Studies on regulation

of the

plantaricin 423 gene

by

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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 entity or in part submitted it at any university for a degree.

Signature:

Date:

SUMMARY

Lactic acid bacteria play an essential role in the majority of fermented foods by producing organoleptic compounds and increasing the shelf life. The best-studied antimicrobial compounds are bacteriocins, i.e. ribosomally synthesized peptides. Most of these peptides have a narrow spectrum of activity and are usually only active against bacteria from the same ecological niche. The fact that all bacteriocins are degraded by proteolytic enzymes enlarges their potential use as natural food preservatives. The ideal would be to replace or reduce chemical preservatives such as sulfur dioxide, nitrates and nitrites.

Bacteriocins are classified into four groups according to their structural and functional characteristics. Plantaricin 423, produced by *Lactobacillus plantarum* 423, is heat stable, plasmid encoded, relatively small (3.5 kDa) and is classified as a class IIa bacteriocin. The peptide is active from pH 1.0 to 10.0 and inhibits Gram-positive bacteria, including *Lactobacillus* spp., *Leuconostoc* spp., *Oenococcus oeni*, *Pediococcus* spp., *Enterococcus* spp., *Propionibacterium* spp. and pathogens such as *Bacillus cereus*, *Clostridium* spp. and *Listeria monocytogenes*.

Production of bacteriocins may occur constitutively or may be regulated by a cell-density dependent system called quorum sensing. Plantaricin 423 is produced throughout logarithmic growth, with no apparent change in production levels when the producer strain is cultured in the presence of plantaricin 423 or *Listeria innocua* and *Lactobacillus sakei*. This led us to believe that plantaricin 423 may be produced constitutively.

A reporter system was constructed which consisted of the plantaricin 423 promoter, P423, fused to the *luxAB* genes and cloned into a shuttle vector, pTRKH2. The newly constructed plasmid, pTAB4, was transformed to a bacteriocin-negative mutant of *L. plantarum* (423 B^-). Despite several repeats, no luciferase activity was recorded and no RNA homologous to the *luxAB* genes was detected.

The region necessary for expression of plantaricin 423 may be located stream-up of the –80 region homologous to the –80 and –40 conserved repeats of regulated class II bacteriocins. Inclusion of the latter region in the reporter construct may result in the successful expression of *luxAB*.

OPSOMMING

Melksuurbakterieë speel 'n belangrike rol in die meeste gefermenteerde voedselsoorte deur die produksie van organoleptiese komponente en die verlenging van rakleeftyd. Van alle antimikrobiese komponente is bakteriosiene (ribosomaal gesintetiseerde peptiede) die beste bestudeer. Hierdie peptiede het gewoonlik 'n nou spektrum van antimikrobiese werking en is meestal aktief teen bakterieë in dieselfde ekologiese nis. Die feit dat bakteriosiene deur proteolitiese ensieme in die spysverteringskanaal vernietig word, verhoog die potensiële gebruik van bakteriosiene as preserveermiddels. Die ideaal sal wees om die konsentrasie van chemiese preserveermiddels soos swaweldioksied, nitrate en nitriete te verlaag of moontlik te vervang met bakteriosiene.

Bakteriosiene word in vier groepe op grond van hul strukturele en funksionele karaktereienskappe geklassifiseer. Plantarisien 423, geproduseer deur *Lactobacillus plantarum* 423, is hitte-stabiel, word deur 'n plasmied gekodeer, is relatief klein (3.5 kDa) en sorteer onder die klas IIa bakteriosiene. Die peptied is aktief oor 'n wye pH-reeks (pH 1.0-10.0) en inhibeer Gram-positiewe bakterieë, insluitend *Lactobacillus* spp., *Leuconostoc* spp., *Oenococcus oeni*, *Pediococcus* spp., *Enterococcus* spp., *Propionibacterium* spp. en patogene soos *Bacillus cereus*, *Clostridium* spp. en *Listeria monocytogenes*.

Produksie van bakteriosiene kan konstitutief plaasvind of kan gereguleer word deur 'n seldigtheids-afhanklike sisteem naamlik "quorum sensing". Plantarisien 423 word regdeur logaritmiese groei geproduseer, met geen verandering in produksievlakke wanneer die produserende stam in die teenwoordigheid van plantarisien 423 of *Listeria innocua* en *Lactobacillus sakei* gekweek word nie. Dit het gelei tot die hipotese dat plantarisien 423 moontlik konstitutief geproduseer word.

'n Verklikkersisteem bestaande uit 'n fusie van die plantarisien 423 promoter, P423, aan die *luxAB* gene is gekonstrueer en in die pendelplasmied pTRKH2 gekloneer. Die nuut-gekonstrueerde plasmied, pTAB4, is na 'n bakteriosien-negatiewe mutant van *L. plantarum* (stam 423 B$^-$) getransformeer. Ten spyte van etlike herhalings kon geen lusiferase-aktiwiteit opgespoor word nie en kon ook geen homologie in die RNA met die *luxAB* gene opgespoor word nie.

Dit is moontlik dat die area nodig vir uitdrukking van plantarisien 423 verder stroom-op van die –80 area, homoloog aan die –80 en –40 gekonserveerde herhalings van reguleerbare klas II bakteriosiene, gesetel is. Insluiting van laasgenoemde area in die verklikker-konstruk mag lei tot die suksesvolle uitdrukking van *luxAB*.

To enjoy your job, you must love the doing and not the secondary consequences.

The Fountainhead

by Ayn Rand

BIOGRAPHICAL SCETCH

Francisca Cohen (née Le Roux) was born in Bloemfontein on 4 June 1977. She matriculated at High School Jim Fouché, Bloemfontein, in 1995. In 1999, she obtained a B.Sc degree at the University of Stellenbosch, majoring in Microbiology and Biochemistry. She was awarded her B.Sc (Hons) in Microbiology *cum laude* in 2000.

Ms Cohen is married to Dr. H.-B. Cohen, a dentist, and resides in Bloemfontein.

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CONTENTS

1. INTRODUCTION

Throughout the history of humankind, lactic acid bacteria (LAB) have played a significant role, whether in the fermentation of food to acquire certain organoleptic qualities or to provide unique health benefits. The identification, characterisation of antimicrobial compounds and production optimisation of industrially important enzymes or metabolic compounds remains a primary focus of several laboratories involved in research related to food safety and quality. During fermentation, LAB produces a few possible antimicrobial substances such as lactic acid, acetic acid, hydrogen peroxide and bacteriocins, which may contribute to the natural preservation of a fermented product (Lindgren and Dobrogosz, 1990; Vandenbergh, 1993; Holzapfel et al., 1995; Stiles, 1996).

Enhanced awareness of food safety is leading towards the diminished use of antibiotics and chemical preservatives such as sulphur dioxide, benzoic acid, sorbic acid, nitrate and nitrite. Natural antimicrobial compounds such as bacteriocins produced by LAB have been consumed for centuries without adverse effects and are accepted as natural and safe biopreservatives (Soomro et al., 2002). Despite some negative reports (eg. Borch et al., 1996; Samelis et al., 2000), LAB still enjoys GRAS (generally recognised as safe) status.

Numerous bacteriocins of LAB have been described over the last two decades. Information on the structure, mode of action, optimisation and quantification of bacteriocins is essential and requires more information on their expression and regulation. Plantaricin 423, produced by *Lactobacillus plantarum* 423, has been characterised and the gene encoding its production has been cloned and expressed in *Saccharomyces cerevisiae* (Van Reenen et al., 1998, 2003). Little is known about the regulation of plantaricin 423. In this study, the bacterial luciferase genes (*luxAB*) from *Vibrio harveyi* were cloned downstream of the plantaricin 423 promoter. The newly constructed vector, pTAB4, was transformed to a bacteriocin-negative strain of *Lactobacillus plantarum* 423 (strain 423 B$^-$) which had been cured from plasmid pPLA4 that harbours the plantaricin 423 operon. Expression of the promoter was studied in the presence of externally added plantaricin 423 and with cells grown in the presence of *Listeria innocua* and *Lactobacillus sakei*, respectively.

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2. THE LACTIC ACID BACTERIA, WITH SPECIAL REFERENCE TO *LACTOBACILLUS PLANTARUM* AND ITS BACTERIOCINS

2.1 INTRODUCTION

Lactic acid bacteria (LAB) are Gram-positive, catalase-negative, non-sporulating and micro-aerophilic (Stiles and Holzapfel, 1997). In 1919 Orla-Jensen divided the Gram-positive, nonmotile and non-spore forming organisms that were capable of fermenting carbohydrates and alcohols to lactic acid, into seven genera (Table 1). The genera have since then been classified as *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Streptococcus*, *Vagococcus, Pediococcus*, *Leuconostoc*, *Weissella*, *Carnobacterium*, *Tertragenococcus* (Klein et al., 1998) and *Oenococcus* (Dicks et al., 1995).

The genus *Lactobacillus* contains by far the largest number of species compared to all other genera in the family *Lactobacillaceae*. Lactobacilli are strictly fermentative and require a complex combination of carbohydrates, amino acids, peptides, fatty acids, esters, salts and vitamins. Lactic acid is the major product from sugar fermentation. Several of the *Lactobacillus* spp. are heterogeneous (Axelsson, 1993), probably because they are found in a range of habitats, *viz.* plant material, manure, sewage, soil, fermented food, the intestine and mucosal membranes of the oral cavity and urogenital pathways (Hammes and Vogel, 1995).

The genus *Lactobacillus* has been divided into three groups (Table 2). Group I comprises the obligately homofermentative species, which lack the enzymes glucose 6-phosphate-dehydrogenase (G-6-PDH), and 6-phosphogluconate-dehydrogenase (6-P-GDH). Species of this group cannot ferment pentoses or gluconate (Pot et al, 1994), but ferment glucose almost exclusively to lactic acid.

Group II contains the facultatively heterofermentative species, metabolising hexoses to lactic acid. They produce gas from gluconate but not from glucose. In contrast to species from the obligately homofermentative group, members of this group contain both dehydrogenase enzymes (G-6-PDH and 6-P-GDH). Pentoses are fermented to lactic and acetic acid via an inducible pentose phosphoketolase pathway (Pot et al., 1994). The best-characterised species within this group are *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum* (Curk et al., 1996).

Group III contains the obligately heterofermentative species lacking FDP-aldolase. These species ferment hexoses to lactic acid, acetic acid and/or ethanol and carbon dioxide. Gas is produced from glucose. Lactic and acetic acids are produced from pentoses via the pentose phosphoketolase pathway (Pot et al., 1994).

Lactobacillus spp. have also been classified into three branches based on 16S rRNA sequence analyses, *viz.* the "full lacto group", the "casei group" and the "lactis group" (Yang and Woese, 1989; Hammes et al., 1999). The genus *Carnobacterium* is only distantly related to lactobacilli, whereas the leuconostocs and pediococci are closer related. These groupings are not according to carbohydrate fermentation reactions and obligately homofermentative and heterofermentative species have been grouped into several branches.

The phylogenetic tree in Fig. 1 illustrates the position of *Lactobacillaceae* and carnobacteria in relation to LAB and closely related genera. Further analyses of the 16S rRNA sequences have indicated seven distinct groups within the lactobacilli (Fig.2). The phylogenetic relatedness within each of these groups is shown in Figs. 3-9.

All species in the *L. buchneri*-group (Fig. 3) are obligately heterofermentative, except for *L. homohiochii*, which is facultatively heterofermentative (Kitahara et al., 1957). The *L. casei*-group (Fig. 4) comprises obligately homofermentative and facultatively heterofermentative species. The *L. delbrueckii*-group (Fig. 5) is consistent with the former *Lactobacillus delbrueckii*-group (Collins et al., 1991), previously also designated the "*L. acidophilus*-group" (Schleifer and Ludwig, 1995). This group is mainly dominated by obligately homofermentative species. The three subspecies of *Lactobacillus delbrueckii* cannot be effectively differentiated by rRNA sequence analysis. The *L. plantarum*-group (Fig. 6) comprises 12 *Lactobacillus* spp. from all three carbohydrate fermentation types. Very high 16S rRNA sequence similarity is observed within this group, namely *L. plantarum*, *L. pentosus* and *L. paraplantarum* (99.7–99.9%), *L. kimchii* and *L. paralimentarius* (99.9%), and *L. mindensis* and *L. farciminis* (99.9%). Species of the latter group share similar G+C contents.

Lactobacillus plantarum and *Lactobacillus pentosus* are difficult to distinguish by simple physiological tests (Kandler and Weiss, 1986). Restriction fragment length polymorphism (RFLP) of the 16S rRNA gene, used by Rodtong and Tannock (1993), is a reliable method for the typing of lactobacilli. Johansson et al. (1995), however, found random amplified polymorphic DNA (RAPD)-PCR a more appropriate method to differentiate strains of *L. plantarum*. Van Reenen and Dicks (1996) successfully differentiated strains of *L. pentosus* and *L. plantarum* with RAPD-PCR.

The *L. reuteri* group (Fig. 7) contains exclusively obligately heterofermentative species with large differences in DNA composition (36–54 mol% G+C). *L. durianis* and *L. vaccinostercus* share a 99.7% 16S rRNA sequence homology, but differ significantly in G+C content (36 and 43 mol%, respectively), as reported by Hammes and Hertel (2003). The latter variation in G+C content is unique to the *L. reuteri* group. The *L. sakei* group (Fig. 8) is the smallest subgroup and contains only facultatively heterofermentative lactobacilli. *L. curvatus* and *L. sakei* are both further divided into two subspecies each.

The *L. salivarius* group (Fig. 9) contains obligate homofermentative and facultative heterofermentative species. *L. animalis* and *L. murinus* share 99.7% 16S rRNA homology (Hammes and Hertel, 2003). The same has been reported for *L. cypricasei* and *L. acidipiscis* (99.7%). *Lactobacillus salivarius* contains two subspecies that cannot be differentiated by rRNA sequence analysis.

Lactic acid bacteria (LAB) provide unique organoleptic and sensorial qualities (Caplice and Fitzgerald, 1999) to food due to the production of stable and safe end products and are used as starter cultures in fermented milk, meat and vegetables (reviewed by De Vuyst and Vandamme, 1994). Many food associated LAB have also been known to be advantageous as live microbial health supplements to improve digestion and the absorption of nutrients, reduce diarrhoea and constipation, and stabilise the intestinal microflora (Gilliland, 1990; Goldin, 1998). Other claimed benefits are the relief from lactose-intolerance, reduction of serum cholesterol levels, stimulation of the immune system, hydrolysis of carcinogens, especially in the colon, and inhibition or exclusion of intestinal pathogens (Havenaar et al., 1992; Bernet et al., 1993; Salminen et al.,1996; Lankaputhra and Shah, 1998;).

Lactobacillus plantarum is one of the most widely distributed species. It is also of industrial importance, being used as starter culture in the production of fermented meat, vegetables, grass silage and certain dairy products (De Vuyst and Vandamme, 1994). Other predominant *Lactobacillus* spp. found in fermented meat products are *Lactobacillus sakei* and *Lactobacillus curvatus* (Hugas et al., 2003). Natural occurring bacteriocin-producing strains of *L. plantarum* have been isolated from many foods, including fermented cucumber (Daeschel et al., 1990; Atrih et al., 1993), olives (Jiménez-Díaz et al., 1993; Duran Quintana et al., 1999), fermented cereal dough (Gobbetti et al., 1994), pineapple (Kato et al., 1994) and grapefruit juice (Kelly et al., 1996), meat (Schillinger and Lücke, 1989; Garriga et al., 1993; Enan et al., 1996), milk (Rekhif et al., 1995) and cheese (González et al., 1994). Many bacteriocins from these strains have been characterised (Schillinger and Lücke, 1989;

Daeschel et al., 1990; Atrih et al., 1993; Jiménez-Díaz et al., 1993; González et al., 1994; Rekhif et al., 1995; Enan et al., 1996; Kelly et al., 1996; Van Reenen et al., 1998; 2003).

Enterococci, especially *Enterococcus faecium*, are also found in relatively high numbers in meat and may contribute to the fermentation process (Hugas et al., 2003). Enterococci have also been used as starter cultures in silage (Seale, 1986) and dairy products (Giraffa et al., 1997), and as probiotics (Fuller, 1989). *Lactobacillus delbrueckii* subsp. *bulgaricus* is widely used in the dairy industry as a starter culture for yoghurt, fermented milk, Italian cheese, and as a probiotic (Fuller, 1989; Goldin, 1998).

LAB and yeasts are the predominant microorganisms in most indigenous African fermented foods (Mugula et al. 2003). *Togwa*, a fermented maize-sorghum gruel, contains *Lactobacillus brevis*, *Lactobacillus cellobiosus*, *Lactobacillus fermentum, L. plantarum* and *Pediococcus pentosaceus* (Mugula et al., 2003).

Another example of LAB associated with African food is the fermentation of cassava dough to produce agbelima, a smooth-textured sour product mainly consumed in Ghana (Mante et al., 2003). Agbelima is cooked with fermented maize dough into a stiff porridge and then consumed with stewed meat. Fermentation is conducted by *Bacillus* spp., lactic acid bacteria, yeasts and, in some cases, moulds. *Lactobacillus plantarum*, *L. brevis*, and *Leuconostoc mesenteroides* lowers the pH and produce antimicrobial compounds active against some of the other microorganisms present (Mante et al., 2003).

Olive fermentation is mainly conducted by naturally occurring strains of *L. plantarum* (Duran Quintana et al., 1999) or *Lactobacillus casei* (Randazzo et al., 2004).

In certain cases, the same LAB that exerts beneficial effects in one product may have negative effects in another. Kimchi, a vegetable dish consumed in Korea, is fermented by naturally occurring LAB of which *Leuconostoc* spp. are the most dominant (Choi and Park, 2000). *Lactobacillus* spp., however, causes over-acidification of kimchi when fermented at high temperatures (Lee et al., 1992; So and Kim, 1995). To combat the problem, Nisin, a lantibiotic produced by *Lactococcus lactis*, is included as preservative (Choi and Park, 2000).

2.2 BACTERIOCINS OF *LACTOBACILLUS PLANTARUM*

2.2.1 CLASSIFICATION OF BACTERIOCINS FROM LACTIC ACID BACTERIA

Klaenhammer (1993) has originally defined four classes of bacteriocins. The first class contains the lantibiotics. They are small, heat-stable peptides containing lanthionine or other thioester amino acids. According to Moll et al. (1999), the lantibiotics can be further subdivided into type A and type B lantibiotics. The type A lantibiotics are elongated, cationic, pore forming peptides. In contrast, type B lantibiotics are compact peptides with globular structure, are immunologically active and inhibit enzymes (De Vuyst and Vandamme, 1994). Small, heat-stable membrane-active peptides that are hydrophobic and antilisterial comprises the second defined class of bacteriocins. The large, heat-labile, hydrophilic proteins represent the third class (Moll et al., 1999). The fourth class, defined by Klaenhammer (1993), comprised complex proteins that depended on a carbohydrate or lipid moiety to be fully functional. This class is no longer recognised, since these complexes may be artefacts of cell constituents or growth medium interacting with regular bacteriocins (Nes et al., 1996).

The best-studied bacteriocins belong to the Class II family. Class II bacteriocins are further subdivided into four groups. Class IIa are bacteriocins that share a YGNGV consensus sequence near their N-termini and are called anti-listerial peptides. Two-peptide bacteriocins are grouped under class IIb. Class IIc are *sec*-dependant bacteriocins. The *sec*-dependant secretion mechanism is, however, no longer used to differentiate non-lantibiotics (Eijsink et al., 2002). Class IId contain bacteriocins that are not defined into any of the latter groups (Nes et al., 1996).

Numerous plantaricins isolated from *L. plantarum* have been described in the literature of which plantaricins C19, C, S, T and 149 are a few examples belonging to class I or II.

Plantaricin C19 (Atrih et al., 1993) is a class IIa bacteriocin produced by *L. plantarum* C19, isolated from fermented cucumbers. Plantaricin C19 is produced during the logarithmic phase and is active against several Gram-positive pathogens, but seems to have no or very weak activity against lactic acid bacteria. The bacteriocin is about 3.5 kDa in size, stable at pH 2-6, heat stable, and is degraded by proteolytic enzymes. The sequence of the first six amino acids is KYYGNG (Atrih et al., 1993).

Plantaricin C (González et al., 1994) is a lantibiotic bacteriocin (Moll et al. 1999), produced by *L. plantarum* LL441 isolated from Cabrales cheese. It inhibits several strains of lactobacilli,

leuconostocs, pediococci and *Streptococcus thermophilus* and is produced during exponential growth, with maximum inhibitory activity at the beginning of the stationary growth phase. The peptide is heat-stable and bacteriocin activity is stable at acid and neutral pHs but not at alkaline pH. The sequence of the first amino acids of plantaricin C is KKTKKNXSGDI (González et al., 1994).

Plantaricin S, a class IIb bacteriocin (Moll et al., 1999) is produced by *L. plantarum* LPCO10 isolated from green olive fermentations (Jiménez-Díaz et al., 1993). Plantaricin S inhibits several species of *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Micrococcus* and *Propionibacterium*, as well as *Enterococcus faecalis* and *Clostridium tyrobutyricum.* The peptide is heat-resistant at pH 4.0, 6.0 and 7.0 and is produced during logarithmic growth. A second bacteriocin, plantaricin T, is secreted once the strain reaches the stationary phase. Plantaricin S is 2.5 kDa in size, with plantaricin T being slightly smaller. The genes encoding the latter two bacteriocins are not located on a plasmid.

L. plantarum NRIC, isolated from pineapple, produces the chromosomally encoded plantaricin-149 (Kato et al., 1994). It is active against strains of *L. plantarum*, *L. delbrueckii*, *L. helveticus*, *L. casei*, *L. fermentum*, *L. mesenteroides*, *P. acidilactici*, *Pediococcus cerevisiae*, *Enterococcus hirae* and *L. lactis*. The bacteriocin is heat-resistant, but sensitive to enzymes such as proteinase K, pronase, papain, pepsin, pancreatin and trypsin. The sequence of the N-terminal amino acids is YSLQMGATAIKQVKKLFKKKGG (Kato et al., 1994)

2.2.2 CLASS IIa BACTERIOCINS

The largest and most extensively studied subgroup of the class II bacteriocins belongs to class IIa. These bacteriocins have a strong inhibitory effect on *Listeria*, the presence of a YGNGVXC-consensus motif in their N-termini and a characteristic disulphide bridge in the same area. They have a similar mode of action, i.e. permeabilizing the membrane of target cells (Bhunia et al., 1991; Bruno and Montville, 1993; Chikindas et al., 1993; Abee, 1995; Jack et al., 1995; Kaiser and Montville, 1996; Chen et al., 1997). Pediocin PA-1, produced by *Pediococcus acidilactici* PAC1.0, is the best-characterised bacteriocin within this group (Hendersen et al., 1992; Marugg et al., 1992; Nieto et al., 1992). Other pediocin-like bacteriocins that have been thoroughly characterised include leucocin A-UAL 187 (Hastings et al., 1991), mecentericin Y105 (Héchard et al., 1992), sakacin P and curvacin A (Holck et al., 1992; Tichaczek et al. 1992). Many other bacteriocins have since been discovered and characterised (Table 3).

Bacillus coagulans strain I_4 produces coagulin (Table 3), which is encoded by an operon with almost exactly the same structure and DNA sequence as the operon encoding pediocin PA-1. Nonlantibiotic bacteriocins, commonly produced by lactic acid bacteria are probably also produced by other Gram-positive bacteria (Nes et al., 1996). Horizontal gene/operon transfer (via plasmids) between bacteria (Lawrence, 1999; De la Cruz and Davies, 2000) is most likely the reason for the presence of the coagulin operon in *Bacillus*. Coagulin is the first and only class II bacteriocin thus far characterised in the genus *Bacillus* (Le Marrec et al., 2000).

Class IIa bacteriocins share considerable sequence similarity. The presence of the YGNGV- and CXXXXCXV-motifs (Table 3) in their N-termini is very characteristic and may be part of a recognition sequence associated with a membrane-bound "receptor" (Fleury et al., 1996). Four genes are usually required for synthesis; processing, secretion and immunity of class IIa bacteriocins (Nes et al., 1996).

2.2.3 REGULATION OF CLASS II BACTERIOCIN PRODUCTION

Bacteriocins from lactic acid bacteria may be chromosomally encoded (Tichaczek et al., 1994; Aymerich et al., 1996), plasmid encoded (e.g. Marugg et al., 1992; Yildirim et al., 1999) or both where more than one bacteriocin is produced (Quadri et al., 1995). The synthesis and extracellular antimicrobial activity of bacteriocins generally depends on the presence of four genes clustered together in one operon, or into two operons controlled by an upstream promoter area (Nes et al., 1996). The structural gene encodes the prebacteriocin, called a precursor or prepeptide. These prepeptides consist of an N-terminal leader sequence and a C-terminal propeptide, which is cleaved from the N-terminal leader sequence to form a mature, antimicrobial peptide (Jack et al., 1995). An immunity gene encodes a protein that protects the producer organism from its own mature bacteriocin (Nes et al., 1996). Immunity genes may be next to, or downstream from, the bacteriocin structural gene and may even not be directly associated with the bacteriocin gene cluster (Eijsink et al., 1998). A third gene encodes a dual function ABC-transporter that facilitates the removal of the leader peptide from its substrate and assists in the transport of the substrate across the cytoplasmic membrane (Håvarstein et al., 1995). The fourth gene, an accessory gene, plays a role in bacteriocin secretion (Nes et al., 1996).

The production of bacteriocins can occur constitutively or could be controlled by a regulating system encoded by a set of genes associated with the gene clusters of bacteriocins. In several cases, the regulation of bacteriocin production depends on a so-called three-component regulatory mechanism, e.g. by the interaction of an induction peptide (IP), a

histidine protein kinase (HPK) and a response regulator (RR) (Nes et al., 1996; Dunny and Leonard, 1997; Kleerebezem et al., 1997). The gene encoding a peptide pheromone or inducing peptide (IP) is on the same transcriptional unit as HPK. All known mature peptide pheromones are derived form prepeptides containing a double-glycine leader, as in the case of most bacteriocins (Nes and Eijsink, 1999). Franz et al. (2000) has developed a simple method to identify the induction peptides, which unlike bacteriocins do not have antimicrobial activity (Kleerebezem, et al., 1997).

This three-component regulatory system acts as a quorum-sensing device, coupling co-ordinated bacteriocin production by a particular strain to its cell density (Saucier et al., 1995; Nes et al., 1996; Kleerebezem et al., 1997). Quorum sensing describes a regulation mechanism which depends on the cell density of a bacterial population. This enables the population to sense its own growth and thus the concentration of surrounding cells, enabling the producing strain to switch on the production of certain proteins at times when competition for nutrients become more severe. This phenomenon has been well described for Gram-negative bacteria where N-acyl homoserine lactones (AHLs) serve as diffusible communication molecules modulating cell-density-dependent phenotypes (Fuqua and Greenberg, 1998). Gram-positive bacteria have shown similar processes which are regulated by cell-density or growth-phase-dependent phenotypes.

The production of antimicrobial peptides (AMPs) by different species of Gram-positive bacteria is an example of a quorum-sensing system (Dunny and Leonard, 1997; Kleerebezem et al., 1997). The general assumption is that the IP (induction peptide) binds to its cognate HPK (histidine protein kinase) receptor, resulting in the activation of the RR (response regulator). The RR is then in part responsible for the transcriptional activation of all the operons involved in bacteriocin production, including the regulatory operon (Diep et al., 1996; Brurberg et al., 1997; Kleerebezem et al., 1997; Quadri et al., 1997; Nes and Eijsink, 1999). Usually when these AMPs act as signal molecules, they are larger precursor peptides which are posttranslationally processed and sometimes modified before secretion. The secreted peptide employed as a pheromone or signal is recognised by the input domain of a typical sensor component of the three-component signal-transduction system and is converted to a functional response by the organism. In cases where modified peptides serve as signals, the structural gene is linked in an operon to one or more genes exerting the export and posttranslational modification function (Fig. 10).

Two well-known examples of bacteriocin production by LAB that are regulated by a three-component systems are the *pln* regulon of *Lactobacillus plantarum* C11 (Diep et al., 1995;

1996) and the *spp* regulon of *Lactobacillus sakei* LTH673 (Eijsink et al., 1996; Hühne et al., 1996; Brurberg et al., 1997).

The *pln* operons have conserved regulatory elements in the promoter regions. These are two 9-bp direct repeats with the consensus sequence 5'-TACGTTAAT-3' in the –80 to –40 region separated by a 12 bp AT-rich spacer to which RRs bind and regulate gene expression (Diep et al., 1996; Nes et al., 1996; Nes and Eijsink, 1999). It has also been shown that the RR encoded by *sppR* in *Lactobacillus sakei* LTH673 binds to characteristic repeats found in the –80 and –40 regions of *spp* operons (Risøen et al., 2000).

2.2.4 PLANTARICIN 423

Plantaricin 423 is a small (approximately 3.5 kDa) plasmid-encoded peptide which is produced by *Lactobacillus plantarum* 423 (Van Reenen et al., 1998), a strain isolated from sorghum beer, a traditional South African alcoholic beverage. Plantaricin 423 inhibits a broad spectrum of bacteria, including *Lactobacillus* spp., *Leuconostoc* spp., *Oenococcus oeni*, *Pediococcus* spp., *Enterococcus* spp. and *Propionibacterium* spp. A few food spoilage and pathogenic bacteria, such as *Staphylococcus, Bacillus, Clostridium* and *Listeria* spp. are also inhibited (Van Reenen et al., 1998). Plantaricin 423 is heat stable and remains active after 30 min at 100°C (Van Reenen et al., 1998). These findings are similar to that reported for other plantaricins, e.g. plantaricin A (Daeschel, 1990), plantaricin C19 (Atrih et al., 1993), plantaricin S (Jiménez-Díaz et al., 1993), plantaricin-149 (Kato et al., 1994) and plantaricin SA6 (Rekhif et al., 1995). Plantaricin 423 is distinguished from other *Lactobacillus plantarum* bacteriocins in that it has strong bactericidal activity against *Listeria innocua* and *Listeria monocytogenes* (Van Reenen et al., 1998). Plantaricin 423 is also sensitive to proteolytic enzymes and is stable over a broad pH range (pH 1.0 – 10.0).

It is interesting to note that bacteriocins from different strains of *Lactobacillus plantarum* do not share sequence homology and classification. Examples of these bacteriocins are plantaricin C19, classified as a class IIa peptide (Atrih et al., 1993), plantaricin C which has been allocated to class I (González et al., 1994), plantaricins EF (Anderssen et al., 1998), JK (Anderssen et al., 1998) and S (Jiménez- Díaz et al., 1993) of class IIb and plantaricin-149, not yet included into a class (Kato et al., 1994).

The approximately 9 kb plasmid pPLA4, one of five plasmids from strain 423, contains a four open reading frame operon structure with high homology to the DNA encoding pediocin PA-1 (Van Reenen et al., 2003). Almost the identical DNA sequence of these genes has recently

been found in *Bacillus coagulans*, a nonlactic acid bacterium (See Table 3). The operon of *B. coagulans* producing the antilisterial bacteriocin, coagulin, has been found to be virtually exactly the same as the pediocin PA-1 operon produced by *Pediococcus acidilactici* (Marugg et al., 1992; Motlagh et al., 1992; Le Marrec et al., 2000).

The first open reading frame (ORF), *plaA*, is designated the structural gene of plantaricin 423 which encodes a 56-amino acid prepeptide consisting of a 37-amino acid mature peptide and a 19-amino acid N-terminal leader peptide. A putative immunity protein with protein sequence similarities to several other bacteriocin immunity proteins is encoded by the second ORF, *plaB*. The *plaC* and *plaD* ORFs have virtually identical DNA sequences to the *pedC* and *pedD* genes of pediocin PA-1/AcH from *Pediococcus acidilactici*, as well as *coaC* en *coaD* of the coagulin operon from *Bacillus coagulans* I_4.

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Table 1

Orla-Jensen's differentiation of lactic acid bacteria from Stiles and Holzapfel (1997)

Genus	Shape	Catalase	Nitrite reduction	Fermentation	Current genera
Betabacterium	Rod	-	-	Hetero-	*Lactobacillus* *Weissella*
Thermobacterium	Rod	-	-	Homo-	*Lactobacillus*
Streptobacterium	Rod	-	-	Homo-	*Lactobacillus* *Carnobacterium*
Streptococcus	Coccus	-	-	Homo-	*Streptococcus* *Enterococcus* *Lactococcus* *Vagococcus*
Betacoccus	Coccus	-	-	Hetero-	*Leuconostoc* *Oenococcus* *Weissella*
Microbacterium	Rod	+	+	Homo-	*Brochotrix* [b]
Tetracoccus	Coccus	+[a]	+	Homo-	*Pediococcus* *Tetragenococcus*

[a] In general pediococci are catalase negative, but some strains produce a pseudocatalase that results in false positives

[b] The genus *Brochotrix* is currently not included in the LAB group

Table 2

Classification of *Lactobacillus* species into three groups

Group	Description	Species	Reference
I	Obligately homofermentative	*L. acidophilus*, *L. amylophilus*, *L. amylovorus*, *L. aviarius* subsp. *araffinosus*, *L. aviarius* subsp. *aviarius*, *L. crispatus*, *L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *delbrueckii*, *L. delbrueckii* subsp. *lactis*, *L. farciminis*, *L. gallinarum*, *L. gasseri*, *L. helveticus*, *L. jensenii*, *L. johnsonii*, *L. kefiranofaciens*, *L. kefirgranum*, *L. mali*, *L. ruminis*, *L. salivarius* subsp. *salicinius*, *L. salivarius* subsp. *salivarius*, *L. sharpeae*, *L. fornicalis*	Stiles and Holzapfel (1997) Dicks *et al.* (2000)
II	Facultatively heterofermentative	*L. acetotolerans*, *L. agilis*, *L. alimentarius*, *L. bifermentans*, *L. casei*, *L. corynefomis* subsp. *corynefomis*, *L. coryneformis* subsp. *torquens*, *L. curvatus*, *L. graminis*, *L. hamsteri*, *L. homohiochii*, *L. intestinalis*, *L. murinus*, *L. paracasei* subsp. *paracasei*, *L. paracasei* subsp. *tolerans*, *L. paraplantarum*, *L. plantarum*, *L. pentosus*, *L. rhamnosus*, *L. sakei*	Stiles and Holzapfel (1997)
III	Obligately heterofermentative	*L. brevis*, *L. buchneri*, *L. collinoides*, *L. fermentum*, *L. bifermentans*, *L. fructivorans*, *L. fructosus*, *L. hilgardii*, *L. kefir*, *L. malefermentans*, *L. oris*, *L. panis*, *L. parabuchneri*, *L. parakefir*, *L. pontis*, *L. reuteri*, *L. sanfranciscensis*, *L. suebicus*, *L. vaccinostercus*, *L. vaginalis*	Stiles and Holzapfel (1997)

Table 3
Sequence alignment of mature class IIa bacteriocins.

Bacteriocin	Producer strain	Mature peptide with consensus sequence	Reference
Acidocin A	*Lactobacillus acidophilus*	KTYYGTNGVHCTKKSLWGKVRLKNVIPGTLCRKQSLPIKQDLKILLGWATGAFGKTFH	Kanatani et al. (1995)
Bacteriocin 31	*Enterococcus faecalis*	ATYYGNGLYCNKQKCWVDWNKASREIGKIIVNGWVQHGPWAPR	Tomita et al. (1996)
Bavaricin MN	*Lactobacillus sakei*	TKYYGNGVYCNSKKCWVDWGQAAGGIGQTVVXGWLGGAIPGK	Kaiser and Montville (1996)
Bifidocin B	*Bifidobacterium bifidum* NCFB 1454	KYYGNGVTCGLHDCRVDRGKATCGIINNGGMWGDIG	Yildirim et al. (1999)
Carnobacteriocin BM1	*Carnobacterium piscicola* LV17B	VNYGNGVSCSKTKCSVNWGQAFQERYTAGINSFVSGVASGAGSIGRRP	Quadri et al. (1994)
Carnobacteriocin B2	*Carnobacterium piscicola* LV17B	AISYGNGVYCNKEKCWVNKAENKQAITGIVIGGWASSLAGMGH	Quadri et al. (1994)
Coagulin	*Bacillus coagulans* I_4	KYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGTHKC	Hyronimus, et al. (1998); Le Marrec et al. (2000)
Curvacin A	*Lactobacillus curvatus* LTH1174	ARSYGNGVYCNNKKCWVNRGEATQSIIGGMISGWASGLAGM	Tichaczek et al. (1992)
Divercin V41	*Carnobacterium divergens* V41	TKYYGNGVYCNSKKCWVDWGQASGCIGQTVVGGWLGGAIPGKC	Métivier et al. (1998); Richard et al.(2003)
Enterocin A	*Enterococcus faecium* CTC492	TTHSGKYYGNGVYCTKNKCTVDWAKATTCIAGMSIGGFLGGAIPGKC	Aymerich et al. (1996)
Enterocin P	*Enterococcus faecium* P13	ATRSYGNGVYCNNSKCWVNWGEAKENIAGIVISGWASGLAGMGH	Cintas et al. (1997)
Lactococcin MMFII	*Lactococcus lactis* MMFII	TSYGNGVHCNKSKCWIDVSELETYKAGTVSNPKDILW	Ferchichi et al. (2001)
Leucocin A	*Leuconostoc gelidum*	KYYGNGVHCTKSGCGVNWGEAFSAGVHRLANGGNGFW	Hastings et al. (1991)
Leucocin C	*Leuconostoc mesenteroides* TA33a	KNYGNGVHCTKKGCSVDWGYAATNIANNSVMNGLTG	Papathanasopoulos et al. (1998)
Mecentericin Y105	*Leuconostoc mesenteroides*	KYYGNGVHCTKSGCSVNWGEAASAGIHRLANGGNGFW	Fleury et al. (1996)
Mundticin	*Enterococcus mundtii*	KYYGNGVSCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWSK	Bennik et al. (1998)
Pediocin PA-1	*Pediococcus acidilactici* PAC 1.0	KYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGNHKC	Nieto et al. (1992)
Piscicocin V1a	*Carnobacterium piscicola* V1	KYYGNGVSCNKNGCTVDWSKAIGIIGNNAAANLTTGGAAGWNKG	Bhugaloo-Vial et al. (1996)
Plantaricin C19	*Lactobacillus plantarum* C19	KYYGNGLSCSKKGCTVNWGQAFSCGVNRVATAGHGK	Atrih et al. (1993); Atrih et al. (2001)
Plantaricin 423	*Lactobacillus plantarum* 423	KYYGNGVTCGKHSCSVNWGQAFSCSVSHLANFGHGKC	Van Reenen et al. (1998)
Sakacin G	*Lactobacillus sake* 2512	KYYGNGVSCNSHGCSVNWGQAWTCGVNHLANGGHGVC	Simon et al. (2002)
Sakacin P	*Lactobacillus sake* LTH673	KYYGNGVHCGKHSCTVTWGTAIGNIGNNAAANWATGGNAGWNK	Tichaczek et al. (1992)

The consensus sequence marked in dark grey shows the residues conserved by more than 90%, and those marked in light grey by at least by 70%. Leucocin A is identical to leucocin B (Felix et al., 1994). Piscicocin V1a is identical to piscicolin126 (Jack et al., 1996), sakacin A is identical to curvacin A (Tichaczek et al., 1993), carnobacteriocin BM1 is identical to piscicocin V1b (Bhugaloo-Vial et al., 1996), and pediocin PA-1 is identical to pediocin AcH (Motlagh et al., 1994) and pediocin SJ-1 (Schved et al., 1994). Bavaricin A (Larson et al., 1993) is probably identical to sakacin P.

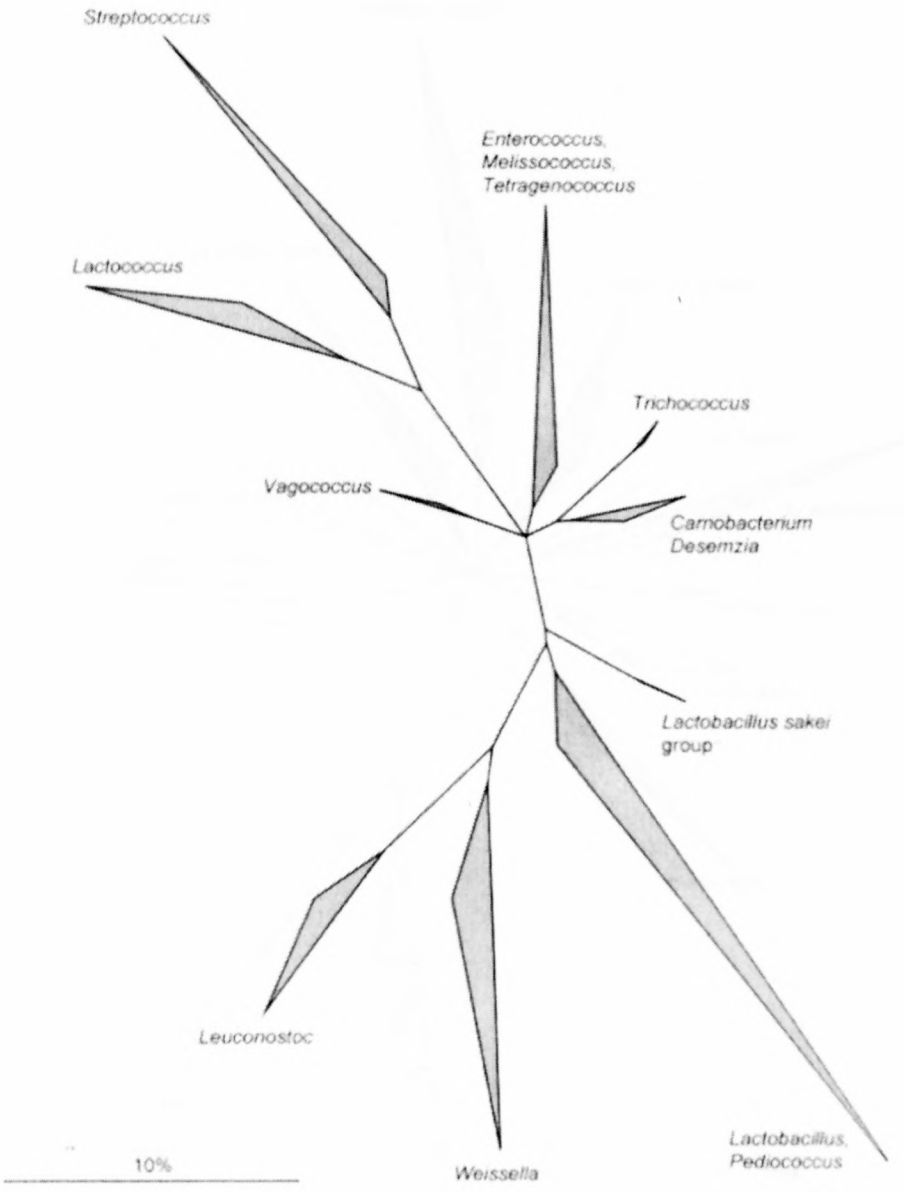

Fig. 1. A phylogenetic tree that illustrates the position of *Lactobacillaceae* and carnobacteria in relation to lactic acid bacteria (LAB) and closely related genera. The consensus tree is based on maximum parsimony analyses of at least 90% completed 16S rRNA sequences of Gram-positive bacteria. The topology was evaluated and corrected according to the results of distance matrix and maximum likelihood analyses with various data sets. Alignment positions that share identical residues in at least 50% of all sequences of the depicted genera were considered. Multifurcations indicate that a common branching order could not be significantly determined or was not supported when performing different alternative treeing approaches. The bar indicates 10% estimated sequence divergence. From Hammes and Hertel (2003).

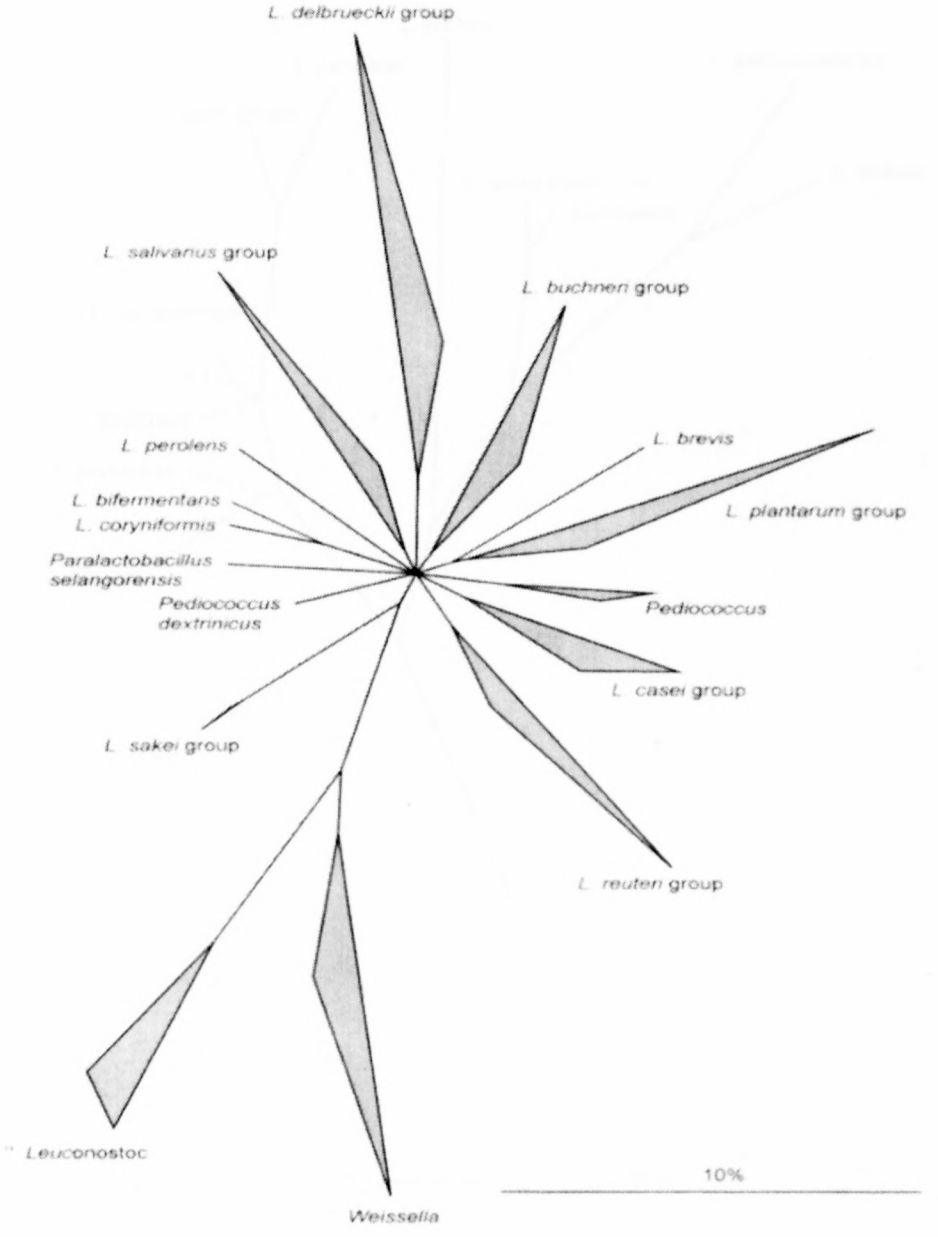

Fig. 2. Phylogenetic relatedness among genera of the family *Lactobacillaceae*. Criteria for the consensus tree are the same as for the tree in Fig. 1. The bar indicates 10% estimated sequence divergence. From Hammes and Hertel (2003).

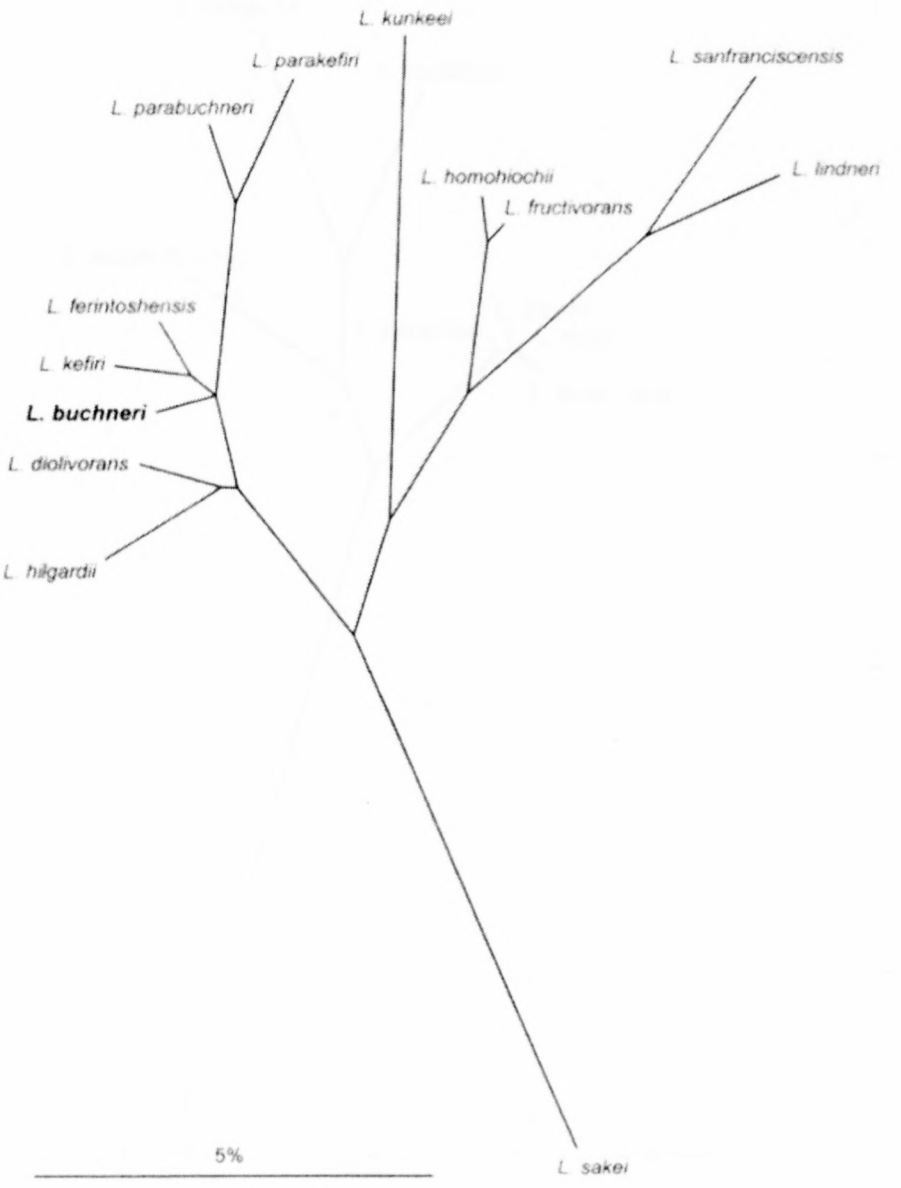

Fig. 3. Maximum likelihood tree, reflecting the relatedness among members of the *L. buchneri*-group. The tree is based on the analyses of at least 90% completed 16S rRNA sequences of the Lactobacillaceae. Alignment positions that share identical residues in at least 50% of all sequences of Lactobacillaceae were considered. The positioning of *L. parabuchneri* is based on partial sequence data and may be subject to changes. The bars indicate 5% estimated sequence divergence. From Hammes and Hertel (2003).

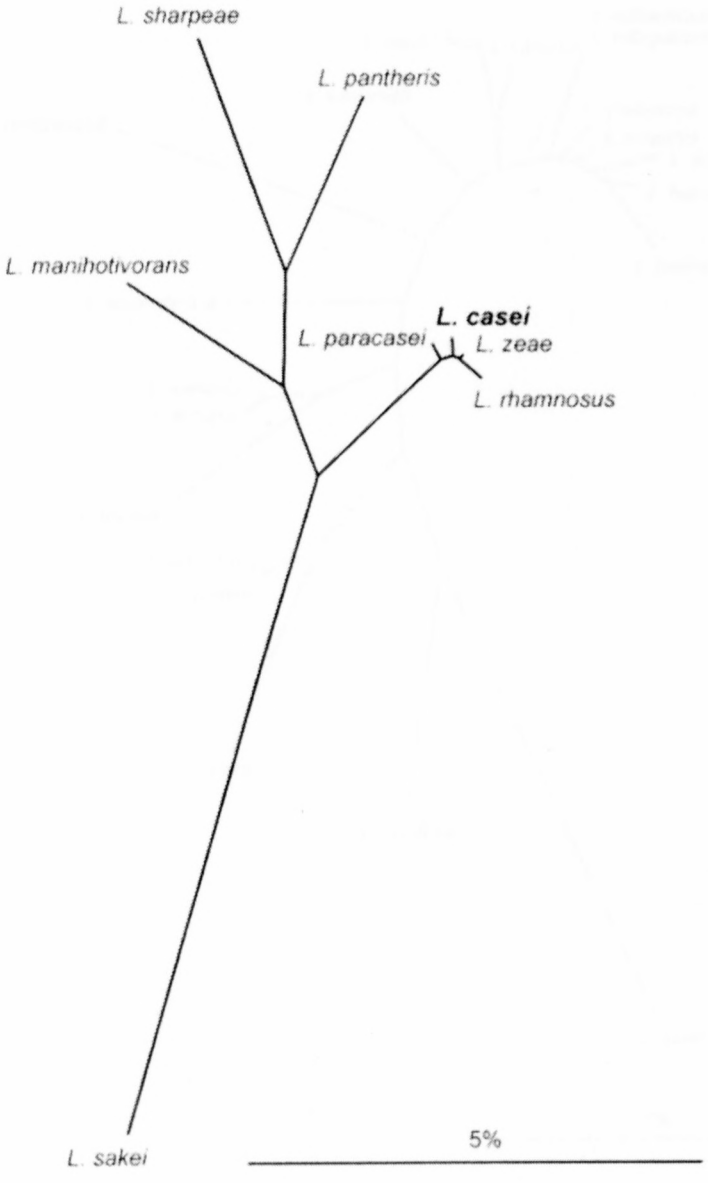

Fig. 4. Maximum likelihood tree, reflecting the relatedness among members of the *L. casei*-group. Sequences and sequence positions were selected as described for Fig. 3. The bars indicate 5% estimated sequence divergence. From Hammes and Hertel (2003).

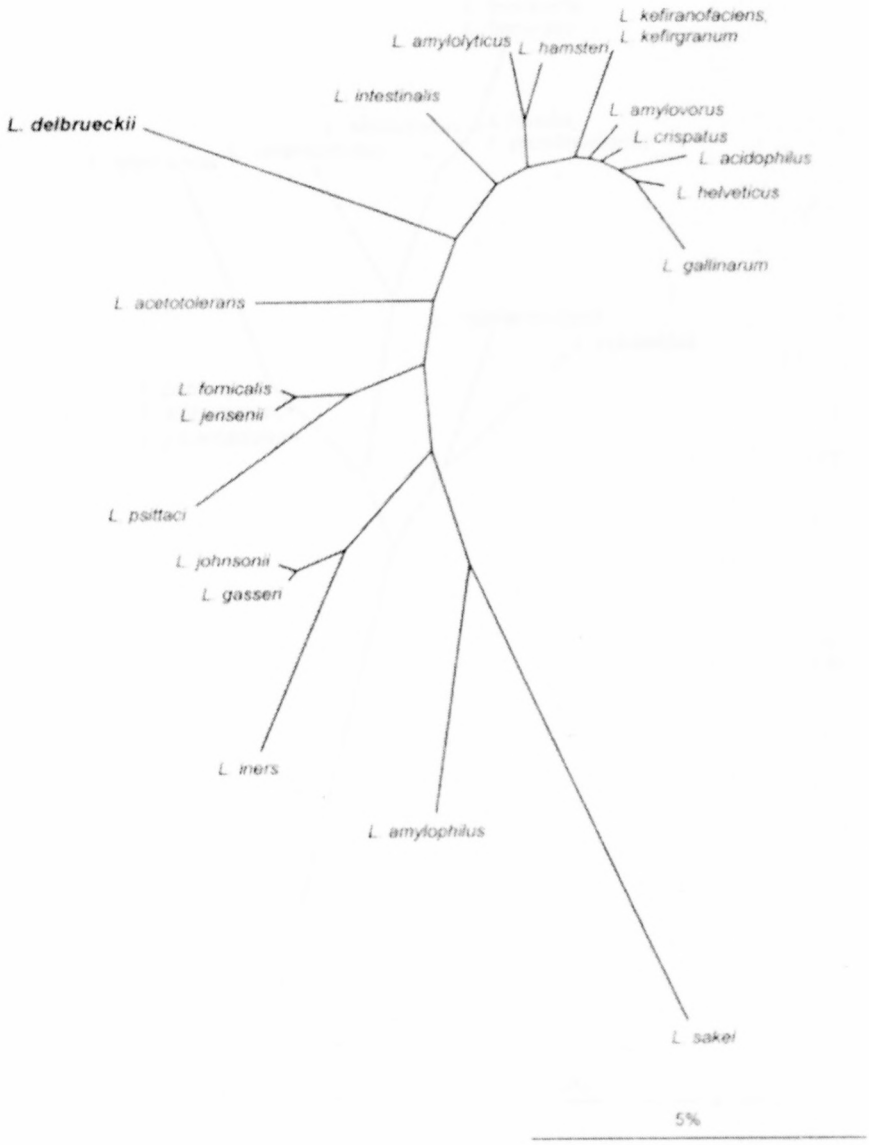

Fig. 5. Maximum likelihood tree, reflecting the relationship among members of the *L. delbrueckii*-group. Sequences and sequence positions were selected as described for Fig. 3. The bars indicate 5% estimated sequence divergence. From Hammes and Hertel (2003).

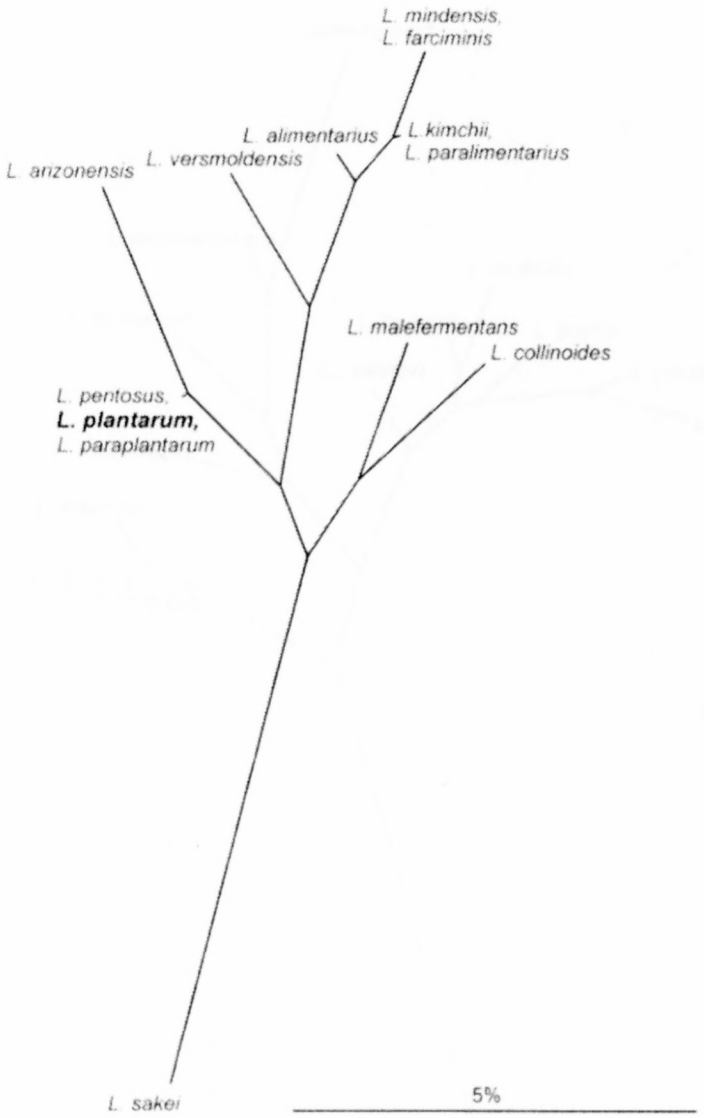

Fig. 6. Maximum likelihood tree, reflecting the relationship among members of the *L. plantarum* group. Sequences and sequence positions were selected as described for Fig. 3. The bars indicate 5% estimated sequence divergence. From Hammes and Hertel (2003).

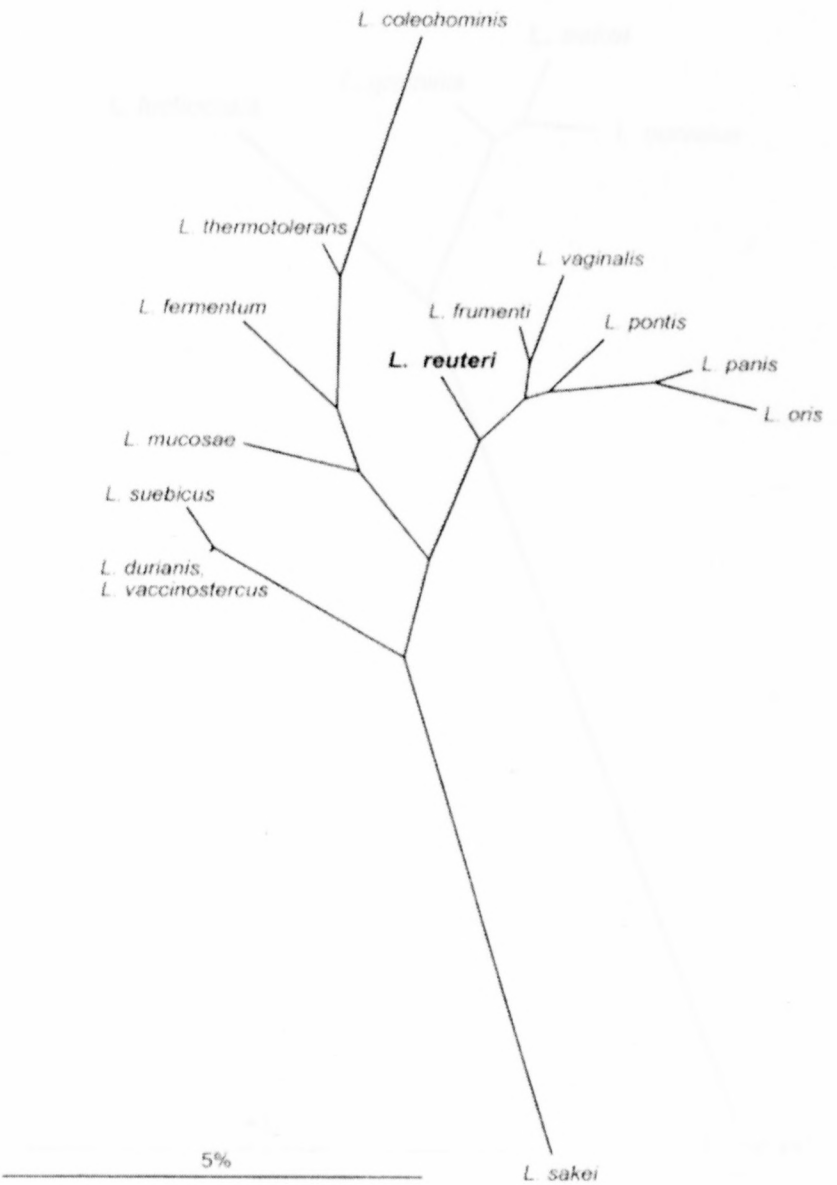

Fig. 7. Maximum likelihood tree, reflecting the relationship among members of the *L. reuteri* group. Sequences and sequence positions were selected as described for Fig. 3. The bars indicate 5% estimated sequence divergence. From Hammes and Hertel (2003).

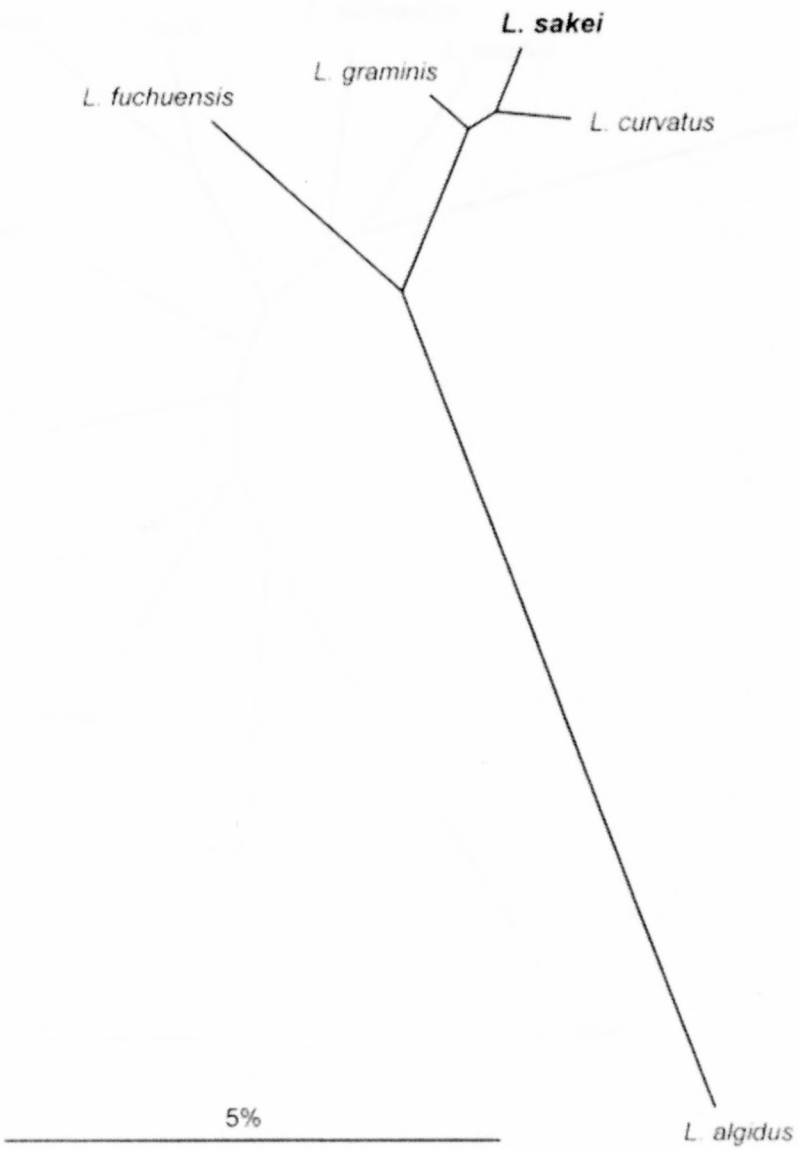

Fig. 8. Maximum likelihood tree, reflecting the relationship among members of the *L. sakei* group. Sequences and sequence positions were selected as described for Fig. 3. The positioning of *L. fuchuensis* is based on partial sequence data and may be subject to changes. The bars indicate 5% estimated sequence divergence. From Hammes and Hertel (2003).

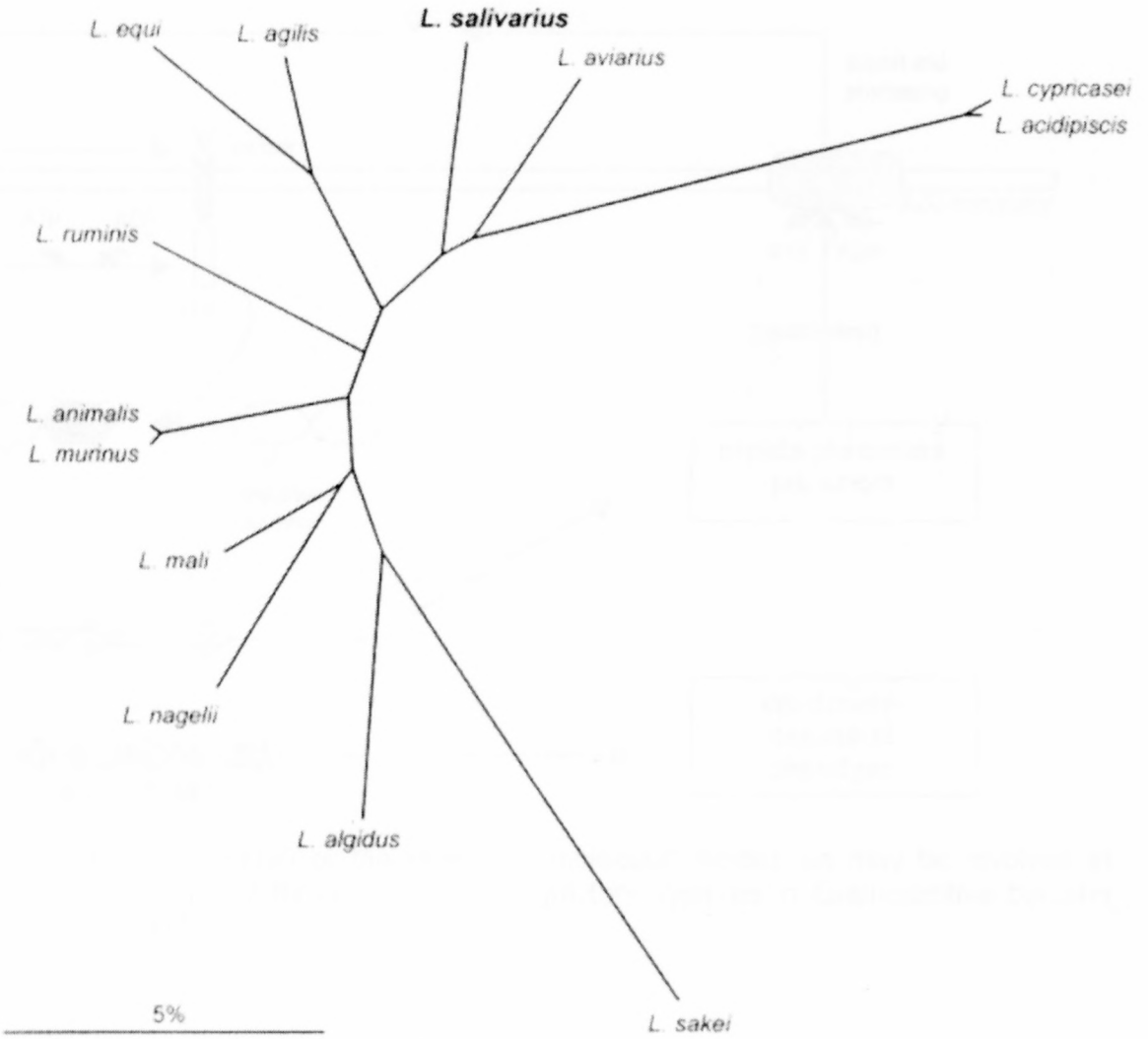

Fig. 9. Maximum likelihood tree, reflecting the relationship among members of the *L. salivarius* group. Sequences and sequence positions were selected as described for Fig. 3. The positioning of *L. acidipiscis* and *L. nagelii* is based on partial sequence data and may be subject to changes. The bars indicate 5% estimated sequence divergence. From Hammes and Hertel (2003).

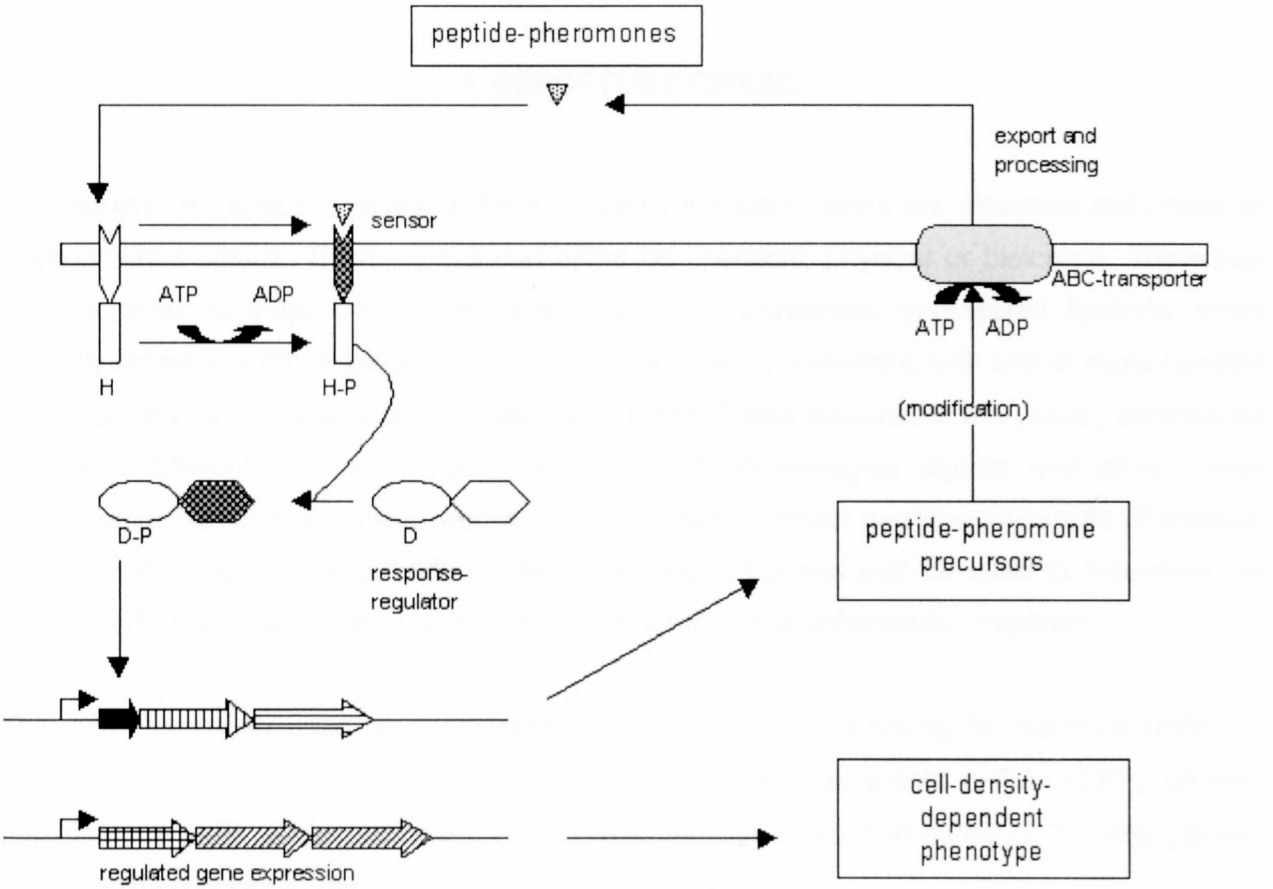

Fig. 10. Schematic presentation of the model for molecular modes, as may be involved in quorum sensing mediated by three-component regulatory systems in Gram-positive bacteria (Kleerebezem et al.,1997).

3. REPORTER SYSTEMS

Bioreporters are effective research tools to gain information about the response cells have to environmental stimuli. These stimuli can either be chemical, physical or biological. There has been a rapid development of biosensors based on genetically engineered bacteria which typically combine a promoter-operator, acting as the sensing element, with one or more reporter genes coding for easily detectable proteins (Fig. 11). These biosensors are usually engineered to detect different parameters such as toxicity, DNA-damaging agents and other stress components. Not only are these reporter systems used to detect negative influences of different organic and inorganic compounds on the environment, but can also be used to determine the regulation behind production of a certain protein, for instance antimicrobial peptides.

The most commonly used reporter genes in bacteria are those coding for bacterial luciferase (lux), insect luciferase (luc), ß-galactosidase (LacZ), green fluorescent protein (GFP), alkaline phosphatase (AP), ß-glucoronidase (GUS), β-lactamase (bla), and catechol 2,3-dioxygenase (C23O). The reporter systems are summarised in Table 4.

When selecting a transcription reporter system several criteria must be taken into account. The protein reporter must be foreign or absent from the host, or easily distinguished from its own protein products. A simple, rapid, sensitive and cost-effective assay should be available to detect the gene expression product. The assay should have a broad linear range to facilitate both small and large changes in the activity of the reporter protein. Expression of the gene may not alter the physiology of the recipient cell or organism.

3.1 BACTERIAL LUCIFERASE (*lux*AB)

Luciferase is a generic name for an enzyme that catalyses a light emitting reaction within a biological organism. Bacterial luciferase is an enzyme that is encoded by the genes *lux*A and *lux*B of the *lux* operon mainly from marine *Vibrio* species. This enzyme catalyses the oxidation of a reduced flavin mononucleotide and a long chain aldehyde to flavin mononucleotide and carboxylic acid (Baldwin et al., 1975). Light is emitted at 490 nm, with a quantum yield of ~ 0.1. The enzymes involved in synthesis of the aldehyde are also coded by genes on the *lux* operon, namely *lux*CDE (Fig. 12). In constructs where only *lux*AB is present the aldehyde needs to be added to the reaction. Luciferases most commonly used are those isolated from *Vibrio harveyi*,

Photobacterium fischeri, and *Photorhabdus luminescens*. Bacterial bioluminescence provides a real-time, non-invasive reporter to determine gene expression. It is a sensitive marker and can be used as a measure of intracellular biochemical function and may thus be used to study cellular viability.

3.2 FIREFLY LUCIFERASE (luc)

The first use of firefly luciferase, encoded by the *luc*-gene, was as a reporter of promoter activity. Detection of ATP made firefly luciferase a popular detection method (Gould and Subramani, 1988). The enzyme luciferase (E) catalyses the oxygen-dependent bioluminescent reaction in which energy is transferred from ATP to the added substrate, D-luciferin (LH_2). This reaction gives yield to oxyluciferin (P), AMP, carbon dioxide and light ($h\nu$) at 560 nm (Fig. 13). A quantum yield of 0.88 is obtained, which is the highest reported for bioluminescent reactions. Luciferin is slowly transported through cell membranes in contrast to the aldehyde substrate used in bacterial bioluminescent reactions (Olsson et al., 1988).

3.3 ß-GALACTOSIDASE (lacZ)

Henrissat and co-workers (Gilkes et al. 1991; Henrissat, 1991; Henrissat and Bairoch, 1993; Durand et al., 1997;) classified enzymes generally referred to as glycosidases into families based on amino acid sequence similarities and hydrophobic cluster analyses. Glycosidases are enzymes that hydrolyse the bond(s) between two or more carbohydrates or the bond between a carbohydrate moiety or a non-carbohydrate moiety. Different combinations of oligosaccharide isomers give rise to a wide variety of hydrolysing enzymes. Glycosidases were originally grouped together, based on the ability to hydrolyse similar substrates; for example enzymes that hydrolyse lactose or related chromogens like ONPG (*o*-nitrophenyl-β-D-galactopyranoside) and X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside), were classified as β-galactosidases. Perhaps the most-studied and useful β-galactosidases are the *lacZ*-encoded enzyme of *Escherichia coli*. Currently, many other important galactosidases from phylogenetically diverse organisms are being discovered.

The activity of the enzyme can be detected in individual cells by using chromogenic (Poulsen et al., 1997), fluorigenic (Rowland et al., 1999), immunofluorescence (Kang et al., 1999; Koch et al., 2001), electrochemical or chemiluminescent substrates (Lewis et al., 1998). This reporter

gene functions in prokaryotic and eukaryotic cells with a high turnover rate, which allows sensitive measurements. Less than 1 amol can be detected, depending upon the substrates used (Köhler et al., 2000).

The limited use of reporter genes such as *lacZ* and *lacG* in LAB is a result of the fact that most LAB produce lactose hydrolases as an important part of their metabolism (Hickey et al., 1986; De Vos and Simons, 1988). It is important to develop a promoter-probe vector which allows direct detection of promoter activity on different substrates and in a wide range of LAB.

3.4 GREEN FLUORESCENT PROTEIN (GFP)

Marine invertebrates are commonly known for their expression of luminescence. Many cnidarians and ctenophores (types of invertebrates) emit light when disturbed. The green fluorescent protein (GFP) is responsible for the green light emitted by the jellyfish *Aequorea victoria* (Morin and Hastings, 1971). GFP is a 238 amino acid protein, which spontaneously fluoresces and has found many applications in molecular biology. For *in situ* expression an intrinsically fluorescent protein can be fused to another protein and used to report on protein expression and transport within cells.

A few characteristics of GFP make it more advantageous as a reporter system over others. Formation of the fluorescent chromophore is not dependent upon species, making it possible to be expressed in a wide range of eukaryotes, e.g., yeasts (Niedenthal et al., 1996), insects (Wang and Hazelrigg, 1994), plants (Casper and Holt, 1996), fish (Moss et al., 1996) and mammals (Ludin et al., 1996). GFP expression is also very sensitive in a broad variety of bacteria (Chalfie et al., 1994; Christensen et al., 1996). Recently, GFP has also been used as a marker for gene expression and as a fluorescent tag for protein translocation in different mycobacteria (Dhandayuthapani et al, 1995; Kremer et al., 1995).

The fluorescent action of GFP does not require additional co-factors or substrates for fluorescence, but only oxygen (Chalfie et al., 1994). Studies on the localisation of protein and DNA molecules in prokaryotes have also been made easier with the use of *Aequorea* GFP (Philips, 2001).

As many other reporter systems, GFP also has a few limitations. GFP is stable at high temperature (T_m = 78°C) and is also stable for several hours (Tombolini et al., 1997). GFP fluorescence might thus only be suitable for measuring the induction of gene expression and less valuable for transient (real-time) gene expression studies. Variant forms of *gfp* with different half-lives have since been constructed (Andersen et al., 1998). The mutant proteins described by Cormack et al. (1996) have a clear advantage over wildtype GFP in studies where excitation in the range of 489 nm is used. Fluorescence from the mutant proteins is detectable within 8 minutes after induction with bacteria growing in log phase at 37°C, whereas the wildtype GFP is only detectable after one to two hours after induction under the same conditions. The mutant protein, Gfpmut3, is approximately 20 times more fluorescent than wild-type GFP when excited at 488 nm, and it is only weakly excited by UV light (Cormack et al., 1996). When pairing these mutants with complimentary GFP mutants, the reduced absorption peak as a result may allow efficient spectral separation of two simultaneously expressed fluorescent tags (Heim et al., 1994).

3.5 ALKALINE PHOSPHATASE (AP) / SECRETED HUMAN PLACENTAL ALKALINE PHOSPHATASE (SEAP)

The use of secreted human placental alkaline phosphatase (SEAP) as a reporter gene is normally associated with eukaryotic systems, e.g. to identify proteins that are exported by oral (Ward et al., 2001) and other pathogens (Lim et al., 1995). The AP gene product (PhoA) derived from *E. coli* has also been successfully expressed in Gram-negative bacteria (Manoil, 2000). An alkaline phosphatase (PhoZ), derived from *Enterococcus faecalis* (Lee et al., 1999), is used as a reporter in Gram-positive bacteria and is also dependent on its export from the cytoplasm.

Alkaline phosphatase (AP) and SEAP are orthophosphoric mono-ester phosphohydrolases with an alkaline pH optimum (Köhler et al., 2000). The enzyme is secreted from the cell through alteration of the coding region for the membrane localisation domain of the normally membrane-bound placental alkaline phosphatase. SEAP detection can therefore be performed with intact cells in a culture medium (Bronstein et al., 1994). AP (encoded by *phoA*) and derived from *E. coli* is activated once transported across the cell membrane into the periplasmic space. The protein is then put under control of the *phoA* promoter area, together with the translational initiation site and its signal peptide.

These are stable enzymes characterised by a high turnover rate. The presence of AP and SEAP are recorded colorimetrically, but requires extremely sensitive fluorescent and luminometric detectors. The sensitivity (0.05-0.01 amol) is similar to, and in some cases better than, that recorded with luciferase (Köhler et al., 2000).

Chemiluminescence also offers highly sensitive detection of SEAP. With the use of a chemiluminescent alkaline phosphatase substrate (CSPD®) as little as 3 fg of the enzyme can be detected (Bronstein et al., 1994). The CSPD substrate is dephosphorylated by alkaline phosphatase (AP). The dioxetane anion which forms is unstable and emits light as it decomposes. Maximum activity is recorded at 477 nm. Furthermore, SEAP is heat stable and resistant to L-homoarginine. This allows specific inhibition of an endogenous enzyme (Bronstein et al. 1994).

3.6 ß-GLUCURONIDASE (GUS)

β-Glucuronidase (GUS), encoded by *gusA*, is a promoterless gene originally identified in *Escherichia coli* (Jefferson et al., 1986). The gene has been expressed by a number of bacterial species (Roberts, et al., 1989; Sharma and Singer, 1990; Feldhaus et al., 1991; Bauer et al., 1993; Tsomlexoglou et al., 2003) and animals (Jefferson, et al., 1987b), but is mainly used in gene expression studies of plants (Jefferson, et al., 1987a). Risøen and co-workers used the *gusA*-gene in studies on the regulation of bacteriocins (Risøen et al., 2001).

The robustness of the enzyme and the simplicity of the GUS assay render this reporter gene in favour above other reporter systems. GUS is a stable gene product which can easily be detected using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc), also in *in situ* studies. The final cleavage product of X-gluc (dichloro-dibromo-indigo) is insoluble in aqueous environments (Pearson et al., 1961; Stomp, 1992), which allows localisation within tissue cells. Very low levels of GUS activity can be detected by a fluorescence assay (Jefferson, 1987a). Another advantage of GUS as a reporter enzyme is its size. Compared to β-GAL, GUS is a smaller molecule (68.2 kDa) which reduces the risk of artefacts in targeting studies (Schmitz et al., 1990).

3.7 β-LACTAMASE (bla)

β-lactamase can be useful in some bacteria, specifically those that are penicillin-resistant. β-lactamase produced by several bacteria is the enzyme that cleaves β-lactame rings in antibiotics, such as penicillin. (Köhler et al., 2000). The use of substrates cleaving fluorescent or colorimetric products allows the detection of this enzyme.

3.8 CHLORAMPHENICOL ACETYLTRANSFERASE (CAT)

One of the very first coding regions that have been used in reporter gene assays is chloramphenicol acetyltransferase or CAT (Alam and Cook, 1990). This reporter enzyme has the disadvantage that only strong promoters can be used since selection for the enzyme production requires very high expression of the *cat* gene (Platteeuw et al., 1994). Assays for CAT expression is also very laborious (extractions and thin-layer chromatography followed by radioisotopic detection) and above all not very sensitive (Platteeuw et al., 1994).

3.9 BIOLUMINESCENCE

Bioluminescence has captivated the interest of scientists for thousands of years. The first description of this phenomenon was made in 384-322 BC by Aristotle whose work was enlarged upon in the first century AD by the Roman scholar Pliny the Elder (23-79 AD) (Anonymous, 1982). One of the first "modern" descriptions of bioluminescence was made by the British scientist Robert Boyle (1627-1691) (Anonymous, 1982). In his studies (1668), Boyle discovered that luminescence is generated virtually without heat and that the intensity of light is dependent on the amount of air (oxygen) present.

Light is the result of vibrating electric and magnetic fields. "Visible light" refers to the portion of the electromagnetic spectrum observed by the naked eye. Bioluminescence is the name given to "an enzymatically catalysed chemiluminescence or a chemical reaction that emits light" (Hastings, 1998).

In some of the earliest studies it was claimed that whole phyla of animals were luminescent, but was later discovered that non-luminous organisms live in symbiosis with luminous bacteria (unknown author, 1982). Luminescent bacteria are of the most abundant and widely distributed

of the light-emitting organisms and are found in marine, fresh water and terrestrial environments. Most commonly these bacteria occur as free-living species in the ocean, as saprophytes living on dead sea animals, as intestinal symbionts of marine fish, as parasites in crustacea and insects, and as light organ symbionts in fish and squid (Hastings, 1986).

The energy required to emit light is significant (Hastings and Presswood, 1978). Light emission can be categorised in three classes, *viz.* offence, defence and communication (Meighen, 1991). These advantageous characteristics are provided to the host by symbiont luminescent bacteria. In turn the host provides a safe environment and stable source of nutrients to the luminescent microorganisms. As offence, light may be used as lure to attract prey. Light may also be used in defence to startle or frighten predators, or may provide camouflage. Communication occurs during courtship and mating displays.

Bioluminescent bacteria emit light of a brilliant blue-green colour (490 nm), although different bacteria can emit light of slightly different colour (Hastings et al., 1969), probably due to the difference in structure and function of the different components necessary for chemiluminescent reaction. It may thus be assumed that these light-emitting systems originated and evolved independently from each other.

3.10 BACTERIAL BIOLUMINESCENCE AND ITS GENETICS

The genetic system within *Vibrio harveyi* required for luminescence is complex. Genes are required for the most important components in bioluminescence and is designated luciferases (catalysing enzymes) and luciferins (their high-energy substrates) (Fisher et al., 1996). Twenty-one different genes from at least three genera of luminescent bacteria are involved in the *lux* system and have been designated as *lux* genes. Only five of these genes have been found in all luminescent bacteria (Meighen, 1991; 1993). The most extensively investigated luminescent bacteria are *Vibrio harveyi, Photobacterium (Vibrio) fischeri, Photobacterium phosphoreum* and *Photobacterium leiognathi.* The terrestrial bacterium, *Photorhabdus (Xenorhabdus) luminescens,* is the third genus of luminescent bacteria that is almost fully investigated (Meighen, 1994). In 1993 *Vibrio fischeri* and *Xenorhabdus luminescens* have been reclassified as *Photobacterium fischeri* (Kita-Tsukamoto et al., 1993) and *Photorhabdus luminescens* (Boemare et al., 1993), respectively. These species are consequently referred to as *V. fischeri* and *X. luminescens* in many publications prior to 1993.

Relatively simple compounds, such as reduced flavin mononucleotide ($FMNH_2$), oxygen and a fatty aldehyde (RCHO), are closely related to the basic metabolites of the cell.

$$FMNH_2 + O_2 + RCHO \rightarrow FMN + H_2O + RCOOH + h\upsilon_{490nm}$$

The common structural genes (Fig. 12) involved in bioluminescence are organised in an operon (*lux*ABCDE), together with several other genes involved in the regulation of bioluminescence.

3.11 BIOLUMINESCENCE OF *VIBRIO HARVEYI*

The *V. harveyi* Lux is encoded by *luxA* and *luxB* cistrons, which are part of the *lux* operon. These genes encode the α- and β-subunits of a heterodimeric oxidase called luciferase (Baldwin et al., 1984). This flavin monooxygenase's α- (≈40 kDa) and β-subunits (≈37 kDa) are 355 and 324 residues, respectively, and together catalyzes the oxidation of a long-chain aldehyde which releases energy in the form of visible blue-green light (490 nm) (Fisher et al., 1996). This reaction provides a very sensitive detection of enzyme reactions (Ziegler and Baldwin, 1981).

It is suggested that the *luxA* and *luxB* have evolved from a single ancestral gene, since the first four amino acid residues of the α- and β-subunits are identical for both *Vibrio harveyi* and *Photobacterium fischeri* and is supported by the findings of Foran and Brown (1988). Luciferase is only active as the αβ-dimer and the enzyme's active site is situated on the α-subunit. Although the precise role of the β-subunit is not clear, its presence is essential for the activity of luciferase (Cohn et al., 1985).

The *lux* operon also comprises three structural genes, *luxC*, *luxD* and *luxE* which encode the subunits of the fatty acid reductase. The *luxR* and *luxI* genes are involved in the regulation of *lux* expression and has been reviewed extensively (Meighen, 1988; 1991; 1993; 1994). A few additional genes (*luxF*, *luxG*, *luxH* and *luxY*) have been identified which form part of the *lux* operons and have been reviewed by Meighen (1991; 1993; 1994).

Most microorganisms have the ability to produce $FMNH_2$. For a foreign bacterium to become bioluminescent, the only requirement would be the transfer of luciferase genes and the addition of a fatty acid reductase. This means that any bacterium expressing only the luciferase

component of the *lux* operon can emit light with the exogenous provision of a long-chain aldehyde such as decanal. Therefore, if the *luxA* and *luxB* genes are expressed and the luciferase is stable, a bioluminescent phenotype may be produced in any prokaryotic organism (Stewart and Williams, 1992).

The unique features of the bacterial bioluminescent reaction provide significant advantages over other reporter genes. A single turnover of the enzyme is directly proportional to the amount of functional luciferase over an unlimited range. This is in great contrast with many enzyme assays in which the initial rate of product formation must be calculated taking into account the restricted conditions in time and the amount of enzyme used. The sensitivity of the measurement and conditions under which the assay is conducted means that only a few seconds are required to obtain a reading. As little as one picogram of luciferase can be detected with a scintillation counter. Costs of materials (aldehyde, $FMNH_2$) are minimal. The most appealing factor of bacterial luciferase assays is the fact that the assay can be performed without destroying the host cell. In contrast with other widely used reporter enzymes such as β-galactosidase and β-glucoronidase, this opens new possibilities to study complex genetic interactions *in vivo*.

The luciferase genes have been exploited in many different molecular biology applications, which includes the development of new plasmid vectors (Farinha and Kropinski, 1990; Sévigny and Gossard, 1990) and as a molecular reporter in gene expression studies (Baldwin et al., 1984; Schauer, 1988; Karp and Meyer, 1989; Guzzo and Dubow, 1991; Wolk et al., 1991). The cloning of the *lux* genes combined with the ability to transfer these genes into almost any biological organism has greatly increased the number of applications which provides opportunities to use light emission for the detection of almost any metabolic process or substance.

One example is the detection of antibacterial activities of lactic acid bacteria against luminescent strains of *Enterococcus faecalis* and *Listeria innocua* (Simon et al., 2001). It has also been used to detect *Listeria monocytogenes* in contaminated foods (Loessner et al., 1997) and has been helpful in monitoring the physiology of bacteria (in this case *Lactococcus lactis*) from the digestive tract (Corthier et al., 1998). Light emitting *Lactobacillus* species have been used to test for antibiotics and/or other compounds in milk that can cause disruption of cellular metabolism and the luminescent reaction (Ahmad and Stewart, 1990). Table 5 shows a summary of some of the Gram-positive bacteria that have been successfully transformed with bacterial luciferase.

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Table 4

Reporter genes and proteins (Köhler et al., 2000)

Reporter protein	Reporter gene	Origin	Potential substrate	Detection method
Bacterial luciferase	*lux*	One of several luminescent bacteria	Long chain aldehydes (C9-C14)	Luminescence
Insect luciferase	*luc*	Fireflies, click beetles	Luciferin	Luminescence
β-Galactosidase	*lacZ*	*E. coli*	Galactopyranosides	Colorimetric, electrochemical, fluorescence, chemiluminescence
Green fluorescent protein	*gfp*	*Aequorea victoria*	No substrate needed	Fluorescence
Alkaline phosphatase	*phoA*	Various	Phosphorylated organic molecules	Colorimetric, chemiluminescence
β-Glucuronidase	*uidA (gusA, gurA)*	*E. coli*	β-Glucuronides	Colorimetric, fluorescence, luminescence
β-Lactamase	*bla*	*E. coli*	Lactamides	Colorimetric

Table 5

Examples of Gram-positive bacteria that have been transformed with bacterial luciferase

Species	Gene transfer system	*lux* genes transferred[a]	Reference
Bacillus megaterium	Transformation	Pf *luxAB*	Carmi et al. (1987)
Bacillus subtilis	Transformation	Pf *luxAB*;	Carmi et al. (1987);
		Vh *luxDAB*	Karp and Meyer (1989)
Clostridium perfringens	Transformation	*luxAB*	Phillips-Jones (2000)
Enterococcus faecalis	Transformation	Vh *luxAB*	Simon et al. (2001)
Lactobacillus casei	Transformation	Pf *luxAB*	Ahmad and Stewart (1990)
Lactococcus lactis	Transformation	Pf *luxAB*	Ahmad and Stewart (1990); Stewart et al. (1989)
Listeria innocua		Vh *luxAB*	Simon et al. (2001)
Listeria monocytogenes	Transformation	Pf *luxAB*	Jassim et al. (1990)
Staphylococcus aureus	Transformation	Pf *luxAB*	Blissett and Stewart (1989)
Streptococcus pneumoniae	Transformation	Phl *luxABCDE*	Beard et al. (2002)
Streptomyces coelicolor	Transformation	Vh *luxAB*	Schauer et al. (1989)

[a]Pf, *Photobacterium fischeri*; Phl, *Photorhabdus luminescens*; Vh, *Vibrio harveyi*

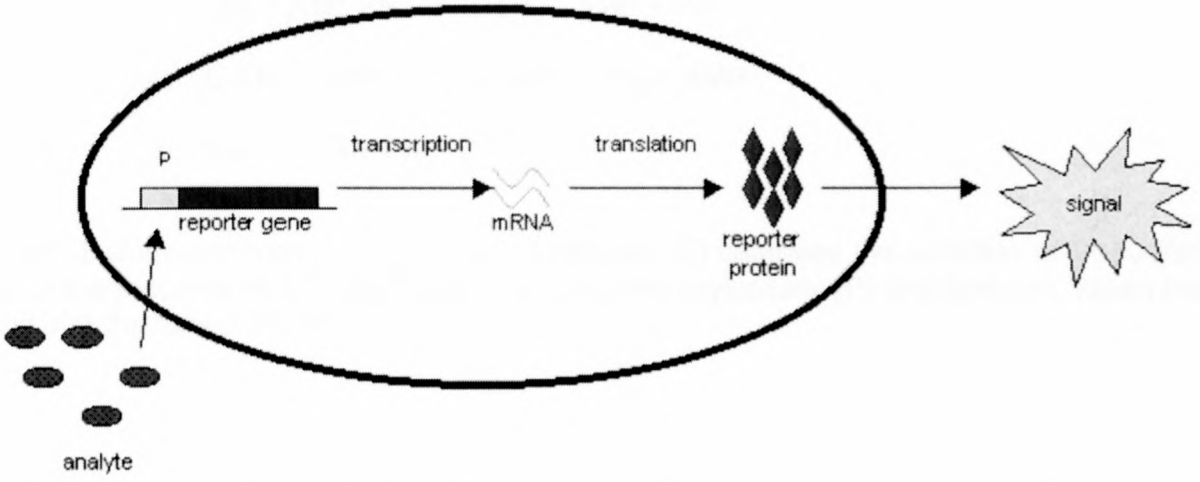

Fig. 11. Reporter gene system. Upon exposure to a specific analyte, the promoter (P)/reporter gene complex is transcribed into messenger RNA (mRNA) and translated into a reporter protein which then produces a measurable signal.

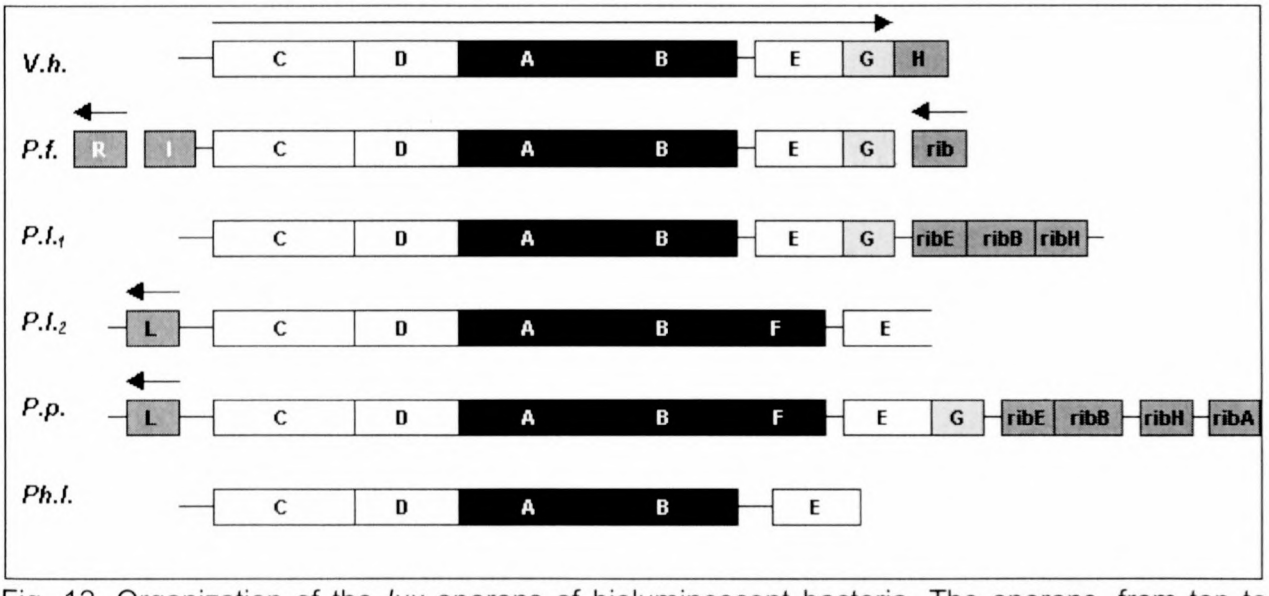

Fig. 12. Organization of the *lux* operons of bioluminescent bacteria. The operons, from top to bottom are those detected in *Vibrio harveyi* (*V.h.*), *Photobacterium fischeri* (*P.f.*), two strains or subspecies of *Photobacterium leiognathi* (*P.l.*$_1$ and *P.l.* $_2$), *Photobacterium phosphoreum* (*P.p.*) and *Photorhabdus luminescens* (*Ph.l.*). Arrows indicate transcription directions. Single letters refer to *lux* genes and linked genes connected to riboflavin synthesis are indicated by "rib".

1. $LH_2 + ATP + E \xrightarrow{Mg^{2+}} E{\bullet}LH_2 - AMP + PP_i$

2. $E{\bullet}LH_2 - AMP + O_2 \rightarrow E{\bullet}P^* + CO_2 + AMP$

3. $E{\bullet}P^* \rightarrow E{\bullet}P + h\nu$

Fig. 13. Bioluminescence reaction. Firefly luciferase (E) catalyses the oxidation of D(-)luciferin (LH_2) in the presence of ATP-Mg^{2+} and O_2 to generate oxyluciferin (P) and light ($h\nu$). Taken from Gould and Subramani (1988).

4. CLONING OF *LUXAB* DOWNSTREAM OF THE PLANTARICIN 423 PROMOTER IN AN ATTEMPT TO STUDY THE REGULATION OF THE PLANTARICIN 423 GENE

Abstract

Plantaricin 423 is a class IIa bacteriocin produced by *Lactobacillus plantarum* 423, with 60 % homology to the structural gene of pediocin PA-1. In a previous study the gene encoding plantaricin 423 was expressed in *Saccharomyces cerevisiae.* However, little is known about the regulation of plantaricin 423. In this study a reporter gene (*luxAB*) was ligated downstream of the plantaricin 423 promoter (P423) and cloned into a shuttle vector, pTRKH2. Despite several assays, which included the growth of the genetically modified strain 423 in the presence of heat-denatured plantaricin 423 or in the presence of *Listeria innocua,* no luciferase activity was detected. Northern blot analysis with luxA and luxB probes revealed no homology signal, suggesting that the *luxAB* gene in *L. plantarum* 423 was not transcribed. However, bioluminescence could be observed in the dark when the vapour from 5 µl decanal was brought into contact with *E. coli* C4 colonies on an agar plate, which served as a positive control. The region necessary for expression of plantaricin 423 may be located upstream of the –80 region homologous to the –80 and –40 conserved repeats of regulated class II bacteriocins.

1. Introduction

The practical application of lactic acid bacteria (LAB) and their metabolic products have been extensively studied (Luchansky, 1999). An important characteristic of LAB is their ability to produce antimicrobial peptides (bacteriocins), which may be used as food preservatives. Nisin, a class I bacteriocin, has been approved to be incorporated in foods in more than 40 countries and has been produced industrially since 1953 (De Vuyst and Vandamme, 1994).

Bacteriocins are small ribosomally synthesised peptides that usually exert antimicrobial activity towards closely related bacteria. These peptides are classified into four groups on the basis of their biochemical and genetic composition (Klaenhammer, 1993). Most bacteriocins belong to class II, which is further subdivided into four groups (Klaenhammer, 1993; Nes et al., 1996). A very important characteristic of class IIa, or pediocin-like, bacteriocins is their strong activity against *Listeria monocytogenes* (Klaenhammer, 1993; Quadri et al., 1994).

Plantaricin 423 is a small, heat-stable, plasmid-encoded, antilisterial bacteriocin from class IIa, produced by *Lactobacillus plantarum* 423 (Van Reenen et al., 1998). Plantaricin 423 also inhibits other closely related *Lactobacillus* spp., Gram-positive foodborne pathogens and spoilage bacteria, including *Bacillus cereus*, *Clostridium sporogenes*, *Enterococcus faecalis* and *Staphylococcus* spp. (Van Reenen et al., 1998). *Lactobacillus plantarum* 423 houses five plasmids, pPLA1 to pPLA5. The 9 kb-plasmid (pPLA4) has been partially sequenced and the genes encoding plantaricin 423 have been characterised (Van Reenen et al., 2003). Four genes in an operon structure were revealed to be almost similar to that of pediocin PA-1 (Van Reenen et al., 2003). The first open reading frame (ORF), *plaA*, codes for a prepeptide that consists of two parts; a 37-amino acid mature molecule joined to a 19-amino acid N-terminal leader peptide. The *plaB* gene encodes a putative immunity protein with an amino acid sequence similar to that of other class IIa bacteriocins. The third and fourth ORFs, *plaC* and *plaD*, are almost identical to *pedC* and *pedD* of pediocin-PA1 (Van Reenen et al., 2003).

Bacteriocins of LAB may be produced constitutively or may be regulated by changes in cell density (Saucier et al., 1995; Nes et al., 1996; Kleerebezem et al., 1997). Some class II bacteriocins are produced by a three-component regulatory system consisting of a bacteriocin-like induction peptide (IP) lacking antimicrobial activity, a histidine protein kinase (HPK) and a response regulator (RR) (Nes et al., 1996; Kleerebezem et al., 1997). Although the genetic organisation of plantaricin 423 has been determined, little is known about its genetic regulation.

Many different reporter systems have been developed to study the expression of promoters. The most commonly used reporter molecules include bacterial luciferase, insect luciferase, β-galactosidase, green fluorescent protein, alkaline phosphatase, β-glucoronidase, β-lactamase and catechol 2,3-dioxygenase (Köhler et al., 2000).

Bacterial luciferase is a heterodimeric enzyme isolated from *Vibrio harveyi*. The α- and β-subunits are encoded by the *luxA* and *luxB* genes in the *lux* operon (Baldwin et al., 1984). Luciferase catalyses the reaction in which a long-chain aldehyde is oxidised and a series of intermediates are formed that lead to the formation of a C4a hydroxyflavin (Fisher et al., 1996). Hydroxyflavin is converted to FMN that leads to the emission of visible light. Luciferase studied from different bacteria appears to be homologous and catalyse the same reaction:

$$FMNH_2 + RCHO^{a} + O_2 \longrightarrow FMN + RCOOH + H_2O + h\nu\ (490\ nm)$$

([a] Long-chain aliphatic aldehyde which is added exogenously) From Fisher et al. (1996)

Bacterial bioluminescence is a sensitive real-time, non-invasive reporter which has been used to monitor gene expression in a number of bacterial species, including Gram-positive bacteria (Carmi et al., 1987; Blissett and Stewart, 1989; Karp and Meyer, 1989; Schauer et al., 1989; Stewart et al., 1989; Ahmad and Stewart, 1990; Jassim et al., 1990; Philip-Jones, 2000; Simon et al., 2001; Beard et al., 2002).

In this study the bacterial *luxAB*-gene (luciferase) from *Vibrio harveyi* was ligated downstream of the plantaricin 423 promoter to determine if luciferase could be used as a reporter system in the expression of plantaricin 423.

2. Materials and methods

2.1. Bacterial strains and plasmids

The bacterial strains and plasmids used are listed in Table 1. *L. plantarum* 423, isolated from sorghum beer (Van Reenen et al., 1998) produces plantaricin 423. *Lactobacillus sakei* LMG 13558 and *Listeria innocua* LMG 13568 (Table 1) were used as indicator strains. Plasmid pLX303ab is a pET3-derivative that harbours the promoterless fusion of *luxAB* isolated from *Vibrio harveyi* (Olsson et al., 1988). The pGEM®-T Easy Vector (Promega Corporation, Madison, USA) was used for cloning and amplification of the *luxAB* construct in *Escherichia coli* TOP 10. *Escherichia coli* JM109 DE3 and a plasmid-free mutant of *L. plantarum* 423 (423 B^-) were used as recipient strains. *E. coli* C4 and *L. plantarum* B11 (see Table 1) are the names given to the transformants containing plasmids pTAB7 and pTAB4 respectively, as shown in Fig. 1.

2.2. Media and growth conditions

Escherichia coli transformants were grown in Luria-Bertani (LB) medium at 37°C, supplemented with ampicillin (100 µg/ml). Blue-white selection of *E. coli* transformed with pGEM®-T Easy (Promega) was possible by adding 40µg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and 10 µg/ml IPTG (isopropylthio-β-D-galactoside) to the agar plates. *E. coli* strains transformed with pTRKH2 were grown in Brain Heart Infusion (BHI) medium (Biolab Diagnostics, Wadeville, South Africa), supplemented with erythromycin (150 µg/ml). *Listeria innocua* LMG 13568 was grown on BHI at 30°C without aeration. *Lactobacillus plantarum* 423 and *L. sakei* LMG 13558 were grown in MRS broth (Biolab) at 30°C without aeration. Recombinant lactobacilli were grown

in MRS broth supplemented with 2.5 µg/ml erythromycin. Agar plates of the appropriate media were prepared with the addition of 1.7% (w/v) bacteriological agar. For the preparation of soft agar, 0.7% (w/v) bacteriological agar was added to the appropriate media.

2.3. Antimicrobial activity

An overnight pre-inoculum of *Lactobacillus plantarum* 423 in MRS, was diluted into a fresh tube of MRS to a concentration of 1% (v/v). After 18 h incubation at 30°C, the cells were harvested (10 000 x *g*, 10 min. at 4°C) and the cell-free supernatant adjusted to pH 7. Serial twofold dilutions of the cell-free supernatant containing plantaricin 423 were spotted (10 µl) onto fresh indicator lawns of *Lactobacillus sakei* LMG 13558. The lawns were prepared by imbedding active growing cells into soft MRS agar (0.7% w/v agar). The seeded plates were incubated at 30°C for approximately 24h. The reciprocal of the highest dilution with a clear zone of inhibition was used to calculate the antimicrobial activity (arbitrary units, AU) per ml, as described by Van Reenen et al. (1998).

To determine if the production of plantaricin 423 is inducible, the following experiments were conducted. Nine sterile Erlenmeyer flasks (250 ml) containing 250 ml sterile MRS each were used. Flasks 1 and 4 to 9 were inoculated with *L. plantarum* 423 to a final optical density (OD_{600}) of 0.05. Flasks 2 and 3 were inoculated with 2 ml heat killed (100° for 20 min.) *Listeria innocua* LMG 13568 and *Lactobacillus sakei* LMG 13558, respectively, which was grown to the OD_{600} of 0.5 before inactivation. Flasks 4 and 5 were in addition inoculated with heat killed *L. innocua* LMG 13568 and *Lb. sakei* LMG 13558, respectively, as for flasks 2 and 3. Flasks 6 and 7 were additionally inoculated with 2 ml live *L. innocua* LMG 13568 and *Lb. sakei* LMG 13558, respectively, which was grown to the OD=0.5. Flask 8 was supplemented with 0.1 AU heat inactivated (autoclaved, 121°C for 20 min.) plantaricin 423 (6400 AU/ml before inactivation). Flask 9 received 0.1 AU active (6400 AU/ml) plantaricin 423. All flasks were incubated at 30°C for 18 h without shaking to an optical density of 3.5 – 4.0 (at 600nm). The number of viable cells (cfu/ml) and plantaricin 423 activity were recorded every two hours until stationary growth.

2.4. DNA isolation

Plasmid DNA was isolated from *E. coli* by using the lysis-by-boiling method (Sambrook et al., 1989). Large scale pure plasmid DNA used for PCR, sequencing and electroporation was isolated from *E. coli* by using the High Pure Plasmid Isolation Kit (Roche, Randburg, RSA). Plasmid DNA (pure, large scale) was isolated from *L. plantarum* with the QIAGEN® Plasmid Midi kit modified for lactobacilli (Southern Cross Biotechnology, Claremont, Cape Town, RSA).

2.5. Construction of the reporter system

Polymerase chain reactions (PCR) were conducted with a Perkin Elmer GeneAmp PCR System 2400 (Perkin-Elmar, Applied Biosystems, California, USA). Oligonucleotide primers (Table 2) were designed based on the sequence accessible from GenBank (accession nr AF304384). The area upstream of the available promoter area (Fig. 2) was further sequenced using the reverse primer SUP-right (Table 2). From this sequence the elongated area containing P423 was used in the reporter construct.

The putative promoter of plantaricin 423 was obtained by PCR with plasmid pPLA4 as template and P423-LN and PLU as forward and reverse primers, respectively (Table 2). The 3' terminal of PLU overlaps with the 5' terminal of *luxAB*. The PCR product (B1) was purified and used as a forward overlap primer in association with LuxB-Right as reverse primer. The plasmid pLX303ab was used as template to obtain the transcriptional fusion of the promoter with the *luxAB* genes (GenBank accession nr M10961). Unique restriction endonuclease sites (*Xba*I and *Sal*I) were inserted in the design of the construct (Fig. 1A) for easy manipulation and directional cloning.

Another construct was made where the strong T7 promoter (Promega Notes for pGEM®-T Easy Vector) from the T7 phage was obtained by PCR with the pGEM®-T Easy Vector as template and T1 and TLU as forward and reverse primers, respectively (Table 2). As with PLU, the 3' terminal of TLU overlapped with the 5' terminal of *luxAB*. The PCR product (C1) was purified and used as a forward overlap primer in association with LuxB-Right as reverse primer.

Plasmid pLX303ab was used as template to obtain the transcriptional fusion of the promoter with the *luxAB* genes. This construct (Fig. 1B) also contains the unique restriction endonuclease

sites, *Xba*I and *Sal*I for easy manipulation and directional cloning. The theoretical design of both constructs is presented in Figs. 3 and 4 respectively. PCR was best performed with *TaKaRa Ex Taq*™ polymerase (TAKARA BIO INC., Kyoto, Japan).

2.6. DNA manipulation

PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and the GFX™ DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, USA). Restriction endonuclease (Roche) and T4 DNA ligase (Roche) were used according to the manufacturer's instructions. Agarose gel electrophoresis and the detection of DNA were performed as described by Sambrook et al. (1989). Plasmid bands were excised from the agarose gels and purified with Agarase (Boehringer Mannheim, Mannheim, Germany) or the QIAquick PCR purification kit (Qiagen). Transformation of *E. coli* was performed using the heatshock method, as described in the Promega Technical Manual (Promega Corporation, Madison, USA). Electroporation of *Lactobacillus plantarum* was performed according to the method described by Bringel and Hubert (1990).

2.7. Cloning and transformation

Ligation of the designed constructs to pGEM®-T Easy (Promega) was performed according to the manufacturer's instructions. Amplification of the newly constructed plasmids (pGEM-P423ab and pGEM-T7ab) was done by transformation in the recombination negative *E. coli* strain TOP10, with ampicillin resistance and blue-white selection. Both *Sal*I / *Xba*I-fragments (reporter constructs) were cloned to pTRKH2 (Fig. 1C). The plasmid containing the P423::*luxAB* construct, pTAB4 (Fig. 1D) was then transformed to *E. coli* TOP10. The T7::*luxAB*-promoter construct, pTAB7 (Fig. 1E), was transformed to *E. coli* JM 109 DE3 which contains the chromosomal T7-phage RNA polymerase gene. The amplified plasmids, pTAB4 and pTAB7, were isolated and sequenced (Core Sequencing Unit of the Genetics Department, University of Stellenbosch, Stellenbosch, RSA) using the oligonucleotides as described in Table 2. Plasmid pTAB4 was electroporated into *L. plantarum* 423 B^- according to the method described by Bringel and Hubert (1990). The resulting transformant was designated *L. plantarum* B11.

2.8. Luciferase assay

Disposable polystyrene measuring cuvettes designed for luminometery were filled with 250 µl of culture samples from different stages of growth. At specific intervals 10 µl of 1% (v/v) n-decyl aldehyde (Sigma-Aldrich, Aston Manor, RSA) in methanol was added and readings taken with a LKB Wallac model 1251 luminometer (Luminescence Photometer, Wallac Oy, Finland). The bioluminescence of *Lactobacillus plantarum* B11 was measured by adding a solution of 0.01% mercaptoethanol, 0.1 M sodium phosphate buffer (pH 6.8), 13 mM NADH and 0.42mM FMN (in a ratio of 1:13:2:2) prior to the addition of decanal. Bioluminescence was measured for whole cells and cell lysates of *L. plantarum* B11 at different stages of growth.

2.9. RNA isolation and Northern blot analysis

Total RNA was isolated from all transformants and control strains (method as described by M. Labuschagne, personal communication). Cells were cultured until late exponential phase and then harvested for 15 min at 9 000 x *g* and 4°C. The pellets were frozen (-20°C) and ground in a pre-cooled mortar to a fine powder. Microtubes (2ml), pre-cooled with liquid nitrogen, were filled up to approximately 0.5 ml with the powdered biomass. The biomass was homogenised in 1 ml TRIzol® (Invitrogen) and incubated at room temperature (25°C) for 5 min. Proteins were removed by chloroform extraction and incubation at room temperature (25°C) for 3 min. Total RNA was precipitated by adding 500 µl isopropanol to the supernatant, followed by centrifugation (12 000 x *g*, 15 min, 4°C). The pellet was washed with 70% ethanol and carefully resuspended in approximately 50 µl formamide. The RNA suspension was stored at -20°C until used.

Gel electrophoresis of the RNA and preparation of Northern blots were performed as described by Köhrer and Domdey (1990). Total RNA was hybridised with randomly labelled α-P^{32} probes prepared from *luxA* and *luxB* specific PCR products. The primers used are listed in Table 2. Hybridisation was performed at 65°C for 16 h in a solution consisting of 0.25 M Na_2HPO_4 and 7% SDS (pH 7.0). The membranes were then washed twice with the hybridisation solution at room temperature (25°C) for 15 minutes, followed by two stringency washes. The first (less stringent) wash was performed at room temperature with a high salt buffer (2 x SSC, 0.1 x SDS) and the second wash at 65°C with a more stringent buffer (0.1 x SSC, 0.1 x SDS).

3. Results

3.1. Bacteriocin activity

Growth of *L. plantarum* 423 in the presence of *L. innocua* LMG 13568 and *L. sakei* LMG 13558, respectively, had no affect on the production of plantaricin 423 (Fig. 5A; Fig. 6). The addition of heat-inactivated and active (0.1 AU) plantaricin 423 (6400 AU/ml) did also not induce plantaricin 423 production (Fig. 5B).

3.2. Construction of reporter system

PCR products were obtained when the *TaKaRa Ex Taq*™ polymerase (TAKARA BIO INC.) enzyme was used. Even better results were obtained when bovine serum albumin (BSA) was added to the reaction mixtures which acts to relieve interference or inhibition of PCR (Kreader, 1996). The PCR products B1 and C1 (Fig. 7A) containing promoters P423 and T7, respectively, were purified and sequenced. In two respective overlap PCR reactions B1 and C1 were used as overlapping left primers obtaining transcriptional fusions between the respective promoters and *luxAB* (Fig. 7B and C). LuxB-right was used as reverse primer in the same two reactions.

The two constructs were cloned in pGEM®-T-Easy Vector and transformed into *E. coli* DH5α™ and XL1-blue, respectively. Both strains support blue/white screening of clones. Although DH5α™ and XL1-blue are both designed to increase stability, repeated instability and recombination of the inserts was observed. This led to the use of *E. coli* TOP10 for the amplification of the constructs, which was designed for stable replication of high-copy-number plasmids (Invitrogen, USA). DNA sequence analysis of positive transformants confirmed the presence of the complete *luxAB* transcriptional unit under control of either the P423 or T7 promoter in TOP10 (Figs. 9 and 10). The constructs were excised with *Xba*I and *Sal*I. These fragments were ligated into plasmid pTRKH2 restricted with *Xba*I/*Sal*I. Plasmid pTAB4 was then transformed to *E. coli* TOP10 and plasmid pTAB7 was transformed to *E. coli* JM109 DE3 for expression of *luxAB* by the T7 promoter. Small-scale plasmid isolation and restriction endonuclease profiles (Fig. 8) confirmed that the pTAB4 constructs were transformed. Plasmid pTAB4 was isolated and transformed to *L. plantarum* 423 B^-. After one week of incubation at 30°C, three colonies were obtained that contained the complete P423::*luxAB* construct. DNA sequences of the inserted fragments were identical to the theoretical designs (Fig. 3; Fig. 4).

3.3. Luciferase activity

E. coli C4 showed the highest level of bioluminescence with a maximum of approximately 1000 RLU (relative light units) and was taken as 100% luciferase activity (Fig. 11A). Bioluminescence could be observed in the dark when the vapour from 5 µl decanal was brought into contact with *E. coli* C4 colonies on an agar plate. However, no luciferase activity was detected when either whole cell extracts or lysates of *Lactobacillus plantarum* B11 was assayed (Fig. 11B). The level of bioluminescence was not affected by the external addition of plantaricin 423 during growth of *Lb. plantarum* B11, nor by the presence of other bacteria (*L. innocua* LMG 13568 and *Lb. sakei* LMG 13558, respectively) that may induce bacteriocin production (Fig. 11B).

3.4. Northern blot analysis

Northern blot analysis was performed to determine whether *luxAB* was expressed by the transformants. No mRNA transcript was present for *L. plantarum* B11 (Fig. 12). *E. coli C4*, which displayed high expression of *luxAB*, was used as positive control.

4. Discussion

Before the reporter gene system was constructed, preliminary studies were performed to determine if the production of plantaricin 423 is autoinducible or induced by the presence of sensitive (target) bacterial cells. In both these cases it seemed that plantaricin 423 is always produced at the same level and growth phase as if only *Lactobacillus plantarum* 423 was present (Fig. 5; Fig. 6). Plantaricin 423 may thus be produced constitutively. The possibility of other factors involved in the induction of plantaricin 423 may very well exist.

Risøen et al. (2000) studied the regulatory promoters of *Lactobacillus sakei* LTH673 and suggested that the response regulators (RRs), which are important factors in quorum sensing-based bacteriocin regulation, are dependent upon characteristic repeats found in the –80 and –40 regions of these operons. Diep et al. (1996) and Brurberg et al. (1997) have also compared the conserved repeats which are separated by an AT-rich spacer region. RRs binds to the repeated regions of the inducible promoters and is crucial for transcriptional activation (Risøen et al., 2000). When these areas are compared to that of the upstream area of P423, a region

almost identical to the left repeat is observed around the –80 region (Fig. 13). Much less conservation is found for the –40 region of P423 as it would be for inducible promoters. Since conserved repeats in the –80 to –40 region upstream of the promoter is a good indication of induced production of bacteriocins, this may confirm our hypothesis that P423 is a constitutive promoter. Previous evidence (Risøen et al., 2000) suggests that the DNA region necessary for constitutive expression is upstream of the –80 and –40 repeats and may be the same for plantaricin 423.

Although no increase in plantaricin 423 production was recorded with preliminary experiments, a reporter system was constructed to try and quantify bacteriocin production. To study expression of the 423 promoter during growth, the reporter plasmid pTAB4 was introduced to *L. plantarum* 423 B^-. The first construction of the P423::*luxAB* contained the promoter area of plantaricin 423, followed by the RBS (ribosomal binding site) of the plantaricin structural gene, the short leader T7-phage polypeptide start codon, the RBS, the natural *lux* RBS, and the start codon. The rationale behind this construction was to obtain the highest possible expression of luciferase, since very low expression of the *lux* genes by Gram-positive bacteria was previously observed. The *luxAB* containing template, pLX303-ab, was originally constructed to contain the additional T7 RBS for higher luciferase activity (Olsson et al., 1988) in *E. coli*. For this study it was decided to simplify the P423::*luxAB* construct to contain one RBS upstream of the *lux* genes.

A control plasmid, pTAB7, was transformed to *E. coli* JM109 DE3. As seen in the results, *E. coli* C4 exhibited significant bioluminescence, whereas no bioluminescence was observed for *L. plantarum* B11 (Fig. 11). The same results were obtained from the cell lysates of *L. plantarum* B11 (results not shown).

Bacteriocin-containing supernatant of the wild type, *L. plantarum* 423, as well as the indicator strain, *L. innocua* LMG 13568 or *L. sakei* LMG 13558, were used to induce the expression of *luxAB* by *L. plantarum* B11. The same results were observed as for the initial experiments, i.e. no increase in the level of bioluminescence was recorded.

The vapour of 5 µl of decanal was enough to observe bioluminescence in the dark with the unaided eye for at least two hours on an LB plate covered with a mat of *E. coli* C4 colonies. Throughout the bioluminescence assays there was no indication of any substrate limitations for

Lb. plantarum B11, since elevated concentrations of the substrate was unable to make a difference in the expression of bioluminescence. However, too high concentrations could strongly inhibit the reaction which could complicate the interpretation of results (Blouin et al., 1996). The assay was carefully calibrated in terms of concentrations before the final results were documented.

Although the *lux* genes have been reported to be expressed in very acidic environments such as found for lactic acid bacteria (Stewart et al., 1989; Ahmad and Stewart, 1990; Drouault et al., 1999; Simon et al., 2001), it may affect the intensity of bioluminescence. To overcome any possibilities of pH having an effect on luciferase activity, the pH was increased to 6.8 (the optimum pH for luciferase activity) with no evident effect. Temperature was also not a factor, since luciferase from *Vibrio harveyi* is stable at temperatures varying from 23°C to 37°C (Eaton et al., 1993).

Transformants contained the complete *luxAB* transcriptional unit under the control of either the T7 or P423 promoter and was confirmed by DNA sequence analysis. One possible explanation for the inability to detect bioluminescence is that one or both of the LuxAB protein subunits were not fully transcribed in *L. plantarum* B11, or incorrect folding of the tertiary protein structure occurred, resulting in the absence of bioluminescence. No mRNA transcript was present for *L. plantarum* B11 in comparison to a highly expressed *luxAB* in *E. coli* C4 (Fig. 12).

Light emission from Gram-positive bacteria is 100-fold less than observed for Gram-negative bacteria (Eaton et al., 1993). This may be due to the weak translation and/or transcription of the Gram-negative genes, a phenomenon which may be solved by using a fusion of *luxA* and *luxB* genes, converting them to a single open reading frame. In this study a 26 bp intergenic region separated the *luxA* and *luxB* genes. The αβ fusion monomer was much more sensitive to elevated temperature *in vivo* than the wild type (Escher et al., 1989) and was therefore not used in this study.

The structural stability of the construct may have influenced the results. The P423::*luxAB* construct was only stably transformed after numerous attempts. Although the PCR products obtained with *TaKaRa Ex Taq*™ polymerase were intact, fragments of the construct were duplicated or deleted after subcloning of the construct to the expression vector pGEM®-T-Easy, and also into the shuttle vector (pTRKH2) that was transformed to *E. coli*. After the first

transformation attempt of the pGEM-P423ab construct into the recombination-negative TOP10 strain, a duplicate of the pGEM-T Easy multiple cloning site was observed in the middle of the construct insert. The T7::*luxAB* construct was never unstable and showed luciferase activity when transformed to JM109 DE3.

After numerous attempts, only three *Lactobacillus plantarum* B11 colonies were obtained with stably transformed and intact *lux*-constructs. This result may indicate that the Gram-positive promoter region, specifically of plantaricin 423, fused to the *luxAB* dicistron, comprises a structurally unstable construct which might be expressed with extreme difficulty with the further set back of low expression of Gram-negative genes in a Gram-positive cell. The possibility also exists that there might be an unknown region upstream of the bacteriocin promoter which may be necessary for constitutive production of the downstream genes (Chikindas, personal communication). The 9 kb plasmid (pPLA4) was only partially sequenced (Van Reenen et al., 1998) and may contain such an unknown region upstream of the sequenced area. The extended area obtained just upstream of the P423 promoter (Fig. 2) might not have the included region.

5. Conclusion

This is the first report of an attempt to use a reporter system to quantify plantaricin 423. The luciferase assay is a very sensitive, non-invasive system to measure *in vivo* promoter activity. The fact that the promoter P423 was unable to express the *luxAB* genes may be due to limited knowledge about the rest of plasmid pPLA4. From these results one may postulate that P423 is a constitutive promoter and that the DNA region necessary for constitutive production may be further upstream than the region that was cloned.

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Table 1

Bacterial strains and plasmid vectors

Strain or plasmid	Characteristics or comments	Source/Reference
Bacterial strains		
Lactobacillus plantarum 423	wild type plantaricin 423 producer isolated from sorghum beer	Van Reenen et al. (1998)
Lactobacillus plantarum 423 B$^-$	mutant strain cured pPLA4; competent cells for reporter plasmid (pTAB4).	Van Reenen et al. (1998)
Lactobacillus plantarum B11	recombinant *L. plantarum* 423 B$^-$ containing pTAB4	this study
Lactobacillus sakei LMG 13558	sensitive to plantaricin 423	LMG[a]
Listeria innocua LMG 13568	sensitive to plantaricin 423	LMG[a]
Escherichia coli DH5α	competent cells for subcloning; *recA1; endA1; lacZΔM15*	Invitrogen, USA
Escherichia coli XL1-blue	competent cells for subcloning; *recA1; endA1; lacI^{q}ZΔM15*	Stratagene, USA
Escherichia coli TOP 10	competent cells for subcloning; *recA1; endA1; lacI^{q}ZΔM15*	Invitrogen, USA
Escherichia coli JM109 DE3	competent cells for pTAB7-construct; carries chromosomal T7-phage RNA polymerase gene.	Promega, USA
Escherichia coli C4	recombinant *E. coli* JM109 DE3 containing pTAB7	this study
Plasmids		
pPLA4	wild type plasmid containing the plantaricin 423 operon; used as template in PCR to obtain promoter P432	Van Reenen et al. (1998)
pGEM® -T Easy	cloning vector with protruding T overhangs; subcloning of PCR products; template in PCR to obtain T7-promoter.	Promega, USA
pLX303ab	carries promoterless *luxAB* dicistron; template in PCR to obtain reporter genes *luxAB*	Olsson et al. (1988)
pTRKH2	high-copy-number (45-80) lactococcal shuttle vector; stable in different lactic acid bacteria; Er^R; 12 unique cloning sites; *lacZα*	O'Sullivan and Klaenhammer (1993)
pTAB4	pTRKH2 with P423::*luxAB* PCR fragment insert	this study
pTAB7	pTRKH2 with T7::*luxAB* PCR fragment insert	this study

[a]LMG, culture collection of the Laboratory of Microbiology, University of Ghent, Ghent, Belgium.

Table 2

Oligonucleotide primers used in this study

Primer name	Sequence in 5'-3' orientation	Restriction sites introduced/ Comments
P423-LN [a,c1]	CCC GGG TCT AGA CAG TTC TTG TTC AGC CTG	*Sma*I; *Xba*I
T1 [b,c1]	TCT AGA GGG CCT CTT CGC TAT TAC	*Xba*I
PLU [a]	AGG AAG TTT CCA AAT TTC ATA AAA ATC TCC TTC TTC TTA AAT CAT T	Overlap primer
TLU [b]	CCA AAT TTC ATA ACA TTT CCT TAG CAT GCG ACG TCG G	Overlap primer
LuxB-right [a, b, c2]	TGG CCA GTC GAC ACG TTA CGA GTG GTA TTT GAC	*MluN*I; *Sal*I; reverse labeling primer for luxB specific probe
SUP-right	GCG GTC ATT CTA GTT CCT AC	Sequencing primer
KL1A [c1]	GAC TGT TGG TAT GAC TTG AT	Sequencing primer
KL2 [c1]	GAA GCT ATT CCA GTC TGA TG	Sequencing primer
KL3 [c1]	CTA AGT TAG GGC TTC CAC TC	Sequencing primer
KL4 [c1]	TAC GAC TCA TAC GTG AAT GC	Sequencing primer
KR1 [c2]	GGC CTA AAT CTC CTT CTT CT	Sequencing primer
KR2 [c2]	GTG TCA AAC TTC AAC TGA TC'	Sequencing primer
KR3 [c2]	CTG GAA TAG CTT CAT AGA TG	Sequencing primer
LAL	GGT CTG AGA TAA CTC AGG TGG CTG ATA AGT G	Forward labeling primer for luxA specific probe
LAR	GCC TTT CAA GAC AAA ATC ACG CCA TTG ACC	Reverse labeling primer for luxA specific probe
LBL	CAA TGG TGT GGT TGG TGC CCC ATT AAC AG	Forward labeling primer for luxB specific probe

[a] Used in the construction of the P423::*luxAB* where PLU served as overlap primer
[b] Used in the construction of the T7::*luxAB* where TLU served as overlap primer
[c1] Used as forward sequencing primers for one or both constructs
[c2] Used as reverse sequencing primers for both constructs

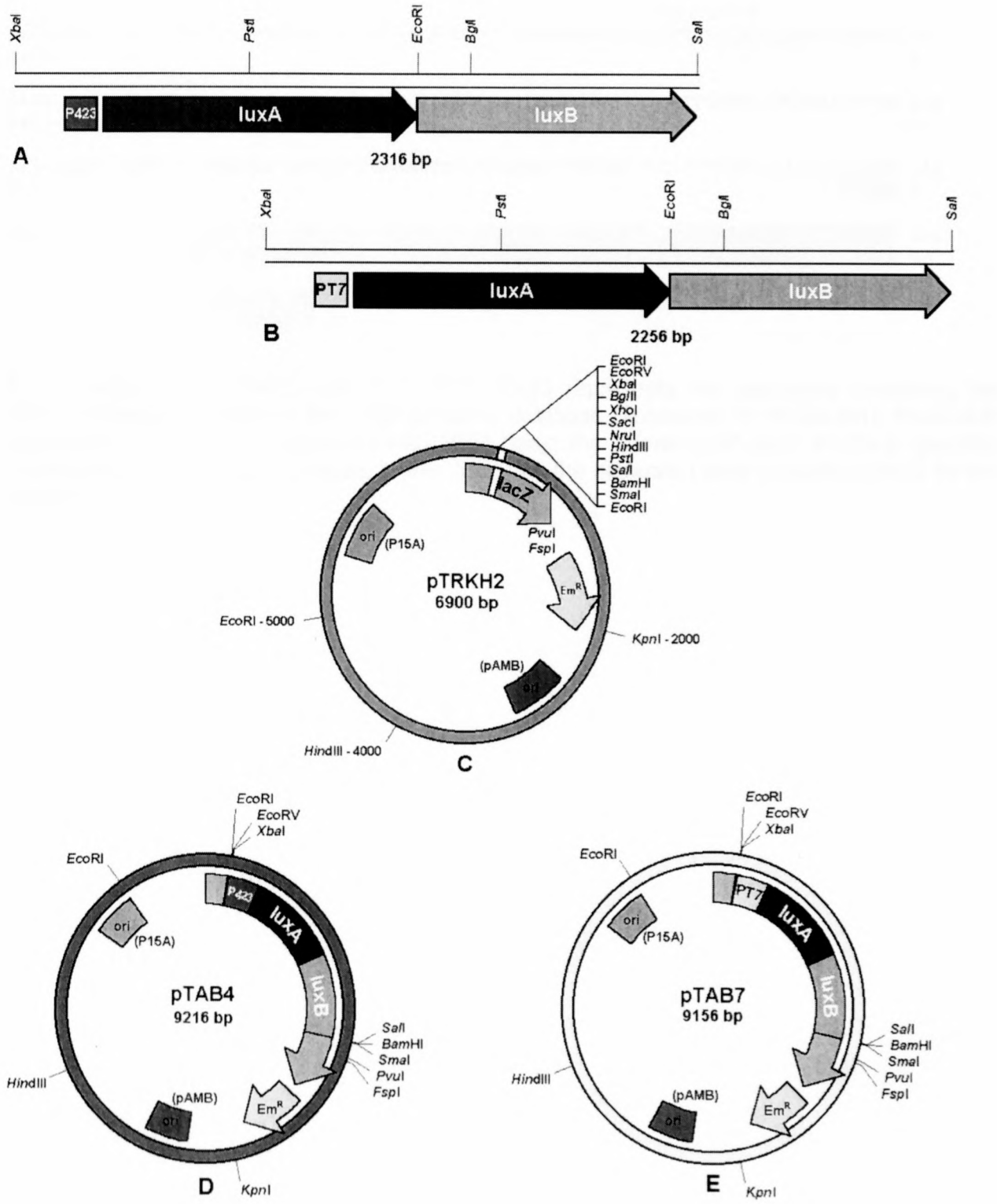

Fig. 1. **(A)** P423::*luxAB* obtained by conventional and overlap PCR. **(B)** T7::*luxAB* obtained by conventional and overlap PCR. **(C)** Shuttle vector pTRKH2. **(D)** P423::*luxAB* cloned as a *Xba*I/*Sal*I fragment in pTRKH2, now designated pTAB4. **(E)** T7::*luxAB* cloned as a *Xba*I/*Sal*I fragment in pTRKH2, now designated pTAB7.

```
                                                          P423-LN →
P423-SUP  1   CCTCGTTTGGTCACTATATCATGACCAATCTTATTAGCCAGTTCTTGTTCAGCCTGTGCTAACC 64
P423      0                                                                   0

P423-SUP  65  GTTGTTTCATAGTGGGAATATCTAAAGCCTTTGACATAATAACCGCTCCTTTTTCAGAATACTG 128
P423      0                                                                   0

P423-SUP  129 CGTTGATAGCAAGGTTTTGGAAATTGGTCAAGATCGTTAACCAGTTTTTATGCAAAATATCTAA 192
P423      1                                                             TCTAA 5

P423-SUP  193 CTAATACTTGACATTTACATTGAGTAGGAACTAGAATGACCGCGTATTAAAAATGATTTAAGAA 256
P423      6   CTAATACTTGACATTTACATTGAGTAGGAACTAGAATGACCGCGTATTAAAAATGATTTAAGAA 69

P423-SUP  257 GAAGGAGATTTTT 269
P423      70  GAAGGAGATTTTT 82
```

Fig. 2. Alignment of P423 with P423-SUP. P423 represents the sequence containing the promoter P423 as obtained from the GenBank database (accession nr AF304384). P423-SUP represents the sequence obtained with PCR using the primer SUP-right. P423-LN (position underlined) was used as a forward primer to obtain the elongated area containing P423 for the reporter construct.

```
CCCGGG
     TCTAGACAGT TCTTGTTCAG CCTG P423-LN >>
1    TCTAGACAGT TCTTGTTCAG CCTGTGCTAA CCGTTGTTTC ATAGTGGGAA TATCTAAAGC CTTTTGACAT AATAACCGCT   80
81   CCTTTTTCAG AATACTGCGT TGATAGCAAG GTTTTGGAAA TTGGTCAAGA TCGTTAACCA GTTTTTATGC AAAATATCTA  160
                                                    << AATGATTT AAGAAGAAGG AGATTTTTAT
161  ACTAATACTT GACATTTACA TTGAGTAGGA ACTAGAATGA CCGCGTATTA AAAATGATTT AAGAAGAAGG AGATTTTTAT  240
           -35                          -10                                  RBS
     GAAATTTGGA AACTTCCT PLU >>
241  GAAATTTGGA AACTTCCTTC TCACTTATCA GCCACCTGAG CTATCTCAGA CCGAAGTGAT GAAGCGATTG GTTAATCTGG  320
321  GCAAAGCGTC TGAAGGTTGT GGCTTCGACA CCGTTTGGTT GCTAGAGCAC CACTTCACTG AATTTGGGTT GTTAGGGAAT  400
401  CCTTATGTTG CTGCCGCACA CCTATTAGGT GCGACAGAAA CGCTCAACGT TGGCACTGCA GCTATCGTAT TGCCGACTGC  480
481  CCATCCGGTT CGACAAGCAG AAGACGTAAA CCTACTGGAT CAAATGTCAA AAGGACGATT CCGTTTTGGT ATTTGTCGCG  560
561  GTTTGTACGA TAAAGATTTT CGTGTCTTTG GTACAGACAT GGATAACAGC CGAGCCTTAA TGGACTGTTG GTATGACTTG  640
641  ATGAAAGAAG GCTTCAATGA AGGCTATATC GCGGCGGATA ACGAACATAT TAAGTTCCCG AAAATCCAAC TGAATCCATC  720
721  GGCTTACACA CAAGGTGGTG CTCCTGTTTA TGTCGTCGCG GAGTCAGCAT CAACGACAGA ATGGGCTGCA GAGCGTGGCC  800
801  TACCAATGAT TCTAAGCTGG ATCATCAACA CTCACGAGAA GAAAGCGCAG CTTGATCTTT ACAACGAAGT CGCGACTGAA  880
881  CATGGCTACG ATGTGACTAA GATTGACCAC TGTTTGTCTT ACATCACCTC CGTCGATCAT GACTCAAATA GAGCCAAAGA  960
961  TATTTGCCGC AACTTCTTGG GCCATTGGTA CGACTCATAC GTGAATGCCA CCAAGATTTT TGACGACTCT GACCAAACAA 1040
1041 AAGGTTACGA CTTCAATAAA GGTCAATGGC GTGATTTTGT GTTGAAAGGC CACAAAGACA CCAATCGCCG AATTGATTAC 1120
1121 AGCTACGAAA TCAACCCAGT AGGGACGCCT GAAGAGTGTA TCGCGATTAT CCAGCAAGAT ATTGATGCGA CGGGTATTGA 1200
1201 CAATATTTGT TGTGGTTTTG AAGCAAACGG TTCTGAAGAA GAAATTATCG CATCTATGAA GCTATTCCAG TCTGATGTGA 1280
1281 TGCCATATCT CAAAGAAAAA CAGTAATTAA TATTTTCTAA AAGGAAAGAG ACATGAAATT TGGATTATTC TTCCTCAATT 1360
                                          RBS
1360 TTATGAATTC AAAGCGTTCT TCTGATCAAG TCATCGAAGA AATGTTAGAT ACCGCACATT ACGTAGATCA GTTGAAGTTT 1440
1441 GACACGTTGG CTGTTTACGA AAACCATTTC TCGAACAATG GTGTGGTTGG TGCCCCACTA ACAGTGGCTG GTTTTTTACT 1520
1521 TGGTATGACA AAGAACGCCA AAGTGGCTTC GTTGAATCAC GTCATTACCA CGCATCATCC AGTACGTGTG GCGGAAGAAG 1600
1601 CGTGTCTACT TGACCAAATG AGTGAAGGCC GTTTTGCCTT TGGCTTTAGT GATTGTGAAA AGAGTGCAGA TATGCGCTTC 1680
1681 TTTAATCGAC CAACGGATTC TCAGTTTCAG TTGTTCAGTG AGTGTCACAA GATCATCAAT GATGCATTCA CTACTGGGTA 1760
1761 CTGCCATCCA AACAATGATT TTTATAGTTT TCCTAAAATC TCCGTTAACC CACACGCGTT CACTGAAGGC GGTCCTGCGC 1840
1841 AATTTGTGAA TGCGACGAGC AAAGAAGTGG TTGAATGGGC GGCTAAGTTA GGGCTTCCAC TCGTGTTTAG ATGGGACGAC 1920
1921 TCAAACGCTC AAAGAAAAGA ATACGCCGGT TTGTACCACG AAGTTGCTCA GGCACATGGT GTCGATGTTA GTCAGGTTCG 2000
2001 ACACAAGCTG ACGCTGCTGG TCAACCAAAA TGTAGATGGT GAAGCAGCAA GGGCAGAAGC TCGCGTGTAT TTGGAAGAGT 2080
2081 TTGTCCGTGA ATCTTACTCA AATACCGACT TTGAGCAAAA AATGGGAGAG CTGTTGTCAG AAAATGCCAT CGGTACTTAT 2160
2161 GAAGAAAGTA CTCAGGCAGC GCGAGTTGCG ATTGAGTGTT GTGGTGCCGC GGACCTATTG ATGTCTTTTG AGTCGATGGA 2240
2241 AGATAAAGCG CAGCAAAGAG CGGTTATCGA TGTGGTAAAC GCCAACATCG TCAAATACCA CTCGTAACGT GTCGAC     2316
                                        << LuxB-right G TCAAATACCA CTCGTAACGT GTCGACTGGCCA
```

Fig. 3. Theoretical design of P423::*luxAB*. The first 241 nucleotides represent the P423 promoter area (GenBank accession AF304384) and the nucleotides from position 242 to 2316 the *luxAB* dicistron (GenBank accession M10961). The –10 and –35 positions and the ribosomal binding sites (RBS) are underlined. Translational start and stop codons of *luxA* and *luxB* are printed in bold. The positions of the primers (P423-LN, PLU and LuxB-right) used in the construction are highlighted. Directions of primer elongation are indicated with arrows. Added restriction endonuclease sites are bold and underlined.

```
     TCTAGAGGGC CTCTTCGCTA TTAC T1 >>
1    TCTAGAGGGC CTCTTCGCTA TTACGCCAGC TGGCGAAAGG GGGATGTGCT GCAAGGCGAT TAAGTTGGGT AACGCCAGGG   80
                                                                          << CCGACGTC
81   TTTTCCCAGT CACGACGTTG TAAAACGACG GCCAGTGAAT TGTAATACGA CTCACTATAG GGCGAATTGG GCCCGACGTC  160
     GCATGCTAAG GAAATGTTAT GAAATT TLU >>
161  GCATGCTAAG GAAATGTTAT GAAATTTGGA AACTTCCTTC TCACTTATCA GCCACCTGAG CTATCTCAGA CCGAAGTGAT  240
           RBS
241  GAAGCGATTG GTTAATCTGG GCAAAGCGTC TGAAGGTTGT GGCTTCGACA CCGTTTGGTT GCTAGAGCAC CACTTCACTG  320
321  AATTTGGGTT GTTAGGGAAT CCTTATGTTG CTGCCGCACA CCTATTAGGT GCGACAGAAA CGCTCAACGT TGGCACTGCA  400
401  GCTATCGTAT TGCCGACTGC CCATCCGGTT CGACAAGCAG AAGACGTAAA CCTACTGGAT CAAATGTCAA AAGGACGATT  480
481  CCGTTTTGGT ATTTGTCGCG GTTTGTACGA TAAAGATTTT CGTGTCTTTG GTACAGACAT GGATAACAGC CGAGCCTTAA  560
561  TGGACTGTTG GTATGACTTG ATGAAAGAAG GCTTCAATGA AGGCTATATC GCGGCGGATA ACGAACATAT TAAGTTCCCG  640
641  AAAATCCAAC TGAATCCATC GGCTTACACA CAAGGTGGTG CTCCTGTTTA TGTCGTCGCG GAGTCAGCAT CAACGACAGA  720
721  ATGGGCTGCA GAGCGTGGCC TACCAATGAT TCTAAGCTGG ATCATCAACA CTCACGAGAA GAAAGCGCAG CTTGATCTTT  800
801  ACAACGAAGT CGCGACTGAA CATGGCTACG ATGTGACTAA GATTGACCAC TGTTTGTCTT ACATCACCTC CGTCGATCAT  880
881  GACTCAAATA GAGCCAAAGA TATTTGCCGC AACTTCTTGG GCCATTGGTA CGACTCATAC GTGAATGCCA CCAAGATTTT  960
961  TGACGACTCT GACCAAACAA AAGGTTACGA CTTCAATAAA GGTCAATGGC GTGATTTTGT GTTGAAAGGC CACAAAGACA 1040
1041 CCAATCGCCG AATTGATTAC AGCTACGAAA TCAACCCAGT AGGGACGCCT GAAGAGTGTA TCGCGATTAT CCAGCAAGAT 1120
1121 ATTGATGCGA CGGGTATTGA CAATATTTGT TGTGGTTTTG AAGCAAACGG TTCTGAAGAA GAAATTATCG CATCTATGAA 1200
1201 GCTATTCCAG TCTGATGTGA TGCCATATCT CAAAGAAAAA CAGTAATTAA TATTTTCTAA AAGGAAAGAG ACATGAAATT 1280
                                                          RBS
1281 TGGATTATTC TTCCTCAATT TTATGAATTC AAAGCGTTCT TCTGATCAAG TCATCGAAGA AATGTTAGAT ACCGCACATT 1360
1361 ACGTAGATCA GTTGAAGTTT GACACGTTGG CTGTTTACGA AAACCATTTC TCGAACAATG GTGTGGTTGG TGCCCCACTA 1440
1441 ACAGTGGCTG GTTTTTTACT TGGTATGACA AAGAACGCCA AAGTGGCTTC GTTGAATCAC GTCATTACCA CGCATCATCC 1520
1521 AGTACGTGTG GCGGAAGAAG CGTGTCTACT TGACCAAATG AGTGAAGGCC GTTTTGCCTT TGGCTTTAGT GATTGTGAAA 1600
1601 AGAGTGCAGA TATGCGCTTC TTTAATCGAC CAACGGATTC TCAGTTTCAG TTGTTCAGTG AGTGTCACAA GATCATCAAT 1680
1681 GATGCATTCA CTACTGGGTA CTGCCATCCA AACAATGATT TTTATAGTTT TCCTAAAATC TCCGTTAACC CACACGCGTT 1760
1761 CACTGAAGGC GGTCCTGCGC AATTTGTGAA TGCGACGAGC AAAGAAGTGG TTGAATGGGC GGCTAAGTTA GGGCTTCCAC 1840
1841 TCGTGTTTAG ATGGGACGAC TCAAACGCTC AAAGAAAAGA ATACGCCGGT TTGTACCACG AAGTTGCTCA GGCACATGGT 1920
1921 GTCGATGTTA GTCAGGTTCG ACACAAGCTG ACGCTGCTGG TCAACCAAAA TGTAGATGGT GAAGCAGCAA GGGCAGAAGC 2000
2001 TCGCGTGTAT TTGGAAGAGT TTGTCCGTGA ATCTTACTCA AATACCGACT TTGAGCAAAA AATGGGAGAG CTGTTGTCAG 2080
2081 AAAATGCCAT CGGTACTTAT GAAGAAAGTA CTCAGGCAGC GCGAGTTGCG ATTGAGTGTT GTGGTGCCGC GGACCTATTG 2160

2161 ATGTCTTTTG AGTCGATGGA AGATAAAGCG CAGCAAAGAG CGGTTATCGA TGTGGTAAAC GCCAACATCG TCAAATACCA 2240
                                                          << LuxB-right G TCAAATACCA
     CTCGTAACGT GTCGACTGGCCA
2241 CTCGTAACGT GTCGAC      2256
```

Fig. 4. Theoretical design of T7::*luxAB*. The first 167 nucleotides represent the T7 promoter area (Promega Notes for pGEM-T-Easy) and the nucleotides from position 168 to 2256 the *luxAB* dicistron (GenBank accession nr M10961). The –10 and –35 positions and the ribosomal binding sites (RBS) are underlined. Translational start and stop codons of *luxA* and *luxB* are printed in bold. The positions of the primers (T1, TLU and LuxB-right) used in the construction are highlighted. Directions of primer elongation are indicated with arrows. Added restriction endonuclease sites are bold and underlined.

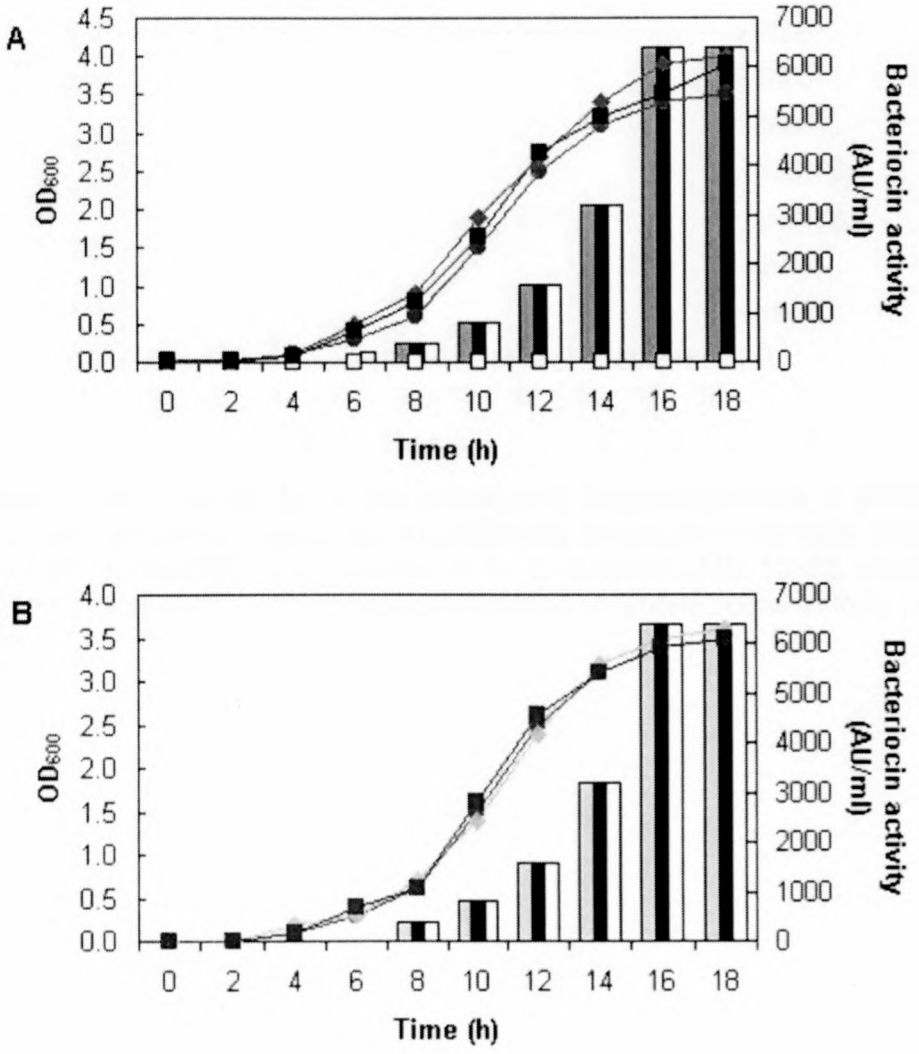

Fig. 5. Production of plantaricin 423 in the presence of (**A**) target organisms and (**B**) plantaricin 423. In graph **A** *L. plantarum* 423 was grown without the addition of target cells (•), and in the presence of heat-inactivated cells of *L. innocua* LMG 13568 (♦) and *Lb. sakei* LMG 13558 (■). Two negative controls show the OD_{600} values of MRS containing heat inactivated *L. innocua* LMG 13568 (◊) and *Lb. sakei* LMG 13558 (□) respectively. In graph **B** *L. plantarum* 423 was grown in the absence of additionally added plantaricin 423 (•), in the presence of heat-inactivated plantaricin 423 (♦) and in the presence of 0.1 AU active plantaricin 423 (■). The bars in graphs **A** and **B** indicate the changes recorded in antimicrobial activity (AU/ml), as per each treatment.

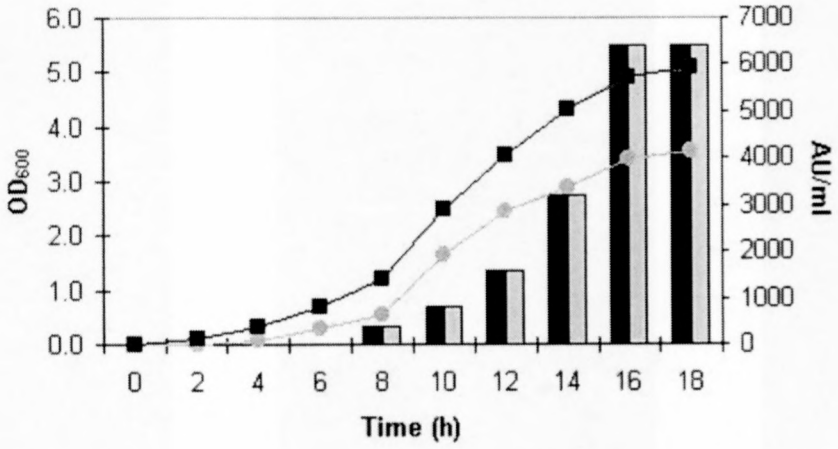

Fig. 6. Production of plantaricin 423 in the presence of target organisms. *L. plantarum* 423 was grown without the addition of target cells (•) and in the presence of live cells of *L. innocua* LMG 13568 (■) and *Lb. sakei* LMG 13558 (same as for *L. innocua* LMG 13568, results not shown). The bars in the graph indicate the changes recorded in antimicrobial activity (AU/ml), as per each treatment.

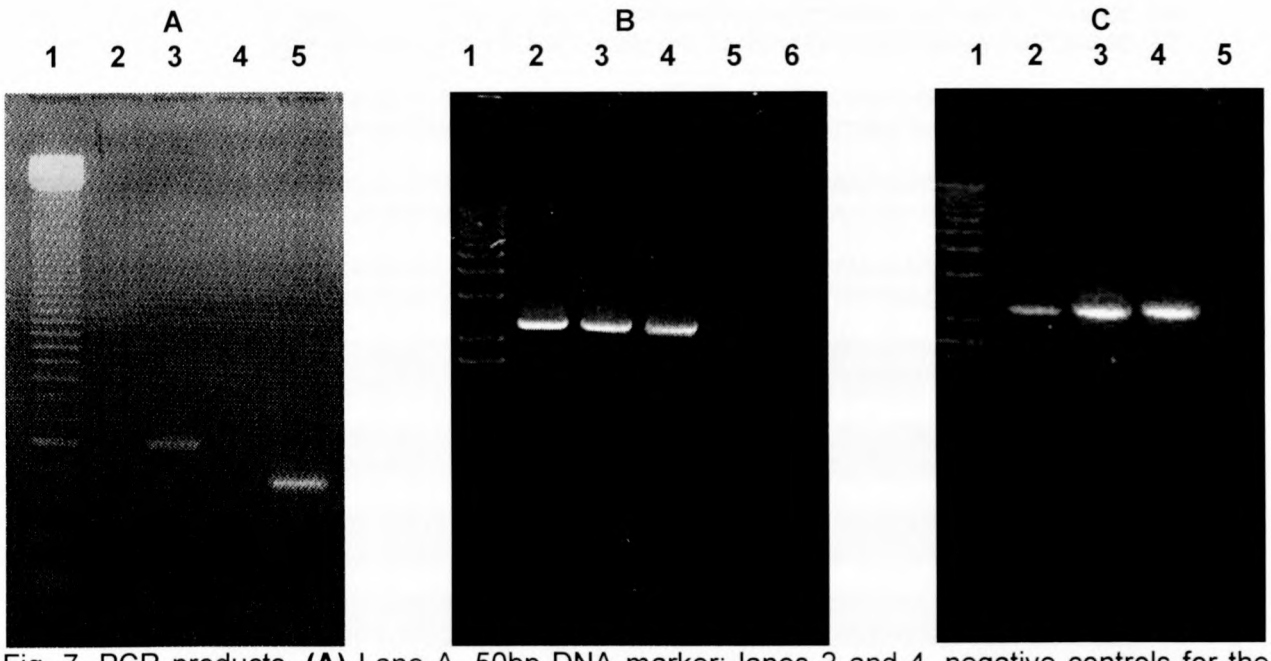

Fig. 7. PCR products. **(A)** Lane A, 50bp DNA marker; lanes 2 and 4, negative controls for the PCR reactions of lanes 3 and 5 respectively; lane 3, 260 bp PCR product (B1) containing P423 promoter; lane 5, 183 bp PCR product (C1) containing T7 promoter. **(B)** Lane 1, 1 kb DNA marker; lanes 2, 3 and 4, 2316 bp PCR products of P423::luxAB obtained by using B1 as overlap forward primer; lane 5, negative control: PCR performed in the presence of plasmid pool of *L. plantarum* 423 B^-; lane 6, negative control: PCR performed without any DNA templates. **(C)** Lane 1, 1 kb DNA marker; lanes 2, 3 and 4, 2256 bp PCR products of T7::luxAB obtained by using C1 as overlap forward primer; lane 5, negative control.

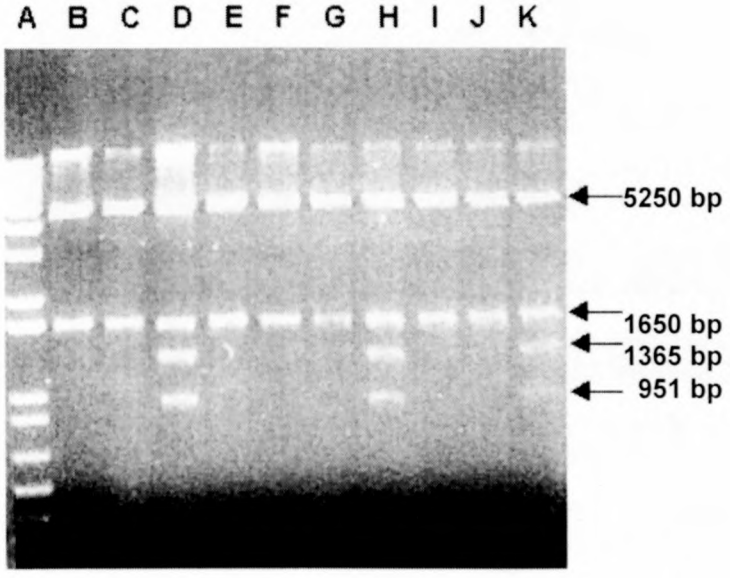

Fig. 8. pTAB4 isolated from *E. coli* TOP 10, digested with *Eco*RI. Lane A, 1 kb DNA marker. Only three of the above 10 isolates (lanes D, H and K) yielded the correct restriction enzyme profile (four fragments) when digested with *Eco*RI.

```
P423::luxAB   1    TCTAGACAGTTCTTGTTCAGCCTGTGCTAACCGTTGTTTCATAGTGGGAATATCTAAAGC 60
PCR-product   1    TCTAGACAGTTCTTGTTCAGCCTGTGCTAACCGTTGTTTCATAGTGGGAATATCTAAAGC 60

P423::luxAB   61   CTTTTGACATAATAACCGCTCCTTTTTCAGAATACTGCGTTGATAGCAAGGTTTTGGAAA 120
PCR-product   61   CTTTTGACATAATAACCGCTCCTTTTTCAGAATACTGCGTTGATAGCAAGGTTTTGGAAA 120

P423::luxAB   121  TTGGTCAAGATCGTTAACCAGTTTTTATGCAAAATATCTAACTAATACTTGACATTTACA 180
PCR-product   121  TTGGTCAAGATCGTTAACCAGTTTTTATGCAAAATATCTAACTAATACTTGACATTTACA 180
                                                          -35
P423::luxAB   181  TTGAGTAGGAACTAGAATGACCGCGTATTAAAAATGATTTAAGAAGAAGGAGATTTTTTAT 240
PCR-product   181  TTGAGTAGGAACTAGAATGACCGCGTATTAAAAATGATTTAAGAAGAAGGAGATTTTTTAT 240
                        -10                                 RBS
P423::luxAB   241  GAAATTTGGAAACTTCCTTCTCACTTATCAGCCACCTGAGCTATCTCAGACCGAAGTGAT 300
PCR-product   241  GAAATTTGGAAACTTCCTTCTCACTTATCAGCCACCTGAGCTATCTCAGACCGAAGTGAT 300

P423::luxAB   301  GAAGCGATTGGTTAATCTGGGCAAAGCGTCTGAAGGTTGTGGCTTCGACACCGTTTGGTT 360
PCR-product   301  GAAGCGATTGGTTAATCTGGGCAAAGCGTCTGAAGGTTGTGGCTTCGACACCGTTTGGTT 360

P423::luxAB   361  GCTAGAGCACCACTTCACTGAATTTGGGTTGTTAGGGAATCCTTATGTTGCTGCCGCACA 420
PCR-product   361  GCTAGAGCACCACTTCACTGAATTTGGGTTGTTAGGGAATCCTTATGTTGCTGCCGCACA 420

P423::luxAB   421  CCTATTAGGTGCGACAGAAACGCTCAACGTTGGCACTGCAGCTATCGTATTGCCGACTGC 480
PCR-product   421  CCTATTAGGTGCGACAGAAACGCTCAACGTTGGCACTGCAGCTATCGTATTGCCGACTGC 480

P423::luxAB   481  CCATCCGGTTCGACAAGCAGAAGACGTAAACCTACTGGATCAAATGTCAAAAGGACGATT 540
PCR-product   481  CCATCCGGTTCGACAAGCAGAAGACGTAAACCTACTGGATCAAATGTCAAAAGGACGATT 540

P423::luxAB   541  CCGTTTTGGTATTTGTCGCGGTTTGTACGATAAAGATTTTCGTGTCTTTGGTACAGACAT 600
PCR-product   541  CCGTTTTGGTATTTGTCGCGGTTTGTACGATAAAGATTTTCGTGTCTTTGGTACAGACAT 600

P423::luxAB   601  GGATAACAGCCGAGCCTTAATGGACTGTTGGTATGACTTGATGAAAGAAGGCTTCAATGA 660
PCR-product   601  GGATAACAGCCGAGCCTTAATGGACTGTTGGTATGACTTGATGAAAGAAGGCTTCAATGA 660

P423::luxAB   661  AGGCTATATCGCGGCGGATAACGAACATATTAAGTTCCCGAAAATCCAACTGAATCCATC 720
PCR-product   661  AGGCTATATCGCGGCGGATAACGAACATATTAAGTTCCCGAAAATCCAACTGAATCCATC 720

P423::luxAB   721  GGCTTACACACAAGGTGGTGCTCCTGTTTATGTCGTCGCGGAGTCAGCATCAACGACAGA 780
PCR-product   721  GGCTTACACACAAGGTGGTGCTCCTGTTTATGTCGTCGCGGAGTCAGCATCAACGACAGA 780

P423::luxAB   781  ATGGGCTGCAGAGCGTGGCCTACCAATGATTCTAAGCTGGATCATCAACACTCACGAGAA 840
PCR-product   781  ATGGGCTGCAGAGCGTGGCCTACCAATGATTCTAAGCTGGATCATCAACACTCACGAGAA 840

P423::luxAB   841  GAAAGCGCAGCTTGATCTTTACAACGAAGTCGCGACTGAACATGGCTACGATGTGACTAA 900
PCR-product   841  GAAAGCGCAGCTTGATCTTTACAACGAAGTCGCGACTGAACATGGCTACGATGTGACTAA 900

P423::luxAB   901  GATTGACCACTGTTTGTCTTACATCACCTCCGTCGATCATGACTCAAATAGAGCCAAAGA 960
PCR-product   901  GATTGACCACTGTTTGTCTTACATCACCTCCGTCGATCATGACTCAAATAGAGCCAAAGA 960

P423::luxAB   961  TATTTGCCGCAACTTCTTGGGCCATTGGTACGACTCATACGTGAATGCCACCAAGATTTT 1020
PCR-product   961  TATTTGCCGCAACTTCTTGGGCCATTGGTACGACTCATACGTGAATGCCACCAAGATTTT 1020

P423::luxAB   1021 TGACGACTCTGACCAAACAAAAGGTTACGACTTCAATAAAGGTCAATGGCGTGATTTTGT 1080
PCR-product   1021 TGACGACTCTGACCAAACAAAAGGTTACGACTTCAATAAAGGTCAATGGCGTGATTTTGT 1080

P423::luxAB   1081 GTTGAAAGGCCACAAAGACACCAATCGCCGAATTGATTACAGCTACGAAATCAACCCAGT 1140
PCR-product   1081 GTTGAAAGGCCACAAAGACACCAATCGCCGAATTGATTACAGCTACGAAATCAACCCAGT 1140

P423::luxAB   1141 AGGGACGCCTGAAGAGTGTATCGCGATTATCCAGCAAGATATTGATGCGACGGGTATTGA 1200
PCR-product   1141 AGGGACGCCTGAAGAGTGTATCGCGATTATCCAGCAAGATATTGATGCGACGGGTATTGA 1200

P423::luxAB   1201 CAATATTTGTTGTGGTTTTGAAGCAAACGGTTCTGAAGAAGAAATTATCGCATCTATGAA 1260
PCR-product   1201 CAATATTTGTTGTGGTTTTGAAGCAAACGGTTCTGAAGAAGAAATTATCGCATCTATGAA 1260
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P423::luxAB   1261 GCTATTCCAGTCTGATGTGATGCCATATCTCAAAGAAAAACAGTAATTAATATTTTCTAA 1320
PCR-product   1261 GCTATTCCAGTCTGATGTGATGCCATATCTCAAAGAAAAACAGTAATTAATATTTTCTAA 1320

P423::luxAB   1321 AAGGAAAGAGACATGAAATTTGGATTATTCTTCCTCAATTTTATGAATTCAAAGCGTTCT 1380
PCR-product   1321 AAGGAAAGAGACATGAAATTTGGATTATTCTTCCTCAATTTTATGAATTCAAAGCGTTCT 1380
                     RBS
P423::luxAB   1381 TCTGATCAAGTCATCGAAGAAATGTTAGATACCGCACATTACGTAGATCAGTTGAAGTTT 1440
PCR-product   1381 TCTGATCAAGTCATCGAAGAAATGTTAGATACCGCACATTACGTAGATCAGTTGAAGTTT 1440

P423::luxAB   1441 GACACGTTGGCTGTTTACGAAAACCATTTCTCGAACAATGGTGTGGTTGGTGCCCCACTA 1500
PCR-product   1441 GACACGTTGGCTGTTTACGAAAACCATTTCTCGAACAATGGTGTGGTTGGTGCCCCACTA 1500

P423::luxAB   1501 ACAGTGGCTGGTTTTTTACTTGGTATGACAAAGAACGCCAAAGTGGCTTCGTTGAATCAC 1560
PCR-product   1501 ACAGTGGCTGGTTTTTTACTTGGTATGACAAAGAACGCCAAAGTGGCTTCGTTGAATCAC 1560

P423::luxAB   1561 GTCATTACCACGCATCATCCAGTACGTGTGGCGGAAGAAGCGTGTCTACTTGACCAAATG 1620
PCR-product   1561 GTCATTACCACGCATCATCCAGTACGTGTGGCGGAAGAAGCGTGTCTACTTGACCAAATG 1620

P423::luxAB   1621 AGTGAAGGCCGTTTTGCCTTTGGCTTTAGTGATTGTGAAAAGAGTGCAGATATGCGCTTC 1680
PCR-product   1621 AGTGAAGGCCGTTTTGCCTTTGGCTTTAGTGATTGTGAAAAGAGTGCAGATATGCGCTTC 1680

P423::luxAB   1681 TTTAATCGACCAACGGATTCTCAGTTTCAGTTGTTCAGTGAGTGTCACAAGATCATCAAT 1740
PCR-product   1681 TTTAATCGACCAACGGATTCTCAGTTTCAGTTGTTCAGTGAGTGTCACAAGATCATCAAT 1740

P423::luxAB   1741 GATGCATTCACTACTGGGTACTGCCATCCAAACAATGATTTTTATAGTTTTCCTAAAATC 1800
PCR-product   1741 GATGCATTCACTACTGGGTACTGCCATCCAAACAATGATTTTTATAGTTTTCCTAAAATC 1800

P423::luxAB   1801 TCCGTTAACCCACACGCGTTCACTGAAGGCGGTCCTGCGCAATTTGTGAATGCGACGAGC 1860
PCR-product   1801 TCCGTTAACCCACACGCGTTCACTGAAGGCGGTCCTGCGCAATTTGTGAATGCGACGAGC 1860

P423::luxAB   1861 AAAGAAGTGGTTGAATGGGCGGCTAAGTTAGGGCTTCCACTCGTGTTTAGATGGGACGAC 1920
PCR-product   1861 AAAGAAGTGGTTGAATGGGCGGCTAAGTTAGGGCTTCCACTCGTGTTTAGATGGGACGAC 1920

P423::luxAB   1921 TCAAACGCTCAAAGAAAAGAATACGCCGGTTTGTACCACGAAGTTGCTCAGGCACATGGT 1980
PCR-product   1921 TCAAACGCTCAAAGAAAAGAATACGCCGGTTTGTACCACGAAGTTGCTCAGGCACATGGT 1980

P423::luxAB   1981 GTCGATGTTAGTCAGGTTCGACACAAGCTGACGCTGCTGGTCAACCAAAATGTAGATGGT 2040
PCR-product   1981 GTCGATGTTAGTCAGGTTCGACACAAGCTGACGCTGCTGGTCAACCAAAATGTAGATGGT 2040

P423::luxAB   2041 GAAGCAGCAAGGGCAGAAGCTCGCGTGTATTTGGAAGAGTTTGTCCGTGAATCTTACTCA 2100
PCR-product   2041 GAAGCAGCAAGGGCAGAAGCTCGCGTGTATTTGGAAGAGTTTGTCCGTGAATCTTACTCA 2100

P423::luxAB   2101 AATACCGACTTTGAGCAAAAAATGGGAGAGCTGTTGTCAGAAAATGCCATCGGTACTTAT 2160
PCR-product   2101 AATACCGACTTTGAGCAAAAAATGGGAGAGCTGTTGTCAGAAAATGCCATCGGTACTTAT 2160

P423::luxAB   2161 GAAGAAAGTACTCAGGCAGCGCGAGTTGCGATTGAGTGTTGTGGTGCCGCGGACCTATTG 2220
PCR-product   2161 GAAGAAAGTACTCAGGCAGCGCGAGTTGCGATTGAGTGTTGTGGTGCCGCGGACCTATTG 2220

P423::luxAB   2221 ATGTCTTTTGAGTCGATGGAAGATAAAGCGCAGCAAAGAGCGGTTATCGATGTGGTAAAC 2280
PCR-product   2221 ATGTCTTTTGAGTCGATGGAAGATAAAGCGCAGCAAAGAGCGGTTATCGATGTGGTAAAC 2280

P423::luxAB   2281 GCCAACATCGTCAAATACCACTCGTAACGTGTCGAC 2316
PCR-product   2281 GCCAACATCGTCAAATACCACTCGTAACGTGTCGAC 2316
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Fig. 9. DNA alignment of the construct PCR-product with P423::*luxAB*. The first 241 nucleotides (not high lighted) represent the extended P423 promoter area (from GenBank accession AF304384 and sequence obtained with SUP-right primer) and the nucleotides from position 242 to 2316 (high lighted) the *luxAB* dicistron (GenBank accession M10961). The –10 and –35 positions and the ribosomal binding sites (RBS) are underlined. Translational start and stop codons of *luxA* and *luxB* are printed in bold. Added restriction endonuclease sites (*Xba*I and *Sal*I) are bold and underlined.

```
T7::luxAB      1   TCTAGAGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGAT 60
PCR-product    1   TCTAGAGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGAT 60

T7::luxAB     61   TAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAAT 120
PCR-product   61   TAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAAT 120

T7::luxAB    121   TGTAATACGACTCACTATAGGGCGAATTGGGCCCGACGTCGCATGCTAAGGAAATGTTATT 180
PCR-product  121   TGTAATACGACTCACTATAGGGCGAATTGGGCCCGACGTCGCATGCTAAGGAAATGTTATT 180
                                                          RBS
T7::luxAB    181   GAAATTTGGAAACTTCCTTCTCACTTATCAGCCACCTGAGCTATCTCAGACCGAAGTGAT 240
PCR-product  181   GAAATTTGGAAACTTCCTTCTCACTTATCAGCCACCTGAGCTATCTCAGACCGAAGTGAT 240

T7::luxAB    241   GAAGCGATTGGTTAATCTGGGCAAAGCGTCTGAAGGTTGTGGCTTCGACACCGTTTGGTT 300
PCR-product  241   GAAGCGATTGGTTAATCTGGGCAAAGCGTCTGAAGGTTGTGGCTTCGACACCGTTTGGTT 300

T7::luxAB    301   GCTAGAGCACCACTTCACTGAATTTGGGTTGTTAGGGAATCCTTATGTTGCTGCCGCACA 360
PCR-product  301   GCTAGAGCACCACTTCACTGAATTTGGGTTGTTAGGGAATCCTTATGTTGCTGCCGCACA 360

T7::luxAB    361   CCTATTAGGTGCGACAGAAACGCTCAACGTTGGCACTGCAGCTATCGTATTGCCGACTGC 420
PCR-product  361   CCTATTAGGTGCGACAGAAACGCTCAACGTTGGCACTGCAGCTATCGTATTGCCGACTGC 420

T7::luxAB    421   CCATCCGGTTCGACAAGCAGAAGACGTAAACCTACTGGATCAAATGTCAAAAGGACGATT 480
PCR-product  421   CCATCCGGTTCGACAAGCAGAAGACGTAAACCTACTGGATCAAATGTCAAAAGGACGATT 480

T7::luxAB    481   CCGTTTTGGTATTTGTCGCGGTTTGTACGATAAAGATTTTCGTGTCTTTGGTACAGACAT 540
PCR-product  481   CCGTTTTGGTATTTGTCGCGGTTTGTACGATAAAGATTTTCGTGTCTTTGGTACAGACAT 540

T7::luxAB    541   GGATAACAGCCGAGCCTTAATGGACTGTTGGTATGACTTGATGAAAGAAGGCTTCAATGA 600
PCR-product  541   GGATAACAGCCGAGCCTTAATGGACTGTTGGTATGACTTGATGAAAGAAGGCTTCAATGA 600

T7::luxAB    601   AGGCTATATCGCGGCGGATAACGAACATATTAAGTTCCCGAAAATCCAACTGAATCCATC 660
PCR-product  601   AGGCTATATCGCGGCGGATAACGAACATATTAAGTTCCCGAAAATCCAACTGAATCCATC 660

T7::luxAB    661   GGCTTACACACAAGGTGGTGCTCCTGTTTATGTCGTCGCGGAGTCAGCATCAACGACAGA 720
PCR-product  661   GGCTTACACACAAGGTGGTGCTCCTGTTTATGTCGTCGCGGAGTCAGCATCAACGACAGA 720

T7::luxAB    721   ATGGGCTGCAGAGCGTGGCCTACCAATGATTCTAAGCTGGATCATCAACACTCACGAGAA 780
PCR-product  721   ATGGGCTGCAGAGCGTGGCCTACCAATGATTCTAAGCTGGATCATCAACACTCACGAGAA 780

T7::luxAB    781   GAAAGCGCAGCTTGATCTTTACAACGAAGTCGCGACTGAACATGGCTACGATGTGACTAA 840
PCR-product  781   GAAAGCGCAGCTTGATCTTTACAACGAAGTCGCGACTGAACATGGCTACGATGTGACTAA 840

T7::luxAB    841   GATTGACCACTGTTTGTCTTACATCACCTCCGTCGATCATGACTCAAATAGAGCCAAAGA 900
PCR-product  841   GATTGACCACTGTTTGTCTTACATCACCTCCGTCGATCATGACTCAAATAGAGCCAAAGA 900

T7::luxAB    901   TATTTGCCGCAACTTCTTGGGCCATTGGTACGACTCATACGTGAATGCCACCAAGATTTT 960
PCR-product  901   TATTTGCCGCAACTTCTTGGGCCATTGGTACGACTCATACGTGAATGCCACCAAGATTTT 960

T7::luxAB    961   TGACGACTCTGACCAAACAAAAGGTTACGACTTCAATAAAGGTCAATGGCGTGATTTTGT 1020
PCR-product  961   TGACGACTCTGACCAAACAAAAGGTTACGACTTCAATAAAGGTCAATGGCGTGATTTTGT 1020

T7::luxAB   1021   GTTGAAAGGCCACAAAGACACCAATCGCCGAATTGATTACAGCTACGAAATCAACCCAGT 1080
PCR-product 1021   GTTGAAAGGCCACAAAGACACCAATCGCCGAATTGATTACAGCTACGAAATCAACCCAGT 1080

T7::luxAB   1081   AGGGACGCCTGAAGAGTGTATCGCGATTATCCAGCAAGATATTGATGCGACGGGTATTGA 1140
PCR-product 1081   AGGGACGCCTGAAGAGTGTATCGCGATTATCCAGCAAGATATTGATGCGACGGGTATTGA 1140

T7::luxAB   1141   CAATATTTGTTGTGGTTTTGAAGCAAACGGTTCTGAAGAAGAAATTATCGCATCTATGAA 1200
PCR-product 1141   CAATATTTGTTGTGGTTTTGAAGCAAACGGTTCTGAAGAAGAAATTATCGCATCTATGAA 1200
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T7::luxAB     1201 GCTATTCCAGTCTGATGTGATGCCATATCTCAAAGAAAAACAGTAATTAATATTTTCTAA 1260
PCR-product   1201 GCTATTCCAGTCTGATGTGATGCCATATCTCAAAGAAAAACAGTAATTAATATTTTCTAA 1260

T7::luxAB     1261 AAGGAAAGAGACATGAAATTTGGATTATTCTTCCTCAATTTTATGAATTCAAAGCGTTCT 1320
PCR-product   1261 AAGGAAAGAGACATGAAATTTGGATTATTCTTCCTCAATTTTATGAATTCAAAGCGTTCT 1320
                      RBS
T7::luxAB     1321 TCTGATCAAGTCATCGAAGAAATGTTAGATACCGCACATTACGTAGATCAGTTGAAGTTT 1380
PCR-product   1321 TCTGATCAAGTCATCGAAGAAATGTTAGATACCGCACATTACGTAGATCAGTTGAAGTTT 1380

T7::luxAB     1381 GACACGTTGGCTGTTTACGAAAACCATTTCTCGAACAATGGTGTGGTTGGTGCCCCACTA 1440
PCR-product   1381 GACACGTTGGCTGTTTACGAAAACCATTTCTCGAACAATGGTGTGGTTGGTGCCCCACTA 1440

T7::luxAB     1441 ACAGTGGCTGGTTTTTTACTTGGTATGACAAAGAACGCCAAAGTGGCTTCGTTGAATCAC 1500
PCR-product   1441 ACAGTGGCTGGTTTTTTACTTGGTATGACAAAGAACGCCAAAGTGGCTTCGTTGAATCAC 1500

T7::luxAB     1501 GTCATTACCACGCATCATCCAGTACGTGTGGCGGAAGAAGCGTGTCTACTTGACCAAATG 1560
PCR-product   1501 GTCATTACCACGCATCATCCAGTACGTGTGGCGGAAGAAGCGTGTCTACTTGACCAAATG 1560

T7::luxAB     1561 AGTGAAGGCCGTTTTGCCTTTGGCTTTAGTGATTGTGAAAAGAGTGCAGATATGCGCTTC 1620
PCR-product   1561 AGTGAAGGCCGTTTTGCCTTTGGCTTTAGTGATTGTGAAAAGAGTGCAGATATGCGCTTC 1620

T7::luxAB     1621 TTTAATCGACCAACGGATTCTCAGTTTCAGTTGTTCAGTGAGTGTCACAAGATCATCAAT 1680
PCR-product   1621 TTTAATCGACCAACGGATTCTCAGTTTCAGTTGTTCAGTGAGTGTCACAAGATCATCAAT 1680

T7::luxAB     1681 GATGCATTCACTACTGGGTACTGCCATCCAAACAATGATTTTTATAGTTTTCCTAAAATC 1740
PCR-product   1681 GATGCATTCACTACTGGGTACTGCCATCCAAACAATGATTTTTATAGTTTTCCTAAAATC 1740

T7::luxAB     1741 TCCGTTAACCCACACGCGTTCACTGAAGGCGGTCCTGCGCAATTTGTGAATGCGACGAGC 1800
PCR-product   1741 TCCGTTAACCCACACGCGTTCACTGAAGGCGGTCCTGCGCAATTTGTGAATGCGACGAGC 1800

T7::luxAB     1801 AAAGAAGTGGTTGAATGGGCGGCTAAGTTAGGGCTTCCACTCGTGTTTAGATGGGACGAC 1860
PCR-product   1801 AAAGAAGTGGTTGAATGGGCGGCTAAGTTAGGGCTTCCACTCGTGTTTAGATGGGACGAC 1860

T7::luxAB     1861 TCAAACGCTCAAAGAAAAGAATACGCCGGTTTGTACCACGAAGTTGCTCAGGCACATGGT 1920
PCR-product   1861 TCAAACGCTCAAAGAAAAGAATACGCCGGTTTGTACCACGAAGTTGCTCAGGCACATGGT 1920

T7::luxAB     1921 GTCGATGTTAGTCAGGTTCGACACAAGCTGACGCTGCTGGTCAACCAAAATGTAGATGGT 1980
PCR-product   1921 GTCGATGTTAGTCAGGTTCGACACAAGCTGACGCTGCTGGTCAACCAAAATGTAGATGGT 1980

T7::luxAB     1981 GAAGCAGCAAGGGCAGAAGCTCGCGTGTATTTGGAAGAGTTTGTCCGTGAATCTTACTCA 2040
PCR-product   1981 GAAGCAGCAAGGGCAGAAGCTCGCGTGTATTTGGAAGAGTTTGTCCGTGAATCTTACTCA 2040

T7::luxAB     2041 AATACCGACTTTGAGCAAAAAATGGGAGAGCTGTTGTCAGAAAATGCCATCGGTACTTAT 2100
PCR-product   2041 AATACCGACTTTGAGCAAAAAATGGGAGAGCTGTTGTCAGAAAATGCCATCGGTACTTAT 2100

T7::luxAB     2101 GAAGAAAGTACTCAGGCAGCGCGAGTTGCGATTGAGTGTTGTGGTGCCGCGGACCTATTG 2160
PCR-product   2101 GAAGAAAGTACTCAGGCAGCGCGAGTTGCGATTGAGTGTTGTGGTGCCGCGGACCTATTG 2160

T7::luxAB     2161 ATGTCTTTTGAGTCGATGGAAGATAAAGCGCAGCAAAGAGCGGTTATCGATGTGGTAAAC 2220
PCR-product   2161 ATGTCTTTTGAGTCGATGGAAGATAAAGCGCAGCAAAGAGCGGTTATCGATGTGGTAAAC 2220

T7::luxAB     2221 GCCAACATCGTCAAATACCACTCGTAACGTGTCGAC 2256
PCR-product   2221 GCCAACATCGTCAAATACCACTCGTAACGTGTCGAC 2256
```

Fig. 10. DNA alignment of the construct PCR-product with T7::*luxAB*. The first 167 nucleotides (not high lighted) represent the T7 promoter area (from Promega Notes for pGEM-T Easy) and the nucleotides from position 168 to 2265 (high lighted) the *luxAB* dicistron (GenBank accession M10961). The ribosomal binding sites (RBS) are underlined. Translational start and stop codons of *luxA* and *luxB* are printed in bold. Added restriction endonuclease sites (*Xba*I and *Sal*I) are bold and underlined.

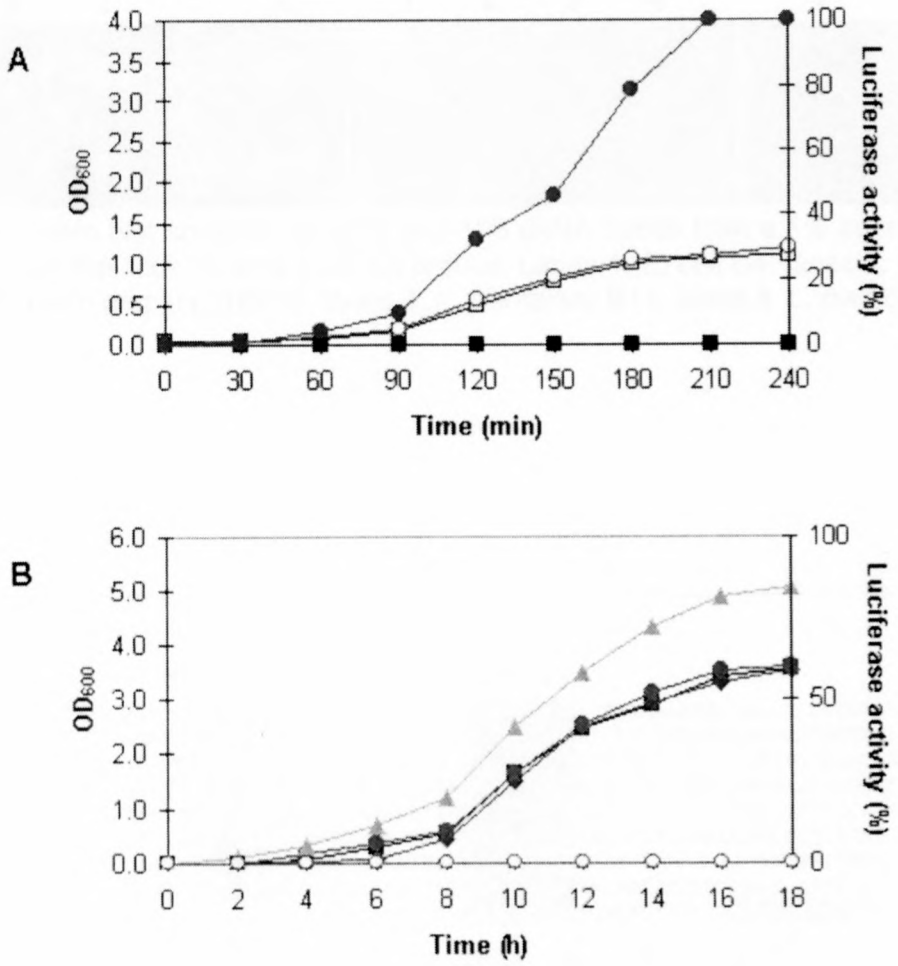

Fig. 11. **(A)** Bioluminescence of *E. coli* strains. Squared markers represent *E. coli* JM 109 DE3 with empty plasmid (pTRKH2). Circular markers represent *E. coli* C4. Open markers (□) and (o) = OD values. Closed markers (■) and (•) = luciferase activity. **(B)** Bioluminescence of *L. plantarum* strains. Squared markers represent the wild type strain, *L. plantarum* 423. Circular markers represent *L. plantarum* 423 B^- with the empty plasmid pTRKH2. Diamond shaped markers represent *L. plantarum* B11 and the triangular markers yield strain B11 grown in the presence of *L. innocua* LMG 13568. Open markers (□), (o), (◊) and (Δ) = luciferase activity. Closed markers (■), (•), (♦) and (▲) = OD values. The external addition of plantaricin 423 had no effect on the luciferase activity (results not shown).

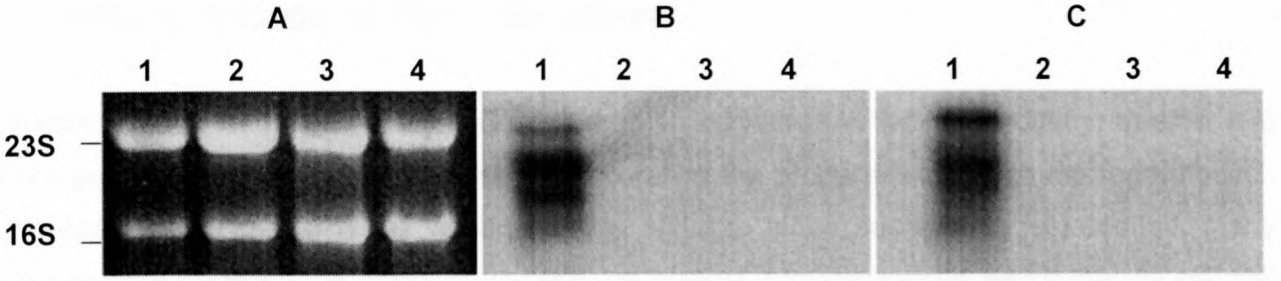

Fig. 12. Northern blot analysis. (A) 23S and 16S rRNA bands from a 1% agarose formamide gel and blots from the *luxA* (B) and *luxB* (C) probes. Lanes 1, *E. coli* C4; lanes 2, *E. coli* JM109 DE3 transformed with empty pTRKH2; lanes 3, *L. plantarum* B11; lanes 4, *L. plantarum* 423

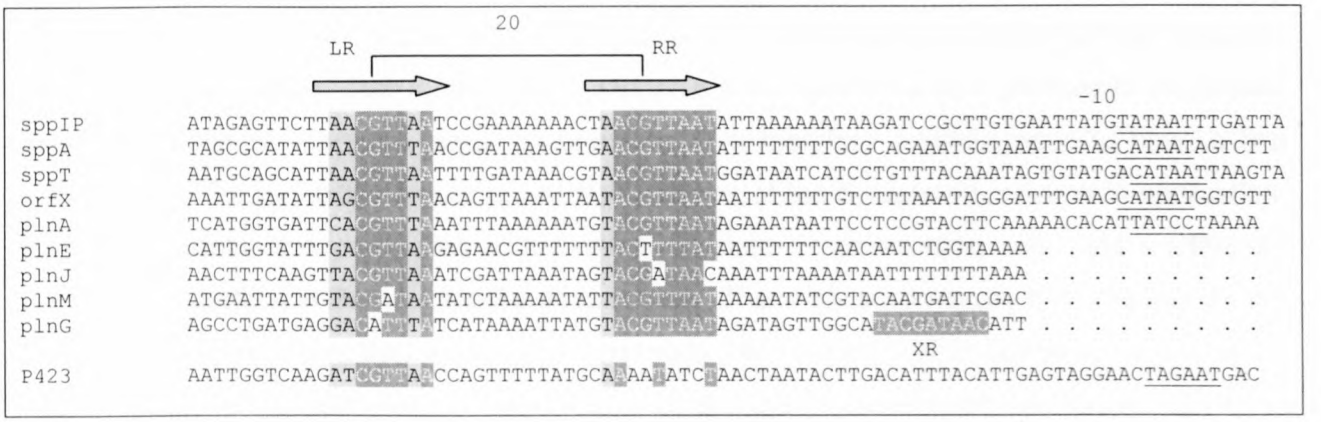

Fig. 13. Inducible promoters from *spp* regulon in *Lb. sakei* LTH673 and the promoter upstream of the *pln* genes in *Lb. plantarum* C11 compared to P423, the promoter upstream of the *plaA* gene in *Lb. plantarum* 423. Arrows and labels, LR (left repeat) and RR (right repeat), indicate repeats, and (putative) –10 regions are underlined. The consensus sequence marked in dark grey (white font) shows the nucleotides conserved by 100% and those in light grey, at least 50%. Inverted and direct repeats can be seen in the various promoters. The distance between individual basepairs observed in the repeated palindromic sequence is 20bp which corresponds to two DNA helical turns (Brurberg, 1997). Note the extra repeat (XR) in plnG, downstream of the right repeat. The sequences shown are: sppIP, sppA, sppT: GenBank accession number AF002276; orfX: Z48542; plnA, plnE, plnJ, plnM, plnG: X75323; P423: P423-SUP, this study.

5. GENERAL DISCUSSION AND CONCLUSIONS

Bacteriocins have the potential to be applied as preservatives in the food industry, whether it is in a pure isolated form or indirectly by incorporating the producer strain into the food product. However, much research lies ahead for this principle to be successfully established. Many factors may influence the action of bacteriocins against microorganisms, which include pH, cell concentration, lipid content, proteolytic enzymes and liquid vs. solid medium (Abee et al., 1995). These are factors that need to be taken into account when applying bacteriocins in the food industry.

The actual amount of bacteriocin produced by the producer strain may pose a problem for their use as biopreservatives. Plantaricin 423 produced by *Lactobacillus plantarum* has been increased in a previous study by optimising its fermentation conditions (Verellen et al., 1998). There are several reports on bacteriocin production by Gram-positive bacteria that are regulated via complex processes. This means that bacteriocin production may be increased or decreased in the presence of a natural trigger mechanism. It is therefore advantageous to have a firm knowledge of the regulation and/or production patterns of the bacteriocin required in application. Examples include the production of two modified class I bacteriocins, nisin and subtilin, by *Lactococcus lactis* and *Bacillus subtilis*, respectively. These bacteriocins are both regulated via a cell-density dependent quorum-sensing mechanism (Klein et al., 1993; Gutowski-Ecked, et al., 1994; Kuipers et al., 1995, 1998; Kleerebezem et al., 1997; Kleerebezem and Quadri 2001). In these cases, the bacteriocins also act as autoinducers. The production of many class II bacteriocins are also regulated via a quorum-sensing mechanism. In these cases peptide pheromones or autoinducer peptides, which lack antimicrobial activity, mediate their regulation. Examples of regulated production of class II bacteriocins include that of plantaricins E/F and J/K by *Lactobacillus plantarum* C11 (Diep et al., 1994, 1995, 1996; Moll et al., 1999), plantaricin ABP-118 by *Lactobacillus salivarius* subsp. *salivarius* UCC118 (Flynn et al., 2002), sakacin A by *L. sakei* Lb706 (Axelsson and Holck 1995; Diep et al., 2000), sakacin P by *Lactobacillus sake* LTH673 (Eijsink et al., 1996; Brurberg et al., 1997), enterocins A and B by *Enterococcus faecium* CTC 492 (Nilsen et al. 1998) and carnobacteriocins A and B2 by *Carnobacterium piscicola* LV17 (Saucier et al. 1995; Quadri et al. 1997; Franz et al. 2000; Kleerebezem et al. 2001).

The results from our study have led to the hypothesis that the production of plantaricin 423 is constitutive. The cell-free, pure culture supernatant of *Lactobacillus plantarum* 423 did not seem

to act either as an autoinducer or as a pheromone type induction factor which should have caused the increased production of plantaricin 423. Neither did the presence of a living or heat killed sensitive strain (*Listeria innocua* LMG 13568 and *Lactobacillus sakei* LMG 13558) induce plantaricin 423 production.

In one of the latest regulation studies the presence of different Gram-positive bacteria have been tested as possible induction stimuli for a class II bacteriocin (Maldonado et al., 2004). The results indicated that the inducer and sensitive phenotypes might not be linked. In other words, there was no apparent relationship between the sensitivity of the strains and their ability to induce the bacteriocin. Maldonado et al. (2004) reported that induction was promoted by both living and heat killed cells of the inducing bacteria. These findings may contribute to our hypothesis of constitutive production since neither living nor heat killed strains were able to induce production of plantaricin 423. On the other hand, there might be specific Gram-positive bacteria that could act as inducing bacteria as was the case for Maldonado et al. (2004).

Temperature, pH, and other growth conditions have been proposed to play an important role in the regulation of bacteriocin production (Diep et al., 1995; Saucier et al., 1995; Nes et al. 1996; Kleerebezem et al., 1997; Nilsen et al., 1998; Kleerebezem and Quadri, 2001), though little or nothing is known about how these factors interact with the regulatory systems controlling bacteriocin production. These reports also suggested that peptide pheromones released during quorum-sensing are not sufficient to either trigger or maintain bacteriocin production when they are highly diluted (as may be the case for media established cultures). Therefore, regulation by other, unknown factors may still exist as was seen for the fermentation optimisation of plantaricin 423 (Verellen et al., 1998). These factors have not been included in our study and might be a suggestion towards future research.

Our study is the first in which an *in vivo* reporter system was applied to quantify the production of plantaricin 423. The *luxAB* genes from *Vibrio harveyi*, which encodes the bacterial bioluminescence enzyme, luciferase, was cloned and electrotransformed into a pPLA4 cured strain of *Lactobacillus plantarum* 423. No bioluminescence was expressed with or without the presence of possible induction factors. This may be due to the unstable construct of Gram-negative genes fused and cloned to a Gram-positive promoter and strain, respectively. Previous reports have shown *luxAB* expression, but only in low levels, in Gram-positive bacteria (Carmi et al., 1987; Blissett and Stewart, 1989; Karp and Meyer, 1989; Schauer et al., 1989; Stewart et al.,

1989; Ahmad and Stewart, 1990; Jassim et al., 1990; Phillips-Jones, 2000; Simon et al., 2001; Beard et al., 2002). Another explanation for the inability to express *luxAB* may be due to an unknown constitutive area of the plantaricin 423 promoter which might have been left out of the construct. Previous evidence (Risøen et al., 2000) suggests that the DNA region necessary for constitutive expression is upstream of the –80 and –40 repeats found in regulated promoters (Diep et al., 1996; Brurberg et al., 1997) which is necessary for the binding of response regulators involved in quorum-sensing regulation. These repeats are also found in the plantaricin 423 promoter, but the –40 is much less conserved, leading to the inability of response regulators to bind to the DNA. This also contributes to the constitutive expression of plantaricin 423 hypothesis. Further sequencing of the pPLA4 plasmid may clarify some unanswered questions.

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