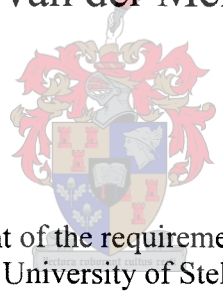
CHARACTERIZATION OF THOENIICIN 447 PRODUCED BY *PROPIONIBACTERIUM THOENII*

by

Iansha van der Merwe



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DECLARATION

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

I.R. van der Merwe

SUMMARY

Antimicrobial peptides continue to be one of the most important classes of food additives. The food industry is especially interested in the application of naturally occuring and biologically derived preservatives. Among the metabolites of industrial importance produced by propionibacteria are peptides called bacteriocins. Bacteriocins are ribosomally synthesized peptides with antagonistic activity against closely related microorganisms. Many microorganisms associated with food produce bacteriocins, which have stimulated interest in the use of these peptides as natural food preservatives. Numerous bacteriocins are produced by lactic acid bacteria, but only a few have been reported for propionibacteria. Since propionic acid bacteria have GRAS (generally regarded as safe) status, their metabolic compounds should be safe for human consumption.

Propionibacterium thoenii 447, isolated from Emmentaler cheese, produces a bacteriocin-like peptide, named thoeniicin 447, with a narrow spectrum of activity. The peptide displays a bactericidal mode of action against *Lactobacillus delbrueckii* subsp. *bulgaricus* and a bacteriostatic action against *Propionibacterium acnes.*

Optimal bacteriocin production was detected during the early stationary growth phase. The peptide is resistant to heat treatments of 60°C and 80°C for 15 and 30 min and to 100°C for 15 min, but loses 80% of its activity after autoclaving (10 min at 121°C). Thoeniicin 447 remains active after incubation in buffers with pH values ranging from 1-10. The peptide is inactivated by pepsin, pronase, α-chymotrypsin, trypsin and Proteinase K. Thoeniicin 447 was partially purified by ammonium sulfate precipitation, followed by SP-Sepharose cation exchange chromatography. The estimated size of thoeniicin 447, according to tricine-SDS-PAGE, is approximately 6 kDa. Based on DNA sequencing, the mature peptide is 7130 Da in size and homologous to propionicin T1 produced by *P. thoenii* strain 419.

Thoeniicin 447 is a relatively small, cationic and heat-stable peptide and can therefor be classified as a member of class II bacteriocins. These features are very similar to those of bacteriocins produced by lactic acid bacteria. However, no unique classification system has been proposed for bacteriocins of propionibacteria.

As a member of the genus *Propionibacterium, P. thoenii* 447 is generally regarded as safe. This, together with the narrow spectrum of activity, particularly the action against *P. acnes,* heat tolerance of thoeniicin 447 and its activity over a wide pH range renders the peptide suitable for possible pharmaceutical applications.

OPSOMMING

Antimikrobiese middels sal deurgaans beskou word as een van die belangrikste klasse van voedsel bymiddels. Die voedselindustrie is veral geïnteresseerd in die toepassing van preserveermiddels van 'n meer natuurlike en biologiese oorsprong. Onder die metaboliese produkte van industriële belang wat deur propionibakterieë geproduseer word is antimikrobiese peptiede (bakteriosiene). Bakteriosiene is ribosomaal-gesintetiseerde peptiede met 'n antagonistiese aktiwiteit teenoor naverwante bakterieë. Verskeie bakteriosiene word deur melksuurbakterieë geproduseer, terwyl slegs enkele vir propionibakterieë beskryf is. Baie van hierdie propionibakterieë word in die algemeen as veilig beskou en het GRAS status. Die metaboliete wat hulle produseer behoort dus veilig vir menslike gebruik te wees.

Propionibacterium thoenii 447 is uit Emmentaler kaas geisoleer en produseer 'n bakteriosien-agtige peptied, naamlik thoeniicin 447 met 'n beperkte spektrum van aktiwiteit. Die peptied het 'n bakteriosidiese werking teenoor *Lactobacillus delbrueckii* subsp. *bulgaricus* en 'n bakteriostatiese werking teenoor *Propionibacterium acnes*.

Optimum bakteriosien produksie is verkry tydens die vroeë stationêre groeifase. Die peptied is bestand teen hittebehandelings van 60°C en 80°C vir 15 en 30 min, asook 100°C vir 15 min, maar verloor 80% van sy aktiwiteit na outoklavering (10 min by 121°C). Die peptied bly aktief na inkubasie in buffers van pH 1-10. Die peptied word deur pepsien, pronase, α-chymotripsien, tripsien en Proteinase K geïnaktiveer.

Thoeniicin 447 is met behulp van ammoniumsulfaat-presipitasie, gevolg deur SP-Sepharose katioon-uitruilchromatografie gedeeltelik gesuiwer. Skeiding op `n trisien-SDS poliakrielamied-jel het `n aktiewe band van ongeveer 6 kDa getoon. Volgens die DNA volgorde bepaling is thoeniicin 447, 7130 Da in grootte en homoloog aan Propionicin T1, geisoleer vanaf *P. thoenii* stam 419.

Thoeniicin 447 is 'n relatiewe klein, kationiese en hitte-bestande peptied en kan op grond hiervan as 'n lid van die klas II bakteriosiene geklassifiseer word. Hierdie eienskappe is soortgelyk aan die eienskappe van bakteriosiene geproduseer deur melksuurbakterieë. Tot op hede is geen klassifikasiesisteem vir die bakteriosiene van propionibakterieë voorgestel nie.

As 'n lid van die genus *Propionibacterium*, word *P. thoenii* 447 in die algemeen as veilig beskou. Dit, tesame met die nou spektrum van aktiwiteit, veral teenoor *P. acnes*, die hittetoleransie van thoeniicin 447, asook die aktiwiteit oor 'n wye pH-grens, maak die peptied geskik vir moontlike farmaseutiese toepassings.

BIOGRAPHICAL SKETCH

Iansha van der Merwe was born on 10 November 1975 in Cape Town. She matriculated at Klein Nederburg Senior Secondary School, Paarl, in 1993. She enrolled at the University of Stellenbosch in 1994 as a B.Sc. student, obtaining her degree in 1998, majoring in Microbiology and Psychology. She completed her B.Sc. (Hons.) degree the following year at the same institute.

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DEDICATION

I dedicate this thesis to my mother.

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CHAPTER 1

INTRODUCTION

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INTRODUCTION

Increased public interest in natural foods and the ongoing outcry against chemical additives shifted the focus of research in the field of food preservation towards natural antimicrobial compounds (Klaenhammer, 1993). Since bacteriocins are natural antimicrobial peptides, and those produced by lactic acid bacteria with GRAS (generally regarded as safe) status regarded as safe for human consumption, they have been targeted as a possible alternative to many chemical food preservatives. Nisin, a lantibiotic produced by *Lactococcus lactis*, is the best known bacteriocin and has been accepted as a preservative in more than 50 countries (Klaenhammer, 1993).

Propionic acid bacteria play an important role in the development of the characteristic flavour and eye formation in Swiss-type cheeses (Sherman and Shaw, 1921). Since these organisms have been consumed by humans and animals for centuries without any adverse effect, they have been classified as GRAS. Fermentation metabolites of *Propionibacterium shermanii* have been used for years to control spoilage caused by *Pseudomonas* spp. and coliforms (Salih et al., 1989; Al-Zoreky et al., 1993). Unlike Nisin, the metabolites produced by propiobibacteria are effective against a variety of Gram-negative bacteria, yeasts and molds and have been used as preservatives in cheese, yoghurt, salad dressings, bakery products, fresh pasta and meats.

Propionibacteria may survive in the gastro-intestinal tract of humans and animals (Montere-Alhonen, 1995) and may even produce bacteriocins active against other intestinal bacteria (Stiles, 1996). One example is the use of dairy propionibacteria, in combination with lactic acid bacteria, in the treatment of intestinal disorders in humans (Sidorchuk and Bondarenko, 1984).

Since bacteriocins are by definition active against species closely related to the producer organism, bacteriocins produced by propionibacteria may also be used in the treatment of acne caused by *Propionibacterium acnes*. *P. acnes* resides in the sebaceous follicles of the human skin (Leyden et al., 1998) and causes the skin disorder acne vulgaris, which commonly occurs during adolescence (Toyoda and Morohashi, 2001). Treatment of acne is usually through antibiotics, either systematically or topically (Eady, 1998). As can be expected, many antibiotic resistant strains of *P. acnes* have been reported.

The aim of this study was to screen several strains of *Propionibacterium* spp. for the production of antimicrobial peptides and to determine if these bacteriocins exhibit antibacterial activity towards *P. acnes*. One such bacteriocin, produced by *Propionibacterium thoenii* 447, was isolated, purified, characterized and named thoeniicin 447.

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CHAPTER 2

THE GENUS *PROPIONIBACTERIUM*

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1. IMPORTANCE OF PROPIONIC ACID BACTERIA

Propionic acid bacteria (PAB) are divided into two groups based on their habitat (Cummins and Johnson, 1986). Species of the first group, the classical or dairy propionibacteria, have been used in the production of cheese since 9000 BC. The main habitat of classical propionibacteria is hard cheese, but they have also been isolated from spoiled olives, spoiled orange juice (Kusano et al., 1997) and anaerobic sewage (Riedel and Britz, 1993). Apart from the production of propionic acid, they produce vitamin B_{12} (Hettinga and Reinbold, 1972c; Janicka et al., 1976; Vorobjeva and Iordan, 1976) and have been used in the production of bread (Spicher, 1983) and in veterinary and medical preparations.

The classical strains have a long history of application in industrial fermentations and dairy products (Rehberger and Glatz, 1990). They play an important role in the development of the characteristic flavour and eye formation in Swiss-type cheeses (Sherman and Shaw, 1921). The sweet flavour in cheese is partly due to proline production, whilst the buttery flavour is caused by the production of diacetyl (Langsrud et al., 1977, 1978).

Apart from their role in dairy fermentations, propionibacteria play a significant role in the production of organic acids for industrial uses, e.g. propionic acid in the production of cellulose plastics, herbicides and perfumes. Propionic acid also acts as a mold inhibitor in silage and grains with a high moisture content (Boyaval et al., 1995).

Certain strains of propionibacteria or their products have medical applications, e.g. nucleotide derivatives that may be used in the prevention and treatment of human thrombotic diseases (Vorobjeva, 1999). Some strains of dairy propionibacteria are resistant to bile and tolerate pH values as low as 2.0, e.g. gastric juice (Perez-Chaia et al., 1999). Viable cells of *Propionibacterium acidipropionici* CRL 198 significantly reduced β-glucoronidase activity and other activities involved in the generation of tumor promoters, carcinogens and mutagens produced by resident flora.

Group two of the genus *Propionibacterium* contains the cutaneous species, also known as anaerobic coryneforms (Cummins and Johnson, 1986). This group is found in a different habitat than the so-called classical group and can be distinguished by a number of

characteristics, notably the pathogenicity of the cutaneous strains. The human skin and intestinal tract are the main habitat of cutaneous propionibacteria. They are often isolated from facial blackheads and, although less frequently, from wounds, bone marrow, and tissue abscesses (Cummins and Johnson, 1986).

2. TAXONOMY OF PROPIONIC ACID BACTERIA

Propionibacteria are Gram-positive, non-sporulating, non-motile, and facultatively anaerobic or aerotolerant (Cummins and Johnson, 1986). Propionic acid and acetic acid are produced from the fermentation of various sugars and lactic acid (Langsrud and Reinbold, 1973).

Propionic acid bacteria are included in the genus *Propionibacterium*, which together with *Eubacterium,* comprises the family *Propionibacteriaceae* (Cummins and Johnson, 1986; Moore and Holdeman, 1986). The dairy species include *Propionibacterium freudenreichii, Propionibacterium jensenii*, *Propionibacterium thoenii, Propionibacterium acidipropionici, Propionibacterium cyclohexanicum* and *Propionibacterium microaerophilicum* (Tables 2a and 2b). Members of the second group, the cutaneous propionibacteria, usually occur on human skin and include species such as *Propionibacterium acnes, Propionibacterium granulosum, Propionibacterium avidum, Propionibacterium lymphophilum* and *Propionibacterium propionicum* (previously *Arachnia propionica*) (Tables 2c and 2d). All the latter species, except *P. propionicum* have been transferred from the genus *Corynebacterium* (Douglas and Gunter, 1946). *Propionibacterium propionicum* has been transferred from the genus *Actinomyces* based on results obtained by 16S rRNA sequence analyses (Charfreitag et al., 1988). The organisms transferred from the genus *Corynebacterium* are anaerobes (most corynebacteria are aerobes) and produce propionic acid as main metabolite (Douglas and Gunter, 1946). The peptidiglycan in their cell wall contains mainly L-diaminopimelic acid (L-DAP), whereas related forms such as aerobic corynebacteria contains meso-DAP. The iso- and anteiso-C_{15} saturated acids are the main fatty acids of cellular lipids and, unlike aerobic corynebacteria, anaerobic species do not contain mycolic acids and arabinogalactan. The closest relative of the genus *Propionibacterium* is *Luteococcus japonicus,* followed by *Luteococcus coccoides,* previously *Propionibacterium coccoides* (Vorobjeva et al., 1983). *Luteococcus coccoides* share many common features with propionibacteria. However, Britz and Riedel (1995) proved that *L. coccoides* are closer related to the genus *Luteococcus*. Due to the close relationship between the various species,

Table 2a. Characteristics of dairy *Propionibacterium* spp.

Characteristic	*Propionibacterium freudenreichii*[a]	*Propionibacterium jensenii*[a]	*Propionibacterium thoenii*[a]	*Propionibacterium acidipropionici*[a]	*Propionibacterium cyclohexanicum*[b]	*Propionibacterium microaerophilicum*[c]
Type strain	ATCC 6207	ATCC 4868	ATCC 4874	ATCC 25562	TA-12	M5
Habitat	Raw milk, Swiss cheese and other dairy products	Dairy products, silage and occasionally from infected lesions	Cheese and other dairy products	Dairy products	Pasteurized, spoiled orange juice	Olive mill wastewater
Catalase	+[d]	d+	+	d+	-	-
Relationship to oxygen	Anaerobic to aerotolerant	Anaerobic to aerotolerant	Less strictly anaerobic	Anaerobic to aerotolerant	Aerotolerant	Microaerophilic facultative anaerobic
pH range for growth	4.5-8.5	ND	ND	ND	3.2-7.5	4.5-9.5
Optimum growth temp (°C)	30-32	30-32	30-32	30-32	35	30
Metabolic products	Propionic acid, acetic acid	Propionic acid, acetic acid	Propionic acid, acetic acid	Propionic acid, acetic acid	Lactic acid, propionic acid, acetic acid	Propionic acid, acetic acid
G+C content (mol%)	64-67	65-68	66-67	66-68	66.8	67.7
Sugars in polysaccharide[e]	Galactose, mannose, rhamnose	Glucose, galactose, mannose	Glucose, galactose	Galactose, glucose, (mannose)	Glucose, galactose, mannose, rhamnose, ribose	ND
Amino acids in cell wall	Alanine, glutamic acid, meso-DAP	Alanine, glutamic acid, glycine, L-DAP	Alanine, glutamic acid, L-DAP	Alanine, glutamic acid, glycine, L-DAP	Alanine, glutamic acid, meso-DAP	ND

[a] Data from Cummins and Johnson (1986)

[b] Data from Kusano et al. (1997)

[c] Data from Koussémon et al. (2001)

[d] -, 90% or more of the strains are negative; +, 90% or more of the strains are positive; d+, 40 to 90% of the strains are positive; d-, 10 to 40% of the strains are positive

[e] Sugars in parenthesis are absent in some strains

ND, no data available

Table 2b. Acid formation from various carbon sources by dairy *Propionibacterium* spp.

Fermentation of carbon source	*Propionibacterium freudenreichii*[a]	*Propionibacterium jensenii*[a]	*Propionibacterium thoenii*[a]	*Propionibacterium acidipropionici*[a]	*Propionibacterium cyclohexanicum*[b]	*Propionibacterium microaerophilicum*[c]
Glycerol	+[d]	+	+	+	+d	ND
Erythritol	+	+	d+	+	-	ND
D-Arabinose	+, -	+	+	+	-	-
Ribose	d+	+	+	+	-	ND
D-Xylose	-	d+	d+	d+	-	-
Adonitol	d+	d+	d+	+	-	ND
Galactose	+	+	+	+	+	ND
D-Glucose	+	+	+	+	+	ND
D-Fructose	+	+	+	+	+	-
D-Mannose	+	+	+	+	+	ND
L-Sorbose	-	-	-	d+	-	+
Rhamnose	-	-	-	-	-	+
Dulcitol	-	-	-	-	-	ND
Inositol	d+	d+	d+	+	-	+
Mannitol	-	+	-	+	-	+
Sorbitol	-	-	d+	+	-	+
Amygdalin	-	d+	d+	-	+	-
Esculin	-	-	+	d+	+	-
Salicin	-	+	d+	+	+	ND
Cellobiose	-	d-	-	+	+	ND
Maltose	-	d+	d+	+	+	ND
Lactose	d-	d+	d-	+	+	-
Melibiose	d-	+	d+	d+	+/-	-
Saccharose	-	+	d+	+	+	ND
Trehalose	-	+	+	+	+	ND
Inulin	-	-	-	-	-	ND
Melezitose	-	d+	d+	+	+	+
D-Raffinose	-	+	d+	d-	-	-
Starch	-	-	+	+	-	+
Glycogen	-	-	d+	-	-	+

[a] Data from Cummins and Johnson (1986)

[b] Data from Kusano et al (1997)

[c] Data from Koussémon et al (2001)

[d] +, 90% or more of the strains are positive; -, 90% or more of the strains are negative; d, 11 to 89% of the strains are positive; d+, 40 to 90% of the strains are positive; d-, 10 to 40% of the strains are positive

ND, no data available

Table 2c. Characteristics of cutaneous *Propionibacterium* spp.

Characteristic	*Propionibacterium acnes*[a]	*Propionibacterium granulosum*[a]	*Propionibacterium avidum*[a]	*Propionibacterium lymphophilum*[a]	*Propionibacterium propionicum*[b]
Type strain	ATCC 6919	ATCC 25564	ATCC 25577	ATCC 27520	ATCC 14157
Habitat	Human skin, comedones of acne vulgaris, intestinal contents, wounds, blood, pus and soft tissue abscesses	Human skin (oily areas), Acne comedones	Vestibule of nose, axilla, perineum, infected sinuses	Urinary tract infections, mesenteric ganglion (monkey), human Hodgkin lymphoma	Human mouth (dental plaque), cervical smears, various organs infected by actinomycoses
Catalase	d +[c]	+	+	d +	-
Relationship to oxygen	Anaerobic to aerotolerant	Anaerobic or microaerophilic	Anaerobic or microaerophilic	Anaerobic	Facultative anaerobic
pH range for growth	ND	ND	ND	ND	ND
Optimum growth temp (°C)	36-37	36-37	36-37	36-37	35-37
Metabolic products	Propionic acid, acetic acid	Propionic acid, acetic acid	Propionic acid, acetic acid	Propionic acid, acetic acid	Lactic acid, propionic acid, acetic acid and succinic acid
Major respiratory quinone	MK-9(H_4)[d]	MK-9(H_4)	MK-9(H_4)	MK-9(H_4)	MK-9(H_4)
G+C content (mol%)	59-60	61-63	62-63	53-54	63-65
Major fatty acid	Branched	Branched	Branched	Branched	Straight
Sugars in polysaccharide[e]	Glucose, mannose, (galactose)	Galactose, mannose	Glucose, mannose, (galactose)	Galactose, glucose, mannose	Glucose, galactose, mannose,
Amino acids in cell wall	Alanine, glutamic acid, glycine, L/meso-DAP	Alanine, glutamic acid, glycine, L-DAP	Alanine, glutamic acid, glycine, L/meso-DAP	Alanine, glutamic acid, lysine	Glycine, glutamic acid, alanine, L-DAP

[a] Data from Cummins and Johnson (1986)

[b] Data from Charfreitag et al. (1988) and Schaal (1986)

[c] -, 90% or more of the strains are negative; +, 90% or more of the strains are positive; d, 11 to 89% of the strains are positive; d +, 40 to 90% of the strains are positive; d -, 10 to 40% of the strains are positive

[d] Data from Jones and Collins (1986)

[e] Sugars in parenthesis are absent in some strains

ND, no data available

Table 2d. Acid formation from various carbon sources by cutaneous *Propionibacterium* spp.

Fermentation of carbon source	*Propionibacterium acnes*[a]	*Propionibacterium granulosum*[a]	*Propionibacterium avidum*[a]	*Propionibacterium lymphophilum*[a]	*Propionibacterium propionicum*[b]
Glycerol	d+[c]	+	+	-	d
Erythritol	d+	-	+	+	d
D-Arabinose	-	-	d+	-	-
Ribose	d+	d-	d+	+	d
D-Xylose	-	-	d-	-	-
Adonitol	d+	-	d+	+	d
Galactose	d+	d-	+	-	d
D-Glucose	d+	+	+	+	+
D-Fructose	d+	+	+	+	+
D-Mannose	d+	+	+	-	d
L-Sorbose	-	-	-	-	-
Rhamnose	-	-	-	-	-
Dulcitol	-	-	-	-	-
Inositol	d-	-	d+	d+	d
Mannitol	d-	d+	d-	-	+
Sorbitol	d+	-	-	-	d
Amygdalin	-	d-	-	-	d
Esculin	-	-	-	-	ND
Salicin	-	-	d+	-	d
Cellobiose	-	-	-	-	-
Maltose	-	d+	+	+	+
Lactose	-	-	d+	-	d
Melibiose	-	d-	d+	-	d
Saccharose	-	+	+	d-	+
Trehalose	-	d+	+	-	d
Inulin	-	-	-	-	-
Melezitose	-	d-	d+	-	-
D-Raffinose	-	d+	d+	-	+
Starch	-	-	-	d+	d
Glycogen	-	-	-	-	-

a Data from Cummins and Johnson (1986)

[b] Data from Charfreitag et al. (1988) and Schaal (1986)

[c] +, 90% or more of the strains are positive; -, 90% or more of the strains are negative; d, 11 to 89% of the strains are positive; d+, 40 to 90% of the strains are positive; d-, 10 to 40% of the strains are positive

ND, no data available

species, differentiation is difficult and separation cannot only rely on the more traditional morphological and physiological differences (Cummins and Johnson, 1986).

Methods for phenotypic and phylogenetic classification include SDS-PAGE of whole cell protein patterns (Baer, 1987), 16S rDNA targeted PCR-RFLP (Riedel et al., 1994), ribotyping (De Carvalho et al., 1994; Riedel and Britz, 1996), 16S and 23S rRNA sequence analyses (Rossi et al., 1997), pulsed field gel electrophoresis (PFGE) (Gautier et al., 1996), randomly amplified polymorphic DNA (RAPD)-PCR and conventional gel electrophoresis restriction endonuclease analysis (CGE-REA) (Rossi et al., 1998). The latter two techniques are very reliable and allow for useful intraspecific differentiation. The DNA base composition of classical propionibacteria is 65-67 mol% G+C, while cutaneous bacteria contains 53-62 mol% G+C (Johnson and Cummins, 1972).

2.1 Propionibacteria from dairy origin

The classical propionibacteria are anaerobic to aerotolerant or microaerophilic (Cummins and Johnson, 1986). Colonies are usually shiny, round or granular, bright and oily. Propionibacteria differ from other bacteria by a peculiar palisade-like arrangement of the cells, sometimes forming short curved chains and hieroglyph-like patterns (Vorobjeva and Iordan, 1976). Their main habitat is hard rennet cheese, but they are also found in other natural fermentations, e.g. in silage and fermenting olives (Plastourgos and Vaughn, 1957) and soil (Van Niel, 1957). More than 60% of propionibacteria isolated from Emmental cheese in Finland have been classified as *P. freudenreichii* subsp. *shermanii* (Meriläinen and Antila, 1976).

2.1.1 *Propionibacterium freudenreichii*

Propionibacterium freudenreichii is usually isolated from Swiss-type cheese, raw milk and other dairy products (Table 2a). Large amounts of propionic acid are produced that confers a specific aroma to cheese. The strains produce large amounts of free proline that are particularly associated with the flavour of Swiss type cheeses (Langsrud et al., 1977, 1978). The cells are generally very short rods, often almost coccal (Cummins and Johnson, 1986). The major fatty acid produced by *P. freudenreichii* is 12-methyltetradecanoic acid (Moss et al., 1969). The major sugars in the peptidoglycans are galactose and mannose (Johnson and Cummins, 1972). Rhamnose is also present, but in smaller amounts and glucose is completely

absent. *Propionibacterium freudenreichii* ferments a number of carbohydrates (Table 2b). The DNA base composition is 64-67 mol% G+C (Johnson and Cummins, 1972). Three subspecies are distinguished on the basis of lactose fermentation and nitrate reduction, viz., *P. freudenreichii* subsp. *freudenreichii, P. freudenreichii* subsp. *shermanii* and *P. freudenreichii* subsp. *globosum.*

2.1.2 *Propionibacterium jensenii*

Propionibacterium jensenii is usually isolated from milk products and silage, and occasionally from infected lesions (Table 2a). The cell wall contains L-DAP, while glucose is the major cell wall sugar, with trace amounts of galactose and mannose (Johnson and Cummins, 1972). The major fatty acid is 13-methyltetradecanoic acid. The G+C content is 65-68 mol%. Pantothenate and biotin are required for growth, while some strains also require *para*-amino benzoic acid (Delwiche, 1949).

2.1.3 *Propionibacterium thoenii*

This species was originally isolated from Emmentaler cheese, but have also been isolated from other cheeses and dairy products (Table 2a). The cells usually form an orange or brownish red colony on solid agar. *Propionibacterium thoenii* causes hemolysis of human, cow, pig, sheep and rabbit blood. The cell wall contains L-DAP and the cell wall sugars glucose and galactose (Johnson and Cummins, 1972). The DNA base composition is 66-67 mol% G+C. Pantothenic acid, biotin, thiamine, and *para*-amino benzoic acid are required for growth (Delwiche, 1949). The major long chain fatty acid produced is 13-methyltetradecanoic acid (Moss et al., 1969).

2.1.4 *Propionibacterium acidipropionici*

Propionibacterium acidipropionici can be isolated from dairy products (Table 2a). Colonies are white, becoming pink after continued incubation (Johnson and Cummins, 1986). The cell wall peptidoglycan contains L-DAP, the sugars glucose and galactose. The cell wall sugar mannose is found in some strains (Johnson and Cummins, 1972). The DNA base composition is 66-68 mol% G+C. Pantothenic acid and biotin are required for growth, whereas thiamine

stimulates growth (Delwiche, 1949). The major long chain fatty acid produced in thioglycollate cultures is 13-methyltetradecanoic acid (Moss et al., 1969).

2.1.5 *Propionibacterium cyclohexanicum*

Propionibacterium cyclohexanicum was isolated from pasteurized, but spoiled, orange juice (Table 2a). This organism is a non-motile, aerotolerant, non-spore-forming, pleiomorphic and rodlike coryneform bacterium (Kusano et al., 1997). The cells are 1.5-3.0 μm long and 1.1-1.6 μm wide. Some cells are club shaped or bent. The colonies are circular, white creamy and translucent and 0.2-0.5 mm in diameter. This species differs from previously described *Propionibacterium* spp. in that it contains cyclohexyl as the main fatty acid. The cells grow at pH 3.2-7.5, with optimal growth at pH of 5.5-6.5. Growth occurs between 20°C and 40°C, with an optimum growth at 35°C. Lactic acid and propionic acid are the major end-products of glucose fermentation, with the occasional formation of acetic acid. The production of lactic acid distinguishes *P. cyclohexanicum* species from all other species, except *P. propionicum* (Charfreitag et al., 1988). *Propionibacterium cyclohexanicum* strains have a MK-9 (H_4) respiratory quinone system (Kusano et al., 1997). They are catalase, oxidase and Voges-Proskauer negative and methyl red positive. The DNA base composition is 66.8 mol% G+C. The cell wall sugars are galactose, mannose, glucose, ribose and rhamnose. The cell wall contains meso-DAP acid, glutamic acid and alanine at a molar ratio of 1:1:2. *Propionibacterium cyclohexanicum* has the highest homology with *P. freudenreichii* DSM 20271 (97%) as determined by 16S rRNA sequence analysis, while homology to other propionibacteria was recorded at 95% (Kusano et al., 1997).

2.1.6 *Propionibacterium microaerophilicum*

Propionibacterium microaerophilicum was isolated from olive mill wastewater (Table 2a). The species is mesophilic, facultative anaerobe and microaerophilic. When grown microaerophilic, the colonies are 2-3 mm in diameter, white and lens-shaped with smooth edges. *Propionibacterium microaerophilicum* is catalase-negative, whereas the propionibacteria are generally catalase-positive, with the exception of *P. propionicum* and *P. cyclohexanicum* (Kusano et al., 1997; Schaal, 1986; Charfreitag et al., 1988). Under anaerobic conditions large amounts of propionate, acetate and CO_2 are formed. No complex

nitrogen compounds such as yeast extract, amino acids or peptides are required (Koussémon et al., 2001). Yeast extract improved the growth of *P. microaerophilicum*, whereas the presence of other growth factors such as pantothenate, biotin or thiamine had no effect. The growth pH ranged from 4.5-9.5, with pH 7.0 as optimum. The growth temperature ranged from 20 to 45°C, with 30°C as optimum. The DNA base composition of *P. microaerophilicum* is 67.7 mol% G+C. Based on 16S rRNA sequence analysis, *P. acidipropionici* is the closest relative with a similarity of 97%. The level of DNA relatedness between *P. microaerophilicum* and *P. acidipropionici* DSM 4900^T is 56 % (Koussémon et al., 2001).

2.2 Cutaneous propionibacteria

Cutaneous propionibacteria are generally isolated from human skin or other epithelial surfaces (Kabongo et al., 1981). Strains of *P. acnes*, *P. granulosum* and *P. avidum* are found on different areas of human skin (McGinley et al., 1978). The disease acne vulgaris is accompanied by a large increase in the number of *P. acnes* on human skin. Cutaneous propionibacteria secrete nucleases, neuraminidases and hyaluronidase, acid phosphatases, lecithinases and other lipases (Ingham et al., 1979, 1980, 1981; Höffler, 1979; Holland et al., 1979; Von Nicolai et al., 1980). Two types of the cell wall are found depending on the presence of galactose, glucose and mannose (Johnson and Cummins, 1972). Diaminoacid is mainly represented by L-DAP. Two serological types, differing in the composition of polysaccharide antigens and the structure of cell walls, are distinguished in *P. acnes*, two in *P. avidum* and one in *P. granulosum*. Most strains contain 31-40% of C_{15} branched-chain fatty acids, while the content of the iso-type fatty acids was reported as ranging from 40 to 50% (Moss et al., 1967). The general characteristic of the genus is the presence of fatty acids of the iso-type.

2.2.1 *Propionibacterium acnes*

Propionibacterium acnes is usually isolated from normal skin, comedones of acne vulgaris (oily areas of the skin), intestinal contents, wounds, blood, pus and soft tissue abscesses (Table 2c). Colonies in deep agar are lenticular, 0.1-4 mm in size and white, with colonies of some strains changing to tan, pink or orange in 3 weeks. On the surface of solid media *P. acnes* grows slowly, with colonies appearing after 4-5 days. Lactate is converted to

propionate by most strains, but only if the initial oxidation-reduction potential of the medium is sufficiently low, or if the initial growth rate is rapid. The main products of fermentation are acetic and propionic acids (Sizova and Arkadjeva, 1968). Succinic acid and traces of lactic and formic acid are also produced (Moore and Cato, 1963). The strains are generally catalase-positive, although cultures need to be exposed to air for a period before testing (McGinley et al., 1978). All strains tested require pantothenate, biotin and thiamine, while nicotinamide, lactate, pyruvate and α-ketoglutarate stimulate growth. Oleate (usually used in the form of Tween 80) is also stimulatory. Some strains produce bacteriocin-like substances inhibitory towards other strains (Fujimura and Nakamura, 1978). A variety of bacteriophages have been recorded (Pulverer and Ko, 1973; Jong et al., 1975; Webster and Cummins, 1978). The major long-chain fatty acid is 13-methyltetradecanoic (Moss et al., 1969). The DNA base composition ranges from 59-60 mol% G+C (Johnson and Cummins, 1972).

2.2.2 *Propionibacterium granulosum*

This species is usually isolated from the sebum rich, oilier areas of the skin but in smaller numbers than *P. acnes* (McGinley et al., 1978). *Propionibacterium granulosum* is found along with *P. acnes* in acne comedones, and play a role in the pathogenesis of acne. Surface colonies are generally white or grayish, smooth, circular, and usually larger and more whitish than colonies of *P. acnes* (Table 2c). This organism is mostly non-hemolytic and contains more active lipases than *P. acnes* (Greenman et al., 1981). The peptidoglycan cell wall contains L-DAP, alanine, glutamic acid and glycine (Johnson and Cummins, 1972). The cell wall polysaccharides are galactose and mannose. *Propionibacterium granulosum* has a low (12-15%) DNA homology with *P. acnes* and other classical propionibacteria. The DNA base composition is 61-63 mol% G+C (Johnson and Cummins, 1972).

2.2.3 *Propionibacterium avidum*

This organism may be isolated from the moist areas of the skin, e.g. the vestibule of the nose, axilla, perineum and chronically infected areas such as sinuses (Table 2c). Surface colonies after 2-3 days are smooth and circular, with a white to light cream color. These strains will grow in a simple medium consisting of salts, glucose and vitamins, while pantothenic acid is an absolute requirement for growth. The cell wall peptidoglycan consists of L-DAP, alanine, glutamic acid and glycine (Johnson and Cummins, 1972), but a few strains of serological type

II have meso-DAP and contain no glycine in the cell wall. This species is hemolytic and produces gelatinase and deoxyribonuclease, but no lecithinase, hyaluronidase and chondroitin sulfites, in contrast with *P. acnes* (Höffler, 1979). Two distinct types of cell wall sugar patterns are found in the polysaccharides. Type I contain glucose, galactose and mannose while type II contains glucose and mannose only (Johnson and Cummins, 1972; Cummins, 1975). A strong serological cross-reaction is observed between *P. avidum* II and *P. acnes* II. The DNA base composition is 62-63 mol% G+C (Johnson and Cummins, 1972).

2.2.4 *Propionibacterium lymphophilum*

Propionibacterium lymphophilum has been isolated from human urinary tract infections, mesenteric ganglion of a monkey and from human Hodgkin lymphoma (Table 2c). The surface colonies after 4 days are punctiform, circular, convex to pulvinate, white, glistening and smooth (Cummins and Johnson, 1986). The species differs from others by its cell wall composition. It contains lysine instead of DAP and glucose, galactose and mannose as the principal cell wall sugars (Johnson and Cummins, 1972). The DNA base composition is 53-54 mol% G+C.

2.2.5 *Propionibacterium propionicum* (*Arachnia propionica*)

Propionibacterium propionicum is a normal inhabitant of the mouth and is isolated from dental plaque. Strains have also been isolated from cervical smears (Table 2c). The species is found in various organs infected by actinomycoses (Brock et al., 1973). The species is characterized by being non-motile, non-acid-fast, branched diphtheroid filamentous rods. The optimum temperature for growth lies between 35-37°C, while some strains grow at 45°C (Holmberg and Nord, 1975). *Propionibacterium propionicum* (previously *Arachnia propionica*) contains iso- and anteiso-branched chain fatty acid components. The major fatty acid components are 12- and 13-methyltetradecanoicacids (C_{15}), with small amounts of C_{16}: 1 and C_{18}: 1 (Cummins and Moss, 1990). In contrast with *P. propionicum*, members of the genus *Actinomyces* contain mainly C_{16}: 0 and C_{18}: 1 acids, either not present or found in trace amounts. These results confirm a close relationship of *Arachnia propionica* with propionic acid bacteria. Comparison of 744 bases of the 16S rRNA sequences (Charfreitag et al., 1988) of *A. propionica, Actinomyces bovis, Actinomyces viscosus*, *P. freudenreichii* and *P. acnes* confirmed that *A. propionica* is closer related to *Propionibacterium* than to any other taxon.

In an unrooted phylogenetic tree *A. propionica* is placed within the genus *Propionibacterium,* being almost equidistant to *P. freudenreichii* and *P. acnes* (Charfreitag et al., 1988). Although these results place *A. propionica* in the genus, the relatedness with *P. freudenreichii* is only 1-5%. A similar low relatedness was reported between *P. freudenreichii* and *P. acnes*. The inclusion of filamentous strains in the genus *Propionibacterium* makes it very heterogeneous with respect to morphology. The major diaminoacids in the peptidoglycan are L-DAP or lysine. The tetrahydrogenated menaquinones with nine isoprene units (MQ-9(H_4) are the major respiratory quinines. The DNA base composition is 63-65 mol% G+C (Schaal, 1986; Charfreitag et al., 1988).

2.2.6 *Propionibacterium innocuum* (renamed *Propioniferax innocua*)

Isolated from human skin (Pitcher and Collins, 1991). *Propionibacterium innocuum* possesses many of the characteristics of the classical propionibacteria, including coryneform morphology, i.e. cells are pleiomorphic rods. The DNA has a high G+C level (approx. 59-63 mol%), close to that of *P. acnes* (59 mol%). *Propionibacterium innocuum* differs from the other propionibacteria inhabiting human skin by its cell wall composition, having polysaccharides in which only arabinose and mannose could be detected but not galactose. The peptidoglycan cell wall is composed of L-DAP. DNA hybridization of *P. innocuum* with other propionibacteria did not reveal significant genomic homologies, but the 16S rRNA sequence of strain NCTC 11082 had the highest homology with *Propionibacterium* spp. However, Yokota et al. (1994) emphasized that the aerobic growth potential of *P. innocuum* and the presence of arabinose in the cell wall indicates that *P. innocuum* should not be classified with the authentic *Propionibacterium* spp. The authors proposed that *P. innocuum* should be transferred to a new genus, *Propioniferax*, and renamed *Propioniferax innocua* (Yokota et al., 1994). The results of 16S rDNA sequence analysis indicate *L. japonicus* as the phylogenetic neighbor of the new species.

3. METABOLISM

3.1 Nutritional requirements

Growth and fermentation of propionibacteria are stimulated by a variety of compounds (Hettinga and Reinbold II, 1972b). Hard rennet cheeses represent a selective habitat for propionic acid bacteria, since they contain lactate formed as the end product of lactose fermentation by lactic acid bacteria. Unlike many other bacteria, propionibacteria can utilize lactate efficiently, and does it the best in the presence of yeast extract (Hettinga and Reinbold I, 1972a). Lactate as a carbon source supports higher growth rates of propionic acid bacteria than lactose (El-Hagarawy et al., 1954). Glucose is the preferred carbon source for biosynthetic processes, although propionibacteria grow equally well on lactose, lactate, pyruvate and glycerol, and can ferment a variety of carbohydrates. They use both organic and inorganic nitrogen sources, and depending on the strain, may require a few or no amino acids (Tatum et al., 1936; Wood et al., 1938). Thiamine, biotin, pantothenic acid, riboflavin and vitamin B_{12} are involved in propionic acid fermentation (Delwiche, 1949). Aspartic and glutamic acids can replace ammonium nitrate as a growth factor, while thiamine may stimulate the growth of certain species (Tatum et al., 1936). Riboflavin stimulates growth, but is not an obligatory requirement (Wood et al., 1938). Most strains require biotin, which plays a major role in transcarboxylation reactions to produce propionic acid.

Complex media used to cultivate propionibacteria typically contain lactic acid as carbon source, a protein source such as tryptone, and yeast extract as additional growth factors. Propionibacteria differ significantly in their growth requirements, while a defined medium usually includes most amino acids, vitamins, purines, and pyrimidines to support growth of all strains (Glatz, 1992). In most laboratory studies, propionibacteria are routinely grown on yeast extract-lactate medium (YEL) described by Malik et al. (1968) or similar media. The classical propionic acid bacteria are cultivated routinely at 28°C-30°C, and the cutaneous strains at 37°C. The optimal pH for growth is between 6.5 and 7.0. The relation of hydrogen-ion concentration to the rate of propionic acid production distinctly show that the optimal growth pH is pH 7.0, while at pH 5 there is practically no growth and little production of propionic acid (Whittier and Sherman, 1923).

3.2 Fermentation and antimicrobial end-products

The Industrial fermentation process to produce propionic acid was first introduced by Sherman and Shaw in 1923. Isolation and identification of the intermediates of glucose fermentation, verification of the expected end-products and analysis of the distribution of labeled products showed that glycolysis is the main pathway of glucose utilization (Wood et al., 1937; Wood and Leaver, 1953). Propionibacteria convert glucose to pyruvate via the Embden-Meyerhof pathway (Fig. 1). Lactate is oxidized to form pyruvate. From pyruvate, acetate is formed through a biochemical pathway that yields one mole of carbon dioxide and one mole of ATP per mole acetate. Through a transcarboxylation reaction, they also convert pyruvate to oxaloacetate, which is then converted to succinate via the enzymes of the dicarboxylic acid pathway. Succinate is converted by methylmalonyl-coenzyme A (CoA) intermediates to propionate, where the carboxyl group removed from methylmalonyl-CoA is transferred to pyruvate to form oxaloacetate.

The key reaction of propionic acid fermentation is the transformation of L-methylmalonyl- CoA to succinyl-CoA, which requires coenzyme B12 (AdoCbl). The uniqueness of propionic acid fermentation is due to the presence of PEP (phospoenolpyruvate) carboxytransphosphorylase, an enzyme not found in other organisms that synthesize propionate. Due to the presence of this enzyme the propionic acid fermentation functions as a cyclic process. Biotin and vitamin B12 are involved in these reactions as cofactors. Biotin has to be added to the growth medium of most strains, while the organism produces vitamin B12 in such copious amounts that it is used to produce the vitamin commercially (Hettinga and Reinbold, 1972c; Janicka et al., 1976; Vorobjeva and Iordan, 1976). Another peculiarity of this fermentation is related to the way propionate is formed, which is coupled with the reduction of fumarate to succinate and the oxidation of pyruvate to acetate and CO_2. The electron transport accompanying these reactions is coupled with oxidative phosphorylation and ATP synthesis (Vorobjeva, 2000). Glucose is phosphorylated, forming hexose monophosphate and hexose diphosphate (Pett and Wynne, 1933; Van Niel, 1957). Transcarboxylase, hexosephosphate isomerase, fructose diphosphate aldolase and triosephosphate dehydrogenase activities are found in the cells of propionibacteria (Sibley and Lehninger, 1949; Wood et al., 1963; Van Demark and Fukui, 1956).

The main fermentation products are propionic acid, acetic acid and CO_2 (Mashur et al., 1971; Foschino et al., 1988). Formic and succinic acids (Mashur et al., 1971), as well as acetoin and diacetyl (Tomka, 1949; Lee et al., 1969, 1970), are also produced, but in smaller

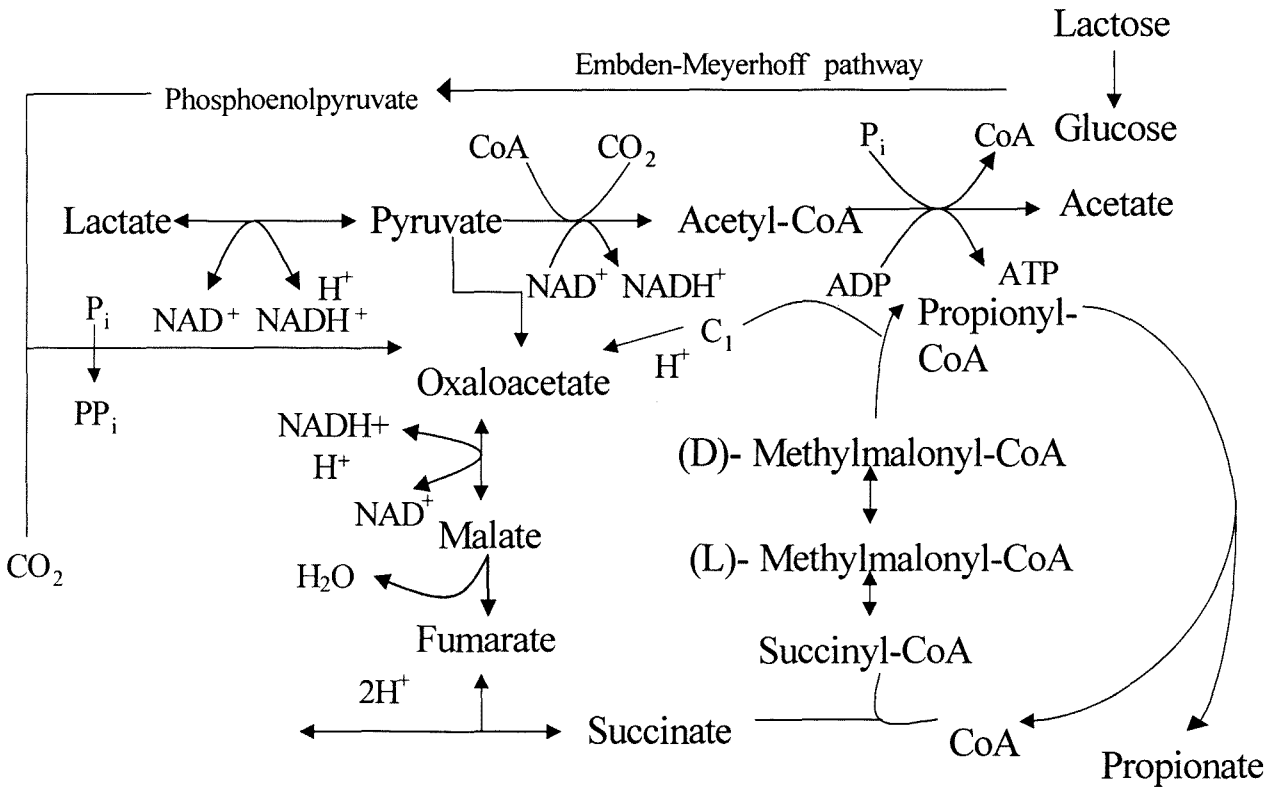

Fig. 1. Pathway of propionic acid fermentation (adapted from Stjernholm and Wood, 1963; Hettinga and Reinbold, 1972b; Vorobjeva and Iordan, 1976)

amounts. Other volatile aromatic substances are dimethylsulfide, acetaldehyde, propionic aldehyde, ethanol and propanol (Keenan and Bills, 1968; Dykstra et al., 1971). Propionic acid fermentation differs from other types of fermentation by the high ATP yield and the unique enzymes and reactions.

3.2.1 Production of organic acids

Propionibacteria produce propionic acid and acetic acid from lactic acid. The inhibitory action of propionic acid is partially caused by the inhibition of nutrient transport (Eklund, 1983), and intracellular accumulation (Lück, 1980). The low pH encountered in Swiss cheese and other fermented products causes the inhibitory effects of propionate and acetate, while the undissociated forms are effective against Gram-negative bacteria (Baird-Parker, 1980). In bakery products propionate inhibits molds when the pH is less than 5.0 (Olson and Mary, 1945). Acetic acid is one of the most used antimicrobials produced by microorganisms. Acetic acid is mainly used as a food additive in the form of vinegar. It is added as a preservative and a flavouring agent to many different foods, including mayonnaise, salad dressing, pickles and mustard. Acetic acid has a wide range of inhibitory activity and inhibits yeast and molds as well as bacteria (Blom and Mortvedt, 1991). The undissociated form penetrates the cell and exerts its inhibitory action, which is consistent with the antimicrobial activity increasing with decreasing pH values. Propionic (pKa = 4.9) and acetic acid (pKa = 4.75) have an optimum antimicrobial function located close to and below their pKa values. At these pKa values a large fraction of the acids are undissociated. They solubilize in the cell membrane, block transport of necessary growth substances, acidify the cytoplasm by dissociation and exert other inhibitory activities on cell growth.

3.2.2 Carbon dioxide production

Low concentrations of carbon dioxide have no effect on the growth of some organisms, while higher concentrations inhibit growth (Lindgren and Dobrogosz, 1990). Propionibacteria produce CO_2 from lactate or glucose (Hettinga and Reinbold, 1972b). The CO_2 creates an anaerobic environment, decreases the intracellular pH and destroys the cell membrane (Clark and Takacs, 1980; Eklund, 1984). Silage and vegetables fermented by propionibacteria are thus protected from contamination by molds (Lindgren and Dobrogosz, 1990).

3.2.3 Diacetyl production

Diacetyl (2,3-butanedione) is responsible for the characteristic aroma and flavour of butter (Jay, 1982). *Propionibacterium shermanii* is known for its production of diacetyl from citrate in milk (Lee et al., 1970). Diacetyl is also produced from lactate by some *Propionibacterium* spp. (Tomka, 1949). In addition to butter and other dairy products, diacetyl is found in red and white wines, brandy, roasted coffee, silage, and many other fermented foods (Jay, 1982). Diacetyl is generally recognized as safe. The application of diacetyl as a food preservative is limited because of the relatively large quantities needed for inhibition (De Vuyst and Vandamme, 1994). The use of diacetyl as an antimicrobial dip for utensils and surfaces has potential application in the food industry due to its high volatility (Jay, 1982). Up to 6 ppm of diacetyl is produced by strains of *P. freudenreichii* subsp. *shermanii* in milk (Lee et al., 1970). However, 172 to 344 ppm are required for inhibition of yeast and non-lactic acid bacteria (Jay, 1982). Production of diacetyl by propionibacteria probably does not contribute to the antimicrobial effect as a whole.

3.2.4 Other compounds

Microgard is a grade A skim milk that has been fermented by *Propionibacterium shermanii* and then pasteurized (Weber and Broich, 1986). The inhibitory action of Microgard has been attributed to diacetyl, propionic, acetic, lactic acid and a small heat-stable peptide of 700 Da (Al-Zoreky et al., 1993). Microgard is antagonistic towards most Gram-negative bacteria, fungi and certain foodborne pathogens (Al-Zoreky et al., 1991). Microgard has been approved by the Food and Drug Administration and is used extensively in about 30% of cottage cheese produced in the United States. Microgard is added at 1% to dairy products such as yogurt and salad dressings. A non-dairy Microgard is used in sausages and bakery products (Salih et al., 1989; Weber and Broich, 1986).

Other inhibitors include propionins, which are antiviral peptides obtained from cellular extracts of *P. freudenreichii* (Ramanathan et al., 1966). Propionin A is a dialyzable peptide demonstrating *in vitro* activity against vaccinia viruses (Ramanathan et al., 1968). Propionins B and C are also dialyzable. They are approximately 1,000 to 2,000 Da in size and demonstrate *in vivo* and *in vitro* activity against the Columbia SK virus.

3.2.5 Bacteriocins

Bacteriocins are antimicrobial substances or biologially active proteins that display a bactericidal mode of action towards the same or closely related species (De Vuyst and Vandamme, 1994). An in depth discussion is given in the next chapter.

4. BACTERIOCINS OF PROPIONIBACTERIA

Bacteriocins produced by food-grade organisms such as lactic acid bacteria and propionibacteria are of special interest due to their potential application in food preservation (Stiles, 1996). Although numerous bacteriocins of Gram-positive bacteria, specifically the genus *Lactobacillus,* have been isolated and characterized (Klaenhammer, 1988), only a few among the genus *Propionibacterium* have been reported. The best-studied bacteriocin from lactic acid bacteria is nisin, which is produced by *Lactococcus lactis* subsp. *lactis* and is approved as a food additive in many countries. The efficiency of nisin in preventing the growth of spoilage bacteria has been proven in a number of food systems. Another important bacteriocin is pediocin PA-1, produced by *Pediococcus acidilactici* PAC 1.0 (Gonzales and Kunka, 1987). Pediocin PA-1 is very active against the food-borne pathogen *Listeria monocytogenes* (Pucci et al., 1988), an organism not very sensitive to nisin (De Vos et al., 1993).

Klaenhammer (1993) originally defined four distinct classes of lactic acid bacteria: class I (lantibiotics), class II (small, < 10 kDa, heat-stable membrane-active peptides), class III (large, > 30 kDa, heat-labile proteins) and class IV (complex bacteriocins). The class II bacteriocins were further divided into *Listeria*-active peptides with N-terminal consensus sequences (class IIa), poration complexes requiring two different peptides for activity (class IIb) and thiol-activated peptides that require reduced cysteine residues for activity (class IIc). Moll et al., (1999) reclassified the bacteriocins: He defined class I as the lantibiotics and divided it into type A lantibiotics and type B lantibiotics. Type A lantibiotics are elongated, cationic, pore forming peptides. Type B lantibiotics are compact, with globular structures, enzyme inhibitors and are immunologically active (De Vuyst and Vandamme, 1994). Class II, the small heat-stable non-lanthionine peptides, is further divided into four groups: Class IIa, which consists of *Listeria*-active peptides with an N-terminal consensus sequence; Class IIb, consisting of two-peptide complexes; Class IIc, consisting of *sec*-dependant bacteriocins

and Class IId, the small heat-stable non-lanthionine bacteriocins that do not belong to any of the first three groups within class II.

4.1 Bacteriocins produced by *Propionibacterium* spp.

Among the dairy propionibacteria, only five bacteriocins have been reported: propionicin PLG-1 isolated from *P. thoenii* P127 (Lyon and Glatz, 1991, 1993); jenseniin G, isolated from *P. thoenii* P126 (previously *P. jensenii*) (Grinstead and Barefoot, 1992); propionicin T1, isolated from *P. thoenii* 419 (Faye et al., 2000); jenseniin P, isolated from *P. jensenii* P1264 (Ratnam et al., 1999); and jenseniin SM1, isolated from *P. jensenii* DF1 (Miescher et al., 2000). Two bacteriocins produced by species within the cutaneous group of propionibacteria have been described, viz. acnecin isolated from *P. acnes* CN-8 (Fujimura and Nakamura, 1978) and an inhibitory compound produced by *P. acnes* RTT 108 (Paul and Booth, 1988).

4.1.1 Bacteriocins produced by *P. thoenii*

4.1.1.1 Propionicin PLG-1

Propionicin PLG-1 is produced by *Propionibacterium thoenii* strain P127 (Lyon and Glatz, 1991, 1993; Paik and Glatz, 1995). It is active against some classical *Propionibacterium* spp. (*P. thoenii*, *P. acidipropionici* and *P. jensenii*), some Gram-positive organisms (*Lactobacillus bulgaricus*, *Lactobacillus casei*, *Pediococcus cerevisiae*, strains of *Lactococcus lactis* subsp. *lactis*), and several Gram-negative organisms (*Campylobacter jejuni*, *Esherichia coli*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa* and *Vibrio parahaemolyticus*). Propionicin PLG-1 inhibits the indicator organism *Lactobacillus delbruekii* ATCC 4797 in buffer, broth and skim milk. Lyon and Glatz (1993) reported maximal production of propionicin PLG-1 (50 AU/ml) at pH 7 after 180 h of growth. Bacteriocin production was found to be much lower in cultures grown at pH 6.0, 6.5 and 7.5. Propionicin PLG-1 adsorbs to sensitive cells of *Propionibacterium acidipropionici*. The peptide does not act by forming pores in cell membranes, but inhibits metabolism, in contrast to most other antibacterial peptides (Gollop and Lindner, 1998). However, the targets of propionicin PLG-1 activity and the mode of action have not been studied in depth. Very low concentrations of propionicin PLG-1 activity is detected when the cells are grown until the late-stationary phase.

Propionicin PLG-1 is sensitive to protease, pronase E, pepsin, trypsin and α-chymotrypsin, but not affected by phospholipase C, lipase or catalase. The molecular mass of purified propionicin PLG-1 is 9328 Da, and the bacteriocin contains 99 amino acids. No homology has been with bacteriocins from lactic acid bacteria (Glatz, 1995). *Propionibacterium thoenii* P127 harbors a single large plasmid of 250 kb, but the genetic determinants for propionicin PLG-1 production or host cell immunity was not found on this plasmid. Methods to produce propionicin PLG-1 in liquid medium in batch and fed-batch fermentations were improved, and large-scale culture protocols to yield high titers were developed (Paik and Glatz, 1997). Propionicin PLG-1 is very stable during storage, especially in the lyophilized state. Incubation at 85°C for 15 min had no effect on activity (Hsieh et al., 1996). Further studies are required to determine the effectiveness of the bacteriocin on the contaminant microflora present in a specific food system before its practical application in that system (Hsieh et al., 1996).

4.1.1.2 Jenseniin G

Jenseniin G is produced by *P. thoenii* P126, previously *P. jensenii* (Grinstead and Barefoot, 1992; Weinbrenner et al., 1997). Jenseniin G is stable during heat treatment at 100°C for 15 min and between pH 3 and 12. The peptide has a molecular size of 4.5 kDa. It is inhibitory towards *P. acidipropionici* P5 and *P. jensenii* P54 and selected lactobacilli (*L. helveticus* NCDO 87, *L. delbrueckii* subsp. *lactis* ATCC 4797 and *L. delbrueckii* subsp. *bulgaricus* NCDO 1489). Jenseniin G effectively limited acid production in yoghurt by inhibiting *L. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* and may be used to extend the shelf life of yoghurt by maintaining the pH within a desirable range of pH 4.2 to 4.3 (Weinbrenner et al., 1997). Jenseniin G is sensitive to proteinase K, pronase E and type 14 protease (Grinstead and Barefoot, 1992; Weinbrenner et al., 1997). No plasmids were detected in *P. thoenii* P126, which suggests a possible chromosomal locus for the genes encoding bacteriocin production. No bacteriocin activity was found in unconcentrated NLB (sodium lactate broth) cultures. Bacteriocin activity was found only when cultures were concentrated 50-100 fold (Grinstead and Barefoot, 1992). The highest jenseniin G activity (160 AU/ml) was detected during stationary phase of growth of the producer in medium maintained at pH 6.4 (Ekinci and Barefoot, 1999). Jenseniin G synthesis and release appear to be dependent on one or more pH sensitive components. Jenseniin G activity in cell-free supernates is stable at –20°C for at least 2 years.

4.1.1.3 Propionicin T1

Propionicin T1 is produced by *P. thoenii* 419. The bacteriocin was purified by ammonium sulfate precipitation and ion exchange- and reverse-phase chromatography (Faye et al., 2000). Propionicin T1 inhibits *P. acidipropionici*, *P. thoenii*, *P. jensenii* and several species of the genera *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Carnobacterium* and *Listeria.* Propionicin T1 is heat-stable (100°C for 30 min) and stable at a pH of 2.5 for 1 hour. It is stable after freezing and thawing. Storage at 4°C or –20°C for up to six months or at room temperature for 24 hours has no effect on activity. Proteinase K inactivates the bacteriocin. The inhibitory activity can be detected in liquid culture, and maximum antimicrobial activity is found in the early stationary growth phase. Propionicin T1 displayed a bactericidal mode of action towards the indicator organism, *P. acidipropionici* ATCC 4965 (Faye et al., 2000).

The calculated molecular mass of the mature propionicin T1 is 7,130.20 Da and the pI calculated at 9.50. The DNA sequence revealed that the bacteriocin is translated as a 96-amino acid prebacteriocin, which is processed to give a mature propionicin T1 of 65 amino acids. No sequence similarity was found with other bacteriocins. Propionicin T1 has several features in common with most bacteriocins isolated from lactic acid bacteria, namely its relatively small size (30-100 amino acids), thermostability and being cationic. Like most bacteriocins from lactic acid bacteria, propionicin T1 is synthesized as a precursor with a N-terminal leader peptide (Faye et al., 2000). The deduced leader sequence of propionicin T1 conforms to the pattern of a typical signal peptide, i.e. it has a positively charged N-terminus, a hydrophobic core and a specific cleavage region. The putative ABC transporter is not likely to be involved in the regular export of propionicin T1, since the bacteriocin shows the characteristics of proteins that are exported by the general secretory pathway. Propionicin T1 may be used to prevent the formation of red spots caused by pigment-forming strains of *P. thoenii* and *P. jensenii*. This is a common problem in the production of Swiss-type cheeses (Faye et al., 2000).

4.1.2 Bacteriocins produced by *P. jensenii*

4.1.2.1 Jenseniin P

Jenseniin P is produced by *P. jensenii* B1264 (Ratnam et al., 1999). Bacteriocin activity was detected during late stationary growth and then in two- to 16-fold concentrated culture

filtrates. The bacteriocin-like substance displayed a bactericidal mode of action against *L. delbrueckii* subsp. *lactis* ATCC 4797 and reduced the viability of the lactobacilli by 90% within 60 min. Treatment with proteinase K, pronase E or type XIV protease eliminated jenseniin P activity (Ratnam et al., 1999). Treatment with trypsin, α-chymotrypsin, type VI-A protease, lysozyme or catalase had no effect on the bacteriocin activity. Adjusting jenseniin P to pH values ranging from 2.2 to 10.2 or treatment with 0.1 to 1.0 mol/L NaCl for up to 225 min did not affect activity. Jenseniin P was stable to heating at 100°C for 0 to 60 min, treatment with 4 mol/l urea for 6 hours and the addition of SDS at final concentrations of 0.1 to 2.0% (m/v). The zone of inhibition corresponded to a silver-stained protein that focused between pH 3.0 and 3.5. The optimization of jenseniin P production has not yet been studied (Ratnam et al., 1999).

4.1.2.2 Propionicin SM1

Propionicin SM1 is produced by *P. jensenii* DF1 and is isolated from Swiss raw milk (Miescher et al., 2000). Treatment of a 100-fold concentrated cell-free supernatant of *P. jensenii* DF1 with proteinase K and a protease from bovine pancreas destroyed all antagonistic activity. The crude inhibitor was not affected by heating at 100°C for 10 min, whereas a 30 min exposure resulted in total loss of activity. Propionicin SM1 activity is stable at 30°C for 14 days and at 4°C for 6 months (Miescher et al., 2000). SDS-PAGE revealed two distinct protein bands, both corresponding to the zone of inhibition against *P. jensenii* DSM 20274. Both these protein bands are regarded as bacteriocins and are termed propionicin SM1 (Ppn A), corresponding to a protein band with a molecular weight of 27 kDa, and propionicin SM2 (Ppn B) with an apparent molecular weight of about 13 kDa. The highest yield of bacteriocin activity was reached during late logarithmic growth. The putative prepeptide of propionicin SM1 has a calculated molecular mass of 22 685 Da, which is processed to form a mature protein of 19 942 Da. No significant homology to any known sequence was found using the SwissProt database or GenEMBL. The N-terminal leader peptide has a length of 27 amino acids (Miescher et al., 2000).

4.1.3 Bacteriocins produced by *P. acnes*

4.1.3.1 Acnecin CN-8

Acnecin CN-8 is produced by a strain of *Propionibacterium acnes* isolated from human oral cavities (Fujimura and Nakamura, 1978). Acnecin CN-8 is non-dialyzable and heating at 60°C for 10 min destroys the activity. Digestion with trypsin, α-chymotrypsin, pronase and lysozyme destroyed the activity of acnecin CN-8. Papain, catalase, lipase, DNase and RNase did not affect the activity of acnecin CN-8. Crude extracts of acnecin inhibited only *P. acnes* and *Corynebacterium parvum*. The molecular weight of acnecin CN-8 is 60 kDa.

4.1.3.2 Bacteriocin-like substance RTT 108

This bacteriocin-like substance RTT 108 is produced by *P. acnes* RTT 108 (Paul and Booth, 1988). The highest yield of bacteriocin activity was recorded from cultures in stationary growth. Trypsin and pronase inhibits the bacteriocin, while α-chymotrypsinogen has no effect. The substance is most stable at pH 7.0. No inhibitory activity is detectable below pH 2.0 or above pH 11.0 after 1 hour of incubation. The substance is completely inactivated when incubated at 55°C for 1 h. The molecular size of the bacteriocin is approximately 78 kDa. The bacteriocin is thermally unstable, which is a common characteristic of large molecular weight proteins. The partially purified bacteriocin can be stored in phosphate buffer (pH 7.0) at 4°C for 90 days with no detectable decrease in activity.

4.1 Mode of action of class II bacteriocins

Although the mode of action of nisin has been studied in detail, much less is known about the interaction between class II bacteriocins and the membrane interactions with their target organisms. The mode of action of the bacteriocins produced by *Propionibacterium* spp. are not as well studied as the bacterocins produced by lactic acid bacteria, particularly the lantibiotic nisin and the class II bacteriocin pediocin PA-1. More recent studies on the mode of action of nisin and pediocin PA-1 indicated that the antimicrobial activity does not require a specific receptor and is enhanced by a membrane potential (Chen et al., 1997; Kaiser and Montville, 1996). Docking molecules may enhance the conductivity and stability of lantibiotic pores, while receptors in the target membrane may determine specificity of class II

bacteriocins. Insertion into the membrane of many bacteriocins is proton motive force driven. Bacteriocins are in general cationic (i.e. they contain an excess of lysyl and arginyl residues), amphipathic molecules composed of 12 to 45 amino acid residues. They are usually unstructured in aqueous solution, but have the propensity to form a α-helical structure when exposed to structure promoting solvents such as trifluoroethanol or when mixed with anionic phospholipid membranes. Some peptides form loop structures owing to a disulphide bridge or a covalent bond (Moll et al., 1999).

Pediocin PA-1 first adhere nonspecifically to the surfaces of the target cells, which is followed by binding to a receptor-like component of the cell membrane. Pediocin may then insert into the membrane and aggregate into oligomeric structures. These structures form hydrophilic pores, which allow the release of ions and small molecules from the target cells, which ultimately leads to cell death with or without lysis (Chikindas et al., 1993). The cell wall of Gram-positive bacteria allows passage of bacteriocins. Anionic cell surface polymers like teichoic and lipoteichoic acids have been suggested to play a role in the initial interaction with cationic bacteriocins (Jack et al., 1995). Evidence suggests that such interactions may be important for the majority of class II bacteriocins, for which receptors have been implicated to explain their narrow target specificity (Van Belkum et al., 1991). Many of these bacteriocins are unable to form pores in liposomes (Zajdel et al., 1985; Moll et al., 1996b, 1999). Membrane insertion is promoted by a $\Delta\psi$ (transmembrane electrical potential) or by the transmembrane pH gradient (ΔpH) (Moll et al., 1996a, 1997).

Various models for pore-formation have been proposed during the years. A wedge-like model for nisin-induced pore formation may involve a proton motive force driven co-insertion of lipids and nisin domains (Fig. 2). The hinges at amino acid position 21 in the nisin molecule might allow bending of the C-terminal part and thus facilitate its insertion into a membrane.

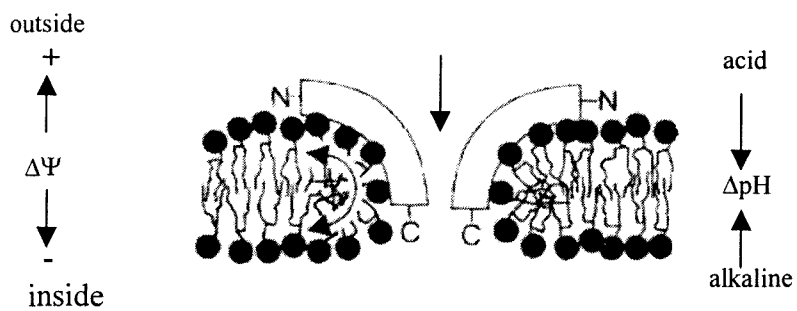

Fig. 2 The Wedge-like pore (adapted from Driessen et al., 1995)

The class II bacteriocins form a bundle of α-helical peptides akin to a barrel-stave like pore (Fig. 3). The presence of a helix-breaking amino acid residue in the center of the molecule may facilitate the insertion of the peptide into the membrane from an initial surface bound state. According to the barrel-stave model, the hydrophilic faces of a bundle of amphipathic α-helical peptides form the inner wall of the water-filled pore. The outer, hydrophobic side of these helical bundles will face the fatty acyl chains of the membrane lipids.

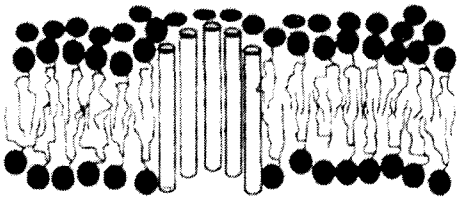

Fig. 3 The Barrel-stave pore (adapted from Moll et al., 1999)

Alternatively, a carpet-like model could explain peptide-induced pore formation (Bechinger, 1997). According to this model, single peptide molecules are oriented parallel to the membrane surface and interfere with the membrane bilayer organization without forming a peptide aggregate. Once the peptides are alined, the membrane will temporarily collapse due to a strong phospholipid mobilizing activity that results in a local and transient permeability (Bechinger, 1997). According to Homblé et al. (1998), the negative charges of the membrane lipids confer cation selectivity to such pores.

4.1 Application of bacteriocins produced by propionibacteria

4.1.1 As food preservatives

A few bacteriocins produced by propionibacteria have been assessed for inhibition of spoilage organisms or pathogens in a food system. Bacteriocins for use as food preservatives must be non-toxic, stable, highly active, inexpensive and simple, with no adverse effect on sensory characteristics (Barefoot and Nettles, 1993). Propionicin PLG-1 can be of use in future food applications because of its broad-spectrum activity, inhibiting various Gram-negative, some

Gram-positive bacteria and yeast and molds (Lyon and Glatz, 1991). Another bacteriocin of possible use to the dairy industry is jenseniin G. This peptide inhibits *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* in yoghurt, thereby controlling acid formation and post acidification during refrigerated storage (Weinbrenner et al., 1997). It can thus be used to extend the shelf life of yoghurt by maintaining the pH within a desirable range (pH 4.2-4.3) (Oberman, 1985). Jenseniin G also delays the outgrowth of *Clostridium botulinum* type A, B and E spores (Garren et al., 1994).

4.3.2 Medical

Acne vulgaris is a skin disorder of the sebaceous follicles that commonly occurs during adolescence (Toyoda and Morohashi, 2001). The development of acne vulgaris requires the occlusion of sebaceous glands, an enhanced secretion of fat and the presence of *P. acnes* (Holland et al., 1981). It is a complex, chronic and common skin disorder of the pilosebaceous units that occurs predominantly in the skin of the face, the upper back and the upper chest (Toyoda and Morohashi, 2001). The sebaceous glands are the most numerous and generally the largest on these areas. Although the disease may be caused by endocrine disorders, there are indications that *P. acnes* could be the primary pathogen (Horner et al., 1992; Ramos et al., 1995). Metabolic products of *P. acnes* can cause skin irritation and inflammation. Four factors appear to play a role in pathogenicity. These factors are 1) an androgen-stimulated increase in the production of sebum, 2) hyperkeratinization and obstruction of sebaceous follicles resulting from abnormal desquamation of follicular epithelium, 3) proliferation of *P. acnes* and 4) inflammation (Berson and Shalita, 1995).

4.3.2.1 Pathological role of *P. acnes* in acne vulgaris

Propionibacterium acnes is a member of the resident flora of the human skin and flourishes beneath the surface in areas rich in sebaceous glands (Kligman et al., 1976; Leyden et al., 1975). Comedones, which are anaerobic sites, create ideal conditions for overgrowth of *P. acnes* filled with a suitable substrate as nutrient source. *Propionibacterium acnes* is not pathogenic by normal standards, because of a minimal correlation between the number of bacteria and the severity and type of acne. A high degree of correlation exists between sebum production and the level of *P. acnes* infection (McGinley et al., 1980). *Propionibacterium acnes* is rare in children from 1-8 years of age, an age when sebum production is low. A

100% colonization of follicles by *P. acnes* is present on the scalp and face that contains large sebaceous glands. On the legs where the follicular structures are small, *P. acnes* colonization occurs in only 20% of the follicles (Strauss, 1998). *Propionibacterium acnes* is most commonly found in the infrainfundibulum. The yeasts *Pitosporum ovale* and *Pitosporum orbiculare* colonizes the upper acroinfundibulum. Aerobic cocci, usually *Staphylococcus epidermis*, reside on the skin surface around the follicles (Marples et al., 1974). Trigliceride (sebum), the major *P. acnes* nutrient, make up 50% of the sebaceous lipid. As triglyceride levels rise, so do *P. acnes* populations (Webster et al., 1980).

Propionibacterium acnes produces lipases and it has been reported that 95% of the free fatty acids at the surface of the skin results from *P. acnes* activity (Freinkel et al., 1965; Marples et al., 1971). Free fatty acids produced by *P. acnes* metabolism contribute to microcomedo formation as well as inflammatory reactions in acne. *Propionibacterium acnes* also produces proteases, chemotactic factors and other extracellular enzymes like hyaluronidase that add to the pathological conditions (Burkhart et al., 1999). *Propionibacterium acnes* sets the stage for the rupture of a comedo by releasing low molecular weight chemotactic factors. The supernatant of *P. acnes* cultures contains cell wall peptidoglycan-polysaccharide fragments. These fragments are small enough to diffuse through the follicular epithelium and attract neutrophils. The cell wall substance is capable of stimulating macrophage production of interleuken 8 and tumor necrosis factor α, both of which upregulate adhesion molecules (Vowels et al., 1995). The neutrophils release inflammatory factors such as lysosomal enzymes and reactive oxygen, which damages the follicle wall and causes it to rupture (Webster et al., 1980; Webster and Kligman, 1979; Miyachi et al., 1986; Akamatsu et al., 1990). The free fatty acids released with the sebum are cytotoxic and probably also contribute to this process. *Propionibacterium acnes* produces C5-derived neutrophil chemotactic factors, which attract leukocytes and further, increases the level of inflammation (Shalita and Wei-Li, 1983).

Propionibacterium acnes ingested by neutrophils are not readily killed or degraded. In the presence of antibodies to *P. acnes*, neutrophil hydrolases are released (Webster et al., 1986). Acne can be further divided into non-inflammatory or inflammatory lesions (Berson and Shalita, 1995). Non-inflammatory acne is characterized by the presence of open (whitehead) or closed (blackhead) comedones, which begin as invisible microcomedones that proceed all other acne lesions. Microcomedo formation is caused by the abnormal keratinization of the infundibular epithelium of hair follicles. In the microcomedo, the sebum is trapped behind a keratin plug. The follicle becomes enlarged and contains a mixture of

sebum and keratinous squamae, which leads to the disruption of the normal architecture of the follicle and to the formation of thin-walled cystic lesions, the comedo (Berson and Shalita, 1995). Inflammatory lesions consist of papules that occur as a result of the closed comedones, and pustules, which are papules with a visible collection of white pus at the surface. These lesions become enlarged and firm and are termed nodules. In inflammatory acne, it is important to reduce the population of *P. acnes* in the follicle and the generation of extracellular products of the organism (Strauss, 1998).

4.3.2.2 Treatment of acne vulgaris

Acne is a chronic disease and treatment is usually necessary for months and often years. Antibiotics are frequently used in acne, either systematically or topically (Gollnick, 1992). The indications for topical therapy are mild and moderate forms of acne vulgaris. In more severe cases, a combined topical and systematic therapy is required (Gollnick, 1992). The spectrum of the most important topical agents in acne therapy includes topical retinoids, azelaic acid, benzoyl peroxide, topical antibiotics, salicyclic acid, zinc and combinations of the above (Gollnick and Schramm, 1998). Those that act in a comedolytic and anti comedogenic manner are the retinoids like tretinoin, isotretinoin, adapalene, tazarotene, and azelaic acid. Azelaic acid and benzoyal peroxide have strong antibacterial potency without inducing bacterial resistance. The appropriate topical agent should be selected in accordance with the predominant type of acne lesions. By combining different topical agents, efficacy of the therapy can be enhanced and toxicity reduced. Topical treatment affects at least three of the four main pathogenic factors (hyperseborrhea, hyperkeratosis, microbial colonization and inflammation) responsible for the development of acne. Systematic antibiotics are indicated for the treatment of moderate and quite severe acne or if acne is considered as very serious by the patient for psychological or social reasons (Meynadier and Alirezai, 1998). A few antibiotics such as tetracyclines (tetracycline, doxycycline, minocycline, limecycline), erythromycin, co-trimoxazole and trimethoprim are useful. During pregnancy the best antibiotic is erythromycin.

The pathogenesis of acne has a well-entrenched hypothesis that bacterium *P. acnes* plays an important role (Leyden et al., 1998). The discovery of antibiotics and the clear-cut clinical improvement seen with agents, such as the tetracyclines, macrolides, sulfonamides and clindamycin which reduce bacterial infection, further strengthens the conclusion that *P. acnes*

plays an important role. More recently, decreased sensitivity of *P. acnes* to antibiotics has developed, particularly in patients treated for prolonged periods (Leyden, 2001).

Propionibacterium acnes resistance to antibiotics is now a worldwide occurrence with long-term implications in acne treatment. Resistance in cutaneous propionibacteria has received scant attention in view of the central role of *Propionibacterium acnes* in inflammatory acne (Eady, 1998). Propionibacteria resistant to erythromycin and clindamycin were first detected in the late 1970s in the USA in comedones extracted from acne patients (Crawford et al., 1979). Carriage of resistant strains results in therapeutic failure by some, but not all antibiotic regiments (Eady, 1998). The likehood of overgrowth of resistant propionibacteria strains increases with duration of therapy (Eady et al., 1989).

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CHAPTER 3

CHARACTERIZATION OF THOENIICIN 447, A BACTERIOCIN ISOLATED FROM *PROPIONIBACTERIUM THOENII* 447

The paper has been written according to the style of International Journal of Food Microbiology and submitted for publication

Characterization of thoeniicin 447, a bacteriocin isolated from *Propionibacterium thoenii* 447

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Abstract

Fifteen strains of propionibacteria, isolated from dairy products, were screened for the production of bacteriocins. *Propionibacterium thoenii* 447 produced an antimicrobial peptide, thoeniicin 447, which acted bactericidal against *Lactobacillus delbrueckii* subsp. *bulgaricus* and bacteriostatic against *Propionibacterium acnes*. Thoeniicin 447 remained stable for 15 min at 100°C. The peptide remained active after 30 min of incubation at pH 1 to 10 and was inactivated when treated with pepsin, pronase, α-chymotrypsin, trypsin and Proteinase K. Optimal bacteriocin production was detected during early stationary growth. The peptide was partially purified by ammonium sulfate precipitation, followed by SP-Sepharose cation exchange chromatography. The estimated size of thoeniicin 447, according to tricine-SDS-PAGE, is 6 kDa. Based on DNA sequencing, the mature peptide is 7130 Da in size and homologous to propionicin T1, produced by *P. thoenii* strain 419 (=NCFB 568^{T}). Strain 447 is phenotypically different from strain 419 and belongs to a separate ribotype cluster.

Keywords: Thoeniicin 447, bacteriocin, characterization

1. Introduction

Antimicrobial peptides produced by microorganisms associated with food are of interest to the food industry for potential use as natural clean-and green preservatives. Bacteriocins are defined as ribosomally synthesized antimicrobial peptides or proteins with antagonistic activity against species genetically closely related to the producer strain (De Vuyst and Vandamme, 1994).

Propionibacterium spp. are used as starter cultures in dairy fermentations, where they play an important role in the development of characteristic flavours and eye production in Swiss-type cheeses (Biede and Hammond, 1979; Langsrud and Reinbold, 1973). Cutaneous strains of propionibacteria are also found on the skin or in the human intestinal tract. Although these clinical strains also produce bacteriocin-like substances, they are involved in certain diseases, e.g. acne vulgaris caused by *Propionibacterium acnes* (Fujimura and Nakamura, 1978; Paul and Booth, 1988).

Numerous bacteriocins from Gram-positive bacteria, mostly lactic acid bacteria, have been isolated and characterized (Klaenhammer, 1988). Only a few bacteriocins have been reported for propionibacteria. Among the dairy propionibacteria, only five bacteriocins have been described, viz. propionicin PLG-1, isolated from *P. thoenii* P127 (Lyon and Glatz, 1991); jenseniin G, isolated from *P. thoenii* P126, previously *P. jensenii* (Grinstead and Barefoot, 1992); propionicin T1, isolated from *P. thoenii* 419 (Faye et al., 2000); a bacteriocin from *P. jensenii* P1264 (Ratnam et al., 1999); and propionicin SM1, isolated from *P. jensenii* DF1 (Miescher et al., 2000). Two bacteriocins produced by cutaneous species have been described, e.g. acnecin, isolated from *P. acnes* CN-8 (Fujimura and Nakamura, 1978) and an inhibitory compound produced by *P. acnes* RTT 108 (Paul and Booth, 1988). Propionicin PLG-1, propionicin T1 and propionicin SM1 have been characterized and their amino acid sequence determined (Lyon and Glatz, 1991; Faye et al., 2000; Miescher et al., 2000). Propionicin T1 is the only bacteriocin which is grouped according to the classification system proposed by Klaenhammer (1993). In this paper we report on thoeniicin 447, a bacteriocin produced by *P. thoenii* 447. Data on the spectrum of antimicrobial activity, production, isolation, purification and mechanism of activity of thoeniicin 447 are presented.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains used are listed in Table 1. The strains were from LMG (Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium), ATCC (American Type Culture Collection, Rockville, MD, USA) and the culture collection of Prof. T.J. Britz (strains numbered "BAC"), Department of Food Science, University of Stellenbosch, Stellenbosch, South Africa. Strain 447 was originally obtained from Prof. G.W. Reinbold, Iowa State University, Iowa, USA. The dairy propionibacteria were cultured in yeast extract lactate (YEL) medium (lactic acid 15ml l^{-1}, yeast extract 5g l^{-1}, peptone 2g l^{-1}, KH_2PO_4 10g l^{-1}, Tween 80 1ml l^{-1}) at 30°C and the *Propionibacterium acnes* strains in RCM medium (Merck, Darmstadt, Germany) at 37°C. All lactic acid bacteria were grown in MRS broth (Biolab Diagnostics, Midrand, South Africa) at 30°C and all other strains in BHI broth (Biolab) at 30°C.

2.2. Inhibitory activity

P. thoenii strain 447 was inoculated (1% v/v) into YEL broth and incubated at 30°C without aeration until late logarithmic growth ($O.D._{600nm}$=2.25). The cell-free supernatant was concentrated 10-times by freeze-drying. The spot-on-lawn method (Mayr-Harting et al., 1972) was used to determine the antimicrobial activity of thoeniicin 447 against the organisms listed in Table 1. The cell-free culture supernatant was adjusted to a pH value between 6.5 and 7.0 with 1M NaOH. An aliquot of 10µl was spotted onto solid medium (0.7% w/v, agar) seeded with active growing cells of the test organism (approx. 10^6 cfu ml^{-1}). The plates were incubated at the optimal growth temperatures as indicated in the respective culture collection catalogues. A clear zone of inhibition of at least 2 mm in diameter was recorded as positive.

2.3. Production of thoeniicin 447

Production of thoeniicin 447 was followed during a 84 h-growth cycle of *P. thoenii* 447 in YEL medium, buffered with phosphate (KH_2PO_4) to pH 7. At specific time intervals, samples of

1 ml were aseptically removed and the number of viable cells determined by plating onto YEL agar. Samples of 10 ml were centrifuged, the cell-free supernatant freeze-dried and concentrated ten-fold by resuspension in sterile distilled water. From the bacteriocin activity, expressed as arbitrary activity units per ml (AU ml^{-1}) and the cell counts (Log_{10} cfu ml^{-1}), the specific bacteriocin activity (AU/Log_{10} cfu) was calculated. The AU was determined according to the method of Henderson et al. (1992). *L. delbrueckii* subsp. *bulgaricus* LMG 13551 was used as indicator organism.

2.4. Isolation and partial purification of thoeniicin 447

P. thoenii 447 was grown in YEL medium at 30°C until late logarithmic growth ($O.D._{600}$ = 2.25). The cells were harvested by centrifugation and thoeniicin 447 isolated from the cell-free supernatant as described by Green et al. (1997). Partial purification was done by ammonium sulfate (60%, w/v, final concentration) precipitation (Bollag and Edelstein, 1991). The precipitate was recovered by centrifugation (11 200 x g, 15min, 4°C), resuspended in sterile MilliQ water and dialysed against sterile MilliQ water at 8°C for 24 h, using a 1 kDa cut-off dialysis membrane (Spectrum Laboratories Inc., Gardena, California, USA). The pH of the desalted sample was adjusted to between 6.5 and 7.0 with 0.1 N NaOH and then freeze-dried.

2.5. Size determination

Freeze-dried samples of thoeniicin 447 were resuspended in sterile distilled water and further purified by SP-Sepharose chromatography (Ausubel et al., 1994), followed by tricine-SDS-PAGE, as described by Schägger and Von Jagow (1987). A low molecular-weight protein marker with a size range from 2.35 to 46 kDa was used. The gels were fixed and one half stained with Coomassie Brilliant Blue R250 (Saarchem, Krugersdorp, South Africa). The other half of the gel was pre-washed with sterile distilled water and overlayed with active growing cells of *P. acnes* ATCC 6919 (approximately 10^6 cfu ml^{-1}), embedded in RCM agar (0.7% w/v). After 1 day of incubation at 37°C, formation of an inhibition zone indicated the position and size of active thoeniicin 447 in the gel.

2.6. Sensitivity to heat, pH and proteolytic enzymes

These tests were done on partially purified samples of thoeniicin 447. *L. delbrueckii* subsp. *bulgaricus* LMG 13551 was used as indicator strain. Aliquots of thoeniicin 447 were exposed to heat treatments of 60, 80 and 100°C for 15 and 30 min, respectively, and 121°C for 10 min, followed by immediate cooling on ice. The samples were tested for antimicrobial activity, as described before. In a separate experiment, samples of thoeniicin 447 were adjusted to pH values ranging from 1 to 10, incubated at 37°C for 30 min, neutralized to pH 7, and tested for bactericidal activity. Resistance of thoeniicin 447 to proteolytic enzymes was determined by incubation of the bacteriocin samples in the presence of pepsin (2500 U mg^{-1} thoeniicin 447), pronase (7 U mg^{-1} thoeniicin 447), α-chymotrypsin (90 U mg^{-1} thoeniicin 447), trypsin (110 Umg^{-1} thoeniicin 447) and Proteinase K (20 U mg^{-1} thoeniicin 447) at 37°C for 2 h. After incubation, the enzymes were heat-inactivated for 3 min at 100°C, and tested for antimicrobial activity as described before.

2.7. Mode of action

Partially purified thoeniicin 447 (128 AU ml^{-1}) was added to mid-logarithmic growth phase cells of *L. delbrueckii* subsp. *bulgaricus* LMG 13551 in 200 ml MRS broth (Biolab) and mid-logarithmic growth phase cells of *P. acnes* ATCC 6919 in 200 ml RCM medium. The controls were ten-fold concentrated medium (MRS or RCM) added to the cultures. Changes in cell density were recorded at 600 nm and the number of viable cells (cfu) determined by plating the samples on MRS or RCM agar (1.4%, w/v).

2.8. Isolation of DNA

Total DNA was isolated according to the method of Dellaglio et al. (1973). Plasmid DNA was isolated using the method described by Burger and Dicks (1994), after which the DNA was further purified by CsCl density gradient centrifugation (Sambrook et al., 1989). The DNA was separated on an agarose gel, according to the method described by Sambrook et al. (1989). Lambda DNA digested with *Eco*RI and *Hind*III (Promega, Madison, USA) was used as molecular

weight marker. From the DNA sequence of propionicin T1 (Faye et al., 2000), two primers were designed; one from the N-terminal end (5' GCTCGAGCACTCAAACCCAT 3') and one from the C-terminal end (5' GCGACCCTCTTCCGGTATCT 3'). The primers for sequencing were obtained from Genosys Biotechnologies (Europe) Ltd. (Pampisford, United Kingdom). The DNA was amplified in 50 µl reaction mixtures containing $MgCl_2$, dNTPs, primers, buffer, Taq polymerase and DNA, as described by Ausubel et al. (1994). Amplification proceeded through 30 cycles with the annealing temperature at 55°C for 30 seconds. The DNA was separated on a 2% low melting point agarose gel (Whitehead Scientific), according to the method described by Sambrook et al. (1989) and purified using a 7 kDa cut-off dialysis membrane (Pierce, Rockford, USA). A PCR product of approx. 460 bp was sequenced on an automatic sequencer (ABI Prism ^{TM} 377, PE Biosystem SA [Pty] Ltd), using dye terminator chemistry (Biosystems, Warrington, England).

2.9. Induction

To determine if induction plays a role in thoeniicin 447 production, a low concentration of thoeniciin 447 (0.0028 AU ml^{-1}) was added to an active growing culture of *P. thoenii* 447. In two separate experiments, approx. 10^7 cfu ml^{-1} of *P. thoenii* 447 and approx. 10^5 cfu ml^{-1} of heat-killed *P. acnes* were added, respectively, to a mid-log phase culture of *P. thoenii.* The results obtained were compared with that recorded for *P. thoenii* 447 grown in YEL medium.

3. Results

All data represents an average of three repeats. The optical density readings and bacteriocin activity tests did not vary by more than 5%.

Among the indicator strains tested, *L. delbrueckii* subsp. *bulgaricus* LMG 13551 was the most sensitive (Table 1). All four strains of *P. acnes* were inhibited. No activity was recorded against the other lactic acid bacteria tested. Maximum specific antimicrobial activity of 10 AU/Log_{10} cfu was recorded during early stationary phase of growth (Fig. 1). The activity remained at 10 AU/Log_{10} cfu for only 4 h, after which it declined to approximately 3 AU/Log_{10}

cfu towards the end of growth (Fig. 1). During growth of *P. thoenii* 447 exopolysaccharides was observed in growth medium.

Thoeniicin 447 was partially purified by ammonium sulfate precipitation (60% saturation), followed by cation exchange chromatography. Separation by tricine-SDS-PAGE revealed an active peptide band in the region of 6 kDa (Fig. 2). Based on DNA sequencing, the mature peptide is 7130 Da in size and homologous to propionicin T1.

Thoeniicin 447 was resistant to heat treatments of 60°C and 80°C for 15 and 30 min and at 100°C for 15 min. However, 80% of the activity was lost after the bacteriocin was autoclaved (121°C for 10 min). Incubation in buffers for 30 min at pH values ranging from 1 to 10 had no effect on the activity of thoeniicin 447. Thoeniicin 447 is sensitive to pepsin, pronase, α-chymotrypsin trypsin and Proteinase K. No rest-activity of thoeniicin 447 was obtained after treatment with these proteolytic enzymes.

The addition of thoeniicin 447 to cells of *L. delbrueckii* subsp. *bulgaricus* LMG 13551 in their mid-logarithmic growth phase resulted in a four log-cycle decrease in the number of viable cells, i.e. from approx. 10^9 to 10^5 cfu ml^{-1} over 10 h (Fig. 3a). In the same experiment, the optical density readings of *L. delbrueckii* subsp. *bulgaricus* LMG 13551 increased for the first hour, but thereafter declined from O.D. $_{600nm}$ 0.6 to 0.3 for the remainder of the incubation period. The addition of thoeniicin 447 to active growing cells of *P. acnes* ATCC 6919 (at 28 h of growth) resulted in a slow decrease in the number of viable cells over a period of 24 h (approx. 10^7 to 10^6 ml^{-1}; Fig. 3b). The optical density readings of *P. acnes* ATCC 6919 remained more-or-less constant at approx. 0.70 for the remainder of the incubation period (Fig. 3b).

A PCR product with a size of approx. 460 bp was found, which contained the structural gene for thoeniicin 447 production (Fig. 4). Deduced from the DNA sequence, the mature bacteriocin contains 65 amino acids, with a total molecular mass of 7130 Da. No significant results were obtained from the induction experiments to conclude that signal transduction could play a role in the production of thoeniicin 447 (results not shown).

4. Discussion

Thoeniicin 447 is a relatively small (7 kDa) and heat-stable peptide and based on DNA sequencing the peptide is cationic and can be regarded as a member of class II bacteriocins,

according to the classification system used for bacteriocins of lactic acid bacteria (Klaenhammer, 1988). Thusfar no classification system has been proposed for bacteriocins produced by propionibacteria.

P. acnes is an opportunistic pathogen found within the human body and plays a significant role in the skin disease, acne vulgaris. Thoeniicin 447 has several features in common with propionicin SM1, propionicin T1 and a bacteriocin produced by *P. jensenii* B1264. All of the latter bacteriocins display a narrow spectrum of activity, whereas propionicin PLG-1 and jenseniin G showed a broad spectrum of activity (Grinstead and Barefoot, 1992).

Thoeniicin 447 is produced during the early stationary phase of growth. However, the culture supernatant had to be concentrated at least ten-fold to detect antimicrobial activity. Propionicin PLG-1 (Lyon and Glatz, 1991) and jenseniin G (Grinstead and Barefoot, 1992) are detected during the late stationary phase of growth. One possible explanation for the decrease in thoeniicin 447 activity could be the adsorbance of the peptide to the exopolysaccharides produced. The adsorbtion to the cell surface could not be due to pH factors, because the culture medium was buffered at pH 7 and remained constant during fermentation. Further research is needed to determine whether other factors are involved in the inactivation of thoeniicin 447.

Thoeniicin 447 was not purified to homogeneity, however with ammonium sulfate precipitation and cation exchange chromatography most of the contaminating proteins were removed. A band ranging in size between 6 and 7 kDa was found on a tricine SDS-PAGE gel, which corresponded to the position of an inhibition zone against *P. acnes* (Fig. 2). According to DNA sequencing the mature thoeniicin 447 is 7.130 kDa in size. Propionicin SM1 is 19.942 kDa in size and is the largest bacteriocin thusfar described for *Propionibacterium* spp., followed by propionicin PLG-1 (9.328 kDa) and a bacteriocin from *P. jensenii* B1264 (between 6 and 9 kDa) (Miescher, 2000; Paik and Glatz, 1995; Ratnam, 1999).

The heat stability of thoeniicin 447 (up to 15 min 100°C) is similar to that reported for the jenseniin G. Thoeniicin 447 remained stable at pH 1 to 10. Similar pH stability results (pH 3 to 9) have been reported for propionicin PLG-1 (Lyon and Glatz, 1991) and for a bacteriocin produced by *P. jensenii* B1264, i.e. pH 3 to 12 (Ratnam et al., 1999).

Thoeniicin 447 displayed a bactericidal mode of action against *L. delbrueckii* subsp. *bulgaricus* LMG 13551 and a bacteriostatic action against *P. acnes* ATCC 6919. The mature peptide displayed complete homology with propionicin T1 produced by *P. thoenii* 419 (Faye et

al., 2000). However, *P. thoenii* 447 is phenotypically different from strain 419 and belongs to a separate ribotype cluster (Riedel and Britz, 1996; Riedel et al., 1998). Four ribotype profiles (ribotype M-P) were identified for strains phenotypically classified as *P. thoenii*. Strain 447 belongs to ribotype N, and strain 419 to ribotype P. No significant homology with other bacteriocins was found using the BLAST (Basic Local Alignment Search Tool) program. According to these observations and the aminoacid sequence of thoeniicin 447, the peptide contains the pattern of a typical signal peptide. A signal peptide consists of a positively charged N-terminus, a central hydrophobic core and a specific cleavage region (Nielsen et al., 1997).

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Table 1
Spectrum of antimicrobial activity of thoeniicin 447, produced by *P. thoenii* 447

Species	Strain	Growth medium	Activity
Bacillus cereus	LMG 13569	BHI	-
Clostridium sporogenes	LMG 13570	RCM	-
Clostridium tyrobutyricum	LMG 13571	RCM	-
Enterococcus faecalis	LMG 13566	BHI	-
Lactobacillus acidophilus	LMG 13550	MRS	-
Lactobacillus bulgaricus	LMG 13551	MRS	+++
Lactobacillus casei	LMG 13552	MRS	-
Lactobacillus fermentum	LMG 13554	MRS	-
Lactobacillus helveticus	LMG 13555	MRS	-
Lactobacillus plantarum	LMG 13556	MRS	-
Lactobacillus reuteri	LMG 13557	MRS	-
Lactobacillus sakei	LMG 13558	MRS	-
Listeria innocua	LMG 13568	BHI	-
Pediococcus pentosaceus	LMG 13560	MRS	-
"	LMG 13561	MRS	-
P. jensenii	BAC 1 S	YEL	-
"	BAC 4 S	YEL	-
"	BAC 5	YEL	-
"	BAC 15	YEL	-
"	BAC 16	YEL	-
P. acidipropionici	BAC 7	YEL	-
P. freudenreichii	BAC 3 S	YEL	-
P. freudenreichii subsp. *shermanii*	BAC 6	YEL	-
"	BAC 2 S	YEL	-
"	BAC 8	YEL	-
"	BAC 9	YEL	-
"	BAC 10	YEL	-
"	BAC 11	YEL	-
"	BAC 12	YEL	-
P. freudenreichii subsp. *freudenreichii*	BAC 13	YEL	-
P. acnes	ATCC 6919	RCM	++
"	ATCC 6922	RCM	++
"	ATCC 11827	RCM	++
"	ATCC 11828	RCM	++

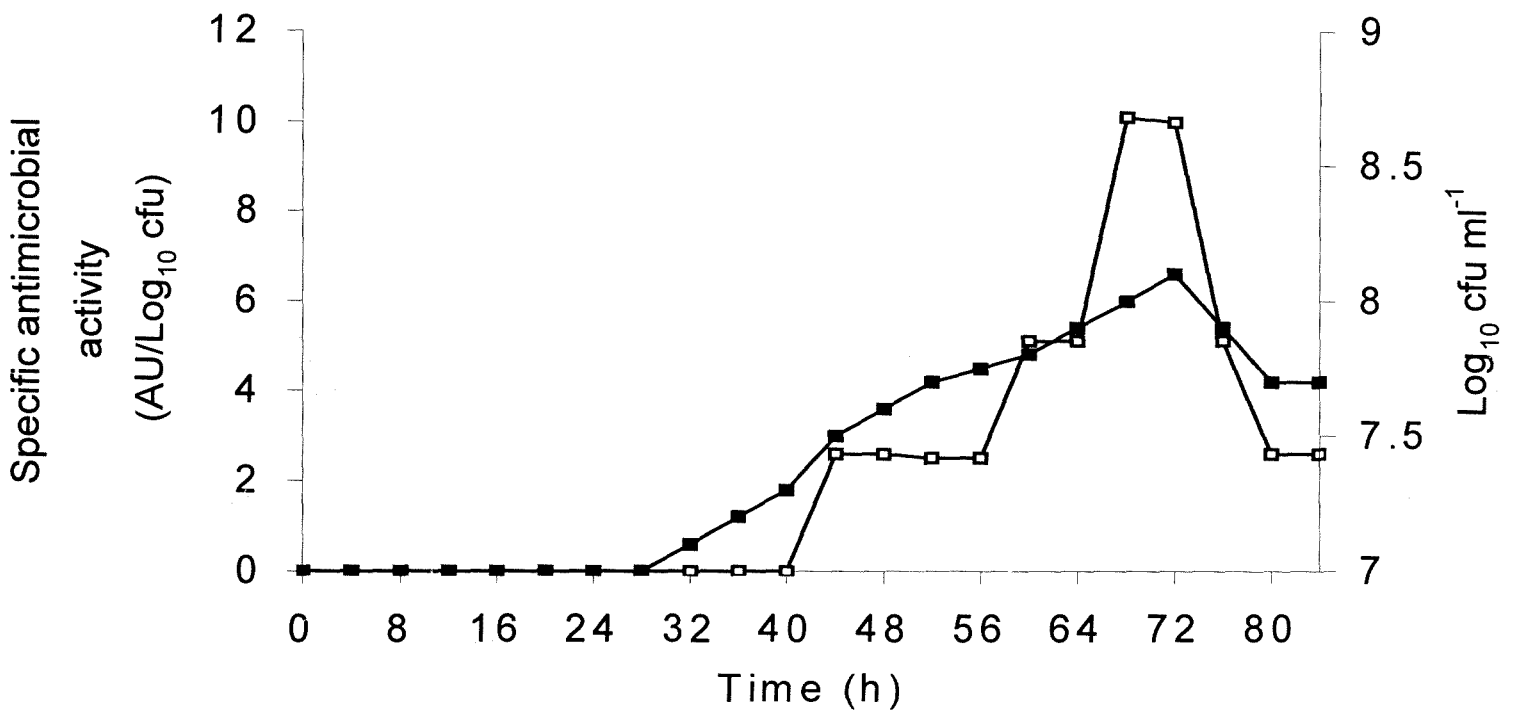

Fig. 1. Production of thoeniicin 447 during growth of *P. thoenii* 447.

Symbols: ■, Log_{10} cfu ml^{-1}; □, specific antimicrobial activity of thoeniicin 447 (AU/Log_{10} cfu).

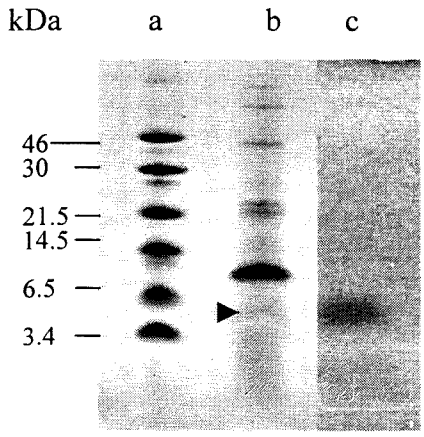

Fig. 2. Separation of thoeniicin 447 by tricine-SDS-PAGE.

(a) Rainbow protein molecular-weight marker; (b) partially purified crude extract and (c) gel overlaid with cells of *Propionibacterium acnes* embedded in RCM agar (0.7%, w/v, agar). The arrow indicates the active protein band.

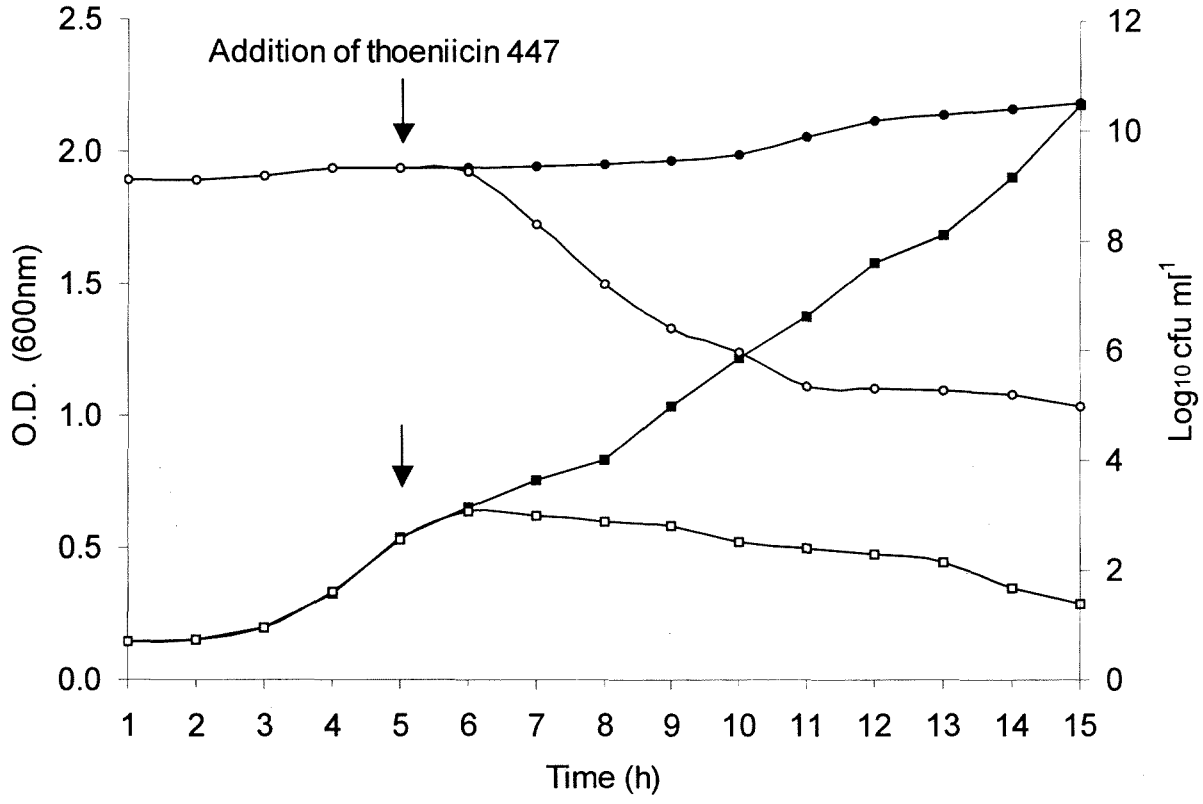

Fig. 3 a. The effect of thoeniicin 447 on the growth of *L. delbrueckii* subsp. *bulgaricus*.

Symbols: ■ and □, turbidity of the cells (measured at O.D.=600nm), in the absence and presence of thoeniicin 447, respectively.

● and ○, viable cell numbers (cfu ml^{-1}), in the absence and presence of thoeniicin 447, respectively.

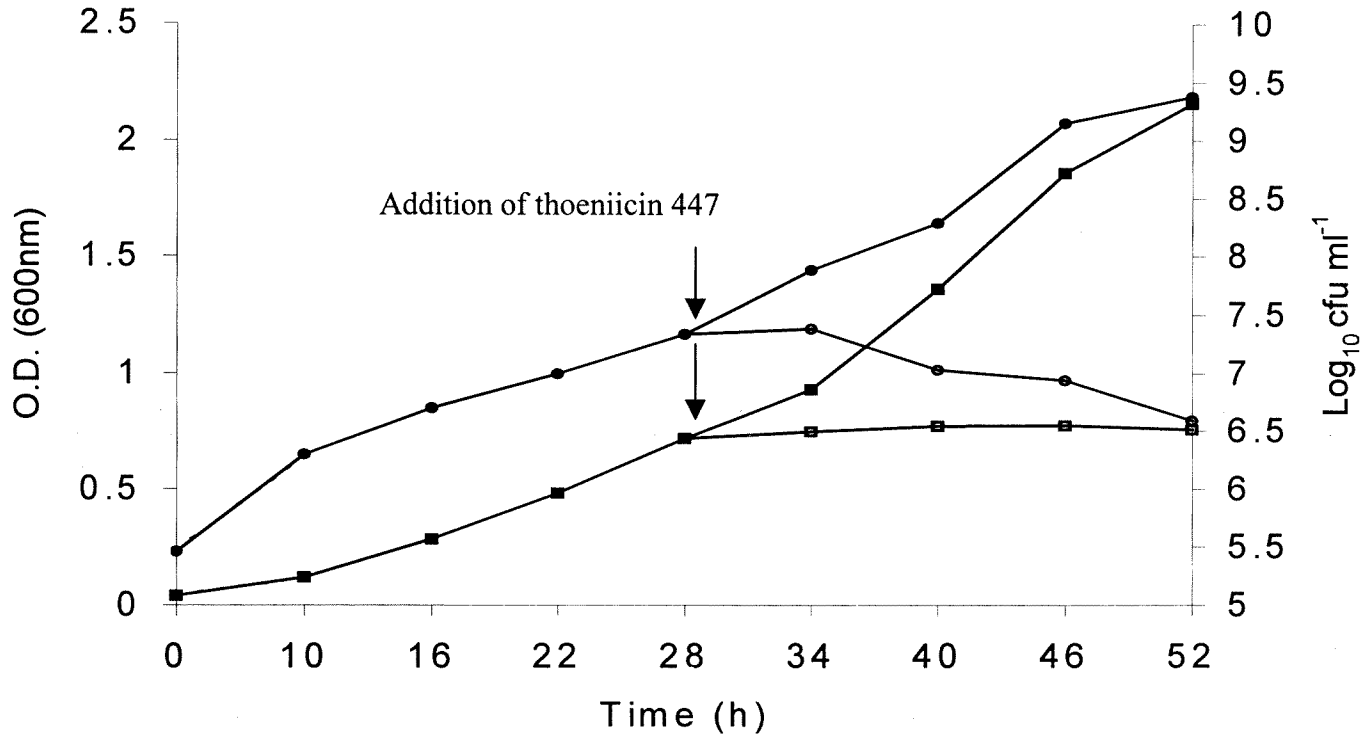

Fig. 3. b The effect of thoeniicin 447 on the growth of *Propionibacterium acnes.*

Symbols: ■ and □, turbidity of the cells (measured at O.D. 600 nm) growing in the absence and presence of thoeniicin 447, respectively.

● and ○, viable cell counts (cfu ml^{-1}) in the absence and presence of thoeniicin 447, respectively.

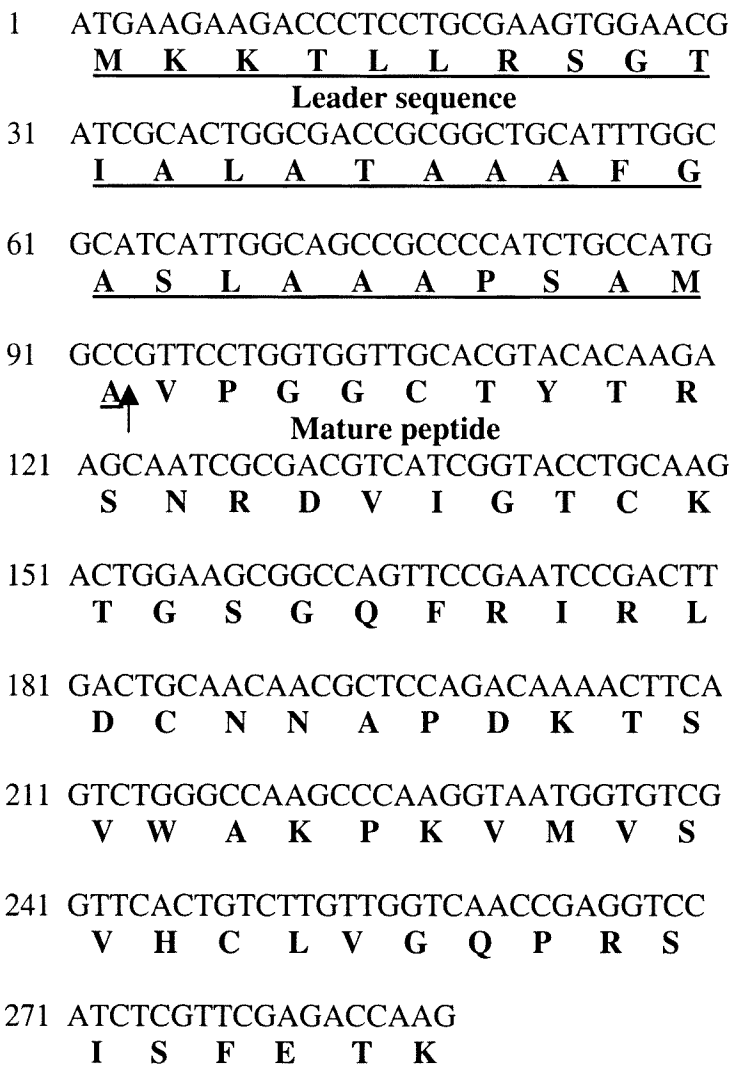

Fig. 4. DNA sequence of thoeniicin 447, showing the cleavage site of the leader sequence (↑) and the amino acid sequence of the mature peptide consisting of 65 amino acids.

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER 4

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Propionibacterium thoenii 447 produces a bacteriocin-like peptide which was named thoeniicin 447. The peptide has a narrow spectrum of activity and inhibited only *Lactobacillus delbrueckii* subsp. *bulgaricus* and a few *P. acnes* strains tested. Thoeniicin 447 was inactivated by the proteolytic enzymes pepsin, pronase, α-chymotripsin, trypsin and Proteinase K. Heat treatments of 60°C, 80°C and 100°C had no effect on the activity of the bacteriocin, but 80% of the activity was lost after autoclaving (121°C, 10 min). The peptide remained active after 30 min of incubation in a pH range 1 to 10. The heat stability and activity over a broad pH range are of industrial importance, as the peptide can be used for possible pharmaceutical applications.

The peptide was partially purified using ammonium sulfate precipitation, followed by cation exchange chromatography. Thoeniicin 447 could not be purified to homogeneity because of the low production of the peptide during growth. Based on results obtained by the tricine-SDS-PAGE, the partially purified bacteriocin was determined to be approximately 6 kDa in size. The DNA sequence of thoeniicin 447 was determined, and based on these results, the molecular size of the mature peptide was determined at 7130 Da. In contrast to the other bacteriocins produced by propionibacteria, thoeniicin 447 is a relatively small peptide.

Deduced from the DNA sequence, mature peptide contains 65 amino acids. Based on the DNA sequencing, thoeniicin 447 is homologous to propionicin T1, produced by *P. thoenii* strain 419 (Faye et al., 2000). However, *P. thoenii* 447 belongs to a different phenotypic subcluster as strain 419 (Britz and Riedel, 1995). Four ribotype profiles (ribotype M-P) were identified for strains originally classified as *P. thoenii* (Riedel and Britz, 1996; Riedel et al., 1998). Strain 447 belongs to ribotype N and strain 419 to ribotype P.

Maximum production of thoeniicin 447 was recorded during early stationary growth. However, the culture supernatant had to be concentrated at least ten-fold to detect antimicrobial activity. The decrease in thoeniicin 447 activity could be due to exopolysaccharides produced during growth. Adsorption to the cell surface could not be due to changes in pH, since the

medium was buffered to pH 7, which then remained constant during the fermentation of *P. thoenii* 447.

Prospective application of thoeniicin 447 in the pharmaceutical industry seems promising. Acne vulgaris is a multifactorial disease in which propionibacteria, which form part of the resident cutaneous microflora, play an important role in the pathogenesis of inflamed lesions (Leyden et al., 1998; Leyden, 2001). *P. acnes* proliferates in the environment of the microcomedo and generates molecules that stimulate both comedogenesis and inflammation. The disease occurs mainly during puberty but also during adolescence. The failure of patients with antibiotic-resistant *P. acnes* to respond to antimicrobial therapy supports the importance of *P. acnes* in the pathogenesis of acne (Eady, 1998). At present antibiotic therapy for acne is very common and frequently used in acne, either systematically or topically.

Unfortunately, bacterial resistance is beginning to emerge as a significant problem. It is therefore important to decrease the number of *P. acnes* in the microcomedo to prevent inflammation of the follicles. Thoeniicin 447 acts bacteriostatic against *P. acnes* and could be used to prevent an increase in opportunistic pathogens such as *P. acnes*.

Thoeniicin 447 may be used in combination with other topical treatments that are currently available. The peptide may be incorporated in a wide range of cosmetics, creams, facial scrubs and soaps. However, it is very important that the peptide remains active and effective. It is therefore important that these products do not contain substances that could have an effect on the activity of the peptide and this must be evaluated.

The specific antimicrobial activity of thoeniicin 447, once concentrated ten-fold, is approximately 10AU/Log_{10} cfu (i.e. 1000AU/1000cfu). Methods to optimize thoeniicin 447 production and identification of factors affecting the production of the peptide have to be developed. Furthermore, *P. thoenii* is an anaerobic, slow growing organism and a well-controlled anaerobic environment is needed. Cost-effective growth media should be developed for better yields of the bacteriocin.

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