print
ISSN 1464-1801
Vol. 13, No. 4, 2007
13 4 07
online
ISSN 1660-2412
www.karger.com/mmb
ISBN 978-3-8055-8353-8

Journal of Molecular Microbiology and Biotechnology

Antimicrobial Peptides: Food, Veterinary and Medical Applications

Editor
Djamel Drider, Nantes

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Journal of
Molecular Microbiology
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27 figures, 2 in color, and 10 tables, 2007

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Printed in Switzerland on acid-free paper by
Reinhardt Druck, Basel
ISBN 978–3–8055–8355–8

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Vol. 13, No. 4, 2007

Journal of
Molecular Microbiology
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Journal of
Molecular Microbiology
and Biotechnology

J Mol Microbiol Biotechnol 2007;13:193
DOI: 10.1159/000107581

Introduction

The first international symposium on *Antimicrobial Peptides: Food, Veterinary and Medical Applications* was organized and presided over by Dr. Djamel Drider and his colleagues Dr. Nathalie Caroff (Nantes University), Dr. Marie-France de la Cochetière (Inserm Nantes), Dr. Gunnar Fimland (Oslo University), Dr. Ismaïl Fliss (Laval University), Dr. Charles Hétru (CNRS Strasbourg), Dr. Pablo Hernandez (Madrid University), Dr. Nacer Lounis (John Hopkins University), Dr. Hervé Prévost (ENITIAA Nantes) and Dr. Françoise Vovelle (Orléans University). This first symposium took place at the Ecole Nationale d'Ingénieurs des Techniques Agricoles et Alimentaires (ENITIAA; National School for Engineers in Agricultural and Food Science, Nantes, France), from 21 to 23 June 2006.

This conference provided an excellent balance, combining fundamental and applied seminars. For this first meeting, more than 170 participants attended from different continents and countries, in order to discuss and illustrate future prospects of antimicrobial peptide science. A new avenue is open in terms of fundamental and applied purposes, and new challenges remain to be overcome, such as the study of resistance mechanisms, the mode of action, and the relation between the structure and function of antimicrobial peptides. There is also a growing and urgent interest in food areas to find new peptides with potential applications, mainly against *Campylobacter jejuni*, a leading cause of food-borne illness in many developed countries. In the medical arena, there is also a need to investigate more antimicrobial peptides and find novel therapeutic agents that could be used to complement conventional antibiotic therapies.

Naturally, the success of this conference was possible thanks to financial support obtained generously from private companies (Biohit, France; Novozymes, Danemark; Bio-Rad, France; Karger, Switzerland; Blackwell Publishing, UK), intellectual groups (The European Peptide Society, French Microbiology Society) and local institutions (ENITIAA Nantes, La Region des Pays de la Loire, Nantes Métropole).

The president of this symposium would like to express his gratitude to the organizers, scientific committee, reviewers, participants, ENITIAA Nantes, who provided materials, and especially to Ms. Béatrice Hélie, for making this conference successful and wishes to update the participants, in due time, on the organization of the second international symposium on antimicrobial peptides.

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Review

Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol 2007;13:194–199
DOI: 10.1159/000104752

Bacteriocins from Lactic Acid Bacteria: Production, Purification, and Food Applications

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Key Words
Bacteriocins · Bacteriocins, food application · Bacteriocins, production · Bacteriocins, purification · Lactic acid bacteria · Lactic acid bacteria, antimicrobial potential

Abstract
In fermented foods, lactic acid bacteria (LAB) display numerous antimicrobial activities. This is mainly due to the production of organic acids, but also of other compounds, such as bacteriocins and antifungal peptides. Several bacteriocins with industrial potential have been purified and characterized. The kinetics of bacteriocin production by LAB in relation to process factors have been studied in detail through mathematical modeling and positive predictive microbiology. Application of bacteriocin-producing starter cultures in sourdough (to increase competitiveness), in fermented sausage (anti-listerial effect), and in cheese (anti-listerial and anti-clostridial effects), have been studied during in vitro laboratory fermentations as well as on pilot-scale level. The highly promising results of these studies underline the important role that functional, bacteriocinogenic LAB strains may play in the food industry as starter cultures, co-cultures, or bioprotective cultures, to improve food quality and safety. In addition, antimicrobial production by probiotic LAB might play a role during in vivo interactions occurring in the human gastrointestinal tract, hence contributing to gut health.

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Introduction

Lactic acid bacteria (LAB) have a long history of application in fermented foods because of their beneficial influence on nutritional, organoleptic, and shelf-life characteristics [1, 2]. They cause rapid acidification of the raw material through the production of organic acids, mainly lactic acid. In addition, their production of acetic acid, ethanol, aroma compounds, bacteriocins, exopolysaccharides, and several enzymes is of importance. Whereas a food fermentation process with LAB is traditionally based on spontaneous fermentation or backslopping, industrial food fermentation is nowadays performed by the deliberate addition of LAB as starter cultures to the food matrix. This has been a breakthrough in the processing of fermented foods, resulting in a high degree of control over the fermentation process and standardization of the end products. Recently, the use of functional starter cultures, a novel generation of starter cultures that offers functionalities beyond acidification, is being explored [2–4]. For instance, LAB are capable of inhibiting various microorganisms in a food environment and display crucial antimicrobial properties with respect to food preservation and safety. In addition, it has been shown that some strains of LAB possess interesting health-promoting properties; one of the characteristics of these probiotics is the potential to combat gastrointestinal pathogenic bacteria such as *Helicobacter pylori*, *Escherichia*

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1464–1801/07/0134–0194$23.50/0

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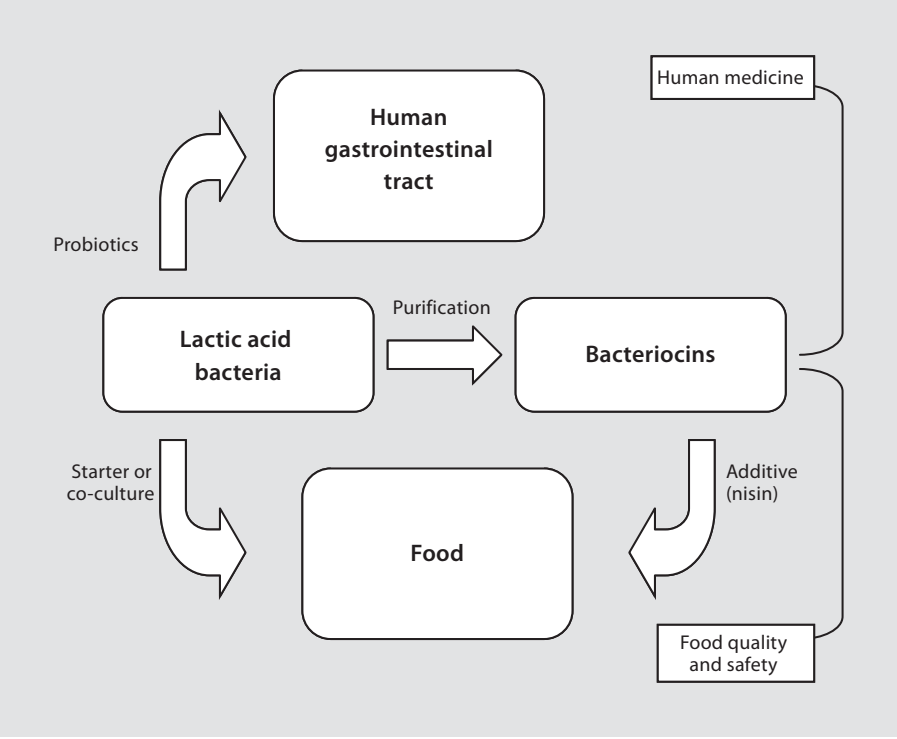

Fig. 1. Overview of the application potential of bacteriocin production by LAB in food quality and safety and in medicine, emphasizing their role as food ingredient and in the human gastrointestinal tract, respectively.

coli, and *Salmonella*. This paper focuses on the role of bacteriocins as fast-acting, antibacterial peptides in both food safety and gastrointestinal health (fig. 1).

Antimicrobial Potential of Lactic Acid Bacteria

LAB display a wide range of antimicrobial activities. Amongst these activities, the production of lactic acid and acetic acid is obviously the most important. However, certain strains of LAB are further known to produce bioactive molecules such as ethanol, formic acid, fatty acids, hydrogen peroxide, diacetyl, reuterin, and reutericyclin. Many strains also produce bacteriocins and bacteriocin-like molecules that display antibacterial activity [5]. Besides the production of bacteriocins, some LAB are able to synthesize other antimicrobial peptides that may also contribute to food preservation and safety. For instance, strains of *Lactobacillus plantarum*, isolated from sourdough and grass silage, display antifungal activity, due to the production of organic acids, other low-molecular-mass metabolites, and/or cyclic dipeptides [6–8]. It is not unlikely that additional, new antimicrobial peptides are to be discovered [9]. Although still in its infancy, there is good reason to believe that genomics will soon become an essential tool for exploring the antimicrobial potency of LAB [10]. Interestingly, bacteriocin screening programs have yielded, during the last decades, a large arsenal of bacteriocins with different properties, target species, and producer organisms [11].

Bacteriocins, a Class of Antibacterial Peptides for Promising Applications

Although bacteriocins may be found in many Gram-positive and Gram-negative bacteria [12], those produced by LAB have received particular attention in recent years due to their potential application in the food industry as natural preservatives [13]. Bacteriocins produced by LAB are small, ribosomally synthesized, antimicrobial peptides or proteins that possess activity towards closely related Gram-positive bacteria, whereas producer cells are immune to their own bacteriocin(s) [5, 11, 14]. The antibacterial spectrum frequently includes spoilage organisms and food-borne pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus*. Besides their antimicrobial action towards undesirable bacteria, bacteriocins are believed to contribute to the competitiveness of the producer cells [15]. Activity against Gram-negative bac-

teria such as *E. coli* and *Salmonella* has been shown, but usually only when the integrity of the outer membrane has been compromised, for example after osmotic shock or low pH treatment, in the presence of a detergent or chelating agent, or after pulsed electric field or high-pressure treatment [16].

Among bacteriocins from LAB, distinction can be made between (i) lantibiotics or small, heat-stable, lanthionine-containing, single- and two-peptide bacteriocins (class I), whose inactive prepeptides are subject to extensive post-translational modification; (ii) peptide bacteriocins or small, heat-stable, non-lanthionine-containing bacteriocins (class II), including pediocin-like or *Listeria*-active bacteriocins (class IIa), two-peptide bacteriocins (class IIb), and circular bacteriocins (class IIc), and, arguably, (iii) bacteriolysins or large, heat-labile, lytic proteins, often murein hydrolases (class III) [14]. Extensive efforts have been made to resolve the relationship between structure and function for both class I and class II bacteriocins [17, 18]. The majority of the class I and class II bacteriocins are active in the nanomolar range, causing membrane permeabilization, leading to the dissipation of membrane potential and the leakage of ions, ATP, and other vital molecules from the target bacteria [14].

Production and Purification of Bacteriocins

Although bacteriocins can be produced in the food matrix during food fermentation, bacteriocins by LAB can be produced in much higher amounts during in vitro fermentations under optimal physical and chemical conditions [19]. The higher in vitro production is due to the absence of limiting factors, such as strong diffusion limitations, inactivation by proteases, and the adsorption to food particles [20]. However, even during controlled fermentor experiments, considerable differences in activity yields are obtained, and an influence of the environmental process conditions on the obtained bacteriocin activity can be seen. For instance, a decrease of pH results in a decreased adsorption of the bacteriocin molecules to the producer cells, and hence in an increased bioavailability [21, 22]. In addition, temperature and pH [23–25] as well as nutrient availability [26–28] seem to play a crucial role in bacteriocin production, whereas the presence of elevated amounts of sodium chloride usually decreases production levels [29, 30]. In general, the cultivation conditions directly affect bacteriocin production as such (specific bacteriocin production in particular) and, indirectly, through biomass production. This is to be explained by the fact that bacteriocin production is a growth-dependent physiological trait and hence follows primary metabolite kinetics [22–24].

Three major methods for the purification of bacteriocins by LAB to homogeneity can be distinguished. First, purification can be done by a conventional method that is based on a rather laborious series of subsequent steps of ammonium sulfate precipitation, ion exchange, hydrophobic interaction, gel filtration, and reversed-phase high-pressure liquid chromatography [31–33]. Second, a simple three-step protocol has been developed [34], including (i) ammonium sulfate precipitation, (ii) chloroform/methanol extraction/precipitation, and (iii) reversed-phase high-pressure liquid chromatography, the sole chromatographic step involved. Third, bacteriocins can be isolated through a unique unit operation, i.e. expanded bed adsorption, using a hydrophobic interaction gel, after maximizing the bioavailable bacteriocin titer through pH adjustment of the crude fermentation medium [35, 36]. Following the latter two methods, which are more rapid than the first conventional method and yet successful, several bacteriocins with interesting industrial potential have been purified, such as the class II bacteriocins amylovorin L (produced by *Lactobacillus amylovorus* DCE 471) and several enterocins (produced by the *Enterococcus faecium* RZS C5, RZS C13, and FAIR-E 406 strains), and the lantibiotic macedocin (produced by *Streptococcus macedonicus* ACA-DC 198) [34, 37–39].

Bacteriocin Production in Foods: Application Possibilities

Bacteriocins can be used as food additives. For instance, nisin is commercially made in a partially purified form [5, 18] and a marketed preparation with the pediocin PA-1 (AcH) producer is available [40]. As an alternative to the addition of bacteriocins to foods, bacteriocins may be produced directly in the food as a result of starter culture or co-culture activity [2]. Several studies have indeed indicated that LAB starter cultures or co-cultures are able to produce their bacteriocins in food matrices, and consequently display inhibitory activity towards sensitive food spoilage or pathogenic bacteria. The latter trait has mainly been documented for fermented sausage, fermented vegetables and olives, and dairy products [2, 13]. For instance, bacteriocin extraction has been demonstrated in the case of Cheddar cheese [41] and fermented

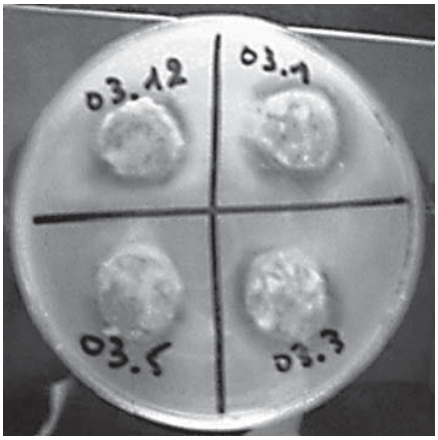

Fig. 2. In situ bacteriocin production by *Lactobacillus curvatus* LTH 1174, as demonstrated by the inhibition zones caused by pieces of Belgian-style fermented sausage on a bacteriocin-sensitive indicator layer containing *Listeria innocua* LMG 13568, in a direct diffusion assay. The pieces of meat were obtained after 1 (03.1), 3 (03.3), 5 (03.5), and 12 (03.12) days of fermentation. The fermented sausages were prepared with a commercial starter culture, supplemented with *L. curvatus* LTH 1174, at an inoculation level of 7.0 log colony-forming units per gram. Control sausages prepared with the same commercial starter culture, but without *L. curvatus* LTH 1174, did not yield inhibition zones.

Lactacin Fa	RNNWQTNVGGAVGSAMIGATVGGTICGPACAVAGAHYLPILWT**A**VTAATGGFGKIRK
Lactacin F	RNNWQTNVGGAVGSAMIGATVGGTICGPACAVAGAHYLPILWT**G**VTAATGGFGKIRK

Fig. 3. Comparison of the amino acid sequence of lactacin F (produced by *Lactobacillus johnsonii* VPI 11088) and lactacin Fa (produced by *L. johnsonii* La1).

sausage and sourdough [Foulquié Moreno MR, Leroy F, De Vuyst L, unpubl. results] (fig. 2). Because of the complexity of the food matrix and the difficulty of quantifying bacteriocin activities in foods, in vitro studies can be performed to simulate and study the in situ functionality of bacteriocinogenic starters [19, 42]. In this way, the kinetics of bacteriocin production by LAB strains in foods have been described in detail, amongst others through mathematical modeling and positive predictive microbiology [43]. Insights in the relationship between the food environment and kinetics of the starter culture have yielded valuable information about the in situ production of bacteriocins and its interactions with the target strains, which will be important if bacteriocins or bacteriocin-producing strains are to be increasingly used in food systems [19, 44, 45]. In particular, such information is essential when dealing with the potential problem of bacteriocin-resistant target bacteria [44, 45]. Application of bacteriocin-producing starter cultures in sourdough (to increase competitiveness and hence establish a desired microbial population), in fermented sausage (anti-listerial effect to meet the zero-tolerance policy in ready-to-eat foods), and in cheese (anti-listerial and anti-clostridial effects), have been studied during in vitro laboratory fermentations as well as on pilot-scale level [19, 41, 44–49]. Results of these studies were highly promising and underline the important role that functional, bacteriocinogenic strains of LAB may play in the food industry as starter cultures, co-cultures, or bioprotective cultures, to improve food quality and safety [2, 3, 13].

Bacteriocin Production by Probiotics

Probiotics are live microorganisms that, when consumed in an adequate amount as part of the food, confer a health benefit on the host [50]. An experimental focus on bacteriocin production by probiotic LAB strains has indicated that this potential might play a considerable role during in vivo interactions occurring in the human gastrointestinal tract, for instance towards *H. pylori* [4, 51–52]. Whereas bacteriocins in food are degraded by the proteolytic enzymes of the stomach, probiotic bacteria may lead to in situ production of bacteriocins in the gastrointestinal tract. Up to now, bacteriocins have been isolated from the commercial probiotic strains *Lactobacillus casei* Shirota and *Lactobacillus johnsonii* La1 [53]. The latter bacteriocin, referred to as lactacin Fa, is a two-peptide bacteriocin, which differs in one amino acid from the well-known bacteriocin lactacin F produced by *L. johnsonii* VPI 11088 (fig. 3). However, only activity towards Gram-positive indicator bacteria has been shown under

the conditions tested [51, 53]. In contrast, the inhibitory role of organic acids produced by probiotics towards Gram-negative pathogenic bacteria has been shown [9, 54].

Conclusions

Bacteriocins produced by LAB have the potential to cover a very broad field of application, including both the food industry and the medical sector. Concerning their use in food, bacteriocin-producing starter or co-cultures have been successfully applied in pilot-scale experiments (cheese, fermented sausage, sourdough, etc.), yielding food quality and food safety advantages. The current bottleneck hampering widespread industrial practice seems to be market implementation rather than scientific evidence or proof-of-concept. With respect to medical applications, antimicrobials produced by probiotic LAB might play a role during in vivo interactions occurring in the human gastrointestinal tract, hence contributing to gut health. Further research is needed to unravel the precise role of LAB bacteriocins in this process.

Acknowledgements

The authors are grateful for their financial support from the Research Council of the Vrije Universiteit Brussel, the Fund for Scientific Research – Flanders, the Institute for the Encouraging of Scientific and Technological Research in the Industry, the Brussels Capital Region, the Ministry of the Flemish Community, the Federal Science Policy, the European Commission, and several (inter)national food companies.

References

1 Wood BJB, Holzapfel WH: The Genera of Lactic Acid Bacteria. London, Blackie Academic & Professional, 1995.

2 Leroy F, De Vuyst L: Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends Food Sci Technol 2004;15:67–78.

3 Leroy F, Verluyten J, De Vuyst L: Functional meat starter cultures for improved sausage fermentation. Int J Food Microbiol 2006;106:270–285.

4 De Vuyst L, Avonts L, Makras L: Probiotics, prebiotics and gut health; in Remacle C, Reusens B (eds): Functional Foods, Ageing and Degenerative Disease. Cambridge, Woodhead Publishing, 2004, pp 416–482.

5 De Vuyst L, Vandamme EJ: Bacteriocins of Lactic Acid Bacteria: Microbiology, Genetics and Applications. London, Blackie Academic & Professional, 1994.

6 Lavermicocca P, Valerio F, Evidente A, Lazzaroni S, Corsetti A, Gobbetti M: Purification and characterization of novel antifungal compounds from the sourdough *Lactobacillus plantarum* strain 21B. Appl Environ Microbiol 2000;66:4084–4090.

7 Strøm K, Sjøgren J, Broberg A, Schnürer J: *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. Appl Environ Microbiol 2002;68:4322–4327.

8 Schnürer J, Magnusson J: Antifungal lactic acid bacteria as biopreservatives. Trends Food Sci Technol 2005;16:70–78.

9 Makras L, Triantafyllou V, Fayol-Messaoudi D, Adriany T, Zoumpopoulou G, Tsakalidou E, Servin A, De Vuyst L: Kinetic analysis of the antibacterial activity of probiotic lactobacilli towards *Salmonella enterica* serovar Typhimurium reveals a role for lactic acid and other inhibitory compounds. Res Microbiol 2006;157:241–247.

10 Nes IF, Johnsborg O: Exploration of antimicrobial potential in LAB by genomics. Curr Opin Biotechnol 2004;15:100–104.

11 Cotter PD, Hill C, Ross RP: Bacteriocins: developing innate immunity for food. Nat Rev Microbiol 2005;3:777–788.

12 Riley MA, Wertz JE: Bacteriocins: evolution, ecology, and application. Annu Rev Microbiol 2002;56:117–137.

13 Ennahar S, Sonomoto K, Ishizaki A: Class IIa bacteriocins from lactic acid bacteria: antibacterial activity and food preservation. J Biosci Bioeng 1999;87:705–716.

14 Klaenhammer TR: Bacteriocins of lactic acid bacteria. Biochimie 1988;70:337–349.

15 Vogel RF, Pohle BS, Tichaczek PS, Hammes WP: The competitive advantage of *Lactobacillus curvatus* LTH1174 in sausage fermentations is caused by formation of curvacin A. Syst Appl Microbiol 1993;16:457–462.

16 Stevens KA, Sheldon BW, Klapes NA, Klaenhammer TR: Nisin treatment for inactivation of *Salmonella* species and other Gram-negative bacteria. Appl Environ Microbiol 1991;57:3613–3615.

17 Ennahar S, Sashihara T, Sonomoto K, Ishizaki A: Class IIa bacteriocins: biosynthesis, structure and activity. FEMS Microbiol Rev 2000;24:85–106.

18 Twomey D, Ross RP, Ryan M, Meaney B, Hill C: Lantibiotics produced by lactic acid bacteria: structure, function and applications. Antonie van Leeuwenhoek 2002;82:165–185.

19 Leroy F, De Vuyst L: Simulation of the effect of sausage ingredients and technology on the functionality of the bacteriocin-producing *Lactobacillus sakei* CTC 494 strain. Int J Food Microbiol 2005;100:141–152.

20 Leroy F, De Vuyst L: Sakacins; in Naidu AS (ed.): Natural Food Antimicrobial Systems. Boca Raton, CRC Press LLC, 2000, pp 589–610.

21 Yang R, Johnson MC, Ray B: Novel method to extract large amounts of bacteriocins from lactic acid bacteria. Appl Environ Microbiol 1992;58:3355–3359.

22 De Vuyst L, Callewaert R, Crabbé K: Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavourable growth conditions. Microbiology 1996;142:817–827.

23 Lejeune R, Callewaert R, Crabbé K, De Vuyst L: Modelling the growth and bacteriocin production by *Lactobacillus amylovorus* DCE 471 in batch cultivation. J Appl Microbiol 1998;84:159–168.

24 Leroy F, De Vuyst L: Temperature and pH conditions that prevail during the fermentation of sausages are optimal for the production of the antilisterial bacteriocin sakacin K. Appl Environ Microbiol 1999;65:974–981.

25 Van den Berghe E, Skourtas G, Tsakalidou E, De Vuyst L: *Streptococcus macedonicus* ACA-DC 198 produces the lantibiotic, macedocin, at temperature and pH conditions that prevail during cheese manufacture. Int J Food Microbiol 2006;107:138–147.

26 Callewaert R, De Vuyst L: Bacteriocin production with *Lactobacillus amylovorus* DCE 471 is improved and stabilized by fed-batch fermentation. Appl Environ Microbiol 2000;66:606–613.

27 Leroy F, De Vuyst L. Growth of the bacteriocin-producing *Lactobacillus sakei* strain CTC 494 in MRS broth is strongly reduced due to nutrient exhaustion: a nutrient depletion model for the growth of lactic acid bacteria. Appl Environ Microbiol 2001;67:4407–4413.

28 Verluyten J, Leroy F & De Vuyst L: Influence of complex nutrient source on growth of and curvacin A production by sausage isolate *Lactobacillus curvatus* LTH 1174. Appl Environ Microbiol 2004;70:5081–5088.

29 Leroy F, De Vuyst L: The presence of salt and a curing agent reduces bacteriocin production by *Lactobacillus sakei* CTC 494, a potential starter culture for sausage fermentation. Appl Environ Microbiol 1999;65:5350–5356.

30 Verluyten J, Messens W, De Vuyst: Sodium chloride reduces production of curvacin A, a bacteriocin produced by *Lactobacillus curvatus* strain LTH 1174, originating from fermented sausage. Appl Environ Microbiol 2004;70:2271–2278.

31 Mørtvedt CI, Nissen-Meyer J, Sletten K, Nes IF: Purification and amino acid-sequence of lactocin S, a bacteriocin produced by *Lactobacillus sake* L45. Appl Environ Microbiol 1991;57:1829–1834.

32 Tichaczek PS, Meyer JN, Nes IF, Vogel RF, Hammes WP: Characterization of the bacteriocins curvacin A from *Lactobacillus curvatus* LTH1174 and sakacin P from *L. sake* LTH673. Syst Appl Microbiol 1992;15:460–468.

33 Parente E, Ricciardi A: Production, recovery and purification of bacteriocins from lactic acid bacteria. Appl Microbiol Biotechnol 1999;52:628–638.

34 Callewaert R, Holo H, Devreese B, Van Beeumen J, Nes I, De Vuyst L: Characterization and production of amylovorin L471, a bacteriocin purified from *Lactobacillus amylovorus* DCE 471 by a novel three-step method. Microbiology 1999;145:2559–2568.

35 Callewaert R, De Vuyst L: Expanded bed adsorption as a unique unit operation for the isolation of bacteriocins from fermentation media. Bioseparation 1999;8:159–168.

36 Foulquié Moreno MR, Callewaert R, De Vuyst L: Isolation of bacteriocins through expanded bed adsorption using a hydrophobic interaction medium. Bioseparation 2001;10:45–50.

37 Foulquié Moreno MR, Leisner JJ, Tee LK, Ley C, Radu S, Rusul G, Vancanneyt M, De Vuyst L: Microbial analysis of Malaysian tempeh, and characterization of two bacteriocins produced by isolates of *Enterococcus faecium*. J Appl Microbiol 2002;92:147–157.

38 Foulquié Moreno MR, Callewaert R, Devreese B, Van Beeumen J, De Vuyst L: Isolation and biochemical characterisation of enterocins produced by enterococci from different sources. J Appl Microbiol 2003;94:214–229.

39 Georgalaki MD, Van den Berghe E, Kritikos D, Devreese B, Van Beeumen J, Kalantzopoulos G, De Vuyst L, Tsakalidou E: Macedocin, a food-grade lantibiotic produced by *Streptococcus macedonicus* ACA-DC 198. Appl Environ Microbiol 2002;68:5891–5903.

40 Rodríguez JM, Martínez MI, Kok J: Pediocin PA-1, a wide-spectrum bacteriocin from lactic acid bacteria. Crit Rev Food Sci Nutr 2002;42:91–121.

41 Foulquié Moreno MR, Rea MC, Cogan TM, De Vuyst L: Applicability of a bacteriocin-producing *Enterococcus faecium* as a co-culture in Cheddar cheese manufacture. Int J Food Microbiol 2003;81:73–84.

42 Leroy F, De Vuyst L: Bacteriocin-producing strains in a meat environment; in Barredo Fuente JL (eds): Methods in Biotechnology – Microbial Products and Biotransformations. Totowa, Humana Press, 2005, pp 369–380.

43 Leroy F, Degeest B, De Vuyst L A novel area of predictive modelling: describing the functionality of beneficial microorganisms in foods. Int J Food Microbiol 2002;73:251–259.

44 Leroy F, Lievens K, De Vuyst L: Modeling bacteriocin resistance and inactivation of *Listeria innocua* LMG 13568 by *Lactobacillus sakei* CTC 494 under sausage fermentation conditions. Appl Environ Microbiol 2005;71:7567–7570.

45 Leroy F, Lievens K, De Vuyst L: Interactions of meat-associated bacteriocin-producing lactobacilli with *Listeria innocua* under stringent sausage fermentation conditions. J Food Prot 2005;68:2078–2084.

46 Verluyten J, Messens W, De Vuyst L: The curing agent sodium nitrite, used in the production of fermented sausages, is less inhibiting to the bacteriocin-producing meat starter culture *Lactobacillus curvatus* LTH 1174 under anaerobic conditions. Appl Environ Microbiol 2003;69:3833–3839.

47 Verluyten J, Leroy F, De Vuyst L: Effects of different spices used in production of fermented sausages on growth of and curvacin A production by *Lactobacillus curvatus* LTH 1174. Appl Environ Microbiol 2004;70:4807–4813.

48 De Vuyst L, Avonts L, Hoste B, Vancanneyt M, Neysens P, Callewaert R: The lactobin A and amylovorin L471 genes are identical, and their distribution seems to be restricted to the species *Lactobacillus amylovorus* that is of interest for cereal fermentations. Int J Food Microbiol 2004;90:93–106.

49 Anastasiou R, Georgalaki M, Manolopoulou E, Kandarakis I, De Vuyst L, Tsakalidou E: The performance of *Streptococcus macedonicus* ACA-DC 198 as starter culture in Kasseri cheese production. Int Dairy J 2006;17:208–217.

50 FAO/WHO: Evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. FAO of the UN and WHO Expert Consultation Report, Cordoba 2001.

51 Avonts L, De Vuyst L: Antimicrobial potential of probiotic lactic acid bacteria. Proc 15th Forum for Applied Biotechnology, Gent, Sept 24–25, 2001, pp 543–550.

52 Kim TS, Hur JW, Yu MA, Cheigh CI, Kim KN, Hwang JK, Pyun YR: Antagonism of *Helicobacter pylori* by bacteriocins of lactic acid bacteria. J Food Prot 2003;66:3–12.

53 Avonts L, Van Uytven E, De Vuyst L: Cell growth and bacteriocin production of probiotic *Lactobacillus* strains in different media. Int Dairy J 2004;14:947–955.

54 Makras L, De Vuyst L: The in vitro inhibition of Gram-negative pathogenic bacteria by bifidobacteria is caused by the production of organic acids. Int Dairy J 2006;16:1049–1057.

Review

Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol 2007;13:200–209
DOI: 10.1159/000104748

Structural and Functional Diversity of Microcins, Gene-Encoded Antibacterial Peptides from Enterobacteria

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Key Words
Microcins · *Escherichia coli* · Siderophore peptide · Nucleotide peptide · Iron siderophore receptors

Abstract
Microcins are a peculiar class of gene-encoded low-molecular-mass antibacterial peptides secreted by enterobacteria. They contribute to the regulation of microbial competitions within the intestinal microbiota. The genetic systems involved in microcin biosynthesis share a conserved organization. Similar to bacteriocins of Gram-positive bacteria, microcins exert potent antibacterial activity directed against phylogenetically-related bacterial strains, with minimal inhibitory concentrations in the nanomolar range. In contrast to bacteriocins, they display a great structural diversity among the few representatives well characterized until now, that makes difficult the description of microcin subclasses. This review focuses on three microcins, MccE492m that carries a *C*-terminal posttranslational modification containing a catechol-type siderophore, MccJ25, a cyclic peptide with a unique 'lasso-type' structure and MccC7 or C51, with a common *N*-formylated heptapeptide-nucleotide structure. We show these microcins exhibit 'Trojan horse' mechanisms of antibacterial activity: either (i) the microcin structure is a mime of an essential element, permitting its recognition by outer membrane receptors used for vital functions in bacteria and further translocation into the periplasmic space, or (ii) it is secreted as a harmless molecule and further processed in susceptible bacteria to form the toxic entity. When inside target bacteria, microcins bind essential enzymes or interact with the inner membrane to form a bacterial killing structure.
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Introduction

The bacterial secretion of peptides or proteins (bacteriocins) to fight against other prokaryotic species has appeared as one of the very first examples of biological warfare. These ribosomally synthesized antimicrobial peptides (AMPs) are considered a successful strategy in maintaining equilibrium within a bacterial ecosystem. They kill other bacteria by several mechanisms, including the modification of membrane permeability and depolarization of membrane ion gradients, or the degradation of nucleic acids or cell walls. In literature, the term bacteriocin is usually restricted to peptides produced by Gram-positive bacteria, while in Gram-negative bacteria, mainly enterobacteria, the toxins are called either colicins (i.e. antibiotic proteins targeting *Escherichia coli*) or microcins (characterized by a lower molecular mass) [for reviews, see 1–3]. Bacteriocins from Gram-positive bacteria, particu-

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larly lactic acid bacteria, and microcins from enterobacteria, mostly *E. coli*, share a number of common characteristics, such as a molecular mass below 10 kDa and a mode of secretion into the extracellular medium involving ABC (ATP-binding cassette)-type transporters. In both cases, the producer has a specific immunity mechanism that protects it from being killed by the newly synthesized antibacterial molecule. Both types of peptide essentially inhibit the growth of phylogenetically related species. However, microcins form a much more restricted group with highly diversified structures and mechanisms of action, as exemplified by the six structurally characterized representatives, compared to the very large number of bacteriocins [1] that can be assembled into subgroups according to common structural features or mechanisms of action.

In fact, most of the bacteriocins of Gram-positive bacteria are described as unmodified peptides/proteins [for reviews, see 4, 5], except the highly modified group of lantibiotics, which contain multiple tethering ether bonds [for reviews, see 6–8]. Bacteriocins are usually classified into three main classes: (i) Lantibiotics, or class I bacteriocins, are peptides <5 kDa that contain the unusual amino acids lanthionine (Lan) and β-methyllanthionine (MeLan) (formed when a dehydrated serine or threonine is covalently bridged with a cysteine through the sulfur atom) and a number of dehydrated amino acids. They include two-component lantibiotics that require the presence of two different peptide components to achieve full antibacterial activity [9]. (ii) Class II bacteriocins (sometimes termed pediocin-like AMPs), also <5 kDa, are heat-stable, non-Lan-containing, cationic membrane-active peptides. This class is subdivided into subclass IIa that concerns *Listeria*-active peptides sharing very similar primary structures, especially in the *N*-terminal part (with the *N*-terminal consensus sequence YGNGV), and subclass IIb requiring two components for antibiotic activity. The subclass IIa is itself subdivided into three subgroups according to similarities and differences in the *C*-terminal part [5]. (iii) Class III bacteriocins are large (>30 kDa) heat-labile proteins. Additionally, a class of circular bacteriocins, exemplified by AS48 and circularin A, has recently been proposed [10]. A great number of members of each of these three main classes, and particularly from classes I and IIa, have been described and studied with regard to their common structural aspects and the mechanisms involved in their antibacterial activity. While the class IIa bacteriocins target the inner membrane through its permeabilization [5], leading to disruption of the protonmotive force, the prototypic lantibiotic nisin has a dual mode of action. Nisin uses lipid II (a crucial precursor in peptidoglycan biosynthesis) as a docking molecule, therefore preventing correct cell wall synthesis, as well as initiating within cell membranes the formation of heteromolecular pores that are made up of lipid II and nisin molecules [11, 12]. Rapid and efficient cell death thus results from such a dual mechanism.

By contrast, microcins form a very restricted group of such defense peptides with only 15 representatives identified [for reviews, see 3, 13–15] since their discovery in 1976 [16]. They all inhibit the growth of a variety of Gram-negative bacteria, including *Escherichia, Salmonella, Enterobacter, Klebsiella, Citrobacter* and *Shigella,* with minimal inhibitory concentrations (MICs) in the nanomolar range. They can be low-molecular-mass peptides <3 kDa (microcins B17, C7/C51, D93 and J25) that are generally highly modified, or polypeptides between 7 and 10 kDa that can be modified or not (microcins E492, L, H47, I47, M, 24 and V). Some of these peptides have only been identified on the basis of genetic studies. Despite a high structural heterogeneity, microcins share a conserved organization of their genetic systems. A typical gene cluster, located either on a plasmid or on the bacterial chromosome, includes open reading frames encoding the precursor of the microcin, secretion factors, immunity proteins and, very frequently, modification enzymes [17].

Presumably related to these various structures, microcins have also diverse mechanisms of action, targeting either the inner membrane of target bacteria, or enzymes involved in DNA or RNA structure and synthesis or in protein synthesis. Moreover, to enter the bacteria more efficiently, these peptides use specific receptors designed by all bacteria for essential nutrient uptake. This seems to be a common feature shared by microcins, which is possibly related to the high selectivity of their antibacterial activity. To exemplify the structural and functional diversity of microcins, we have focused on three of them that have been deeply characterized from a biochemical and functional point of view, and that will be discussed in this review: microcins E492 (MccE492), J25 (MccJ25) and C7/C51 (MccC7/C51).

Structural Diversity of Microcins

Among the eleven microcins identified until now, only six have been structurally characterized (MccB17, MccC7/C51, MccE492, MccJ25, MccL and MccV [also known as ColV, since initially described as a colicin]), while the others (MccD93, MccH47, MccI47, MccM, Mcc24) have been essentially studied through genetical approaches.

MccB17 is a 43-residue peptide characterized by post-translational modifications consisting of thiazole and oxazole rings [18]. MccC7/C51 is a short nucleotide heptapeptide [19–21] and MccJ25 a cyclic 21-amino-acid peptide [22–25], while MccE492 [26, 27], MccL [28] and MccV [29] are linear polypeptides with molecular masses between 8 and 10 kDa. The structures of microcins have often been the subject of debates in the literature. This is the case of MccE492, MccJ25 and MccC7/C51 that have been selected in this review.

MccE492 is a chromosomally-determined microcin initially characterized from a *Klebsiella pneumoniae* strain [30]. Further cloning in *E. coli* [31] allowed its purification from culture supernatants and primary structure determination [26]. It was identified by mass spectrometry as an unmodified 84-residue peptide [26], in agreement with the amino acid sequence deduced from the gene encoding its precursor MceA [32, 33]. The amino acid residues are mainly uncharged and hydrophobic, with the exception of one histidine, three aspartic acids and one glutamic acid (fig. 1a). Therefore, this peptide is slightly anionic. That differentiates it from both the known bacteriocins from Gram-positive bacteria and most of the AMPs from vertebrates, invertebrates and plants, which all exhibit a strong cationic character. However, we have recently shown that MccE492 could be secreted by both the wild-type *K. pneumoniae* and the recombinant *E. coli* strains under two forms, the unmodified polypeptide previously described and a posttranslationally modified variant that we called MccE492m [27]. This microcin variant, which bears a catechol-type siderophore comprising the linear trimer of dihydroxybenzoylserine (DHBS), exhibits enhanced activity. The siderophore moiety is anchored to the peptide *C*-terminus through a β-D-glucose, which in turn is linked to the Ser84 carboxylate through an *O*-glycosidic bond at C6, and to the first DHBS entity via a *C*-glycosidic bond at C1 (fig. 1a) [27]. This novel posttranslational modification is reminiscent of siderophores, the molecules designed by bacteria to chelate Fe(III), enabling its uptake through the outer membrane via specific receptors [34, 35]. Moreover, mass spectrometry has shown that the modified microcin MccE492m selectively binds ferric iron through the catecholate moiety, confirming it operates as a siderophore and thus establishing a new type of AMP, the siderophore peptides [27]. This modification is in good accordance with the presence of several genes in the genetic system involved in MccE492 biosynthesis, the role of which remained speculative [36] until the finding of this new form of MccE492. In particular, three gene products show sequence homologies to glycosyltransferases (MceC), acyltransferases (MceI) and enterobactin esterases (MceD), which cleaves enterobactin, the cyclic trimer of DHBS, into its monomer, dimer and linear trimer [37], thus providing the corresponding genes *mceC, mceI and mceD* with a very likely role in the acquisition of the posttranslational modification.

MccJ25 has been first described as a head-to-tail macrocyclic linear peptide [38, 39]. The very high stability of the peptide towards high temperatures associated with high concentrations of chaotropic agents has been largely studied in our group [40]. Later on, the peptide was identified as a 'lasso' peptide, with an extraordinary three-dimensional structure, showing that the ring was actually a small cycle resulting from a linkage between the *N*-terminal amino group and the Glu8 side-chain carboxylate (fig. 1b) [22–25]. The 13-residue linear *C*-terminal tail is threaded into the ring, in such a manner similar to the thread through a needle eye, thus forming a loop. Two aromatic bulky side chains from Phe19 and Tyr20, which are each positioned on one side of the ring, tightly lock the tail into the ring (fig. 1b). The tail thus remains firmly entrapped within the ring through this strong steric effect, in combination with non-covalent interactions, in such a manner that it can only be released via the cleavage of the ring. This cleavage is particularly hard to perform, since it can only be accomplished in basic medium [24]. An inference of this peculiar structure is that the ring closure should have been accomplished after acquisition of the spatial structure of the molecule and almost a correct positioning of both (i) the carboxylate and the amino groups involved in the ring closure and (ii) the aromatic side chains responsible for the strong steric hindrance that locks the tail into the ring. Endopeptidases and strong acidic media do not target the ring, but only cleave bonds in the loop that is subsequently opened. The resulting entities are two-chain peptides [41], the tail (or a shortened tail) remaining firmly anchored to the ring. Such a typical and original lasso structure has never been identified previously among AMPs including bacteriocins and microcins. However, it appears in some enzyme inhibitors synthesized by *Streptomyces* that may be either additionally stabilized or not by a disulfide bonding [25, 42]. Moreover, MccJ25 is the first lasso-type peptide with a glutamic acid involved in the ring closure, rather than an aspartic acid, as found in the *Streptomyces* peptides.

Two microcins sharing a nucleotide peptide character, MccC7 and MccC51, have been described from two different *E. coli* strains [19, 43]. The genetic systems involved in the biosynthesis of these two microcins show only few

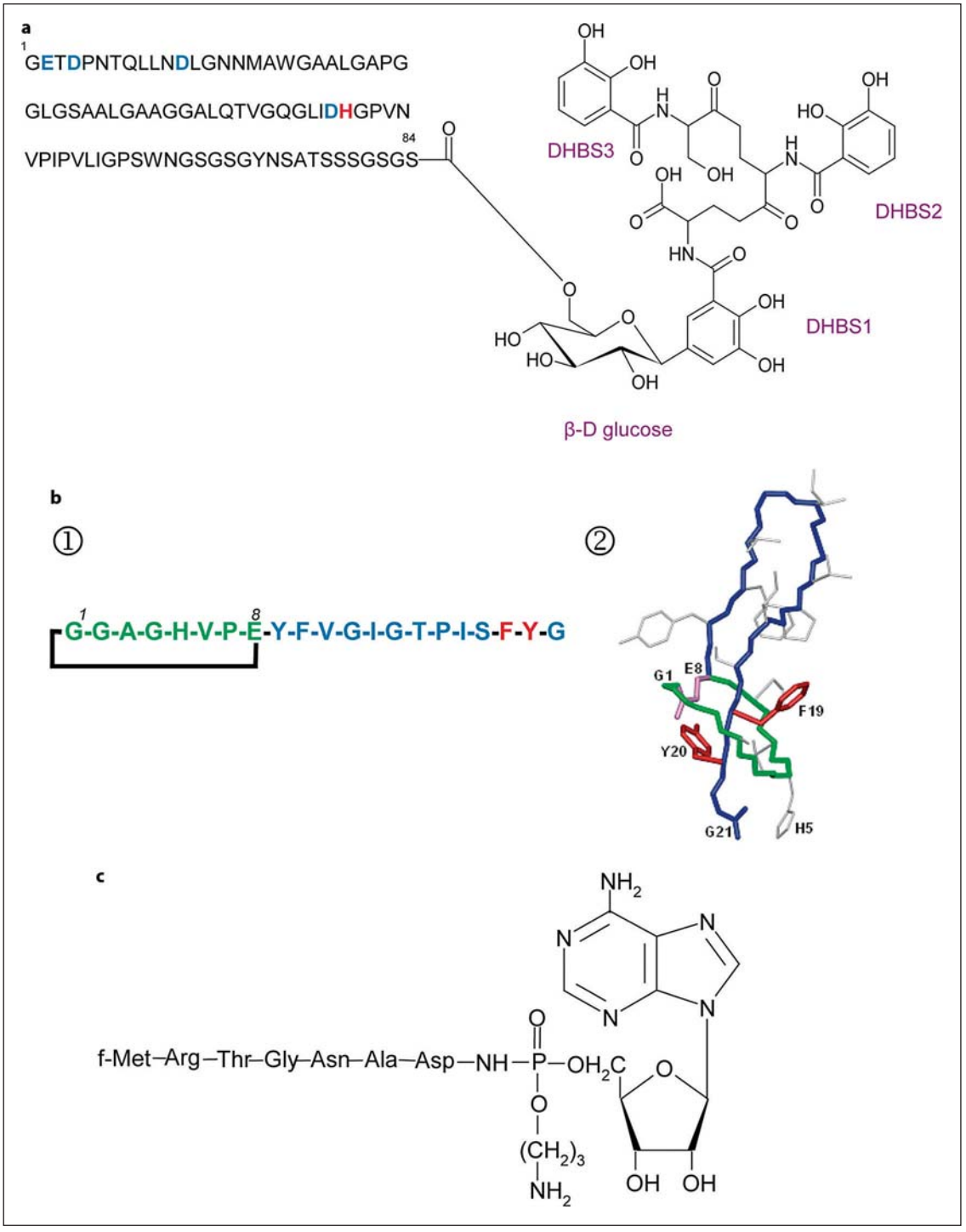

Fig. 1. The structures of MccE492/MccE492m, MccJ25 and MccC7/C51. **a** The amino acid sequence common to MccE492/MccE492m is indicated in one-letter code and the positively and negatively charged amino acids are figured red and blue, respectively. The posttranslational modification carried by the siderophore peptide MccE492m consists of a trimer of the catechol-type siderophore 2,3-dihydroxybenzoyl serine (DHBS) linked to Ser84 via a *β*-D-glucose. **b** (1) The cyclic structure of MccJ25 comprises an *N*-terminal ring followed by a *C*-terminal linear tail; the amino acids are colored according to the 'lasso-type' three-dimensional structure (2) that shows the steric hindrance due to two aromatic side chains (Phe19, Tyr20) maintaining the tail entrapped into the ring. **c** Structure of the nucleotide peptide MccC7/C51: the *N*-formylated heptapeptide (in three-letter code) is linked to the adenosine monophosphate moiety through a phosphoramidate bond; the phosphate group bears an *n*-aminopropanol chain.

differences that are located in a gene involved in the immunity to the microcin [21]. A common *N*-formylated heptapeptide had been identified in the two molecules, which differed in both the linkage between the peptide and nucleotide parts and in the nucleotide structure. MccC51 was described as a nebularin 5′-monophosphate *C*-terminus linked to the Asp7 side chain through three methylene bonds. MccC7 contained a modified adenosine monophosphate (AMP) covalently attached to the *C*-terminal aspartic acid residue through a phosphoramidate bond. The phosphoramidate group showed a chiral phosphorus atom, since it was substituted by an *n*-aminopropanol chain. We have optimized the production of MccC51 to reinvestigate its structure and have evidenced that it is actually similar to that of MccC7 [20, 21]. The critical points of the structure, i.e. the presence of the phosphoramidate bond acting as a linker between the heptapeptide and the nucleotide and the location of the *n*-aminopropanol chain, have been unambiguously assigned through typical cross-peaks in two-dimensional ^{1}H-^{31}P NMR spectra [20]. Therefore, MccC7 and MccC51, which arise from two distinct *E. coli* strains that bear closely related genetic systems, share a common structure that will be henceforth denominated MccC7/C51 (fig. 1c). MccC7/C51 is the smallest microcin isolated to date and the only microcin known to carry a nucleotide as a post-translational modification; its three-dimensional structure has never been described.

As previously announced, and now exemplified throughout the description of MccE492, MccJ25 and MccC7/C51 structural characteristics, microcins offer a wide array of peculiar structures. As correlated, a broad diversity of mechanisms of action is exploited by microcins to kill their bacterial targets.

From Structural Diversity to Diverse and Complex Mechanisms of Action

The great differences between eukaryotic and prokaryotic AMPs concern the antibiotic efficacy and the microbial target specificity. The AMPs secreted by bacteria (bacteriocins and microcins) appear to be overall more potent than those from Eukaryota, since they are active in the pico- to nanomolar concentration range, while the latter are active at micromolar concentrations. In addition, they have a narrow spectrum of activity, susceptible strains being essentially closely related to the producing strain, while AMPs from multicellular organisms more often have a broad spectrum of activity directed against Gram-positive and -negative bacteria, yeasts and fungi. It is thus of importance to elucidate the reasons of such an efficiency and selectivity. More refined and complex mechanisms of antibacterial activity, including either a dual mode of action or the involvement of specific receptors could thus be expected for AMPs of bacterial origin.

MccE492 and its siderophore peptide counterpart MccE492m both inhibit selectively the growth of Gram-negative enterobacteria, such as *E. coli*, *Salmonella enterica, Enterobacter cloacae* and *K. pneumoniae*. MccE492m is 2–8 times more active than MccE492 with MICs ranging from 40 to 160 nM against *E. coli* and *S. enterica* strains. The activity of both MccE492 and MccE492m is bactericidal with minimal bactericidal concentration values similar to MIC values [27, 44].

Both MccE492 and MccE492m form ion channels in artificial planar lipid bilayers [44, 45] and permeabilize the inner membrane of Gram-negative bacteria, as shown in *E. coli* ML35 [44, 46]. Interaction with the inner membrane and subsequent permeabilization were considered as responsible for the bactericidal effect [45]. However, we hypothesized that this membrane activity was not sufficient by itself to account for the lethal effect of the microcins [44]. Indeed, despite its more potent antibacterial activity against *E. coli*, MccE492m is less efficient in permeabilizing the membrane bilayer than MccE492, indicating the absence of direct correlation between the rapid decrease in cell viability induced by MccE492m and the rate of membrane permeabilization [46]. Moreover, electron microscopy showed that treated and killed *E. coli* had still intact inner and outer membranes and only showed a swelling of the periplasmic space [46]. Therefore, permeabilization of the inner membrane does not lead to membrane disruption, but rather to a more subtle mechanism, such as the formation of pores, similar to those observed in artificial bilayers [44, 45]. Consequently, we suspect that an additional step shall be involved in the mechanism of antibacterial activity of MccE492/MccE492m, which could be either the formation of a lethal complex within the bilayer or the interaction of the microcin with a cytoplasmic target (fig. 2a).

Since mutations in *tonB* have resulted in resistance to MccE492, this protein has been suggested to be involved in the mechanism of antibacterial activity of this microcin [47]. TonB, which spans the periplasmic space, is generally found in complex with two other partners ExbB and ExbD, located in the inner membrane, thus forming the TonB-dependent energy transduction system [48–50]. TonB acts as the energy transducer, using the proton-

motive force; the role of ExbB and ExbD is less clear. The TonB system is used by bacteria for the uptake of vitamin B_{12} and iron chelators, the siderophores [34, 51], which are recognized at the outer membrane by specific receptors. We have shown that the *tonB* mutation induces a high resistance to MccE492 and MccE492m, and that the antibacterial activity of both microcins is completely restored in the TonB-complemented strain, unambiguously indicating that TonB is required for the antibacterial property of both MccE492 [44] and MccE492m [27]. Moreover, MccE492 and MccE492m activities require the three outer membrane receptors, FepA, Fiu and Cir. These are catechol-type siderophore receptors associated to the TonB system [for reviews, see 34, 52]. At this time, there is still no evidence of the direct interaction of MccE492/MccE492m with any of the three iron siderophore receptors, neither is it not explicitly demonstrated that the microcins are translocated into the periplasmic space through the FepA/Fiu/Cir receptors. Indeed, the translocation step could occur using either a porin, or membrane defects such as membrane islands, which have different lipid compositions, that result in a loose membrane bilayer packing (fig. 2). However, since MccE492/MccE492m are recognized by the receptors involved in the uptake of enterobactin and its breakdown products, the linear trimer, dimer, and monomer of DHBS, which are imported through a TonB- and energy-dependent mechanism [for reviews, see 34, 35], it is highly probable that the microcins undergo the same uptake mechanism across the outer membrane of *E. coli*, involving the TonB/ExbB/ExbD complex and the FepA/Cir/Fiu receptors.

MccJ25 exhibits a potent bactericidal activity with MICs in the 5–500 nM range against a number of enterobacteria including pathogenic *Salmonella, Escherichia* and *Shigella* strains [53, 54]. MccJ25 has been shown to inhibit transcription by binding bacterial RNA polymerase (RNAP), which is targeted at the level of the β′ subunit [55, 56]. MccJ25 binds the RNAP secondary channel, obstructing it and thus preventing the correct positioning of the nucleoside triphosphate substrates, which are therefore prevented from reaching the catalytic center of the enzyme [57, 58]. Indeed, the MccJ25 binding instantaneously stops RNAP progression and thus prevents further elongation of transcription [57]. The first studies on MccJ25 have shown that mutants resistant to MccJ25 were impaired in *fhua*, *tonB* and *sbmA*, indicating that the outer-membrane iron transporter FhuA (the receptor for ferrichrome) and the inner membrane proteins TonB and SbmA were likely involved in the microcin uptake [59, 60]. We recently demonstrated the direct interaction between FhuA and MccJ25 by size-exclusion chromatography and isothermal titration calorimetry, showing that MccJ25 binds to FhuA with a 2:1 stoichiometry and a K_d of 1.2 μM [61]. The differential scanning calorimetry revealed that the interaction of MccJ25 with its receptor involves the external loops of FhuA [61]. To go further in the whole process used by MccJ25 to exert its antibacterial activity, it thus remained to identify both the regions of the molecule involved in the different steps of the mechanism (i.e. recognition, translocation and RNAP targeting) and the other protein partners. The two-chain peptide resulting from MccJ25 thermolysin cleavage (t-MccJ25), which keeps the intact spatial structure of the ring and the positioning of the trapped chain, but does not maintain anymore the β-hairpin loop, was used together with other shorter truncated variants in order to identify the MccJ25 regions involved either in the recognition step by FhuA or in the interaction with RNAP [61, 62]. Since disruption of the Val11-Pro16 β-hairpin did not affect the inhibition of transcription by MccJ25, contribution of this loop to the interaction with RNAP was excluded [62]. Furthermore, t-MccJ25 was unable to bind FhuA, affording direct evidence of the involvement of this region in the recognition step of MccJ25 by the outer membrane receptor FhuA [61]. Before reaching RNAP, MccJ25 should be transported through *E. coli* membranes. Possible partners for this uptake are the outer membrane receptor FhuA itself, and the inner membrane protein SbmA, whose function is still unknown, but which has been shown to be involved in the import of MccB17 [63], MccJ25 [60] and bleomycin into susceptible bacteria. The *E. coli* SbmA protein is homolog of BacA from *Rhizobium meliloti* (64% identity), which is crucial for the complex association between bacteria of the genus *Rhizobium* and leguminous plants [64]. Recently, the His5 residue of MccJ25 has been shown to be critical for the SbmA-dependent transport of MccJ25, and not to be required for RNAP inhibition. Indeed, MccJ25 variants where single amino acids in the ring, and in particular His5, were mutated became unable to kill cells, while still inhibiting RNAP [65]. It was thus proposed that the single histidine residue, which is positioned in the ring, is crucial for the specific interaction of MccJ25 with SbmA, which ensures crossing of the inner membrane, thus achieving the uptake of MccJ25 into the cytoplasm. Therefore, the MccJ25 uptake comprises a first recognition by FhuA, the iron siderophore receptor of the hydroxamate type, and subsequent translocation into the periplasmic space through the TonB/ExbB/ExbD complex, followed by recognition by the inner membrane

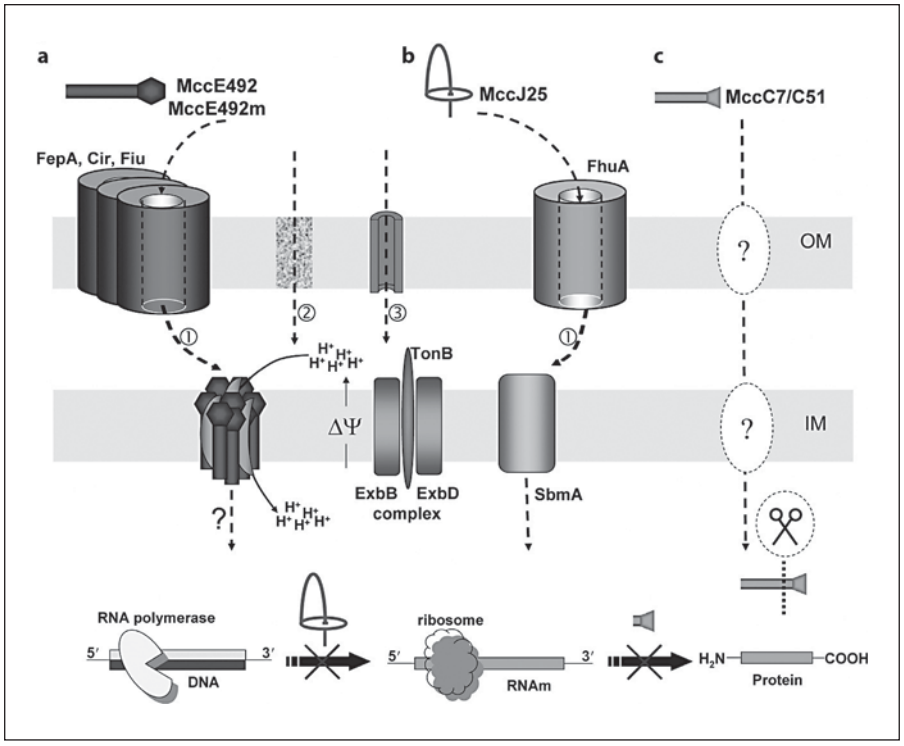

Fig. 2. The 'Trojan horse' mechanisms of action of MccE492/MccE492m, MccJ25 and MccC7/C51. **a** After recognition by the FepA, Cir and Fiu catecholate siderophore receptors, MccE492/MccE492m are translocated across the outer membrane (OM) through an energy- and TonB-dependent mechanism. Translocation may occur via the receptor β-barrel (1), a region with different lipid composition such as a 'membrane island' (2), or a porin (3). Once in the periplasmic space, MccE492/MccE492m insert into the inner membrane (IM) inducing proton leakage and subsequent drop of the IM potential (ΔΨ). At this stage, it is not known whether the microcins recruit other partners, such as inner membrane proteins, to form a toxic supramolecular edifice; it is not known either if they reach an intracytoplasmic target. **b** MccJ25 is recognized by FhuA, the hydroxamate siderophore receptor, before translocation through a TonB-dependent mechanism and further transport through the IM using the inner membrane protein SbmA. Once internalized in the cytoplasm, MccJ25 reaches RNAP, which is inhibited through interaction with the β′ subunit and further obstruction of the secondary channel, which in turn prevents the correct positioning of nucleoside triphosphate substrates. **c** MccC7/C51 is internalized in susceptible bacteria through an unknown mechanism, before being processed by an unknown peptidase that targets the ultimate MccC7/C51 peptide bond (Ala-Asp), leading to a modified unhydrolyzable aspartyl adenylate analogue that contains an *N*-acyl phosphoramidate linkage. The modified aspartyl adenylate thus generated inside the susceptible bacteria inhibits translation by specifically blocking the function of aspartyl-tRNA synthetase via a mechanism that remains to be elucidated.

protein SbmA and further transport through the inner membrane to the cytoplasm, where it can reach RNAP (fig. 2b).

At this stage, it is striking to note that both MccJ25 and MccE492, which do not have any apparent structural similarity to catecholate or hydroxamate siderophores respectively, are however recognized by high-affinity receptors specific for these ligands. This could be made possible by acquisition of specific three-dimensional structures that bring strategic chemical groups at the correct positioning required for recognition by the receptor. The siderophore moiety carried by MccE492m should then allow a double recognition, resulting in an improved uptake, in agreement with the higher antibacterial activity of MccE492m compared to its unmodified counterpart MccE492.

MccC7/C51 is active against several genera and species of enterobacteria such as *Escherichia*, *Klebsiella*, *Salmonella*, *Shigella, Yersinia* and *Proteus* [66–68]. It inhibits the growth of *E. coli* strains at 100–500 nM. The activity is bactericidal [S. Rebuffat et al., unpubl. results]. MccC7/C51 has been initially proposed to target protein synthesis, inhibiting translation [19]. Since a synthetic peptide with the heptapeptide sequence devoid of the nucleotide

moiety mildly inhibited translation in vitro, it was concluded that the peptide was responsible for translation inhibition and that the nucleotide was involved in the recognition and transport into susceptible cells [19]. However, it was recently demonstrated that MccC7/C51 is degraded inside the target bacteria, leading to a modified aspartyl adenylate. This unhydrolyzable aspartyl adenylate strongly inhibits translation by preventing the synthesis of aminoacylated tRNA-Asp by aspartyl-tRNA synthetase, making the nucleotide part of the molecule responsible for cell killing after being processed by a specific protease [69] (fig. 2c). Such a mechanism is reminiscent of that used by other antibiotics containing a nucleotide part such as albomycin, which is taken inside the cells through the ferrichrome uptake system FhuA, before being converted into an active form by peptidase N [70]. Therefore, MccC7/C51 constitutes the first example of a bacterial AMP (either a bacteriocin or a microcin) being processed and converted into the active molecule inside the target bacteria rather than in the producer strain, as it is generally observed. Furthermore, the peptide moiety should consequently play a role in the microcin uptake into susceptible cells. However this remains to be evidenced, as well as the system involved in recognition and uptake.

Concluding Remarks

Despite their completely unrelated structures, the three microcins taken as examples in this paper share a common 'Trojan horse' strategy for killing other bacteria. Either the microcin bears a likeness to essential molecules through its three-dimensional structure, or it disguises itself with a mime of such a useful molecule. This allows MccJ25, MccE492 and MccE492m recognition by naturally specific receptors designed by bacteria for the uptake of essential elements. Alternatively, the microcin is secreted as a harmless molecule (MccC7/C51) that is friendly allowed to enter the susceptible bacteria; but when inside, the inactive microcin is further transformed by the host bacterium itself into a poison fatal to the misled bacterium. Such subtle strategies differ from those adopted by Gram-positive bacteriocins, which prefer to combine two different efficient mechanisms rather than to use dupery and duplicity in order to kill the competitors in their microbial ecosystem. With their outstanding stratagems, microcins and bacteriocins could thus inspire the design of antimicrobial molecules.

References

1 Sablon E, Contreras B, Vandamme E: Antimicrobial peptides of lactic acid bacteria: mode of action, genetics and biosynthesis. Adv Biochem Eng Biotechnol 2000;68:21–60.

2 Gillor O, Kirkup BC, Riley MA: Colicins and microcins: the next generation antimicrobials. Adv Appl Microbiol 2004;54:129–146.

3 Baquero F, Moreno F: The microcins. FEMS Microbiol Lett 1984;23:117–124.

4 Ennahar S, Sashihara T, Sonomoto K, Ishizaki A: Class IIa bacteriocins: biosynthesis, structure and activity. FEMS Microbiol Rev 2000;24:85–106.

5 Fimland G, Johnsen L, Dalhus B, Nissen-Meyer J: Pediocin-like antimicrobial peptides (class IIa bacteriocins) and their immunity proteins: biosynthesis, structure, and mode of action. J Pept Sci 2005;11:688–696.

6 McAuliffe O, Ross RP, Hill C: Lantibiotics: structure, biosynthesis and mode of action. FEMS Microbiol Rev 2001;25:285–308.

7 Patton GC, van der Donk WA: New developments in lantibiotic biosynthesis and mode of action. Current Opin Microbiol 2005;8:1–9.

8 Chatterjee C, Paul M, Xie L, van der Donk WA: Biosynthesis and mode of action of lantibiotics. Chem Rev 2005;105:633–683.

9 Garneau S, Martin NI, Vederas JC: Two-peptide bacteriocins produced by lactic acid bacteria. Biochimie 2002;84:577–592.

10 Kemperman R, Kuipers A, Karsens H, Nauta A, Kuipers O, Kok J: Identification and characterization of two novel clostridial bacteriocins, circularin A and closticin 574. Appl Environ Microbiol 2003;69:1589–1597.

11 Breukink E, van Heusden HE, Vollmerhaus PJ, Swiezewska E, Brunner L, Walker S, Heck AJ, de Kruijff B: Lipid II is an intrinsic component of the pore induced by nisin in bacterial membranes. J Biol Chem 2003;278:19898–19903.

12 Hasper HE, de Kruijff B, Breukink E: Assembly and stability of nisin-lipid II pores. Biochemistry 2004;43:11567–11575.

13 Destoumieux-Garzón D, Peduzzi J, Rebuffat S: Focus on modified microcins: structural features and mechanisms of action. Biochimie 2002;84:511–519.

14 Pons AM, Lanneluc I, Cottenceau G, Sable S: New developments in non-post translationally modified microcins. Biochimie 2002;84:531–537.

15 Poey ME, Azpiroz MF, Laviña M: Comparative analysis of chromosome-encoded microcins. Antimicrob Agents Chemother 2006;50:1411–1418.

16 Asensio C, Pérez-Diaz JC, Martinez MC, Baquero F: A new family of low-molecular-weight antibiotics from enterobacteria. Biochem Biophys Res Commun 1976;69:7–14.

17 Moreno F, Gonzalez-Pastor JE, Baquero MR, Bravo D: The regulation of microcin B, C and J operons. Biochimie 2002;84:521–529.

18 Bayer A, Freund S, Jung G: Post-translational heterocyclic backbone modifications in the 43-peptide antibiotic microcin B17. Structure elucidation and NMR study of a ^{13}C,^{15}N-labelled gyrase inhibitor. Eur J Biochem 1995;234:414–426.

19 Guijarro JI, González-Pastor JE, Baleux F, San Millán JL, Castilla MA, Rico M, Moreno F, Delepierre M: Chemical structure and translation inhibition studies of the antibiotic microcin C7. J Biol Chem 1995;270:23520–23532.

20 Blond A, Goulard C, Fomenko DE, Metliskaya AZ, Peduzzi J, Barthélémy M, Katrukha G, Khmel I, Rebuffat S: Structure-activity relationship of the antibiotic nucleotide-peptide microcin C51; in Martinez J, Fehrenz JA (eds): Peptides 2000. Proc 26th European Peptide Symposium, EDK, Paris 2000, pp 601–602.

21 Fomenko DE, Metlitskaya AZ, Peduzzi J, Goulard C, Katrukha GS, Gening LV, Rebuffat S, Khmel IA: Microcin C51 plasmid genes: possible source of horizontal gene transfer. Antimicrob Agents Chemother 2003;47:2868–2874.

22 Bayro MJ, Mukhopadhyay J, Swapna GV, Huang JY, Ma LC, Sineva E, Dawson PE, Montelione GT, Ebright RH: Structure of antibacterial peptide microcin J25: a 21-residue lariat protoknot. J Am Chem Soc 2003;125:12382–12383.

23 Rosengren KJ, Clark RJ, Daly NL, Goransson U, Jones A, Craik DJ: Microcin J25 has a threaded side chain-to-backbone ring structure and not a head-to-tail cyclized backbone. J Am Chem Soc 2003;125:12464–12474.

24 Wilson KA, Kalkum M, Ottesen J, Yuzenkova J, Chait BT, Landick R, Muir T, Severinov K, Darst SA: Structure of microcin J25, a peptide inhibitor of bacterial RNA polymerase, is a lassoed tail. J Am Chem Soc 2003;125:12475–12483.

25 Rebuffat S, Blond A, Destoumieux-Garzfin D, Goulard C, Peduzzi J: Microcin J25, from the macrocyclic to the lasso structure: implications for biosynthetic, evolutionary and biotechnological perspectives. Curr Protein Pept Sci 2004;5:383–391.

26 Pons AM, Zorn N, Vignon D, Delalande F, Van Dorsselaer A, Cottenceau G: Microcin E492 is an unmodified peptide related in structure to colicin V. Antimicrob Agents Chemother 2002;46:229–230.

27 Thomas X, Destoumieux-Garzón D, Péduzzi J, Afonso C, Blond A, Birlirakis N, Goulard C, Dubost L, Thai R, Tabet JC, Rebuffat S: Siderophore peptide, a new type of post-translationally modified antibacterial peptide with potent activity. J Biol Chem 2004;279:28233–28242.

28 Pons AM, Delalande F, Duarte M, Benoit S, Lanneluc I, Sable S, Van Dorsselaer A, Cottenceau G: Genetic analysis and complete primary structure of microcin L. Antimicrob Agents Chemother 2004;48:505–513.

29 Håvarstein LS, Holo H, Nes IF: The leader peptide of colicin V shares consensus sequences with leader peptides that are common among peptide bacteriocins produced by Gram-positive bacteria. Microbiology 1994;140:2383–2389.

30 De Lorenzo V, Pugsley AP: Microcin E492, a low-molecular-weight peptide antibiotic which causes depolarization of the *Escherichia coli* cytoplasmic membrane. Antimicrob Agents Chemother 1985;27:666–669.

31 Wilkens M, Villanueva JE, Cofre J, Chnaiderman J, Lagos R: Cloning and expression in *Escherichia coli* of genetic determinants for production of and immunity to microcin E492 from *Klebsiella pneumoniae*. J Bacteriol 1997;179:4789–4794.

32 Lagos R, Villanueva JE, Monasterio O: Identification and properties of the genes encoding microcin E492 and its immunity protein. J Bacteriol 1999;181:212–217.

33 Lagos R, Baeza M, Corsini G, Hetz C, Strahsburger E, Castillo JA, Vergara C, Monasterio O: Structure, organization and characterization of the gene cluster involved in the production of microcin E492, a channel-forming bacteriocin. Mol Microbiol 2001;42:229–243.

34 Braun V, Braun M: Active transport of iron and siderophore antibiotics. Curr Opin Microbiol 2002;5:194–201.

35 Andrews SC, Robinson AK, Rodríguez-Quiñones F: Bacterial iron homeostasis. FEMS Microbiol Rev 2003;27:215–237.

36 Corsini G, Baeza M, Monasterio O, Lagos R: The expression of genes involved in microcin maturation regulates the production of active microcin E492. Biochimie 2002;84:539–544.

37 Hantke K: Dihydroxybenzoylserine – a siderophore for E. coli. FEMS Microbiol Lett 1990;55:5–8.

38 Blond A, Peduzzi J, Goulard C, Chiuchiolo MJ, Barthélémy M, Prigent Y, Salomón RA, Farías RN, Moreno F, Rebuffat S: The cyclic structure of microcin J25, a 21-residue peptide antibiotic from *Escherichia coli*. Eur J Biochem 1999;259:747–755.

39 Blond A, Cheminant M, Segalas-Milazzo I, Peduzzi J, Barthelemy M, Goulard C, Salomón R, Moreno F, Farías R, Rebuffat S: Solution structure of microcin J25, the single macrocyclic antimicrobial peptide from *Escherichia coli*. Eur J Biochem 2001;268:2124–2133.

40 Blond A, Cheminant M, Destoumieux-Garzón D, Segalas-Milazzo I, Peduzzi J, Goulard C, Rebuffat S: Thermolysin-linearized microcin J25 retains the structured core of the native macrocyclic peptide and displays antimicrobial activity. Eur J Biochem 2002;269:6212–6222.

41 Rosengren KJ, Blond A, Afonso C, Tabet JC, Rebuffat S, Craik DJ: Structure of thermolysin cleaved microcin J25: extreme stability of a two-chain antimicrobial peptide devoid of covalent links. Biochemistry 2004;43:4696–4702.

42 Frechet D, Guitton JD, Herman F, Faucher D, Helynck G, Monegier du Sorbier B, Ridoux JP, James-Surcouf E, Vuilhorgne M: Solution structure of RP 71955, a new 21 amino acid tricyclic peptide active against HIV-1 virus. Biochemistry 1994;33:42–50.

43 Metlitskaya AZ, Katrukha GS, Shashkov AS, Zaitsev DA, Egorov TA, Khmel IA: Structure of microcin C51, a new antibiotic with a broad spectrum of activity. FEBS Lett 1995;357:235–238.

44 Destoumieux-Garzón D, Thomas X, Santamaria M, Goulard C, Barthélémy M, Boscher B, Bessin Y, Molle G, Pons AM, Letellier L, Peduzzi J, Rebuffat S: Microcin E492 antibacterial activity: evidence for a TonB-dependent inner membrane permeabilization on *Escherichia coli*. Mol Microbiol 2003;49:1031–1041.

45 Lagos R, Wilkens M, Vergara C, Cecchi X, Monasterio O: Microcin E492 forms ion channels in phospholipid bilayer membrane. FEBS Lett 1993;321:145–148.

46 Destoumieux-Garzón D, Peduzzi J, Thomas X, Djediat C, Rebuffat S: Parasitism of iron-siderophore receptors of *Escherichia coli* by the siderophore-peptide microcin E492m and its unmodified counterpart. Biometals 2006;19:181–191.

47 Pugsley AP, Moreno F, de Lorenzo V: Microcin-E492-insensitive mutants of *Escherichia coli* K12. J Gen Microbiol 1986;132:3253–3259.

48 Braun V: Energy-coupled transport and signal transduction through the gram-negative outer membrane via TonB-ExbB-ExbD-dependent receptor proteins. FEMS Microbiol Rev 1995;16:295–307.

49 Postle K: TonB protein and energy transduction between membranes. J Bioenerg Biomembr 1993;25:591–601.

50 Peacock RS, Weljie AM, Howard SP, Price FD, Vogel HJ: The solution structure of the *C*-terminal domain of TonB and interaction studies with TonB box peptides. J Mol Biol 2005;345:1185–1197.

51 Neilands JB: Siderophores: structure and function of microbial iron transport compounds. J Biol Chem 1995;270:26723–26726.

52 Faraldo-Gomez JD, Sansom MS: Acquisition of siderophores in Gram-negative bacteria. Nat Rev Mol Cell Biol 2003;4:105–116.

53 Salomón RA, Farías RN: Microcin 25, a novel antimicrobial peptide produced by *Escherichia coli*. J Bacteriol 1992;174:7428–7435.

54 Portrait V, Gendron-Gaillard S, Cottenceau G, Pons AM: Inhibition of pathogenic *Salmonella enteritidis* growth mediated by *Escherichia coli* microcin J25 producing strains. Can J Microbiol 1999;45:988–994.

55 Delgado MA, Rintoul MR, Farías RN, Salomón RA: *Escherichia coli* RNA polymerase is the target of the cyclopeptide antibiotic microcin J25. J Bacteriol 2001;183:4543–4550.

56 Yuzenkova J, Delgado M, Nechaev S, Savalia D, Epshtein V, Artsimovitch I, Mooney RA, Landick R, Farías RN, Salomón R, Severinov K: Mutations of bacterial RNA polymerase leading to resistance to microcin J25. J Biol Chem 2002;277:50867–50875.

57 Adelman K, Yuzenkova J, La Porta A, Zenkin N, Lee J, Lis JT, Borukhov S, Wang MD, Severinov K: Molecular mechanism of transcription inhibition by peptide antibiotic Microcin J25. Mol Cell 2004;14:753–762.
58 Mukhopadhyay J, Sineva E, Knight J, Levy RM, Ebright RH: Antibacterial peptide microcin J25 inhibits transcription by binding within and obstructing the RNA polymerase secondary channel. Mol Cell 2004;14:739–751.
59 Salomón RA, Farías RN: The FhuA protein is involved in microcin 25 uptake. J Bacteriol 1993;175:7741–7742.
60 Salomón RA, Farías RN: The peptide antibiotic microcin 25 is imported through the TonB pathway and the SbmA protein. J Bacteriol 1995;177:3323–3325.
61 Destoumieux-Garzón D, Duquesne S, Péduzzi J, Goulard C, Desmadril M, Letellier L, Rebuffat S, Boulanger P: The iron-siderophore transporter FhuA is the receptor for the AMP microcin J25: role of the microcin Val11-Pro16 β-hairpin region in the recognition mechanism. Biochem J 2005;389:869–876.
62 Semenova E, Yuzenkova Y, Peduzzi J, Rebuffat S, Severinov K: Structure-activity analysis of microcin J25: distinct parts of the threaded lasso molecule are responsible for interaction with bacterial RNA polymerase. J Bacteriol 2005;187:3859–3863.
63 Laviña M, Pugsley AP, Moreno F: Identification, mapping, cloning and characterization of a gene (sbmA) required for microcin B17 action on *Escherichia coli* K12. J Gen Microbiol 1986;132:1685–1693.
64 Ferguson GP, Jansen A, Marlow VL, Walker GC: BacA-mediated bleomycin sensitivity in *Sinorhizobium meliloti* is independent of the unusual lipid A modification. J Bacteriol 2006;188:3143–3148.
65 De Cristobal RE, Solbiati JO, Zenoff AM, Vincent PA, Salomón RA, Yuzenkova J, Severinov K, Farías RN: Microcin J25 uptake: His5 of the MccJ25 lariat ring is involved in interaction with the inner membrane MccJ25 transporter protein SbmA. J Bacteriol 2006;188:3324–3328.
66 González-Pastor JE, San Millán JL, Castilla MA, Moreno F: Structure and organization of plasmid genes required to produce the translation inhibitor microcin C7. J Bacteriol 1995;177:7131–7140.
67 Khmel IA, Bondarenko VM, Manokhina IM, Basyuk EI, Metlitskaya AZ, Lipasova VA, Romanova YM: Isolation and characterization of *Escherichia coli* strains producing microcins B and C types. FEMS Microbiol Lett 1993;111:269–274.
68 Kurepina NE, Basyuk EI, Metlitskaya AZ, Zaitsev DA, Khmel IA: Cloning and mapping of the genetic determinants for microcin C51 production and immunity. Mol Gen Genet 1993;241:700–706.
69 Metlitskaya A, Kazakov T, Kommer A, Pavlova O, Praetorius-Ibba M, Ibba M, Krasheninnikov I, Kolb V, Khmel I, Severinov K: Aspartyl-tRNA synthetase is the target of peptide-nucleotide antibiotic microcin C. J Biol Chem 2006;281:18033–18042.
70 Braun V, Gunthner K, Hantke K, Zimmerman L: Intracellular activation of albomycin in *Escherichia coli* and *Salmonella typhimurium*. J Bacteriol 1983;156:308–315.

Review

Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol 2007;13:210–219
DOI: 10.1159/000104750

The Two-Peptide Class II Bacteriocins: Structure, Production, and Mode of Action

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Key Words
Bacteriocins · Antimicrobial peptides · Immunity proteins · Two-peptide bacteriocins

Abstract
The two-peptide class II bacteriocins consist of two different unmodified peptides, both of which must be present in about equal amounts in order for these bacteriocins to exert optimal antimicrobial activity. These bacteriocins render the membrane of target cells permeable to various small molecules. The genes encoding the two peptides of two-peptide bacteriocins are adjacent to each other in the same operon and they are near the genes encoding (i) the immunity protein that protects the bacteriocin-producing bacteria from being killed by their own bacteriocin, (ii) a dedicated ABC transporter that transports the bacteriocin out of the bacteriocin-producing bacteria, and (iii) an accessory protein whose specific role is not known, but which also appears to be required for secretion of the bacteriocin. The production of some two-peptide bacteriocins is transcriptionally regulated through a three-component regulatory system that consists of a membrane-interacting peptide pheromone, a membrane-associated histidine protein kinase, and response regulators. Structure analysis of three two-peptide bacteriocins (plantaricin E/F, plantaricin J/K, and lactococcin G) by CD (and in part by NMR) spectroscopy reveal that these bacteriocins contain long amphiphilic α-helical stretches and that the two complementary peptides interact and structure each other when exposed to membrane-like entities. Lactococcin G shares about 55% sequence identity with enterocin 1071, but these two bacteriocins nevertheless kill different types of bacteria. The target-cell specificity of lactococcin G-enterocin 1071 hybrid bacteriocins that have been constructed by site-directed mutagenesis suggests that the β-peptide is important for determining the target-cell specificity.
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Introduction

Many bacteria produce ribosomally synthesized antimicrobial peptides, often referred to as peptide bacteriocins [1–5]. There is great interest in developing these peptides into agents for treatment of infections and for preservation of food and animal feed, not the least because of the alarming increase in antibiotic-resistant pathogenic bacteria and the undesirable side effects that many chemical preservatives may have. There has especially been great interest in the peptide bacteriocins produced by lactic acid bacteria (LAB) because of the 'food grade quality' and industrial importance of these bacteria. The LAB peptide bacteriocins are grouped into two main classes.

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Table 1. An overview of genetically and biochemically characterized two-peptide bacteriocins

Bacteriocin[1]	Producer	Ref.
Lactococcin G	*Lactococcus lactis* LMGT2081	6
Enterocin 1071[2]	*Enterococcus faecalis* FAIR-E 309	7
	Enterococcus faecalis BFE 1071	8, 9
Lactococcin Q	*Lactococcus lactis* QU 4	10
Plantaricin E/F	*Lactobacillus plantarum* C11	11, 12
Plantaricin J/K	*Lactobacillus plantarum* C11	11, 12
Plantaricin S	*Lactobacillus plantarum* LCP010	13, 14
Plantaricin NC8	*Lactobacillus plantarum* NC8	15
Lactacin F	*Lactobacillus johnsonii* VPI11088	16, 17
Brochocin C	*Brochothrix campestris* ATCC 43754	18
Thermophilin 13	*Streptococcus thermophilus* Sfi13	19
ABP-118	*Lactobacillus salivarius* subsp. *salivarius* UCC118	20
Mutacin IV	*Streptococcus mutans* UA140	21
Lactococcin MN	*Lactococcus lactis* 9B4	22
Lactocin 705	*Lactobacillus casei* CRL 705	23
Leucocin H[3]	*Leuconostoc* MF215B	24

[1] Enterocin L50 has two peptides [25], but is not included in the list as it is not considered a bona fide two-peptide bacteriocin, since the two peptides have similar sequences and they have potent antimicrobial activity when assayed individually. Lactococcin MMT24 [26] is not included in the list since its sequence is unknown and it is therefore uncertain whether it might be identical to one of the above bacteriocins.

[2] Refer to reference 7 for correct sequence of enterocin 1071.

[3] Leucocin H has not been completely sequenced and it is consequently uncertain whether or not it is an unmodified class II bacteriocin or a two-peptide lantibiotic (i.e. class I bacteriocin).

Class I consists of the post-translationally modified peptide bacteriocins, often referred to as lantibiotics. Class II consists of the peptide bacteriocins without modified residues, and has been divided into three subclasses, class IIa, IIb and IIc. The antilisterial one-peptide *pediocin-like* bacteriocins that have very similar amino acid sequences are allocated to class IIa, whereas non-pediocin-like one-peptide bacteriocins that show no sequence similarity to the pediocin-like bacteriocins are placed in class IIc. Class IIb contains the two-peptide bacteriocins. These bacteriocins are unique in that they consist of two very different peptides and optimal antimicrobial activity requires the presence of both peptides in about equal amounts [1–4]. Since the first isolation of a two-peptide bacteriocin, lactococcin G, in 1992 [6], at least 14 two-peptide bacteriocins produced by LAB have been identified, isolated and characterized (table 1). The focus in this article will be on the two-peptide (class IIb) bacteriocins. It should be mentioned that two-peptide lantibiotics have also recently been identified and characterized, but these class I bacteriocins will not be discussed.

The Two Peptides of Two-Peptide Bacteriocins Function as One Antimicrobial Unit

The individual peptides of two-peptide bacteriocins share many characteristics with one-peptide bacteriocins; they are all usually cationic and contain hydrophobic and/or amphiphilic regions. One or both peptides of some two-peptide bacteriocins (such as lactacin F [16] and plantaricin E/F, and J/K [11]) may in fact individually display some – although low – antimicrobial activity. Two-peptide bacteriocins should, however, not be thought of as simply two synergistically-acting one-peptide bacteriocins. A peptide from a two-peptide bacteriocin displays high antimicrobial activity only in combination with the complementary peptide from the same two-peptide bacteriocin (or in some cases in combination with a peptide from a homologous two-peptide bacteriocin; see discussion below dealing with enterocin 1071 and lactococcin G and Q). For instance, the two complementary peptides that constitute lactococcin G are active at pico- to nanomolar concentrations when combined, but show

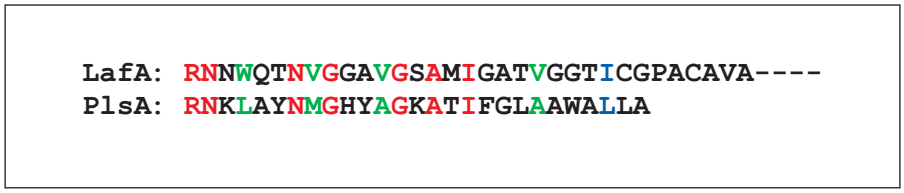

Fig. 1. Sequence alignment of the LafA peptide of lactacin F and the PlsA peptide of plantaricin S. Amino acids are colored in the two sequences as follows: amino acids that are identical in the two sequences are in red; the amino acids where both residues are similar with respect to either charge (R and K; D and E), hydrophobicity (V, I, L), hydrophilicity (N and Q; S and T) or aromatic character (W, F and Y) are in blue; amino acids that have the same polarity but are structurally somewhat dissimilar (A, M, W or F together with either I, V, L; N or Q together with either E or D) are in green; all other residues are in black. PlsA contains 27 residues whereas LafA contains 57, but only the first 33 are shown. The sequences are from references 14 and 16.

no activity when tested individually at concentrations as high as 50 μM [27], nor when combined with either the E- or F-peptide of plantaricin E/F or the J- or K-peptide of plantaricin J/K [11]. The requirement of both complementary peptides for a potent antimicrobial effect clearly indicates that the two peptides of two-peptide bacteriocins function together as one antimicrobial entity. Also the facts that (i) the genes encoding the two peptides of two-peptide bacteriocins are found next to each other on the same operon [7–10, 12, 14–23, 28] and that (ii) there is only one immunity gene for each two-peptide bacteriocin, indicate that the peptides of two-peptide bacteriocins function together as one unit. Moreover, structural studies of three two-peptide bacteriocins (lactococcin G, and plantaricin E/F and J/K) revealed a direct physical interaction between complementary peptides when they exert their bactericidal effect [29, 30] (see below).

Although two-peptide bacteriocins are not two synergistically-acting one-peptide bacteriocins, two-peptide bacteriocins may have evolved from two synergistically-acting one-peptide bacteriocins. If two synergistically-acting one-peptide bacteriocins were produced by the same bacteria, there would clearly be a selection pressure for the enhancement of the synergistic effect of the two peptides – possibly at the expense of the activity of the individual peptides – and this would in turn create selection pressure for genetically linking the two peptides, with the formation of a two-peptide bacteriocin. It should be noted in this context that one of the peptides (LafA) of the two-peptide bacteriocin lactacin F [16] shows sequence similarity to one of the peptides (PlsA) of the two-peptide bacteriocin plantaricin S [14] (fig. 1). It thus seems that one bacteriocin-like peptide might have become genetically linked with another bacteriocin-like peptide at two different occasions, resulting in the formation of the two two-peptide bacteriocins, lactacin F and plantaricin S. There is also some sequence similarity between the A-peptide of mutacin IV and the A-peptide of thermophilin 13, and between the B-peptide of mutacin IV and LafA of lactacin F [4, 21], suggesting that the two peptides that evolved into the mutacin IV A and B peptides also became separately linked to other bacteriocin-like peptides with the formation of the two two-peptide bacteriocins thermophilin 13 and lactacin F.

Two-Peptide Bacteriocins Permeabilize the Membrane of Target Cells

All two-peptide bacteriocins whose mode of action has been studied, this includes lactococcin G [27, 31], plantaricin E/F and J/K [32], lactacin F [33], thermophilin 13 [19], and lactocin 705 [34], render membranes permeable to various small molecules. The studies suggest that these two-peptide bacteriocins show some specificity with respect to which small molecules they conduct across membranes, and that the specificity varies among the various two-peptide bacteriocins. Lactococcin G permeabilizes target-cell membranes for a broad range of monovalent cations, such as Na^+, K^+, Li^+, Cs^+, Rb^+ and choline, but not for H^+, nor divalent cations (Mg^{2+}) or anions, such as phosphate [27, 31]. Plantaricin E/F and plantaricin J/K also permeabilize membranes for monovalent cations, including H^+ (in contrast to lactococcin G), but not for divalent cations (Mg^{2+}) nor phosphate [32]. It appears as if plantaricin J/K conducts anions more efficiently than plantaricin E/F and vice versa for cations [32]. Lactacin F makes the membrane permeable to K^+ and phosphate [33]. Thermophilin 13 also permeabilizes cell membranes, as it dissipates both the transmembrane electrical potential and pH gradient, but its specificity with respect to compounds it conducts across membranes has not been char-

acterized [19]. The bacteriocin-induced permeabilization of cell membranes for various ions has clearly a detrimental effect on cells, as it destroys the proton motive force by dissipation of the transmembrane electrical potential and/or the transmembrane pH gradient.

Genes and Proteins Involved in Production of Two-Peptide Bacteriocins

Five genes are the minimum required to produce two-peptide bacteriocins and these genes are usually found in either one or two operons [1–5]. There are two structural genes that encode the preforms of the two peptides that constitute the bacteriocin, one immunity gene that encodes the immunity protein that protects the bacteriocin producer from its own bacteriocin, one gene that encodes the dedicated ABC transporter that transfers the bacteriocin across the membrane concomitantly with removal of the leader sequence, and one gene that encodes an accessory protein whose specific role is not known, but which also appears to be required for secretion of the bacteriocin. For all genetically characterized two-peptide bacteriocins, the two structural genes are found next to each other on the same operon and the two peptides that constitute the bacteriocin thus appear to be produced in approximately equal amounts. This operon also contains the gene that encodes the immunity protein. The genes encoding the dedicated ABC transporter and the accessory protein are usually found in either the operon that contains the structural and immunity genes (as is the case for lactococcin G [28]) or on a separate operon nearby the operon that contains the structural and immunity genes (as is the case for enterocin 1071 and plantaricin E/F and J/K [7–9, 12]).

All two-peptide bacteriocins that have been characterized (table 1) are synthesized with a 15- to 30-residue N-terminal leader sequence of the so-called double glycine type that is cleaved off at the C-terminal side of two glycine residues by the dedicated ABC transporter upon export of the bacteriocin from cells [1–5]. A novel feature of the dedicated ABC transporter is an N-terminal extension of about 150 residues not present on other ABC transporters. Functional studies using the N-terminal region of the lactococcin G ABC transporter showed that this region specifically cleaves off the lactococcin G leader sequence at the C-terminal side of the double glycine motif [35]. The leader thus seems to facilitate interaction with the ABC transporter and may perhaps also function to keep the bacteriocin inactive until it has been secreted.

It is presently not clear how immunity proteins protect cells from bacteriocins. Structure predictions suggest that many immunity proteins have transmembrane helices. The putative immunity proteins of the two-peptide bacteriocins thermophilin 13 [3, 19], plantaricin S [3, 14] and brochocin C [3, 18] appear to contain two transmembrane helices, while the putative immunity proteins of lactococcin MN [3, 22], lactococcin G [3, 28], and plantaricin E/F and J/K [3, 12] may contain 4–5 transmembrane helices. The number of transmembrane helices thus seems to differ, but a common mechanism for bacteriocin immunity involving interactions with membrane components may nevertheless exist.

Production of Some Two-Peptide Bacteriocins Is Regulated through a Three-Component Regulatory System

The production of some two-peptide bacteriocins, such as ABP-118 [20] and plantaricin E/F, J/K, [12, 36–41] and NC8 [42], is transcriptionally regulated through a three-component regulatory system that consists of a peptide pheromone, a membrane-associated histidine protein kinase, and response regulators [43–45]. The regulation of bacteriocins by three-component regulatory systems has perhaps been most thoroughly characterized in *Lactobacillus plantarum* C11 which produces the two two-peptide bacteriocins plantaricin E/F and J/K and the peptide pheromone plantaricin A, which induces the production of these two bacteriocins [11, 12, 36–41]. Plantaricin A is encoded by a gene located in a regulatory operon that also contains the genes encoding the histidine protein kinase and two response regulators. The production of plantaricin E/F and J/K is apparently activated when the concentration of plantaricin A reaches a threshold value as a result of high cell density. In addition to activating bacteriocin production, plantaricin A also induces its own production. An autoinduction loop is thus triggered, resulting in a rapid increase in the transcription of all genes involved in the production of plantaricin E/F and J/K.

Signal transduction is apparently initiated when plantaricin A binds to the membrane-associated histidine protein kinase, as this triggers the kinase to phosphorylate the two response regulators, which in turn activate the genes encoding plantaricin E/F and J/K, as well as the gene encoding plantaricin A [38–41]. A recent study on the structure and mode of action of plantaricin A revealed a novel mechanism whereby plantaricin A (and

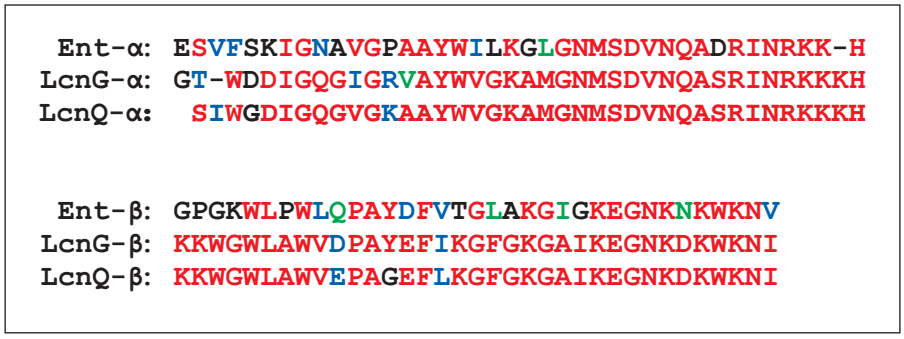

Fig. 2. Sequence alignment of the α- and β-peptides of enterocin 1071 and lactococcin G and Q. Amino acids are colored in the sequences as described in legend to figure 1. The sequences are from references 6, 7 and 10.

Table 2. The activity[1] of lactococcin G and enterocin 1071 against four different indicator strains

Organism	Strain	MIC for lactococcin G nM	MIC for enterocin 1071, nM
Enterococcus faecalis	NCDO 581	>50	0.2
Enterococcus faecalis	LMGT2333	>50	0.3
Lactobacillus casei ssp. *casei*	NCDO 161	>50	>50
Lactococcus	LMGT2077	0.2	4.7

[1] Activity is quantitated in terms of minimum inhibitory concentrations (MIC) of the two peptides in nM (the sum of both complementary peptides (1:1 ratio) concentrations) measured against four different strains as described in references 6 and 27.

perhaps other membrane active peptide pheromones/hormones) functions [46]. It seems that plantaricin A interacts initially in a non-chiral manner with membrane lipids and that this interaction induces α-helical structuring in part of the peptide [46]. The peptide thus becomes sufficiently structured and properly positioned in the membrane and this enables it to subsequently interact in a chiral manner with the receptor in or near the membrane-water interface. This membrane-interacting mode of action may explain why peptide pheromones that are part of three-component regulatory systems are often cationic with the potential to form an amphiphilic helix upon interacting with membranes.

Sequence Similarities between the Two-Peptide Bacteriocins Enterocin 1071 and Lactococcin G and Q

Three of the two-peptide bacteriocins that have been identified and characterized, lactococcin G [6, 27, 29, 31], lactococcin Q [10], and enterocin 1071 [7–9], show marked sequence similarity and they are thus clearly evolutionary related (fig. 2). Despite about 55% sequence identity between lactococcin G and enterocin 1071, they appear to differ in their relative potencies to different target cells [6, 8] (table 2). These two bacteriocins (along with lactococcin Q) consequently represent an excellent system for correlating the structure of two-peptide bacteriocins with target-cell specificity, potency, and interactions with immunity proteins. We are presently studying the structure-function relationship of these two two-peptide bacteriocins by analyzing their NMR structure and by constructing and testing the activity of modified bacteriocin variants, of which hybrids of lactococcin G and enterocin 1071 are of particular interest. We will in the rest of this review especially focus on lactococcin G and enterocin 1071 (along with homologues lactococcin Q), but we will also refer to other two-peptide bacteriocins whenever this is relevant for highlighting general differences and similarities between the various two-peptide bacteriocins.

Lactococcin G is produced by a few strains of *Lactococcus lactis* and is perhaps presently the best characterized class II two-peptide bacteriocin [6, 27–29, 31]. Enterocin

1071 is produced by strains of *Enterococcus faecalis* [7–9], while lactococcin Q was recently isolated from a *L. lactis strain* [10]. One of the peptides in these three bacteriocins contains 39 residues and is termed α (termed Ent1071A in references 7–9) and the other peptide contains 35 residues and is termed β (termed Ent1071B in references 7–9) (fig. 2). As is the case with most, if not all two-peptide bacteriocins, the complementary α- and β-peptides of these bacteriocins are initially synthesized as preforms with double glycine type leaders [7–10, 28, 47], and the genes that encode the two complementary peptides are adjacent to each other in the same operon, which in case of lactococcin G and enterocin 1071 also contains the gene encoding the immunity protein (this has not been determined for lactococcin Q), and in the case of lactococcin G, also the genes encoding the ABC transporter and the accessory protein [7–10, 28]. In contrast to lactococcin G, the genes encoding the ABC transporter and the accessory protein for enterocin 1071 are located on another operon that is transcribed in the opposite direction of the operon that contains the structural genes [9].

These three bacteriocins (enterocin 1071 and lactococcin G and Q) represent an exception to the rule that only combinations of complementary peptides from one and the same two-peptide bacteriocin display marked antimicrobial activity, since high activity is obtained upon combining one peptide from one of these two-peptide bacteriocins with the complementary peptide from one of the two other bacteriocins (fig. 3; see reference 10 for combinations of peptides from lactococcin G and Q). The high sequence similarity among these three bacteriocins thus enables peptides from two different bacteriocins to function together; although the activity is often reduced, but there are some interesting exceptions. For instance, the hybrid combination LcnG-β + Ent-α was about 4-fold more potent than enterocin 1071 (Ent-α + Ent-β) against *Lactococcus* LMGT2077, although it was about 5-fold less potent than lactococcin G (LcnG-α + LcnG-β) – perhaps not surprising considering that lactococcin G is about 20-fold more potent than enterocin 1071 against these cells (fig. 3). The hybrid combination LcnG-α + Ent-β was inactive. Taken together, the results suggest that the β-peptide of these bacteriocins is especially important in determining the target-cell specificity. This conclusion is supported by results we have obtained by determining the potencies against various target cells of all combinations of 15 different LcnG-α/Ent-α hybrid peptides and more than 20 different LcnG-β/Ent-β hybrid peptides (i.e. more than 15 × 20 combinations of various complementary hybrid peptides [48].

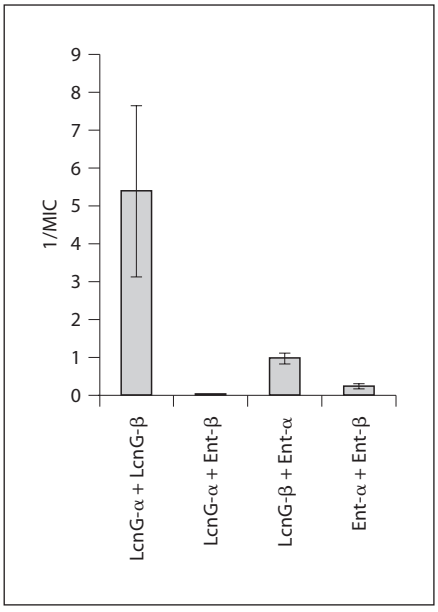

Fig. 3. The activity obtained upon combining one peptide from enterocin 1071 with the complementary peptide from lactococcin G (i.e. LcnG-α + Ent-β and LcnG-β + Ent-α). The activity of wild-type combinations (i.e. LcnG-α + LcnG-β and Ent-α + Ent-β) are also shown for comparison. The activity is quantitated in terms of 1/MIC where MIC is the minimum inhibitory concentration of the two peptides in nM measured against *Lactococcus* LMGT 2077 as described in references 6 and 27. The 1/MIC values of the combination LcnG-α + Ent-β was <0.02 nM^{-1}.

Secondary Structure and Interaction with Target-Cell Membranes

The α- and β-peptides of enterocin 1071 and lactococcin G and Q have putative amphiphilic α-helices in their N-terminal and mid region (residues 3–27 in α and 8–25 in β) as judged by displaying their sequences on an Edmundson α-helical wheel [6, 8, 9]. The non-polar amino acids are found almost exclusively on one side of the helix while the polar residues are found on the opposite side. CD structural studies of the lactococcin G α- and β-peptides and fragments thereof show that the α- and β-peptides are unstructured in water and that there is no structural interaction between them when they are free in aqueous solution, but they become structured upon exposure to membrane-like entities, such as micelles and negatively charged liposomes [29]. Moreover, the studies revealed that membrane-like entities induced the formation of an amphiphilic α-helix in the N-terminal and mid region of both peptides, showing that this region in both peptides does indeed form an amphiphilic α-helix [29]

Table 3. α-Helical content[1] in the α- and β-peptides of lactococccin G, and in various fragments of these peptides, all in the presence of micelles or liposomes

Peptide	α-Helical content in the presence of dodecylphosphocholine micelles[2]		α-Helical content in the presence of 1.4 mM dioleoylglycerophosphoglycerol liposomes	
	% helicity	residue number	% helicity	residue number
α	42	16–17	44	17
α(1–26)	62	16–17	67	17–18
α(19–39)	8	1–2	6	1–2
β	25	8–9	36	12–13
β(1–26)	26	6–7	33	8–9
β(6–26)	30	6–7	37	7–8
β(10–26)	9	1–2	20	3–4
β(10–35)	8	2	27	7
β(21–35)	5	0–1	12	1–2

[1] Data are from reference 29.
[2] The concentration of dodecylphosphocholine was 4 mM in all cases except α(19–39) and β(21–35) for which 12 mM was used.

(table 3). Recent NMR analysis of the α-peptide in micelles indicates an α-helical region from about residue 3 to 22, with a possible break in the glycine-rich region between residue 7 and 12 [Rogne et al., unpubl. data]. For the β-peptide, the NMR data suggest an α-helical region that includes the proline residue in position 11 and stretches about 3–5 residues to each side [Rogne et al., unpubl. data]. Interestingly, in the presence of liposomes, the α- and β-peptides induce additional α-helical structuring in each other [29] (table 4), indicating that the peptides interact upon contact with target membranes. The additional structuring occurred only when the two peptides were added simultaneously to liposomes, not when one peptide was added before the other, nor when two liposome samples, each containing a peptide, were mixed [29] (table 4).

CD structural studies similar to those done on lactococcin G have also been carried out with two other two-peptide bacteriocins, plantaricin E/F and plantaricin J/K and basically the same results were obtained [30]. Membrane-like entities induced amphiphilic α-helices in the peptides of plantaricin E/F and J/K and additional structuring was obtained when complementary peptides were exposed simultaneously to the membrane entities [30] (table 4). One would expect similar results also for the α- and β-peptides of enterocin 1071 and lactococcin Q, since their sequence similarities to lactococcin G indicate that enterocin 1071 and lactococcin G and Q have similar three-dimensional structures and mode of action. For enterocin 1071 and lactococcin G and Q, as well as for plantaricin E/F and J/K, the two complementary peptides thus appear to interact in a structure-inducing manner upon arrival at the target membrane, resulting in the formation of an antimicrobial peptide complex with amphiphilic α-helical regions. The synergistic antimicrobial effect obtained with the two complementary peptides of two-peptide bacteriocins is thus apparently due to inter-peptide interactions, rather than to the two complementary peptides interacting separately at different sites on the target cell. It is not clear exactly at what stage this inter-peptide interaction occurs when peptides are exposed to sensitive cells, but it presumably occurs after the peptides come in contact with the target cells and before they become fully embedded in the lipids of the cell membrane. The peptides might first bind individually to an entity on the cell wall or membrane and then interact before penetrating further into the hydrophobic part of the cell membrane. Such a sequence of events is consistent with the following observations: toxicity is observed when sensitive cells are first treated with one lactococcin G peptide, washed and then treated with the other lactococcin G peptide, whereas no toxicity is observed when cells treated with one peptide are mixed with cells treated with the other peptide [31]. This indicates that both peptides can separately interact stably with the target-cell surface without losing their potential bacteriocin activity

Table 4. Percent helical content[a] in lactococcin G peptides and the plantaricin E/F and J/K peptides upon combining complementary peptides in 1.4 mM dioleoylglycerophosphoglycerol liposomes

Mixing procedure	% helical content of peptide combination		
	LcnG-α + LcnG-β	PlnE + PlnF	PlnJ + PlnK
Mixing two peptides before adding liposomes	52	38	42
Mixing two liposomes-peptide samples	41	32	30
Calculated average based on individual measurements	40	29	30

[a] Data are from references 29 and 30.

in this '*dormant state*' (i.e. when bound separately to the cell surface), but they are unable to diffuse to another cell once bound to the cell surface [31].

The sequences of lactacin F [16] and plantaricin S [14] and NC8 [15] suggest that also these two-peptide bacteriocins contain relatively long amphiphilic α-helical segments. The amphiphilic α-helix thus appears to be an important structural motif in many two-peptide bacteriocins, but there are some exceptions. CD and NMR structural analysis of the brochocin C α- and β-peptides suggest a high content of β-sheet structure in these peptides [49], and this is probably also the case for the two peptides that constitute thermophilin 13, since it has marked sequence similarity with brochocin C [18, 19] and thus presumably also has similar three-dimensional structure. Moreover, both the complementary peptides of brochocin C and thermophilin 13 contain long hydrophobic segments with a cysteine residue near each end [18, 19], and it is consequently difficult to envisage how these dominantly hydrophobic peptides can form amphiphilic (helical) segments of any length. Also both peptides of AMP-118 contain hydrophobic segments (30–40 residues) with cysteine residues near each end [20]. One of the peptides of lactococcin MN has also a hydrophobic segment of about 40 residues [22], but without cysteine residues, whereas both peptides of mutacin IV have (somewhat shorter and not as marked) hydrophobic segments that contain two cysteine residues [21].

The hydrophobic and or amphiphilic character of two-peptide bacteriocins presumably enables their membrane-interacting mode of action. The peptides with a long hydrophobic segment presumably insert the hydrophobic segment into the hydrophobic phase of the target membrane, and those with an amphiphilic α-helix are thought to initially interact with the target membrane according to the 'carpet mechanism', which entails that the amphiphilic helix lies parallel to the plane of the membrane in the interface region, with the hydrophobic side of the helix facing towards and shallowly penetrating into the hydrophobic phase of the membrane. It is uncertain to what extent this 'carpet mechanism' is sufficient to destabilize the phospholipid packing of membranes and cause membrane permeabilization at pico- to nanomolar concentrations of peptide, and several features suggest that the amphiphilic helical region is likely to eventually insert obliquely or more perpendicularly into membranes. The fact that the two complementary peptides function together synergistically through structure-stabilizing interactions indicates that membrane permeabilization is much more complex than simply the horizontal binding of amphiphilic helical structures to the cell surface according to the 'carpet mechanism'. Moreover, the high potency of the two-peptide bacteriocins suggests that membrane permeabilization depends on a relative low number of peptides that may interact with membrane proteins, unlike what is expected if permeabilization occurs through the 'carpet mechanism'. Finally, the fact that two-peptide bacteriocins are relatively specific with respect to the molecules they conduct across membranes (see discussion above on permeabilization of target-cell membrane) suggests that they form specific pores, rather than cause unspecific disintegration of membranes through the 'carpet mechanism'.

A fusion protein consisting of the immunoglobulin-binding domain of streptococcal protein G (GB1 domain) linked to the N-terminal residues of the lactococcin G α- and β-peptide exhibited bacteriocin activity when combined with the lactococcin G β- and α-peptide, respectively, which could indicate that the N-terminal parts of the lactococcin G α- and β-peptide are not the regions that penetrate into or through the target-cell membrane. This might, however, be an overinterpretation, since the

fused β-peptide was about 5,000-fold less potent than the unfused β-peptide and the fused α-peptide was 20,000- to 30,000-fold less active than the unfused α-peptide [Rogne and Oppegård, unpubl. results].

Concluding Remarks

More detailed structural analysis of two-peptide bacteriocins combined with construction and analysis of genetically modified variants is clearly necessary in order to gain more insight into the structure and structural features that are important for the mode of action and potency of two-peptide bacteriocins. Such insight is invaluable for future rational design of more optimal bacteriocin variants and for optimal use of bacteriocins in medical and biotechnological applications. The future use of LAB bacteriocins for preservation of food and animal feed and for treatment of infections looks promising, since these bacteriocins exert their antimicrobial activity very differently from antibiotics and preservatives, and might thus complement and possibly substitute antibiotics and preservatives. Moreover, these bacteriocins may be considered as relatively safe, since LAB are part of the natural microbial flora in food humans consume, they constitute a significant part of our indigenous flora, and they are used in food and feed production.

Acknowledgment

This work has been supported by the Norwegian Research Council.

References

1 Nes IF, Diep DB, Håvarstein LS, Brurberg MB, Eijsink V, Holo H: Biosynthesis of bacteriocins in lactic acid bacteria. Antonie van Leeuwenhoek 1996;70:113–128.
2 Nissen-Meyer J, Nes IF: Ribosomally synthesized antimicrobial peptides: their function, structure, biogenesis, and mechanism of action. Arch Microbiol 1997;167:67–77.
3 Nes IF, Holo H, Fimland G, Hauge HH, Nissen-Meyer J: Unmodified peptide-bacteriocins (class II) produced by lactic acid bacteria; in Dutton CJ, Haxell MA, McArthur HAI, Wax RG (eds): Peptide Antibiotics, Discovery, Modes of Action, and Application. New York, Dekker, 2002, pp 81–115.
4 Garneau S, Martin NI, Vederas JC: Two-peptide bacteriocins produced by lactic acid bacteria. Biochimie 2002;84:577–592.
5 Fimland G, Johnsen L, Dalhus B, Nissen-Meyer J: Pediocin-like antimicrobial peptides (class IIa bacteriocins) and their immunity proteins: biosynthesis, structure, and mode of action. J Pept Sci 2005;11:688–696.
6 Nissen-Meyer J, Holo H, Håvarstein LS, Sletten K, Nes IF: A novel lactococcal bacteriocin whose activity depends on the complementary action of two peptides. J Bacteriol 1992;174:5686–5692.
7 Franz CMAP, Grube A, Herrmann A, Abriouel H, Stärke J, Lombardi A, Tauscher B, Holzapfel WH: Biochemical and genetic characterization of the two-peptide bacteriocin enterocin 1071 produced by *Enterococcus faecalis* FAIR-E 309. Appl Environ Microbiol 2002;68:2550–2554.
8 Balla E, Dicks LMT, Du Toit M, van der Merwe MJ, Holzapfel WH: Characterization and cloning of the genes encoding enterocin 1071A and enterocin 1071B, two antimicrobial peptides produced by *Enterococcus faecalis* BFE 1071. Appl Environ Microbiol 2000;66:1298–1304.
9 Balla E, Dicks LMT: Molecular analysis of the gene cluster involved in the production and secretion of enterocins 1071A and 1071B and of the genes responsible for the replication and transfer of plasmid pEF1071. Int J Food Microbiol 2005;99:33–45.
10 Zendo T, Koga S, Shigeri Y, Nakayama J, Sonomoto K: Lactococcin Q, a novel two-peptide bacteriocin produced by *Lactococcus lactis* QU 4. Appl Environ Microbiol 2006; 72:3383–3389.
11 Anderssen EL, Diep DB, Nes IF, Eijsink VGH, Nissen-Meyer J: Antagonistic activity of *Lactobacillus plantarum* C11: two new two-peptide bacteriocins, plantaricin EF and JK, and the induction factor, plantaricin A. Appl Environ Microbiol 1998;64:2269–2272.
12 Diep DB, Håvarstein LS, Nes IF: Characterization of the locus responsible for the bacteriocin production in *Lactobacillus plantarum* C11. J Bacteriol 1996;178:4472–4483.
13 Jiménez-Diaz R, Ruiz-Barba JL, Cathcart DP, Holo H, Nes IF, Sletten KH, Warner PJ: Purification and partial amino acid sequence of plantaricin S, a bacteriocin produced by *Lactobacillus plantarum* LPCO10, the activity of which depends on the complementary action of two peptides. Appl Environ Microbiol 1995;61:4459–4463.
14 Stephens SK, Floriano B, Cathcart DP, Bayley SA, Witt VF, Jiménez-Díaz R, Warner PJ, Ruiz-Barba JL: Molecular analysis of the locus responsible for production of plantaricin S, a two-peptide bacteriocin produced by *Lactobacillus plantarum* LPCO10. Appl Environ Microbiol 1998;64:1871–1877.
15 Maldonado A, Ruiz-Barba JL, Jiménez-Diaz R: Purification and genetic characterization of plantaricin NC8, a novel coculture-inducible two-peptide bacteriocin from *Lactobacillus plantarum* NC8. Appl Environ Microbiol 2003;69:383–389.
16 Allison GE, Fremaux C, Klaenhammer TR: Expansion of bacteriocin activity and host range upon complementation of two peptides encoded within the lactacin F operon. J Bacteriol 1994;176:2235–2241.
17 Fremaux C, Ahn C, Klaenhammer TR: Molecular analysis of the lactacin F operon. Appl Environ Microbiol 1993;59:3906–3915.
18 McCormick JK, Poon A, Sailer M, Gao Y, Roy KL, McMullen LM, Vederas JC, Stiles ME, van Belkum MJ: Genetic characterization and heterologous expression of brochocin-C, an antibotulinal, two-peptide bacteriocin produced by *Brochothrix campestris* ATCC 43754. Appl Environ Microbiol 1998;64:4757–4566.
19 Marciset O, Jeronimus-Stratingh MC, Mollet B, Poolman B: Thermophilin 13, a nontypical antilisterial poration complex bacteriocin, that functions without a receptor. J Biol Chem 1997;272:14277–14284.

20 Flynn S, van Sinderen D, Thornton GM, Holo H, Nes IF, Collins JK: Characterization of the genetic locus responsible for the production of ABP-118, a novel bacteriocin produced by the probiotic bacterium *Lactobacillus salivarius* subsp. *salivarius* UCC118. Microbiology 2002;148:973–984.

21 Qi F, Chen P, Caufield PW: The group I strain of *Streptococcus mutans*, UA140, produces both the lantibiotic mutacin I and a nonlantibiotic bacteriocin, mutacin IV. Appl Environ Microbiol 2001;67:15–21.

22 Van Belkum MJ, Hayema BJ, Jeeninga RE, Kok J, Venema G: Organization and nucleotide sequence of two lactococcal bacteriocin operons. Appl Environ Microbiol 1991;57: 492–498.

23 Cuozzo SA, Sesma F, Palacios JM, de Ruiz Holgado AP, Raya RR: Identification and nucleotide sequence of genes involved in the synthesis of lactocin 705, a two-peptide bacteriocin from *Lactobacillus casei* CRL 705. FEMS Microbiol Lett 2000;185:157–161.

24 Blom H, Katla T, Holck A, Sletten K, Axelsson L, Holo H: Characterization, production, and purification of leucocin H, a two-peptide bacteriocin from *Leuconostoc* MF215B. Curr Microbiol 1999;39:43–48.

25 Cintas LM, Casaus P, Holo H, Hernandez PE, Nes IF, Håvarstein LS: Enterocins L50A and L50B, two novel bacteriocins from *Enterococcus faecium* L50, are related to staphylococcal hemolysins. J Bacteriol 1998;180: 1988–1994.

26 Ghrairi T, Frére J, Berjeaud JM, Manai M: Lactococcin MMT24, a novel two-peptide bacteriocin produced by *Lactococcus lactis* isolated from rigouta cheese. Int J Food Microbiol 2005;105:389–398.

27 Moll G, Ubbink-Kok T, Hauge HH, Nissen-Meyer J, Nes IF, Konings WN, Driessen AJM: Lactococcin G is a potassium ion-conducting, two-component bacteriocin. J Bacteriol 1996;178:600–605.

28 Nes IF, Håvarstein LS, Holo H: Genetics of non-lantibiotic bacteriocins; in Ferretti JJ, Gilmore MS, Klaenhammer TR, Brown F (eds): Genetics of Streptococci, Enterococci and Lactococci. Developments in Biological Standards. Basel, Karger, 1995, vol 85, pp 645–651.

29 Hauge HH, Nissen-Meyer J, Nes IF, Eijsink VGH: Amphiphilic α-helices are important structural motifs in the α and β peptides that constitute the bacteriocin lactococcin G: enhancement of helix formation upon α-β interaction. Eur J Biochem 1998;251:565–572.

30 Hauge HH, Mantzilas D, Eijsink VGH, Nissen-Meyer J: Membrane-mimicking entities induce structuring of the two-peptide bacteriocins plantaricin E/F and plantaricin J/K. J Bacteriol 1999;181:740–747.

31 Moll G, Hauge HH, Nissen-Meyer J, Nes IF, Konings WN, Driessen AJM: Mechanistic properties of the two-component bacteriocin lactococcin G. J Bacteriol 1998;180:96–99.

32 Moll GN, van den Akker E, Hauge HH, Nissen-Meyer J, Nes IF, Konings WN, Driessen AJM: Complementary and overlapping selectivity of the two-peptide bacteriocin EF and JK. J Bacteriol 1999;181:4848–4852.

33 Abee T, Klaenhammer TR, Letellier L: Kinetic studies of the action of lactacin F, a bacteriocin produced by *Lactobacillus johnsonii* that forms poration complexes in the cytoplasmic membrane. Appl Environ Microbiol 1994;60:1006–1013.

34 Cuozzo SA, Castellano P, Sesma FJM, Vignolo GM, Raya RR: Differential roles of the two-component peptides of lactocin 705 in antimicrobial activity. Curr Microbiol 2003; 46:180–183.

35 Håvarstein LS, Diep DB, Nes IF: A family of bacteriocin ABC transporters carry out proteolytic processing of their substrates concomitant with export. Mol Microbiol 1995; 16:229–240.

36 Diep DB, Håvarstein LS, Nes IF: A bacteriocin-like peptide induces bacteriocin synthesis in *Lactobacillus plantarum* C11. Mol Microbiol 1995;18:631–639.

37 Hauge HH, Mantzilas D, Moll GN, Konings WN, Driessen AJ, Eijsink VGH, Nissen-Meyer J: Plantaricin A is an amphiphilic α-helical bacteriocin-like pheromone which exerts antimicrobial and pheromone activities through different mechanisms. Biochemistry 1998;37:16026–16032.

38 Diep DB, Myhre R, Johnsborg O, Aakra Å, Nes IF: Inducible bacteriocin production in *Lactobacillus* is regulated by differential expression of *pln* operons and by two antagonizing response regulators, the activity of which is enhanced upon phosphorylation. Mol Microbiol 2003;47:483–494.

39 Risøen PA, Håvarstein LS, Diep DB Nes IF: Identification of the DNA-binding sites for two response regulators involved in control of bacteriocin synthesis in *Lactobacillus plantarum* C11. Mol Gen Genet 1998;259: 224–232.

40 Risøen PA, Johnsborg O, Diep DB, Hamoen L, Venema G, Nes IF: Regulation of bacteriocin production in *Lactobacillus plantarum* depends on a conserved promoter arrangement with consensus binding sequence. Mol Genet Genomics 2001;265:198–206.

41 Johnsborg O, Diep DB, Nes IF: Structural analysis of the peptide pheromone receptor plnB, a histidine protein kinase from *Lactobacillus plantarum*. J Bacteriol 2003;185: 6913–6920.

42 Maldonado A, Jiménez-Diaz R, Ruiz-Barba JL: Induction of plantaricin production in *Lactobacillus plantarum* NC8 after coculture with specific Gram-positive bacteria is mediated by an autoinduction mechanism. J Bacteriol 2004;186:1556–1564.

43 Kleerebezem M, Quadri LE, Kuipers OP, de Vos WM: Quorum sensing by peptide pheromones and two component signal-transduction systems in Gram-positive bacteria. Mol Microbiol 1997;24:895–904.

44 Nes IF, Eijsink VGH: Regulation of group II peptide bacteriocin synthesis by quorum-sensing mechanisms; in Danny GM, Winans SC (eds): Cell-Cell Signalling in Bacteria. Washington, ASM Press, 1999, pp 175–192.

45 Kleerebezem M, Quadri LE: Peptide pheromone-dependent regulation of antimicrobial peptide production in Gram-positive bacteria: a case of multicellular behavior. Peptides 2001;22:1579–1596.

46 Kristiansen PE, Fimland G, Mantzilas D, Nissen-Meyer J: Structure and mode of action of the membrane-permeabilizing antimicrobial peptide pheromone plantaricin A. J Biol Chem 2005;280:22945–22950.

47 Håvarstein LS, Holo H, Nes IF: The leader peptide of colicin V shares consensus sequences with leader peptides that are common among peptide bacteriocins produced by Gram-positive bacteria. Microbiol 1994; 140:2383–2389.

48 Oppegård C, Fimland G, Thorbak L, Nissen-Meyer J: Analysis of the two-peptide bacteriocins lactococcin G and enterocin 1071 by site-directed mutagenesis. App Environ Microbiol 2007;73:2931–2938.

49 Garneau S, Ference CA, van Belkum MJ, Stiles ME, Vederas JC: Purification and characterization of brochocin A and brochocin B(10–43), a functional fragment generated by heterologous expression in *Carnobacterium piscicola*. Appl Environ Microbiol 2003;69:1352–1358.

Research Article

Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol 2007;13:220–225
DOI: 10.1159/000104751

Amphiphilic Dendrimeric Peptides as Model Non-Sequential Pharmacophores with Antimicrobial Properties

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Key Words
Antibacterial peptides · Dendrimeric peptides · Non-sequential pharmacophore

Abstract
A concept of application of dendrimer chemistry for construction of 'non-sequential pharmacophore', mimicking active conformation of linear antimicrobial peptides, is introduced. It resulted in the synthesis of a family of low-molecular-weight basic peptide dendrimers with antimicrobial properties against *Staphylococcus aureus* (Gram-positive), *Escherichia coli* (Gram-negative) and *Candida albicans*.

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Introduction

Epidemiologic studies performed in European countries report an increase in infectious diseases and bacterial resistance to antibiotics. This has resulted in intensive worldwide research focusing on the use of natural, linear antimicrobial peptides and their derivatives. Antimicrobial peptides are 10- to 50-amino-acid-long peptide antibiotics that have been recently discovered in many living organisms including humans. They are an important part of the innate defense system, possessing a high potency and broad spectrum of activity against prokaryotic cells with only a minor impact on eukaryotic cells. Such properties raised some hope that natural antimicrobial peptides and their synthetic analogs may be adaptable for use in vivo as new generation antibiotics. Among them a large group, presently ca. 600 compounds, are qualified as 'cationic peptides', defined as peptides with an overall positive charge, imparted by the presence of multiple lysine and arginine residues, and a substantial portion (50% or more) of hydrophobic/aromatic residues. During interactions with biological membranes they adopt α-helical, β-sheet or both β-sheet and α-helical structures with the first two classes being the most common in nature. Structure-activity studies of these peptides reveal two main requirements for the antimicrobial activity: a cationic charge and an induced amphipathic conformation [1–3].

The knowledge of structural elements required for expressing antimicrobial properties allows us to develop a concept that the active structure of linear peptides could be modeled by application of dendrimer chemistry. Dendrimers are macromolecules of nanoscopic dimensions which are characterized by a combination of a high number of functional groups on the surface and have a compact molecular structure. They are synthesized using organic chemistry methods and have well-defined, discrete structures which can be precisely controlled throughout the synthesis. The multivalent nature of these compounds and unambiguous composition, reliability and versatility

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1464–1801/07/0134–0220$23.50/0

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of their synthesis make this type of carrier well suited for various biotechnological and biochemical applications such as diagnostic purposes [4], protein mimetics [5], antiviral agents and vaccines [6, 7], or drug and gene delivery vehicles [8]. If biomedical applications of dendrimers are involved, the well-recognized polydispersity of a higher generation of dendrimers makes them hard to synthesize and purify. Therefore, we synthesized a series of low-molecular-mass peptide dendrimers, based on lysine used both as a trivalent core and branching element, and confirmed their antimicrobial properties [9] and low cytoxicity of some structural classes [10].

The present article focuses on the synthesis and antimicrobial properties of a particular class of these amphiphilic dendrimers with the C-end modified by non-amino acid residues.

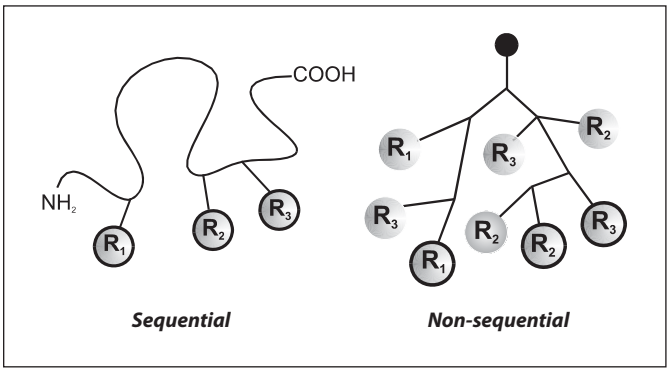

Fig. 1. Illustration of a general concept of 'non-sequential pharmacophore'.

Results and Discussion

Synthesis and Antimicrobial Activity

The basic idea of this research is to transfer antimicrobial properties between two structurally different classes of molecules – linear peptides and dendrimers. In solution, linear peptides exist as a dynamic composition of various conformations, from which only a small population has structures suitable for interactions with biological membranes. This active conformation is directly associated with a particular sequence of amino acids constituting the chain which we call the 'sequential pharmacophore'. For example, most abundant natural linear lytic peptides are believed to undergo conformational changes in the vicinity of the membrane, leading to adoption of an amphipathic α-helical structure [1, 11]. The helical structure enables clustering of basic and hydrophobic amino acid residues in order to interact with complementary elements of the membrane followed by its disruption through several postulated mechanisms [12]. However, very potent membrane-active antimicrobial peptides with shorter chains (e.g. indolicidine) are known where the amphipathic structure is achieved due to another process.

In order to obtain compounds with amphiphilic properties, we designed peptide dendrimers from amino acids containing basic and hydrophobic residues that are required for expressing antimicrobial properties in the majority of natural peptides. In such molecules the key role is the spatial distribution of the amino acid residues. Therefore, we decided to synthesize branched compounds with a collection of basic and hydrophobic/aromatic groups located not only on the surface but within the whole dendrimer tree. Although conformational flexibility of branched molecules is lower than their linear precursors, receptors can recognize and bind one out of several active groups of the same type, distributed in a controlled fashion. Contrary to the sequence-dependent active conformation of the linear peptides, we called this construction a 'dispersed' or 'non-sequential' pharmacophore (fig. 1). Using lysine as a core and branching unit and retaining 2-Cl-benzyloxycarbonyl (2-Cl-Z) or other protecting groups at α- or ε-positions allowed us to obtain molecules with positively charged amino groups and a number of hydrophobic/aromatic residues. Versatility of the peptide synthetic procedures (orthogonal approach) allowed to us extensively modify their three-dimensional structures. In addition, modifications of the dendrimer C-end enabled the introduction of residues of peptidic and non-peptidic character with varying lypophilicity and spatial requirements.

The designed dendrimers retain the overall (+2) charge located at the two unsubstituted α-amino groups of lysines and belong to three dendrimer families. In group 1, compounds D/1–D/4 are the first-generation lysine dendrimers functionalized at the C-end by tryptamine, (*R*)- or (*S*)-methylbenzylamine (MBA) or benzylamine (BA). In group 2, compounds D/5–D/7, C-terminal benzylamine is attached to the lysine core via amino acid spacers of various side chain polarity. In group 3, compounds D/9–D/10, the amino acid spacer is located between core and the first-generation lysines. Examples of molecular formulas of the compounds D/1, D/5 and D/9, belonging to different structural classes, along with abbreviated structure and molecular mass, are shown in figure 2.

According to the published data, the majority of dendrimers containing amino acids were synthesized on sol-

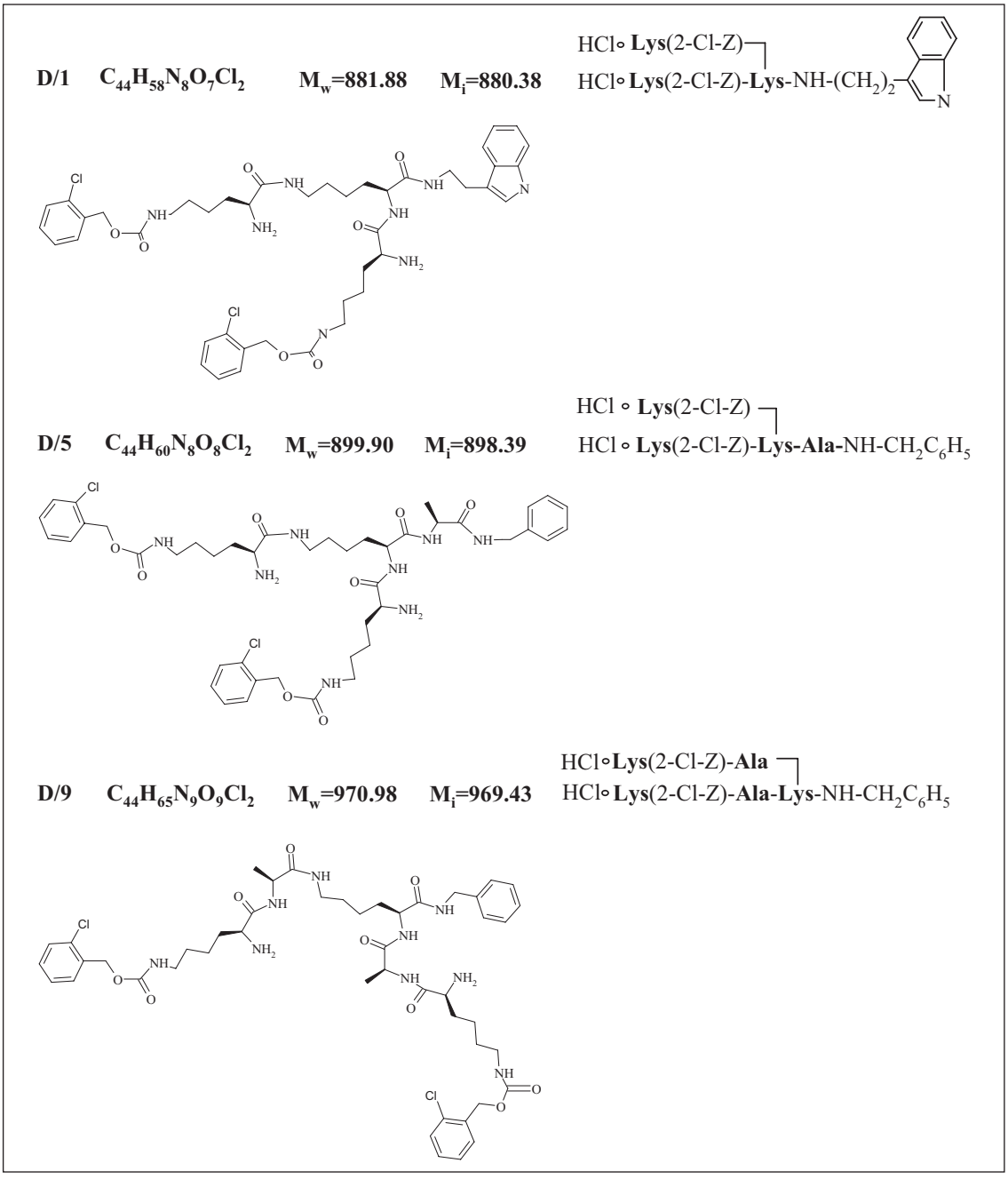

Fig. 2. Molecular structures of D/1, D/5 and D/9 dendrimers belonging to different structural classes. Molecular formula is followed by theoretical and experimental mass (from ESI MS).

id support. In the present case, all dendrimers were synthesized in solution by DCC coupling. The purification procedure, i.e. gel filtration on Sephadex LH-20 followed by preparative HPLC, enabled to obtain gram quantities of each dendrimer for structural and biological studies. The fraction containing pure peptide was lyophilized twice and purity was confirmed by analytical HPLC. Electrospray ionization mass spectrometry (ESI-MS) was a particularly valuable technique for characterization of these multiply charged low-molecular-mass dendrimers. To elucidate the influence of dendrimer's hydrophobicity and conformational flexibility on their membrane activ-

Table 1. Antimicrobial properties for dendrimers D1-D10

No.	Formula	MIC, μM *S. aureus*	MIC, μM *E. coli*	MIC, μM *C. albicans*
D/1	HCl ◦ **Lys**(2-Cl-Z) ┐ HCl ◦ **Lys**(2-Cl-Z)-**Lys**-NC-$(CH_2)_2$ 	5.1	2.6	167
D/2	HCl ◦ **Lys**(2-Cl-Z) ┐ HCl ◦ **Lys**(2-Cl-Z)-**Lys**-NH-$CH(CH_3)C_6H_5$*(S)*	11	55	11
D/3	HCl ◦ **Lys**(2-Cl-Z) ┐ HCl ◦ **Lys**(2-Cl-Z)-**Lys**-NH-$CH(CH_3)C_6H_5$*(R)*	11	55	22
D/4	HCl ◦ **Lys**(2-Cl-Z) ┐ HCl ◦ **Lys**(2-Cl-Z)-**Lys**-NH-$CH_2C_6H_5$	22	22	44
D/5	HCl ◦ **Lys**(2-Cl-Z) ┐ HCl ◦ **Lys**(2-Cl-Z)-**Lys**-**Ala**-NH-$CH_2C_6H_5$	77	154	77
D/6	HCl ◦ **Lys**(2-Cl-Z) ┐ HCl ◦ **Lys**(2-Cl-Z)-**Lys**-**D**-**Ala**-NH-$CH_2C_6H_5$	77	77	77
D/7	HCl ◦ **Lys**(2-Cl-Z) ┐ HCl ◦ **Lys**(2-Cl-Z)-**Lys**-**Ser(Bzl)**-NH-$CH_2C_6H_5$	64	64	64
D/8	HCl ◦ **Lys**(2-Cl-Z) ┐ HCl ◦ **Lys**(2-Cl-Z)-**Lys**-**Pro**-NH-$CH_2C_6H_5$	75	75	75
D/9	HCl ◦ **Lys**(2-Cl-Z)-**Ala**┐ HCl ◦ **Lys**(2-Cl-Z)-**Ala**-**Lys**-NH-$CH_2C_6H_5$	144	144	288
D/10	HCl ◦ **Lys**(2-Cl-Z)-**D**-**Ala** ┐ HCl ◦ **Lys**(2-Cl-Z)-**D**-**Ala**-**Lys**-NH-$CH_2C_6H_5$	288	288	144

ity and selectivity, we designed analogs with the C-end modified by various amines – tryptamine, benzylamine, and both chiral and racemic methylbenzylamine. The antimicrobial activities of dendrimers D/1–D/10 against *Staphylococcus aureus* ATCC 25923 (Gram-positive), *Escherichia coli* NCTC 8196 (Gram-negative) and *Candida albicans* NCTC 8196 are shown in table 1. It appears that first-generation dendrimers with the C-end modified by non-peptidic aromatic residues (group 1) have a ca. 5- to-10 fold higher activity when compared to the formerly studied dendrimeric peptides with Phe or Tyr located at the C-end [9]. Among these compounds, derivative D/1, with a bulky tryptamine residue, is as active as indolicidine (Ile-Leu-Pro-Trp-Lys-Trp-Pro-Trp-Trp-Pro-Trp-Arg-Arg-NH_2) – a small natural antibacterial peptide synthesized in our laboratory and used previously as reference compound (minimum inhibitory concentration (MIC) equals 1.5 and 3.0 for *S. aureus* and *E. coli*, respectively). This analog is also the least active towards *C. albicans*. Elongation of the C-end of dendrimers by L- or D-Ala, or (Bzl)Ser (group 2), gave compounds three times less active than those in group 1. Elongation of lysine arms by an additional L- or D-Ala (group 3) resulted in a 5- to 10-fold decrease in bacteriostatic potency. Analysis of antimicrobial potency showed that introduction of additional aliphatic amino acid in the vicinity of the free α-amino groups of lysines significantly reduces activity. We assume that bulky Ala, Ser or Pro residues make these groups sterically hindered and therefore less accessible for interactions with membrane components. On the other side, aromatic groups located at the C-terminus significantly enhance antimicrobial activity. There is a 5-fold shift in selectivity towards the Gram-positive strain in the case of diastereoisomeric compounds D/2 and D/3. However, opposite chirality at one stereogenic center does not influence their activity.

In summary, the results presented confirm our hypothesis that the dendrimer structure can be used for construction of branched peptides, interacting with biological membranes. Although lysine dendrimers have been used before as synthetic scaffolds for the preparation of multiple antigen peptides [13] or for attachment of 2–8 copies of tetrapeptide R4 (RLYR) or an octapeptide R8 (RLYRKVYG) – fragments of antimicrobial peptide [14] – a concept of non-sequential pharmacophore allows to synthesize simple compounds with high antimicrobial potency and low molecular weight. From a practical point of view, such compounds are much easier to synthesize and purify. They represent a new class of membrane-active dendrimers where the dendrimer tree is used not

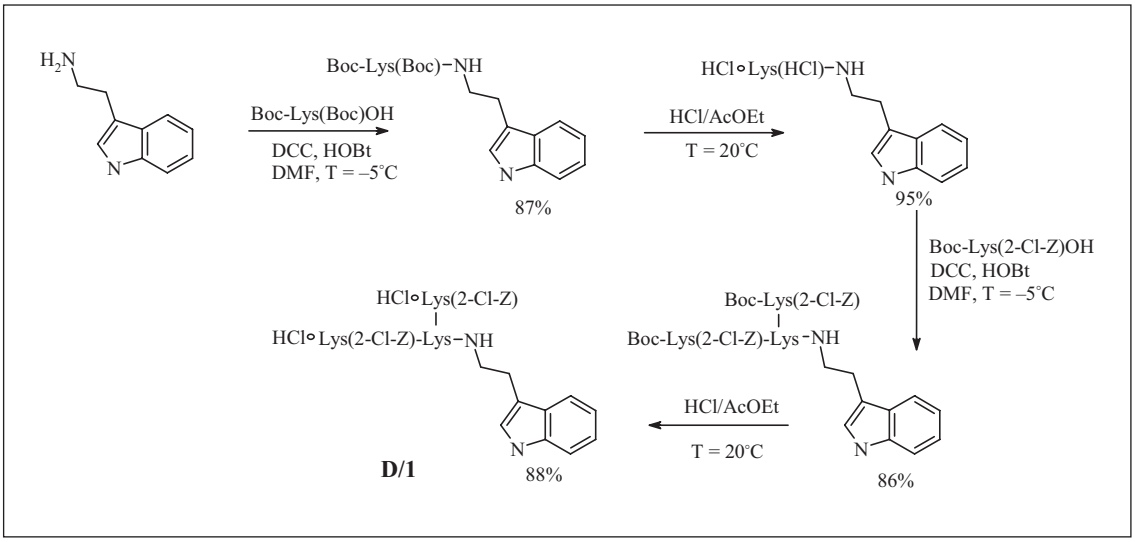

Fig. 3. Synthesis of compound D/1.

only for multiplication of active elements but also for spatial distribution of cationic and aromatic (hydrophobic) groups that is essential for interactions with bacterial membranes. Although both natural peptides and branched analogs are built from the same elements (amino acids with positively charged and hydrophobic side chains), assignment of a particular mechanism of interactions with biological membranes is at this point not justified. We have been able to exclude a channel mechanism by observing that MIC values were the same for a family of isomeric compounds with the same overall charge but with introduction of D-amino acids at various positions in comparison with an all-L-derivative [9]. Molecular mechanic calculations performed for some of these molecules showed that planar structures with a wide distribution of branches are the lowest energy conformations, thus suggesting that a 'carpet mechanism' is highly probable. On the other side, data on interactions of our peptides with model lipid bilayers using the DSC method explain that no other receptors are required in order to effectively disintegrate such membranes [15].

Experimental Procedure

All solvents and reactants were of analytical grade and were used without further purification. Mass spectra were recorded with a Mariner ESI time-of-flight mass spectrometer (PerSeptive Biosystems). The needle voltage was adjusted to 5 kV; declustering potential was varied from 20 to 220 V. Samples were prepared in MeOH.

Synthesis and Assay of Antimicrobial Activity

All dendrimers were synthesized by the Boc chemistry in solution. A representative example of the synthesis of the dendrimer D/1 from tryptamine, Nα,Nε-bis-Boc lysine and Nε-(2-Cl-Z)-lysine (Z = benzyloxycarbonyl) is outlined in figure 3. Other compounds were similarly prepared except that the starting materials were appropriate. All crude peptide dendrimers were purified by gel filtration on Sephadex LH-20, followed by preparative HPLC. The fraction containing pure peptide was lyophilized twice and purity was confirmed by analytical HPLC and mass spectrometry. Supplementary data contain ESI-MS spectra of the studied compounds.

Antimicrobial activity was assayed against *S. aureus* ATCC 25923, *E. coli* NCTC 8196 and *C. albicans* NCTC 8196. To determine the MIC, the microdilution broth method was used. Briefly, cells of each bacterial strain were collected in the logarithmic phase of growth and suspended in nutrient broth (Biotest AG, Germany). The concentration of colony-forming units (CFU) was quantified by measuring absorbancy at 600 nm (A_{600}). Dendrimer samples were dissolved in nutrient broth (pH 7.0) and diluted serially. The sample solution (100 μl) was mixed with the diluted bacterial suspension (100 μl). Mixtures containing 10^5 bacterial CFU and from 1 to 0.003% of the test peptides were incubated for 24 h at 37°C. Antimicrobial activities were expressed as the IC – the concentration at which 100% inhibition of growth was observed. Three independent experiments were averaged and calculated deviations were <15%.

Acknowledgements

Authors are grateful to Prof. A.W. Lipkowski for valuable discussions. This work was supported by the Ministry of Education and Science, Grant No. 3T09B 115 28.

References

1 Lequin O, Bruston F, Convert O, Chassaing G, Nicolas P: Helical structure of dermaseptin B2 in a membrane-mimetic environment. Biochemistry 2003;42:10311–10323.

2 Papo N, Shai Y: Exploring peptide membrane interaction using surface plasmon resonance: differentiation between pore formation versus membrane disruption by lytic peptides. Biochemistry 2003;42:458–466.

3 Powers JPS, Hancock REW: The relationship between peptide structure and antibacterial activity. Peptides 2003;24:1681–1691.

4 Misselwitz B, Schmidt-Willich H, Ebert W, Frenzel T, Weinmann HJ: Pharmacokinetics of gadomer-17, a new dendritic magnetic resonance contrast agent. Magn Res Mater Phys Biol Med 2001;B12:128–134.

5 Ramshaw JAH, Werkmeister, JA, Glattauer V: Collagen-based biomaterials. Biotechnol Genet Eng Rev 1996;13:335–382.

6 Witvrouw M, Fikkert V, Pluymers W, Matthews B, Mardel K, Schols D, Raff J, Debyser Z, De Clercq E, Holan G, Pannecouque C: Polyanionic (i.e., polysulfonate) dendrimers can inhibit the replication of human immunodeficiency virus by interfering with both virus adsorption and later steps (reverse transcriptase/integrase) in the virus replicative cycle. Mol Pharmacol 2000;58:1100–1108.

7 Ben-Yedidia T, Arnon R: Design of peptide and polypeptide vaccines. Curr Opin Biotechnol 1997;8:442–448.

8 Dufe C, Uchegbu IF, Schatzlein AG: Dendrimers in gene delivery. Adv Drug Deliv Rev 2005;57:2177–2202.

9 Janiszewska J, Switon J, Lipkowski AW, Urbanczyk-Lipkowska Z: Low molecular mass peptide dendrimers that express antimicrobial properties. Bioorg Med Chem Lett 2003; 13:3711–3713.

10 Klajnert B, Janiszewska J, Urbanczyk-Lipkowska Z, Bryszewska M, Epand RM: DSC studies on interactions between low molecular mass peptide dendrimers and model lipid membranes. Int J Pharm 2006;327:145–152.

11 Simmaco M, Mignogna G, Barra D: Antimicrobial peptides from amphibian skin: What do they tell us? Biopolymers 1998;47:435–450.

12 Chen CZ, Cooper SL: Interactions between dendrimer biocides and bacterial membranes. Biomaterials 2002;23:3359–3368.

13 Tam JP: Recent advances in multiple antigen peptides. J Immunol Methods 1996;196:17–32.

14 Tam JP, Lu Y-A, Yang J-L: Antimicrobial dendrimeric peptides. Eur J Biochem 2002; 269:923–932.

15 Klajnert B, Janiszewska J, Urbanczyk-Lipkowska Z, Bryszewska M, Shcharbin D, Labieniec M: Biological properties of low molecular mass peptide dendrimers. Int J Pharm 2006;309:208–217.

Research Article

Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol 2007;13:226–234
DOI: 10.1159/000104747

A System for the Random Mutagenesis of the Two-Peptide Lantibiotic Lacticin 3147: Analysis of Mutants Producing Reduced Antibacterial Activities

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Key Words
Random mutagenesis · Lacticin 3147 · Antimicrobial peptides · Lantibiotics · Peptide engineering

Abstract
Lantibiotics are antimicrobial peptides that contain several unusual amino acids resulting from a series of enzyme-mediated posttranslational modifications. As a consequence of being gene-encoded, the implementation of peptide bioengineering systems has the potential to yield lantibiotic variants with enhanced chemical and physical properties. Here we describe a functional two-plasmid expression system which has been developed to allow random mutagenesis of the two-component lantibiotic, lacticin 3147. One of these plasmids contains a randomly mutated version of the two structural genes, *ltnA1* and *ltnA2*, and the associated promoter, Pbac, while the other encodes the remainder of the proteins required for the biosynthesis of, and immunity to, lacticin 3147. To test this system, a bank of ~1,500 mutant strains was generated and screened to identify mutations that have a detrimental impact on the bioactivity of lacticin 3147. This strategy established/confirmed the importance of specific residues in the structural peptides and their associated leaders and revealed that a number of alterations which mapped to the –10 or –35 regions of Pbac abolished promoter activity.

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Introduction

The lantibiotics are an ever-expanding family of antimicrobial peptides that are produced by a diverse range of bacteria [for reviews, see 1–3]. They display several distinctive characteristics and exhibit activity toward Gram-positive bacteria that can be several orders of magnitude more potent than classical antibiotics. These gene-encoded, ribosomally derived peptides are exemplified by the presence of unique modified amino acids, predominantly dehydrated amino acids (e.g. dehydroalanine (Dha) and dehydrobutyrine (Dhb)), and the thioether-containing amino acids lanthionine (Lan) and β-methyllanthionine (MeLan). These unusual amino acids are introduced via a series of posttranslational modification reactions that include the dehydration of serine and threonine residues followed by intramolecular addition of cysteines to the resultant unsaturated amino acids. The latter event results in the formation of internal ring structures, conferring structure and function to the previously inactive peptide. Lacticin 3147 is a two-component lantibiotic produced by *Lactococcus lactis* subsp. *lactis* that is active against a wide variety of clinically significant Gram-positive organisms including methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecalis*, penicillin-resistant *Pneumo-*

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coccus, and *Propionibacterium acnes* in addition to food-borne pathogens such as *Listeria monocytogenes* and *Bacillus cereus* [4, 5]. The two structural lanthionine-containing peptides, designated LtnA1 and LtnA2, act synergistically to form pores in the membranes of sensitive bacterial cells [6, 7], an action that is facilitated by binding of the essential precursor of peptidoglycan synthesis, lipid II. This binding of lipid II also results in the inhibition of cell wall synthesis in the target strain [8]. An unusual feature of lacticin 3147 is the presence of three D-alanine residues (D-Ala) [9–11] making it one of only a few examples of prokaryotic gene-encoded peptides in which such residues have been identified [12, 13]. The recent structural elucidation of both peptides [10] has revealed that the C-terminus of LtnA1 bears a striking resemblance to another lipid II-binding lantibiotic, mersacidin. Indeed, a number of lantibiotics that include mersacidin, LtnA1 and actagardine contain a conserved stretch of amino acids, CTLTXEC, that could comprise the core site for binding lipid II [14, 15]. The LtnA2 peptide forms a structure more typical of linear cationic peptides and comparison with closely related peptides reveals a highly conserved C-terminus, potentially representing the site of interaction with LtnA1.

Unlike classical peptide antibiotics that are produced by multienzyme complexes, the gene-encoded nature of lantibiotics has permitted the development of a number of mutagenic systems to produce novel structural variants. While these systems have predominantly been used to reveal information about the structure-function relationships of lantibiotics they have, on rare occasions, resulted in an enhancement of their chemical and antimicrobial properties. Since multiple genes are required for lantibiotic synthesis and immunity, the in vivo bioengineering of the structural peptide has occurred in the background of the original producer or derivatives thereof. Such systems have been developed for nisin [16–18], subtilin [19], Pep5 [20], mutacin II [21], mersacidin [22], cinnamycin [23] and lacticin 3147 [9].

To date, 69 derivatives of the lacticin 3147 peptides (including complete alanine scanning mutagenesis) have been generated utilizing a system reliant on the modification of the *ltnA1A2* genes on a shuttle vector by site-directed mutagenesis and incorporation of the engineered genes into the native lacticin 3147-encoding plasmid, pMRC01, in *L. lactis* MG1363 by double crossover homologous recombination [9, 15]. While use of this system ensures that the sole alteration within the bioengineered strains is the replacement of a single amino acid, it suffers in being relatively time consuming and unsuitable for random mutagenesis approaches. Consequently, the main aim of this study was to develop a system in which randomly mutagenized *ltnA1* and *ltnA2* genes could be expressed *in trans* in a background capable of the biosynthesis of fully modified lacticin 3147 peptides. In addition to achieving this goal, a proof of concept exercise was carried out whereby an initial bank consisting of ~1,500 strains was generated and screened for mutations that result in diminished lacticin 3147 bioactivity.

Results

Complementation of a Strain Lacking ltnA1A2

L. lactis MGMRΔA1A2 is a transconjugant harbouring a derivative of the large natural lacticin 3147-encoding plasmid pMRC01 in which *ltnA1* and *ltnA2* have been deleted. Initial attempts to complement the lacticin 3147-negative phenotype of this strain *in trans* focused on the provision of the *ltnA1A2* genes under the control of their natural promoter Pbac on a high copy shuttle vector, i.e. pDF01. As this did not result in a bacteriocin-positive (Bac^+) phenotype (data not shown), a low copy vector, pPTPL, was used as an alternative. pPTPL was originally generated for use as a promoter reporter vector [24] via transcriptional fusion to a β-galactosidase-encoding *lacZ* gene and thus, in addition to facilitating the introduction of randomly mutagenized Pbac*ltnA1A2* fragments into a strain, had the added potential to reveal information regarding the functionality of Pbac in each case. However, when a derivative of this plasmid carrying an unmutated Pbac*ltnA1A2* fragment, i.e. pDF02, was introduced into MGMRΔA1A2, it too resulted in a Bac^- phenotype. To determine whether the host strain, rather than the complementing plasmid, was responsible for the Bac^- phenotypes, an alternative host, i.e. *L. lactis* MG1363 pOM44 [25], was used. pOM44 is a derivative of the shuttle vector pCI372 into which all of the lacticin 3147 determinants except *ltnA1* and *ltnA2* had been cloned. As both pOM44 and pDF01 are pCI372 derivatives, pDF02 was used as the complementing plasmid (fig. 1). The MG1363 pOM44 pDF02 combination successfully yielded a Bac^+ phenotype, albeit at slightly lower bioactivity levels than MG1363 pMRC01 (fig. 2a). The reason behind our inability to complement MGMRΔA1A2 is not apparent. Notably, the introduction of the pDF01 plasmid into MG1614 pMRC01ΔA1 also restored a Bac^+ phenotype, thus establishing that pMRC01/pCI372 plasmid incompatibility is not an issue (data not shown).

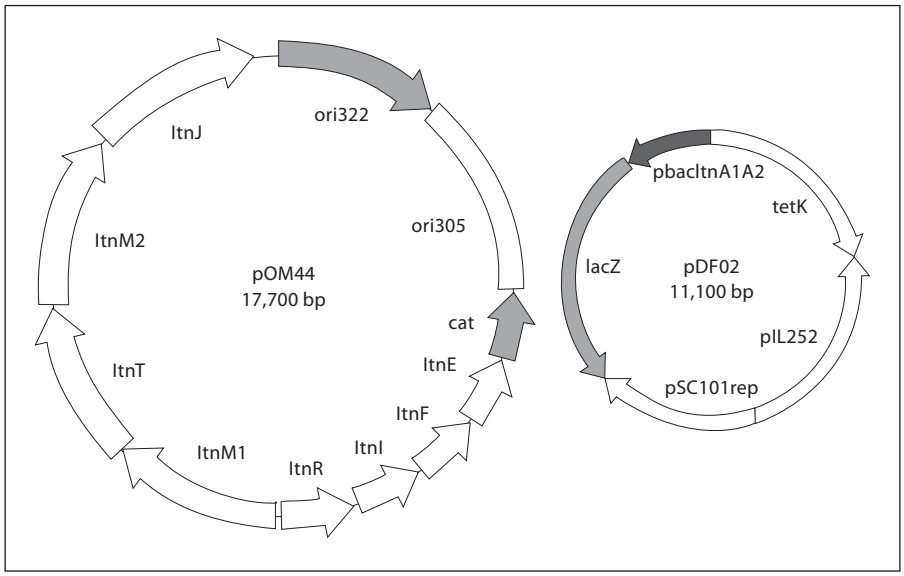

Fig. 1. Production of lacticin 3147 by a two-plasmid system. The genes encoding *ltnA1A2* are present, under the control of their natural promoter, on pDF02 and all other genes associated with production of and immunity to lacticin 3147 are located on pOM44.

Creation and Screening of a Bank of Lacticin 3147 Derivatives

We sought to determine whether this complementation system could be applied, when used in combination with error-prone PCR (ep-PCR), to the creation of a bank of randomly mutated lacticin 3147 derivatives. pDF01 was used as a template for the generation of randomly mutated Pbac*ltnA1A2* fragments containing an average of one nucleotide change. These fragments were cloned into pPTPL and ultimately introduced into MG1363 pOM44. Generally, large numbers of clones are required to provide a comprehensive spectrum of mutations to increase the probability of finding mutants with enhanced activity. As our investigations represented an initial proof of concept trial, a relatively small bank consisting of approximately 1,500 MG1363 pOM44 pDF02 variants was generated. DNA sequencing of a number of these revealed that mutations appeared at random locations throughout the sequence and at a desired low rate of 0–3 mutations/kb. Given the size of the bank, the likelihood of identifying mutants with enhanced activity was low. Thus, to demonstrate the effectiveness of the system, we proceeded to identify mutant clones that exhibited reduced or a complete loss of bioactivity (i.e. the combined impact on production and activity). This strategy had the potential to reveal additional information pertaining to essential, immutable residues within LtnA1 and LtnA2 and in addition, by comparing the consequences of individual mutations with those previously generated by site-directed mutagenesis it was possible to determine the reliability of the system.

Colonies that were white/light blue on X-gal-containing plates revealed mutations that resulted in reduced bioactivity as a consequence of a significant reduction in Pbac activity. Sequence analysis revealed that reduced promoter activity was due to the presence of point mutations in the –35 and –10 promoter regions (fig. 3). The Pbac promoter is typical of most strong lactococcal promoters, i.e. TTGACA (–35) and TATAAT (–10) separated by a 17-bp spacer region [26], though it differs in the –10 region by one nucleotide (TAAAAT). A previous study of the divergent promoters Pbac and Pimm identified in the lacticin 3147 gene cluster has shown that while Pimm is regulated by the transcriptional repressor LtnR, Pbac appears to be constitutive [27]. Reduced bioactivity was detected by means of the deferred antagonism method. DNA sequencing of blue colonies exhibiting reduced or abolished activity revealed that several contained more than one mutation within the structural genes, e.g. A1: A17V, E-25D which exhibited a reduction in activity (data not shown) and A1:V-19A, A2:P21E that was devoid of activity (data not shown). In these instances the impact on bioactivity could not be attributed to a single change in the DNA sequence and were not further characterized. In addition, other mutations leading to a loss of activity were attributed to the introduction of a stop codon within the structural genes or, in one case, the alteration of the ribosome-binding site from GGAGG to GGTGG. Of the blue colonies tested, approximately 40% had a reduction or abolition of activity that could be traced to changes within the DNA fragment. A number of mutants which exhibited reduced bioactivity and contained only one

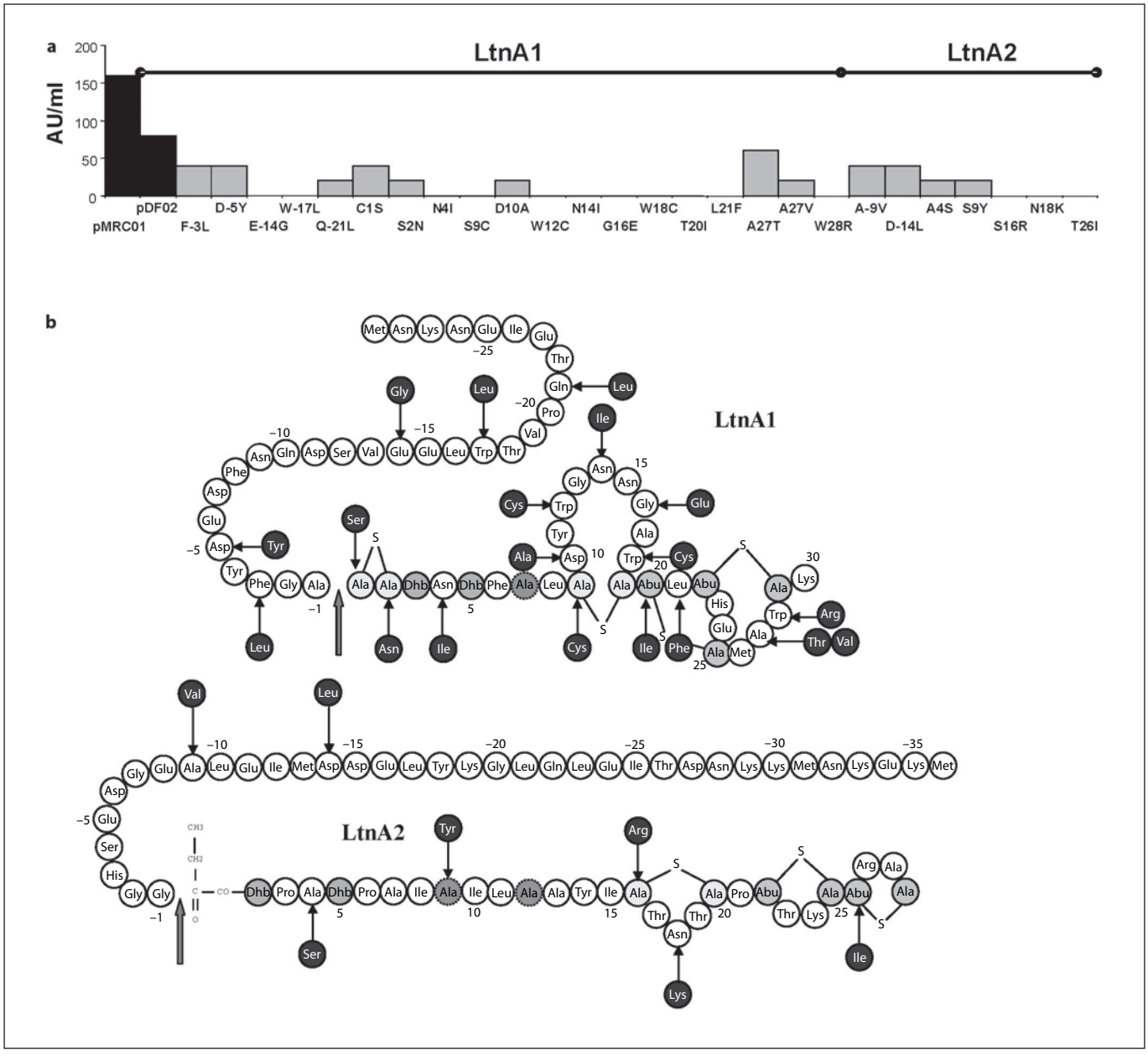

Fig. 2. a Bioactivity results of lacticin 3147 mutants as determined by well-diffusion assays. **b** Lacticin 3147 mutants created by random mutagenesis. Black circles indicate the mutations made within the LtnA1 and LtnA2 peptides as well as the leader regions. Posttranslational modifications are indicated in grey. Dhb: Dehydrobutyrine, Abu: 2-aminobutyric acid, Ala-S-Ala: lanthionine, Abu-S-Ala: 3-methyllanthionine; D-alanine is indicated by broken line. Arrow indicates the processing site.

point mutation (fig. 2b) were further assessed by well-diffusion assays (fig. 2a). These results were compared with the corresponding data generated from alanine scanning mutagenesis [15] and confirmed/revealed a number of trends. No bioactivity was observed from mutants with the amino acid substitutions A1:S9C, A1:T20I, A2:S16R and A2:T26I. This is as expected since these residues are involved in Lan or MeLan formation (fig. 2b). The impact of mutagenesis on amino acids involved in lanthionine bridges (Cys, Ser, and Thr) has been well documented, and in all cases the outcome has been a severe decrease or total loss of activity [21, 28]. It is all the more notable

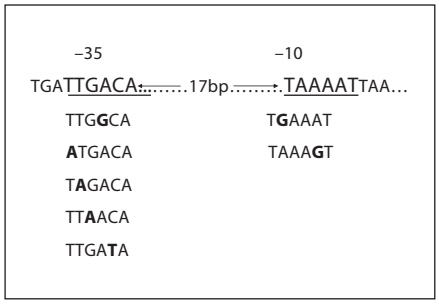

Fig. 3. Random mutants created within the promoter region Pbac. The conserved –35 and –10 regions are underlined. Point mutations are in bold.

therefore that two mutants, in which residues involved in the formation of the first lanthionine bridge of LtnA1 are altered, retain bioactivity (fig. 2b). A1:C1S and A1:S2N displayed 40 and 20 AU/ml respectively, indicating that antimicrobial activity is still present despite abolition of the N-terminal lanthionine bridge of LtnA1. While it has been established that mutants, in which these residues have been respectively converted to alanine, retain relatively high bioactivity [15], the retention of bioactivity as a consequence of these more extreme changes emphasizes the flexibility of this bridge. It is also apparent that other modified residues are tolerant of change. Recently, the significance of the three D-Ala residues (1 in LtnA1 and 2 in LtnA2) for production and antimicrobial activity of lacticin 3147 was established [9]. While A2:S9A, A2:S9G, A2:S9V and A2:S9T mutants retained activity, it was still surprising that the random mutant A2:S9Y retained activity (20 AU/ml) despite conversion of a D-Ala to a bulky tyrosine residue. This is especially significant as it has been postulated that the residues in this region of LtnA2 may form an α-helix that could contribute to the functionality of the peptide [10]. One would expect that any such α-helix would be dramatically affected by the presence of a tyrosine residue. A number of other alterations (A1:D10A, A1:A27T and A1:A27V) that result in the reduction, but not elimination, of bioactivity were apparent. The effect of the A1:D10A substitution results in overall charge of +1 in the LtnA1 peptide (which normally has a net neutral charge) and is seen to impact greatly on bioactivity (fig. 2a). Interestingly, two variants at Ala27 in LtnA1 were isolated that differ in observed bioactivities. A1:A27T and A1:A27V displayed 60 and 20 AU/ml, respectively. Interestingly, the A1:A27T change results in a derivative which more closely resembles the related C55α peptide [29] that has a Ser residue at this location while A1:A27V mimics a natural variation between LtnA1 and Plwα [30]. A1:W12C, A1:N14I, A1:W18C, A1:L21F, A1:W28R, and A2:N18K all lacked detectable bioactivity. These results are particularly reassuring as in each case the corresponding alanine residues are also devoid of activity, thus indicating that the tolerance of individual residues to conversion to alanine is an indicator of the flexibility of a residue in general. The data generated also emphasizes the greater impact of mutation on the bioactivity of LtnA1 mutants (20 with reduced activity), relative to LtnA2 (7 mutants). Thus, LtnA1 is less tolerant to amino acid change and contains several core residues essential for structure-function interaction. On the other hand, the relatively few LtnA2 mutants detected would suggest that this peptide appears to be more tolerant to residue substitution, though residues involved in (β-methyl)lanthionine bridging are an exception.

In addition to revealing information about Pbac and the structural propeptides, the random mutagenesis strategy highlighted important residues within the leader regions of the two peptides. This is the first occasion upon which mutants of the leaders of LtnA1, LtnA2 or any closely related lantibiotic have been generated. The leader peptides of lantibiotics are thought to represent recognition sites for modification enzymes and are ultimately cleaved, often concomitantly with export. Previously, the amino acid substitution of conserved residues within the leader regions of nisin, Pep5 and mutacin II has shown that even minor amino acid changes can have a dramatic impact on the production of, and biosynthesis rate of, the mature peptide [31–33]. In the case of mutacin II, it has been suggested that the amino acids corresponding to positions –8, –12 and –13 are important for optimal rates of biosynthesis [33]. Significantly, it has also been established that the presence of residues –1 to –13 of the lacticin 481 leader are sufficient for in vitro recognition and modification by LctM [2]. The consequences of mutating the LtnA1 and LtnA2 leaders were variable. It was interesting to note that A1:E-14G lacked activity (fig. 2a). An alignment of the leader peptide regions of lantibiotics with similarities to LtnA1 (fig. 4) demonstrates the presence of a number of conserved residues, which include this glutamate. Although a conservative change (E-13D) at this location in the mutacin II leader did not have any detectable effect on mutacin II production, another mutation E-13K reduced production levels by 90%. Of the other leader mutants (A1:Q-21L, A1:W-17L, A1:D-5Y, A1:F-3L, A2:A-9V and A2:D-14L), only A1:W-17L lacked detectable bioactivity and would appear to be an essential residue in lacticin 3147 biosynthe-

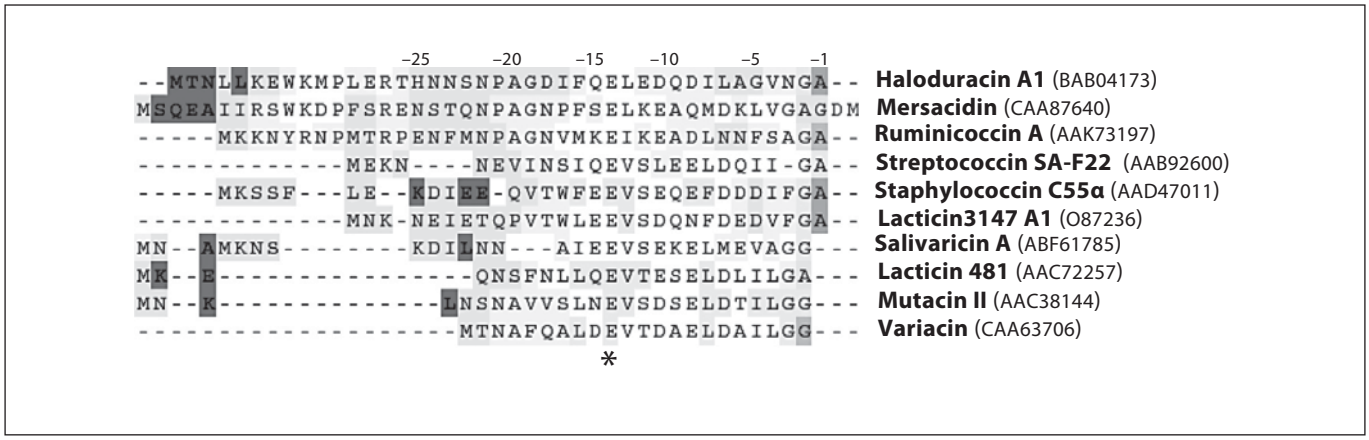

Fig. 4. Multiple sequence alignment of the leader peptides of lantibiotics with similarities to LtnA1. Residue numbers correspond to the LtnA1 leader sequence only and the conserved glutamic acid residue (E) that corresponds to position –14 in the LtnA1 leader is indicated by *. The accession number for the lantibiotic sequences are given in parentheses. The alignment was generated by TCoffee [40].

Table 1. Cloning strains and plasmids used in this study

Strain/plasmids	Relevant characteristics	Reference
Strains		
L. lactis		
MG1363 pMRC01	MG1363 transconjugant harbouring pMRC01	41
MG1363 pOM44	MG1363 harbouring pOM44	25
MGMRΔA1A2	MG1614 transconjugant harbouring pMRC01 with deletions in *ltnA1* and *ltnA2*	42
MGMRΔA1	MG1614 transconjugant harbouring pMRC01 with a deletion in *ltnA1*	42
HP	Indicator organism	DPC collection
E. coli		
E. coli MC1000 pPTPL	*E. coli* harbouring pPTPL	24
E. coli XL-1 Blue	Intermediate cloning host	Stratagene
Plasmids		
pMRC01	60.2-kb natural lactococcal plasmid containing lacticin 3147 biosynthetic operon	5
pCI372	Cm^R; high-copy number cloning vector	43
pOM44	pCI372-*ltnEFIRM1TM2J*	25
pPTPL	Tet^R; lacZ; low-copy number cloning vector	24
pDF01	pCI372 with *ltnA1A2* under associated promoter Pbac	25
pDF02	pPTPL with *ltnA1A2* under associated promoter Pbac	This study

sis. If the data pertaining to lacticin 481 is representative of the situation for LtnA1, the distal location of this mutated residue would indicate that the alteration impacts on LtnT cleavage/exportation, rather than LtnM1 modification. It is hoped that with the help of these and other mutants it will be possible to generate the sort of information required to better understand the interactions involved in the peptide modification, cleavage and transport processes.

Discussion

The inaccurate amplification of genes by ep-PCR is a powerful and versatile technique to produce molecular diversity and has been used successfully in directed evolution experiments [34–36]. Though several ep-PCR methods exist, a simple and flexible method is the use of mutagenic polymerases to introduce random mutations within a target DNA sequence. Such an ep-PCR strategy

was employed to bring about mutagenesis of the lacticin 3147 structural genes. Essential to the success of the system however was the complementation of a strain lacking *ltnA1* and *ltnA2* through the reintroduction of these genes on a plasmid, thereby restoring lacticin 3147 activity and establishing a method for rapidly screening randomly mutated genes cloned into the same plasmid. While similar such strategies have not always been successful when applied to lantibiotic systems, e.g. subtilin and Pep5 [37], the introduction of pDF02 into MG1363 pOM44 successfully reinstated lacticin 3147 activity (fig. 2a). The bioactivity observed, around 50% of that of the wild-type pMRC01, was considered sufficient to permit the screening of random mutants with altered bioactivity. Following introduction of the ep-PCR amplified products into pPTPL and the subsequent establishment of the constructs in MG1363 pOM44, deferred antagonism assays were carried out to detect β-gal$^+$ colonies that had reduced or were deficient in bioactivity. Sequence analysis revealed a desired low rate of mutation, with the result that in the majority of cases a reduction/lack of activity was attributable to a single nucleotide change. It should be noted that, given the relatively lower activity of MGMRΔA1A2 pDF02 relative to MG1363 pMRC01, mutations that abolish bioactivity when the structural genes are present *in trans* may result in the retention of a low amount of bioactivity were they to be located in pMRC01. The corollary is also likely to be true and thus, should further screening identify derivatives with activity greater than that observed with pDF02, it is highly likely that this impact can be enhanced further by recreation of the mutation within the native plasmid. Recently, a complete scanning mutagenesis of lacticin 3147 was carried out whereby each of the 59 amino acids of LtnA1 and LtnA2 was converted to an alanine (or glycine instead of existing alanine residues) to identify key residues and domains across both peptides [15]. This detailed map, which allows one to discriminate between essential and variable residues across both peptides, was of considerable advantage as comparisons could be made between the altered bioactivity of alanine scanning mutants and that of the random mutants generated in this study. It was thus reassuring that the data generated from both strategies was in close agreement and thus served to validate both systems.

The investigation of structure-function relationships within lantibiotics is principally motivated by the desire to create new and more effective antimicrobial peptides. The continuation of rational and random mutagenesis strategies will be central to the efforts in unlocking the structure-function relationships within lantibiotics and thus provide blueprints for the design and optimization of more potent antimicrobial peptides. To this end, the development of in-vivo expression systems could play a key role in the production and screening of such peptides. In-vivo-based systems have several advantages including well-characterized expression hosts and cloning vectors and once combined with routine cloning procedures are capable of producing rapidly renewable sources of manipulated genes. One could claim that a limitation associated with in vivo protein engineering is the abolition, or reduction in production, of novel structural variants as a result of the intolerance of the biosynthetic machinery to certain amino acid substitutions [21, 28]. However, we would argue that this perceived limitation is actually quite beneficial, i.e. when screening for novel variants, the most interesting will be those that are highly produced, do not overwhelm the immunity systems of the producing host and have enhanced activity.

While only a limited number of bioengineered lantibiotics have been created to date, it is encouraging that the use of site-directed mutagenesis has already resulted in the creation of lantibiotic peptide variants that display not only enhanced antimicrobial activity [19, 38], but also improved chemical properties including solubility and stability [19, 39]. These significant findings would suggest that a certain degree of flexibility with regard to amino acid substitution resides within wild-type lantibiotic peptides and, as a consequence, the facility to widen the scope of their physical, chemical and antimicrobial properties is a tantalizing prospect. Accordingly, the implementation of random strategies on lantibiotic structural genes has the potential to generate vast numbers of structural variants. The fact that the bactericidal action of lacticin 3147 results from the synergistic activity of two peptides means that an even larger canvas of substitutable amino acids than is available in singularly active lantibiotics. It is also noteworthy that random mutagenesis of lacticin 3147 benefits from the fact that unlike some other lantibiotics, such as nisin and subtilin, which autoregulate their own biosynthesis there is no evidence to date that mutation of the structural genes severely impacts on Pbac activity. This fact is further supported by the strong β-galactosidase activity of all pDF02 variants but for those in which either the –35 or –10 regions of the promoter are altered.

Our initial proof-of-concept investigations presented here focused on the creation of a relatively small bank of mutants and the identification of those exhibiting reduced bioactivity. While the resultant findings have been

significant in their own right, it is likely that the realization of an expression system for the random mutagenesis of lacticin 3147 will have even greater long-term implications. Our further efforts will focus on the creation of a much larger bank of mutants and the rapid screening thereof, involving high-throughput screening technologies and robotic systems, to identify derivatives with enhanced phenotypes. Given the number of interesting mutants that have already been generated by the relatively few site-directed studies carried out to date, we are optimistic that we will be successful in this regard.

Experimental Procedures

Bacterial Strains, Plasmids, and Growth Conditions

Bacterial strains and plasmids used in this study are listed in table 1. *L. lactis* strains were grown in M17 broth (Oxoid) supplemented with 0.5% glucose (GM17) or GM17 agar at 30°C and without aeration. *Escherichia coli* was grown in Luria-Bertani broth or agar at 37°C. Chloramphenicol and tetracycline were used at 5 and 10 μg/ml respectively for *L. lactis* and at 20 and 10 μg/ml respectively for *E. coli*. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used at a concentration of 40 μg/ml.

Random Mutagenesis

DNA extracted from *L. lactis* MG1363 pMRC01 was used as template for amplification of *ltnA1* and *ltnA2* and the associated promoter region Pbac with Vent polymerase (New England Biolabs) using the primers ltnA1soeA 5′ AACTGCAGTTATATATTTGCGGC and ltnA2soeD 5′ ACGAATTCTCTTACAGAGTT. PCR amplicons were purified using the QIAquick PCR purification kit (Qiagen Inc.), digested with *Pst*I and *Eco*RI (Roche) and cloned into similarly digested pCI372. Following introduction into *E. coli* XL-1 Blue cells, plasmid was isolated from one clone and was sequenced (MWG Biotech, Germany) using the primers pCI372For 5′ CGGGAAGCTAGAGTAAGTAG and pCI372Rev 5′ CCTCTCGGTTATGAGTTAG to ensure its integrity. This plasmid, pDF01, was used as a template DNA for ep-PCR using the Genemorph random mutagenesis kit (Stratagene) according to the manufacturer's guidelines. To introduce an average of one base change in the 847-bp cloned fragment, amplification was performed in a 50-μl reaction containing approximately 100 ng of target DNA (pDF01), 2.5 units Mutazyme DNA polymerase, 1 mM dNTPs and 200 ng each of primers pPTPLA1A2 Forward BglII 5′ TCAGATCTTATATACAGAGTTACTA and pPTPLA1A2 Reverse XbaI 5′ TGTCTAGATAATTTCTGGAAAAAC. The reaction was preheated at 96°C for 1 min, and then incubated for 22 cycles at 96°C for 1 min, 49°C for 1 min and 72°C for 1 min, and then finished by incubating at 72°C for 10 min. Amplified products were purified by gel extraction using the QIAquick gel extraction kit (Qiagen Inc.), digested with *Bgl*II and *Xba*I (Roche), ligated with similarly digested pPTPL and introduced into *E. coli* MC1000. To determine if the correct rate of mutation had been achieved, recombinant plasmid DNA was isolated from selected clones using the QIAprep Spin miniprep kit (Qiagen Inc.) and sequenced (MWG Biotech). Transformants were pooled and stored in 80% glycerol at –20°C. Plasmid DNA isolated from the mutant bank was used to transform *L. lactis* MG1363 pOM44.

Bioassays for Antimicrobial Activity

Deferred antagonism assays were performed by spotting strains on GM17 agar plates and allowing them to grow overnight before overlaying with GM17 agar (0.75% w/v) seeded with the lacticin 3147-sensitive indicator strain *L. lactis* HP (approx. 10^6 fresh overnight-grown cells/ml). Well-diffusion assays were also carried out as follows: molten agar was cooled to 48°C and seeded with *L. lactis* HP (approx. 10^6 fresh overnight-grown cells/ml). The inoculated medium was rapidly transferred into sterile Petri plates in 50-ml volumes, allowed to solidify and dried. Wells (4.6 mm in diameter) were then made in the seeded plates. 50-μl volumes of cell-free supernatant from overnight cultures were then added to the wells and the plates incubated at 30°C overnight. To determine the bioactivity of overnight cultures, the cultures were centrifuged and the cell-free supernatant was serially diluted in one-quarter-strength Ringer's solution and dispensed into the 4.6-mm diameter wells. Bioactivity was expressed as arbitrary units/ml (AU/ml) and calculated as the reciprocal of the highest dilution that gave a definite zone multiplied by the conversion factor (i.e. 20 when 50 μl was used) [5].

Acknowledgement

This work was supported by the Irish Research Council for Science Engineering and Technology (IRCSET).

References

1 Cotter PD, Hill C, Ross RP: Food microbiology: bacteriocins: developing innate immunity for food. Nat Rev Microbiol 2005;3:777–788.

2 Chatterjee C, Paul M, Xie L, van der Donk WA: Biosynthesis and mode of action of lantibiotics. Chem Rev 2005;105:633–684.

3 Guder A, Wiedemann I, Sahl HG: Posttranslationally modified bacteriocins – the lantibiotics. Biopolymers 2000;55:62–73.

4 Galvin M, Hill C, Ross RP: Lacticin 3147 displays activity in buffer against Gram-positive bacterial pathogens which appear insensitive in standard plate assays. Lett Appl Microbiol 1999;28:355–358.

5 Ryan MP, Rea MC, Hill C, Ross RP: An application in cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, lacticin 3147. Appl Environ Microbiol 1996;62:612–619.

6 McAuliffe O, Ryan MP, Ross RP, Hill C, Breeuwer P, Abee T: Lacticin 3147, a broad-spectrum bacteriocin which selectively dissipates the membrane potential. Appl Environ Microbiol 1998;64:439–445.

7 Morgan SM, O'Connor PM, Cotter PD, Ross RP, Hill C: Sequential actions of the two component peptides of the lantibiotic lacticin 3147 explain its antimicrobial activity at nanomolar concentrations. Antimicrob Agents Chemother 2005;49:2606–2611.

8 Wiedemann I, Bottiger T, Bonelli RR, Wiese A, Hagge SO, Gutsmann T, Seydel U, Deegan L, Hill C, Ross P, Sahl HG: The mode of action of the lantibiotic lacticin 3147 – a complex mechanism involving specific interaction of two peptides and the cell wall precursor lipid II. Mol Microbiol 2006;61:285–296.

9 Cotter PD, O' Connor PM, Draper LA, Lawton EM, Deegan LH, Hill C, Ross RP: Post-translational conversion of L-serines to D-alanines is vital for optimal production and activity of the lantibiotic Lacticin 3147. Proc Natl Acad Sci USA 2005;102:18584–18589.

10 Martin NI, Sprules T, Carpenter MR, Cotter PD, Hill C, Ross RP, Vederas JC: Structural characterization of lacticin 3147, a two-peptide lantibiotic with synergistic activity. Biochemistry 2004;43:3049–3056.

11 Ryan MP, Jack RW, Josten M, Sahl HG, Jung G, Ross RP, Hill C: Extensive post-translational modification, including serine to D-alanine conversion, in the two-component lantibiotic, lacticin 3147. J Biol Chem 1999; 274:37544–37550.

12 Kawai Y, Ishii Y, Arakawa K, Uemura K, Saitoh B, Nishimura J, Kitazawa H, Yamazaki Y, Tateno Y, Itoh T, Saito T: Structural and functional differences in two cyclic bacteriocins with the same sequences produced by lactobacilli. Appl Environ Microbiol 2004; 70:2906–2911.

13 Skaugen M, Nissen-Meyer J, Jung G, Stevanovic S, Sletten K, Inger C, Abildgaard M, Nes IF: In vivo conversion of L-serine to D-alanine in a ribosomally synthesized polypeptide. J Biol Chem 1994;269:27183–27185.

14 Breukink E, de Kruijff B: Lipid II as a target for antibiotics. Nat Rev Drug Discov 2006;5: 321–332.

15 Cotter PD, Deegan L, Lawton EM, Draper LA, O'Connor PM, Hill C, Ross RP: Complete alanine scanning of the two-component lantibiotic lacticin 3147: generating a blueprint for rational drug design. Mol Microbiol 2006;62:735–747.

16 Dodd HM, Hao Z, Horn N, Gasson MJ: A Lactococcal expression system for engineered nisins. Appl Environ Microbiol 1992; 58:3683–3693.

17 Kuipers OP, Beerthuyzen MM, Siezen R, De Vos WM: Characterization of the nisin gene cluster nisABTCIPR of *Lactococcus lactis*. Requirement of expression of the *nisA* and *nisI* genes for development of immunity. Eur J Biochem 1993;216:281–291.

18 Kuipers OP, Rollema HS, Yap WMGJ, Boot HJ, Seizen RJ, de Vos WM: Engineering dehydrated amino acids residues in the antimicrobial peptide nisin. J Biol Chem 1992;267: 24340–24346.

19 Liu W, Hansen JN: Enhancement of the chemical and antimicrobial properties of subtilin by site-directed mutagenesis. J Biol Chem 1992;267:25078–25085.

20 Bierbaum G, Reis M, Szekat C, Sahl HG: Construction of an expression system for engineering of the lantibiotic Pep5. Appl Environ Microbiol 1994;60:4332–4338.

21 Chen P, Novak J, Kirk M, Barnes S, Qi F, Caufield PW: Structure-activity study of the lantibiotic mutacin II from *Streptococcus mutans* T8 by a gene replacement strategy. Appl Environ Microbiol 1998;64:2335–2340.

22 Szekat C, Jack RW, Skutlarek D, Farber H, Bierbaum G: Construction of an expression system for site-directed mutagenesis of the lantibiotic mersacidin. Appl Environ Microbiol 2003;69:3777–3783.

23 Widdick DA, Dodd HM, Barraille P, White J, Stein TH, Chater KF, Gasson MJ, Bibb MJ: Cloning and engineering of the cinnamycin biosynthetic gene cluster from *Streptomyces cinnamoneus cinnamoneus* DSM 40005. Proc Natl Acad Sci USA 2003;100:4316–4321.

24 O'Driscoll J, Glynn F, Cahalane O, O'Connell-Motherway M, Fitzgerald GF, van Sinderen D: Lactococcal plasmid pNP40 encodes a novel, temperature-sensitive restriction-modification system. Appl Environ Microbiol 2004;70:5546–5556.

25 Cotter PD, Draper LA, Lawton EM, McAuliffe O, Hill C, Ross RP: Overproduction of wild-type and bioengineered derivatives of the lantibiotic lacticin 3147. Appl Environ Microbiol 2006;72:4492–4496.

26 Jensen PR, Hammer K: Artificial promoters for metabolic optimization. Biotechnol Bioeng 1998;58:191–195.

27 McAuliffe O, O'Keeffe T, Hill C, Ross RP: Regulation of immunity to the two-component lantibiotic, lacticin 3147, by the transcriptional repressor LtnR. Mol Microbiol 2001;39:982–993.

28 Ottenwalder B, Kupke T, Brecht S, Gnau V, Metzger J, Jung G, Gotz F: Isolation and characterization of genetically engineered gallidermin and epidermin analogs. Appl Environ Microbiol 1995;61:3894–3903.

29 Navaratna MA, Sahl HG, Tagg JR: Identification of genes encoding two-component lantibiotic production in *Staphylococcus aureus* C55 and other phage group II *S. aureus* strains and demonstration of an association with the exfoliative toxin B gene. Infect Immun 1999;67:4268–4271.

30 Holo H, Jeknic Z, Daeschel M, Stevanovic S, Nes IF: Plantaricin W from *Lactobacillus plantarum* belongs to a new family of two-peptide lantibiotics. Microbiology 2001;147: 643–651.

31 Van der Meer JR, Rollema HS, Siezen RJ, Beerthuyzen MM, Kuipers OP, de Vos WM: Influence of amino acid substitutions in the nisin leader peptide on biosynthesis and secretion of nisin by *Lactococcus lactis*. J Biol Chem 1994;269:3555–3562.

32 Neis S, Bierbaum G, Josten M, Pag U, Kempter C, Jung G, Sahl HG: Effect of leader peptide mutations on biosynthesis of the lantibiotic Pep5. FEMS Microbiol Lett 1997;149: 249–255.

33 Chen P, Qi FX, Novak J, Krull RE, Caufield PW: Effect of amino acid substitutions in conserved residues in the leader peptide on biosynthesis of the lantibiotic mutacin II. FEMS Microbiol Lett 2001;195:139–144.

34 Brouns SJJ, Wu H, Akerboom J, Turnbull AP, De Vos WM, van der Oost J: Engineering a selectable marker for hyperthermophiles. J Biol Chem 2005;280:11422–11431.

35 Delmas J, Robin F, Carvalho F, Mongaret C, Bonnet R: Prediction and evolution of ceftazidime resistance in extended-spectrum β-lactamase CTX-M-9. Antimicrob Agents Chemother 2006;50:731–738.

36 Wurth C, Guimard NK, Hecht MH: Mutations that reduce aggregation of the Alzheimer's Aβ42 peptide: an unbiased search for the sequence determinants of Aβ amyloidogenesis. J Mol Biol 2002;319:1279–1290.

37 Pag U, Sahl HG: Multiple activities in lantibiotics – models for the design of novel antibiotics? Curr Pharm Des 2002;8:815–833.

38 Yuan J, Zhang ZZ, Chen XZ, Yang W, Huan LD: Site-directed mutagenesis of the hinge region of nisinZ and properties of nisinZ mutants. Appl Microbiol Biotechnol 2004; 64:806–815.

39 Rollema HS, Kuipers OP, Both P, de Vos WM, Siezen RJ: Improvement of solubility and stability of the antimicrobial peptide nisin by protein engineering. Appl Environ Microbiol 1995;61:2873–2878.

40 Poirot O, O'Toole E, Notredame C: Tcoffee@igs: a web server for computing, evaluating and combining multiple sequence alignments. Nucleic Acids Res 2003;31:3503–3506.

41 Coakley M, Fitzgerald G, Ros RP: Application and evaluation of the phage resistance- and bacteriocin-encoding plasmid pMRC01 for the improvement of dairy starter cultures. Appl Environ Microbiol 1997;63: 1434–1440.

42 Cotter PD, Hill C, Ross RP: A food-grade approach for functional analysis and modification of native plasmids in *Lactococcus lactis*. Appl Environ Microbiol 2003;69:702–706.

43 Hayes F, Daly C, Fitzgerald GF: Identification of the minimal replicon of *Lactococcus lactis* subsp. *lactis* UC317 plasmid pCI305. Appl Environ Microbiol 1990;56:202–209.

Research Article

Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol 2007;13:235–242
DOI: 10.1159/000104749

Lantibiotic Engineering: Molecular Characterization and Exploitation of Lantibiotic-Synthesizing Enzymes for Peptide Engineering

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Key Words
Lantibiotics · Post-translational modification · Unusual amino acid · Peptide engineering

Abstract
Lanthionine-containing peptide antibiotics called lantibiotics are produced by a large number of Gram-positive bacteria. Nukacin ISK-1 produced by *Staphylococcus warneri* ISK-1 is type-A(II) lantibiotic. Ribosomally synthesized nukacin ISK-1 prepeptide (NukA) consists of an N-terminal leader peptide followed by a C-terminal propeptide moiety that undergoes several post-translational modification events including unusual amino acid formation by the modification enzyme NukM, cleavage of leader peptide and export by the dual functional ABC transporter NukT, finally yielding a biologically active peptide. Unusual amino acids in lantibiotics contribute to biological activity and also structural stability against proteases. Thus, lantibiotic-synthesizing enzymes have a high potentiality for peptide engineering by introduction of unusual amino acids into desired peptides with altering biological and physicochemical properties, e.g., activity and stability, termed lantibiotic engineering. We report the establishment of a heterologous expression of nukacin ISK-1 biosynthetic gene cluster by the nisin-controlled expression system and discuss our recent progress in understanding of the biosynthetic enzymes for nukacin ISK-1 such as localization, molecular interaction in biophysical and biochemical aspects. Substrate specificity of the lantibiotic-synthesizing enzymes was evaluated by complementation of the biosynthetic enzymes (LctM and LctT) of closely related lantibiotic lacticin 481 for nukacin ISK-1 biosynthesis. We further explored a rapid and powerful tool for introduction of unusual amino acids by co-expression of hexa-histidine-tagged NukA and NukM in *Escherichia coli*.

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Introduction

The emergence of antibiotic resistance, such as vancomycin-resistant enterococci and methicillin-resistant staphylococci, has been of great concern in recent years, so there is a pressing need for development of novel antimicrobial agents. Among many candidates, interest in working with antimicrobial peptides is being inspired by the possibility of using them as alternative antibiotics.

Lantibiotics are ribosomally synthesized post-translationally modified antimicrobial peptides that contain unusual amino acids such as lanthionine and/or 3-methyllanthionine and dehydrated amino acids [1–3]. Various biological activities and significant structural stability of lantibiotics have been reported and all the activities depend on the presence of unusual amino acids [4, 5]. Lantibiotics therefore have a high possibility for the application in medical and food industries due to their

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1464–1801/07/0134–0235$23.50/0

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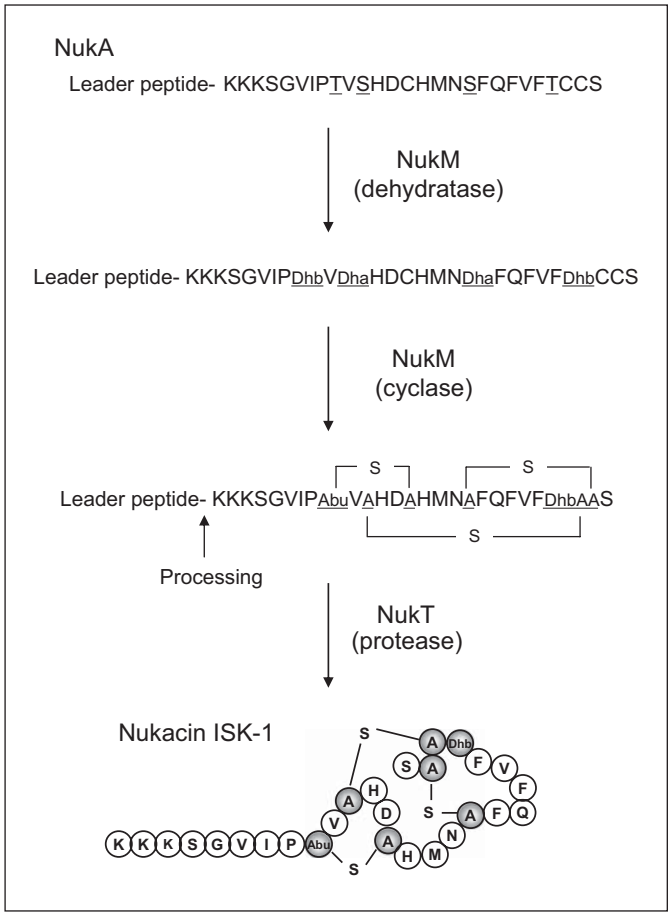

Fig. 1. Post-translational modification process of lantibiotic nukacin ISK-1. Underlined serine and threonine residues in nukacin ISK-1 prepeptide (NukA) are dehydrated and cyclized by NukM [17, 23]. After dehydration/cyclization, the leader peptide is removed proteolytically by the N-terminal protease domain of NukT [Shioya et al., unpubl. data]. A-S-A, lanthionine; Abu-S-A, 3-methyllanthionine; Dha, dehydroalanine; Dhb, dehydrobutyrine.

excellent properties. The lantibiotic prepeptide consists of an N-terminal leader peptide followed by a C-terminal propeptide moiety that undergoes several post-translational modification events including unusual amino acids formation, cleavage of leader peptide, and export [6]. Serine and threonine residues at specific positions in the propeptide moiety are dehydrated to dehydroalanine (Dha) and dehydrobutyrine (Dhb) residues respectively, and the thioether rings of lanthionine and 3-methyllanthionine are formed by the cyclization of Dha and Dhb residues with cysteine residues [7]. Thus, enzymes involved in lantibiotic biosynthesis may have high potentiality for peptide engineering by introduction of unusual amino acids into desired peptides, which might envisage the founding of a universal approach to advance for structural design of novel peptides, termed lantibiotic engineering.

Staphylococcus warneri ISK-1 was isolated from a well-aged *Nukadoko*, a bed of fermented rice bran [8]. This bacterium produces a novel type-A(II) lantibiotic, nukacin ISK-1 [9] (fig. 1). The gene cluster of nukacin ISK-1 consists of at least *nukAMTFEGH*, and ORF1 encoded on plasmid, having been sequenced [10–12] (fig. 2a). From the sequence similarity to known proteins involved in lantibiotic biosynthesis, NukA, NukM, and NukT are predicted to be the nukacin ISK-1 prepeptide, the modification enzyme, and the processing and transport enzyme, respectively (fig. 1). Incorporation of unusual dehydro- and lanthionine-type amino acids depends on the ability of the lantibiotic-synthesizing enzyme, however, little has been known about the molecular characterization of the biosynthetic enzymes in lantibiotics. Here, we report characterization of the nukacin ISK-1 biosynthetic enzymes and development of novel system for introduction of unusual amino acids into peptides for lantibiotic engineering.

Heterologous Expression of the Biosynthetic Gene Cluster for Nukacin ISK-1

Lantibiotics are gene-encoded and, therefore, can be readily manipulated by genetic engineering, which provides an efficient means for constructing and producing large numbers of structural analogs needed for structure-function studies and rational design. So far, various expression and gene replacement strategies have been developed to produce engineered lantibiotics [13–16].

We demonstrated the heterologous expression and gene replacement system for nukacin ISK-1 biosynthetic gene cluster in *Lactococcus lactis* under the control of the nisin-controlled expression system. We successively cloned the *nukAMTFEGH* genes downstream of the nisin-inducible promoter P*nisA* in pNZ8048, resulting in plasmid pInuk (table 1; fig. 2a). The recombinant *L. lactis* harboring pInuk showed antimicrobial activity against *Pediococcus pentosaceus* JCM 5885 as an indicator strain (fig. 2b). Electrospray ionization-mass spectrometry (ESI-MS) analysis demonstrated the presence of nukacin ISK-1 in the culture supernatant. These results suggest that the recombinant *L. lactis* harboring *nukAMTFEGH* produced nukacin ISK-1 heterologously. We further examined the effects of inactivation of *nukA*, *-M*, or *-T* on

Table 1. Bacterial strains and plasmids used in this study

		Description[a]	Reference and source
Strains	*Staphylococcus warneri* ISK-1	Wild-type, nukacin ISK-1 producer	8
	Lactococcus lactis NZ9000	MG1363 derivative; *nisRK::pep*	28
	NZ9000 recombinant[b]		
	(pInuk)	Em^r, NZ9000 derivative, *nukAMTFEGH*, nukacin ISK-1 producer, Nuk$^+$	17
	(pInukdA)	Disruption of *nukA* in pInuk, Nuk$^-$	17
	(pInukdM)	Disruption of *nukM* in pInuk, Nuk$^-$	17
	(pInukdT)	Disruption of *nukT* in pInuk, Nuk$^-$	17
	Lactococcus lactis CNRZ481	Wild-type, lacticin 481 producer	19
	Pediococcus pentosaceus JCM 5885	Indicator strain of nukacin ISK-1	JCM
	Lactobacillus sakei subsp *sakei* JCM 1157^T	Indicator strain of nukacin ISK-1	JCM
	Escherichia coli BL21(DE.3)	Expression host *F*$^-$, *ompT*, *hsdS*$_B$($r_B^-m_B^-$) *gal*, *dcm* (DE3)	Novagen
	E. coli BL21(DE3) recombinant[b] (pETnukAM)	Ap^r, BL21(DE3) carrying pETnukAM	23
Plasmids	pNZ8048	Cm^r, *L. lactis* expression vector with P*nisA*	27
	pNZM	pNZ8048 derivative containing *nukM* downstream of P*nisA*	17
	pNZT	pNZ8048 derivative containing *nukT* downstream of P*nisA*	17
	pNZLCTA	pNZ8048 derivative containing *lctA* downstream of P*nisA*	17
	pNZLCTM	pNZ8048 derivative containing *lctM* downstream of P*nisA*	This study
	pNZLCTT	pNZ8048 derivative containing *lctT* downstream of P*nisA*	This study

[a] Ap^r = ampicillin resistance; Cm^r = chloramphenicol resistance; Em^r = erythromycin resistance. P*nisA* indicates the *nisA* promoter that is used for the nisin-controlled expression system [27, 28]. Nuk$^+$/Nuk$^-$, phenotypes of nukacin ISK-1 production or not, respectively.

[b] Plasmids introduced in strains are indicated in parentheses.

the production of nukacin ISK-1. Inactivation of *nukA*, *-M*, or *-T* resulted in the complete loss of the nukacin ISK-1 production phenotype. These findings indicate that *nukAMT* are indispensable for the biosynthesis of nukacin ISK-1 [17].

Localization and Interaction of the Biosynthetic Enzymes for Nukacin ISK-1

Knowledge on the nukacin ISK-1 biosynthetic enzymes would thus be important for understanding the properties as well as the possibility of their application for lantibiotic engineering. We therefore characterized biophysical and biochemical aspects of the nukacin ISK-1 biosynthetic enzymes.

Localization analysis of NukM and NukT in the wild-type strain by Western blotting indicated that both proteins were located at the cytoplasmic membrane. NukM expressed heterologously in *Staphylococcus carnosus* TM300 was also located at the cytoplasmic membrane even in the absence of NukT. The hydrophobic profile of NukM by several prediction programs suggested the lack of transmembrane segments and therefore indicated that NukM would be associated with the cytoplasm membrane. In vivo interaction analysis by yeast two-hybrid assay showed the presence of a complex comprising at least two each of NukM and NukT which are associated with a single copy of NukA. In vitro interaction analysis by surface plasmon resonance biosensor further suggested that membrane-associated NukM interacts with NukA. These results indicate that NukM and NukT

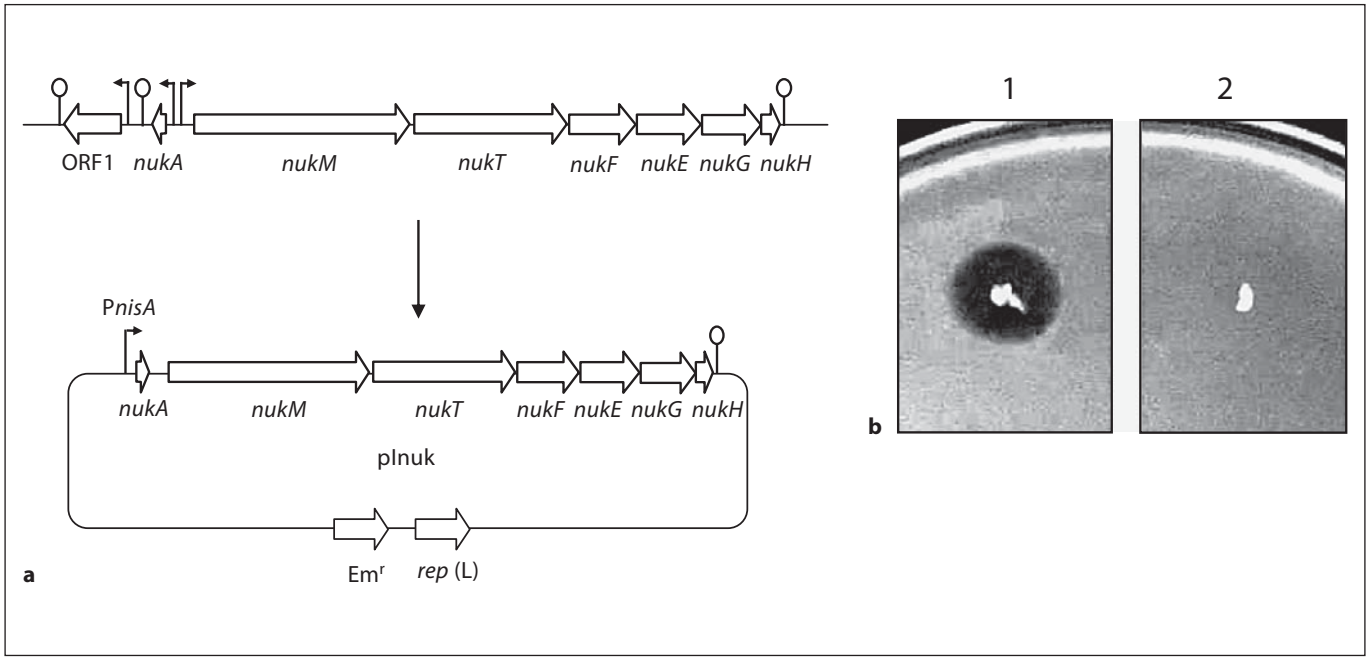

Fig. 2. a Construction of pInuk. The *nukAMTFEGH* genes were successively cloned downstream of the nisin-inducible promoter P*nisA* in pNZ8048. P*nisA* represents the nisin-inducible promoter derived from pNZ8048. Putative promoters and ρ-independent terminators are indicated by arrows and lollipop-like symbols, respectively. *rep* (L) indicates replicon in lactococci. Em^r indicates resistance gene for erythromycin. **b** Bioassay for the production of nukacin ISK-1 in *L. lactis* NZ9000 (pInuk). (1) The induced culture of recombinant *L. lactis* NZ9000 (pInuk) was spotted on a GM17 agar plate containing nisin (10 μg/l). (2) The uninduced culture of recombinant *L. lactis* NZ9000 (pInuk) was spotted on a GM17 agar plate. The plates were incubated at 30°C overnight. MRS soft agar seeded with *P. pentosaceus* JCM 5885 as an indicator strain was overlaid on the plates and further incubated overnight.

Table 2. Overview of complementation test of the biosynthetic enzymes for nukacin ISK-1 and lacticin 481

Strain characteristic	Prepeptide	Modification enzyme	Processing and transport enzyme	Antimicrobial activity and detected mass[a]	Reference
NZ9000 derivative containing the plasmids, pInukdA and pNZA	NukA	NukM	NukT	+ (2,960 Da)	17
NZ9000 derivative containing the plasmids, pInukdM and pNZLCTM	NukA	LctM	NukT	– (ND[b])	This study
NZ9000 derivative containing the plasmids, pInukdT and pNZLCTT	NukA	NukM	LctT	+ (2,960 Da)	This study
L. lactis (CNRZ481, wild type	LctA	LctM	LctT	+ (2,901 Da)	19
NZ9000 derivative containing the plasmids, pInukdA and pNZLCTA	LctA	NukM	NukT	– (ND[b])	17

[a] Nukacin ISK-1 and lacticin 481 production in culture supernatant of the wild type and the obtained recombinant strains was analyzed by bioassay using Tricine SDS-PAGE and overlay assay [21], and by LC/MS. *L. sakei* was used as an indicator strain. The mass corresponding to the mature nukacin ISK-1 and lacticin 481 in parentheses was determined by LC/MS analysis.

[b] ND = not detected.

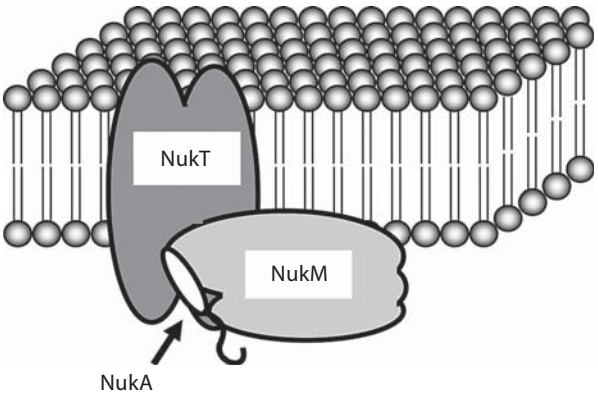

Fig. 3. Proposed membrane-located multimeric enzyme complex comprising at least two each of NukM and NukT which are associated with NukA [18].

form a membrane-located multimeric protein complex as shown in figure 3, and that post-translational modification of nukacin ISK-1 would occur at the cytoplasm membrane [18]. Our surface plasmon resonance analysis implied that NukM independently recognized NukA without complex formation with NukT. Additionally, we confirmed the function of NukM as a modification enzyme independently of NukT as described in a later section. Hence, we propose that NukM and NukT form a well-governed complex for the efficient production of nukacin ISK-1. Such an architecture may prevent the diffusion-controlled state of the substrate in active cells.

Substrate Recognition of the Biosynthetic Enzymes

The versatility of the exploitation of lantibiotic-synthesizing enzymes for the incorporation of unusual amino acids into lantibiotic analogs or non-lantibiotic polypeptides aimed at lantibiotic engineering depends on the substrate specificity of these enzymes.

The most similar lantibiotic to nukacin ISK-1 is lacticin 481 [19] produced by *L. lactis* CNRZ481. They exhibit 64% identity with the prepeptides NukA and LctA. In contrast, the modification enzymes (NukM and LctM) and the processing and transport enzymes (NukT and LctT) show less identity, 40 and 46%, respectively. In fact, we found that the biosynthetic enzymes (NukM and NukT) for nukacin ISK-1 were not capable of processing the lacticin 481 prepeptide (LctA) [17] (table 2). We therefore determined as to whether the lacticin 481 biosynthetic enzymes can complement for nukacin ISK-1 biosynthesis. Complementation test of the biosynthetic enzymes for lacticin 481, LctM and LctT, in place of NukM and NukT, was investigated using *L. lactis* (pInukdM) and *L. lactis* (pInukdT) strains respectively in terms of nukacin ISK-1 production.

Construction of plasmids for expression of LctM or LctT, was done as given below. Each gene fragment of *lctM* or *lctT* was amplified by PCR with the plasmid extracted from *L. lactis* CNRZ481 as a template using sets of appropriate primers as follows: 5′-GGACAAAATCATGAAAAAAAAGACTTACC-3′ for the 5′ region of *lctM*; 5′-TAAACTCGAGCTCATATTAATCAACATATG-3′ for the 3′ region of *lctM*; 5′-GAGTTTACCATGGAAATAGTTTTACAAAA-3′ for the 5′ region of *lctT*; 5′-CGATACGGAGCTCTTTATTTATTCATTAA-3′ for the 3′ region of *lctT*. The amplified fragments of *lctM* and *lctT* were digested with *Pag*I and *Sac*I and *Nco*I and *Sac*I, and cloned under a nisin-inducible promoter in pNZ8048 at the *Nco*I and *Sac*I sites, resulting in plasmids pNZLCTM and pNZLCTT, respectively (table 1). The constructed plasmids pNZLCTM and pNZLCTT were introduced in *L. lactis* (pInukdM) and *L. lactis* (pInukdT) strains respectively, according to the method described previously [17]. *L. lactis* recombinant strains were grown at 30°C in GM17 medium [17] for bioassay and in chemically defined medium (CDM) [20] for detection of produced nukacin ISK-1 by liquid chromatography/mass spectrometry (LC/MS). CDM was used to reduce the amounts of interfering proteinaceous impurities, which improved analysis of expressed peptides in the culture supernatant. Supernatants of CDM culture were prepared by centrifugation at 6,500 *g* for 15 min. Ten milliliters of supernatants were loaded onto a Sep-Pak C_{18} cartridge (100 mg; Waters, Milford, Mass., USA), washed with 2 ml of water/0.1% trifluoroacetic acid and eluted with 3 ml of 50% acetonitrile/0.1% trifluoroacetic acid. Eluates were dried, dissolved in 200 μl of distilled water, and 50 μl of solutions were applied to LC/MS device (LC; Agilent HP1100, JEOL, Tokyo, Japan: MS; Accutof T100LC, JEOL). Eluates were directly loaded into ESI-MS. The mass spectrometer was operated under the following conditions: positive polarity, capacity temperature 250°C, needle voltage of 2.0 kV, orifice voltage of 92 V, and ring voltage of 10 V. After scanning molecular ions derived from column eluates in the m/z range of 500–3,000, ion chromatograms extracted were plotted with detector counts at indicated m/z, with a window of 1 mass unit. Nukacin ISK-1 activity in the GM17 culture supernatants of *L. lactis* recombinant

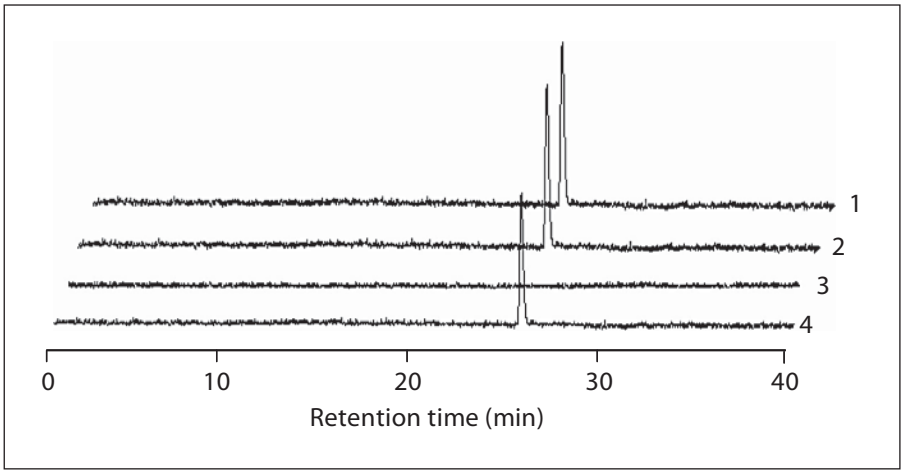

Fig. 4. Complementation test of the biosynthetic enzyme of lacticin 481 for nukacin ISK-1 biosynthesis. Plasmids pNZM and pNZLCTM were each introduced in *L. lactis* (pInukdM), and pNZT and pNZLCTT were each in *L. lactis* (pInukdT) strains. Production of nukacin ISK-1 was monitored by LC/MS analysis of culture supernatant of *L. lactis* recombinant strains carrying pInukdM/pNZM (1), pInukdT/pNZT (2), pInukdM/pNZLCTM (3) and pInukdT/pNZLCTT (4). The extracted ion chromatogram at m/z = 1,480 corresponding to double charge of nukacin ISK-1 (2,960 Da), was displayed for each analysis.

strains was analyzed by Tricine SDS-PAGE and overlay assay [21] using *Lactobacillus sakei* subsp. *sakei* JCM 1157^T as an indicator strain.

Complete restore of nukacin ISK-1 production was observed in complementation tests with LctT but not LctM, indicating that NukT can be replaced by LctT in processing and transport of nukacin ISK-1. However, NukM cannot be replaced by LctM in modification of nukacin ISK-1 (table 2; fig. 4). The reason why the lacticin 481 prepeptide LctA cannot be processed by the nukacin ISK-1 biosynthetic enzymes (NukM and NukT) [17] may arise from the substrate specificity of NukM. The conserved domains found among LanM proteins in type-A(II) lantibiotics [22] are highly conserved in both NukM and LctM (data not shown), suggesting the existence of a recognizable specific sequence and/or conformation in prepeptides by each modification enzyme. Further studies are required to identify the essential determinants for the substrate recognition by the biosynthetic enzymes of lantibiotics.

Development of Novel System for Introduction of Unusual Amino Acids

We demonstrated a novel strategy for introduction of unusual amino acids into hexa-histidine-tagged NukA (His-NukA) by co-expression with NukM in the Gram-negative bacterium, *Escherichia coli* BL21(DE3). The gene fragment encoding NukA and NukM was successively amplified from the template plasmid pInuk [17] and cloned under a T7 promoter in pET-14b at *Bam*HI site. The resultant plasmid pETnukAM (table 1) was in-frame translated with hexa-histidine tag sequence at N-terminus of NukA. Co-expression of NukA and NukM was induced with 1 mM isopropyl 1-thio-β-D-galactoside for 20 h at 20°C. Nickel-affinity chromatography of the resulting His-tagged prepeptide and subsequent ESI-MS spectrometry analysis showed that the prepeptide is converted into a postulated peptide with decrease in mass of 72 Da, which indicates the dehydration of four amino acids. The molecular mass data described above does not allow for the identification of the presence of lanthionine or 3-methyllanthionine, because intramolecular cyclization of Dha or Dhb residues with cysteine does not produce a change in mass. We therefore characterized the modified His-NukA by a further physicochemical analysis such as peptide mapping with the protease lysyl endopeptidase, amino acid sequencing and amino acid composition analysis. The characterization of the resultant prepeptide indicated the presence of unusual amino acids in its C-terminal propeptide moiety (fig. 1). The modified prepeptide encompassing the leader peptide attached to the post-translationally modified propeptide moiety was readily obtained by one-step purification. The lanthionine-introducing system in *E. coli* as developed in this study has many advantages such as easy manipulation of genetic engineering and rapid and instant usages. We would like to emphasize that considerable amounts of modified His-tagged-labeled NukA (1.5 mg/l of culture) was readily obtained by one-step affinity chromatography. These findings will be useful for further peptide engineering by introduction of unusual amino acids and it will facilitate our insights in the properties of the modification enzyme [23].

Conclusion and Perspective

Recent advances in peptide chemistry, molecular biology and genetics have expanded the possibility for the engineering of peptides and proteins. Amino acids substitution, D-amino acid replacement, chemical modification, glycosylation, and backbone cyclization are the present technological advancement that can ameliorate the potentiality of antimicrobial peptides and proteins. In the post-genomic era, molecular design and alteration of protein structure and function by introducing unusual amino acids enjoy an immense interest and are highly forthcoming in order to develop new biological tools for producing novel therapeutic agents. In particular, the engineering of new peptides is a promising technique to create potential antibiotics, since the traditional antibiotics are diminishing in the race of increasing antibiotic resistance among microbial populations. Thus, exploitation of lantibiotic-synthesizing enzymes has high possibility for peptide engineering. Recent reports in type-A(I) lantibiotic nisin showed that non-lantibiotic peptides fused to the C-terminus of the nisin leader peptide are successfully expressed as a dehydrated or cyclized forms via the nisin-synthesizing enzymes [24, 25]. These findings would pave the way for generating a wide range of dehydrated or cyclized peptides with novel biological activities by lantibiotic-synthesizing enzymes.

In this report, we have described the heterologous expression of the type-A(II) lantibiotic nukacin ISK-1 and the characterization of nukacin ISK-1 biosynthetic enzymes (NukM and NukT) such as localization, molecular interaction and substrate recognition. Furthermore, we have successfully developed a novel tool for introduction of unusual amino acids into prepeptides. Our findings are helpful to provide new insight into lantibiotic-synthesizing enzymes and will further open up a way for architecture of physiologically active peptides by introducing unusual amino acids. Based on the knowledge obtained in nukacin ISK-1 research, the rational and tailor-made design of a wide range of novel peptides and proteins would be accomplished in near future. However, there are also remaining questions to be solved, e.g.: How do the modifying enzymes interact with their substrates? Why are specific serine and threonine residues modified? How can the direction of dehydrated amino acids formation be controlled as well as the regiospecificity of lanthionine bridges? Comprehensive works and integration of chemical, biological, enzymatical and structural approaches would thus further revolutionize and expand the possibility of lantibiotic engineering. We have recently delineated the determinants governing to structure-function relationships on nukacin ISK-1 with the fragments and the structural mutants generated [26], and are now trying to shed light on the further means of lantibiotic engineering by enzymatic incorporation of unusual dehydro-, lanthionine-type amino acids with lantibiotic-synthesizing enzymes based on lantibiotic nukacin ISK-1.

Acknowledgements

Our work on lantibiotic nukacin ISK-1 has been partially supported by grants from the following organizations: the Japan Society for the Promotion of Science, the Japan Science Society, the Novartis Foundation (Japan) for the Promotion of Science, the Novozymes Japan Research Fund, and the Nagase Science and Technology Foundation.

References

1 De Vos WM, Kuipers OP, van der Meer JR, Siezen RJ: Maturation pathway of nisin and other lantibiotics: post-translationally modified antimicrobial peptides exported by Gram-positive bacteria. Mol Microbiol 1995; 17:427–437.

2 Chatterjee C, Paul M, Xie L, van der Donk WA: Biosynthesis and mode of action of lantibiotics. Chem Rev 2005;105:633–684.

3 McAuliffe O, Ross RP, Hill C: Lantibiotics: structure and mode of action. FEMS Microbiol Rev 2005;25:285–308.

4 Van Kraaij C, Breukink E, Rollema HS, Bongers RS, Kosters HA, de Kruijff B, Kuipers OP: Engineering a disulfide bond and free thiols in the lantibiotic nisin Z. Eur J Biochem 1995;230:827–853.

5 Bierbaum G, Szekat C, Josten M, Heidrich C, Kempter C, Jung G, Sahl H-G: Engineering of a novel thioether bridge and role of modified residues in the lantibiotic Pep5. Appl Environ Microbiol 1996;62:385–392.

6 Sahl H-G, Jack RW, Bierbaum G: Biosynthesis and biological activities of lantibiotics with unique post-translational modifications. Eur J Biochem 1995;230:827–853.

7 Ingram LC: Synthesis of the antibiotic nisin: formation of lanthionine and β-methyl-lanthionine. Biochim Biophys Acta 1969;184: 216–219.

8 Ishizaki A, Takese E, Ikai T, Kumai S, Nagano R, Sonomoto K, Doi K, Ogata S, Kawamura Y, Ezaki T: Taxonomic position of new bacteriocin (nukacin ISK-1) producer isolated from long-aged *Nukadoko*. J Gen Appl Microbiol 2001;47:143–147.

9 Kimura H, Matsusaki H, Sashihara T, Sonomoto K, Ishizaki A: Purification and partial identification of bacteriocin ISK-1, a new lantibiotic produced by *Pediococcus* sp. ISK-1. Biosci Biotechnol Biochem 1998;62:2341–2345.

10 Sashihara T, Kimura H, Higuchi T, Adachi A, Matsusaki H, Sonomoto K, Ishizaki A: A novel lantibiotic, nukacin ISK-1, of *Staphylococcus warneri* ISK-1: cloning of the structural gene and elucidation of the structure. Biosci Biotechnol Biochem 2000;64:2420–2428.
11 Aso Y, Sashihara T, Nagao J, Kanemasa Y, Koga H, Hashimoto T, Higuchi T, Adachi A, Nomiyama H, Ishizaki A, Nakayama J, Sonomoto K: Characterization of a gene cluster of *Staphylococcus warneri* ISK-1 encoding the biosynthesis of and immunity to the lantibiotic, nukacin ISK-1. Biosci Biotechnol Biochem 2004;68:1663–1671.
12 Aso Y, Koga H, Sashihara T, Nagao J, Kanemasa Y, Nakayama J, Sonomoto K: Description of complete DNA sequence of two plasmids from the nukacin ISK-1 producer, *Staphylococcus warneri* ISK-1. Plasmid 2005;53:164–178.
13 Bierbaum G, Reis M, Szekat C, Sahl HG: Construction of an expression system for engineering of the lantibiotic Pep5. Appl Environ Microbiol 1994;60:4332–4338.
14 Kuipers OP, Rollema HS, Yap WMGJ, Boot HJ, Siezen RJ, de Vos WM: Engineering dehydrated amino acid residues in the antimicrobial peptide nisin. J Biol Chem 1992;267:24340–24346.
15 Szekat C, Jack RW, Skutlarek D, Färber H, Bierbaum G: Construction of an expression system for site-directed mutagenesis of the lantibiotic mersacidin. Appl Environ Microbiol 2003;69:3777–3783.
16 Chen P, Novak J, Kirk M, Barnes S, Qi F, Caufield PW: Structure-activity study of the lantibiotic mutacin II from *Streptococcus mutans* T8 by a gene replacement strategy. Appl Environ Microbiol 1998;64:2335–2340.
17 Aso Y, Nagao J, Koga H, Okuda K, Kanemasa Y, Sashihara T, Nakayama J, Sonomoto K: Heterologous expression and functional analysis of the gene cluster for the biosynthesis of and immunity to the lantibiotic, nukacin ISK-1. J Biosci Bioeng 2004;98:429–436.
18 Nagao J, Aso Y, Sashihara T, Shioya K, Adachi A, Nakayama J, Sonomoto K: Localization and interaction of the biosynthetic proteins for the lantibiotic, nukacin ISK-1. Biosci Biotechnol Biochem 2005;69:1341–1347.
19 Piard J-C, Kuipers OP, Rollema HS, Desmazeuad MJ, de Vos WM: Structure, organization and expression of the *lct* gene for lacticin 481, a novel lantibiotic produced by *Lactococcus lactis*. J Biol Chem 1993;268:16361–16368.
20 Jensen PR, Hammer K: Minimal requirements for exponential growth of *Lactococcus lactis*. Appl Environ Microbiol 1993;59:4363–4366.
21 Miller KW, Schamber R, Chen Y, Ray B: Production of active pediocin AcH in *Escherichia coli* in the absence of processing and secretion genes from the *Pediococcus pap* operon. Appl Environ Microbiol 1998;64:14–20.
22 Uguen P, Le Pennec JP, Dufour A: Lantibiotic biosynthesis: interaction between prelacticin 481 and its putative modification enzyme, LctM. J Bacteriol 2000;182:5262–5266.
23 Nagao J, Harada Y, Shioya K, Aso Y, Zendo T, Nakayama J, Sonomoto K: Lanthionine introduction into nukacin ISK-1 prepeptide by co-expression with the modification enzyme NukM in *Escherichia coli*. Biochem Biophys Res Commun 2005;336:507–513.
24 Kuipers A, de Boef E, Rink R, Fekken S, Kluskens LD, Driessen AJM, Leenhouts K, Kuipers OP, Moll GN: NisT, the transporter of the lantibiotic nisin, can transport fully modified, dehydrated and unmodified prenisin and fusions of the leader peptide with non-lantibiotic peptides. J Biol Chem 2004;279:22176–22182.
25 Kluskens LD, Kuipers A, Rink R, de Boef E, Fekken S, Driessen AJM, Kuipers OP, Moll GN: Post-translational modification of therapeutic peptides by NisB, the dehydratase of the lantibiotic nisin. Biochemistry 2005;44:12827–12834.
26 Asaduzzaman SM, Nagao J, Aso Y, Nakayama J, Sonomoto K: Lysine-oriented charges trigger the membrane binding and activity of nukacin ISK-1. Appl Environ Microbiol 2006;72:6012–6017.
27 De Ruyter PG, Kuipers OP, de Vos WM: Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. Appl Environ Microbiol 1996;62:3662–3667.
28 De Ruyter PG, Kuipers OP, Beerthuyzen MM, van Alen-Boerrigter I, de Vos WM: Functional analysis of promoters in the nisin gene cluster of *Lactococcus lactis*. J Bacteriol 1996;178:3434–3439.

Research Article

Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol 2007;13:243–247
DOI: 10.1159/000104753

The Antifungal Activity of RsAFP2, a Plant Defensin from *Raphanus sativus*, Involves the Induction of Reactive Oxygen Species in *Candida albicans*

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Key Words
Ascorbic acid · Glucosylceramide · *Raphanus sativus* antifungal plant defensin · Reactive oxygen species · Small unilamellar vesicle · *Candida albicans*

Abstract
RsAFP2 (*Raphanus sativus* antifungal peptide 2), an antifungal plant defensin isolated from seed of *R. sativus*, interacts with glucosylceramides (GlcCer) in membranes of susceptible yeast and fungi and induces membrane permeabilization and fungal cell death. However, using carboxyfluorescein-containing small unilamellar vesicles containing purified GlcCer, we could not observe permeabilization as a consequence of insertion of RsAFP2 in such vesicles. Therefore, we focused on a putative RsAFP2-induced signaling cascade downstream of RsAFP2-binding to GlcCer in fungal membranes. We show that RsAFP2 induces reactive oxygen species (ROS) in *Candida albicans* wild type in a dose-dependent manner, but not at all in an RsAFP2-resistant Δ*gcs C. albicans* mutant that lacks the RsAFP2-binding site in its membranes. These findings indicate that upstream binding of RsAFP2 to GlcCer is needed for ROS production leading to yeast cell death. Moreover, the antioxidant ascorbic acid blocks RsAFP2-induced ROS generation, as well as RsAFP2 antifungal activity. These data point to the presence of an intracellular plant defensin-induced signaling cascade, which involves ROS generation and leads to fungal cell growth arrest.
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Introduction

Plant defensins are small, basic, cysteine-rich peptides that possess antifungal and in some cases also antibacterial activity [reviewed in 1, 2]. They are not only active against phytopathogenic fungi (such as *Fusarium culmorum* and *Botrytis cinerea*), but also against human pathogenic fungi (such as *Candida albicans*) and baker's yeast. However, they are non-toxic to human and plant cells [2]. Plant defensins are part of the innate immune system of plants and protect them against invading fungal and bacterial pathogens. In this respect, it was demonstrated by various research groups that overexpression of plant defensins resulted in increased fungal resistance in various crops [1].

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The molecular mechanism of fungal growth inhibition by plant defensins is far from clarified. We previously demonstrated that RsAFP2 (*Raphanus sativus* antifungal peptide 2), a plant defensin isolated from seeds of *R. sativus* [3], interacts with glucosylceramides (GlcCer) in the plasma membrane of susceptible fungi and yeast strains [4]. This interaction results in hyperpolarization of the fungal membrane potential [5], permeabilization of the cell and cell growth arrest [6]. Fungal and yeast mutants with membranes (i) lacking GlcCer due to a nonfunctional gene encoding GlcCer synthase *(GCS)* gene [7], or (ii) composed of GlcCer with structurally altered ceramide moiety [8] are resistant to RsAFP2-induced membrane permeabilization and cell death [4, 9]. Whether the RsAFP2-induced membrane permeabilization results from direct insertion of RsAFP2 in the fungal membrane or from induction of an intracellular signaling cascade is currently not clear.

We therefore analyzed in a first instance the permeabilization capacity of RsAFP2 on carboxyfluorescein-containing small unilamellar vesicles (SUVs) containing purified GlcCer. Next, we set out to identify a putative RsAFP2-induced signaling cascade leading to fungal cell death. To this end, we assessed a putative induction of endogenous reactive oxygen species (ROS) by RsAFP2 in susceptible fungi.

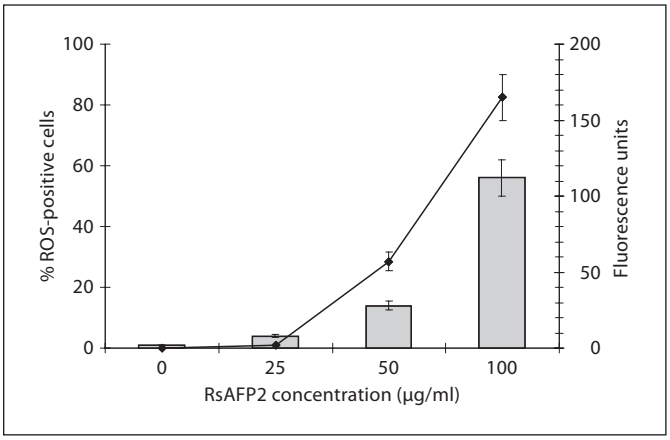

Fig. 1. RsAFP2-induced increase of endogenous ROS in *C. albicans*. Logarithmically growing *C. albicans* cells were suspended in YPD/PDB, pH 7.0, preincubated with RsAFP2 for 3 h at 30°C, washed with PBS and incubated with DCFHDA for 1.5 h at 30°C. Fluorescence emitted by the cells was measured using fluorescence spectrometer (λ_{ex} = 485 nm and λ_{em} = 540 nm) (line, fluorescence units on right y-axis) and microscopically (bars, % ROS-positive cells (n = 500) on left y-axis). This figure is a representative of 3 experiments.

Results

RsAFP2 Does Not Induce Permeabilization in GlcCer-Containing Vesicles

In all the experiments described below, the stability of the liposomes was checked by measuring the leakage of the entrapped self-quenched carboxyfluorescein (CF). An increase in CF fluorescence intensity implies that the membrane stability was compromised. Since membranes of the various yeast species typically contain 0.9–2.0 mol% GlcCer [10], we prepared SUVs containing GlcCer concentrations ranging from 0.1 to 2.5 mol% to best mimic in vivo conditions.

For a first series of experiments, SUVs containing GlcCer concentrations ranging from 0.1 to 2.5 mol% in a background of (i) 100 mol% phosphatidylcholine (PC) or (ii) 80 mol% PC and 20 mol% cholesterol were prepared. After addition of RsAFP2 at different concentrations (20–50 μg/ml) exceeding those minimally required for antifungal activity on *C. albicans* (i.e. 15 μg/ml [4]), no CF leakage could be observed from these vesicles as there was no increase in CF fluorescence intensity (data not shown). Since the presence of negatively charged phospholipids such as phosphatidic acid (PA) and phosphatidylserine (PS) can be important for pore formation [11, 12], SUVs containing 1.5–2.5 mol% GlcCer in a background of (i) 20 mol% cholesterol and 80 mol% PS or (ii) 20 mol% cholesterol, 25 mol% PA and 55 mol% PC were used. However, no CF leakage could be observed of these SUVs upon addition of 20–50 μg/ml RsAFP2. In conclusion, it seems that the interaction between GlcCer and RsAFP2 does not lead directly to membrane permeabilization, via e.g. direct insertion of RsAFP2 in the membrane and pore formation.

After each of the above experiments, SUVs were lysed with 1% of the membrane-solubilizing agent, Triton X-100, as a positive control. Fluorescence intensity of Triton-induced CF leakage was found to be around 700–800 fluorescence units. Background intensity values were typically around 50 fluorescence units.

RsAFP2 Induces Endogenous ROS in C. albicans

Since RsAFP2 interacts with GlcCer in the fungal plasma membrane and ceramide-induced signaling cascade frequently involves ROS generation [13, 14], we further assessed a putative effect of RsAFP2 on endogenous ROS levels in *C. albicans*. Using a fluorimetric and microscopic assay, we could demonstrate a dose-dependent

ROS induction in *C. albicans* cells incubated with 20–100 μg/ml RsAFP2 for 3 h (fig. 1), thereby correlating the concentration at which the induction of ROS species occurs (i.e. 20 μg/ml) and the MIC of RsAFP2 on *C. albicans* (i.e. 15 μg/ml [4]). In contrast, incubation of a *C. albicans gcs*-deletion mutant that lacks GlcCer [7], the RsAFP2-binding site [4], with RsAFP2 (up to 100 μg/ml) did not result in induction of endogenous ROS levels (results not shown) nor in cell death [4].

Ascorbic Acid Abolishes RsAFP2-Induced Endogenous ROS and Antifungal Action of RsAFP2

To determine whether the observed ROS induction by RsAFP2 is linked to its antifungal activity and is not merely a secondary effect of RsAFP2 action, we assessed the effect of the antioxidant, ascorbic acid (AA), on RsAFP2-induced ROS generation and RsAFP2 antifungal activity against *C. albicans*. Addition of 10 mM AA can prevent RsAFP2-induced ROS generation (fig. 2a) and mitigate the RsAFP2 antifungal activity (fig. 2b), both at RsAFP2 concentrations up to 100 μg/ml RsAFP2. These data point to a causal link between RsAFP2-induced ROS generation in susceptible yeast and antifungal action of RsAFP2, respectively. A concentration of AA up to 100 mM alone did not affect survival of yeast cell cultures nor ROS generation (results not shown).

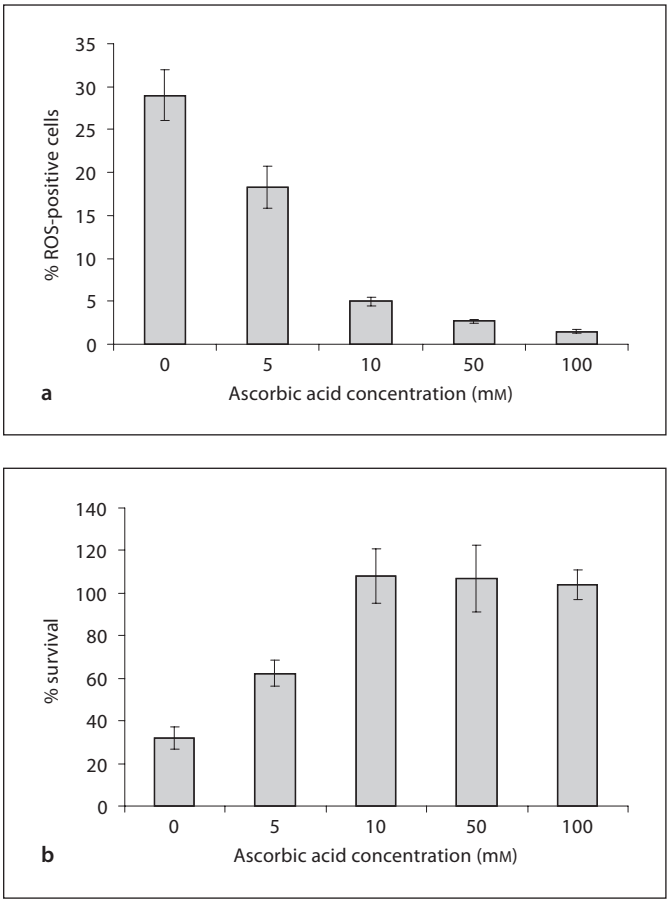

Fig. 2. Effect of the antioxidant AA on (**a**) RsAFP2-induced ROS generation and (**b**) antifungal activity. Logarithmically growing *C. albicans* cells were suspended in PDB/YPD, pH 7.0, preincubated with 100 μg/ml RsAFP2 in the presence of various AA concentrations for 3 h whereafter cells were washed in PBS. **a** Percentage ROS-positive cells (n = 500) determined after 1.5 h incubation with DCFHDA (30°C). **b** Percentage survival of the *C. albicans* cultures relative to the initial inoculum. This figure is a representative of 3 experiments.

Discussion

The present study distinguishes between various models for the mode of action of the antifungal plant defensin RsAFP2, which interacts with GlcCer in fungal membranes. First, in our experiments with GlcCer-containing SUVs, we could not detect any membrane permeabilization upon treatment with RsAFP2. This makes the hypothesis of RsAFP2-induced antifungal activity via a direct insertion of RsAFP2 upon its interaction with GlcCer in the fungal membrane unlikely. Instead, we demonstrate a causal link between RsAFP2-induced ROS generation in susceptible fungi and the antifungal action of RsAFP2. ROS, such as hydrogen peroxide and hydroxyl radical, are produced as by-products of aerobic respiration and cause damage to proteins, lipids, and DNA, resulting in mutation and loss of viability [reviewed in 15]. In mammalian cells, there is substantial evidence that an increase in ceramide content in membranes, e.g. as a consequence of sphingomyelin breakdown by sphingomyelinases or via de novo synthesis, can induce ROS generation in mitochondria [13, 14]. In this respect, we observed that the antifungal action of RsAFP2 does not require de novo ceramide generation, since the de novo ceramide synthesis inhibitor myriocin (ranging from 0.1 to 10 μg/ml) does not antagonize RsAFP2 antifungal action (results not shown). Whether RsAFP2 induces breakdown of GlcCer after its initial interaction, resulting in elevated ceramide levels, needs to be investigated further. However, in contrast to mammalian cells, enzymes involved in breakdown of GlcCer to ceramide have not yet been identified in yeast.

ROS induction capacity of various antifungals has been previously reported. Miconazole [16, 17], belonging

to the azole antifungals and inhibiting ergosterol biosynthesis, as well as the polyenes amphotericin B [18], nystatin [16] and niphimycin [19], which interact with ergosterol in the fungal membrane and cause membrane permeabilization, induce ROS in susceptible fungi. In addition, the benzo[a]naphthacenequinone antibiotic pradimicin A [20], natural perylenequinonoid pigments [21], the isoprenoid alcohol farnesol [22] and several antifungal peptides/proteins have been demonstrated to induce ROS in yeast species. These ROS-inducing antifungal peptides/proteins include the antifungal tobacco defense protein osmotin [23], the antifungal protein PAF produced by *Penicillium chrysogenum* [24] and lactoferrin, a cationic amphipathic peptide that is part of the innate immunity [25].

Yeast cells undergoing apoptosis or programmed cell death display several characteristic markers, including the induction of endogenous ROS [26]. Some of the above-described ROS-inducing antifungals, such as amphotericin B [18], pradimicin A [20], osmotin [23] and PAF [24], have been shown to induce apoptosis in yeast cells. Whether the RsAFP2-induced ROS are intracellular messengers stimulating the proapoptotic regulatory machinery in yeast needs to be investigated further.

In conclusion, we show here for the first time that the antifungal activity of a plant defensin, in casu RsAFP2, is causally linked with an intracellular process in susceptible yeast species, being the induction of endogenous ROS. It seems that RsAFP2 exerts its antifungal action through activation of a plant defensin-induced signaling cascade, involving ROS production, resulting in membrane permeabilization, rather than inserting itself in the fungal plasma membrane. It is currently not clear whether RsAFP2 (i) is internalized upon its interaction with GlcCer and affects an intracellular target, resulting in ROS induction, or (ii) is not internalized but induces ROS through activation of a signaling cascade starting from its GlcCer interaction. In addition, whether the RsAFP2-induced ROS production leads to apoptotic cell death in the fungal pathogen *C. albicans* needs to be investigated further.

Experimental Procedures

Materials and Microorganisms

RsAFP2 was isolated as described previously [3]. The yeast strain used in this study is *C. albicans* strain SC5314 CAI4 [27]. GlcCer was purified from *Pichia pastoris* plasma membranes as described previously [4]. All chemicals were purchased from Sigma (St. Louis, Mo., USA).

CF Leakage Assay

CF-encapsulated SUVs composed of egg PC, PA, PS, cholesterol and GlcCer were prepared by the method of sonication as described previously [28]. After sonication, the liposomes were purified on Sephadex G-50 gel filtration chromatography column to eliminate the unencapsulated fluorescence dye. Samples were applied to the column and eluted with a CF-free buffer (150 mM NaCl, 10 mM Tris-HCl, 0.01% NaN_3, pH 7.4). Fractions (1 ml) were collected and those containing liposomes were pooled to a final volume of 3 ml. The time-course leakage of CF from SUVs was monitored with a PerkinElmer LS-50B spectrofluorometer. The excitation and emission wavelengths were at 492 and 520 nm, respectively.

ROS Induction Assay

Fluorimetric measurement of endogenous ROS in *C. albicans* using 2′,7′-dichlorofluorescin diacetate (DCFHDA; Molecular Probes, Inc., Eugene, Oreg., USA) was performed as described previously [16]. Briefly, logarithmically growing *C. albicans* cells were treated with various concentrations of RsAFP2 (ranging from 10 to 100 μg/ml) or water in the presence or absence of the antioxidant AA (ranging from 5 to 100 mM) in PDB/YPD (18 g/l potato dextrose broth, Difco; yeast extract 2 g/l, peptone 4 g/l; glucose 4 g/l, pH 7.0 [4]) for 3 h at 30°C, whereafter cells were washed with PBS and DCFHDA was added (final concentration of 10 μM in PBS). After 1.5 h incubation at 30°C, fluorescence of the cells was determined using PerkinElmer LS-50B fluorescence spectrometer. Fluorescence values of the samples were corrected by subtracting the fluorescence value of RsAFP2 ± antioxidant in the corresponding concentration without cells but with DCFHDA. Additionally, determination of the percentage ROS-positive yeast cells within a RsAFP2 ± antioxidant- or water-treated yeast culture was performed microscopically (about 500 cells for each treatment) using an Optiphot-2 fluorescence microscope (Nikon).

Antifungal Activity Assay

Logarithmically growing *C. albicans* culture was treated with 100 μg/ml RsAFP2 in the presence or absence of various concentrations of the antioxidant AA (ranging from 5 to 100 mM) in PDB/YPD, pH 7.0. After 0 and 3 h of incubation at 30°C, 100-μl aliquots were plated on YPD plates and colony-forming units (CFUs) were counted after 2 days of incubation at 30°C. Percentage survival was calculated as the ratio of the number of CFUs after treatment with the compound(s) as compared to the initial inoculum.

Acknowledgements

Purified glucosylceramide from membranes of *Pichia pastoris* were kindly provided by Dr. Warnecke (University of Hamburg, Germany). This work was supported by a grant from FWO-Vlaanderen to B.P.A.C., and a travel grant from FWO-Vlaanderen to K.T. Predoctoral fellowship to A.M.A. from IWT-Vlaanderen and postdoctoral fellowship to K.T. from K.U. Leuven (IOF) are gratefully acknowledged.

References

1 Lay FT, Anderson MA: Defensins, components of the innate immune system in plants. Curr Prot Pept Sci 2005;6:85–101.

2 Thevissen K, Francois IE, Aerts AM, Cammue BP: Fungal sphingolipids as targets for the development of selective antifungal therapeutics. Curr Drug Targets 2005;6:923–928.

3 Terras FR, Schoofs HM, De Bolle MF, Van Leuven F, Rees SB, Vanderleyden J, Cammue BP, Broekaert WF: Analysis of two novel classes of plant antifungal proteins from radish (*Raphanus sativus* L.) seeds. J Biol Chem 1992;267:15301–15309.

4 Thevissen K, Warnecke DC, François IE, Leipelt M, Heinz E, Ott C, Zahringer U, Thomma BP, Ferket KK, Cammue BP: Defensins from insects and plants interact with fungal glucosylceramides. J Biol Chem 2004; 279:3900–3905.

5 Thevissen K, Ghazi A, De Samblanx GW, Brownlee C, Osborn RW, Broekaert WF: Fungal membrane responses induced by plant defensins and thionins. J Biol Chem 1996;271:15018–15025.

6 Thevissen K, Terras FR, Broekaert WF: Permeabilization of fungal membranes by plant defensins inhibits fungal growth. Appl Environ Microbiol 1999;65:5451–5458.

7 Leipelt M, Warnecke D, Zahringer U, Ott C, Muller F, Hube B, Heinz E: Glucosylceramide synthases, a gene family responsible for the biosynthesis of glucosphingolipids in animals, plants, and fungi. J Biol Chem 2001; 276:33621–33629.

8 Park C, Bennion B, Francois IE, Ferket KK, Cammue BP, Thevissen K, Levery SB: Neutral glycolipids of the filamentous fungus *Neurospora crassa:* altered expression in plant defensin-resistant mutants. J Lipid Res 2005;46:759–768.

9 Ferket KK, Levery SB, Park C, Cammue BP, Thevissen K: Isolation and characterization of *Neurospora crassa* mutants resistant to antifungal plant defensins. Fungal Genet Biol 2003;40:176–185.

10 Sakaki T, Zahringer U, Warnecke DC, Fahl A, Knogge W, Heinz E: Sterol glycosides and cerebrosides accumulate in *Pichia pastoris*, *Rhynchosporium secalis* and other fungi under normal conditions or under heat shock and ethanol stress. Yeast 2001;18:679–695.

11 Valcarcel CA, Dalla Serra M, Potrich C, Bernhart I, Tejuca M, Martinez D, Pazos F, Lanio ME, Menestrina G: Effects of lipid composition on membrane permeabilization by sticholysin I and II, two cytolysins of the sea anemone *Stichodactyla helianthus*. Biophys J 2001;80:2761–2774.

12 Zhao H, Mattila JP, Holopainen JM, Kinnunen PK: Comparison of the membrane association of two antimicrobial peptides, magainin 2 and indolicidin. Biophys J 2001; 81:2979–2991.

13 Therade-Matharan S, Laemmel E, Carpentier S, Obata Y, Levade T, Duranteau J, Vicaut E: Reactive oxygen species production by mitochondria in endothelial cells exposed to reoxygenation after hypoxia and glucose depletion is mediated by ceramide. Am J Physiol 2005;289:R1756–R1762.

14 Moussavi M, Assi K, Gomez-Munoz A, Salh B: Curcumin mediates ceramide generation via the de novo pathway in colon cancer cells. Carcinogenesis 2006;27:1636–1644.

15 Ames BN, Shigenaga MK, Hagen TM: Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci USA 1993;90:7915–7922.

16 François IEJA, Cammue BPA, Borgers M, Ausma J, Dispersyn GD, Thevissen K: Azoles: mode of antifungal action and resistance development. Effect of miconazole on endogenous reactive oxygen species production in *Candida albicans*. Anti-Infect Agents Med Chem 2006;5:3–13.

17 Kobayashi D, Kondo K, Uehara N, Otokozawa S, Tsuji N, Yagihashi A, Watanabe N: Endogenous reactive oxygen species is an important mediator of miconazole antifungal effect. Antimicrob Agents Chemother 2002;46:3113–3117.

18 Phillips AJ, Sudbery I, Ramsdale M: Apoptosis induced by environmental stresses and amphotericin B in *Candida albicans*. Proc Natl Acad Sci USA 2003;100:14327–14332.

19 Nakayama K, Yamaguchi T, Doi T, Usuki Y, Taniguchi M, Tanaka T: Synergistic combination of direct plasma membrane damage and oxidative stress as a cause of antifungal activity of polyol macrolide antibiotic niphimycin. J Biosci Bioeng 2002;94:207–211.

20 Hiramoto F, Nomura N, Furumai T, Oki T, Igarashi Y: Apoptosis-like cell death of *Saccharomyces cerevisiae* induced by a mannose-binding antifungal antibiotic, pradimicin. J Antibiot (Tokyo) 2003;56:768–772.

21 Xing MZ, Zhang XZ, Sun ZL, Zhang HY: Perylenequinones act as broad-spectrum fungicides by generating reactive oxygen species both in the dark and in the light. J Agric Food Chem 2003;51:7722–7724.

22 Machida K, Tanaka T, Fujita K, Taniguchi M: Farnesol-induced generation of reactive oxygen species via indirect inhibition of the mitochondrial electron transport chain in the yeast *Saccharomyces cerevisiae*. J Bacteriol 1998;180:4460–4465.

23 Narasimhan ML, Damsz B, Coca MA, Ibeas JI, Yun DJ, Pardo JM, Hasegawa PM, Bressan RA: A plant defense response effector induces microbial apoptosis. Mol Cell 2001;8:921–930.

24 Leiter E, Szappanos H, Oberparleiter C, Kaiserer L, Csernoch L, Pusztahelyi T, Emri T, Pocsi I, Salvenmoser W, Marx F: Antifungal protein PAF severely affects the integrity of the plasma membrane of *Aspergillus nidulans* and induces an apoptosis-like phenotype. Antimicrob Agents Chemother 2005; 49:2445–2453.

25 Vorland LH: Lactoferrin: a multifunctional glycoprotein. APMIS 1999;107:971–981.

26 Madeo F, Fröhlich E, Ligr M, Grey M, Sigrist SJ, Wolf DH, Fröhlich K-U: Oxygen stress: a regulator for apoptosis in yeast. J Cell Biol 1999;145:757–767.

27 Fonzi WA, Irwin MY: Isogenic strain construction and gene mapping in *Candida albicans*. Genetics 1993;134:717–728.

28 Thulborn KR, Sawyer WH: Properties and the locations of a set of fluorescent probes sensitive to the fluidity gradient of the lipid bilayer. Biochim Biophys Acta 1978;511:125–140.

Research Article

Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol 2007;13:248–254
DOI: 10.1159/000104754

Nisin Biosynthesis in vitro

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Key Words
Nisin · Rapid Translation System

Abstract
The lantibiotic nisin is produced by *Lactococcus lactis*. In the biosynthesis of nisin, the enzyme NisB dehydrates nisin precursor, and the enzyme NisC is needed for lanthionine formation. In this study, the *nisA* gene encoding the nisin precursor, and the genes *nisB* and *nisC* of the lantibiotic modification machinery were expressed together in vitro by the Rapid Translation System (RTS). Analysis of the RTS mixture showed that fully modified nisin precursor was formed. By treating the mixture with trypsin, active nisin was obtained. However, no nisin could be detected in the mixture without zinc supplementation, explained by the fact that NisC requires zinc for its function. The results revealed that the modification of nisin precursor, which is supposed to occur at the inner side of the membrane by an enzyme complex consisting of NisB, NisC, and the transporter NisT, can take place without membrane association and without NisT. This in vitro production system for nisin opens up the possibility to produce nisin variants that cannot be produced in vivo. Moreover, the system is a promising tool for utilizing the NisB and NisC enzymes for incorporation of thioether rings into medical peptides and hormones for increased stability.
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Introduction

Lantibiotics are a unique class of antimicrobial peptides produced by bacteria [1]. They are post-translationally modified and contain unusual amino acids such as dehydroalanine, dehydrobutyrine, lanthionine and β-methyllanthionine [2]. The most studied member of lantibiotics is nisin, produced by some *Lactococcus lactis* and *Streptococcus uberis* strains [3]. Nisin kills Gram-positive bacteria by binding to the membrane-bound cell wall precursor lipid II, leading to inhibition of peptidoglycan synthesis and formation of a membrane pore [4]. Nisin is the only bacteriocin allowed as a preservative in food products (E234) [5].

The 11 genes needed for the nisin biosynthesis, regulation, and immunity are located in a conjugative transposon in the chromosome of *L. lactis* [2]. After ribosomal translation, nisin precursor is modified at the inner side of the membrane by an intracellular membrane-associated enzyme complex consisting of NisB, NisC, and NisT (fig. 1) [2]. The enzyme NisB dehydrates serines and threonines, NisC is responsible for lanthionine ring formation by coupling the dehydrated residues with cysteines, and the transporter NisT excretes the fully modified nisin out of the cell. Besides nisin, the NisBTC complex is able to dehydrate and secrete non-lantibiotic peptides as nisin leader fusions [7]. Thus, the presence of the leader sequence is essential for targeting peptides to the lantibi-

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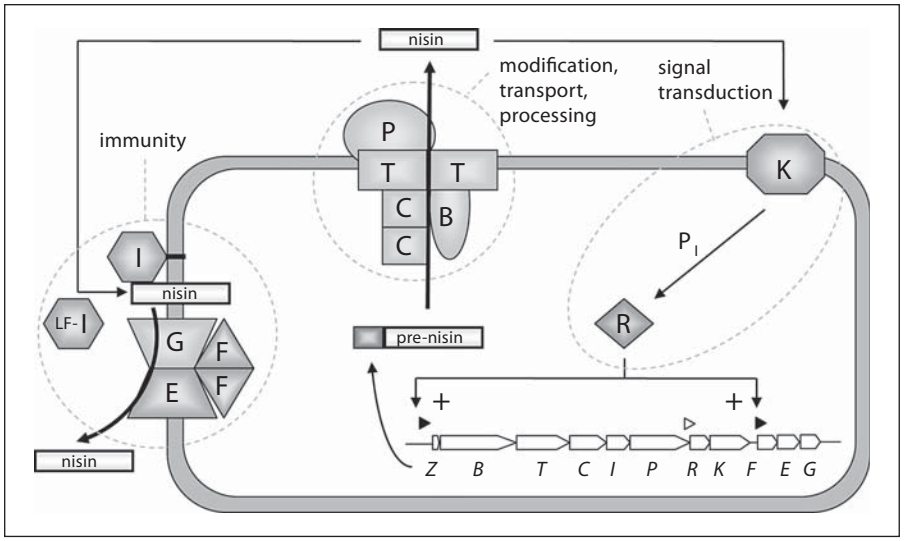

Fig. 1. A model for nisin Z biosynthesis, regulation, and immunity in *L. lactis*. *Biosynthesis:* After ribosomal synthesis of the nisin structural gene *nisZ*, the pre-pronisin is modified at the inner side of the membrane by the dehydratase NisB and the cyclase dimer NisC, and translocated through the membrane by the transporter dimer NisT. The protease NisP removes the leader from pre-nisin, and releases active nisin. *Regulation:* Nisin binds to the sensor histidine kinase NisK. Autophosphorylation of NisK leads to phosphorylation (P_I) of the regulator NisR. Phosphorylated NisR activates nisin-inducible promoters of *nisZBTCIPRK* and *nisFEG*-operons. *Immunity:* The immunity protein NisI binds nisin. NisI exists in a membrane-anchored, and in a secreted lipid-free form (LF-I). The transporter complex NisFEG exports cell-associated nisin to the external environment. Filled and open triangles indicate nisin-inducible and constitutive promoters, respectively [modified from 6].

otic enzymes. In the last step of nisin biosynthesis, the leader peptide is cleaved by the cell-wall-anchored serine protease NisP, releasing active nisin. However, NisP is not coupled with the NisBTC complex, as it can be functionally expressed in *L. lactis* without other lantibiotic enzymes [8].

The LanB dehydratases, e.g. NisB and EpiB, are mostly hydrophilic, but they also contain hydrophobic regions suggesting membrane-associated segments [9, 10]. The involvement of NisB in the dehydration of nisin was shown by overexpressing *nisB* gene in engineered nisin producing lactococci [11]. Further experimental evidence of the dehydration by NisB in vivo was provided by expressing His-tagged nisin precursor in a NisB mutant strain, resulting in the accumulation of the unmodified precursor [12]. However, despite attempts with EpiB and SpaB, there is still no in vitro verification of the activity of LanB proteins [10, 13].

The LanC enzymes bind the dehydrated prepeptide and stabilize a conformation that enables the thiol groups of cysteines to react with the dehydrated amino acids. NisC and SpaC require zinc for their function [14]. The activity of NisC was shown with a *nisC* mutant strain, which dehydrated nisin precursor but was unable to form lanthionine rings and did not produce antimicrobially active nisin [12]. Recently, the in vitro activity of NisC was demonstrated by using dehydrated prenisin, produced in *L. lactis*, as substrate [15].

The active lantibiotic lacticin 481 has been produced by using purified lantibiotic synthetase LctM, an enzyme which combines the activities of LanB and LanC [16]. The His-tagged LctA substrate was expressed in *Escherichia coli* followed by the post-translational modifications executed in vitro by LctM.

To date, no experimental evidence for in vitro dehydration activity by any LanB protein has been reported. Also, the in vitro productions of the two lantibiotics, nisin and lacticin 481, have used in vivo synthesized prepeptides for their substrates. Applying a lantibiotic modification system in vitro would make it possible to produce lantibiotic variants, which cannot be produced in living cells, and even to design novel modified peptides displaying unique properties.

In this study, we produced fully modified prenisin in the in vitro transcription and translation system using *nisA*, *nisB*, and *nisC* genes as templates. Active nisin was obtained by cleaving the leader peptide with trypsin treatment.

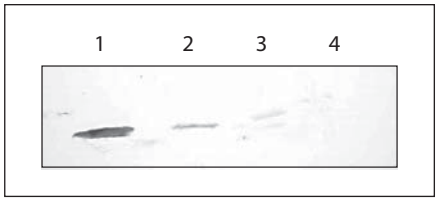

Fig. 2. Western blot analysis of RTS mixtures with nisin A antibody. Lanes: 1, 0.5 μg nisin; 2, RTS reaction mixture treated with trypsin; 3, RTS mixture without trypsin treatment; 4, RTS mixture without *nis* gene templates.

Fig. 3. Antimicrobial activity of the RTS reaction mixture. RTS mixtures and nisin controls were spotted onto the *M. luteus* plate. Spots: 1–3, nisin controls of 0.5, 1.25 and 2.5 μg/ml; 4, RTS mixture with *nisABC* templates treated with trypsin; 5, RTS mixture with *nisABC* templates but without trypsin treatment; 6, RTS mixture without *nisABC* templates but with trypsin treatment.

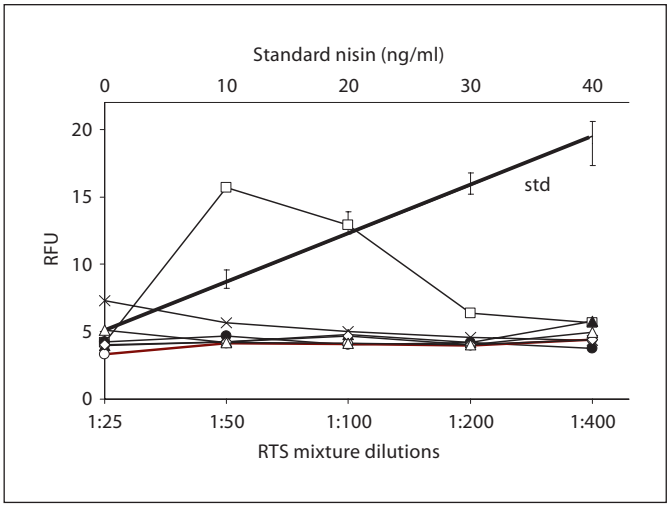

Fig. 4. Detection of nisin from RTS reaction mixtures with nisin-inducible GFP bioassay. Standard (std) curve for nisin-induced fluorescence of *L. lactis* LAC240 cells. The concentrations for the nisin standard curve are 0, 10, 20, 30, and 40 ng/ml. The data shown are the mean ± SD (error bars) from four parallel experiments. The R^2 for the curve was 0.9898. The RTS samples were diluted from 1:25 to 1:400. According to the standard curve, the concentration of nisin in trypsin-treated reaction mixture containing zinc and the *nisABC* templates was approximately 2 μg/ml (estimated from dilution 1:100). The nisin concentration of trypsin-treated reaction mixture containing zinc and the *nisABC* templates in dilutions 1:25 and 1:50 inhibited the growth of LAC240 and cannot be used for estimation of nisin concentration. The concentrations of the other reaction mixtures were below the detection limit (10 ng/ml). □ = *nisABC* +zinc +trypsin; ▲ = *nisABC* +zinc –trypsin; × = *nisABC* –zinc +trypsin; ● = *nisABC* –zinc –trypsin; ○ = *nisAC* +zinc +trypsin; ◇ = *nisAB* +zinc +trypsin; △ = no templates +zinc +trypsin.

Results

Construction of nisABC *Gene Expression Templates*

The primary objective of this study was to test if the fully modified nisin precursor containing dehydrated residues and lanthionine rings could be produced in vitro by applying genes *nisA*, *nisB*, and *nisC* from the nisin operon, into the *E. coli* in vitro Rapid Translation System (RTS). Since the cloning of the *nisB* and *nisC* genes into a plasmid vector caused instability problems (data not shown), the genes were applied to the RTS as linear DNA. The gene templates were constructed with RTS *E. coli* Linear Template Generation Set. The genes *nisA*, *nisB*, and *nisC* were amplified from plasmid pNZ9111 by PCR, using gene-specific primers containing overlapping sequences to the T7 regulatory region. The T7 promoter and terminator were added to the nisin genes by a second PCR. The resulting linear expression constructs of *nisA*, *nisB*, and *nisC* were mixed with the *E. coli* in vitro protein expression mixture and incubated for 6 h in RTS. Since NisC requires zinc for its activity, the RTS reaction mixture was supplemented with 1 μM zinc sulfate.

Detection of Nisin by Immunoblotting

To investigate if the RTS reaction produced the fully modified nisin precursor, the reaction mixture was analyzed by Western blotting using nisin A-specific antibody. Prior to the Western analysis, the RTS reaction mixture was treated with trypsin to cut the leader peptide from the presumed nisin precursor releasing active nisin. The nisin A antibody recognized a protein of the same size as nisin in the trypsin-treated RTS reaction mixture

(fig. 2). In addition, a weaker band with a slightly larger mass than nisin was detected in non-trypsin-treated RTS mixture, suggesting the presence of fully modified nisin with the leader still attached.

Antimicrobial Activity of the RTS Reaction Mixture

The next objective was to test if the produced putative nisin recognized by anti-nisin A in the RTS reaction mixture would have antimicrobial activity. The RTS reaction mixture was treated with trypsin to cut the leader peptide. Then the antimicrobial activity was determined by agar diffusion using *Micrococcus luteus* as the indicator bacteria. The trypsin-treated RTS reaction mixture produced a large inhibition zone against *M. luteus* (fig. 3). Reaction mixture without trypsin treatment or the RTS mixture without the *nis* gene templates did not inhibit *M. luteus*. According to the control spots, the concentration of the putative nisin in the RTS reaction mixture was approximately 5 μg/ml. In conclusion, it was made evident that the in vitro produced presumed nisin was antimicrobially active.

Confirmation of Nisin by Nisin-Inducible GFP Bioassay

To conclude that the antimicrobial substance in the RTS mixture was actually fully modified nisin, the mixture was analyzed by nisin-inducible bioassay. Again, prior to the bioassay, the mixture was treated with trypsin to liberate active nisin. After trypsin treatment, the reaction mixture containing zinc sulfate, and the templates *nisA*, *nisB*, and *nisC*, induced the nisin-inducible *nisF* promoter in the LAC240 indicator strain, resulting in GFP fluorescence (fig. 4). According to the standard curve, the nisin concentration in the mixture was approximately 2 μg/ml. Nisin could not be detected in the reaction mixture without template *nisB* or *nisC*, demonstrating that both enzymes are required for complete nisin modification. As expected, the mixture without trypsin treatment could not induce the GFP expression. Interestingly, the reaction mixture without zinc sulfate supplementation showed no nisin inducibility verifying the central role of zinc in nisin biosynthesis. In summary, we conclude that fully modified nisin precursor was produced in vitro via the coupled transcription/translation of nisin structural gene *nisA*, followed by dehydration by NisB and lanthionine ring formation by NisC.

Discussion

In this study, fully modified nisin precursor was produced in the in vitro RTS by using gene expression templates *nisA*, *nisB*, and *nisC*. Antimicrobially active nisin was obtained from the RTS mixture by trypsin treatment. The results revealed that all modifications of nisin precursor can take place in vitro without membrane association and the transporter protein NisT.

First, we tested the RTS reaction mixture with Western analysis using nisin A-specific antibody. A protein of same size as commercial nisin control was recognized, indicating that the mixture probably contained modified nisin. The mixture was further characterized for its antimicrobial activity against *M. luteus*. Previous studies have revealed that non-modified or incorrectly modified nisin has no or strongly reduced antimicrobial activity [15, 17], providing more evidence that the antimicrobially active agent in this mixture would be correctly modified nisin. Finally, this antimicrobial substance was shown to activate the nisin-inducible *nisF* promoter in the GFP fluorescence-based nisin bioassay. Since the nisin-inducible gene regulation by signal transduction is specific only to correctly modified nisin [18, 19], we conclude that the RTS reaction mixture contained fully modified prenisin. This could be achieved only when active NisB and NisC had modified nisin precursor translated from *nisA* template. From fully modified prenisin, active nisin was released by trypsin treatment.

Thus far, the attempts for testing the dehydratase activity of purified LanB proteins in vitro have been unsuccessful [10, 13]. The epidermin dehydratase EpiB was successfully purified from *Staphylococcus carnosus*, but it could not dehydrate purified epidermin precursor [10]. The subtilin dehydratase SpaB from *Bacillus subtilis* was overexpressed in *E. coli*, and it was shown to interact with SpaC [13]. However, no dehydratase activity was displayed for SpaB. Thus, the prenisin modification by NisB shown here in our study is the first experimental evidence of LanB activity in vitro. LanB proteins are generally regarded as membrane-associating proteins. However, according to hydrophobicity studies, LanB proteins are rather hydrophilic and contain no trans-membrane domains [2]. To produce membrane proteins in RTS, a detergent, such as Tween 20 or CHAPS, is needed in the reaction mixture [20]. Here, we did not use any detergent in the RTS reaction, suggesting that NisBC can function also without membrane interaction.

The LanC enzymes bind the dehydrated prepeptides and link the cysteines with dehydrated amino acids [2].

Moreover, NisC and SpaC have been shown to be zinc metalloproteins [14]. Recently, by using dehydrated prenisin as a substrate, the in vitro activity of NisC was demonstrated [15]. The crystal structure of NisC revealed that a single zinc ion is situated at the top face of the α, α barrel in NisC. The zinc ion may activate the cysteine thiol of the peptide substrate towards intramolecular Michael addition to the dehydrated residues [14]. Here, when the RTS reaction lacked zinc, the *nisF* promoter in the GFP nisin bioassay could not be induced, explained by deficiency of NisC activity, thus confirming the indispensable role of zinc in nisin biosynthesis. In addition, the necessity of zinc for the formation of active nisin by RTS emphasizes the involvement of NisC activity in RTS reaction.

Previously, lantibiotics have been synthesized in vitro only by using in vivo produced prepeptides as substrates [15, 16]. In this study, we used the in vitro synthesized nisin propeptide to demonstrate NisB and NisC modification activity. In fact, this paper presents the first example of the complete in vitro biosynthesis of a lantibiotic.

LanT proteins are not necessary for all lantibiotic systems. For example, Pep5 and epicidin 280 do not require LanT for their extracellular transport [21, 22]. In these cases, host cell transporters probably complement the specific transporter deficiency. In nisin biosynthesis, although NisT forms a membrane-associated complex with NisBC [2], it is not crucial for processing nisin [23, 24], as also confirmed in this study.

In vivo genetic engineering studies aimed to improve lantibiotic properties have focused on site-directed mutagenesis of the precursor [25–28]. These investigations have had their limitations, including loss of lantibiotic production, degradation of the product or intermediate, breakdown of immunity system, or perturbation of signal pathways in cases where the bacteriocin acts as a quorum sensor controlling its own expression [18, 29, 30]. In vitro engineering of the lantibiotic biosynthetic processes can overcome these disadvantages. First, the structures of the prepeptides are not limited by the amino acids of natural proteins. Second, regulatory properties, cytotoxicity, or degradation of the products are not problems as in in vivo approaches. Third, it is possible to use non-peptide structures to produce even more stable molecules. The in vitro production system for nisin presented here opens up the possibility to produce nisin variants that cannot be produced in living cells, due to incompatibility of the transport, regulation, or the immunity systems.

The potential of in vitro use of lantibiotic modifying enzymes is not limited to lantibiotics production. These enzymes may also find applications in adding dehydro amino acids and lanthionine rings into other targets for increased chemical and proteolytic stability [31]. Indeed, when fused to the nisin leader, non-lantibiotic peptides could be dehydrated by NisB [8, 32]. The findings presented in this paper could be exploited to utilize the NisB and NisC enzymes for incorporation of the dehydrated and cyclic amino acids into medical peptides and hormones for increased stability, making in vitro engineering of dehydrated amino acids and lanthionine rings for biotechnological and medical applications possible.

Experimental Procedures

Bacterial Strains and Growth Conditions

L. lactis MG1614 (pNZ9111) [33] was used as the source of *nisABC* genes. *L. lactis* LAC240 [34] was used as the indicator strain in nisin detection by nisin-inducible GFP fluorescence. Lactococci were grown at 30°C in M17 medium (Oxoid) containing 0.5% (w/v) glucose and 5 μg/ml erythromycin. *M. luteus* A1 NCIMB86166 (National Collection of Industrial and Marine Bacteria) was used as the indicator bacteria in agar diffusion bioassay of nisin. *M. luteus* was cultivated in LB medium at 37°C.

Production of Nisin Precursor Expression Templates by PCR

The gene templates were constructed by two sequential PCRs with RTS *E. coli* Linear Template Generation Set (Roche Applied Science). In the first PCR, the genes *nisA*, *nisB*, and *nisC* were amplified directly from colonies of *L. lactis* MG1614 (pNZ9111) using gene-specific primers containing overlapping sequences to the T7 regulatory regions. The PCR cycles for *nisB* and *nisC* templates were as follows: 95°C 5 min; 30 cycles consisting of 94°C 1 min, 50°C 1 min, 72°C 3 min; final 10 min extension at 72°C. The PCR program for *nisA* template was the same, except the polymerization step at 72°C was 1 min. The primers for amplifications were: *nisA*-forward (5′-CTTTAAGAAGGAGATATACCATGAGTACAAAAGATTTTAAC-3′), *nisA*-reverse (5′-TGATGATGAGAACCCCCCCTTATTTGCTTACGTGAATAC-3′), *nisB*-forward (5′-CTTTAAGAAGGAGATATACCATGATAAAAAGTTCATTTAAAGCTCAA-3′), *nisB*-reverse (5′-TGATGATGAGAACCCCCCCCTTATTTCATGTATTCTTCCGAAACAAA-3′), *nisC*-forward (5′-CTTTAAGAAGGAGATATACCATGAATAAAAAAAATATAAAAAGAAATGTT-3′), and *nisC*-reverse (5′-TGATGATGAGAACCCCCCCCTTATTTCCTCTTCCCTCCTT-3′). Nis-specific regions of the primers are shown underlined. Each 50-μl PCR mixture contained: 1 h Pfx buffer (Invitrogen), 1 mM $MgCl_2$, 400 μM dNTP mixture (Promega), 0.5 μM of each primer, and 0.5 U Platinum Pfx DNA polymerase (Invitrogen). The PCR products were analyzed in 0.8% agarose gel.

In the second overlap extension PCR, the T7 promoter and terminator were added to the nisin genes. The products of the first PCR were annealed with the T7 regulatory elements, the 3′ ends

were extended, and the final expression constructs were amplified with the flanking primers. Each 50-μl PCR mixture contained: 2 μl of the first PCR products, 480 nM T7 promoter primer, 480 nM T7 terminator primer, the DNA fragments coding for the T7 regulatory elements, 1 Pfx buffer, 1 mM $MgCl_2$, 400 μM dNTP mixture, and 0.5 U Platinum Pfx DNA polymerase. The PCR cycles for *nisA* and *nisC* templates were as follows: 95°C 5 min; 25 cycles consisting of 94°C 1 min, 60°C 1 min, 72°C 2 min, final 10-min extension at 72°C. The PCR program for *nisB* template was the same, except the polymerization step at 72°C was 4 min. The PCR products were analyzed in 0.8% agarose gel.

Rapid Translation System

After purification with QIAquick PCR Purification Kit (Qiagen), the linear expression constructs of *nisA*, *nisB*, and *nisC* were mixed with the *E. coli* in vitro protein expression mixture supplemented with 1 μM zinc sulfate, using RTS 100 or RTS 500 *E. coli* HY kit (Roche Applied Science). The reaction was performed at 30°C for 6 h in RTS with a stirring speed of 120 rpm. Prior to nisin detection bioassays, the RTS reaction mixture was treated with 0.5 mg/ml trypsin (Sigma-Aldrich) for 1 h at 37°C.

Western Analysis

The RTS reaction mixture was run in 4–20% SDS-polyacrylamide gradient gel (Mirador DNA Design), followed by transfer onto the Immobilin-P PVDF membrane (Millipore) using a Bio-Rad electroblot. The membrane was probed for 2 h with nisin A antibody [35], and the proteins were detected by colorimetric reaction with alkaline phosphatase-conjugated anti-mouse IgG and NBT/BCIP substrate (Promega).

Inhibition Bioassay by Agar Diffusion

The antimicrobial activity of the RTS reaction mixture was tested with agar diffusion assay using *M. luteus* as indicator bacteria on LB agar. 3-μl droplets of RTS mixture with and without trypsin treatment, RTS mixture without *nis* gene templates, and nisin controls (Sigma) were added onto the agar, and the plate was grown overnight at 37°C.

Nisin-Inducible GFP Bioassay

The presence of nisin in the RTS mixture was determined by using the nisin-inducible GFP bioassay [34]. The indicator strain *L. lactis* LAC240 is a derivative of MG1614 carrying the plasmid pLEB599. The plasmid pLEB599 provides constitutive expression of the nisin regulatory genes *nisRK*. The green fluorescent protein gene *gfp* is cloned into pLEB599 under the control of the nisin-inducible *nisF* promoter. The RTS mixtures were boiled for 5 min prior to GFP bioassay. GFP fluorescence from the nisin-induced cells was detected as relative fluorescence units with a Fluoroscan Ascent 374 fluorometer (Labsystems). The excitation and emission filters used were 485 and 538 nm.

Acknowledgements

This work was supported by the Academy of Finland, Project No. 177321, and by a grant from Centre for International Mobility (CIMO) in Finland to Fang Cheng.

References

1 Patton GC, van der Donk WA: New developments in lantibiotic biosynthesis and mode of action. Curr Opin Microbiol 2005;8:543–551.
2 Chatterjee C, Paul M, Xie L, van der Donk WA: Biosynthesis and mode of action of lantibiotics. Chem Rev 2005;105:633–683.
3 Wirawan RE, Klesse NA, Jack RW, Tagg JR: Molecular and genetic characterization of a novel nisin variant produced by *Streptococcus uberis*. Appl Environ Microbiol 2006;72:1148–1156.
4 Bauer R, Dicks LM: Mode of action of lipid II-targeting lantibiotics. Int J Food Microbiol 2005;101:201–216.
5 Thomas LV, Clarkson MR, Delves-Broughton J: Nisin; in Naidu AS (ed): Natural Food Antimicrobial Systems. Boca Raton, CRC Press, 2000, pp 463–524.
6 Quadri LEN: Regulation of antimicrobial peptide production by autoinducer-mediated quorum sensing in lactic acid bacteria. Antonie van Leeuwenhoek 2002;82:133–145.
7 Kluskens LD, Kuipers A, Rink R, de Boef E, Fekken S, Driessen AJ, Kuipers OP, Moll GN: Post-translational modification of therapeutic peptides by NisB, the dehydratase of the lantibiotic nisin. Biochemistry 2005;44:12827–12834.
8 Kuipers A, de Boef E, Rink R, Fekken S, Kluskens LD, Driessen AJ, Leenhouts K, Kuipers OP, Moll GN: NisT: the transporter of the lantibiotic nisin, can transport fully modified, dehydrated, and unmodified prenisin and fusions of the leader peptide with non-lantibiotic peptides. J Biol Chem 2004;279:22176–22182.
9 Engelke G, Gutowski-Eckel Z, Hammelmann M, Entian KD: Biosynthesis of the lantibiotic nisin: genomic organization and membrane localization of the NisB protein. Appl Environ Microbiol 1992;58:3730–3743.
10 Peschel A, Ottenwalder B, Götz F: Inducible production and cellular location of the epidermin biosynthetic enzyme EpiB using an improved staphylococcal expression system. FEMS Microbiol Lett 1996;137:279–284.
11 Karakas Sen A, Narbad A, Horn N, Dodd HM, Parr AJ, Colquhoun I, Gasson MJ: Post-translational modification of nisin. The involvement of NisB in the dehydration process. Eur J Biochem 1999;261:524–532.
12 Koponen O, Tolonen M, Qiao M, Wahlström G, Helin J, Saris PEJ: NisB is required for the dehydration and NisC for the lanthionine formation in the post-translational modification of nisin. Microbiology 2002;148:3561–3568.
13 Xie L, Chatterjee C, Balsara R, Okeley NM, van der Donk WA: Heterologous expression and purification of SpaB involved in subtilin biosynthesis. Biochem Biophys Res Commun 2002;295:952–957.
14 Okeley NM, Paul M, Stasser JP, Blackburn N, van der Donk WA: SpaC and NisC, the cyclases involved in subtilin and nisin biosynthesis, are zinc proteins. Biochemistry 2003;42:13613–13624.
15 Li B, Yu JP, Brunzelle JS, Moll GN, van der Donk WA, Nair SK: Structure and mechanism of the lantibiotic cyclase involved in nisin biosynthesis. Science 2006;311:1464–1467.

16 Xie L, Miller LM, Chatterjee C, Averin O, Kelleher NL, van der Donk WA: Lacticin 481: in vitro reconstitution of lantibiotic synthetase activity. Science 2004;303:679–681.
17 Van Kraaij C, Breukink E, Rollema HS, Bongers RS, Kosters HA, de Kruijff B, Kuipers OP: Engineering a disulfide bond and free thiols in the lantibiotic nisin Z. Eur J Biochem 2000;267:901–909.
18 Kuipers OP, Beerthuyzen MM, de Ruyter PG, Luesink EJ, de Vos WM: Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction. J Biol Chem 1995;270:27299–27304.
19 Qiao M, Ye S, Koponen O, Ra R, Usabiaga M, Immonen T, Saris PEJ: Regulation of the nisin operons in *Lactococcus lactis* N8. J Appl Bacteriol 1996;80:626–634.
20 Betton JM: Rapid Translation System: a promising alternative for recombinant protein production. Curr Protein Pept Sci 2003;4:73–80.
21 Meyer C, Bierbaum G, Heidrich C, Reis M, Süling J, Iglesias-Wind MI, Kempter C, Molitor E, Sahl HG: Nucleotide sequence of the lantibiotic Pep5 biosynthetic gene cluster and functional analysis of PepP and PepC. Evidence for a role of PepC in thioether formation. Eur J Biochem 1995;232:478–489.
22 Peschel A, Schnell N, Hille M, Entian KD, Götz F: Secretion of the lantibiotics epidermin and gallidermin: sequence analysis of the genes *gdmT* and *gdmH*, their influence on epidermin production and their regulation by EpiQ. Mol Gen Genet 1997;254:312–318.
23 Qiao M, Saris PEJ: Evidence for a role of NisT in transport of the lantibiotic nisin produced by *Lactococcus lactis* N8. FEMS Microbiol Lett 1996;144:89–93.
24 Abbas Hilmi HT, Kylä-Nikkilä K, Ra R, Saris PEJ: Nisin induction without nisin secretion. Microbiology 2006;152:1489–1496.
25 Liu W, Hansen JN: Enhancement of the chemical and antimicrobial properties of subtilin by site-directed mutagenesis. J Biol Chem 1992;267:25078–25085.
26 Kuipers OP, Bierbaum G, Ottenwalder B, Dodd HM, Horn N, Metzger J, Kupke T, Gnau V, Bongers R, van den Bogaard P, Kosters H, Rollema HS, de Vos WM, Siezen RJ, Jung G, Götz F, Sahl HG, Gasson MJ: Protein engineering of lantibiotics. Antonie Van Leeuwenhoek 1996;69:161–169.
27 Chen P, Novak J, Kirk M, Barnes S, Qi F, Caufield PW: Structure-activity study of the lantibiotic mutacin II from *Streptococcus mutans* T8 by a gene replacement strategy. Appl Environ Microbiol 1998;64:2335–2340.
28 Szekat C, Jack RW, Skutlarek D, Farber H, Bierbaum G: Construction of an expression system for site-directed mutagenesis of the lantibiotic mersacidin. Appl Environ Microbiol 2003;69:3777–3783.
29 Van der Meer JR, Rollema HS, Siezen RJ, Beerthuyzen MM, Kuipers OP, de Vos WM: Influence of amino acid substitutions in the nisin leader peptide on biosynthesis and secretion of nisin by *Lactococcus lactis*. J Biol Chem 1994;269:3555–3562.
30 Bierbaum G, Reis M, Szekat C, Sahl HG: Construction of an expression system for engineering of the lantibiotic Pep5. Appl Environ Microbiol 1994;60:4332–4338.
31 Bierbaum G, Szekat C, Josten M, Heidrich C, Kempter C, Jung G, Sahl HG: Engineering of a novel thioether bridge and role of modified residues in the lantibiotic Pep5. Appl Environ Microbiol 1996;62:385–392.
32 Rink R, Kuipers A, de Boef E, Leenhouts KJ, Driessen AJ, Moll GN, Kuipers OP: Lantibiotic structures as guidelines for the design of peptides that can be modified by lantibiotic enzymes. Biochemistry 2005;44:8873–8882.
33 Van der Meer JR, Polman J, Beerthuyzen MM, Siezen RJ, Kuipers OP, de Vos WM: Characterization of the *Lactococcus lactis* nisin A operon genes *nisP*, encoding a subtilisin-like serine protease involved in precursor processing, and *nisR*, encoding a regulatory protein involved in nisin biosynthesis. J Bacteriol 1993;175:2578–2588.
34 Reunanen J, Saris PEJ: Microplate bioassay for nisin in foods based on nisin induced GFP-fluorescence. Appl Environ Microbiol 2003;69:4214–4218.
35 Suárez AM, Rodríguez JM, Hernández PE, Azcona-Olivera JI: Generation of polyclonal antibodies against nisin: immunization strategies and immunoassay development. Appl Environ Microbiol 1996;62:2117–2121.

Short Communication

Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol 2007;13:255–258
DOI: 10.1159/000104755

Anticytomegaloviral Activity of Esterified Milk Proteins and L-Polylysines

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Key Words
Cytomegalovirus · Esterification · Milk · Proteins · Peptic peptides · Polylysines · Antiviral activity

Abstract
MRC-5 fibroblasts infected with human cytomegalovirus (HCMV) reference strain AD 169 were treated with different concentrations of methylated α-lactalbumin (Met-ALA) or methylated β-lactoglobulin (Met-BLG), as well as with their peptic hydrolysates, and with the highly basic polypeptides such as are L-polylysines (4–15 kDa). The antiviral activity was calculated by comparing the number of infected cells in the presence and absence of the tested substances. Both Met-ALA and Met-BLG, as well as their peptic hydrolysates, decreased the infectious activity of cytomegalovirus in fibroblast cells. As expected, L-polylysines showed the highest antiviral activity. However, the tested basic proteins and polypeptides despite their lower antiviral activities might be potentially quite useful in fight of arising drug resistance activities and the persistence capacities of this virus.

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Human cytomegalovirus (HCMV) belonging to the Herpesviridae family is a serious life-threatening opportunistic pathogen in immunosuppressed or immunocompromised individuals but innocuous in immunocompetent persons. Although efficient antiviral drugs against HCMV infection exist, like ganciclovir [1], cidofovir [2] and foscarnet [3], they present poor bioavailability, induce multiple side effects, provoke dose-dependent toxicity, and induce easily the development of single and multiple drug resistances [4–6]. Several HCMV mutants resistant to antiviral agents were isolated from immunocompromised patients treated with antiviral drugs [7–9]. Hence, the need for new antiviral agents devoid of side effects still exists.

HCMV may be not only resistant to the traditional antiviral drugs but it can also display a life-long persistence in a healthy host in spite of host cellular and humoral antiviral immune responses [10]. Persistence is characterized by the presence of latent viral genomes, which periodically activate and produce infectious virus [11]. Persistence strategies may include novel patterns of gene expression and expression of viral gene products, which can modulate the immune responses of the host [12–15]. Hence, new anti-CMV agents are needed to counteract the persistence of this virus and of many others.

HCMV genome has a coding capacity of about 200 proteins, some of which are eliciting antiviral reactions of CD8 T cells [16]. CMV glycoprotein B has a cluster of acidic amino acids acting as a signal recognized by cellular proteins. This acidic cluster plays a pivotal role in

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1464–1801/07/0134–0255$23.50/0

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Table 1. Viral inhibitory effect of esterified milk proteins and L-polylysines against cytomegalovirus AD 169 infected into MRC-5 fibroblast confluent cells

	Viral inhibition, %				
	Met-ALA[1]	Met-ALA P[2]	Met-BLG[3]	Met-BLG P[4]	PL[5]
Concentration					
4 μg/ml	71 ± 6	64 ± 6	47 ± 5	67 ± 6	96 ± 4
20 μg/ml	82 ± 7	65 ± 6	72 ± 7	82 ± 6	98 ± 3
100 μg/ml	85 ± 5	89 ± 5	94 ± 6	85 ± 5	99 ± 2

[1, 2] Met-ALA and its peptic hydrolysate, respectively.
[3, 4] Met-BLG and its peptic hydrolysate, respectively.
[5] L-Polylysines (molecular masses 4–15 kDa).

viral invasion of epithelial cells functioning as a signal for entry into the endocytic pathway [17]. It is also an important mediator of virus entry and cell-to-cell dissemination of infection [18].

It was demonstrated recently that esterified whey proteins could protect *Lactococcus lactis* against bIL66, bIL67 and bIL170 bacteriophage infection [19]; the same proteins inhibit bacteriophage M13 replication [20]. Consequently, basic esterified milk proteins may present antiviral activities inactivating either acid proteins of the glycoprotein B type essential for viral propagation and replication or binding to crucial fragments of naked viral nucleic acids. The aim of this study was to investigate the anticytomegaloviral activity of methylated α-lactalbumin (Met-ALA) or methylated β-lactoglobulin (Met-BLG), as well as their peptic hydrolysates, as compared with the highly basic polypeptide, L-polylysines (4–15 kDa).

Data presented in table 1 show the inhibitory effect of esterified milk proteins and L-polylysines on HCMV type AD 169 during its infectious action on fibroblast confluent cells (MRC-5) after 2 days of incubation at 37°C. Met-ALA and Met-BLG, as well as their peptic hydrolysates, decreased the infectious activity of cytomegalovirus in fibroblast cells. When using the lowest concentration of esterified proteins (4 μg/ml), the antiviral activity of ALA was higher than that of BLG. In the case of higher concentrations of esterified proteins (20 and 100 μg/ml), the antiviral activity was similar for both esterified proteins. Generally, antiviral activities of peptic hydrolysates of esterified proteins were lower or equal to that of the entire proteins, except for peptic hydrolysate of BLG tested in the lowest concentration. However, the hydrolyzed forms of esterified proteins can still be recommended as antiviral agents since they may be preferred over the intact proteins, which often precipitate. The antiviral effect of positively charged esterified proteins or of their peptic hydrolysates may be due to their DNA-binding properties [21]. Esterified proteins could make a complex with the viral genomic DNA perturbing the replication or transcription activities of the virus. Alternatively, these positively charged proteins could either interfere or interact with viral proteins perturbing viral life cycle. Since glycoprotein B of cytomegalovirus has an acidic amino acid cluster [17], possible electrostatic interactions between glycoprotein B and esterified proteins could attenuate or stop its activities perturbing consequently viral life cycle. However, the methodology used in this study is not sufficient to give quantitative data. Methodology is based on the visual observation of infected fibroblast cultures which might be hampered by some alterations in the cellular layers or artifacts. For a better quantitative evaluation, other methodologies, e.g. flow cytometry, real-time PCR, or hybrid capture CMV DNA assays, may be recommended.

L-Polylysines (4–15 kDa) showed highest antiviral activities against cytomegalovirus infectiousness. This demonstrates the importance of the basicity of the antiviral compounds in antiviral activity.

The obtained results agree well with previously observed antiviral activity of esterified milk whey proteins and L-polylysines against herpes simplex virus [unpubl. results], which is a member of the same viral family. Hence, L-polylysines and esterified milk proteins use probably similar viral inhibitory mechanisms, which may consist in direct interactions with exposed fragments of viral DNA or mRNA or in direct inhibitions of functional viral proteins. This may enable them to counteract emerging viral drug resistances against drugs such as ganciclovir [22].

BLG (Protarmor 907 NK) was purchased from Armor Protéines (Saint-Brice-en-Coglès, France) and purified

according to Mailliart and Ribadeau Dumas [23]. ALA (Armor Protéines) was further purified by ion-exchange chromatography on a DEAE-Sepharose Fast Flow column (50 × 300 mm). The elution was performed with a 50-mM Tris, 10 mM $CaCl_2$ buffer at pH 8, by using two steps at 10 and 40 mM NaCl. Porcine pepsin (EC 3.4.23.1; 4,550 U/mg) and L-polylysines (molecular masses of 4–15 kDa) were purchased from Sigma Chemical Co.

For protein esterification the general procedure of Sitohy et al. [24] was used. To quantify the extent of esterification of proteins, the color reaction using hydroxylamine hydrochloride developed by Halpin and Richardson [25] was used with modification according to Bertrand-Harb et al. [26]. Peptic hydrolysis was performed as previously described [27, 28].

HCMV reference strain AD 169 was propagated on MRC-5 fibroblasts (BioMérieux, Lyon, France). Confluent MRC-5 cells were conserved on Eagle's minimum essential medium (Eurobio) containing 2% fetal calf serum (Eurobio).

The culture medium was withdrawn from all the wells of the plates and replaced with 1 ml of fresh culture medium in the wells serving as cellular control. In the case of the wells serving as virus control, 0.1 ml of virus suspension with a known titer was added to 0.9 ml of the culture medium. For the wells designated for evaluation of the antiviral activity, 0.1 ml of virus and 0.1 ml of the tested substance solutions were added and the volume was completed to 1 ml with the culture medium. All controls were performed in 4 replicates (4 wells). The plates were incubated at 37°C in the presence of 5% CO_2 for 48 h. After incubation, the media were removed and the cells were fixed by addition of acetone (at –20°C) and incubated during 10 min. After drying, the cells were incubated with E13 monoclonal antibodies at 37°C for 30 min. After rinsing with PBS, the IgG antiglobulin coupled with isothiocyanate fluorescent agent (Bioatlantic) mixed with blue coloration solution was added and the cells were reincubated for 30 min. Finally, the cells were washed with PBS, dried and covered with 90% glycerol. The plates were read with an inverted microscope (Leitz) equipped with an UV lamp. The number of the fluorescent nuclei was taken as an index of the HCMV-infected cells. The antiviral activity was calculated as follows:

Number of infected cells (Cv) – number of the infected and treated cells (T)/number of infected cells (Cv) × 100.

Acknowledgements

The authors thank INRA and the Région Pays de la Loire for the fellowship awarded to M.S., in the scope of the program 'Valorisation Alimentaire et Non Alimentaire des Macromolécules'. The authors are very grateful to the Ministère des Affaires Etrangères for the financial support of the Partenariat Hubert Curien 'Imhotep' no. 12455RC 'Propriétés antivirales et hypoallergéniques des protéines laitières modifiées'.

References

1 Crumpacker CS: Ganciclovir. Drug Ther 1996;335:721–729.

2 Lalezari JP, Stagg RJ, Jaffe HS, Hitchcock MJ, Drew WL: Preclinical and clinical overview of the nucleotide-based antiviral agent cidofovir (HPMPC). Adv Exp Med Biol 1996;394:105–115.

3 Chrisp P, Clissold SP: Foscarnet: a review of its antiviral activity, pharmacokinetic properties and therapeutic use in immunocompromised patients with cytomegalovirus retinitis. Drugs 1991;41:104–129.

4 Field AK, Biron KK: 'The end of innocence' revisited: resistance of herpes viruses to antiviral drugs. Clin Microbiol Rev 1994;7:1–13.

5 Sarasini A, Baldanti F, Furione M, Percivalle E, Brerra R, Barbi M, Gerna G: Double resistance to ganciclovir and foscarnet of four human cytomegalovirus strains recovered from AIDS patients. J Med Virol 1995;47:237–244.

6 Harada K, Eizuru Y, Isashiki Y, Ihara S, Minamishima Y: Genetic analysis of a clinical isolate of human cytomegalovirus exhibiting resistance against both ganciclovir and cidofovir. Arch Virol 1997;142:215–225.

7 Drew WL, Miner RC, Busch DF, Follansbee SE, Gullett J, Mehalko SG, Drobyski WR, Knox KK, Carrigan DR, Ash RC: Foscarnet therapy for ganciclovir-resistant cytomegalovirus in marrow transplantation. Transplantation 1991;52:155–157.

8 Slavin MA, Bindra RR, Gleaves CA, Pettinger MB, Bowden RA: Ganciclovir sensitivity of cytomegalovirus at diagnosis and during treatment of cytomegalovirus pneumonia in marrow transplant recipients. Antimicrob Agents Chemother 1993;37:1360–1363.

9 Wolf DG, Yaniv I, Honigman A, Kassis I, Schonfel T, Ashkenazi S: Early emergence of ganciclovir-resistant human cytomegalovirus strains in children with primary combined immunodeficiency. J Infect Dis 1998;178:535–538.

10 Alford CA, Britt WJ: Cytomegalovirus; in Roizman B, Whitley RJ, Lopez D (eds): The Herpes Viruses. New York, Raven Press, 1993, pp 227–255.

11 Mocarski ESJ: Cytomegalovirus biology and replication, in Roizman B, Whitley RJ, Lopez D (eds): The Herpes Viruses. New York, Raven Press, 1993, pp 173–226.

12 Kondo KH, Kaneshima H, Mocarski ES: Human cytomegalovirus latent infection of granulocyte-macrophage progenitors. Proc Natl Acad Sci USA 1994;91:11879–11883.

13 Kondo KH, Xu J, Mocarski ES: Human cytomegalovirus latent gene expression in granulocyte-macrophage progenitors in culture and in seropositive individuals. Proc Natl Acad Sci USA 1996;93:11137–11142.
14 Jones TR, Wiertz EJ, Sun L, Fish KN, Nelson JA, Ploegh HL: Human cytomegalovirus US3 impairs transport and maturation of major histocompatibility complex class I heavy chains. Proc Natl Acad Sci USA 1996; 93:11327–11333.
15 Jones TR, Sun L: Human cytomegalovirus US2 destabilizes major histocompatibility complex class I heavy chain. J Virol 1997;71: 2970–2979.
16 Reddehase MJ: The immunogenicity of human and murine cytomegaloviruses. Curr Opin Immunol 2000;12:390–396.l
17 Tugizov S, Maidji E, Xiao J, Pereira A: An acidic cluster in the cytosolic domain of human cytomegalovirus glycoprotein B is a signal for endocytosis from plasma membrane. J Virol 1999;73:8677–8688.
18 Boyle KA, Compton T: Receptor-binding properties of a soluble form of human cytomegalovirus glycoprotein B. J Virol 1998;72: 1826–1833.
19 Sitohy M, Chobert J-M, Karwowska U, Gozdzicka-Jozefiak A, Haertlé T: Inhibition of bacteriophage M13 replication with esterified milk proteins. J Agric Food Chem 2006; 54:3800–3806.
20 Sitohy M, Chobert J-M, Haertlé T: Esterified whey proteins can protect *Lactococcus lactis* against bacteriophage infection. Comparison with the effect of native basic proteins, and L-polylysines. J Agric Food Chem 2005; 53:3727–3734.
21 Sitohy M, Chobert J-M, Gaudin J-C, Renac T, Haertlé T: When positively charged milk proteins can bind to DNA. J Food Biochem 2002;26:511–532.
22 Sullivan V, Talarico CL, Stanat SC, Davis M, Coen DM, Iron KK: A protein kinase homologue controls phosphorylation of ganciclovir in human cytomegalovirus-infected cells. Nature 1992;358:162–164.
23 Mailliart P, Ribadeau Dumas B: Preparation of β-lactoglobulin and β-lactoglobulin-free proteins from whey retentate by NaCl salting out at low pH. J Food Sci 1988;53:743–752.
24 Sitohy M, Chobert J-M, Haertlé T: Simplified short-time method for the esterification of milk proteins. Milchwissenschaft 2001;56: 127–131.
25 Halpin MI, Richardson T: Elected functionality changes of β-lactoglobulin upon esterification of side chain carboxyl groups. J Dairy Sci 1985;68:3189–3198.
26 Bertrand-Harb C, Chobert J-M, Dufour E, Haertlé T: Esterification of food proteins: characterization of the derivatives by a colorimetric method and by electrophoresis. Sci Aliments 1991;11:641–652.
27 Sitohy M, Chobert, JM, Haertlé T: Peptic hydrolysis of methyl-, ethyl- and propyl-esters of β-casein and α-lactalbumin: Milchwissenschaft 2001;56:303–307.
28 Sitohy M, Chobert JM, Dalgalarrondo M, Haertlé T: Factors influencing pepsinolysis of methyl-, ethyl- and propyl-ester derivatives of β-lactoglobulin. J Food Biochem 2001;25:181–198.

Short Communication

Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol 2007;13:259–263
DOI: 10.1159/000104756

Antimicrobial Activity of Divercin RV41 Produced and Secreted by *Lactococcus lactis*

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Key Words
Divercin RV41 · Divercin RV41, antimicrobial activity · *Carnobacterium divergens* V41 · *Lactococcus lactis* · Nisin-controlled expression system

Abstract
Divercin V41 is a class IIa bacteriocin produced by *Carnobacterium divergens* V41 with a strong anti-*Listeria* activity. We have previously produced a recombinant form of divercin V41 (DvnRV41) in *Escherichia coli* strain Origami, by cloning a synthetic gene that codes for a mature divercin RV41 peptide. In this work we describe the inducible expression and secretion of DvnRV41 in the food-grade lactic acid bacterium, *Lactococcus lactis*. The production of DvnRV41 by recombinant *L. lactis* was confirmed and quantified by Western blot and ELISA assays. In addition, anti-*Listeria* activity of DvnRV41 was determined using an agar diffusion test. Although the levels of DvnRV41 produced by recombinant *L. lactis* were similar to those produced by the natural host, *C. divergens* V41, the specific activities were lower. In conclusion, our data show that the bacteriocin DvnRV41 is produced and secreted in an active form by *L. lactis* and that this approach may have important applications in the preservation of foods.
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Bacteriocins are ribosomally synthesized antimicrobial peptides of proteinaceous nature produced by numerous Gram-positive and Gram-negative bacteria that can kill or inhibit the growth of other bacteria [Tagg et al., 1976]. Lactic acid bacteria (LAB) are Gram-positive usually non-motile and non-spore-forming rods and cocci found in fermented products which produce a variety of bacteriocins [Cleveland et al., 2001; Cotter et al., 2005; Drider et al., 2006]. Bacteriocins produced by these food-grade LAB represent potential natural and non-toxic candidates for use in food preservation, as they can control the growth of some pathogenic strains such as *Listeria monocytogenes* in food products.

We have previously reported that inhibition of *L. monocytogenes* in cold smoked salmon was clearly attributed to in situ production of divercin V41 (DvnV41), rather than nutritional competition [Richard et al., 2003]. DvnV41 is a class IIa bacteriocin produced by *Carnobacterium divergens* V41 that was previously isolated and

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1464–1801/07/0134–0259$23.50/0

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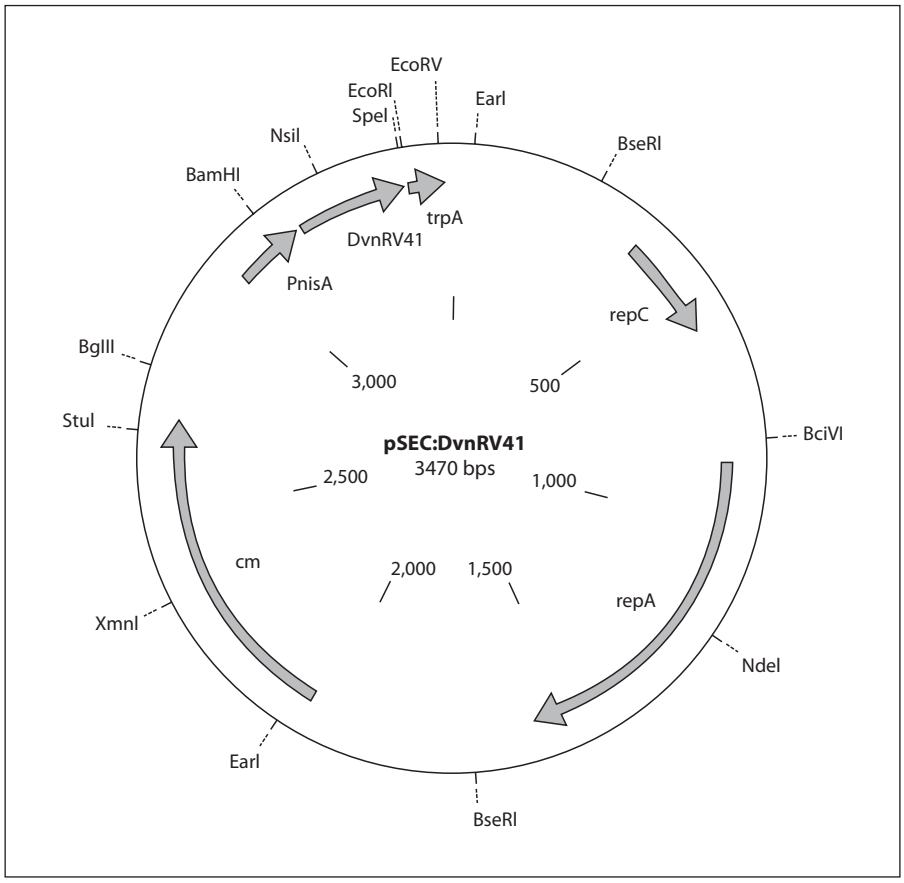
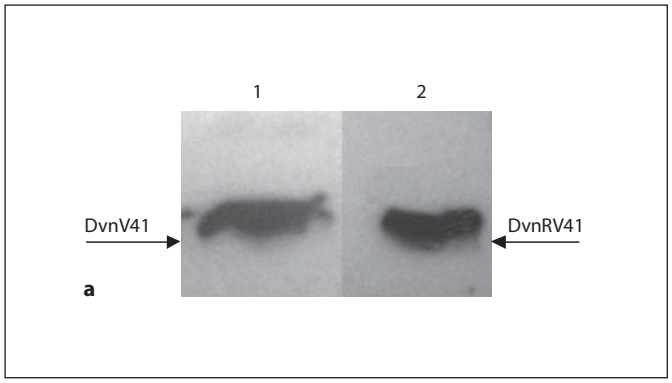


Fig. 1. Schematic representation of the pSEC:DvnRV41 vector.

characterized in our laboratory as having a strong anti-*Listeria* activity [Pilet et al., 1995; Métivier et al., 1998; Duffes et al., 1999; Richard et al., 2006]. Mature DvnV41 is composed of 43 amino acids with a molecular mass of 4.5 kDa, containing two disulfide bonds which are important for anti-*Listeria* activity [Métivier et al., 1998].

Recently, we have overexpressed a recombinant form of DvnV41 (DvnRV41) by cloning a synthetic *dvnRV41* gene into *Escherichia coli* strain Origami [Richard et al., 2004a]. In the present study, the *dvnRV41* gene was expressed in the food-grade LAB, *Lactococcus lactis* using the nisin-controlled expression system [de Ruyter et al., 1996; Kuipers, 1998]. DvnRV41 expression and secretion by *L. lactis* was confirmed by Western blot and ELISA assays. Finally, the biological activity of recombinant DvnRV41 produced and secreted by *L. lactis* was demonstrated by its anti-*Listeria* activity [Pilet et al., 1995; Richard et al., 2003].

To achieve DvnRV41 production and secretion by *L. lactis*, we used the Sec pathway, which recognizes proteins synthesized as precursors containing a mature protein and an N-terminal signal peptide that is essential for precursor secretion [for review, see van Wely et al., 2001]. For this, the *dvnRV41* gene was fused in frame with a DNA fragment containing the Usp45 signal peptide, derived from the predominant *L. lactis*-secreted protein [van Asseldonk et al., 1990] and placed under the control of the P_{nisA}-inducible promoter, whose expression depends on the nisin concentration used [de Ruyter et al., 1996]. To accomplish this, the following steps were carried out: (i) a 159-bp DNA fragment encoding for mature DvnRV41 peptide was PCR amplified from the pET32b: Div-Rec vector, using *Nsi*I-RV41 (5′-CCA***ATGCAT***CAGATCCGACCAAA TATTACGGCAACG-3′) and *Spe*I-RV41 (5′-GG***ACTAGT***CCTTAGCATTTGCCCGGAAT CGCACCGC-3′) primers; (ii) the resulting fragment was directly digested with *Nsi*I and *Spe*I enzymes and cloned into purified *Nsi*I-*Spe*I-cut pSEC-E7 vector [Bermúdez-Humarán et al., 2002] which contains a ρ-independent transcription terminator (trpA) for clone stability [Christie et al., 1981], resulting in the pSEC:DvnRV41 plasmid (fig. 1). This plasmid was introduced into *L. lactis* strain



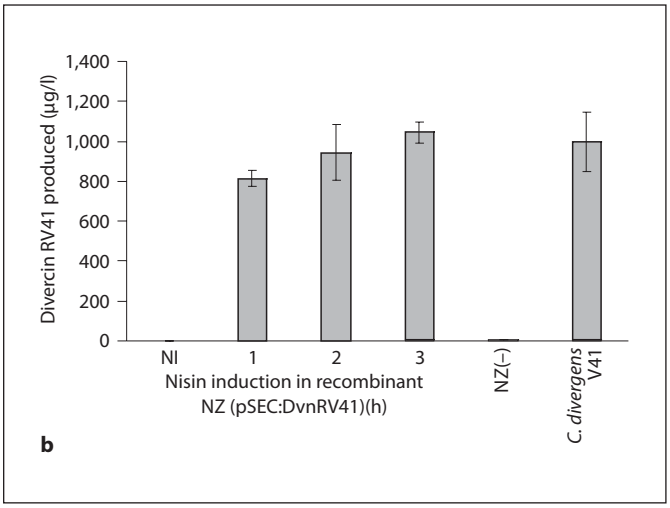


Fig. 2. Expression and characterization of DvnRV41 produced by *L. lactis*. **a** Production and secretion of DvnRV41 by recombinant *L. lactis* was analyzed by Western blot on induced cultures of NZ(pSEC:DvnRV41) strain (lane 2). DvnV41 produced by *C. divergens* V41 was used as the positive control for Western blot assays (lane 1). A quantity of 35 µl of each culture supernatant was loaded. **b** ELISA assessment of DvnRV41 produced by *L. lactis* NZ(pSEC:DvnRV41). NI = Non-induced; 1, 2, 3 = time in hours after nisin induction. *C. divergens* V41 was used as the positive control.

NZ9000 carrying regulatory genes *nisR* and *nisK* [de Ruyter et al., 1996] to obtain the strain NZ(pSEC:DvnRV41). As a negative control, *L. lactis* NZ9000 was transformed with an empty vector to generate NZ(–). Recombinant clones were selected by addition of 10 µg of chloramphenicol per ml of medium. Recombinant *L. lactis* strains were grown in M17 medium supplemented with 1% glucose (GM17) at 30°C without agitation.

The capacity of strain NZ(pSEC:DvnRV41) to produce and accumulate DvnRV41 in either the cytoplasm or the extracellular medium was determined. Non-induced and induced culture samples were examined by Western blotting using anti-DvnV41 antibodies [Richard et al., 2004b]. Induction for DvnRV41 expression was performed using nisin (10 ng/ml; Sigma) for a 3-hour period as previously described [Bermúdez-Humarán et al., 2003]. As expected, in the absence of nisin, no DvnRV41 signal was detected (data not shown). Analysis of induced cultures of *L. lactis* strain NZ(pSEC:DvnRV41) revealed a single band of ~4.5 kDa in the supernatant, whereas no signal was detected in the cell fraction (fig. 2a). This result suggests that secretion of DvnRV41 by *L. lactis* is very efficient, as most of the recombinant protein is detected in the supernatant. In parallel, we analyzed supernatant samples of the natural host of DvnV41, *C. divergens* V41. As expected, Western blot analysis reveal only a one major band of ~4.5 kDa corresponding to DvnV41 (fig. 2a).

The listericidal activity of DvnV41 and DvnRV41 produced by the recombinant lactococcal strain was determined using the agar diffusion test [Pilet et al., 1995; Richard et al., 2003]. The highest listericidal activity was obtained with DvnRV41 against *L. innocua* (800 UA/ml; table 1); this value corresponds to 1/30th of the activity of the native peptide DvnV41 (25,600 UA/ml; table 1).

It has been reported that heterologous production and secretion of two other bacteriocins in *L. lactis*, pediocin PA-1 and enterocin A, was lower when compared to those obtained by the wild-type producers [Martinez et al., 2000]. The authors attributed this drawback to a low efficiency of transport and/or maturation of these bacteriocins by the chromosomally encoded bacteriocin translocation machinery of *L. lactis* [Martinez et al., 2000]. Recently, heterologous production of enterocin P (EntP), a *sec*-dependent class IIa bacteriocin from *Enterococcus faecium* P13 in *L. lactis* IL1403 and *L. lactis* MG1363, was shown to be dependent on the host strain, the expression vector, and the presence of the EntP immunity gene (*entiP)* in the constructs of recombinant *L. lactis* strains [Gutierrez et al., 2006]. The highest amount of EntP was produced with derivatives containing *entP* and *entiP*, for both *L. lactis* strains, leading to 5- to 6-fold more EntP than *E. faecium* P13 [Gutierrez et al., 2006].

To understand the lower anti-*Listeria* activity observed with the DvnRV41 produced by recombinant lactococci

Table 1. Indicator organisms used in this work

Species	Reference	Source	Activity of the divercin RV41[a], AU/ml	Activity of the divercin V41[b], AU/ml	Growth medium	Growth temperature °C	Time of growth h
Lactobacillus casei	20011	DSMZ	0	0	MRS	30	20
Lactobacillus plantarum	14917	ATCC	0	0	MRS	30	20
Lactotoccus lactis	1075	CNRZ	0	0	Elliker	30	16
Leuconostoc mesenteroides ssp. *cremoris*	20200	DSMZ	0	0	MRS	30	17
Listeria innocua	F	DSV	800	25,600	Elliker	30	20
Listeria monocytogenes EGDe	107776	CIP	400	12,800	Elliker	37	16
Listeria monocytogenes Scott A	103.575	CIP	0	4,800	Elliker	37	16
Listeria monocytogenes	19115	ATCC	400	12,800	Elliker	37	16
Staphylococcus aureus	25923	ATCC	0	0	BHI	30	18
Streptococcus thermophilus			0	0	MRS	42	20
Streptococcus thermophilus			0	0	MRS	42	20
Bacillus cereus	78.3	CIP	0	0	BHI	30	16
Lactobacillus delbrueckii ssp. *bulgaricus*	20074	DSMZ	0	0	MRS	42	20

DSMZ = Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; ATCC = American Type Culture Collection, Rockville, Md., USA; CNRZ = Centre National de Recherches Zootechniques, Jouy-en-Josas, France; DSV = Direction des Services Vétérinaires, Nantes, France; CIP = Collection Institut Pasteur, France.

[a] Divercin RV41 is produced by *L. lactis* NZ(pSEC:DvnRV41).

[b] Divercin V41 is produced by *C. divergens* V41.

when compared with DvnV41 obtained from *C. divergens* V41, we introduced plasmid pSEC:DvnRV41 in three different strains of *L. lactis* deficient in its major proteases: a *clpP-L. lactis* strain, deficient in its major intracellular housekeeping protease [Frees and Ingmer, 1999], an *htrA-L. lactis* strain, deficient in its major surface housekeeping protease [Poquet et al., 2000], and a *clpP-HtrA-L. lactis* strain, deficient in both ClpP and HtrA proteases [Cortes-Perez et al., 2006]. The anti-*Listeria* activity of each recombinant strain was determined in triplicate by agar diffusion test [Pilet et al., 1995; Richard et al., 2003].

The anti-*Listeria* activity displayed by each mutant was similar to that obtained by wild-type *L. lactis* (data not shown), suggesting that these proteases do not degrade the DvnRV41 peptide. In our opinion, the difference of antimicrobial activity observed between natural DvnV41 and DvnRV41 produced by *L. lactis* (DvnRV41 30-fold under that of natural DvnV41) could be explained by (i) the folding of some DvnRV41 peptides, which is not accurately performed, thus perhaps impeding the formation of the two disulfide bonds; (ii) the peptide is not correctly processed in the heterologous host; (iii) possibility of a synergistic or additive effect of DvnRV41 with other antimicrobial substances, which are absent in the lactococcal host; (iv) the sequence optimization of the synthetic gene is more suitable to expression in *E. coli* strain Origami [Richard et al., 2004a]. Finally, another hypothesis to explain this low specific activity might be cysteinylation of the cysteines in our bacteriocin. Kuipers et al. [2006] observed that many *sec*-secreted peptides were partially cysteinylated. This cysteinylation was not observed by incubating the peptide in the culture medium [Gert Moll, pers. commun.], hence it was related to *L. lactis*.

Further experiments for determining the relative amount of DvnRV41 present in the supernatant of *L. lactis* carrying pSEC:DvnRV41 were performed. This was assessed by the non-competitive indirect enzyme-linked immunosorbent assay against specific anti-DvnRV41 antibodies [Richard et al., 2004b]. The graduated scale of standards was established with pure DvnRV41 previously obtained [Richard et al., 2004a]. The highest amount of DvnRV41 produced by *L. lactis* was estimated to be 1,041 ± 51 μg/ml, which is relatively similar to the amount of DvnV41 produced by *C. divergens* V41 (fig. 2b). Nevertheless, the relative amount of DvnRV41 was not dependent on plasmid copy number since DNA plasmid pSEC-DvnRV41 remained stable during the entire nisin induction process.

The spectrum of activity of DvnV41 and DvnRV41 was determined against different indicator strains (ta-

ble 1). Both bacteriocins DvnV41 and DvnRV41 displayed a similar spectrum of activity, but the specific activity of DvnRV41 was 30-fold lower for reasons cited above. In the spectral analysis, we remarked that DvnRV41 was not active against *L. monocytogenes* Scott A, suggesting that *L. monocytogenes* Scott A is likely less sensitive to certain other bacteriocins.

In this study we show that the anti-*Listeria* bacteriocin DvnRV41 can be produced and secreted in an active form by *L. lactis* by the cloning of a DvnV41 synthetic gene. Although the quantification of natural DvnV41 and recombinant DvnRV41 led to similar amounts, the specific activity obtained with DvnRV41 was lower than DvnV41. Nevertheless, the antilisterial activity displayed by the recombinant strain remains satisfactory and our approach may have important applications in the preservation of smoked foods, such as smoked salmon.

Acknowledgments

The authors are indebted to Dr. Gisèle LaPointe (Université Laval, Québec, Canada) for critical reading of the manuscript. Work carried out at ENITIAA was supported by Région des Pays de la Loire and European Union through VANAMII and SEAFOOD plus programs. J.R. is a recipient of a PhD scholarship awarded by the French Government.

References

Bermúdez-Humarán LG, Langella P, Miyoshi A, Gruss A, Taméz-Reyes RS, Montes de Oca-Luna R, Le Loir Y: Production of human *Papillomavirus* type 16 E7 protein in *Lactococcus lactis*. Appl Environ Microbiol 2002;68:917–922.

Bermúdez-Humarán LG, Langella P, Commissaire J, Gilbert S, Le Loir Y, L'Haridon YR, Corthier G: Controlled intra- or extracellular production of staphylococcal nuclease and ovine omega interferon in *Lactococcus lactis*. FEMS Microbiol Lett 2003;224:307–313.

Christie GE, Farnham PJ, Platt T: Synthetic sites for transcription termination and a functional comparison with tryptophan operon termination sites in vitro. Proc Natl Acad Sci USA 1981;78:4180–4184.

Cleveland J, Montville TJ, Nes IF, Chikindas ML: Bacteriocins: safe, natural antimicrobials for food preservation. Int J Food Microbiol 2001;71:1–20.

Cortes-Perez NG, Poquet I, Oliveira M, Gratadoux JJ, Madsen SM, Miyoshi A, Corthier G, Azevedo V, Langella P, Bermúdez-Humarán LG: Construction and characterization of a *Lactococcus lactis* strain deficient in intracellular ClpP and extracellular HtrA proteases. Microbiology 2006;152:2611–2618.

Cotter PD, Hill C, Ross RP: Bacteriocins: developing innate immunity for food. Nat Rev Microbiol 2005;3:777–788.

De Ruyter PGGA, Kuipers OP, de Vos WM: Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. Appl Environ Microbiol 1996;62:3662–3667.

Drider D, Fimland G, Hechard Y, McMullen L, Prévost H: The continuing story of class IIa bacteriocins. Microbiol Mol Biol Rev 2006;70:564–582.

Duffes F, Leroi F, Boyaval P, Dousset X: Inhibition of *Listeria monocytogenes* by *Carnobacterium* spp. strains in a simulated cold smoked fish system stored at 4°C. Int J Food Microbiol 1999;47:33–42.

Frees D, Ingmer H: ClpP participates in the degradation of misfolded protein in *Lactococcus lactis*. Mol Microbiol 1999;31:79–87.

Gutierrez J, Larsen R, Cintas LM, Kok J, Hernandez PE: High-level heterologous production and functional expression of the *sec*-dependent enterocin P from *Enterococcus faecium* P13 in *Lactococcus lactis*. Appl Microbiol Biotechnol 2006;72:41–51.

Kuipers OP: Quorum sensing-controlled gene expression in lactic acid bacteria. J Biotech 1998;64:15–21.

Kuipers A, Wierenga J, Rink R, Kluskens LD, Driessen AJ, Kuipers OP, Moll GN: Sec-mediated transport of posttranslationally dehydrated peptides in *Lactococcus lactis*. Appl Environ Microbiol 2006 72;7626–7633.

Martinez JM, Kok J, Sanders JW, Hernandez PE: Heterologous co-production of enterocin A and pediocin PA-1 by *Lactococcus lactis*: detection by specific peptide-directed antibodies. Appl Environ Microbiol 2000;66:3543–3549.

Métivier A, Pilet MF, Dousset X, Sorokine O, Anglade P, Zagorec M, Piard JC, Marion D, Cenatiempo Y, Frémaux C: Divercin V41, a new bacteriocin with two disulphide bonds produced by *Carnobacterium divergens* V41: primary structure and genomic organization. Microbiology 1998;144:2837–2844.

Pilet MF, Dousset X, Barré R, Novel G, Desmazeaud M, Piard JC: Evidence for two bacteriocins produced by *Carnobacterium piscicola* and *Carnobacterium divergens* isolated from fish and active against *Listeria monocytogenes*. J Food Prot 1995;58:256–262.

Poquet I, Saint V, Seznec E, Simoes N, Bolotin A, Gruss A: HtrA is the unique surface housekeeping protease in *Lactococcus lactis* and is required for natural protein processing. Mol Microbiol 2000;35:1042–1051.

Richard C, Brillet A, Pilet MF, Prévost H, Drider D: Evidence on inhibition of *Listeria monocytogenes* by divercin V41 action. Lett Appl Microbiol 2003;36:288–292.

Richard C, Drider D, Elmorjani K, Marion D, Prévost H: Heterologous expression and purification of active divercin V41, a class IIa bacteriocin encoded by a synthetic gene in *Escherichia coli*. J Bacteriol 2004a;186:4276–4284.

Richard C, Drider D, Fliss I, Denery S, Prévost H: Generation and utilization of polyclonal antibodies to a synthetic C-terminal amino acid fragment of divercin V41, a class IIa bacteriocin. Appl Environ Microbiol 2004b;70:248–254.

Richard C, Canon R, Naghmouchi K, Bertrand D, Prévost H, Drider D: Evidence on correlation between number of disulfide bridge and toxicity of class IIa bacteriocins. Food Microbiol 2006; 23:175–183.

Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual, ed 2. Cold Spring Harbor, Cold Spring Harbor Laboratory, 1989.

Tagg JR, Dajani AS, Wannamaker LW: Bacteriocins of Gram-positive bacteria. Bacteriol Rev 1976;40:722–756.

Van Asseldonk MG, Rutten M, Oteman RJ, Siezen RJ, de Vos WM, Simons G: Cloning, expression in *Escherichia coli* and characterization of *usp45*, a gene encoding a highly secreted protein from *Lactococcus lactis* MG1363. Gene 1990;95:155–160.

Van Wely KHM, Swaving J, Freudl R, Driessen AJM: Translocation of proteins across the cell envelope of Gram-positive bacteria. FEMS Microbiol Rev 2001;25:437–454.

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No. 1–3

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No. 4

Antimicrobial Peptides: Food, Veterinary and Medical Applications

Editor: Drider, D. (Nantes)