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Review

Inhibiting factors produced by lactic acid bacteria. 2. Bacteriocins and other antibacterial substances

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Summary — Lactic acid bacteria can produce a variety of substances with antibacterial activity which are described in this article. Non-peptide antibacterial substances are distinguished from bacteriocins, which have a proteinaceous active site. Among the former, reuterin produced by *Lactobacillus reuteri* is a broad spectrum inhibitor active against Gram-positive and Gram-negative bacteria, yeasts, molds and protozoas. It is a glycerol derivative, β-hydroxypropionaldehyde. Bacteriocins can be produced by most lactic acid bacteria species and their spectrum of activity is generally restricted to organisms taxonomically close to the producer. The biochemical properties of bacteriocins, their structure and nature of their genetic determinants are highly variable. This literature review discusses the similarities and differences existing in this group of substances.

lactic acid bacteria / antimicrobial substance / bacteriocin

Résumé — Les facteurs inhibiteurs produits par les bactéries lactiques : 2. Bactériocines et autres substances antibactériennes. *Des bactéries lactiques sont capables de produire une variété de substances à activité antibactérienne décrites dans le présent article. On distingue les antibactériens non peptidiques des bactériocines dotées d'un site actif de nature protéique. Parmi les premiers, la reuterine produite par* Lactobacillus reuteri *est un antibiotique à large spectre, actif vis-à-vis des bactéries Gram-positives et Gram-négatives, des levures, des moisissures et des protozoaires. Il s'agit d'un dérivé du glycérol, le β-hydroxypropionaldéhyde. Les bactériocines peuvent être produites par la plupart des espèces de bactéries lactiques et leur spectre d'activité est restreint en général aux bactéries taxonomiquement proches du microorganisme producteur. Les propriétés biochimiques des bactériocines, leur structure et la nature de leur déterminant génétique sont d'une grande variabilité. Cette revue bibliographique fait le point des similitudes et des disparités qui existent au sein de ce groupe de susbstances.*

bactérie lactique / substance antimicrobienne / bactériocine

INTRODUCTION

Lactic acid bacteria used as the transformation agent for milk and other food products have several technological roles. In the case of milk transformation, they participate in the formation of flavors and rheological qualities of cheeses and fermented milks. They also play an important hygienic role by synthesizing a variety of inhibitory compounds which prevent the development of undesirable or pathogenic bacteria. In the first part of this review, the inhibitory factors resulting from the metabolism of oxygen in lactic acid bacteria and the cellular catabolism products of these bacteria have been described (Piard and Desmazeaud, 1991).

In this second part, we will distinguish 2 types of substances: antibacterial agents which are often non-proteinaceous and protein bacteriocins. This category of substances which can be produced by most lactic acid bacteria species has sparked new interest in the last several years. As a result, some of the molecular bases concerning the structure and genetics of bacteriocins are beginning to be understood.

For convenience, *Lactococcus lactis* subsp *lactis*, *L lactis* subsp *lactis* biovar *diacetylactis* and *L lactis* subsp *cremoris* will be designated *L lactis*, *L diacetylactis* and *L cremoris* respectively.

SMALL ANTIMICROBIAL SUBSTANCES

A number of authors have reported the capacity of *L diacetylactis* and *Lc citrovorum* to inhibit undesirable microorganisms, notably *Pseudomonas* sp (Mather and Babel, 1959; Daly *et al,* 1972; Genske and Branen, 1973). Although these studies established the role of organic acids in the observed inhibitions, it was suggested that other antibacterial factors must also be involved (Mather and Babel, 1959; Pinheiro *et al,* 1968). Continued studies by Branen *et al* (1975) showed that *L diacetylactis* produced a small peptide of 100–300 Da, active against *P fluorescens*, *P fragi*, *P putrefaciens* and *E coli*.

Relatively similar results were obtained in terms of the antimicrobial activity of *S thermophilus*. Using the milk fermented by this species, Pulusani *et al* (1979) successively extracted a material with methanol and then acetone (M–A extract), which reacted with ninhydrin and which was inhibitory towards *L lactis*, *E coli*, *S typhimurium*, *Shigella* sp, *P fluorescens*, *P aeruginosa* and *Bacillus* sp. Sikes and Hilton (1987) extended the study of this spectrum and showed that the M–A extract was also active against *Clostridium perfringens*, *Staphylococcus aureus* and *Salmonella enteritidis*. The partial purification of inhibitory substances by gel filtration led to elution of a 700–Da molecular weight substance with antibacterial activity (Pulusani *et al,* 1979). The results, however, did not enable the authors to determine whether 1 or 2 compounds were involved in the inhibitory activity. The composition of the substances was not determined, but the authors believed that an aromatic moiety was present.

Abdel-Bar *et al* (1987) came to the same conclusion in a study on the inhibitory factor produced by *Lb bulgaricus* 7994, active against *P fragi* and to a lesser extent against *S aureus*. The optimum pH for activity is at pH 4.0 and the inhibitor is stable for 1 h at 100 °C. Purification by reversed-phase chromatography enabled the elution of a chromatographically pure peak which was not a polypeptide as shown by the negative biuret reaction. Mass spectrometry and the study of the UV/Vis absorption spectrum were in agreement, enabling the authors to conclude that the

substance contained an aromatic moiety. The molecular weight of the inhibitor is around 700 Da.

Considerable studies have recently led to the identification of reuterin, an inhibitor produced by *Lb reuteri*, a resident bacterium in the digestive tract of humans and animals. This substance is produced by *Lb reuteri* grown anaerobically in the presence of glycerol (Talarico *et al,* 1988). Its spectrum of activity is exceptionally broad and includes Gram-positive and Gram-negative bacteria, yeasts, molds and protozoa.

The chemical characterization of reuterin showed that it is a dehydrated derivative of glycerol, β-hydroxypropionaldehyde. This compound can occur in solution in 3 forms, one of which is a cyclized dimer. It has not been determined if all 3 forms are active (Talarico and Dobrogosz, 1989). Reuterin could exert its activity on ribonucleotide reductase (Talarico and Dobrogosz, 1989). In the living world, this enzyme universally catalyzes the first step in DNA synthesis, which could explain reuterin's broad spectrum of activity. Talarico and Dobrogosz (1989) observed that another enzyme with a sulfhydryl group was inactivated by reuterin, indicating that this type of enzyme could be one of the targets of the antimicrobial substance. The wide spectrum of activity of this substance justifies further study, especially as regards the safety of reuterin and its values in the food and agricultural industry, as a result of the restrictive conditions of production by *Lb reuteri*.

BACTERIOCINS AND LACTIC ACID BACTERIA

Bacteriocins are protein substances which are bactericidal towards bacteria taxonomically close to the producer (Tagg *et al,* 1976). They were initially demonstrated in *E coli* (see colicins; Hardy, 1975) and then later in Gram-positive bacteria (Tagg *et al,* 1976). Bacteriocins do not respond to well defined criteria. Rather, their biochemical properties, molecular weight, spectrum of activity, mode of action and genetic support are very heterogeneous (Reeves, 1972; Tagg *et al,* 1976). The study of bacteriocins in lactic acid bacteria has been actively developed over the past few years (Klaenhammer, 1988; Schillinger, 1990) and these investigations have shown that bacteriocin production is an extensively prevalent phenotype in this group of bacteria.

Spectrum of activity

The spectrum of activity of the main bacteriocins described in lactic acid bacteria is listed in table I. Most lactic acid bacterial species can produce bacteriocins which are active against the lactic acid flora itself. Bacteriocin production has not been studied much in leuconostocs, but Orberg and Sandine (1984) have demonstrated the production of antimicrobial species in 4 strains of *Leuconostoc*. Harris *et al* (1989) also showed the capacity of *Leuconostoc* sp UAL14 to produce a protein inhibitor which was active against *L monocytogenes* and Rammelsberg and Radler (1990) noted that *Leuconostoc oenos* produced an inhibitor with a narrow spectrum of activity. Finally, mesentericin Y105 is a bacteriocin which is being characterized in *Lc mesenteroides* Y105 and is active against *L monocytogenes* (Hechard and Cenatiempo, personal communication).

Nisin is unusual in that it can inhibit most Gram-positive bacteria. Interest in this substance initially arose from its bactericidal activity against spores of *Clostridium* sp and of *Bacillus* sp (Hurst, 1978; Somers and Taylor, 1987). These bacteria are responsible for most of the deterioration af-

Table I. Spectrum of activity of bacteriocins produced by lactic acid b a.
Spectre d'activité des bactériocines de bactéries lactiques.

Bacteriocin	*Producer organism*	*Spectrum of activity*	*References*
Nisin	*L lactis*	Gram-positive bacteria	Hurst (1981)
Diplococcin	*L cremoris*	*Lactococcus* sp	Davey and Richardson (1981)
Lactostrepcins	*L lactis*	*Lactococcus* sp, β-haemeolytic streptococci *Lb helveticus*, *Leuconostoc* sp, *Clostridium* sp	Kozak *et al* (1978)
Bac	*Lactococcus* sp	*Lactococcus* sp, *Lactobacillus* sp, *E faecalis*, *Pediococcus* sp, *Leuconostoc* sp, *Clostridium* sp	Geis *et al* (1983)
Bac	*L lactis*	*Lb helveticus*, *L monocytogenes*	Carminati *et al* (1989)
Lacticin 481	*L lactis*	*Lactococcus* sp, *Lb helveticus*, *Lb bulgaricus* *Leuconostoc* sp, *S thermophilus*, *C tyrobutyricum*	Piard *et al* (1990)
Lactococcin A	*L cremoris*	*Lactococcus* sp	Holo *et al* (1991)
Lactocin 27	*Lb helveticus*	*Lb helveticus*, *Lb acidophilus*	Upreti and Hinsdill (1973)
Helveticin J	*Lb helveticus*	*Lb helveticus*, *Lb bulgaricus*, *Lb casei*	Joerger and Klaenhammer (1986)
Bac	*Lb fermentii*	*Lactobacillus* sp	De Klerk and Smit (1967)
Lactacin B	*Lb acidophilus*	*Lb leichmanii*, *Lb bulgaricus*, *Lb helveticus*, *Lb casei*	Barefoot and Klaenhammer (1983)
Lactacin F	Lb acidophilus	*Idem* + *Lb fermentum* + *E faecalis*	Muriana and Klaenhammer (1987)
Plantaricin A	*Lb plantarum*	*Lactobacillus* sp, *Pediococcus* sp, *Leuconostoc* sp, *E faecalis*	Daeschel *et al* (1990)
Plantaricin B	*Lb plantarum*	*Lb plantarum*, *Lc mesenteroides*, *P damnosus*	West and Warner (1988)
Plantaricin S	*Lb plantarum*	*Lactobacillus* sp, *Leuconostoc* sp *Lactococcus* sp, *Pediococcus* sp	Jimenez-Diaz *et al* (1990)
Sakacin A	*Lb sake*	*Lactobacillus* sp, *L monocytogenes*	Schillinger and Lücke (1989)
Lactocin S	*Lb sake*	*Lactobacillus* sp, *Lc mesenteroides* *P acidilactici*, *P pentosaceus*	Mørtvedt and Nes (1990)
Caseicin 80	*Lb casei*	*Lb casei*	Rammelsberg *et al* (1990)
Brevicin 37	*Lb brevis*	*Lactobacillus* sp, *Leuconostoc* sp *Pediococcus* sp	Rammelsberg and Radler (1990)
Pediocin A	*P pentosaceus*	Lactic acid bacteria, *Clostridium* sp, *S aureus*, *L monocytogenes*, *B cereus*	Daeschel and Klaenhammer (1985) Spelhaug and Harlander (1989)
Pediocin AcH	*P acidilactici*	Idem	Ray *et al* (1989a)
Pediocin PA-1	*P acidilactici*	Lactic acid bacteria + *L monocytogenes*	Pucci *et al* (1988)
Bac	*P acidilactici*	*Pediococcus* sp, *E faecalis*, *L monocytogenes*	Hoover *et al* (1988)
Bac	*P acidilactici*	*L ivanovii*, *L monocytogenes*	Nielsen *et al* (1990)

fecting canned foods and the addition of nisin to these products can reduce the doses of nitrite used (Rayman *et al,* 1981). In addition, nisin prevents late blowing phenomena (Hurst, 1981) caused by the development of *Clostridium tyrobutyricum* in pressed cheeses. Recently, more attention has been paid to nisin because of its antibacterial activity against *L monocytogenes* (Mohamed *et al,* 1984), a pathogen capable of causing outbreaks of listeriosis after consumption of contamined cheeses (Doyle, 1985). These different observations have led to the authorization to use nisin as a food additive in Europe (Hurst, 1981) and recently in the United States (FDA, 1988).

Other bacteriocins, such as the pediocins and sakacin A (table I) can also inhibit *L monocytogenes*, although some serotypes are resistant (Hoover *et al,* 1988). In lactococci Carminati *et al* (1989) characterized several bacteriocins active against *L monocytogenes* but further studies have recently shown that these were actually similar to nisin (Giraffa, personal communication). Bacteriocins produced by lactic acid bacteria may thus present a spectrum of activity which is not always confined to bacteria of the same species or the same genus. On the other hand, bacteriocins isolated from alimentary lactic acid bacteria active against Gram-negative bacteria have not yet been characterized. These substances may be characterized in the future by the study of strains obtained from other environments. For example, Mc Groarty and Reid (1988) characterized an inhibitor produced by a *Lactobacillus* isolated from the female genital tract and the substance was found to be inhibitory towards *E coli.* It is not clear, however, whether this substance is a bacteriocin or another inhibitory agent. Klaenhammer (1988) defined 2 classes of bacteriocins according to their spectrum of activity. One included bacteriocins active against bacteria taxonomically close to the producer and the other was composed of bacteriocins (nisin, pediocins) with a relatively broad spectrum of activity against Gram-positive bacteria. Various possibilities of use can be envisaged according to the spectrum of activity of bacteriocins. Those of the first type are likely candidates for favoring the growth of a given bacterial strain in competition with the natural flora. This is interesting in the case of empirical fermentations, in which starters are not used, *eg* the fermentation of olives. This type of bacteriocin could also be used in certain food products in which development of lactic acid bacteria is detrimental (certain alcoholic beverages, products of the 4th range, meat products, refined sugar). Bacteriocins of the second type, on the other hand, have an additional value since they combat certain pathogenic flora.

Biochemical properties

Tolerance to heat, to acid and sensitivity to enzymes

As a result of the definition attributed to bacteriocins, all are inactivated by at least one protease (table II). Most authors differentiate nisin from the other lactococcal bacteriocins by the fact that α-chymotrypsin is the only proteolytic enzyme to which nisin is sensitive (Jarvis and Mahoney, 1969). This property is to be regarded with caution, however, since others have reported that nisin can be inactivated by pronase-type proteases or by proteinase K (Rammelsberg and Radler, 1990; Giraffa, personal communication; Piard *et al,* unpublished results). Nisinase is another enzyme which inactivates nisin and is important, since it can be produced by certain strains of *B cereus,* a species con-

Table II. Sensitivity to enzymes, thermotolerance and acidotolerance of bacteriocins produced by lactic acid bacteria.
Sensibilité aux enzymes, thermotolérance et acidotolérance des bactériocines de bactéries lactiques.

Bacteriocin	*Inactivating enzymes*	*Thermotolerance * acidotolerance*	*References*
Nisin	α-Chymotrypsin nisinase	+ (115 °C, pH 2) – (neutral pH)	Hurst (1981)
Lactostrepcins	α-Chymotrypsin trypsin, pronase, phospholipase D	+ (100 °C, 10 min) – (neutral pH)	Kozack *et al* (1978)
Diplococcin	α-Chymotrypsin, trypsin, pronase	unpurified: + (1 h, 100 °C, pH 5.0) – (1 h, 100 °C, alkaline pH) Purified: – (1 min, 100 °C)	Davey and Richardson (1981)
Bac	α-Chymotrypsin, pronase, proteinase κ	+ (30 min, 100 °C, pH 4.5 and 7.0) +/– (30 min, 100 °C, pH 9.4)	Geis *et al* (1983)
Lacticin 481	α-Chymotrypsin, pronase, ficin, proteinase κ, rennet	+ (1 h, 100 °C, pH 4.5 and 7.0) Stable from pH 2 to pH 8	Piard *et al* (1990)
Lactocin 27	Trypsin, pronase	+ (1 h, 100 °C)	Upreti and Hinsdill (1973)
Helveticin J	Trypsin, pronase, ficin, proteinase κ, pepsin, subtilisin	– (30 min, 100 °C)	Joerger and Klaenhammer (1986)
Bac	Trypsin, pepsin	+ (30 min, 96 °C)	De Klerk and Smit (1967)
Lactacin B	Proteinase κ	Unpurified: + (1 h, 100 °C) Purified: + (3 min, 100 °C)	Barefoot and Klaenhammer (1983, 1984)
Lactacin F	Trypsin, ficin, proteinase κ	+ (15 min, 121 °C)	Muriana and Klaenhammer (1987)
Plantaricin A	Proteolytic preparation	+ (30 min, 100 °C)	Daeschel *et al* (1990)
Plantacin B	α-Chymotrypsin, trypsin, pronase, pepsin, lipase, α-amylase		West and Warner (1988)
Plantaricin S	α-Chymotrypsin, ficin pronase, proteinase κ trypsin, thermolysin, dextranase, α-amylase lipase, phospholipase	+ (1 h, 100 °C)	Jimenez-Diaz *et al* (1990)
Sakacin A	Trypsin, pepsin	+ (20 min, 100 °C)	Schillinger and Lücke (1989)
Lactocin S	Trypsin, protease type XIV	50% inactivated after 1 h, 100 °C	Mørtvedt and Nes (1990)
Caseicin 80	Trypsin, α-chymotrypsin pronase, proteinase κ, pepsin	– (10 min, 60 °C, pH 2)	Rammelsberg *et al* (1990)
Brevicin 37	Trypsin, pronase	+ (1 h, 121 °C, pH 2 to 4) Stable 24 h from pH 1 to 11	Rammelsberg and Radler (1990)
Pediocin A	Pronase	+ (1 h, 100 °C)	Daeschel and Klaenhammer (1985)
Pediocin AcH	Trypsin	+ (1 h, 100 °C, pH 7.0) Unstable at pH 11.0	Ray *et al* (1989a)
Pediocin PA-1	α-Chymotrypsin, pepsin, papain	+ (10 min, 100 °C) – (15 min, 121 °C)	Pucci *et al* (1988)

* +: Stable, –: unstable.

sidered to be a nisin target in canned food products. It is a dehydroalanine reductase and is thus active against nisin-type peptides (see *Elements of composition and/or structure*) (except the D form of nisin) and subtilin (Jarvis and Farr, 1971). Other bacteriocins are generally inactivated by an array of proteolytic enzymes (table II), including those of pancreatic origin (trypsin and α-chymotrypsin) and sometimes of gastric origin (pepsin). This high sensitivity of lactic acid bacterial bacteriocins to metabolic proteolytic enzymes is very interesting with respect to food safety, since it means that the ingestion of bacteriocins will not alter digestive tract ecology and also will not cause risks related to the use of common antibiotics. Few authors have investigated the sensitivity of bacteriocins to rennet. Piard *et al* (1990) reported that lacticin 481 was resistant to the concentrations of rennet usually employed in cheese making, which is an important property from a technological standpoint. Some bacteriocins produced by bacteria of the genus *Lactobacillus* are sensitive to non-proteolytic enzymes. Plantaricin B is inactivated by a lipase and by an α-amylase, and plantaricin S is inactivated by glycolytic, lipolytic and phospholipolytic enzymes (table II). These observations indicate that the active part of bacteriocins of lactobacilli may be chemically heterogeneous, which could signify that the term bacteriocin covers a set of chemically varied substances.

The heat tolerance of lactic acid bacteria bacteriocins is generally high (table II), even though it may be significantly reduced after purification (Davey and Richardson, 1981; Barefoot and Klaenhammer, 1984). In all cases, this heat stability enables bacteriocins to resist a heat treatment equivalent to the pasteurization of milk (63 °C for 30 min or 72 °C for 15 s). This heat resistance also suggests that the activity of these substances is based on molecular structures which are relatively small and uncomplicated, probably lacking a tertiary structure. Nevertheless, helveticin J is distinguished from the other bacteriocins by a relatively lower heat stability which could indicate the presence of a more elaborate protein structure.

Lactic acid bacteria bacteriocins are generally stable at acid or neutral pH, indicating that the substances are well adapted to the environment of the bacteria producing them. Nisin and lactostrepcins are particularly sensitive to these factors. The maximum solubility and stability of nisin is at pH 2.0, and these parameters decrease significantly as the pH increases. The solubility of nisin decreases from 57 mg/ml at pH 2 to about 1.5 mg/ml at pH 6, which is a considerable disadvantage for the use of nisin as an additive in non-acidic foods (canned foods, cheeses during ripening). This drawback to the use of nisin may be overcome by engineering more soluble molecular forms which are also more resistant to neutral pH. Also, lactostrepcins are stable at pH 4.6–5.0 and are reversibly inactivated at pH 6.0 and higher (Kozak *et al*, 1978).

Biosynthesis and conditions of maximal production

Bacteriocin biosynthesis occurs during or at the end of exponential growth phase (table III). Generally, they are cellular excretion products but a part of antimicrobial activity may be retained within the cell (Kozak *et al*, 1978; Rammelsberg *et al*, 1990). In the case of nisin, excretion occurs when the pH of the culture is unregulated (Hurst, 1966b) or when the culture is regulated at pH 6.0 (Cheeseman and Berridge, 1957), while activity remains inside producing cells grown at pH 6.7–6.8 (Bailey and Hurst, 1971). The fact that nisin synthesis begins only after half the biomass has

Table III. Biosynthesis of bacteriocins and conditions for maximum production.
Biosynthèse des bactériocines et conditions de production maximale.

Bacteriocin	*Biosynthesis*	*Conditions for maximum production*	*References*
Nisin	Starts when half of the biomass has been synthesized Excreted in the medium or cell-associated according to pH	Complex medium buffered at pH 5.9–6.1	Hurst (1981)
Lactostrepcins	Late log phase of growth, 3–5% of intracellular activity	Non-pH-regulated cultures	Kozak *et al* (1978)
Diplococcin	Parallel to growth curve	ND	Davey and Richardson (1981)
Bac	Log phase of growth	Elliker > M17 > milk	Geis *et al* (1983)
Lacticin 481	*Idem*	cultures maintained at pH 5.5 in Elliker broth	Piard *et al* (1990)
Lactocin 27	*Idem* No intracellular activity	Cultures in APT medium held at 37 °C	Upreti and Hinsdill (1973)
Helveticin J	Late log phase of growth	Anaerobic cultures maintained at pH 5.5 in MRS broth	Joerger and Klaenhammer (1986)
Lactacin B	Early log phase of growth	Cultures held at pH 6.0 in MRS broth	Barefoot and Klaenhammer (1984)
Lactacin F	*Idem*	Cultures held at pH 7.0 in MRS broth	Muriana and Klaenhammer (1987)
Plantaricin S	Parallel to growth curve	Cultures maintained at pH 5.0 in MRS broth	Jimenez-Diaz *et al* (1990)
Caseicin 80	2% of intracellular activity	Cultures in tomato juice-medium Increase in caseicin production upon induction with mitomycin C	Rammelsberg *et al* (1990)
Sakacin A	Late log phase of growth	ND	Schillinger and Lücke (1989)
Lactocin S	*Idem*	ND	Mørtvedt and Nes (1990)

ND: not determined
NS : non déterminé

formed and that this bacteriocin contains modified amino acids (see *Elements of composition and/or structure*) suggested that nisin could be formed *via* a multienzyme synthesis process, as is the case for certain bacterial antibiotics such as gramicidin (Consden *et al,* 1947) or bacitracin (Craig *et al,* 1952). Hurst (1966a) in fact showed that chloramphenicol, an inhibitor of RNA synthesis, inhibited nisin synthesis

after 60 min of contact with producing cells. Ingram (1970) later showed that nisin biosynthesis is a 2-step process, the first involving the ribosomal synthesis of pronisin, the second the maturation of this propeptide into active nisin (see *Elements of composition and/or structure*). This biosynthetic pathway has been confirmed recently by genetic studies showing that RNA corresponding to the nisin gene were present in nisin producing cells taken at any stage of growth whereas mature nisin could only be detected in the supernatant after 5 hours of growth (Buchman *et al,* 1988). Some preliminary work (Hurst and Paterson, 1971) suggested that a membrane-associated enzyme catalyzed these reactions. This enzymatic activity appeared only very progressively during the growth of the producing bacteria, which thus explained the late synthesis of nisin. However, these statements have not been confirmed and remain speculative.

With the aim of optimizing bacteriocin purification, several groups have studied the culture conditions of the producing strain which would lead to maximal bacteriocin production (table III). Most authors have noted that bacteriocin production is correlated with the quantity of biomass produced. As a result of this, maximal bacteriocin production could be obtained by supplementing a given culture medium with growth-limiting factors, such as sugars, vitamins, nitrogen sources (Hurst, 1981), by regulating bacterial cultures at a given pH or by choosing the most well adapted culture medium. Concerning this, Geis *et al* (1983) reported that Elliker medium is better for bacteriocin production than M17 or milk. Piard *et al* (1990) observed that Elliker medium buffered with sodium β-glycerophosphate led to higher production of lacticin 481 than M17 medium. The latter authors also assayed lacticin 481 excreted during the experimental manufacture of cheese with *L lactis* 481 Bac^+ (bacteriocin producer) Bac^r (bacteriocin resistant). They showed that bacteriocin production in the milk and later in the cheese curd after coagulation is equivalent to production in synthetic media.

Plantaricin S is produced by *L plantarum* LPCO-10, isolated during the fermentation of olives. Maximal synthesis of plantaricin S occurs in cultures regulated at pH 5.5 in MRS medium (table III) and enables a single peak of antimicrobial activity to be detected. In the presence of 4% salt, however, *Lb plantarum* LPCO-10 successively produces 2 bacteriocins with different properties (Jimenez-Diaz *et al,* 1990). This finding shows the value of working in conditions close to those encountered in the environment of the producing bacterial strain.

In order to facilitate the purification steps, some groups have attempted to use a semi-defined culture medium for bacteriocin production. These attempts have led to highly reduced bacteriocin production in some cases (Geis *et al,* 1983; Zajdel and Dobrezanski, 1983; Rammelsberg *et al,* 1990; Piard *et al,* unpublished results), but in other cases have led to bacteriocin concentrations in the culture supernatant equivalent to those in cultures grown in rich media (Barefoot and Klaenhammer, 1984; Joerger and Klaenhammer, 1986). Based on the postulate that bacteriocin production would be an inducible factor of dominance for a strain competing with other strains (Hirsch, 1952), we examined bacteriocin production by *L lactis* CNRZ 481 Bac^+ Bac^r when the strain was grown in association with another strain, *L cremoris* CNRZ 117 Bac^- Bac^s. No increase in the apparent concentration of bacteriocin in the culture supernatant was detected, even though it was possible that the higher levels of bacteriocin synthesis in these conditions were masked by the adsorption

of a part of the bacteriocin produced on *L cremoris* CNRZ 117 (unpublished results). Also, Upreti and Hinsdill (1973) grew *Lb helveticus* LP27 Bac^+ Bac^r in stressful conditions, at 45 or 55 °C, with no positive effect on lactocin 27 production.

Concerning colicins, it has been reported that bacteriocin producing strains could be induced by treating with mitomycin C or with UV (Hardy, 1975). In this context, Rammelsberg *et al* (1990) observed that treating *Lb casei* B 80 with UV had no effect on bacteriocin production, but that treatment with mitomycin C, on the other hand, induced a 5- to 7-fold increase in caseicin 80 synthesis . The authors did not state, however, whether or not they had checked that the increase in bacteriocin concentrations arose from the release of phage particles. To our knowledge, this report is the first to show this type of phenomenon in lactic acid bacteria. In experiments analogous to that with lacticin 27 (Upreti and Hinsdill, 1973) or with lacticin 481 (Piard, unpublished results), no variations in bacteriocin production were observed after treating producing strains with varying concentrations of mitomycin C. These tests should be extended to other lactic acid bacteria, since if confirmed they would indicate that, as in the case of *E coli* (Salles *et al,* 1987), lactic acid bacteria synthesis of bacteriocins could be induced by the SOS system.

Mode of action

According to Tagg *et al* (1976), the action of bacteriocins on sensitive cells is a 2-step process. The first phase is the adsorption of bacteriocins on specific or non-specific receptors on the cell envelopes of host bacteria. At this time, bacteriocins are sensitive to proteases. The second irreversible phase involves pathological changes in the target cell, specific to each bacteriocin. This process has been described for Gram-positive bacteria but it is not clear whether it is applicable to the bacteriocins of lactic acid bacteria.

Most published work has shown that bacteriocins produced by lactic acid bacteria are non-specifically adsorbed to Gram-positive bacteria, whether they are sensitive or resistant to the bacteriocin studied (Upreti and Hinsdill, 1975; Davey, 1981; Zajdel *et al,* 1985; Bhunia *et al,* 1991). In the case of Gram-negative bacteria, on the other hand, there is no adsorption, as shown by Andersson *et al* (1988) for nisin and by Bhunia *et al* (1991) for pediocin AcH. These data suggest that: i), the activity of bacteriocins does not depend on their specific adsorption to the surface of sensitive cells or/and that: ii), the immunity of Bac^r strains is rather due to the production of an immunity protein than to an alteration of receptor. This is reminiscent of the immunity system of colicins (Konisky, 1982). However, the propensity of bacteriocins from lactic acid bacteria to adsorb non-specifically on cell envelopes could arise from the hydrophobic nature of bacteriocins (see *Purification of lactic acid bacteria bacteriocins*) which would hide the presence of specific receptors. Zajdel *et al* (1985) showed that lactostrepcin 5 is 10 times less active against indicator cells previously treated with trypsin and that it is totally inactive against the protoplasts of sensitive cells. The authors concluded that the cell wall is involved in the antibacterial action of lactostrepcin 5, probably *via* proteinaceous receptors. Inversely, Muriana and Klaenhammer (1991a) reported that lactacin F was bactericidal towards protoplasts, while intact cells of the corresponding strain were immune to the bacteriocin. This suggests that the cell wall is involved in immunity. Bhunia *et al* (1991) observed that the receptor sites for pediocin AcH were not proteins, sugars, lipids or phos-

phate groups. The authors treated cell walls with methanol/chloroform/TCA and extracted a fraction containing lipoteichoic acids (LTA) which seemed to be responsible for the attachment of pediocin AcH. LTA are present in the walls of Gram-positive bacteria and are absent in those of Gram-negative bacteria. The involvement of these acids in the adsorption of pediocin AcH would thus be consistent with the fact that it adsorbs only on Gram-positive bacteria.

With the exception of lactocin 27, which is bacteriostatic towards sensitive strains (Upreti and Hinsdill, 1975), the activity of lactic acid bacteria bacteriocins is *via* a lytic (Andersson *et al,* 1988; Pucci *et al,* 1988; Bhunia *et al,* 1991) or non-lytic bactericidal effect (Kozak *et al,* 1978; Davey, 1981; Barefoot and Klaenhammer, 1983; Piard *et al,* 1990). Their action is rapid, as shown by the decrease in the viable population within several minutes following cell/bacteriocin contact (Barefoot and Klaenhammer, 1983; Zajdel *et al,* 1985; Piard *et al,* 1990) and their lethal effect is higher on exponentially growing cells than on stationary phase cells (Davey, 1981; Zajdel *et al,* 1985). These observations can be explained by the type of alteration caused by bacteriocins. Several authors have reported that bacteriocins cause the leaking of K^+, ATP and sometimes UV absorbing substances from target cells (Upreti and Hinsdill, 1975; Sahl and Brandis, 1983; Zajdel *et al,* 1985; Bhunia *et al,* 1991). The loss of ATP and the efflux of K^+ ions, which have a role in the maintenance of intracellular pH (Otto *et al,* 1983) and thus of the ATP-generating electrochemical gradient in lactic acid bacteria, lead to the exhaustion of energy reserves in sensitive bacteria. These phenomena result in decreased synthesis of macromolecules (DNA, RNA, proteins). These observations suggest that the membrane is the principal bacteriocin target. Considerable work along this line has been done with nisin and lactococcin A. Nisin acts as a depolarization agent on energized bacterial membranes (Ruhr and Sahl, 1985) and creates pores in lipid bilayers (Sahl *et al,* 1987). The formation of pores requires the existence of a negative transmembrane potential (the negative pole must be in the compartment opposite that containing nisin) and/or of a pH gradient (alkaline inside) in the cells or in the bilayers (Gao *et al,* 1991). We have seen above that the antibacterial activity of some bacteriocins was lower in stationary phase indicator cells than in exponentially growing indicator cells. This is consistent with the fact that both the membrane potential and the pH gradient of the former are lower than that of the latter. The ability of nisin to create pores in liposomes clearly established that no membrane receptor is required for activity. The activity of nisin is dependent on the nature of the phospholipids composing the membrane and therefore, the threshold potentials necessary for nisin activity vary from –30 to –100 mV according to the phospholipids used (Sahl *et al,* 1987; Gao *et al,* 1991). Pore sizes vary from 0.2 to 1 nm and their lifetime is several ms to 100 ms. It is probable that the size of nisin oligomers determines the size of the pores formed and thus the size of the molecules which can cross the membrane. Compounds with a molecular weight of 500 Da can cross 1-nm diameter pores.

Lactococcin A acts similarly to nisin in that it also increases the permeability of the cytoplasmic membrane and dissipates the membrane potential of sensitive cells (van Belkum *et al,* 1992b). No lysis of the indicator cells is visible by electron microscopy and therefore the permeabilization of the membrane seems to occur *via* pore formation. In contrast with nisin, and with colicins (Koninsky, 1982), lactococcin A is ac-

tive in absence of a proton motive force. Also, lactococcin A is unable to dissipate membrane potential in liposomes prepared from *L lactis* phospholipids which suggests that, in contrast to nisin, lactococcin A activity is mediated by a receptor present in the membrane of the sensitive cells. The colicins acting by forming ion channels in the cytoplasmic membrane show some similarities with nisin's mode of action. Recently, Pattus *et al* (1990) proposed a model in 3 stages for the mechanism of action of colicin A. The active fragment of colicin A is composed of 10 α helices, 2 of which contain hydrophobic residues only, while the remainder contain a majority of positively charged residues. i) The first step occurs *via* electrostatic interactions of the positively charged domains of colicin A and the polar headgroups of the negatively charged phospholipids. This orientates the hydrophobic hairpin formed by the 2 hydrophobic α-helices so that its axis is perpendicular to the membrane surface. ii) The hairpin penetrates into the membrane leading to a conformational change within the protein which opens like an umbrella, leaving the positively charged domain of the protein on the surface of the bilayer. This is the closed state of the channel. (iii) Upon a *trans*-negative potential, insertion of further helical hairpins occurs leading to an open state of the channel. The molecularity of the proposed model is 3.

The phenomena of lysis observed with some bacteriocins, however, result from another type of mechanism. Nisin has been shown to be able to induce 2 autolytic systems in *Staphylococcus cohnii*, a muramidase-type enzyme and a glucosamidase-type enzyme (Bierbaum and Sahl, 1985). These enzymes bind with LTA by affinity and are thus inhibited. Nisin-type cationic peptides, which also have a high affinity for LTA, compete with these enzymes, which are displaced and thus activated (Bierbaum and Sahl, 1987). These findings could explain those of Henning *et al* (1986), who reported that nisin inhibited murein synthesis. This could in fact involve the induction of a muramidase by nisin. The protective effect of LTA observed in cells exposed to pediocin AcH (Bhunia *et al,* 1991) could thus indicate a similar mechanism of action for this bacteriocin, which also causes lytic phenomena.

Purification of lactic acid bacteria bacteriocins

The purification of several bacteriocins has been performed and the most important findings are listed in table IV. The relatively high heat resistance of bacteriocins has in some cases enabled the active supernatants of cultures to be heat treated, thereby inactivating enzymes which denature bacteriocins (Davey and Richardson, 1981; Piard *et al,* 1990). Most authors have initially used a pre-purification by fractionated precipitation with salts or solvents. In 2 cases, for lacticin 481 (Piard *et al,* 1991) and lactacin B (Muriana and Klaenhammer, personal communication), treating the active supernatants of cultures with high concentrations of ammonium sulphate led to an ≈ 450-fold increase in total biological activity. The reasons for this increase are unknown, but the authors believe that the salt could have an effect both on the dissociation of native bacteriocins into more active monomers and on the conformation of bacteriocins. Several reports have dealt with the propensity of lactobacilli bacteriocins in the native form to form molecular aggregates larger than 100 000 to 300 000 Da (table IV). These aggregates were disrupted by ultrafiltration (Muriana and Klaenhammer, 1991a) or by treatment with SDS (Upreti and Hinsdill, 1973; Joerger and Klaenhammer, 1986; Muriana and

Table IV. Purification and structure data of bacteriocins produced by lactic acid bacteria.
Purification et éléments de structure des bactériocines de bactéries lactiques.

Bacteriocin	*Purification protocol*	*Chemical composition structure data*	*Remarks*	*References*
Nisin	Organic solvent precipitations (1), or counter current distribution (2) or anion exchange chromatography + pH gradient chromatography (3) or hydrophobic interaction chromatography + reversed-phase HPLC (5)	34 Amino acids peptide containing α,β-unsaturated acids, lanthionine (Lan) and β-methyllanthionine (4);	Purified from culture supernatant (1) or from producing cells (3); 5 types of nisin (A,B,C,D,E) differing slightly in amino acid composition (2); nisin Z (5): see text	(1) Cheeseman and Berridge (1957) (2) Berridge *et al* (1952) (3) Bailey and Hurst (1971) (4) Gross and Morel (1971) (5) Mulders *et al* (1991)
Diplococcin	$(NH_4)_2SO_4$ Precipitation + cation exchange chromatography	51 Amino acids (5 300 Da), no Lan or α,β-unsaturated acids, high glycine content	Inactivation of proteinases by heat treatment, purification yield 45%	Davey and Richardson (1981)
Lacticin 481	$(NH_4)_2SO_4$ Precipitation + gel filtration + C_{18}RP-HPLC	21 Amino acids (1 700 Da), contains 1 or 2 Lan per mol; N-terminal sequence: KGGSGVI	Increase (450x) of total biological activity upon treatment with $(NH_4)_2SO_4$, high hydrophobicity of lacticin 481	Piard *et al* (1991)
Bac	Dialysis + gel filtration + adsorption chromatography	Lipocarbohydrate protein, protein 24%, carbohydrate 53%, lipid 21%	Carbohydrate moiety involved in biological activity, uncharged molecule	De Klerk and Smit (1967)
Lactococcin A	$(NH_4)_2SO_4$ Precipitation + cation exchange chromatography + HIC	54 Amino acids (5778 Da), pHi = 9.2; high glycine and alanine content;	Hydrophobic N- and C-terminus, possible membrane-spanning helice	Holo *et al* (1991)
Lactocin 27	Organic solvent precipitations + gel filtration with SDS in the running buffer	Glycoprotein of 12 400 Da, protein: carbohydrate, 1.75:1, high content of glycine and alanine;	Aggregates of more than 200 000 Da before purification, amino acid composition close to that of *Lb fermenti* bacteriocin;	Upreti and Hinsdill (1973)
Helveticin J	$(NH_4)_2SO_4$ Precipitation + gel filtration + SDS–PAGE	Protein of 37 000 Da;	Aggregates of more than 300 000 Da before purification, solubilization using SDS	Joerger and Klaenhammer (1986)
Lactacin B	Cation exchange chromatography + ultrafiltration + gel filtration	Peptide of 6 500 Da, max = 211 nm, minimal absorbance at 280 nm	Lactacin B produced in semi-defined medium, loss of 97% of biological activity upon ion exchange chromatography, disruption of aggregates with urea 8 M	Barefoot and Klaenhammer (1984)
Lactacin F	$(NH_4)_2SO_4$ Precipitation + gel filtration + C_{18}RP-HPLC	Peptide of 57 amino acids (6 300 Da), molecular weight estimated by SDS–PAGE: 2 500 Da	Lactacin F recovered in a floating pellicle formed upon $(NH_4)_2SO_4$ treatment of culture supernatants	Muriana and Klaenhammer (1991a)
Caseicin 80	Ultrafiltration + cation exchange chromatography + gel filtration	Protein of 40 000–42 000 Da, pHi = 4,5	Loss (76%) of caseicin 80 activity upon UF, caseicin 80 inactivated during anion exchange chromatography	Rammelsberg *et al* (1990)
Lactocin S	$(NH_4)_2SO_4$ Precipitation + ion exchange chromatography + HIC + GF + HIC–FPLC + C_{18} RP–HPLC	Peptide of 33 amino acids: MELLPTAAVLYXDVAGXFKYXAKHH + 8 amino acids (A and V);	X are unidentified residues, partial homology of the sequence of lactocin S with the signal sequence of the precursors of 2 other proteins	Mørtvedt *et al* (1991)
Pediocin AcH	$(NH_4)_2SO_4$ Precipitation + GF + anion exchange chromatography	Peptide of 2 700 Da		Bhunia *et al* (1988)

$(NH_4)_2SO_4$ Ammonium sulphate; FPLC/HPLC fast/high performance liquid chromatography; HIC hydrophobic interaction chromatography; GF gel filtration; UF ultrafiltration.

Klaenhammer, 1991a), urea (Barefoot and Klaenhammer, 1984) or Nonidet P-40 (Muriana and Klaenhammer, 1991a).

Further purification of active extracts by ion exchange chromatography led to both good resolution and good recovery of the bacteriocin in the case of nisin (Bailey and Hurst, 1971), of diplococcin (Davey and Richardson, 1981) and of lactococcin A (Holo *et al,* 1991). On the other hand, this is not the method of choice for other bacteriocins such as lactacin B, with only 3% recovery (Barefoot and Klaenhammer, 1984) and lacticin 481 (Piard *et al,* 1992). The choice of the ion exchanged can affect the level of activity recovered, as noted by Rammelsberg *et al* (1990) for caseicin 80, which is totally inactivated after passing through an anion exchange resin but which is satisfactorily purified on a cation exchange resin.

Chromatographic methods involving hydrophobic interaction have been successfully used to purify nisin, lactococcin A and lactocin S. This method is not interesting for certain bacteriocins with elevated hydrophobicity (lacticin 481 and plantaricin S), which in the absence of salt react spontaneously with the gel matrix (Piard *et al,* 1992; Jimenez-Diaz and Piard, unpublished results). Some authors have thus used C18-reversed phase chromatography which is more adapted to hydrophobic molecules. This method was shown to be satisfactory for the final purification of nisin, lactacin F, lacticin 481 and lactosin S (table IV). In order to demonstrate the correlation between the absorbance peak containing the antimicrobial activity and the bacteriocin studied, some groups have applied the same purification protocol to supernatants of cultures of an isogenic strain, cured of Bac^+ Bac^r phenotypes (Muriana and Klaenhammer, 1991a; Piard *et al,* 1992). These extracts exhibited the same elution profile as the active extracts, except for the peak containing antimicrobial activity.

Elements of composition and/or structure

The analysis of amino acid composition and of the structure of lactic acid bacteria bacteriocins has shown that 3 groups of molecules exist: i) lantibiotics, low molecular weight peptides (< 50 amino acids) which contain lanthionine (nisin, lacticin 481; see table IV), ii) low molecular weight peptides without lanthionine (diplococcin, lactococcin A, lactacin F), iii) proteins with molecular weights from 12 400 (lactocin 27) to 42 000 Da (caseicin 80).

Nisin was the first lantibiotic studied. First reports indicated the existence of 5 types of nisin (A, B, C, D and E), which could be produced by the same strain of *L lactis* (table IV). These 5 types were distinguished by their amino acid composition which differed by several residues, as well as by the level of their specific biological activity (Berridge *et al,* 1952). However, these statements have not been confirmed and it is not clear whether nisin B, C, D, E are deleted variants of nisin A (Chan *et al,* 1989) or if different nisin variants can occur. Only recently, a new natural nisin variant, designated nisin Z, was characterized (Mulders *et al,* 1991). Nisin Z has a unique His27Asn as compared with nisin A. Nisin A is a peptide containing 34 amino acids (3 354 Da), with 1 lanthionine (Lan) residue, 4 β-methyllanthionine (β-CH_3Lan) residues and α-, β-unsaturated acids (2-dehydroalanine and 1-dehydrobutyrine) (fig 1). The study of the breakdown products of nisin, $nisin^{1-32}$ (nisin lacking the 2 C-terminal residues) and (des-Ala5) $nisin^{1-32}$ ($nisin^{1-32}$ lacking dehydroalanine in position 5) showed that the biological activity of the first product was comparable to

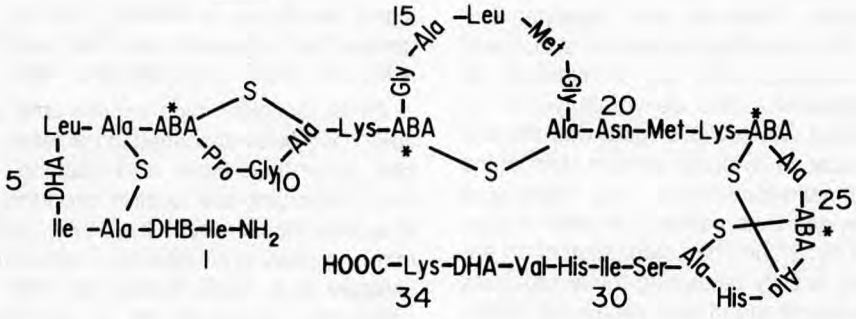

Fig 1. The structure of nisin A. Abbreviations: Abu, aminobutyric acid; Dha, dehydroalanine; Dhb, dehydrobutyrine. Lanthionine and β-methyllanthionine residues are shown in figure 2 (after Gross and Morel, 1971).

La structure de la nisine A. Abréviations : Abu, acide aminobutyrique; Dha, Dehydroalanine; Dhb, dehydrobutyrine; les résidus lanthionine et β-méthyllanthionine sont représentés figure 2 (d'après Gross et Morel, 1971).

that of native nisin, while the second was totally inactive (Chan *et al,* 1989). Dehydroalanine 5 or the corresponding ring is thus apparently one of the determinants for biological activity. These observations are consistent with the characterization of nisinase produced by some strains of *B cereus* and which is believed to be a dehydroalanine reductase (Jarvis and Farr, 1971; see *Tolerance to heat, to acid and sensitivity to enzymes*). Since nisin is synthesized by a ribosomal process (see *Biosynthesis and conditions of maximal production*), Ingram (1969) proposed a synthesis path which could explain the appearance of these residues (fig 2). This path suggests

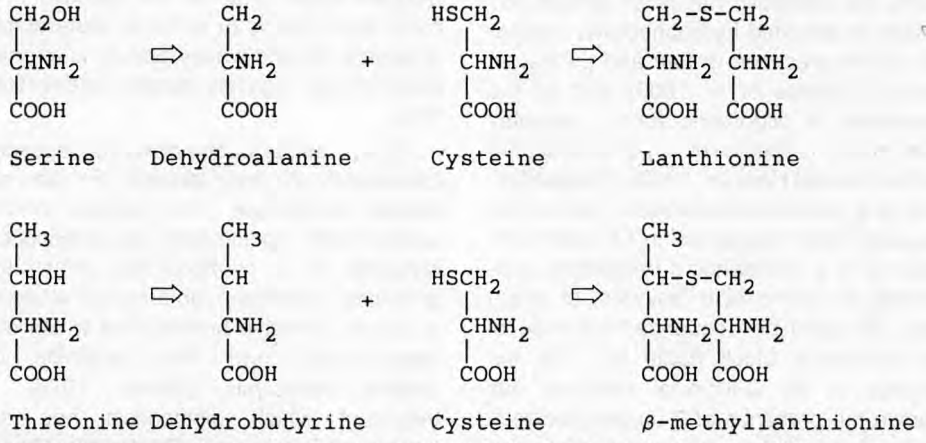

Fig 2. Pathway for synthesis of lanthionine and β-methyllanthionine (after Ingram, 1969).

Voie de synthèse des résidus lanthionine et β-méthyllanthionine (d'après Ingram, 1969).

the synthesis of a nisin precursor containing serine, threonine and cysteine residues. It is possible that serine and threonine residues may be dehydrated to dehydroalanine and dehydrobutyrine, respectively, which would react with the sulfur groups of cysteine to form lanthionine and β-methyllanthionine. This hypothesis for the synthesis pathway of nisin is supported by: i), the characterization of an enzymatic activity catalyzing these reactions in the membrane of nisin producing strains (see *Biosynthesis and conditions of maximal production*); and ii) sequencing the gene coding for the presumed propeptide (see *Genetic determinants of bacteriocins*). This pathway of post-transcriptional modification is now well established.

Lacticin 481 produced by *L lactis* CNRZ 481 (table IV) is another lantibiotic, smaller than nisin (21 amino acids identified and a molecular weight of 1 700 Da, estimated by SDS–PAGE) and whose N-terminal amino acid sequence does not show any resemblance to nisin. The analysis of the amino acid composition has shown the presence of 1 or 2 lanthionine residues per molecule and a majority of hydrophobic or uncharged amino acids. As in the case of nisin, the lantibiotic nature of lacticin 481 results in elevated hydrophobicity, imputable to the presence of Lan and β-CH_3Lan groups (Kaletta *et al,* 1989) and by the presence of oligomeric forms, probably due to the reactivity of α-, β-unsaturated acids (Liu and Hansen, 1990). The production of a lantibiotic bacteriocin, lactocin S, has also been suggested in *Lb sake*. Lactocin S is a hydrophobic bacteriocin containing 33 amino acid residues, of which only 25 could be sequenced because of an N-terminal block (table IV). The sequence of 25 C-terminal residues has shown the presence of 3 unidentified residues, X, which have the same properties as lanthionine. The sequence of lactocin S is unique, but shows homologies with the signal sequences of 2 other proteins, suggesting that a possible target of lactocin S is the membrane (Mørtvedt *et al,* 1991).

Other lantibiotic type antimicrobial peptides have been described in bacterial species other than lactic acid bacteria. The most important are subtilin produced by *B subtilis* (Gross and Kiltz, 1973), epidermin and pep5 produced by *S epidermidis* (Allgaier *et al,* 1986; Weil *et al,* 1990) and gallidermin produced by *S gallinarium* (Kellner *et al,* 1988). The examination of the propeptide sequences of the lantibiotics shows considerable analogies within this group of substances (table V). These observations raise several comments. These substances are produced by taxonomically distant strains and which on the one hand are generally recognized as safe (GRAS) type bacteria *(L lactis)* and on the other hand undesirable or pathogenic bacteria (*Staphylococcus* sp). The antibacterial activity of these peptides differs and is exerted preferentially against strains belonging to species close to the producer. As a result of this, these substances are excellent candidates for structure/function studies. For example, the study of nisin analogues could lead to the discovery of more active forms or of forms more stable at neutral pH and whose activity is exerted preferentially against certain undesirable flora.

Other peptide bacteriocins (diplococcin, lactococcin A and lactacin F) do not contain lanthionine. The analysis of the amino acids constituting the diplococcin produced by *L cremoris* has shown the presence of ornithine, an amino acid usually absent in native proteins but which can nevertheless form from ariginine by alkaline hydrolysis (Gross, 1977), a treatment which diplococcin has not undergone in this case. On the other hand, the amino acid has been detected in

Table V. Comparison of amino acid sequence of the main lantibiotics; producer strain of the lantibiotic: nisin *(Lactococcus lactis)*; subtilin *(Bacillus subtilis)*; epidermin *(Staphylococcus epidermis)*; gallidermin *(Staphylococcus gallinarum)*; Pep 5 *(Staphylcoccus epidermis)*.
Séquence d'acides aminés des propeptides des principaux lantibiotiques; souche productrice du lantibiotique : nisine (Lactococcus lactis)*; subtiline* (Bacillus subtilis)*; épidermine* (Staphylococcus épidermis)*, gallidermine* (Staphylococcus gallinarum)*; Pep 5* (Staphylococcus epidermis).

Lantibiotic	*Amino acid sequence* *
Nisin	I T S I T L C T P G C K T G A L M G C N M K T A T C H C S I H V S K
Subtilin	W K S E S L C T P G C V T G A L Q T C F L Q T L T C N C K I S K
Epidermin	I A S K F I C T P G C A K T G S F N S Y C C
Gallidermin	I A S K F L C T P G C A K T G S F N S Y C C
Pep5	T A G P A I R A S V A Q C Q K T L K A T R L F T V S C K G K N G C K

* Amino acids which are homologous to nisin amino acids are underlined.
Les acides aminés identiques à ceux de la nisine sont soulignés.

gramicidin (Synge, 1945) and in bacitracin (Craig *et al,* 1952), 2 bacterial antibiotics produced by a non-ribosomal multienzymatic process. The modalities of diplococcin synthesis are unknown but they may belong to those of these antibiotics and thus explain the presence of ornithine. Lactacin F produced by *Lb acidophilus* 11088 is a peptide estimated at 2 500 Da by SDS–PAGE and at 6 200 Da by amino acid analysis (table IV). The observed difference arises from the highly hydrophobic nature of the substance (Muriana and Klaenhammer, 1991a). The sequence of the 25 N-terminal residues (RNNWQTNVGGAVGS/
CAMIGATVGGTI...) did not enable Lan or β-CH_3Lan to be identified, an observation which is consistent with the fact that several cysteine and threonine residues are included in the amino acid composition of lacticin F. Sequencing data have nevertheless shown that lactacin F contains one arginine residue at its N-terminal extremity, atypical in non-mature proteins. These data have been confirmed by the study of the gene coding for lactacin F (Muriana and Klaenhammer, 1991b; see *Genetic determinants of bacteriocins*). Lactacin F is a peptide containing 57 amino acids (6 300 Da) with a high proportion (21%) of glycine. Predictions of the secondary structure of non-matured lactacin F show a propensity for an α-helical structure at the N-terminal end, followed by a β-sheet and β-turn structure which could explain the heat stability of lactacin F. Finally the characterization of lactococcin A (table IV) has shown that it is a 54-amino acid peptide rich in alanine and glycine, whose hydrophobicity and structure prediction calculations suggest that it is a membrane peptide.

The last category of bacteriocins includes molecules > 10 000 Da. They are bacteriocins produced by *Lb helveticus* (lactocin 27 and helveticin J), *Lb fermenti* and *Lb casei* (caseicin 80) (table IV). Lac-

tocin 27 and the bacteriocin produced by *Lb fermenti* are glycoproteins and the latter also has a lipid moiety which cannot be dissociated. The carbohydrate fraction is composed of 4 sugars (galactose, glucose, mannose and rhamnose) and is indispensable for the maintenance of biological activity (De Klerk and Smit, 1967). The amino acid composition of these 2 bacteriocins is close and they contain large quantities of glycine, alanine and aspartate.

The comparative analysis of the compositional and structural elements of lactic acid bacteria bacteriocins shows that these molecules exhibit a relatively broad chemical heterogeneity. They are peptides or proteins which may contain cycling Lan or β-CH_3Lan residues and sometimes contain a carbohydrate or lipid moiety. It may nevertheless be asked in the latter case if a sufficient degree of purity had been reached. Indeed, Muriana and Klaenhammer (1991a) reported the presence of lipids combined with lactacin F, but this fraction could be eliminated by reversed phase HPLC. The several publications described above show considerable hydrophobicity of bacteriocins produced by lactic acid bacteria. This results from the high proportion of glycine or from the presence of thioether bridges in the lantibiotics. This hydrophobicity is characteristic of proteins which interact with membranes (von Heijne, 1988) and is thus consistent with observations on the mode of action of certain of these substances (see *Mode of action*).

There is still little data available on the amino acid sequences of bacteriocins, undoubtedly due to the difficulties encountered by several groups when attempting to purify them. Nevertheless, active research is underway in this field and the comparison of the biological and biochemical properties among different structural analogues should enable structure/function relationships to be identified in these molecules.

Genetic determinants of bacteriocins

In their definition of the bacteriocins of Gram-positive bacteria, Tagg *et al* (1976) specified that the genetic determinant of these substances is a plasmid. This is a frequent but not general property of bacteriocins produced by lactic acid bacteria. Table VI lists the most important work carried out on localizing and even characterizing the genes coding for Bac^+ Bac^r phenotypes. Several authors initially noted the instability of Bac^+ Bac^r phenotypes, whose high frequency of spontaneous loss (Fuchs *et al*, 1975; Mørdvedt and Nes, 1990) or loss caused by plasmid curing agents (Kozak *et al*, 1974; Davey and Pearce, 1980; Daeschel and Klaenhammer, 1985; Gonzalez and Kunka, 1987; Hoover *et al*, 1988; Ray *et al*, 1989a) suggested that the genetic determinant was a plasmid. Subsequently, several authors attempted to transfer plasmids bearing genes coding for the Bac^+ Bac^r phenotypes by conjugation. Based on the postulate that these 2 phenotypes are genetically linked, some groups selected transconjugants in a bacteriocin-enriched medium (Gonzalez and Kunka, 1985; Tsai and Sandine, 1987; Ray *et al*, 1989b). This assertion is unjustified, however, as shown by Gonzalez and Kunka (1987) for pediocin PA-1, whose resistance genes are not on the plasmid bearing the production genes. Conjugation phenomena require physical contact between the donor strains (Bac^+ Bac^r) and recipient strains (Bac^- Bac^s) and so there is a bactericidal risk of the donor towards the recipient. To avoid this, donor suspensions were first treated with a protease which degrades bacteriocins (Steele and McKay, 1986), leading to an increased conjugation

Table VI. Genetic determinant of bacteriocins produced by lactic acid bacteria.
Déterminants génétiques des bactériocines de bactéries lactiques.

Bacteriocin	*Plasmids involved in* Bac+	Bac^r	*Plasmid size (MDa)*	*Gene characterization and/or remarks*	*References*
Nisin (Nis)	+/–	+/–	17.5	*Nis* associated with sucrose fermenting abilities, *nis* gene can be plasmid or chromosome-borne due to an insertion sequence adjacent to *nis* gene, *nis* structural gene cloned, sequenced but not expressed.	Tsai and Sandine (1987) Dodd *et al* (1990) Steen *et al* (1991)
Diplococcin	+	+	54	10^{-1} T/donor, 54 MDa plasmid absent from the donor strain.	Davey (1984)
Bac1	+	+	39.6; 75	10^{-4}–10^{-7} T/donor, high homology between plasmids carrying the Bac+ determinants; 3 *bac* genes from the 39.6 MDa plasmid were cloned, expressed and sequenced, high homology between the upstream region of the 3 genes.	Neve *et al* (1984) van Belkum *et al* (1989, 1991a, 1991b) van Belkum (1991)
Bac2	+	+	88	Self-transmissible (Tra+) plasmid, cloning of *bac* gene resulted in higher *Bac* production possibly due to higher plasmid copy number.	Scherwitz *et al* (1983) Scherwitz-Harmon and McKay (1987)
Lactococcin A (LCN-A)	+	+	36	*lcnA* gene cloned, sequenced and expressed using a synthetic DNA probe *lcnA* gene located in an ORF coding for a 75-amino acid precursor including a 21-amino acid extension;	Holo *et al* (1991)
Helveticin J (hlv)	–	–		*hlv* gene cloned using λgt11 and λEMBL3 vectors, sequenced and expressed, possible signal sequence upstream of *hlv* gene.	Joerger and Klaenhammer (1986, 1990)
Lactacin B	–	–		Chromosomic determinant.	Barefoot and Klaenhammer (1983)
Lactacin F (laf)	+	+	52.68	Episomal plasmid, gene cloned, expressed and sequenced, λαφ gene located in an ORF coding for an N-terminal extension.	Muriana and Klaenhammer (1987, 1991)
Plantaricin A	–	–		Same plasmid profile in Bac+ and Bac– derivatives.	Daeschel *et al* (1990)
Plantaricin S	+	+	nd	80% Bac–Bac^r derivatives after novobiocin treatment of the producer.	Jimenez-Diaz *et al* (1990)
Sakacin A	+	+	18	Plasmid curing using acriflavine.	Schillinger and Lücke (1989)
Lactocin S	+	+	32.7	Plasmid prone to instability and deletion.	Mørtvedt and Nes (1990)
Pediocin A	+	+	13.6	Plasmid curing in glucose-limited chemostat cultures.	Daeschel and Klaenhammer (1985)
Pediocin PA-1	+	–	6.2	Plasmid cured Bac– derivatives remained Bac^r.	Gonzales and Kunka (1987)
Pediocin AcH	+	+	7.4	Conjugative plasmid.	Ray *et al* (1989a, b)

T: Transconjugant, ORF: open reading frame.

yield (Broadbent and Kondo, 1991). It is also possible to harvest the donor suspension at the beginning of exponential growth and carry out the conjugation matings on agar in conditions prohibiting bacteriocin production. Muriana and Klaenhammer (1987) used MRS agar at pH 5.5, a pH value which prohibits the production of lactacin F. Recently, Broadbent and Kondo (1991) developed a new, direct plate conjugation method, enabling the Nip$^+$ (nisin production) Suc$^+$ (sucrose fermentation) traits to be transferred with a frequency 4–100 times higher than with conventional methods of conjugation on agar. In some cases, there have been difficulties in correlating the acquisition of Bac$^+$ Bac^r phenotypes with the appearance of a plasmid in transconjugants. In the case of lactacin F, the 2 plasmids of 52 and 68 MDa detected in a Laf$^+$ Laf^r transconjugant (T–E) were absent in the donor strain and were very unstable. Nevertheless, transferring T–E type strains in the presence of lactacin F led to the appearance of a second type of transconjugant (T–89), in which the Laf$^+$ Laf^r phenotypes were maintained in spite of the loss of the 2 plasmids of 52 and 68 MDa (Muriana and Klaenhammer, 1987). The authors suggested that the genetic determinant of lactacin F is chromosomal and it is mobilized from the donor strain in plasmids. These plasmids would be capable of being reintegrated in the chromosome, except in the case of unstable T–E type transconjugants where mobilization would have been defective or incomplete. In the case of nisin, several reports have described the conjugative and simultaneous transfer of the Nip$^+$ Nis^r Suc$^+$ phenotypes in *L lactis*, suggesting that the genes in question are adjacent and on a plasmid, but the acquisition of these characters could not be correlated with the acquisition of a plasmid (Gasson, 1984; Gonzalez and Kunka, 1985; Steele and McKay, 1986). Steele and McKay (1986) ruled out the possibility of the chromosomal integration of the Nip$^+$ Nis^r Suc$^+$ determinants by homologous recombination but did suggest the possibility of transposition or insertion events. Finally, Tsai and Sandine (1987) succeeded in transferring the Nip$^+$ Nis^r Suc$^+$ phenotypes by conjugation in *Lc dextranicum* and successfully correlated this transfer with that of a 17.5-MDa plasmid. Nisin levels produced by the transconjugant were 1 000 times higher than those produced by the donor. These phenotypes, however, could not be preserved after transfers (Sandine, cited by Klaenhammer, 1990).

Following this work on the localization of the genes coding for bacteriocins, several groups began characterizing these genes. The structural gene of nisin could be cloned and sequenced by the use of a synthetic DNA probe corresponding to the sequence of pronisin (Buchman *et al*, 1988; Kaletta and Entian, 1989; Dodd *et al*, 1990). It was localized in an open reading frame (ORF) coding for an N-terminal extension which led to the synthesis of a prepropeptide containing 57 amino acids. The C-terminal portion containing 34 residues matured according to the scheme proposed by Ingram (1970; see *Elements of composition and/or structure*) and facilitated the formation of pronisin by the incorporation of dehydroalanine, dehydrobutyrine, lanthionine and β-methyllanthionine residues. The last step involved the cleavage of 23 amino acids, leading to the release of active nisin (Kaletta and Entian, 1989). By comparing with epidermin, the authors concluded that cleavage occurred at the $Pro^{-2}X^{-1}\backslash\,/Y^{+1}$ site, where X is a basic amino acid and Y is a hydrophobic residue. It is extremely interesting to note the analogies between the N-terminal extension of nisin and those of other lantibiotics (subtilin, epidermin), as well as among their hy-

dropathic profiles (Buchman *et al,* 1988; Kaletta and Entian, 1989). These observations suggest that these antibacterial peptides all evolved from a common origin and that their leader peptide plays a role in maturation. However, the role of this leader peptide is still unknown. It escapes the rules of signal sequences in that it is quite hydrophilic and does not respect the "–3, –1" rule (Von Heijne, 1984, 1988). It can therefore be speculated that nisin secretion relies on the involvement of a translocation protein. The search for maturation enzyme systems and for the nisin immunity system led to the study of regions adjacent to the nisin structural gene. No promotor region has been identified in the region upstream from the *nis* gene but several groups (Buchman *et al,* 1988; Dodd *et al,* 1990; Rauch *et al,* 1991) characterized an open read frame coding for a 253 amino acid protein, having significant homology with *E coli* IS3 transposase and named IS*904*. Gasson *et al* (1990) showed that the *nis* gene is located in a 70-kb element that has the properties of a transmissible transposon. However the role of IS*904* in the mobility of the *nis* gene in the genome is not actually demonstrated since the left end of the transposon is about 200-bp beyond IS*904* and analysis of the opposite (right end) of this element does not reveal the presence of a second copy of IS*904*. The presence of a 70-kb conjugative transposon, encoding production of and immunity to nisin, has been demonstrated recently (Horn *et al,* 1991; Rauch and De Vos, 1992) and it has been clearly shown that the nisin genes are located on the chromosome. This transposon has been found to insert at various locations in the chromosome of the recipient strain. It lacks inverted repeats in its termini and it is flanked by a direct hexanucleotide repeat. Rauch and De Vos (1992) mapped this transposon. It was confirmed that the structure gene for nisin is located in the upstream region of the transposon and that the gene specifying sucrose fermentation was present in the opposite orientation, suggesting that the 2 genes are transcribed separately. Another open reading frame downstream from the *nis* gene codes for a putative protein of 851 amino acids (Dodd *et al,* 1990; Steen *et al,* 1991). This sequence shares homologies with membrane proteins and structural prediction studies have shown that the protein contains several amphipathic α-helices and a membrane attachment helix. This suggests a putative role in nisin processing (Steen *et al,* 1991). This ORF is followed by a putative ρ-independent terminator, suggesting that this is the end of an operon.

Although the mechanisms of immunity exhibited by nisin producing strains have not been characterized, the nisin resistance phenotype (Nis^r) is encountered in lactic acid bacteria not producing nisin. In the lactococci, the Nis^r phenotype coded by resident plasmids of *L diacetylactis* not producing nisin has been cloned (Froseth *et al,* 1988; Simon and Chopin, 1988; von Wright *et al,* 1990). Froseth and McKay (1991) have recently shown that the gene coding for the Nis^r phenotype is borne by a 1.2-kb *Dra*I fragment. The nucleotide sequence showed the presence of a large open reading frame designated *nsr* composed of 957 nucleotides which can code for a 35 035 Da protein containing 318 amino acids. These data agree with *in vitro* transcription–translation studies which have led to the identification of a 37-kDa protein. Analysis of the putative sequence of the protein has shown that it has no homology with known proteins and that it contains a highly hydrophobic region at its N-terminal extremity, analogous to membrane integration proteins.

Hybridization studies have shown that the 1.2-kb *Dra*I fragment does not hybridize with genome DNA of *L lactis* 11454

Nip$^+$ Nis^r, which indicates that the 2 systems of resistance are different. This is consistent with the fact that the nisin resistance conferred by the system described here is only 1/10 that of nisin producing strains and is thus different from the immune system of Nip$^+$ Nis^r strains.

In their study on the bacteriocin-producing character of *L cremoris* 9B4, van Belkum *et al* (1989) cloned and identified 2 regions of p9B4-6, one 1.8-kb fragment coding for a low activity bacteriocin and another 1.3-kb fragment coding for another bacteriocin with higher activity. The analysis of sequences showed that the 1.8-kb fragment contained 3 ORFs (ORF–A1, –A2, –A3) coding for products containing 69, 77 and 154 amino acids respectively, and that the 1.3-kb fragment contained 2 ORFs (ORF–B1 and –B2) coding for 75 and 98 amino acid proteins respectively (van Belkum *et al,* 1991a). It is interesting to note that the regions upstream from ORF–A1 and ORF–B1, as well as the first 20 nucleotides of these 2 ORFs (for a total of 378 bp) are identical. The different activities of the 2 bacteriocins is thus not due to the different strength of promoters. On the other hand, the similarity of these upstream sequences could mean that these operons may move in the genome by transposition events. The organization of the 2 operons was analyzed by mutations/deletions of the ORFs identified. In the first operon, the first 2 ORFs (ORF–A1, –A2) are involved in the Bac$^+$ phenotype, while ORF–A3 specifies immunity. In the second operon, ORF–B1 and ORF–B2 determine bacteriocin production and bacteriocin immunity, respectively. The ORF–B1 product could be detected after migration in an SDS–PAGE gel. An inhibition zone was detected in a position close to 3.4 kDa, to be compared with an expected molecular weight of 8.1 kDa. This indicates that the substance examined exhibited aberrant mobility in SDS–PAGE gels, or that it had matured. The products of ORF–A1 and ORF–B1 had a certain degree of homology and so the authors believed that ORF–A2 could play a role in maturation and/or secretion of the bacteriocin produced by ORF–A1, or that it could form a heterodimer with the ORF–A1 product.

In another study, Holo *et al* (1991) characterized lactococcin A (LCN-A) produced by *L cremoris* LMG 2130. The amino acid sequence and the *lcnA* gene sequence showed that lactococcin A is identical to the bacteriocin coded by ORF–B1 in the prior study. It is a peptide containing 54 amino acids, with a 21 residue N-terminal extension. This leader peptide is composed of a positively charged N-terminal extremity and a hydrophobic zone characteristic of the signal peptides of Gram-positive bacteria. However, it escapes the "–3, –1" rule of von Heijne (1984) concerning signal peptidases.

Analysis of the sequence downstream of the lactococcin A operon showed that a third bacteriocin determinant was present (van Belkum, 1991). A 1.2-kb *CelI* II–*Sca*I fragment carrying the information for production of and immunity to this bacteriocin (designated lactococcin B) was analyzed. Two genes transcribed as an operon were found. Deletion and mutation analyses revealed that the upstream ORF, coding for a 7.5-kDa product and designated *lcnB*, is involved in bacteriocin production and that the second ORF, designated *lciB*, encodes immunity to lactococcin B. Upstream of the first ORF, a stretch of 123 bp appeared almost identical to the nucleotide sequences upstream of lactococcin *M* (the bacteriocin encoded by ORF-A1, –A2) and lactococcin *A* genes. In addition, the 5' nucleotide sequence of *lcnB* shows similarity with the first 20 bp of *lcnMa* (the gene coding for lactococcin M) and with the first 64 bp of *lcnA*. This suggests that the processing

site of these 3 bacteriocins might be at the same position. Using a T7 RNA polymerase-specific promotor, lactococcin B operon could be expressed in *E coli*. SDS–PAGE analysis revealed that the size of lactococcin B was 3.4 kDa when detected from *L lactis* supernatant and 6.5 kDa when isolated from *E coli* lysates. This suggest that: i) *E coli* is unable to process the bacteriocin precursor; ii) processing is not necessary for biological activity of lactococcin B. From comparison with lactococcin A, processing of pre-lactococcin B was assumed to remove the N-terminal 21 amino acids. A construct was made by deleting the 5' part of *lcnB* to obtain the mature lactococcin B as the primary translation product. No expression of antagonistic activity was detected in cells or lysates of *E coli* or *L lactis*. This suggests that the N-terminal extension is necessary either for gene expression or for maturation to an active product.

Muriana and Klaenhammer (1991b) recently cloned the *laf* gene encoding lactacin F. As in the studies discussed above (van Belkum *et al*, 1989; Holo *et al*, 1991), the phenotype is not expressed in *E coli* but a synthetic DNA probe was used to screen clones having acquired an insert bearing the *laf* gene. The gene sequence showed the presence of an ORF coding for lactacin F plus an N-terminal extension of 18 amino acids. This sequence had the hydropathy characteristics of signal sequences and complied with the "–3,–1" rule. Even though immune functions were coded by the cloned 2.2-kb fragment, an ORF corresponding to these functions remains to be identified.

The genetic determinant of helveticin J borne by the chromosome of *Lb helveticus* 481 was recently characterized (Joerger and Klaenhammer, 1990). A gene bank was created in λgt11 and a polyclonal anti-helveticin J antibody was used to screen phages producing β-galactosidase-helveticin fusion proteins. Two recombinant phages (HJ1 and HJ4), containing 350- and 600-bp inserts respectively, specifically hybridized with the genomic DNA of the helveticin producing strain. The 600-bp insert of HJ4 was used as a probe to screen a chromosomal DNA bank from *Lb helveticus* 481, prepared in EMBL3. Sequencing a 3364-bp fragment of DNA showed the presence of 2 ORFs (ORF2 and ORF3), of which ORF3 could code for a 37 511 Da protein, possibly helveticin J since its molecular weight after purification was estimated to be 37 000 Da (Joerger and Klaenhammer, 1986). Inserts of HJ1 and HJ4 are localized in this ORF. Upstream from ORF3, ORF2 could code for a 11 808 Da protein, with an N-terminal region characteristic of signal peptides. Signals –10 and –35 of a putative promoter and a possible ribosome binding site were identified upstream from ORF2, suggesting that these 2 ORFs are transcribed as an operon. As a result of this, this product could be an immunity and a transport protein, as is the case for certain colicins (Konisky, 1982). Helveticin J is not expressed in *E coli*, but cloning a fragment containing the 2 ORFs in *Lb acidophilus* led to the excretion of a bacteriocin having the same properties as helveticin J.

Finally, the genetic organization of pediocin PA-1 biosynthesis genes was recently presented in a meeting (Marugg *et al*, personal communication).

CONCLUSION

In addition to their capacity to produce organic acids and hydrogen peroxide (Piard and Desmazeaud, 1991), lactic acid bacteria can produce antimicrobial peptides or proteins. The development of our know-

ledge of these substances may lead to an additional increase in the role of the microorganisms in the preservation of foods.

Considerable effort has recently been focussed on the understanding of the structure, the genetic organization and the mode of action of several bacteriocins. There has been a concomitant development in the description of new bacteriocins, whose biochemical and genetic characterization should lead to the discovery of important elements for the elucidation of structure/function relationships in these substances.

The ideal alimentary antimicrobial substance should respond to the following requirements: i) a spectrum of activity directed against harmful flora; ii) physicochemical properties enabling it to resist heat treatments and pH changes encountered in the food industry; iii) a small size, consistent with rapid diffusion in semi-solid systems, which characterize most alimentary products. Among the bacteriocins described above, several respond individually to 1 or 2 of these criteria and may thus constitute good material for study and comparison. The field of action for modifying these substances is broad, notably as a result of maturation systems such as those encountered in the lantibiotics and which circumvent the restrictions imposed by the genetic code for the incorporation of modified amino acids (Buchman *et al,* 1988).

Certain domains remain to be further explored. We have only little data available on the mode of action of bacteriocins and none whatsoever on the systems conferring immunity to these substances. These data are necessary if we want to understand the elements governing the specificity of the spectrum of bacteriocins better and also to be able to render useful lactic acid flora immune.

The current interest in the study of bacteriocins is proportional to the stakes represented by these substances. Several sectors of the food industry are preoccupied with the possibility of being able to offer products with increased hygienic qualities. In addition, these "natural" substances, biodegradable in the human digestive tract, are good candidates for replacing certain usual antibiotics used for pharmaceutical purposes.

Egg-white lysozyme is another antibacterial agent, active against *Listeria monocytogenes*, *Clostridium* sp and *B stearothermophilus* (Hughey and Johnson, 1987). The genetic determinant of this enzyme was recently cloned in *L lactis* (van de Guchte *et al,* 1989). It is thus another potential inhibitor whose utilization could be facilitated by the possibility of obtaining resistant variants in lactic acid bacteria (Neviani *et al,* 1991; Veaux *et al,* 1991).

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