenicol acetyltransferase gene (de Vos, 1987), the E. coli β-glucoronidase gene (Plateeuw et al., 1994), Leuconostoc mesenteroides β-galactosidase gene (Israelson et al., 1995), the Bacillus licheniformis α-amylase gene (Hols et al., 1992), the Staphylococcus aureus nuclease gene (Poquet et al., 1998) and the Vibrio fisheri luciferase gene (Eaton et al., 1993). The phenotypic tests linked to these systems require the addition of exogenous substrates for the detection of recombinant strains expressing the reporter genes. Addition of exogenous substrates may present limitations for in vivo studies.

A system based on the GFP from A. victoria circumvented the drawback of addition of substrates or co-factors. This system has been used successfully in lactic acid bacteria such as Streptococcus thermophilus (Solaiman and Somkuti, 1997) and L. lactis (Scott et al., 1998). Scott et al. (1998) demonstrated that a plasmid-encoded gfp gene expressed from a lactococcal P32 promoter gave sufficient fluorescence in several species of lactic acid bacteria for detection at the level of individual cells and colonies. It was also shown that highly anaerobic environments prevented GFP fluorescence, but not GFP expression.

According to Hansen et al. (2001), the use of GFP as a molecular reporter is restricted by its requirements for oxygen in the development of the fluorophore and its poor fluorescence at low pH. Under strictly anaerobic conditions (0.025 p.p.m dissolved oxygen) cells expressing

GFP did not fluoresce. However, in conventially prepared anaerobic medium (0.1 p.p.m dissolved oxygen) cells were fluorescent. *Streptococcus gordonii* DL1 cells that expressed GFP were grown in batch culture. Production of lactic acid and the subsequent acidification (pH 4.5) of the culture led to a decrease in fluorescence intensity. When these cells were resuspended in fresh growth medium (pH 7.0) within 23 hours after exposure to the previously acidic environment, the fluorescence intensity recovered within 5 minutes.

The validity of GFP as a marker for visualizing interactions of organisms expressing GFP with specific immune cells and monitoring their fate *in vivo* was determined by Geoffrey *et al.* (2000). They reported that GFP constituted an adequate reporter for both applications. Their model strain was *L. plantarum* containing a plasmid with the *gfp* gene under the inducible promoter *nisA*. These bacteria were released in a mixed community. Flow cytometry was used to discriminate between fluorescent and non-fluorescent cells, therefore opening the way to quantitative detection of bacteria expressing GFP in complex mixed communities. Macrophage cells, which had taken up lactobacilli expressing GFP, could also be analyzed and counted by flow cytometry.

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Cloning of the *gfp* (green fluorescent protein) gene downstream of the *ldh* promoter in *Lactobacillus sakei* DSM 20017 to serve as a reporter strain in bacteriocin studies

This manuscript was prepared for publication in International Journal of Food Microbiology.

Cloning of the *gfp* (green fluorescent protein) gene downstream of the *ldh* promoter in *Lactobacillus sakei* DSM 20017 to serve as a reporter strain in bacteriocin studies

F.P. Liss, C.A. Van Reenen, L.M.T. Dicks[*]

Department of Microbiology, University of Stellenbosch, Stellenbosch 7600, South Africa

Abstract

Lactobacillus plantarum 285 (isolated from sorghum beer) produces bacteriocin 285, which displays activity against several food pathogens. For future application of bacteriocin 285 in the food industry, it is important to characterize the peptide and identify the genes encoding its production. The effect of bacteriocin 285 on sensitive cells was determined through the use of an indicator (sensitive) organism, *Lactobacillus sakei* DSM 20017. This indicator strain was genetically modified to express GFP (green fluorescent protein), with the aim of quantifying the antibacterial activity of bacteriocin 285 against the sensitive organism directly as a function of GFP fluorescence. Bacteriocin 285 was found to be identical to plantaricin 423, produced by *L. plantarum* 423. Plantaricin 423 is a class IIa bacteriocin that displays antibacterial activity towards a broad spectrum of bacteria, including several food pathogens. The sensitivity of *L. sakei* DSM 20017 towards antibacterial peptides produced by *Lactobacillus curvatus* DF38, *L. plantarum* 423, *Lactobacillus casei* LHS, *Lactobacillus salivarius* 241 is not limited to the growth stage of the organism. Cells remained sensitive to all four of the latter bacteriocins, from lag phase to late exponential growth phase. To inhibit growth of up to 90% of the cells of *L. sakei* DSM 20017, 1 AU/ml bacteriocin 285 (7 ng/ml of partially purified bacteriocin 285) is required. However, to kill 99.99% of *L. sakei* DSM 20017 cells, 16 AU/ml (110 ng/ml of partially purified bacteriocin 285) is required. GFP_{uv} fluorescence cannot be used as a direct method to quantify antimicrobial activity towards sensitive cells, since cells remained fluorescent for prolonged periods after treatment with lethal concentrations of bacteriocin 285. However, the non-viability of the cells was confirmed through the use of epifluorescence microscopy and a LIVE/DEAD® *Baclight*™ Bacterial Viability Probe. Cultures stained with the viability probe indicated that the majority of untreated *L. sakei* DSM 20017 cells were viable. However, treatment with 16 AU/ml bacteriocin 285 rendered all visible cells non-viable.

Keywords: Bacteriocin 285, Plantaricin 423, *Lactobacillus sakei* DSM 20017, GFP, Epifluorescence Microscopy

[*] Corresponding author. Tel.: +27-21-8085849; fax: +27-21-8085846.

E-mail address: lmtd@sun.ac.za (L.M.T. Dicks)

1. Introduction

Bacteriocins are small, ribosomally synthesized antimicrobial peptides (Héchard and Sahl, 2002). Most bacteriocins produced by Gram-positive bacteria exhibit activities towards a wide range of Gram-positive species (De Vuyst and Vandamme, 1994). Although structurally different, bacteriocins produced by lactic acid bacteria are subdivided into three distinct classes, namely class I, class II and class III (Nes *et al.*, 1996). Class I bacteriocins (lantibiotics) undergo posttranslational modifications. Bacteriocins of class II undergo no posttranslational modifications and are small heat-stable membrane-active peptides. Class III bacteriocins are large, heat-labile proteins (Nes *et al.*, 1996).

Most bacteriocins belong to class II, which is subdivided in four groups. Class IIa bacteriocins are the major subgroup of bacteriocins because of their variation, activities and potential applications (Ennahar *et al.*, 2000). Class IIa bacteriocins are also the most extensively studied group of bacteriocins. Their strong inhibitory effects towards the foodborne pathogen *Listeria monocytogenes* grants these bacteriocins the possibility to be used in a variety of foods as biopreservatives. Interestingly, all class IIa bacteriocins are produced by lactic acid bacteria associated with food. Only nanomolar quantities of class IIa bacteriocins are needed to be inhibitory towards sensitive organisms. Class II bacteriocins mainly induce membrane permeabilisation and leakage of molecules. They show a narrow inhibition spectrum, mostly limited to species or strains, which are related to bacteriocin producers. Class IIa bacteriocin activity is mainly directed against low G+C Gram-positive bacteria such as *Listeria*, *Enterococcus* and *Clostridium* (Héchard and Sahl, 2002).

The preservative role of lactic acid bacteria in fermented foods and their production of organoleptic compounds have been well documented. Most of the antimicrobial substances such as lactic acid, acetic acid, hydrogen peroxide and bacteriocins are produced *in situ* by starter cultures, rendering the product less perishable. Use of bacteriocins could replace unwanted chemical additives such as sulfur dioxide, benzoic acid, sorbic acid, nitrate and nitrite, or reduce the concentration needed. Although sulfur dioxide, sodium benzoate and sorbic acid are considered to be safe food preservatives, the general tendency is to avoid chemical additives. In the United States of America, 0.05% of the population are sulfite-sensitive (Lester, 1995).

Bacteriocin 285, which is produced by *Lactobacillus plantarum*, has an inhibition spectrum similar to plantaricin 423. Plantaricin 423 has previously been described and its sequence has been determined (Van Reenen and Dicks, 1996). For future application of bacteriocin 285 in the food industry, it was important to characterize the peptide, identify the genes encoding its production, and study its mode of action. We determined that bacteriocin 285 is identical to plantaricin 423. Bacteriocin 285 is thus, a class IIa bacteriocin of approximately 3.5 kDa, resistant to heat, stable over a pH range of 1-10 and sensitive to proteolytic enzymes (Van Reenen *et al.*, 1998).

Viable cell counts and optical density readings of cultures are often not sensitive enough to detect small changes in the target cell brought about by bacteriocins. The effect of bacteriocin 285 on sensitive cells was studied by using an indicator sensitive strain of *Lactobacillus sakei* (DSM 20017). *Lactobacillus sakei* DSM 20017 was chosen as indicator organism, since it is sensitive to several bacteriocins, including bacteriocin 285. Furthermore, the sequence of the lactate dehydrogenase (*ldh*) gene of *L. sakei* has been published (Van den Berg *et al.*, 1995). The lactate dehydrogenase enzyme plays a critical role in the metabolism of *L. sakei*, where it reduces pyruvate to lactate. The aims of this study were to construct a reporter strain of *L. sakei* DSM 20017 with *gfp* (green fluorescent protein) gene under control of the *ldh* promoter, to evaluate GFP as a reporter system in *L. sakei* and to investigate alternative systems aimed at direct detection. Strain 20017 was genetically modified to express a mutant form of GFP, namely GFP$_{uv}$ containing contains three amino acid substitutions. These mutations make GFP$_{uv}$ fluoresce 18 times brighter than wild type GFP (Scholz *et al.*, 2000). The inhibition of *L. sakei* DSM 20017 would then, theoretically, result in a decrease in GFP$_{uv}$ fluorescence. A similar approach was taken by Simon *et al.* (2001). In the latter case, the authors have used luciferase luminescence to quantify the effect of bacteriocins on a number of bacteria. A decrease in luminescence was observed when sensitive cells were treated with the bacteriocins. Use of GFP as a reporter has several advantages. GFP fluorescence is relatively easily detectable, requires no addition of exogenous substrate or energy source, the cells need no processing and individual cells can be monitored (Bloemberg *et al.*, 1997). A reporter strain containing the *gfp* gene could be useful as a tool for rapid identification and quantification of bacteriocin activities by fluorescence microscopy, fluorimetry or flow cytometry.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The organisms included in this study are listed in Table 1. The lactic acid bacteria were cultured in De Man-Rogosa-Sharpe (MRS) medium (Biolab Diagnostics, Midrand, South Africa) at 30°C. Transformants of *L. sakei* were grown in MRS broth containing erythromycin (10 µg/ml). *Escherichia coli* DH5α was grown in Luria-Bertani (LB) medium (tryptone 1% m/v, yeast extract 0.5% m/v, NaCl 1% m/V) at 37°C. Transformants of *E. coli* with plasmids bearing an ampicillin resistance gene were grown in LB broth containing ampicillin (100 µg/ml). Transformants of *E. coli* with plasmids bearing an erythromycin resistance gene were grown in Brain Heart Infusion (BHI) medium (Biolab Diagnostics, Midrand South Africa) containing erythromycin (150 µg/ml).

2.2. Isolation of plasmid encoding bacteriocin 285

Isolation and purification of plasmid DNA from *L. plantarum* 285 was performed through a modified alkaline lysis method (Sambrook, 2001) and as described by Van Reenen *et al.* (1998). The structural gene of bacteriocin 285 was amplified by PCR. The primers that were used for amplification were:

423A5' (5′-GTCGCCCGGGAAATACTATGGTAATGGGG-3′) and

423A3' (5′-GCGTCCCGGGGTTAATTAGCACTTTCCATG-3′)

The primers were obtained from Genosys Biotechnologies (Europe) (Pampisford, United Kingdom). The product was sequenced on an automatic sequencer (ABI Prism^TM, PE Biosystems SA Ltd.) using the primers discussed above. Computer alignment of the sequence to that of plantaricin 423 was facilitated using DNAMAN *for Windows*® (Lynnon Biosoft, Quebec, Canada).

2.3. Inhibitory spectrum of bacteriocin 285

Antibacterial activity of bacteriocin 285 was determined against *Lactobacillus casei* ATCC 334, *Streptococcus thermophilus* LMG 13564, *Bacillus cereus* LMG 13569, *Lactobacillus curvatus* LMG 13553, *Lactobacillus fermentum* LMG 13554, *Pediococcus pentosaceus* LMG 13560, *Pediococcus acidilactici* ATCC 12687, *L. sakei* LMG 13558 and *L.*

sakei DSM 20017 by using the spot-on-lawn method (Van Reenen *et al.*, 1998). The filter-sterilised supernatant (pH 7) of an active culture (18 hours) of *L. plantarum* 285 was used to spot on MRS agar plates containing one of the above mentioned strains. The plates were incubated at 30°C for up to 4 days.

2.4. Amplification of gfp$_{uv}$ *gene and* ldh *promoter*

To obtain a template for amplification of the *gfp$_{uv}$* gene, plasmid pBadGFP$_{uv}$ was isolated from *E. coli* JB 361 using the alkaline sodium dodecyl sulfate lysis method (Sambrook, 2001). The primers were obtained from Genosys Biotechnologies (Pampisford, United Kingdom). Primers used in PCR amplification of the *gfp$_{uv}$* gene were pBadF (5'-GATCACTAGTTCTGTATCAGGCTGAAAATC-3') containing a *Spe*I restriction endonuclease site (underlined) and pBadR (5'-CAGATCTAGAATACATATGGCTAGCAA AGG-3') containing a *Xba*I restriction site (underlined) Primers were designed from the sequence of the *gfp$_{uv}$* gene as published by Crameri *et al.* (1996). A template for the amplification of the *ldh* promoter was obtained by isolating genomic DNA from *L. sakei* DSM 20017 using the method as described by Sambrook (2001). Primers used in the amplification of the *ldh* promoter were ldh5' (5'CAGAGAGCTCCCATACCCGTTT TTCTTTC-3') with a *Xba*I restriction endonuclease site (underlined) and LDHProm (5'-*ACTAGT*GTCGACCCAATATAATGACGTCCTTT -3') which included restriction endonuclease sites for *Spe*I (italics) and *Sal*I (underlined). The primers were designed from the sequence of the *ldh* gene as published by Van den Berg *et al.* (1995). The following concentrations of reagents were used in the PCR reactions: 1.5 mM Mg^{2+}, 50 mM KCl, 0.2 mM dNTP´s and 1 μM of each primer. In each PCR reaction 2.25 to 3.75 units of Taq DNA polymerase and between 1 and 50 ng of template DNA was used. During the thermal amplification cycles, DNA was denatured first for 2 min at 94°C, followed by 30 cycles with the following steps: denaturation for 30 sec at 94°C, annealing for 30 sec at 55°C and polymerization of 80 sec at 72°C. The last cycle had a polymerization step of 3 min.

2.5. Cloning of the gfp$_{uv}$ *gene and* ldh *promoter*

Standard techniques were used for DNA restriction enzyme digestion, blunt-end generation of 3'-recessed ends of restricted DNA fragments and ligations (Ausubel *et al.*, 1994). The enzymes used were from Roche Molecular Chemicals, Mannheim, Germany. DNA was

sequenced on an automatic sequencer (ABI Prism™, PE Biosystems SA Ltd.) using the primers previously described (section 2.4). Standard DNA manipulation, cloning of DNA fragments, and *E. coli* transformation methods were used (Ausubel *et al.*, 1994).

The cloning steps were carried out as follows: pBluescript SK (Fig. 1 A) was cut at the *BamH*I site and blunt-ends were generated from the 5′-overhangs created by *BamH*I. The plasmid DNA was then digested with *Xba*I (Fig. 1 C). The *ldh* promoter fragment that was obtained from PCR amplification (Fig. 1 B) was digested with *Spe*I to generate blunt-ends. The *ldh* promoter-fragment was then digested with *Xba*I and inserted in pBluescript SK- at the *Xba*I site (Fig. 1 B and C). pBlue31 (pBluescript SK- containing *ldh* promoter at the *Xba*I restriction site) was digested at the *Eco*RV and at the *Pst*I sites (Fig. 1 D). Purified amplified *gfp*uv gene product (Fig. 1 E) was digested with *Spe*I and blunt-ends were generated. The *gfp* gene was then digested with *Pst*I and was ligated to pBlue31 at the *Pst*I site (Fig. 1 F). The *ldh* promoter-*gfp*uv fragment in pBlue315 (pBlue31 containing the *gfp*uv gene downstream of the *ldh* promoter) was excised from pBlue315 using the restriction enzymes *Not*I and *Pst*I (Fig. 1 F and G). The shuttle vector pTRKH2 (Fig. 1 H) (O′Sullivan *et al.* 1993) was digested at the *Eco*RV and *Pst*I sites. The *ldh* promoter-*gfp*uv fragment (Fig. 1 I) was ligated to the shuttle vector pTRKH2 at the *Eco*RV and *Pst*I sites to form pTRK315 (Fig. 1 J and K).

2.6. Curing of plasmids from L. sakei *DSM 20017*

A 2% (v/v) inoculum from an overnight culture of *L. sakei* was used to inoculate MRS broth in different samples with increasing concentrations of novobiocin (Sigma), ranging from 0 to 150 µg/ml. The cultures were incubated at 37°C for four days. The cultures with the highest concentration of novobiocin where growth was still detected were selected for further studies. Plasmid loss was evaluated through plasmid isolations as described by Van Reenen *et al.* (1998).

2.7. Electroporation of L. sakei *DSM 20017 cells*

Competent cells for transformation by electroporation were generated using the method described by Berthier *et al.* (1996). Subsequent electroporation procedures were carried out as described by Berthier *et al.* (1996) and Wei *et al.* (1995).

2.8. Plasmid stability of pTRK315 in L. sakei *DSM 20017*

Lactobacillus sakei DSM 20017 cells with plasmid pTRK315 were grown in MRS broth containing erythromycin (5 µg/ml). The culture was serially diluted and the cells plated onto MRS agar. Incubation was at 30°C for two days. Colonies were selected from the plates and transferred to MRS agar plates containing erythromycin (5 µg/ml). These steps were repeated until no colonies were present on plates containing erythromycin (5 µg/ml).

2.9. Plasmid isolation from L. sakei *DSM 20017*

Plasmid isolations were performed from cured, normal and electroporated *L. sakei* DSM 20017 cells using the method described by Van Reenen *et al.* (1998). Normal and cured *L. sakei* DSM 20017 cells were inoculated in MRS medium and electroporated *L. sakei* DSM 20017 cells were inoculated into MRS containing erythromycin (5 µg/ml). The cultures were incubated for approximately 20 hours. Plasmid DNA isolated from electroporated *L. sakei* DSM 20017 and pTRK315 (isolated from *E. coli*) were subjected to electrophoresis on an agarose gel. The plasmids from cured, electroporated *L. sakei* DSM 20017 that displayed the same size as pTRK315 were excised from the agarose gel. Plasmid DNA was purified from the agarose with the use of a Qiagen PCR purification kit (Qiagen). The plasmid DNA from *L. sakei* strains and pTRK315 from *E. coli* were digested with *EcoR*I for 2 hours to confirm the identity of the isolated plasmid as pTRK315.

2.10. Bacteriocin production, purification and activity determination

Six organisms (*Enterococcus faecalis* BFE 1071, *Lactobacillus curvatus* DF38, *Lactobacillus plantarum* 423, *Lactobacillus casei* LHS, *Lactobacillus salivarius* 241 and *Pediococcus pentosaceus* ATCC 43200) were each inoculated in 100 ml MRS broth and incubated at 30°C (*E. faecalis* BFE 1071 at 37°C) for 24 hours. The cultures were centrifuged and the cell-free supernatants (containing bacteriocins) were filter-sterilized through sterile 0.2 µm filters. The filter-sterilized supernatants were freeze-dried and resuspended in 10 ml sterile distilled water. The pH from each of the redissolved supernatants was adjusted to 7.0 with sterile 1 M NaOH. The supernatants were filter-sterilized again through 0.44 µm filters and the antimicrobial activity of each of the supernatants determined against *L. sakei* DSM 20017. Antibacterial activities of the concentrated supernatants were tested against *L. sakei* DSM

20017 using the spot-on-lawn method (Van Reenen *et al.*, 1998). A two-fold serial dilution of each of the supernatants of the bacteriocin-producing strains and of ammonium precipitated bacteriocin 285 was done. Ten microliters of each dilution (undiluted to 1:1224 diluted) of each of the supernatants from the bacteriocin-producing strains was spotted on the plates and incubated for 24 hours at 30°C. Bacteriocins that caused inhibition zones larger than 1 mm were regarded as antimicrobially active.

2.11. Ammonium sulfate precipitation of bacteriocin 285

The concentrated, freeze-dried cell-free supernatant from *L. plantarum* 285 was diluted ten times in distilled deionised water. All subsequent steps were carried out at 4°C. Ammonium sulphate (final concentration 75 %, w/v) was added to the supernatant and stirred gently for 5 hours. It was then centrifuged for 30 minutes at 10 000 g. The pellet was dissolved in two pellet volumes of distilled water and the pH was adjusted to 7.0. The solution was dialysed overnight in distilled deionised water through a 1000-Dalton molecular weight cut-off membrane. The dialysate was added to 25 volumes of 1:2 methanol:chloroform and stirred gently for 1 hour. To obtain the precipitated protein, the sample was centrifuged for 30 minutes at 10 000 g. The pellet was dissolved in the original volume of the freeze-dried supernatant.

2.12. Protein assay

The protein concentrations of the partially purified supernatant from *L. plantarum* 285 and the cell-free supernatants from *E. faecalis* BFE 1071, *L. curvatus* DF38, *L. plantarum* 423, *L. casei* LHS, *L. salivarius* 241 and *P. pentosaceus* ATCC 43200 were determined using the Bio-Rad protein assay, a modified Bradford method (Table 4) (Bradford, 1976). Bovine serum albumin was used as protein standard for the colorimetric assay reagent. The absorbance wavelength used was 595 nm.

2.13. Bacteriocin sensitivity of L. sakei *DSM 20017 as a function of growth stage*

MRS broth was inoculated with an overnight culture of *L. sakei* DSM 20017 (2%, v/v, inoculum) and incubated at 30°C. At different absorbance values (OD_{600} = 0.05, 0.24, 0.35, 0.54, 0.811, 1.05, 1.195) 5 ml samples were taken from the inoculated culture. The samples

were incubated in the presence of one of six different bacteriocin-containing solutions for 1 hour, 2 hours, 4 hours and 6 hours, respectively, after which absorbance values for the samples were determined. The concentrations of the bacteriocins added are shown in Table 2. The number of viable cells were determined by serial dilution and plating onto MRS agar. Incubation was for 24 hours at 30°C.

2.14. Effect of bacteriocin 285 concentration on L. sakei growth

MRS broth was inoculated with an active (18 hour-old) culture of L. sakei DSM 20017 (2%, v/v, inoculum) and incubated at 30°C until the absorbance (at 600 nm) was 0.34. The culture was divided in 24 samples of 5 ml each. To each sample (in duplicate), a different concentration of bacteriocin 285 (0, 220 ng/ml, 110 ng/ml, 55 ng/ml, 27.5 ng/ml, 13.75 ng/ml, 6.9 ng/ml, 3.4 ng/ml, 1.7 ng/ml, 0.8 ng/ml or 0.4 ng/ml) or nisin (4 mg/ml) was added and incubated at 30°C. Absorbance readings of samples were taken (600 nm) every 30 min for 15 hours and at 25 hours after incubation with bacteriocins. Two hours after incubation, 1 ml was taken from each sample, serially diluted, plated onto MRS agar in duplicate and incubated at 30°C for up to two days.

2.15. Fluorescence Microscopy

Microscopy was performed with a Nikon Eclipse E 400 epifluorescent microscope with a 60 X oil immersion objective and pictures were taken with a COHU high performance CCD camera (model no. 4912-5010/0000).

2.16. Effect of bacteriocin 285 on gfp-expressing L. sakei cells

MRS broth, containing erythromycin (5 µg/ml) was inoculated with 2% (v/v) inoculum of an actively growing culture of L. sakei that contained plasmid pTRK315. The culture was incubated at 30°C until the absorbance was 0.3 (600 nm). The pH was adjusted to 6.5 with 1M NaOH. The culture was treated with nisin (4 mg/ml, 4000 U/ml), bacteriocin 285 (110 ng/ml, 16 AU/ml) or ten times concentrated MRS broth (1 %, v/v). Immediately thereafter (0 min) and after 1, 5, 15, 30, 45, 60, 80 and 240 min, respectively, samples were viewed with an epifluorescent microscope. Fluorescein and dual filters were used.

In a second experiment, the pH from *L. sakei* culture (OD = 0.3) that was incubated with nisin (4 mg/ml, 4000 U/ml), bacteriocin 285 (110 ng/ml, 16 AU/ml) or concentrated MRS broth for 30 min at 30°C, was adjusted to 5.0 to prevent GFP fluorescence. The cells were viewed with an epifluorescence microscope through the FITC filter to verify that they were not fluorescent. The cells were then stained with a LIVE/DEAD® *Bac*light™ Bacterial Viability probe (Molecular Probes Inc., Eugene, Oregon, USA) for 30 min and washed with distilled water to remove excess probe. The cells were viewed under the fluorescence microscope through the dual, rhodamine and fluorescein filters, respectively.

MRS broth, containing erythromycin (5 µg/ml), was inoculated with *L. sakei* DSM 20017 containing pTRK315 and incubated to an OD_{600} of 0.3. Cells were treated with different concentrations of bacteriocin 285 (1.75 ng/ml, 3.5 ng/ml, 7 ng/ml, 14 ng/ml and 20 ng/ml, respectively), nisin (100 mg/l, 100 U/ml) and bacteriocin 285 that was subjected to the following treatments:

- incubation at different pH values (pH 1, pH 3, pH 8 and pH 10),

- heat treatments (30 min at 60 °C, 30 min at 80°C, 30 min at 100°C, and 20 min at 121°C) and

- bacteriocin 285 treated with benzoic acid (804 mg/l) (Tfouni *et al.*, 2002).

The cells were treated with these bacteriocins for 2 hours, after which samples were analysed by flow cytometry. Samples were analyzed using a Becton Dickinson FACScalibur flow cytometer with an air-cooled argon ion laser (488 nm). From each sample, 200 000 cells were acquired. Further analysis was performed using WinMDI software, version 2.8 (http//:facs.scripps.edu/software.html).

3. Results and discussion

3.1. Isolation of plasmid encoding bacteriocin 285 gene

Plasmid isolation from *L. plantarum* 285 yielded 5 plasmids, which displayed the same plasmid profile as plasmids from *L. plantarum* 423 (results not shown). The plasmids from *L. plantarum* 285 that corresponded in size to pBAC 4 from *L. plantarum* 423 were isolated and used as a template for PCR. The product obtained by PCR was sequenced and revealed an identical sequence to the mature peptide from bacteriocin 423, which has previously been

sequenced (van Reenen *et al.*, 1998). In Fig. 2, the sequence of the fragment obtained by PCR is shown (blue text) and is aligned with the sequence of the *plaA* gene from the plantaricin 423 operon (Genbank, Los Alamos, USA; Accession number AF304384). Since the mature peptide from bacteriocin 423 and bacteriocin 285 possessed identical sequences, it was therefore deduced, that the two bacteriocins were identical. Bacteriocin 285 is, therefore, a small, plasmid-encoded protein of approximately 3.5 kDa. Bacteriocin 285 is sensitive to proteolytic enzymes and stable over a pH range of 1-10.

3.2. Inhibitory spectrum of bacteriocin 285

Bacteriocin 285 inhibited *B. cereus* LMG 13569, *L. curvatus* LMG 13553, *L. fermentum* LMG 13554, *P. pentosaceus* LMG 13560, *P. acidilactici* ATCC 12687, *L. sakei* LMG 13558 and *L. sakei* DSM 20017 (Table 3) to varying degrees. Of these organisms, the *ldh* gene sequence of only *L. sakei* was known (van den Berg *et al.*, 1995). The clear region that formed on the plates where bacteriocin 285 was tested against *L. sakei* LMG 13558 were smaller than the clear regions on the plates where *L. sakei* DSM 20017 was present in the soft MRS agar. This lead to the assumption that *L. sakei* DSM 20017 is more sensitive to bacteriocin 285 than *L. sakei* LMG 13558. *Lactobacillus sakei* DSM 20017 was, therefore, chosen as the sensitive organism in which the *gfp* gene would be inserted under the control of the *ldh* promoter.

3.3. Cloning of the gfp$_{uv}$ gene and ldh *promoter*

A vector that contained the *gfp*$_{uv}$ gene under the control of the *ldh* promoter was constructed in pBluescript SK- and was named pBlue315. Sequencing of pBue315 confirmed the presence of both genes (Fig. 3).

The *ldh* promoter-*gfp*$_{uv}$ fragment was excised from pBlue315, inserted in the shuttle vector pTRKH$_2$ and transformed to *E. coli* cells. The *E. coli* transformants were fluorescent when viewed under the epifluorescent microscope (Fig. 4), indicating not only that pTRK315 was indeed present in the cells, but also that the *L. sakei* L-lactate dehydrogenase promoter is active in *E. coli* as previously described by Malleret *et al.* (1998).

3.4. Transformation of L. sakei *DSM 20017 with pTRK315*

Lactobacillus sakei DSM 20017 cells were not easily transformable. Transformants were obtained at extremely low frequencies. The improved procedure for efficient transformation of *L. sakei* described by Berthier *et al.* (1996) was less effective with strain DSM 20017. Berthier *et al.* (1996) reported transformation frequencies of up to 10^7 transformants per μg supercoiled DNA. Transformation frequencies of strain DSM 20017 with plasmids pTRKH$_2$ and pTRK315 were lower than 200 transformants per μg DNA.

3.5. Plasmid stability

The instability of plasmid-marked constructs that are used in Gram-positive hosts in the absence of antibiotic selection makes the differentiation between plasmid loss and loss of the introduced strain from the system impossible. It was important to determine whether plasmid pTRK315 was stable in electroporated *L. sakei* cells, i.e., whether the plasmid in *L. sakei* transformants could be maintained in the absence of erythromycin. Plasmid pTRK315, however, was lost within three generations of growth in the absence of erythromycin. It was, therefore, decided to culture the *L. sakei* transformants in the presence of erythromycin in all subsequent experiments.

3.6. Plasmid isolations from L. sakei

To verify the presence of pTRK315 in *L. sakei*, plasmid isolations were performed on a number of *L. sakei* transformants. A plasmid corresponding in size to pTRK315 was present in the electroporated *L. sakei* (Fig. 5, lane 3 and 6). When the plasmid indicated with an arrow in lane 6 was isolated from an agarose gel and digested with *Eco*RI, fragments were generated that appeared to have the same sizes as pTRK315 (digested by *Eco*RI) on an agarose gel (Fig. 5, lane 4 and 5). The band corresponding in size to pTRK315 was not present in the cured *L. sakei* cells (Fig. 5, lane 7). It was, therefore, accepted that the additional plasmid that was present in *gfp*-expressing *L. sakei* cells was pTRK315. Furthermore, without pTRK315 (Fig. 5, lane 6 arrow), *L. sakei* cells were not fluorescent as they were when this plasmid was present. As can be seen in lane 7, some of the natural plasmids from *L. sakei* DSM 20017 remained present after the plasmids were cured. It may be possible that *L. sakei* DSM 20017 requires these plasmids for growth.

3.7. Bacteriocin activity tests against L. sakei *DSM 20017*

The effect of different bacteriocins on *L. sakei* DSM 20017 was determined by producing six bacteriocins and by determining the activity of these bacteriocins toward *L. sakei* DSM 20017 cells. The concentration of protein in each of the bacteriocin-containing supernatants was determined (Table 4). The arbitrary units of bacteriocin activity were calculated according to the lowest dilution where activity was evident against *L. sakei* DSM 20017. The cell-free supernatants of six bacteriocin-producing strains were used to test for activity against *L. sakei* DSM 20017. *Lactobacillus sakei* DSM 20017 was not sensitive to any of the bacteriocins produced by *E. faecalis* BFE 1071 (Fig. 6A). However, bacteriocins produced by *L. curvatus* DF38, *L. plantarum* 285, *L. casei* LHS and *L. salivarius* 241 were very active against *L. sakei* DSM 20017 (Fig. 6B-F). Clear regions were formed in the areas where these bacteriocins were spotted even up to the ninth dilution of a two-fold serial dilution where the lowest concentration of protein in the active dilutions was between 0.35 ng/ml and 0.50 ng/ml. Bacteriocins produced by *P. pentosaceus* ATCC 43200 were less active against *L. sakei* DSM 20017. The lowest concentration of protein where clear regions formed was 21 ng/ml (third dilution in a two-fold serial dilution). *Lactobacillus sakei* cells containing pTRK315 plasmids displayed the same sensitivity towards all the bacteriocins. It was, therefore, assumed that the *gfp*-expressing *L. sakei* cells would behave in the same way in subsequent experiments as those cells not bearing the plasmids in respect to their sensitivity to bacteriocins.

3.8. Bacteriocin sensitivity of L. sakei *DSM 20017 as a function of growth stage*

To determine whether *L. sakei* DSM 20017 cells were sensitive to antimicrobials during all growth stages, antimicrobial activity of concentrated cell-free supernatants from *E. faecalis* BFE 1071 (control, < 1 AU/ml), *L. curvatus* DF38 (256 AU/ml), *L. plantarum* 423 (256 AU/ml), *L. casei* LHS (256 AU/ml), *L. salivarius* 241 (256 AU/ml) and *P. pentosus* ATCC 43200 (4 AU/ml) were tested against *L. sakei* cells at different growth stages (Table 2). The effect of these cell-free supernatants on *L. sakei* DSM 20017 cells at specific growth stages are shown in Fig. 7A-G. Bacteriocins from *L. curvatus* DF38, *L. plantarum* 285, *L. casei* LHS and *L. salivarius* 241 inhibited the growth of *L. sakei* DSM 20017. However, bacteriocins from *P. pentosaceus* ATCC 43200 only partially inhibited the growth of *L. sakei* DSM 20017 (Fig. 7A-G). Bacteriocins from *E. faecalis* BFE 1071 were added as control, since these bacteriocins displayed no visible activity towards *L. sakei* DSM 20017 (Table 2). *Lactobacillus sakei* DSM 20017 cells remained sensitive to bacteriocins from lag phase to late

exponential growth phase. During the first 2 hours after addition of bacteriocins to *L. sakei* DSM 20017 culture at exponential growth, early stationary and stationary phase, the optical density of the culture still increased. It is possible that the bacteriocin required about an hour for its action to be observed as decrease in absorbance.

3.9. Effect of bacteriocin 285 concentration on L. sakei growth

Upto approximately 7 ng/ml (1 AU/ml) of bacteriocin 285 caused a decrease in turbidity of *L. sakei* DSM 20017, as seen in Fig. 8A. The absorbance of samples treated with concentrations of 6.9 ng/ml (1 AU/ml) to 220 ng/ml (32 AU/ml) of bacteriocin 285, decreased over time. Plate counts (Fig. 8 B) showed that 6.9 ng/ml of bacteriocin 285 was required to kill more than 90% of *L. sakei* cells. To kill more than 99,99% of *L. sakei* cells, at least 110 ng/ml (16 AU/ml) bacteriocin 285 was required. The decreases in optical density and in cell numbers caused by bacteriocin 285 might indicate that bacteriocin 285 is bactericidal, as previously suggested by van Reenen *et al.* (1998).

3.10. GFP-fluorescence and bacterial viability probe

Cells that were incubated with lethal concentrations of nisin (4 mg/ml, 4000 U/ml) and bacteriocin 285 (110 ng/ml, 16 AU/ml) remained as fluorescent as the untreated cells (Fig. 9). Even after two hours of incubation with concentrations of nisin and bacteriocin 285 that were high enough to kill more than 99,99% of *L. sakei* cells, the cells remained fluorescent. In Fig. 9 A, B and D, cells were viewed through epifluorescence microscopy with the fluorescein filter. In Fig. 9 C fluorescence microscopy was combined with light microscopy to confirm that all cells were green fluorescent and that the fluorescent cells in Fig. 8 A, B and D represented the state of all cells. Although the treated cells were fluorescent, the cells were not viable (Fig. 10), as determined through the use of a LIVE/DEAD® *Baclight*™ Bacterial Viability Probe where viable cells stained green and non-viable cells stained red. In Fig. 10 A and B, untreated cells were viewed through the fluorescein filter and the dual filter, respectively. The dual filter allows simultaneous evaluation of cells with different fluorescent wavelengths, i.e., allows simultaneous view of viable (green) and non-viable (red) cells. In Fig. 10 B, where both viable (green) and non-viable cells (red) are visible, it was evident that the majority of the cells were viable, as only a few red cells were detected. When the cells were viewed through the rhodamine filter, less than 10 red cells were visible. However, when

bacteriocin 285-treated cells were viewed through the rhodamine filter, a vast majority of red cells (non-viable) were visible. In Fig. 10 D, bacteriocin 285-treated cells were viewed through the dual filter. No green cells (viable) were visible.

Lactobacillus sakei cells that were treated with lethal concentrations of bacteriocins (16 AU/ml) were green-fluorescent for prolonged periods of time although the cells were not viable. The non-viability of the cells was confirmed through the use of the Baclight probe. The probe utilizes mixtures of a green fluorescent nucleic acid stain (SYTO®9) and a red fluorescent nucleic acid stain (propidium iodide). These stains differ in their abilities to penetrate healthy cells. The SYTO®9 stain generally stains both cells with intact cell membranes and cells with compromised membranes. Propidium iodide, however, penetrates only bacteria with damaged membranes and causes reduction in the green fluorescence from SYTO®9. With an appropriate mixture of the two dyes, bacteria with intact cell membranes stain green fluorescent and bacteria with damaged membranes stain red fluorescent. However, cells having damaged membranes may be able to recover and reproduce, but will be scored as non-viable with the assay. Nevertheless it is safe to assume that most cells that stained red were non-viable, as results from plate counts confirmed that more than 99,99% of the cells were killed by the concentration of bacteriocin 285 (16 AU/ml) that was used. It seems therefore, that GFP-fluorescence can not be used as a direct mean to study bacteriocin activity, as cells that are dead remain fluorescent. A possible solution could be to find a GFP variant that has a shorter half life.

3.11. Flow cytometry

Flow cytometry was evaluated as a method to confirm viability/non-viability of *L. sakei* cells (expressing GFP) that were treated with bacteriocins. Advantage was taken of GFP fluorescence to analyse the cells. Although GFP fluorescence requires an excitation wavelength of 388 nm, the wavelength of 488 nm of the FACS (Fluorescent Activated Cell Sorter) laser provides a sufficient signal for cytometrical analysis of GFP_{uv}-expressing cells (Shapiro, 1995). The absorbance at 488 nm is about one third of the maximum absorbance at 388 nm of GFP and GFP_{uv}.

The mean values of side scatter (SSC), forward scatter (FSC) and FL1 (fluorescence intensity) were calculated for all samples. As observed by using fluorescence microscopy, GFP_{uv} fluoresced equally strong in healthy and in competent cells. The mean fluorescence intensity of cells was similar in all samples (2.43 ± 0.4). The differences in SSC and FSC recorded of

samples towards the control (untreated) cells are presented in Fig. 10 A and B. Side scatter gives an indication of the granularity of the cells, whereas forward scatter indicates the relative size of cells.

The mean SSC (Fig. 11 A) and FSC (Fig. 11 B) of differently treated samples are compared as a fraction of the mean of the control. The cells become less granular (SSC) and smaller (FSC) when lethal concentrations of bacteriocins are present. Cells treated with bacteriocin 285 (autoclaved for 20 minutes) showed only a slight decrease in mean SSC and FSC compared to the control (untreated) cells. This may indicate that autoclaving of bacteriocin 285 for 20 minutes destroys a significant proportion of the antimicrobial peptides. This agrees with results from Van Reenen *et al.* (1998), where it was shown that autoclaving of plantaricin 423 destroys aout 80 percent of the activity of the bacteriocin. However, bacteriocin 285 that was treated with different pH values (pH1, pH3, pH8, pH10) and temperatures (60°C, 80°C and 100°C for 30 minutes) caused a decrease in SSC and FSC of treated samples, which may indicate that the bacteriocin can sustain these treatments. Bacteriocin 285 was incubated with benzoic acid at a concentration that is used in food as a preservative to observe whether benzoic acid might damage the peptide if it should be used with benzoic acid as a preservative in food. However, as seen in Fig. 11, it seems that cells that were subjected to treatment with bacteriocin 285 that was treated with benzoic acid are affected similar to cells treated with untreated bacteriocin 285. It is, therefore, possible that benzoic acid does not affect bacteriocin 285 activity negatively.

In Figs. 12 and 13, typical flow cytometry data are shown. Fig. 12 shows contour plots of fluorescence intensity (FL1) versus granularity (SSC) of *L. sakei* DSM 20017 cells that were untreated (A) and cells that received different concentrations of bacteriocin 285 (B, 4 AU/ml; C, 2 AU/ml; D, 1 AU/ml; E, 0.5 AU/ml; F, 0.25 AU/ml). Different colours represent different quantities of bacteria. The changes that the different concentrations of bacteriocins 285 caused in the population of cells are obvious. The population in B, where cells were treated with 4 AU/ml seem to be very different to the population in A (untreated cells). As the bacteriocin concentration decreases, the population starts to look increasingly like the untreated population (A). The population that was treated with 0.25 AU/ml looks nearly exactly like the control population in A.

In Fig. 13, dot plots of forward scatter (relative size) versus side scatter (relative granularity) are shown of untreated cells (A) and cells treated with different concentrations of bacteriocins (B, 4 AU/ml; C, 2 AU/ml; D, 1 AU/ml; E, 0.5 AU/ml; F, 0.25 AU/ml). Each dot represents a cell. The area marked with R2 represents the healthy, viable cells of the population. Once

again, treatment with 4 AU/ml causes a definate shift in the population. As the concentration of bacteriocin 285 decreases, the population starts to appear increasingly like the untreated population.

Flow cytometry allowed analysis of a great number of cells. However, the method did not appear to be as sensitive as optical density measurements and plate counts to evaluate effects of bacteriocins on *L. sakei* cells. For example in Fig. 9B, cells treated with 4 AU/ml bacteriocin 285 show only a 20% difference towards the untreated cells. According to plate counts, however, more than 99 % cells are killed by 4 AU/ml bacteriocin 285. This discrepancy may have occurred as only SSC and FSC could be used to characterize the state of the population. Since there was no difference in mean fluorescence intensity between treated and untreated cells, it may be suitable to stain non-fluorescent bacteria with a fluorescent stain, such as propidium iodide, so that only compromised bacteria are fluorescent. The differentiation between damaged and healthy bacteria might then be evaluated by using SSC, FSC and fluorescent intensity.

4. Conclusions

Bacteriocin 285, which is produced by *Lactobacillus plantarum* 285, is identical to plantaricin 423, produced by *L. plantarum* 423. Bacteriocin 285 is thus, a class IIa bacteriocin that displays antibacterial activity towards a broad spectrum of bacteria, including several food pathogens. *Lactobacillus sakei* DSM 20017 is sensitive to various bacteriocins, including bacteriocin 285. Furthermore, *L. sakei* DSM 20017 cells remain sensitive to bacteriocins from lag phase to late exponential growth phase. There appears to be no stage where the cells are more less sensitive towards bacteriocins.

The GFP$_{uv}$–expressing plasmid that was transformed to *L. sakei* was unstable. It was therefore necessary to grow cells bearing the plasmid in the presence of an antibiotic. To inhibit growth of up to 90% of *L. sakei* DSM 20017 cells, 1 AU/ml bacteriocin 285 (7 ng/ml of partly purified bacteriocin 285) is required. However, to kill more than 99,99% of *L. sakei* DSM 20017 cells, 16 AU/ml (110 ng/ml of partly purified bacteriocin 285) is required.

GFP$_{uv}$ fluorescence cannot be used directly to quantify antimicrobial activities towards sensitive cells, as cells remain fluorescent for prolonged periods after treatment with lethal concentrations of bacteriocins. GFP$_{uv}$ fluorescence can be used like turbidity, to monitor cells as a function of time. By using a fluorimeter, it may be possible to monitor the effect of

bacteriocins on GFP$_{uv}$–expressing cells. However, a suitable fluorimeter was not available for such studies. There may be no advantage of using fluorimetry to study decreases in fluorescence intensity as opposed to standard turbidity measurements.

Flow cytometry as analytical method of bacteriocin activity was not as suitable as plate counts and optical density measurements, as there was no difference in fluorescent intensity in treated and untreated cells. However, flow cytometry was valuable in confirming results obtained from fluorescence microscopy, namely, that cells remained fluorescent after treatment with lethal concentrations of bacteriocins. Furthermore, a definite shift in the population after treatment with bacteriocins could be observed. However, this shift could not be evaluated quantitatively.

For further studies, a stable plasmid could be generated with a reporter that can be used more quantitatively, such as luciferase luminescence. Recently, mutants of GFP have been generated, which display shorter half lifes (Anderson *et al.*, 1998). These unstable variants of GFP may be valuable in bacteriocin activity studies when the action of the bacteriocin is visible as a decrease in fluorescence. Another alternative could be to treat non-fluorescent cells with bacteriocins, to stain the bacteria with propidium iodide, and to evaluate the effect of the bacteriocins through fluorescence microscopy and flow cytometry.

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Figures
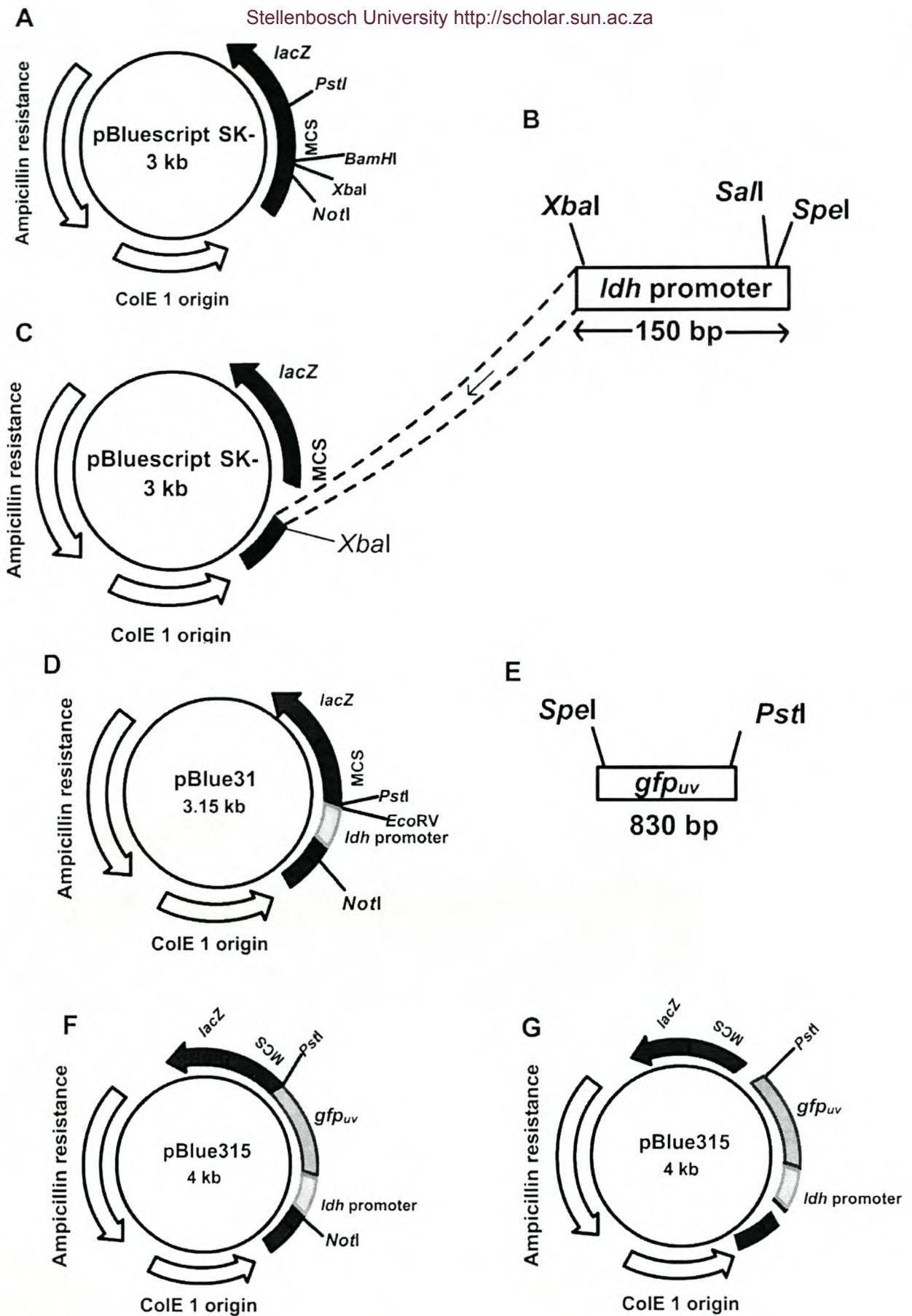
Fig. 1 A - G. Schematic representation of plasmids and fragments that were used to construct pTRK315. The *ldh* promoter fragment (B) was inserted in pBluescript SK- (A), which was digested by restriction enzymes (C) to form pBlue31 (D). A fragment containing the *gfp_uv* gene (E) was inserted in pBlue31 to form pBlue315 (F). A fragment containing the *ldh* promoter and the *gfp_uv* gene was excised from pBlue315 (G).
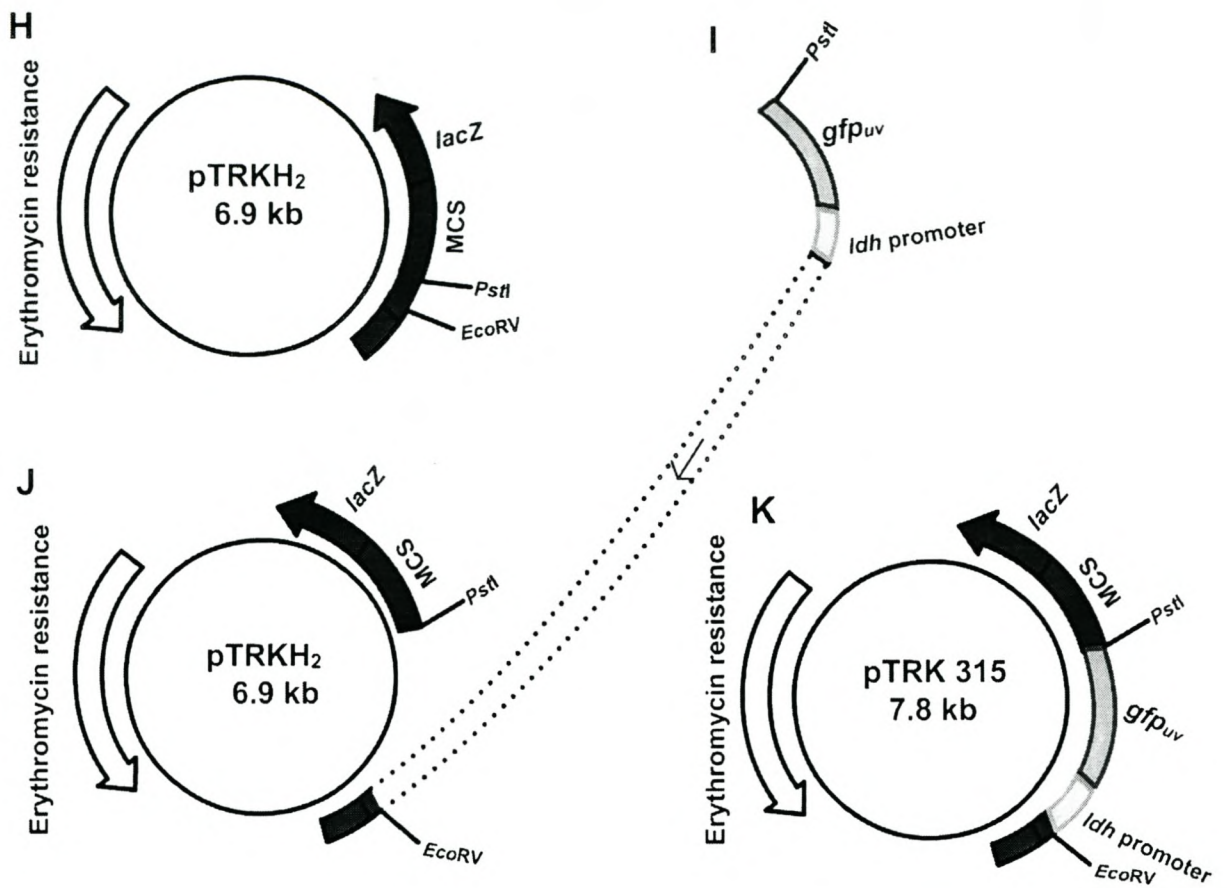
Fig. 1 H - K. Schematic representation of plasmids and fragments that were used to construct pTRK315. The shuttle vector pTRKH₂ (H) was cut (J) and the fragment containing the *ldh* promoter and the *gfp$_{uv}$* gene (I) was inserted in pTRKH₂ (J) to form pTRK315 (K).

```
  1 tctaactaat acttgacatt tacattgagt aggaactaga atgaccgcgt attaaaaatg
 61 atttaagaag aaggagattt ttatgatgaa aaaaattgaa aaattaactg aaaaagaaat
121 ggccaatatc attggtggta aatactatgg taatggggtt acttgtggta aacattcctg
                                                  tt acttgtggta aacattcctg
181 ctctgttaac tggggccaag cattttcttg tagtgtgtca catttagcta acttcggtca
    ctctgttaac tggggccaag cattttcttg tagtgtgtca catttagcta acttcggt
241 tggaaagtgc taa
```

Fig. 2. Alignment of the mature peptide of bacteriocin 285 (blue text) with the sequence of the *plaA* gene from the plantaricin 423 operon.

```
1     tctagaccat acccgttttt ctttctatat taggaaataa atagttttct

51    taaaatttaa acaaaattag cgaactttca tgcaaattat gctatactgg

101   ttttcgaaat gaagaattgt tttacagaaa ggacgtcatt atattggtct

151   aga


154   actagttaca tatggctagc aaaggagaag aacttttcac tggagttgtc

204   ccaattcttg ttgaattaga tggtgatgtt aatgggcaca aattttctgt

254   cagtggagag ggtgaaggtg atgctacata cggaaagctt acccttaaat

304   ttatttgcac tactggaaaa ctacctgttc catggccaac acttgtcact

354   actttctctt atggtgttca atgcttttcc cgttatccgg atcatatgaa

404   acggcatgac ttttttcaaga gtgccatgcc cgaaggttat gtacaggaac

454   gcactatatc tttcaaagat gacgggaact acaagacgcg tgctgaagtc

504   aagtttgaag gtgataccct tgttaatcgt atcgagttaa aaggtattga

554   ttttttaaaga gatggaaaca ttctcggaca caaactcgag tacaactata

604   actcacacaa tgtatacatc acggcagaca aacaaaagaa tggaatcaaa

654   gctaacttca aaattcgcca caacattgaa gatggatccg ttcaactagc

704   agaccattat caacaaaata ctccaattgg cgatggccct gtccttttac

754   cagacaacca ttacctgtcg acacaatctg ccctttcgaa agatcccaac

804   gaaaagcgtg accacatggt ccttcttgag tttgtaactg ctgctgggat

854   tacacatggc atggatgagc tctacaaata atgaattcga gctcggtacc

904   cgggggatcct ctagagtcga c
```

Fig. 3. Sequence of the *ldh* promoter-*gfp*uv fragment cloned into pBluescript SK. The *ldh* promoter stretches from bases 1 to 151, while the *gfp*uv gene stretches from bases 154 to 904.

Fig. 4. Transformants of *E. coli* with pTRK315 plasmids. The plasmid contained the *ldh* promoter-*gfp*$_{uv}$ fragment.
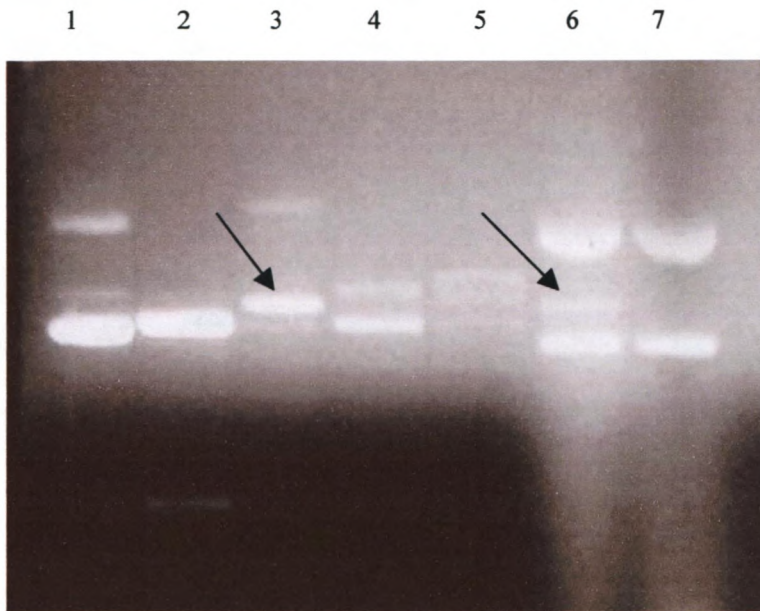
Fig. 5. Agarose gel electrophoresis of plasmid DNA isolated from *E. coli* (lanes 1 to 4) and *L. sakei* (lanes 5 to 7). pTRKH$_2$ (lane 1), pTRKH$_2$ digested with *Eco*RI (lane 2), pTRK315 (lane 3), pTRK315 digested with *Eco*RI (lane 4), pTRK315 isolated from total plasmid isolation of electroporated *L. sakei* that was digested with *Eco*RI (lane 5), plasmid DNA from electroporated *L. sakei* (lane 6) and plasmid DNA from cured *L. sakei* (lane 7). Arrows indicate DNA fragments with similar sizes (7.8 kb).
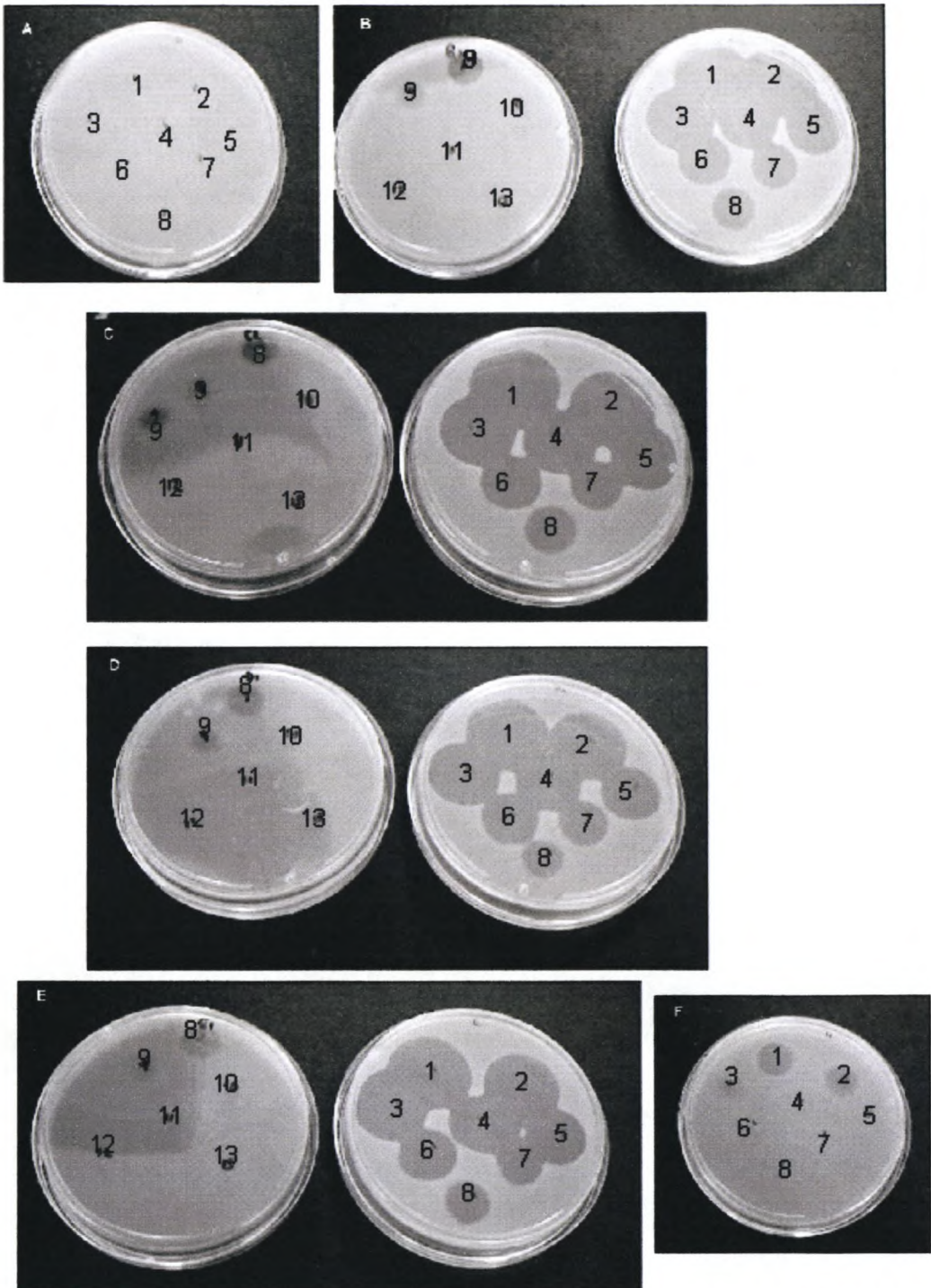
Fig. 6. MRS agar plates overlayed with MRS soft agar containing 2% (v/v) active *Lactobacillus sakei* DSM 20017 culture and spotted with a two-fold serial dilution of concentrated cell-free supernatants of *E. faecalis* BFE 1071 (A), *L. curvatus* DF38 (B), *L. plantarum* 423 (C), *L. casei* LHS (D), *L. salivarius* 241 (E) and *P. pentosus* ATCC 43200 (F). Clear regions indicate antimicrobial activity.
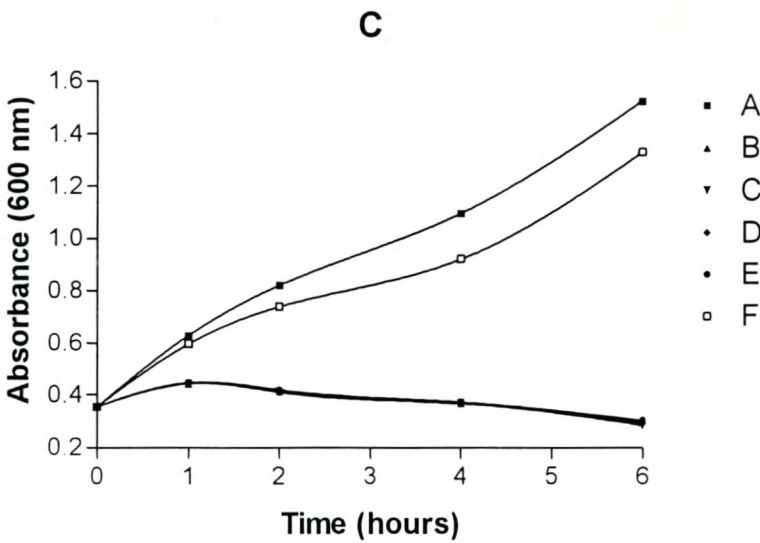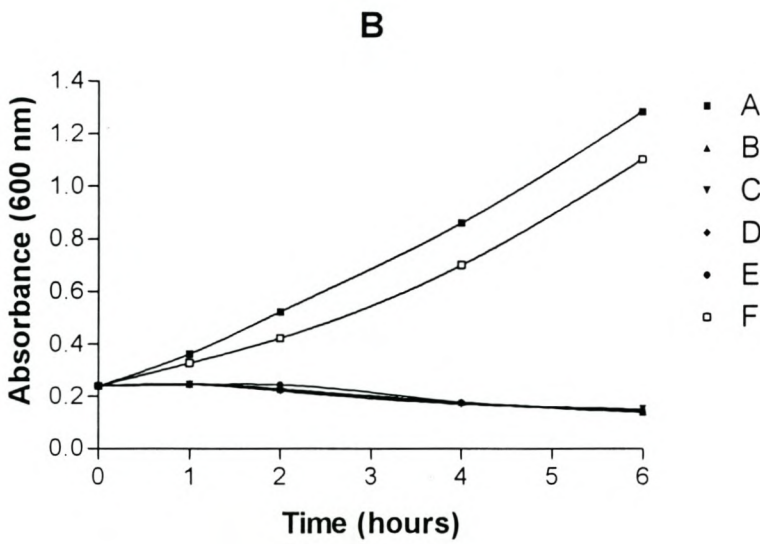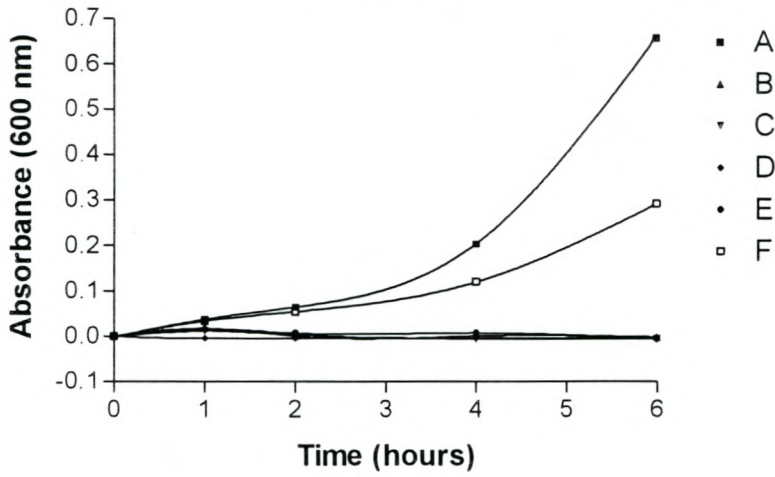
Fig. 7 A. Effect of bacteriocins on *L. sakei* at OD$_{600}$ = 0.05 (A), 0.24 (B), 0.35 (C). The legend refers to cell-free supernatants from *E. faecalis* BFE 1071 (A), *L. curvatus* DF38 (B), *L. plantarum* 423 (C), *L. casei* LHS (D), *L. salivarius* 241 (E) and *P. pentosaceus* ATCC 43200 (F).
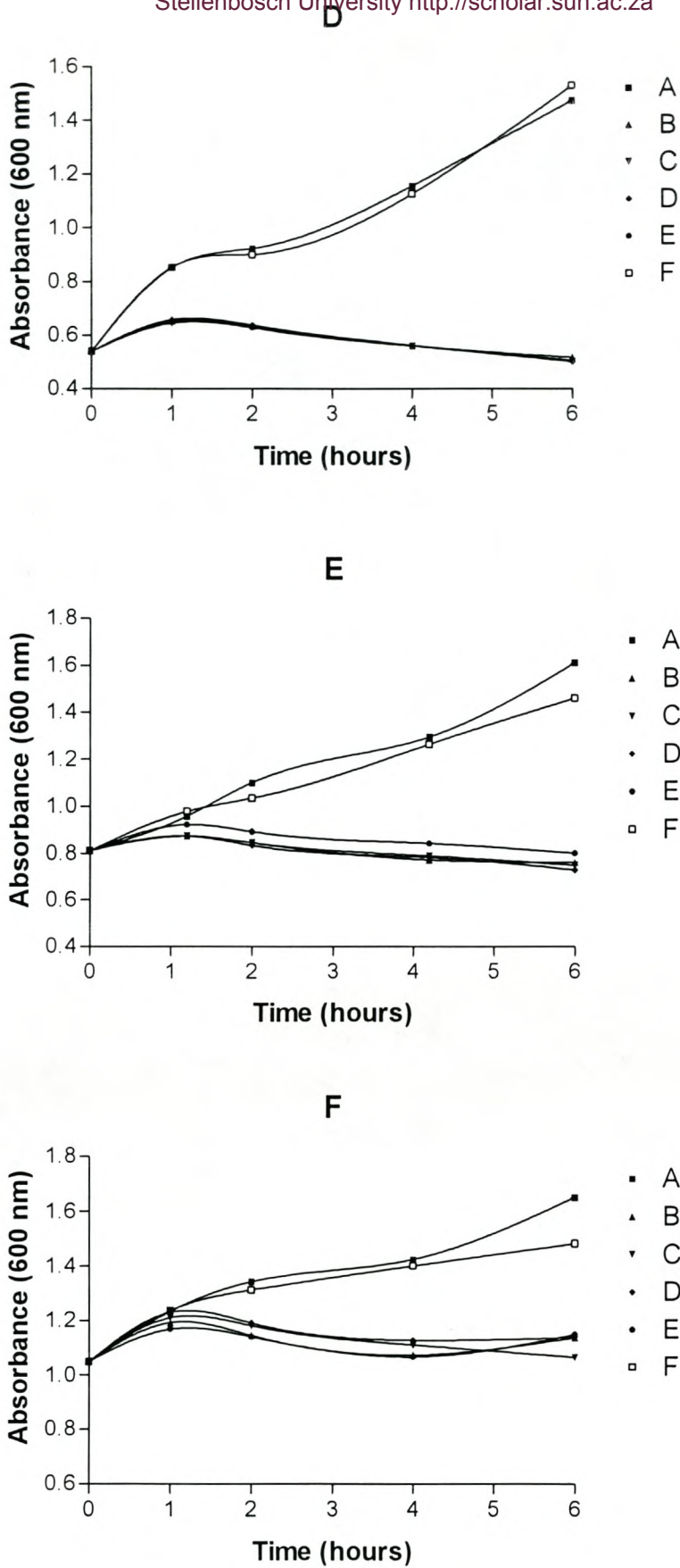
Fig. 7 B. Effect of bacteriocins on *L. sakei* at OD_{600} = 0.54 (D), 0.81 (E), 1.05 (F). The legend refers to cell-free supernatants from *E. faecalis* BFE 1071 (A), *L. curvatus* DF38 (B), *L. plantarum* 423 (C), *L. casei* LHS (D), *L. salivarius* 241 (E) and *P. pentosceus* ATCC 43200 (F).
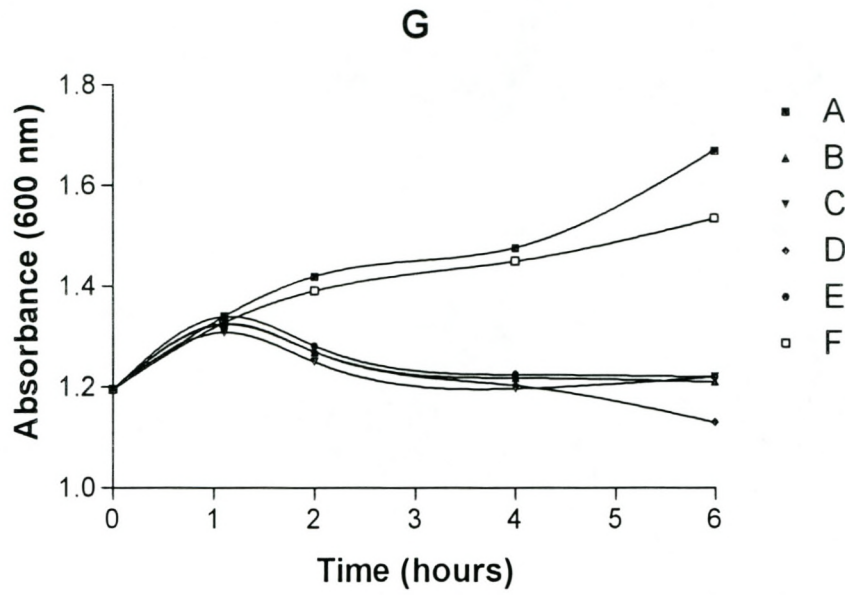
Fig. 7 C. Effect of bacteriocins on *L. sakei* at $OD_{600} = 1.2$ (G). The legend refers to cell-free supernatants from *E. faecalis* BFE 1071 (A), *L. curvatus* DF38 (B), *L. plantarum* 423 (C), *L. casei* LHS (D), *L. salivarius* 241 (E) and *P. pentosceus* ATCC 43200 (F).

A

B
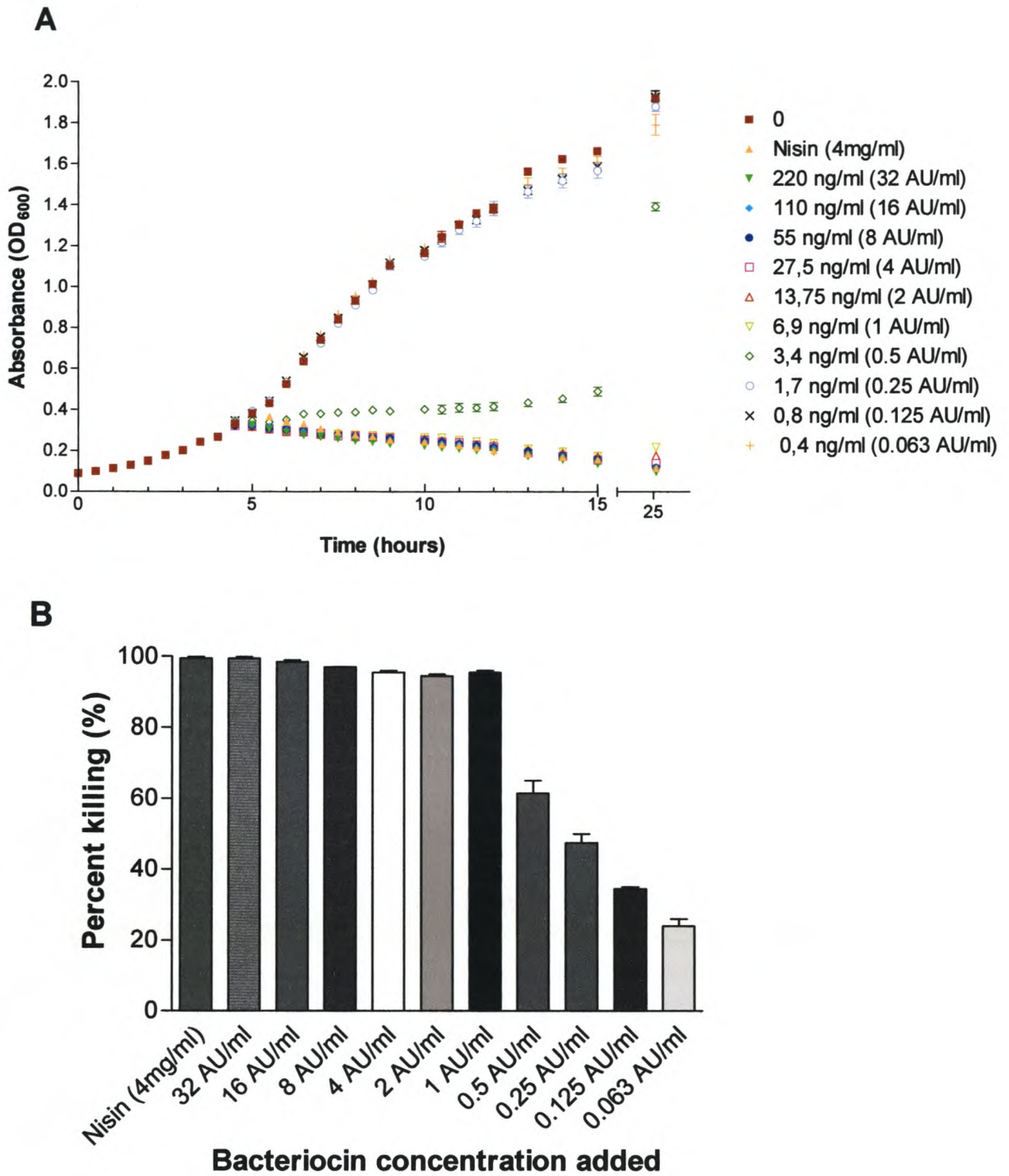
Fig. 8. Effect of bacteriocin 285 concentration on *L. sakei* DSM 20017 cells. Results shown are averages of duplicate samples with error bars indicating the lowest and highest values obtained. In graph A, the effect of bacteriocin 285 concentration on *L. sakei* cells at an optical density of 0.35 is shown up to 25 hours. In graph B, killing of *L. sakei* cells by the different concentrations of bacteriocin 285 is shown.
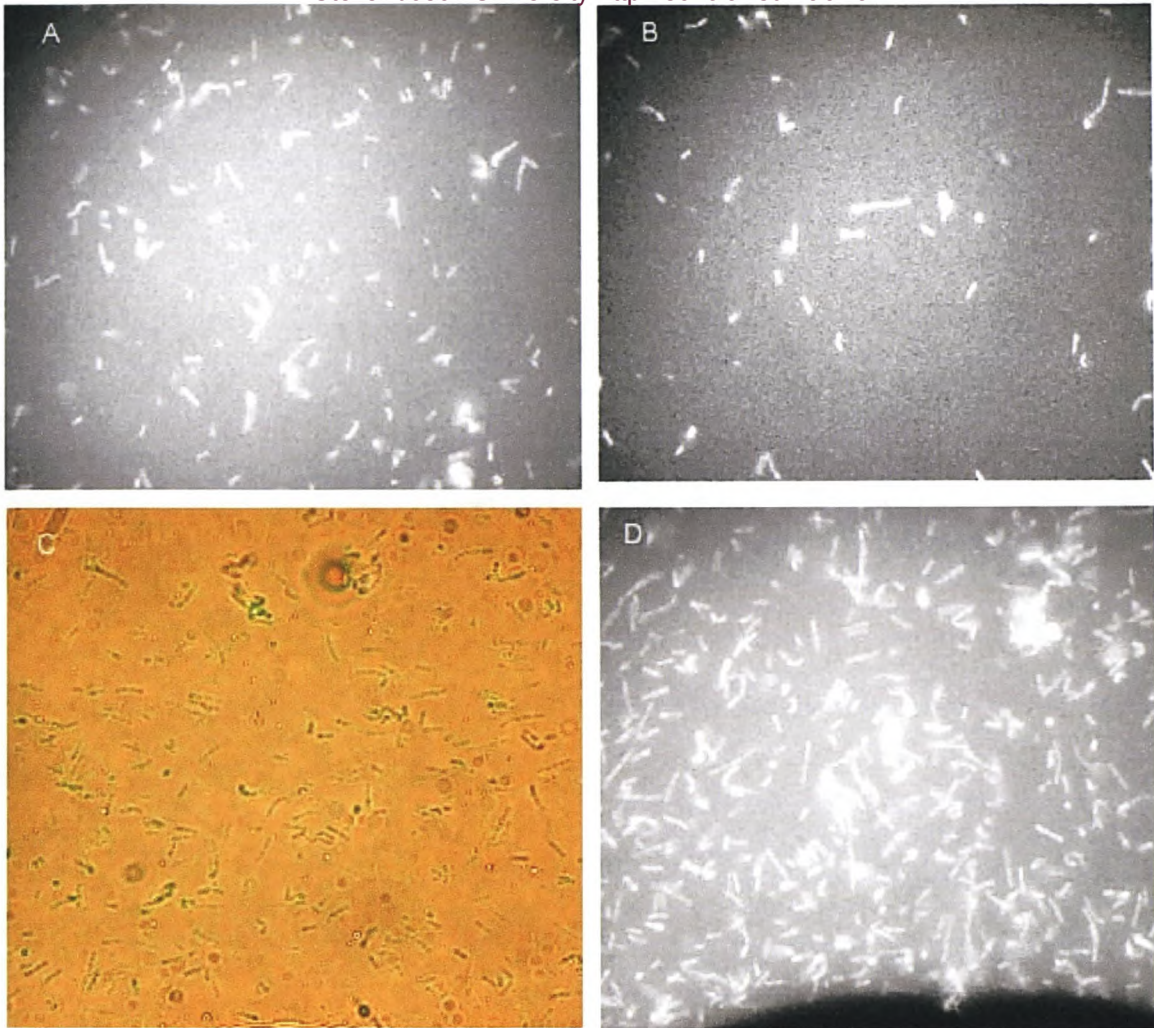
Fig. 9. Fluorescence microscopy (A, B and D) and fluorescence microscopy combined with light microscopy (C) of *L. sakei* DSM 20017 containing the GFP-producing plasmid pTRK315. Cells were treated with nisin (A) and bacteriocin 285 (C and D) for 45 minutes or untreated (B).
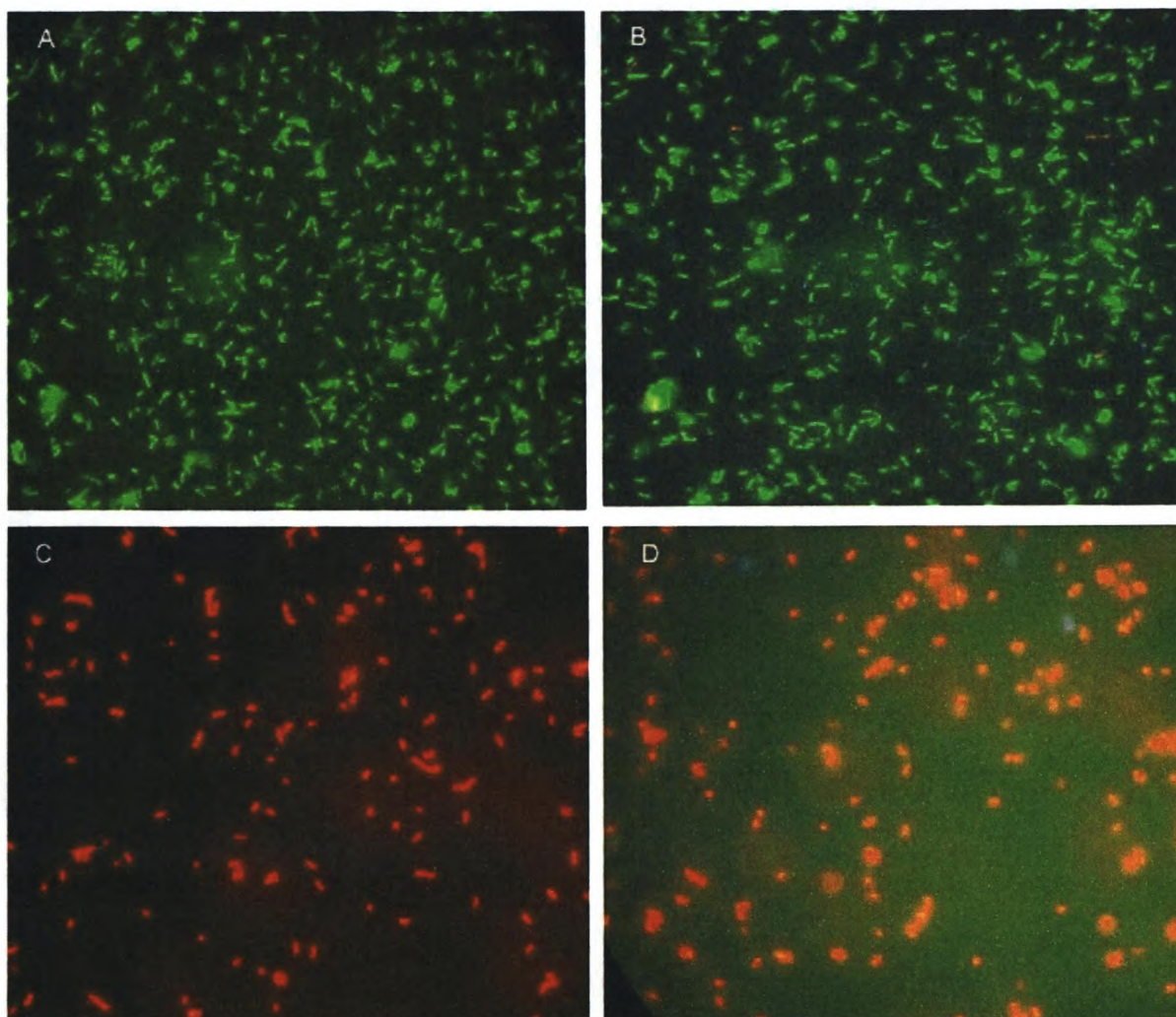
Fig. 10. *Lactobacillus sakei* DSM 20017 cells treated with bacteriocin 285 (110 ng/ml) for 30 minutes (C and D) and untreated (A and B). Cells were stained with Baclight viability probe. Green cells are viable and red cells are non viable.
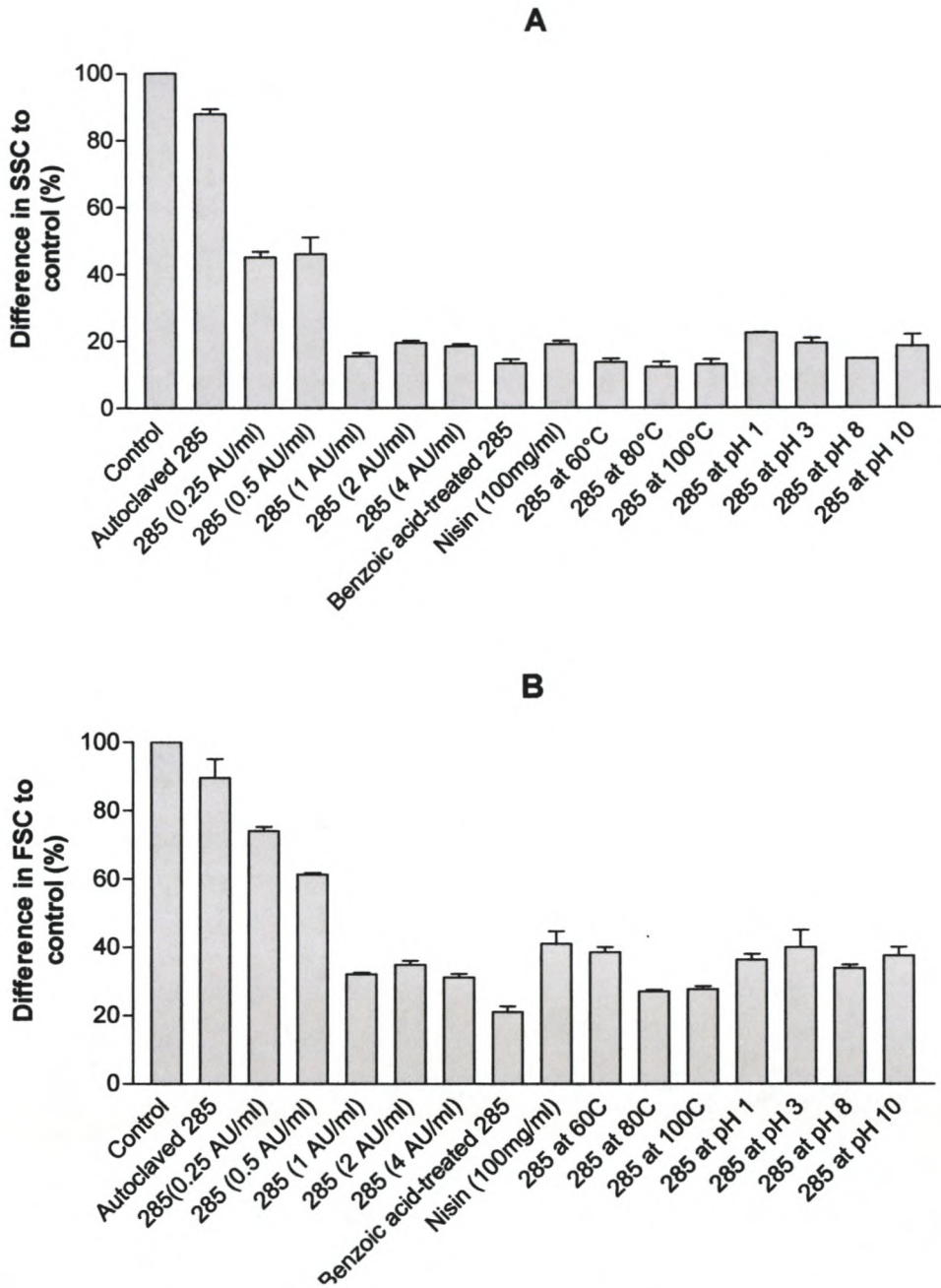
Fig. 11. Percent difference in SSC (side scatter) (A) and FSC (forward scatter) (B) of treated and untreated (Control) *L. sakei* DSM 20017 cells containing the GFP-expressing plasmid pTRK315. Results shown are averages of duplicate samples.
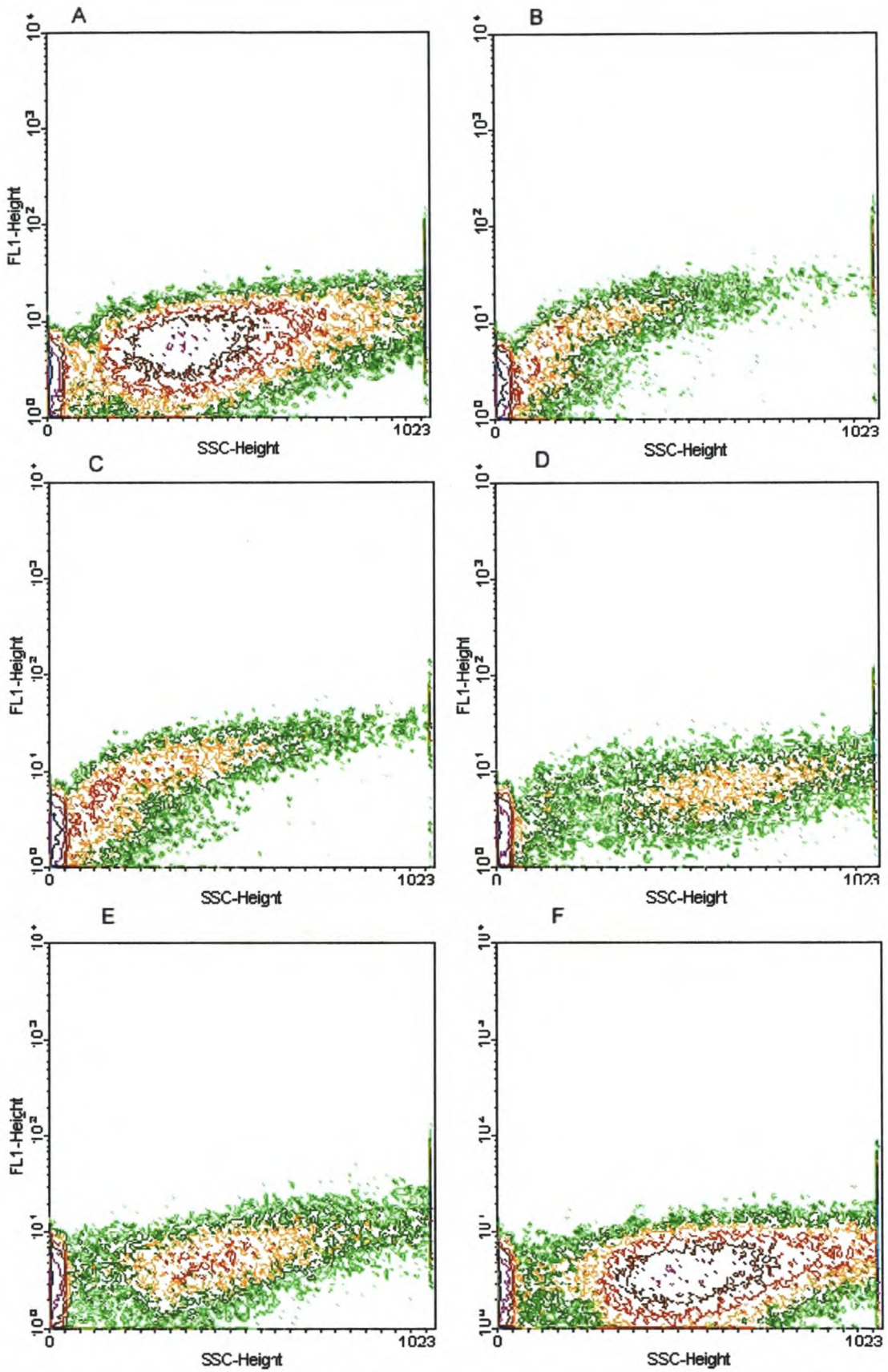
Fig. 12. Contour plot of fluorescence intensity (FL1) versus granularity (SSC) of *L. sakei* DSM 20017 cells that were untreated (A) and cells that received different concentrations of bacteriocin 285 (B, 4 AU/ml; C, 2 AU/ml; D, 1 AU/ml; E, 0.5 AU/ml; F, 0.25 AU/ml). Different colours represent different amounts of bacteria.
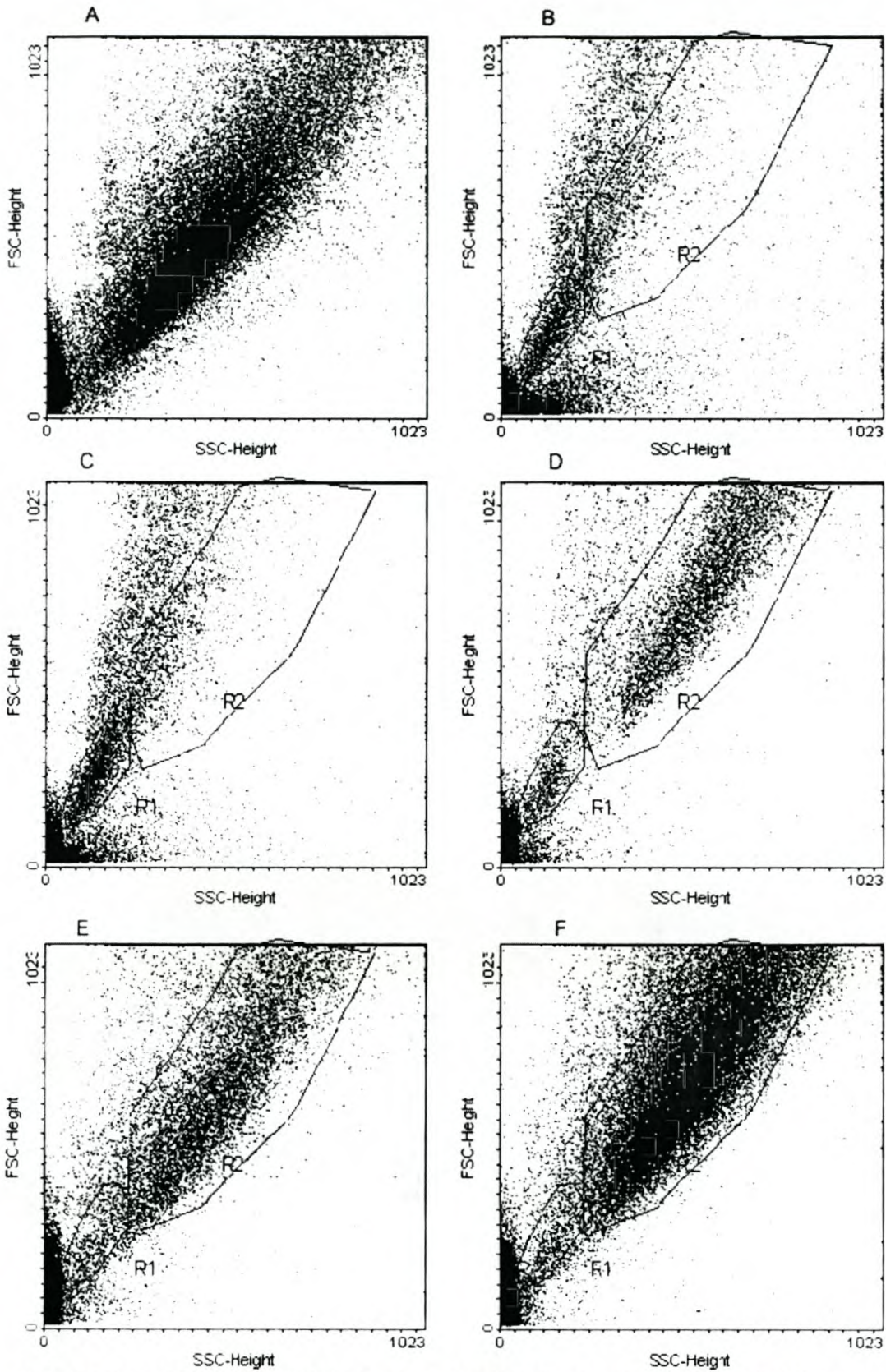
Fig. 13. Dot plot of FSC versus SSC of untreated cells (A) and cells treated with different concentrations of bacteriocins (B, 4 AU/ml; C, 2 AU/ml; D, 1 AU/ml; E, 0.5 AU/ml; F, 0.25 AU/ml). Each dot represents a cell.

Tables

Table 1

Bacterial strains and plasmids

Strain or plasmid	Reference
Bacterial strain	
Lactobacillus plantarum 285	Van Reenen *et al.* (1998)
Lactobacillus plantarum 423	"
Lactobacillus casei ATCC 334	ATCC[a]
Streptococcus thermophilus LMG 13564	LMG[b]
Bacillus cereus LMG 13569	"
Lactobacillus curvatus LMG 13553	"
Lactobacillus fermentum LMG 13554	"
Pediococcus pentosaceus LMG 13560	"
Pediococcus acidilactici ATCC 12687	"
Lactobacillus sakei LMG 13558	"
Lactobacillus sakei DSM 20017	DSM[c]
Escherichia coli DH5α	Ausubel *et al.* (1994)
Escherichia coli JB 361	Søren Molin[d]
Plasmid	
pBadGFPuv	Søren Molin[d]
pBluescript SK-	Stratagene[e]
pTRKH$_2$	O'Sullivan *et al.* (1993)

[a] ATCC. American Type Culture Collection, Rockville USA.

[b] LMG. Laboratorium voor Mikrobiologie, University of Ghent, Ghent, Belgium.

[c] DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig Germany.

[d] Prof. Søren Molin, Technical University of Denmark, Denmark.

[e] Stratagene, Whitehead Scientific, South Africa.

Table 2

Concentration of protein added to each sample to study the effect of bacteriocins on the growth stage of *L. sakei* DSM 20017

Bacteriocin-producing culture	Concentration protein (μg/ml)	Arbitrary activity units/ml[a]
Enterococcus faecalis BFE 1071	1.52	< 1
Lactobacillus curvatus DF38	2.55	256
Lactobacillus plantarum 423	2.6	256
Lactobacillus casei LHS	1.78	256
Lactobacillus salivarius 241	1.87	256
Pediococcus pentosaceus ATCC	1.67	4

[a] Determination of arbitrary activity units (AU) of bacteriocin per milliliter was based on the reciprocal of the greatest dilution of the concentrated bacteriocin solution that showed activity against *L. sakei* DSM 20017.

Table 3

Inhibitory spectrum of bacteriocin 285

Strain	Sensitivity to bacteriocin 285[a]
Lactobacillus casei ATCC 334	-
Streptococcus thermophilus LMG 13564	-
Bacillus cereus LMG 13569	++
Lactobacillus curvatus LMG 13553	+++
Lactobacillus fermentum LMG 13554	++
Pediococcus pentosaceus LMG 13560	+
Pediococcus acidilactici ATCC 12687	++
Lactobacillus sakei LMG 13558	++
Lactobacillus sakei DSM 20017	+++

[a] Sensitivity recorded as: − (not sensitive), + (low sensitivity), ++ (intermediate sensitivity), +++ (very sensitive).

Table 4

Protein concentration and antimicrobial activity of concentrated cell-free supernatants of bacteriocin-producing organisms

Organism from which bacteriocin-containing supernatant originates	Protein concentration (mg/ml)	Bacteriocin activity (Arbitrary units/mg protein)
Enterococcus faecalis BFE 1071	0.15	< 1
Lactobacillus curvatus DF38	0.25	100400
Lactobacillus plantarum 285	0.26	98500
Lactobacillus plantarum 285[a]	0.11	145500
Lactobacillus casei LHS	0.18	143800
Lactobacillus salivarius 241	0.19	136900
Pediococcus pentosaceus ATCC 43200	0.17	2400

[a] Partially purified supernatant