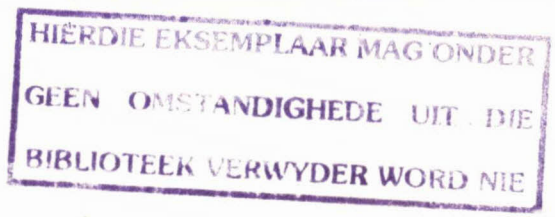

A TAXONOMIC RE-EVALUATION OF "*Propionibacterium coccoides*"

by

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Submitted in fulfilment of the requirements
for the degree of

MASTER OF SCIENCE

in the

Department of Microbiology and Biochemistry, Faculty of Natural Science
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November 1998

To my parents, especially my mother

"It is, however, important to realise that species are entities created by man to group organisms into, and that these groupings are based on hypotheses which are subject to continual re-evaluation"

K-H.J. Riedel (1996)

Acknowledgements

I wish to express my sincere gratitude and appreciation to the following persons for their contributions to the successful completion of this study:

Dr. K-H.J. Riedel, Department of Microbiology and Biochemistry, University of the Orange Free State, for his adequate guidance, continual patience and for his valuable and constructive criticism of the manuscript;

Prof. B.D. Wingfield, Department of Genetics, University of Pretoria, for her advice and for granting a post-graduate bursary through the Foundation of Research Development;

Prof. L.I. Vorobjeva, Department of Microbiology, Moscow State University, Russia, for the two "*Propionibacterium coccoides*" strains and sincere interest throughout this study;

Prof. J.P. van der Walt, Department of Microbiology and Biochemistry, University of the Orange Free State, for his invaluable assistance in the nomenclature of the two "*P. coccoides*" strains;

The staff of the Department of Microbiology and Biochemistry, University of the Orange Free State, and numerous people not mentioned by name who in some way have contributed to this study;

To my parents, for their love, continuous encouragement, moral support as well as financial support throughout my entire university career;

To my friend, Jakkie van Eden, for his love, patience, interest, encouragement and support throughout the study; and

Above all, to Him who made it all possible.

Table of Contents

Language and style used in this dissertation are in accordance with the requirements of the journal Systematic and Applied Microbiology)

Chapter 1

Introduction

The genus *Propionibacterium* consist of two principal groups from different habitats, namely the "classical" or dairy and the "cutaneous" or clinical propionibacteria. The classical group is isolated primarily from cheese and dairy products. One species of the classical group namely *Popionibacterium freudenreichii* is used as a starter culture during the manufacture of Swiss-type cheese. It has been reported that the propionic acid bacteria contribute to the sweet nutty, as well as the buttery flavour of these cheese (*Glatz*, 1992; *Hettinga* and *Reinbold*, 1972; *Langsrud* and *Reinbold*, 1973). Propionibacteria are also responsible for the characteristic "eye" formation in Swiss-type cheese through the production of carbon dioxide (*Langsrud* and *Reinbold*, 1974; *Chaia* et al., 1990). The classical propionibacteria may also contribute to the natural fermentation of silage and olives (*Woolford*, 1975), although spoilage in the olive industry by members of the genus *Propionibacterium* has also been reported (*Vaughn*, 1981). The propionibacteria have, however, also been reported to be the causative agents of some defects, including split and late blowing of Swiss-type cheese, due to the excessive production of CO_2 (*Langsrud* and *Reinbold*, 1974; *Hettinga* and *Reinbold*, 1975; *Massa* et al., 1992). The presence of pigmented propionibacteria can also be responsible for a spoilage condition in the food industry which is often referred to as red or brown spotting (*Britz* and *Jordaan*, 1976; *Baer* and *Ryba*, 1992; *Baer* et al., 1993). The production of propionic and other minor organic acids, CO_2, bacteriocins, vitamins (*Mantere-Alhonen*, 1995) and the antimutagenic properties displayed by propionibacteria (*Vorobjeva* et al., 1991:1996) may be of importance in the application of these organisms as probiotic agents in foods. The propionibacteria are also receiving increasing attention due to the fact that they rank amongst the most potent immunomodulatory stimulating cell populations involved in non-specific resistance (*Roszkowski* et al., 1990).

The genus *Propionibacterium* was first described in 1906 by *Von Freudenreich* and *Orla-Jensen*. In 1957 *Breed* et al. described 11 classical species within this genus. These 11 species were later consolidated in 4 species namely:

P. acidipropionici; *P. freudenreichii*; *P. jensenii;* and *P. thoenii* (*Moore* and *Holdeman*, 1974). Recently a fifth classical species namely *P. cyclohexanicum* (*Kusano* et al., 1997) was also described which was isolated from spoiled orange juice.

Members of the cutaneous group have primarily been isolated from the intestine and upper respiratory tract of humans (*Cummins* and *Johnson*, 1986). Species was, however, also isolated from dogs (*Goodacre* et al., 1994), pigs (*Benno* and *Mitsuoka*, 1982) and paddy soils (*Hayashi* and *Furusaka*, 1979). Although these organisms are generally considered opportunistic pathogens, recent reports published by *Brooke* and *Frazier* (1991), *Horner* et al. (1992) and *Ramos* et al. (1995) indicate the possibility that the species of the cutaneous group could be primary pathogens. The cutaneous group was initially included in the genus *Corynebacterium*. In 1974, *Moore* and *Holdeman* transferred four *Corynebacterium* to form the cutaneous members of the genus *Propionibacterium*. These species include: *P. acnes; P. avidum; P. granulosum;* and *P. lymphophilum* (*Moore* and *Holdeman*, 1974). In 1988, *Charfreitag* et al. proposed that *Arachnia propionicus* be transferred to the genus *Propionibacterium* as *P. propionicus*. This was proposed based on sequence analysis done on long regions of the 16S rRNA that indicates a more reliable relationship with *P. freudenreichii* and *P. acnes* than with members of the genus *Arachnia*.

In 1983, *Vorobjeva* et al. described a propionic acid producing coccus, which was isolated from Soviet hard cheese in the early stage of ageing. When compared to the other propionic acid bacteria, this propionic acid producing bacterium was shown to be similar in fatty acid composition to the genus *Propionibacterium*, especially *P. jensenii* (the old "*P. technicum*" strain). This bacterium also contained the *anteiso* isomer of the C_{15}-saturated fatty acid (12-methyltetradecanoic) as the major type of cellular lipids, which is characteristic of the other members of the genus *Propionibacterium*. During lactate fermentation, propionic acid was also produced as the major metabolic end-product, which is characteristic of the genus *Propionibacterium*. Furthermore, considerable quantities of vitamin B_{12}, catalase,

superoxide dismutase and peroxidase are synthesised by both the propionibacteria and this propionic acid coccus (*Vorobjeva* et al., 1983; *Cummins* and *Johnson*, 1992).

Based on the high degree of similarity in phenotypic characteristics, data on fatty acid composition, DNA nucleotide composition and the high DNA:DNA homology values observed between this propionic acid producing coccus and members of the genus *Propionibacterium*, *Vorobjeva* et al. (1983) subsequently proposed that this propionic acid coccus be included in the genus *Propionibacterium* as an independent species under the name "*Propionibacterium coccoides*". Despite the fact that various attempts have been made at solving the taxonomic dilemma in the genus *Propionibacterium*, no further comparative evaluations of "*P. coccoides*" and the propionic acid bacteria have ever been undertaken. Subsequently "*P. coccoides*" has never been officially recognised as a new species within the genus *Propionibacterium*. No mention of this strain or the proposal of *Vorobjeva* et al. (1983) was made in Bergey's Manual of Systematic Bacteriology (*Cummins* and *Johnson*, 1986). Furthermore, "*P. coccoides*" was also not included in Bergey's Manual of Determinative Bacteriology (*Holt* et al., 1994) despite the fact that *Cummins* and *Johnson* (1992) stated that there was sufficient evidence to include this species in the genus *Propionibacterium.*

The objectives of this study were therefore, to firstly characterise "*P. coccoides*" and the various classical species within the genus *Propionibacterium* phenotypically, in order to determine to what extent the "*P. coccoides*" strains could be equated with the existing classical species. The next objective was to determine the relationship of the "*P. coccoides*" strains to known *Propionibacterium* marker strains using various molecular techniques, based on the 16S ribosomal RNA region, as well as DNA:DNA hybridisation. Using the information gained from these studies, as well as that of various other studies (*Britz* and *Riedel*, 1994; *Riedel* and *Britz*, 1993:1996 and *Riedel* et al., 1994), the systematic position of "*P. coccoides*" relative to the existing *Propionibacterium* species would be evaluated.

References

Baer, A., Ryba, I.: Serological identification of propionibacteria in milk and cheese samples. Int. Dairy J. 2, 299-310 (1992).

Baer, A., Ryba, I., Grand, M.: Study on the appearance of brown-spotting in cheese. Schweiz. Milchw. Forschung 22, 3-7 (1993).

Benno, Y., Mitsuoka, T.: Anaerobic bacteria isolated from abscesses in pigs. Jap. J. Vet. Sci. 44, 309-315 (1982).

Breed, R.S., Murray, E.G.D., Smith, N.R.: Genus 1. *Propionibacterium* Orla-Jensen 1909, pp. 569-577. In: Bergey's Manual of Determinative Bacteriology (R.S. Breed, E.G.D. Murray, N.R. Smith, eds.) 7th ed., Bailliére, London, Tindall and Cox 1957.

Britz, T.J., Jordaan, H.F.: The rapid presumptive detection of propionibacteria as the causative organisms of defects in Gouda cheese. S. Afr. J. Dairy Technol. 8, 79-83 (1976).

Britz, T.J., Riedel, K-H.J.: *Propionibacterium* species diversity in Leerdammer cheese. Int. J. Food Microbiol. 22, 257-267 (1994).

Brooke, I., Frazier, E.H.: Infections caused by *Propionibacterium* species. Rev. Infect. Dis. 13, 819-822 (1991).

Chaia, A.P., De Ruiz Holgado, A.P., Oliver, G.: Peptide hydrolyses of propionibacteria: Effect of pH and temperature. J. Food Protect. 53, 237-240 (1990).

Charfreitag, O., Collins, M.D., Stackebrandt, E.: Reclassification of *Arachnia propionica* as *Propionibacterium propionicus* comb. nov. Int. J. Syst. Bacteriol. 38, 354-357 (1988).

Cummins, C.S., Johnson, J.L.: Genus I. *Propionibacterium* Orla-Jensen 1909, pp. 1346-1353. In: Bergey's Manual of systematic Bacteriolgy (P.H.A. Sneath, N.S. Mair, M.E. Sharpe, J.G. Holt, eds.) vol. 2. Baltimore, Williams and Wilkins 1986.

Cummins , C.S., Johnson, J.L.: The Genus *Propionibacterium*, pp. 834-849. In: The Prokaryotes (A. Balows, H.G. Truper, M. Dworkin, W. Harder and K-H. Schleifer, eds.) vol. 1. New York, Springer-Verlag 1992.

Glatz, B.A.: The classical propionibacteria: their past, present and future as industrial organisms. ASM News 58, 197-201 (1992).

Goodacre, R., Neal, M.J., Kell, D.B., Greenham, L.W., Noble, W.C., Harvey, R.G.: Rapid identification using pyrolysis mass spectrometry and artificial neural networks of *Propionibacterium acnes* isolated from dogs. J. Appl. Bacteriol. 76, 124-134 (1994).

Hayashi, S., Furusaka, C.: Studies of *Propionibacterium* isolated from paddy soils. Antonie van Leeuwenhoek 45, 565-574 (1979).

Hettinga, D.H., Reinbold, G.W.: The propionic acid bacteria - a review. I. Growth. J. Milk Food Technol. 35, 295-301 (1972).

Hettinga, D.H., Reinbold, G.W.: Split defect of Swiss cheese. II. Effect of low temperatures on the metabolic activities of *Propionibacterium*. J. Milk Food Technol. 38, 31-35 (1975).

Holt, J.G., Krieg, N.R., Sneath, P.H., Staley, J.T., Williams, S.T.: Genus *Propionibacterium*, p. 580. In: Bergey's Manual of Determinative Bacteriology (J.G. Holt, N.R. Krieg, P.H. Sneath, J.T. Staley, S.T. Williams, eds.) 9th ed., Baltimore, Williams and Wilkins 1994.

Horner, S.M., Sturridge, M.F., Swanton, R.H.: *Propionibacterium acnes* causing an aortic root abscess. Br. Heart J. 68, 218-220 (1992).

Kusano, K., Yamada, H., Niwa, M., Yamasato, K.: *Propionibacterium cyclohexanicum* sp. nov., a new acid-tolerant ω-cyclohexyl fatty acid-containing *Propionibacterium* isolated from spoiled orange juice. Int. J. Syst. Bacteriol. 47, 825-831 (1997).

Langsrud, T., Reinbold, G.W.: Flavour development and microbiology of Swiss Cheese. A review. II. Starters, manufacturing processes and procedures. J. Milk Food Technol. 36, 531-542 (1973).

Langsrud, T., Reinbold, G.W.: Flavour development and microbiology of Swiss Cheese. A review. IV. Defects. J. Milk Food Technol. 37, 26-41 (1974).

Mantere-Alhonen, S.: Propionibacteria used as probiotics - A review. Lait 75, 447-452 (1995).

Massa, S., Gardini, F., Sinigaglia, M., Guerzoni, M.E.: *Klebsiella pneumonia* as a spoilage organism in Mozzarella cheese. J. Dairy Sci. 75, 1411-1414 (1992).

Moore, W.E.C., Holdeman, L.V.: Genus I. *Propionibacterium* Orla-Jensen 1909, pp. 633-641. In: Bergey's Manual of Determinative Bacteriology (R.E. Buchanan, N.E. Gibbons, eds.) 8th ed., Baltimore, Williams and Wilkins 1974.

Ramos, J.M., Esteban, J., Soriano, F.: Isolation of *Propionibacterium acnes* from central nervous system infections. Anaerobe 1, 17-20 (1995).

Riedel, K-H.J., Britz, T.J.: *Propionibacterium* species diversity in anaerobic digesters. Biodiv. Conserv. 2, 400-411 (1993).

Riedel, K-H.J., Britz, T.J.: Justification of the "classical" *Propionibacterium* species concept by ribotyping. System. Appl. Microbiol. 19, 370-380 (1996).

Riedel, K-H.J., Wingfield, B.D., Britz, T.J.: Justification of the "classical" *Propionibacterium* species concept by restriction analysis of the 16S ribosomal RNA genes. Syst. Appl. Microbiol. 17, 536-542 (1994).

Roszkowski, W., Roszkowski, K., Ko, H.L., Beuth, J., Jeljaszewicz, J.: Immunomodulation by propionibacteria. Zbl. Bakt. 274, 289-298 (1990).

Van Niel, C.B.: The propionic acid bacteria. Thesis. Haarlem, Delft, J.W. Boissevain & Co 1928.

Vaughn, R.H.: Lactic acid fermentation of cabbage, cucumbers, olives and other products, pp. 220-224. In: Prescott & Dunn's Industrial Microbiology. (G. Reed, ed.) Connecticut, Saybrook Press 1981.

Von Freudenreich, E., Orla-Jensen, S.: Über die in Emmentalerkäse stattfindende Propionisäuregarung. Zentralbl. Bakteriol. Parasitenk. Infekt. Hyg. Abt. II 17, 529-546 (1906). (As cited by Van Niel, 1928).

Vorobjeva, L.I., Turova, T.P., Kraeva, N.I., Alekseeva, M.A.: Propionic acid cocci and their systematic position. Mikrobiol. 52, 368-373 (1983).

Vorobjeva, L.I., Cherdinceva, T.A., Abilev, S.K., Vorobjeva, N.V.: Antimutagenicity of propionic acid bacteria, Mutat. Res. 251, 233-239 (1991).

Vorobjeva, L.I., Khodjeav, E.Yu., Cherdinceva, T.A.: The study of induced antimutagenesis of propionic acid bacteria. J. Microbiol. Methods 24, 249-258 (1996).

Woolford, M.K.: The significance of *Propionibacterium* species and *Micrococcus lactolyticus* to the ensiling process. J. Appl. Bacteriol. 39, 301-306 (1975).

Chapter 2

Literature Review

(Note: All the taxonomic and spelling styles used in this chapter are as reported in the original scientific papers)

Characteristics of the genus *Propionibacterium*

Two principal groups of organisms from different habitats are currently described within the genus *Propionibacterium*. The first group namely the "classical" or dairy propionibacteria, was originally described by *Von Freudenreich* and *Orla-Jensen* in 1906. The classical propionibacteria are isolated primarily from cheese and dairy products, although they have also been isolated from soil (*Van Niel*, 1928; *Hayashi* and *Furusaka*, 1979), silage (*Beerens* et al., 1986), natural fermentations (*Gonzalez-Cancho* et al., 1970), anaerobic digesters (*Dubourgier* et al., 1988; *Riedel* and *Britz*, 1993; *Sarada* and *Joseph*, 1994) and nematodes recovered from the hind gut of zebra (*Krecek* et al., 1992). In contrast to the classical group, the "cutaneous" or clinical group are most frequently isolated from the human skin, especially areas rich in sebaceous glands such as the alae nasi, as well as moist rather than oily regions, such as the axilla, perineum and anterior nares (*Cummins* and *Johnson*, 1981). Members of the cutaneous group have also been isolated from the intestine and the upper respiratory tract of humans (*Cummins* and *Johnson*, 1986), as well as from dogs (*Goodacre* et al., 1994), pigs (*Benno* and *Mitsuoka*, 1982) and paddy soils (*Hayashi* and *Furusaka*, 1979). Although these organisms are generally considered opportunistic pathogens, recent reports indicate that species of the cutaneous group could be primary pathogens (*Brooke* and *Frazier*, 1991; *Horner* et al., 1992; *Ramos* et al., 1995)

The genus *Propionibacterium* is characterised as pleomorphic rods, 0,5 - 0,8 μm in diameter x 1 - 5 μm in length. They are often diptheroid or club-shaped with one end rounded and the other tapered or pointed. The cells may, however, also be

coccoid, bifid or even branched. The cells may occur singly, in pairs of short chains, in V or Y configuration, or in clumps with "chinese character" arrangements (*Cummins* and *Johnson*, 1986; *Holt* et al., 1994). Morphologically members of the genus *Propionibacterium* are irregularly staining, Gram-positive, non-motile, nonsporing rods. In general the classical propionibacteria tend to be shorter and rather thicker than the cutaneous propionibacteria, although all strains may be very variable in morphology, especially in early log phase cultures. Some strains may produce extracellular slime consisting primarily of carbohydrates. The propionibacteria are chemo-organotrophs and fermentation products include large amounts of propionic, acetic, iso-valeric, formic, succinic or lactic acids and carbon dioxide (*Cummins* and *Johnson*, 1986). These anaerobic mesophiles are generally catalase-positive, they grow most rapid at 30 - 37 °C and form small raised colonies that are cream, yellow, orange, white, grey, pink or deep red (*Cummins* and *Johnson*, 1986; *Glatz*, 1992). The mol% G + C content of their DNA varies from 53 - 67 mol% (T_m).

The cell walls of most of the strains in the genus typically contain peptidoglycan in which L-DAP is the diamino acid and a polysaccharide consisting of hexosamines and some combination of glucose, galactose and mannose. *Propionibacterium freudenreichii* and certain strains of *P. acnes*, and *P. avidum* contain *meso*-diaminopimelic acid, while *P. granulosum* has been reported to contain lysine rather than glycine as the interpeptide bridge between DAP and the D-alanine of the adjacent chain, as observed for the other species (*Charfreitag* and *Stackebrandt*, 1989). The various species of the genus *Propionibacterium* display rather uniform menaquinone and fatty acid composition, with the cellular lipids of both the classical and cutaneous species being characterised by large amounts of C_{15} branched-chain fatty acids (principally iso- and anteiso-C_{15} acids). Complex lipids that have chemoattractant properties for phagocytes have also been extracted from strains of *P. acnes* (*Russel* et al., 1976).

The nutritional requirements of both groups of propionibacteria are rather similar, suggesting a close resemblance between the overall metabolism of the two groups. Pantothenate is required by all strains and many also require biotin (*Delwiche*,

1949). Biotin plays a major role in the transcarboxylation reactions in the pathways to propionic acid (*Glatz*, 1992). Thiamine and nicotinamide have been reported to improve growth whilst some strains require *p*-aminobenzoic acid (*Cummins* and *Johnson*, 1981). A number of other unknown factors in potato or yeast extract are also stimulatory (*Hettinga* and *Reinbold*, 1972a). Although in general the nutritional requirements of the two groups are very similar, the amino acid requirements of the cutaneous group are more complex (*Ferguson* and *Cummins*, 1978). Many strains of propionibacteria will, however, grow in a basal medium without the addition of amino acids. Various organic and inorganic nitrogen sources can also be utilised. Some of the classical propionibacteria can grow with ammonium sulphate as nitrogen source. Due to the fact that the nutritional requirements of *Propionibacterium* strains may differ significantly, defined media have generally included most amino acids, vitamins, purines and pyrimidines to support the growth of all strains. The propionibacteria can utilise a variety of sugars, including trioses, tetroses, pentoses, hexoses and their corresponding polyols as well as lactic acid as carbon sources. To differentiate phenotypically between species within the genus *Propionibacterium*, *Holt* et al., 1994 published an identification key consisting of nine characteristics (Table 1), where some of these sugars are of significance.

Although initially considered strictly anaerobic, *De Vries* et al. (1972) demonstrated that they were also able to grow under aerobic conditions. In terms of metabolism, the propionibacteria have a fermentative metabolism and characteristically produce large amounts of propionic acid as their fermentation end products via the dicarboxylic acid pathway. Lesser amounts of acetic, iso-valeric, formic and succinic acids as well as carbon dioxide are also generally formed (*Cummins* and *Johnson*, 1986). Generally the ratio of propionic acid to acetic acid is approximately 2:1, but the ratio may vary widely and be as high as 5:1 or more (*Cummins* and *Johnson*, 1981). By using the Embden-Meyerhof-Parnas pathway, propionibacteria convert glucose to pyruvate. Another carbon-source namely lactate, which are commonly found in milk, can be used preferentially to glucose as a substrate by most propionic acid producing bacteria. *Propionibacterium freudenreichii* and *P. acidipropionici*, however, do not produce propionic acid from lactate under aerobic conditions but only acetic acid (*Pritchard* et al., 1977). Lactate can be oxidised directly by a lactate dehydrogenase to

Table 1. Differential characteristics of species within the genus *Propionibacterium* (*Holt* et al., 1994)

Organism	Acid from:					Pigmentation of colonies	β-Hemolysis	Hydrolysis of:	
	Sucrose	Maltose	L-Arabinose	Cellobiose	Glycerol			Esculin	Gelatin
P. acnes	-	-	-	-	D	White to grey	d	-	+
P. avidum	+	+	d	-	+	White to cream	+	+	+
P.granulosum	+	+	-	-	+	White to grey	-	-	d
P. freudenreichii	-	-	+	-	+	May be tan or pink	-	+	-
P. jensenii	+	+	-	d	+	White to pink	-	+	-
P. thoenii	+	+	-	-	+	Orange to red-brown	+	+	-
P. acidipropionici	+	+	+	+	+	White	-	+	-
P. lymphophilum	d	+	-	-	-	White	-	-	d

Symbols: +, 90% or more of strains are positive; (+), 80-89% of strains are positive; (-), 11-20% of strains are positive; -, 90% or more of strains are negative; d, 21-79% of strains are positive.

pyruvate in a reaction requiring a flavoprotein as H-acceptor. The pyruvate is then further transformed to form acetate and carbon dioxide but only in the presence of the enzyme pyruvate dehydrogenase (a biochemical pathway that yields 1 mole of carbon dioxide and 1 mole of ATP per mole of acetate). The reduction of lactate or pyruvate to propionate follows the methylmalonyl-CoA pathway (Fig. 1) and occurs according to the following overall equation (*Schlegel*, 1992):

$$3\ CH_3\text{-}CHOH\text{-}COOH \longrightarrow 2\ CH_3\text{-}CH_2\text{-}COOH + CH_3\text{-}COOH + CO_2 + H_2O$$

Oxaloacetate is also carboxylated from pyruvate by methylmalonyl CoA carboxytransferase, in a transcarboxylation reaction with (S)-methylmalonyl-coenzyme A (CoA) as the CO_2-donor and biotin as the CO_2-carrier. The reaction of malate dehydrogenase and fumarase yields fumarate, which is reduced to succinate by fumarate reductase. This reaction is coupled to an electron transport phosphorylation with fumarate as the final electron acceptor and can thus be classified as anaerobic respiration, called fumarate respiration. Thus, although succinate is generally an intermediate, it can also be produced as a metabolic end product.

Succinyl-CoA is then formed in a CoA transferase (succinyl-CoA:propionate CoA transferase) reaction and the rearrangement as catalysed by the coenzyme B_{12} (cyanocobalamin) containing methylmalonyl-CoA mutase, leads to (R)-methylmalonyl-CoA. The (R)-methylmalonyl-CoA is, however, not a substrate for the transcarboxylase. The (S)-enantiomer is subsequently formed by a specific racemerase. It is this intermediate which loses carbon dioxide to yield propionyl-CoA by the CoA transferase, whilst the methylmalonyl-CoA carboxytransferase mentioned above acquires the CO_2. Propionate is liberated from propionyl CoA by the CoA transferase, which transfers CoA to succinate. One NADH is consumed during the oxidation of lactate to acetate.

The enzyme (S)-methylmalonyl-CoA-pyruvate transcarboxylase is the key to the cyclic nature of this pathway, since it enables a carboxyl group to be transferred from (S)-methylmalonyl-CoA to pyruvate to form oxaloacetate and propionyl-CoA (*Hettinga* and *Reinbold*, 1972b; *Gottschalk*, 1986; *Schlegel*, 1992). It should be noted

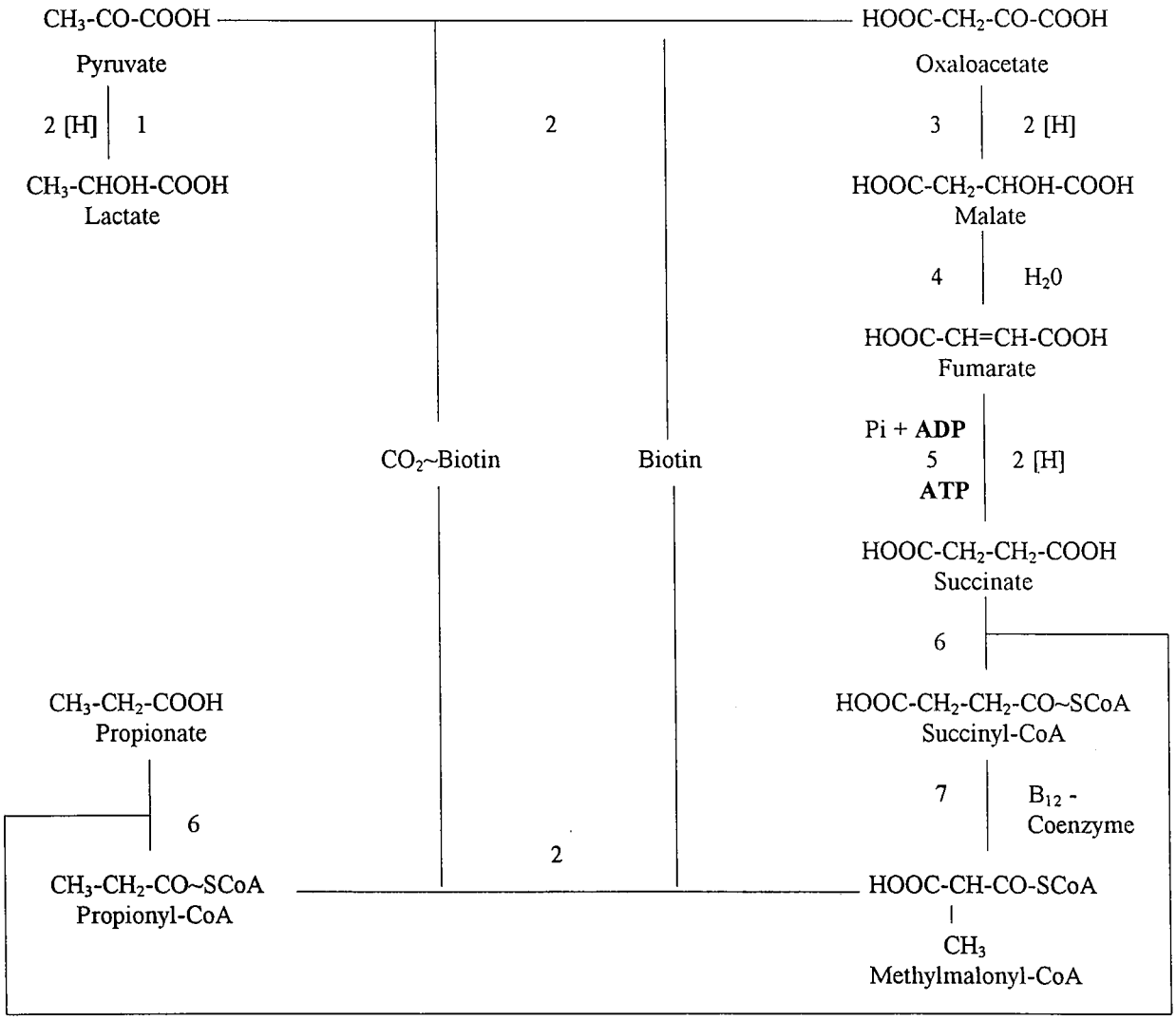


Fig 1. The Methylmalonyl-CoA pathway of propionate formation (*Schlegel*, 1992)

Enzymes:
1: Lactate dehydrogenase
2: Methylmalonyl-CoA carboxytransferase
3: Malate dehydrogenase
4: Fumarase
5: Fumarate reductase
6: CoA transferase
7: Methylmalonyl-CoA mutase

that in this process of propionate formation, two groups namely, CO_2 and CoA, are transferred back from product to precursor without occurring in the free form. Noteworthy feature of the metabolic pathway is the participation of three cofactors (biotin, coenzyme A and coenzyme B_{12}). Whilst biotin must be added to the growth medium, most of the classical *Propionibacterium* strains produce vitamin B_{12} and some, especially *P. freudenreichii* strains, produce such excessive quantities that these strains can be used to produce this vitamin commercially (*Glatz*, 1992). The production of vitamin B_{12} by the cutaneous propionibacteria has, however, never been reported (*Cummins* and *Johnson*, 1986).

The propionibacteria can also utilise aspartate, which is also available in cheese, as an energy source. Propionibacteria in the presence of both lactate and aspartate, ferment lactate to propionate combined with the fermentation of aspartate to succinate (*Crow*, 1986a:1986b). Due to the fact that aspartate is rapidly metabolised during the ripening of Emmentaler cheese (*Crow*, 1986b), the theoretical propionate / acetate ratio established in the propionic acid fermentation is modified to the disadvantage of propionate. During this coupled fermentation reaction the production of acetate, carbon dioxide and succinate is, however, enhanced.

Although the genus *Propionibacterium* is generally considered to have low proteolytic activity, two types of activity have been found in all species of propionibacteria, one acting on β-casein and the other on α_{s1}-casein as substrate. The typical nutty flavour of Emmentaler cheese is also an important contribution made by *Propionibacterium freudenreichii,* through the production of a minimum amount of proline (*Hettinga* and *Reinbold*, 1972a; *Langsrud* and *Reinbold*, 1973). Propionibacteria can also degrade various amino acids, especially aspartate, alanine, serine and glycine, depending on the species. This results in additional CO_2 production in cheese (*Langsrud* et al., 1995; *Tobiassen* et al., 1996). Although lipases and esterases may play an important role in the hydrolysis of fat and in the flavour composition of Emmentaler cheese, knowledge of these activities is rather limited. In 1967, *Oterholm* compared the lipolytic activity of *P. freudenreichii* subsp. *shermanii* with that of lactic acid bacteria. He reported that *P. freudenreichii* subsp. *shermanii* displayed a hundred

fold higher lipolytic activity than lactic acid bacteria. Between three to six different esterase activities were detected, two of them common to various strains of *P. freudenreichii* subsp. *freudenreichii*.

The cutaneous *Propionibacterium* species were formerly, described in the genus *Corynebacterium*. Based essentially on the work of *Johnson* and *Cummins*, 1972, they were transferred to the genus *Propionibacterium* primarily due to the fact that they were: anaerobic; produced propionic acid as a major end product of metabolism; contain mostly L-diaminopimelic acid (L-DAP) as the diamino acid of peptidoglycan; contained C_{15} *iso*- and *anteiso*-acids as the principal fatty acids of cell lipids; and they lacked mycolic acids and the arabingalactan which was characteristic of *Corynebacterium* sensu stricto. This transfer was, however, not without controversy. *Prévot* (1976) proposed that this cutaneous group of organisms should not be transferred to the genus *Propionibacterium* but should instead be accommodated in a separate subgenus *Coryneformis* in the family *Corynebacteriaceae*.

The designation of *Propionibacterium freudenreichii* as the type species of the genus by *Van Niel* in 1928 is today perhaps rather anomalous in the light of more recent information. *Propionibacterium freudenreichii* is rather atypical of the genus in that: a) strains of *P. freudenreichii* contain *meso*-DAP instead of the LL-isomer found in all other strains; b) it has a cell wall polysaccharide containing rhamnose; and c) it is more distantly related to other *Propionibacterium* species by DNA:DNA homology (*Johnson* and *Cummins*, 1972).

In 1993, *Sutcliffe* and *Shaw* reported on proportions and types of phospholipids in *Propionibacterium freudenreichii*. The major phospholipids identified were bis(phosphatidyl)glycerol, phosphatidylglycerol and phosphatidylinositol, with minor components identified as phosphatidylethanolamine and lysophosphatidylinositol. In contrast to the results of *Prottey* and *Ballou* (1968), no phosphatidylinositol mannosides could be detected. The presence of this mannose containing phospholipid in *P. freudenreichii* as reported by *Prottey* and *Ballou* (1968), was considered as an additional factor indicating a close relationship between the genera *Propionibacterium* and *Mycobacterium* (*Hettinga* and *Reinbold*, 1972c). The presence of

phosphatidylinositol, minor amounts of phosphatidylethanolamine and high bis(phosphatidyl)glycerol and phosphatidyl glycerol contents in *P. freudenreichii* is, however, consistent with the relative taxonomic position of the propionibacteria between the other actinomycetes and the *Clostridium-Bacillus-Streptococcus* subdivision of the Gram-positive eubacteria (*Charfreitag* and *Stackebrandt*, 1989; *Goodfellow*, 1989; *Sutcliffe* and *Shaw*, 1993). Members of this genus are readily confused with some species of *Corynebacterium* or *Clostridium* (*Holt* et al., 1994). Genera, which are phylogenetically related to the genus *Propionibacterium*, include the members of the genera *Corynebacterium*, *Clostridium*, *Arachnia* (*Cummins* and *Johnson*, 1986), *Luteococcus*, *Aeromicrobium*, *Nocardioides* (*Yokota* et al., 1994) and *Terrabacter* (*Kusano* et al., 1997).

Industrial applications

The classical propionibacteria, especially *Propionibacterium freudenreichii* are used extensively as starter cultures in the manufacture of Swiss-type cheeses. The French cheese industry, for example, produced more than 240 000 tonnes of Swiss-type cheese during 1994 (SIGF, 1994). The propionibacteria can use both the lactose present in the milk and the lactic acid produced by the lactic acid starter cultures as fermentation substrates. Lactic acid is, however, the preferred substrate. Through the production of propionic and acetic acid, diacetyl and various amino acids, especially leucine and proline, these organisms add to the characteristic sweet nutty flavour of Swiss-type cheese (*Langsrud* and *Reinbold*, 1973; *Chaia* et al., 1990). In 1992, *Glatz* reported that the sweet flavour of the cheese could be partly attributed to proline production by these organisms, whereas the buttery flavour is caused by the production of diacetyl. Propionibacteria also occur in some other cheese-types such as Appenzell, Tilsit and Sbrinz (*Baer* et al., 1993), Parmiggiano, Reggiano and Grana Padano (*Thompson* and *Marth*, 1985; *Carcano* et al., 1995) as well as Mozzarella (*Champagne* and *Lange*, 1990). In these latter cheeses the conditions for their proliferation are, however, not favoured, and the propionibacteria subsequently do not contribute significantly to the ripening of these cheeses.

Propionibacteria are also responsible for the characteristic "eye" formation in Swiss-type cheese through the production of carbon dioxide. Excessive carbon dioxide production by propionibacteria, from both lactose and lactic acid fermentation as well as amino acid metabolism can, however, result in split and late blowing defects in Swiss cheese (*Langsrud* and *Reinbold*, 1974; *Hettinga* and *Reinbold*, 1975). This phenomenon has also been observed in other types of cheese, such as Gouda (*Britz* and *Jordaan*, 1976) and an American-type Mozzarella (*Massa* et al., 1992). Similarly, insufficient CO_2 production, due to the inhibition of propionibacterial growth, may also result in inadequate "eye" formation in Swiss-type cheese (*Langsrud* and *Reinbold*, 1974).

With the technical improvement of cheese manufacturing today, the role of the natural propionic acid bacteria in raw milk seems to have become less important (*Meriläinen* and *Antila*, 1976). The presence of pigmented *Propionibacterium* strains in foods may also cause a spoilage condition referred to as "red" or "brown spot" (*Baer* and *Ryba*, 1992; *Baer* et al., 1993). The development of "brown spots" caused by *P. freudenreichii*, in Emmentaler cheese can be suppressed by adding increased amounts of propionibacteria to the milk. Complete inhibition, however requires salt concentrations of 15-20g/kg cheese (*Sollberger*, 1996), which greatly diminishes the sensorial properties of the cheese. This defect is visible only after three months of ripening, initially in the peripheral area and then in the whole cheese. Although very rare, "red spots" can be attributed to the presence of red-pigmented "*P. rubrum*" colonies in Swiss-cheese (*Baer* and *Ryba*, 1992; *Baer* et al., 1993). The classical propionibacteria also contribute to the natural fermentation of silage and olives (*Woolford*, 1975), although spoilage in the olive industry by members of the genus *Propionibacterium* has also been reported (*Vaughn*, 1981).

The propionibacteria are further also important in the food industry, where they are responsible for the production of organic acids, biomass, vitamin B_{12} and other specific metabolites (*Marcoux* et al., 1992). Although the classical propionibacteria were extensively used for the commercial production of vitamin B_{12}, this vitamin is currently produced using a faster growing *Pseudomonas* strain which has a higher yield (*Glatz*, 1992). Vitamin B_{12} is an important co-factor for the metabolism of

carbohydrates, lipids, amino acids, nucleic acids and it is also reported to have been used in chemotherapy (*Quesda-Chanto* et al., 1994).

Propionic acid is an important compound used in the production of thermo- and cellulose-plastics, herbicides, flavours, perfumes, antiarthritic drugs and solvents. The calcium, sodium and potassium salts of this acid are widely used as food and feed preservatives (*Lewis* and *Yang*, 1992; *Paik* and *Glatz*, 1994). Although propionic acid is currently produced mainly by chemical synthesis from petroleum feedstock, interest in an alternative route for production of this acid using microbiological processes and cheap renewable material such as whey, waste molasses or corn steep liquor is increasing (*Boyaval* and *Corre*, 1995).

The production and application of bacteriocins may also have great potential as natural food preservatives. *Klaenhammer*, in 1993 defined bacteriocins as proteins or peptides, which are bactericidal to other, usually closely related bacteria. Further characteristics are that bacteriocins have a narrow spectrum of activity and are mostly plasmid-borne. Both classical and cutaneous propionibacteria have been reported to produce bacteriocins.

Two bacteriocin-like substances were partially purified from a *Propionbacterium acnes* strain isolated from plaque. The activity of these two bacteriostatic substances namely: acnecin CN-8 (*Fujimura* and *Nakamura*, 1978); and bacteriocin-like substance RTT 108 were both inhibited by lysozyme at low ionic strength. The bacteriocin-like substance RTT 108 had a rather large activity spectrum in that it was active against both Gram-negative and Gram-positive anaerobes, while acnecin had a relatively narrow host range, inhibitory only to other strains of *P. acnes* and *Corynebacterium parvum*.

The first bacteriocin purified and characterised within the classical propionic acid bacteria group was PLG-1 from *P. thoenii* (*Lyon* and *Glatz*, 1991). Characteristics of this propionicin included heat lability, sensitivity to different proteolytic enzymes and its stability between pH 3 and 9. This bacteriocin was also not inhibitory to the strain of *P. thoenii* that produced propionicin PLG-1. In 1993,

Lyon and *Glatz* further characterised PLG-1 and they reported that this bacteriocin was produced during the stationary growth phase of *P. thoenii* and that it was not plasmid-coded. Propionicin PLG-1's spectrum of activity ranges from several Gram-positive bacteria, including lactic acid bacteria, some Gram-negative bacteria as well as yeasts and moulds. Inhibitory activity within the genus *Propionibacterium* was detected against *P. thoenii* and *P. jensenii*, but no inhibitory effect against either three subspecies of *P. freudenreichii* was observed.

Another bacteriocin similar to PLG-1, which was also coded for on the chromosome rather than plasmid-borne, was isolated and described in 1992 by *Grinstead* and *Barefoot*. This bacteriocin known as jenseniin G due to the fact that it was isolated from a *P. jensenii* strain, was active at pH 7, sensitive to proteolytic enzymes, heat stable and resistant to freezing and cold storage. Only three species were, however, included in its activity spectrum namely: *P. acidipropionici*; *P. jensenii*; and *Lactobacillus delbrueckii* subsp. *lactis*. (*Paul* and *Booth*, 1988).

A product such as "Microgard" (Wesman Foods, Inc., Beaverton, Oregon USA), which has been approved by the Food and Drug Administration, contains a small heat stable bacteriocin, produced by fermenting skimmed milk with *Propionibacterium freudenreichii* subsp. *shermanii*. The product is used as a preservative in about 30% of the cottage cheese produced in the United States (*Grinstead* and *Barefoot*, 1992). Microgard has an antagonistic effect against Gram-negative bacteria and some yeasts and moulds, but not against Gram-positive bacteria (*Lyon* and *Glatz*, 1991).

Another application of the genus *Propionibacterium* is their use as probiotics. Probiotics are defined as a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance (*Fuller*, 1989). In order to exert a probiotic impact in the intestine, bacteria have to survive the passage through the stomach and duodenum and adhere to the intestinal mucosae. The probiotic effect is based on the production of beneficial metabolites and antimicrobial compounds for their host. As a consequence, higher resistance to harmful bacteria responsible for

intestinal disorders, stabilisation of the intestinal microflora, anticarcinogenic and anticholesterolemic influences have been postulated (*Fessler*, 1997).

In 1995, *Mantere-Alhonen* proposed propionibacteria as probiotics for humans and as probiotic growth promotors for pigs. As probiotic agents, propionibacteria in combination with other bacteria such as lactobacilli, lactococci and enterococci can be used to increase the weight gain of calves (*Cerna* et al., 1991) and curing intestinal disorders in calves, piglets and hens (*Vladimirov* et al., 1977; *Antipov* and *Subbotin*, 1980; *Tuikov* et al., 1980). According to *Mantere-Alhonen* (1982) *P. freudenreichii* subsp. *shermanii* was observed to stimulate the growth of piglets. It has, however, been suggested (*Mantere-Alhonen*, 1982; *Fessler*, 1997) that the high mineral content (Mn, Zn, Cu, Fe) of propionibacteria could be partly responsible for the reported probiotical effect, since no colonisation or adhesion of propionibacteria to the intestinal mucosae of the piglets could be found.

Perez Chaia et al. (1994) reported that propionibacteria useful in the dairy industry, could also favourably effect the lipid metabolism in the gut and the immune system of mice. Probiotic food products in which propionibacteria were combined with bifidobacteria or lactic acid bacteria, have also been described (*Fessler*, 1997). An example is a sour milk product which was developed by *Mantere-Alhonen* (1987) containing *P. freudenreichii* subsp. *shermanii* and *Lactobacillus acidophilus*. The product was reported to exhibit favourable sensory quality, mild flavour, good consistency and storage properties.

During the manufacturing process of Swiss-type cheese, interactions between the lactic acid bacteria and the propionibacteria have both favourable and unfavourable impacts on each other (*Perez Chaia* et al., 1982:1994; *Beide* et al., 1977; *Jimeno* et al., 1995). *Perez Chaia* et al. (1994) reported that the rapid pH reduction by the growth of *L. helveticus* and consequently lactate accumulation in the cheese due to the slow lactate utilisation by *P. acidipropionici* was the major reason observed for the inhibition of propionibacteria in mixed cultures. When propagating *L. helveticus* and another species of the genus *Propionibacterium* namely *P. freudenreichii* subsp. *shermanii* together, a lower biomass yield of lactobacilli, and an increase in the

fermentation activities of both micro-organisms with an increase in propionic acid production, was observed (*Perez Chaia* et al., 1982). A mixed liquid cultivation of *L. acidophilus* and *P. freudenreichii* subsp. *shermanii* also seems to be beneficial for the proliferation of both bacteria (*Liu* and *Moon*, 1982). *Beide* et al. (1977), however, reported that the addition of increasing amounts of *L. bulgaricus* to cheese resulted in a lower pH, lower levels of acetyl production and proteolysis and a decrease in the number of propionibacteria. *Jimeno* et al. (1995) suggested that the relative amount of copper and citrate in cheese could play an important role in *L. rhamnosus* and *L. casei* both having an inhibitory effect on *P. freudenreichii.* This suggestion was verified when they found that the inhibition of *P. freudenreichii* growth could not be reproduced in cultures with the usual media (*Jimeno* et al., 1995).

The production of propionic and other minor organic acids, CO_2, bacteriocins, vitamins (*Mantere-Alhonen*, 1995) and the antimutagenic properties displayed by propionibacteria (*Vorobjeva* et al., 1991:1996) may be of importance in the application of these organisms as probiotic agents in foods. The propionibacteria are also receiving increasing attention due to the fact that they rank amongst the most potent immunomodulatory stimulating cell populations involved in non-specific resistance (*Roszkowski* et al., 1990).

Early isolations

Despite the initial reports by *Fitz* in 1878 and 1879 that organisms from cheese could ferment lactate to propionic and acetic acids and liberate carbon dioxide in the process, the first pure cultures of propionic acid producing bacteria were isolated and described in 1906 by *Von Freudenreich* and *Orla-Jensen*. The organisms isolated by *Von Freudenreich* and *Orla-Jensen* in 1906 from Emmentaler cheese were named "*Bacterium acidi propionici a*", "*Bacterium acidi propionici b*" and "*Bacillus acidi propionici*" based on Ferdinand Cohn's taxonomic system of classification. This classification system concentrated primarily upon structural characteristics and in particular the shape of the bacterial cells. The short rods were classified in the genus *Bacterium* and the longer rods in the genus *Bacillus*.

In 1908, *Thöni* and *Alleman* isolated two more species from the cut surface of Emmentaler cheese. Using the same nomenclatural system, they named their isolates "*Bacterium acidi propionici* var. *rubrum*" and "*Bacterium acidi propionici* var. *fuscum*". The variants were differentiated on the basis of red and brown pigmentation, respectively. A year later *Troili-Petersson* (1909) while studying a Swedish type of Emmentaler cheese discovered yet another propionic acid producing bacterium. Due to it's fermentative behaviour and clumping characteristics displayed in liquid medium which differed from the already reported propionic acid producing bacteria, she named this organism "*Bacterium acidi propionici c*".

Orla-Jensen in 1909 was the first researcher to indicate a relationship between the propionic acid bacteria and other groups of microbes. The propionic acid bacteria were considered as a separate genus under the order *Peritrichinae*. *Orla-Jensen* (1909) proposed a new genus *Propionibacterium* with "*Bacterium acidi propionici a*", "*Bacterium acidi propionici b*" and "*Bacillus acidi propionici*" being the only species within this genus.

This genus, was, however, not recognised by the committee on the characterisation and classification of bacterial types of the Society of American Bacteriologists (1917). Criticising their lack of recognition of the genus *Propionibacterium*, *Orla-Jensen* (1921) stated that if morphological characteristics were of such significance then a division in the genus requires that besides the genus *Propionibacterium*, a genus "*Propionicoccus*" should also be created for the spherical forms. In the same year, *Sherman* (1921) reported the isolation of another type of propionic acid bacterium which he named "*Bacterium acidi propionici d*". The various propionic acid producing bacteria which had been described before 1921 are presented in Fig. 2.

No mention of the genus *Propionibacterium* or the genus "*Propionicoccus*" was made in the first and second edition's of Bergey's Manual of Determinative Bacteriology (*Bergey*, 1923:1925). *Buchanan* (1925) in his "General Systematic Bacteriology" merely stated that a genus *Propionibacterium* as well as "*Propionicoccus*" had been proposed by *Orla-Jensen* in 1909 and 1921, respectively,

"***Bacterium acidi propionici* var. *rubrum***"
(*Thöni* and *Alleman*, 1908)
"***Bacterium acidi propionici* var. *rubrum***"
(*Thöni* and *Alleman*, 1908)
"***Bacillus acidi propionici***"
(*Von Freudenreich* and *Orla-Jensen*, 1906)

"***Bacterium acidi propionici b***"
(*Von Freudenreich* and *Orla-Jensen*, 1906)
"***Bacterium acidi propionici c***"
(*Troili-Petersson*, 1909)

"***Bacterium acidi propionici* var. *fuscum***"
(*Thöni* and *Alleman*, 1908)
"***Bacterium acidi propionici a***"
(*Von Freudenreich* and *Orla-Jensen*, 1906)
"***Bacterium acidi propionici d***"
(*Sherman*, 1921)

Fig. 2. A list of the species of propionic acid bacteria described before 1921 and the literature citations of the descriptions.

but emphasised that the status of the genus was doubtful since no species had been described or referred to.

Primarily due to the work of *Orla-Jensen* (1909) on the metabolic processes of the propionic acid bacteria, *Kluyver* and *Donker* (1925), were able to place the propionic acid bacteria between the lactic acid bacteria and the butyl alcohol and butyric acid bacteria. In 1927 *Rippel*, however, placed the propionic acid bacteria in a subgroup of the heterofermentative lactic acid bacteria. This placement was undertaken based primarily on the results of *Virtanen* (1923) and his own findings on the intermediate fermentation products, such as lactic acid. This was the start of the problems concerning the taxonomic placement of the propionic acid bacteria.

The Van Niel era

The propionic acid producing bacteria were grouped together in a systematic unity for the first time under the generic name *Propionibacterium* in the family *Lactobacillaceae* when *Van Niel* (1928) published a comprehensive treatise on the propionibacteria. *Orla-Jensen*'s (1921) proposal of a second genus "*Propionicoccus*" was, however, rejected as *Van Niel* (1928) was convinced that different cultural conditions which could change the morphology of an organism, could result in the same species being classified under different genera. Despite this fact, *Van Niel* (1928) used cell morphology successfully in differentiating the various groups. Other characteristics which played a significant role in the placement of the groups in the genus *Propionibacterium* were the fact that they were all nonmotile, Gram-positive, formed no spores, grew into short rods under anaerobic conditions in a neutral medium and into irregular long rod shaped cells under aerobic conditions, catalase positive, producing propionic acid, acetic acid and carbon dioxide during the fermentation of lactic acid, carbohydrates and polyalcohols. Complex nitrogen compounds were also required for growth and development.

Van Niel (1928) designated *Propionibacterium Freudenreichii* as the type species and recognised eight species (Fig. 3). *Van Niel* (1928) also included a concise description of each species, with the properties summarised in the form of a key. This

"*Bacterium acidi propionici* var. *rubrum*" - **1) "*P. rubrum*"**
(*Thöni* and *Alleman*, 1908)
"*Bacterium acidi propionici* var. *rubrum*" - **2) "*P. Thönii*"**
(*Thöni* and *Alleman*, 1908)
"*Bacillus acidi propionici*" - **3) "*P. pentosaceum*"**
(*Von Freudenreich* and *Orla-Jensen*, 1906)

- **4i) "*P. Jensenii* var. *raffinosaceum*"**

"*Bacterium acidi propionici b*" - **4ii) "*P. Jensenii*"**
(*Von Freudenreich* and *Orla-Jensen*, 1906)
"*Bacterium acidi propionici c*" - **5) "*P. Peterssonii*"**
(*Troili-Petersson*, 1909)

- **6) "*P. technicum*"**

"*Bacterium acidi propionici* var. *fuscum*" - **7ii) *P. Freudenreichii***
(*Thöni* and *Alleman*, 1908) (Synonym)
"*Bacterium acidi propionici a*" - **7i) *P. Freudenreichii***
(*Von Freudenreich* and *Orla-Jensen*, 1906)
"*Bacterium acidi propionici d*" - **8) "*P. Shermanii*"**
(*Sherman*, 1921)

(1-8 = *Van Niel*, 1928)

Fig. 3. A list of the *Propionibacterium* species as proposed by *Van Niel* (1928).

key was based on morphological characteristics, fermentation patterns, pigment production and growth in liquid or stab culture. *Orla-Jensen* (1909) proposal to use differences in acid formation as a distinguishing characteristic was rejected.

"*Bacterium acidi propionici* var. *rubrum*" isolated by *Thöni* and *Alleman* (1908), was represented by four strains, two distinguishable from the other two. *Van Niel* (1928) named the one group "*Propionibacterium rubrum*" and the other "*Propionibacterium Thönii*". "*Propionibacterium acidi propionici* var. *fuscum*" (*Thöni* and *Alleman*, 1908) was, however, considered to be a synonym of *Propionibacterium Freudenreichii*. *Van Niel* (1928) further suggested that the genus *Propionibacterium* should rather be placed between the bacteria of the colon group and the lactic acid bacteria, rather than between the latter and the butyric acid and butyl alcohol bacteria due to the characteristic presence of the catalase enzyme in the propionic acid bacteria, despite their strictly anaerobic nature.

The genus *Propionibacterium* was subsequently officially recognised for the first time, as a legitimate genus in the third publication of Bergey's Manual of Determinative Bacteriology (*Bergey*, 1930). The genus was placed in the family *Bacteriaceae*, as one of the genera of the tribe *Propionibacteriaceae*, using *Van Niel*'s (1928) method for differentiation.

Further isolations: 1931 - 1944

Werkman and *Kendell* (1931) raised "*P. Jensenii* var. *raffinosaceum*" to specific rank as "*P. raffinosaceum*" (Fig. 4). They criticised *Van Niel* (1928) for using pigmentation to differentiate between the species of the propionic acid bacteria. In 1934, *Hitchner* described two new species isolated while studying the physiological characteristics of the propionic acid producing bacteria. These two isolates were named "*P. zeae*" and "*P. arabinosum*" (Fig. 4). In 1933, the validity of the eleven *Propionibacterium* species was re-evaluated by *Werkman* and *Brown*. They reported that without exception it was possible to recognised the various species individually, whatever criterion for classification was used. Three subgeneric groups were formed based on serological differentiation (Fig. 4). "*Propionibacterium zeae*" and

"*Bacterium acidi propionici* var. *rubrum*" - **1)** **"*P. rubrum*"**
(*Thöni* and *Alleman*, 1908)

"*Bacterium acidi propionici* var. *rubrum*" - **2)** **"*P. Thönii*"**
(*Thöni* and *Alleman*, 1908)

"*Bacillus acidi propionici*" - **3)** **"*P. pentosaceum*"**
(*Von Freudenreich* and *Orla-Jensen*, 1906)

- **"*P. arabinosum*"**

- **4i) "*P. Jensenii* var. *raffinosaceum*"**
→ **"*P. raffinosaceum*"**
(*Werkman* and *Kendell*, 1931)

"*Bacterium acidi propionici b*" - **4ii)** **"*P. Jensenii*"**
(*Von Freudenreich* and *Orla-Jensen*, 1906)

"*Bacterium acidi propionici c*" - **5)** **"*P. Peterssonii*"**
(*Troili-Petersson*, 1909)

- **6)** **"*P. technicum*"**

- **"*P. zeae*"**

"*Bacterium acidi propionici* var. *fuscum*" - **7ii)** ***P. Freudenreichii***
(*Thöni* and *Alleman*, 1908) (Synonym)

"*Bacterium acidi propionici a*" - **7i)** ***P. Freudenreichii***
(*Von Freudenreich* and *Orla-Jensen*, 1906)

"*Bacterium acidi propionici d*" - **8)** **"*P. Shermanii*"**
(*Sherman*, 1921)

(1-8 = *Van Niel*, 1928)

(___ / ___ / ..... = *Werkman* and *Brown*, 1933)

Fig. 4. The three subgeneric groups formed within the genus *Propionibacterium* (*Werkman* and *Brown*, 1933).

"*P. rubrum*" were found to show relatively little relationship to any of the three groups. *Werkman* and *Brown* (1933) also proposed an identification key based primarily on carbohydrate fermentation. Pigment production, catalase and nitrate reduction were also mentioned, but to a lesser degree.

Controversies experienced during the placement of strains in specific species

The first indication that the 11 published and accepted species did not include all forms of the propionic acid bacteria arose when *Rodenkirchen* (1938) found that three of his 25 isolates could not be identified using the *Werkman* and *Brown* (1933) classification system.

In 1941, *Sakaguchi* and co-workers described five new species and one new variant which they had isolated from Japanese cheese. They named their species "*P. globosum*", "*P. amylaceum*", "*P. japonicum*", "*P. orientum*" and "*P. coloratum*" (Fig. 5). The new variant was named "*P. amylaceum* var. *auranticum*" (Fig. 5). Using the classification systems of both *Van Niel* (1928) and *Werkman* and *Brown* (1933), *Sakaguchi* et al., (1941) reported that a revision of the classification systems would be required to establish the taxonomic position of their newly isolated species.

Furthermore, *Janoschek* (1944) reported that of his 200 isolates, mostly isolated from milk and cheese, 53 could not be identified further than the generic level. *Janoschek* (1944) subsequently described three more species, namely: "*P. casei*"; "*P. pituitosum*"; and "*P. sanguineum*" (Fig. 5). The new species described by both *Sakaguchi* et al. (1941) and *Janoschek* (1944) were clearly separable from the 11 previously described species in the 5th edition of Bergey's Manual (*Bergey* et al., 1939). These species subsequently constituted valid descriptions, since in both cases, the newly proposed species were clearly described and correctly named (*Buchanan* et al., 1966). In the next edition of Bergey's Manual (*Bergey*, 1948), the newly described species were, however, considered as variants of existing species and were only mentioned in the appendix.

"*Bacterium acidi propionici* var. *rubrum*" (*Thöni* and *Alleman*, 1908)	**- 1)**	**"*P. rubrum*"**
"*Bacterium acidi propionici* var. *rubrum*" (*Thöni* and *Alleman*, 1908)	**- 2)**	**"*P. Thönii*"**
"*Bacillus acidi propionici*" (*Von Freudenreich* and *Orla-Jensen*, 1906)	**- 3)**	**"*P. pentosaceum*"**
	-	**"*P. arabinosum*"**
	- 4i)	**"*P. Jensenii* var. *raffinosaceum*"** → **"*P. raffinosaceum*"** (*Werkman* and *Kendell*, 1931)
"*Bacterium acidi propionici b*" (*Von Freudenreich* and *Orla-Jensen*, 1906)	**- 4ii)**	**"*P. Jensenii*"**
"*Bacterium acidi propionici c*" (*Troili-Petersson*, 1909)	**- 5)**	**"*P. Peterssonii*"**
	- 6)	**"*P. technicum*"**
	-	**"*P. zeae*"**
	-	**"*P. amylaceum*"**
	-	**"*P. amylaceum* var. *auranticum*"**
	-	**"*P. japonicum*"**
	-	**"*P. pituitosum*"**
"*Bacterium acidi propionici* var. *fuscum*" (*Thöni* and *Alleman*, 1908)	**- 7ii)**	***P. Freudenreichii*** (Synonym)
"*Bacterium acidi propionici a*" (*Von Freudenreich* and *Orla-Jensen*, 1906)	**- 7i)**	***P. Freudenreichii***
"*Bacterium acidi propionici d*" (*Sherman*, 1921)	**- 8)**	**"*P. Shermanii*"**
	-	**"*P. casei*"**
(1-8 = *Van Niel*, 1928)		
	-	**"*P. globosum*"**
(___ / ___ / ..... = *Werkman* and *Brown*, 1933)		
(*Hitchner*, 1934)	-	**"*P. orientum*"**
(*Sakaguchi et al.*, 1941)	-	**"*P. coloratum*"**
(*Janoschek*, 1944)		
	-	**"*P. sanguineum*"**

Fig. 5. The eight five new species and one new variant isolated by *Sakaguchi* et al. (1941) and *Janoschek*, 1944.

The family *Propionibacteriaceae*

In the 7th edition of Bergey's Manual (*Breed* et al., 1957), the propionic acid producing bacteria were placed in the order *Eubacteriales*, in a new family *Propionibacteriaceae* as proposed by *Delwiche* (1954). The family consisted of the genera *Propionibacterium*, *Butyribacterium* and *Zymobacterium*. This family was placed between the *Lactobacillaceae* and the *Corynebacteriaceae* because of its relationships to these families. The existing eleven species (*Van Niel*, 1928) were retained with only minor changes to the identification key. The species described by *Sakaguchi* et al., (1941) and *Janoschek* (1944) were, however, once again not mentioned by *Breed* et al. (1957).

In 1963, *Antila* and *Gyllenberg* while doing a study on the propionic acid producing bacteria of dairy origin found that the species "*P. casei*" of *Janoschek* (1944) should be given specific rank. Using the identification key of *Janoschek* (1944) they were, however, not able to separate "*P. Peterssonii*" and *P. Jensenii*, but divided "*P. arabinosum*" into two subgroups. They further differentiated the genus *Propionibacterium* into four main groups (Fig. 6), namely: "*P. arabinosum*", *P. Freudenreichii*, "*P. Shermanii*" and "*P. casei*"; "*P. pentosaceum*" and "*P. technicum*"; *P. Jensenii* together with "*P. raffinosaceum*" and "*P. Peterssonii*"; and finally "*P. Thönii*". *Antila* and *Gyllenberg* (1963) did, however, not find "*P. arabinosum*" and "*P. pentosaceum*" to be closely related. They also concluded that species consolidation was required.

Seyfried again studied the position of the family *Propionibacteriaceae* relative to that of *Lactobacillaceae* in 1968 while doing a computer aided classification of the lactobacilli. She found that the propionibacteria were clearly separated from the lactic acid bacteria. Furthermore, she reported that the propionibacteria clustered together in one group, with two unnamed cultures clearly distinguishable from the existing species. Other cultures that could not be associated with known species of propionibacteria were also reported by *Malik* et al. (1968). Whilst in studying the spoilage of pickled-olives, *Gonzalez-Cancho* et al. (1970) also reported that certain cultures identified in their study, using the identification key of *Breed* et al. (1957), could not be grouped

"***Bacterium acidi propionici* var. *rubrum***" - 1) "***P. rubrum***"
(*Thöni* and *Alleman*, 1908)

"***Bacterium acidi propionici* var. *rubrum***" - 2) "***P. Thönii***" (d)
(*Thöni* and *Alleman*, 1908)

"***Bacillus acidi propionici***" - 3) "***P. pentosaceum***" (b)
(*Von Freudenreich* and *Orla-Jensen*, 1906)

\- "***P. arabinosum***" (a)

\- 4i) "***P. Jensenii* var. *raffinosaceum***" (c)
→ "***P. raffinosaceum***"
(*Werkman* and *Kendell*, 1931)

"***Bacterium acidi propionici b***" - 4ii) "***P. Jensenii***" (c)
(*Von Freudenreich* and *Orla-Jensen*, 1906)

"***Bacterium acidi propionici c***" - 5) "***P. Peterssonii***" (c)
(*Troili-Petersson*, 1909)

\- 6) "***P. technicum***" (b)

\- "***P. zeae***"

\- "***P. amylaceum***"

\- "***P. amylaceum* var. *auranticum***"

\- "***P. japonicum***"

\- "***P. pituitosum***"

"***Bacterium acidi propionici* var. *fuscum***" - 7ii) ***P. Freudenreichii*** (a)
(*Thöni* and *Alleman*, 1908) (Synonym)

"***Bacterium acidi propionici a***" - 7i) ***P. Freudenreichii*** (a)
(*Von Freudenreich* and *Orla-Jensen*, 1906)

"***Bacterium acidi propionici d***" - 8) "***P. Shermanii***" (a)
(*Sherman*, 1921)

\- "***P. casei***" (a)

(1-8 = *Van Niel*, 1928)

\- "***P. globosum***"

(___ / ___ / ..... = *Werkman* and *Brown*, 1933)

(*Hitchner*, 1934) - "***P. orientum***"

(*Sakaguchi et al.*, 1941) - "***P. coloratum***"
(*Janoschek*, 1944)

\- "***P. sanguineum***"

(a-d) = *Antila* and *Gyllenberg*, 1963)

Fig. 5. The four main groups as described by *Antila* and *Gyllenberg* (1963)

within the existing eleven species of the genus *Propionibacterium*. It was thus evident that not all propionibacteria had been studied and described, and that the existing identification system of *Breed* et al. (1957) would have to be amended to incorporate these unnamed strains.

The anaerobic coryneforms

Unna (1916) made the first observation of the organism originally referred to in medical literature as the "acne bacillus". In 1900, *Gilchrist* named this bacterium *Bacillus acnes*. *Bacillus acnes* was subsequently placed within the genus *Corynebacterium* by *Bergey* (1923) primarily due to similarity in morphological relationships between these two groups. Since the inclusion of this organism in the genus *Corynebacterium* seemed questionable, *Douglas* and *Gunter* (1946) considered it worth while to make a comparative study of the series of isolates.

They found that on a morphological basis *C. acnes* might be placed in either the genus *Corynebacterium* or the genus *Propionibacterium*, for these two groups have certain morphological features in common. Two characteristics, however, indicated that the *C. acnes* strains were more related to the propionic acid bacteria than to the corynebacteria. These characteristics were the nature of the catabolic process and the effect of oxygen which either completely or strongly inhibited the growth of *C. acnes*. This characteristic distinguished *C. acnes* from the typically aerobic corynebacteria. *Douglas* and *Gunter* (1946) subsequently suggested that *C. acnes* should be transferred to the genus *Propionibacterium* as *Propionibacterium acnes* inspite of the fact that their data indicated that the organism did not ferment lactate to propionate.

Furthermore, *Douglas* and *Gunter* (1946) proposed that the definition of the genus *Propionibacterium* should be modified to include non-lactate-fermenting species. In addition, they found that the strains of *C. acnes* formed a homogeneous group based on the following characteristics: optimum temperature; gelatin liquefaction; action on milk; nitrate reduction; lactate fermentation; and habitat.

The nomenclatural changes proposed by *Douglas* and *Gunter* (1946) were, however, not accepted, since the description of *C. acnes* under the genus *Corynebacterium* was maintained in the 7th edition of Bergey's Manual (*Breed* et al., 1957). In 1980, *Skerman* accepted the fermentation of lactate as a diagnostic criterion for the genus *Propionibacterium*. Lactate fermenting cultures, which were apparently otherwise similar to *C. acnes*, were isolated from the rumen of cattle by *Gutierrez* (1953). *Gutierrez* (1953) subsequently assigned these isolates to *C. acnes*.

The validity of *Corynebacterium acnes* was tested in 1963 by *Moore* and *Cato*. They demonstrated that the strains of *C. acnes* consistently fermented lactate with the production of propionic acid under anaerobic conditions, thereby fulfilling the diagnostic criteria as prescribed for members of the genus *Propionibacterium*. Their results subsequently supported the proposal of *Douglas* and *Gunter* (1946) to reclassify *C. acnes* to the genus *Propionibacterium*. Furthermore, their transfer would not require a redefinition of the genus to include non-lactate-fermenting species as had originally been suggested.

It wasn't until 1968 that any investigation was again initiated to evaluate this observation. In this year *Zierdt* et al., proposed that *C. acnes* should be retained in the genus *Corynebacterium*. This was based on differences they observed between these two genera. Their motivation was founded on the morphological differences observed between *C. acnes* and members of the genus *Propionibacterium*. *Corynebacterium acnes* was delicate, of quite uniform bacillary morphology, occurred in pairs and single and stained unevenly during Gram-staining. In contrast, species of the genus *Propionibacterium* were robust, almost coarse, pleomorphic, occurred singly and stained uniformly.

They further stated that it should be noted that *C. acnes* requires anaerobic conditions for production of propionic acid from glucose or lactate, while *Propionibacterium* produce it aerobically as well as anaerobically. To confirm their results, they also named three other organisms which produced similar products during their fermentation. These bacteria included *Corynebacterium diptheriae*, which produced propionic acid from glucose (*Tasman* and *Brandwijk*, 1938); *Veillonella*

gazogenes (*Johns*, 1951); and an even more closely resembled example, a unidentified Gram-positive coccus which was isolated from the rumen of sheep which was found to produce propionic acid, acetic acid and CO_2 from lactate (*Elsden*, 1945).

In 1972, *Johnson* and *Cummins* observed that several of the species classified in the genus *Corynebacterium* were similar in their cell wall composition and the mol% G+C content of their DNA to members of the genus *Propionibacterium*. *Moore* and *Holdeman* (1973) subsequently reclassified several of the anaerobic to aerotolerant members of the genus *Corynebacterium* as members of the genus *Propionibacterium*. These bacteria were anaerobic, produced propionic acid as a major end-product of their metabolism, contained mostly L-DAP as the diamino acid of peptidoglycan, produced C_{15} *iso*-and *anteiso*-acids as the principle fatty acids of cell lipids and lacked mycolic acids and the arabinogalactan which was characteristic of the genus *Corynebacterium*. These anaerobic coryneforms were subsequently reclassified as *Propionibacterium acnes*, *P. avidum*, *P. granulosum* and *P. lymphophilum*. This was partially in agreement with the proposal of *Douglas* and *Gunther* (1946), that *C. acnes* should be transferred to the genus *Propionibacterium*.

The transfer of these anaerobic coryneforms to the genus *Propionibacterium* was, however, very controversial. Some workers were in favour of the transfer (*Johnson* and *Cummins*, 1972), while others considered the dissimilarities between the two groups sufficient enough to support their separation (*Langsrud* and *Reinbold*, 1974). *Prévot* (1976) proposed that the *P. acnes* group of organisms should not be transferred to the genus *Propionibacterium*, but should instead be accommodated in a separate subgenus *Coryneformis* in the family *Corynebacteriaceae*, primarily due to their pathogenic and reticulostimulatory properties.

These four species currently form the cutaneous members of the genus *Propionibacterium* (*Holt* et al., 1994). The proposal of a new species *Propionibacterium propionicus* (*Charfreitag* et al., 1988), which was closely related to these four cutaneous members of the genus *Propionibacterium*, was only mentioned by *Holt* et al. (1994) in the ninth edition of Bergey's Manual of Determinative

Bacteriology, although it was retained in the genus *Arachnia* for determinative purposes.

Changes in the current taxonomy

A major change in the taxonomic status and the species arrangement of the genus *Propionibacterium* occurred in the 8th edition of Bergey's Manual (*Moore* and *Holdeman*, 1974). According to this system the propionibacteria were part of the *Actinomycetales* and related organisms (Part 17) of Class 1, the Bacteria, in the Kingdom *Procaryotae*. The family *Propionibacteriaceae* was retained with two genera, the *Propionibacterium* and the *Eubacterium*.

Based essentially on cell wall composition and deoxyribonucleic acid similarities as reported by *Johnson* and *Cummins* (1972), *Moore* and *Holdeman* (1974) consolidated and reduced the 21 species within the genus *Propionibacterium* to eight species. The species proposed by *Moore* and *Holdeman* (1974) were: *P. acidipropionici*; *P. freudenreichii*; *P. jensenii*; and *P. thoenii*, which formed the classical propionic acid bacteria group as well as *P. acnes*; *P. avidum*; *P. granulosum*; and *P. lymphophilum*, which formed the cutaneous group. The species "*P. pentosaceum*", "*P. arabinosum*" and "*P. rubrum*", "*P. Thönii*" were consolidated to form the species *P. acidipropionici* and *P. thoenii* respectively (Fig. 7).

Furthermore, nine species namely: "*P. raffinosaceum*"; *P. Jensenii*; "*P. Peterssonii*"; "*P. technicum*"; "*P. zeae*"; "*P. amylaceum*"; "*P. amylaceum* var. *auranticum*"; "*P. japonicum*"; and "*P. pituitosum*" were consolidated to form the species *P. jensenii* (Fig. 7). *Propionibacterium Freudenreichii*; "*P. Shermanii*"; "*P. casei*"; "*P. globosum*"; "*P. orientum*"; "*P. coloratum*"; and "*P. sanguineum*" were consolidated to form the fourth species of the classical propionic acid bacteria group, namely *P. freudenreichii* (Fig. 7). *Propionibacterium freudenreichii* was further differentiated into three subspecies primarily due to their significance in the cheese industry, namely: *P. freudenreichii* subsp. *freudenreichii*; *P. freudenreichii* subsp. *shermanii*; and *P. freudenreichii* subsp. *globosum*. *Propionibacterium acnes* was divided into two subgroups on the basis of cell wall composition and cell wall

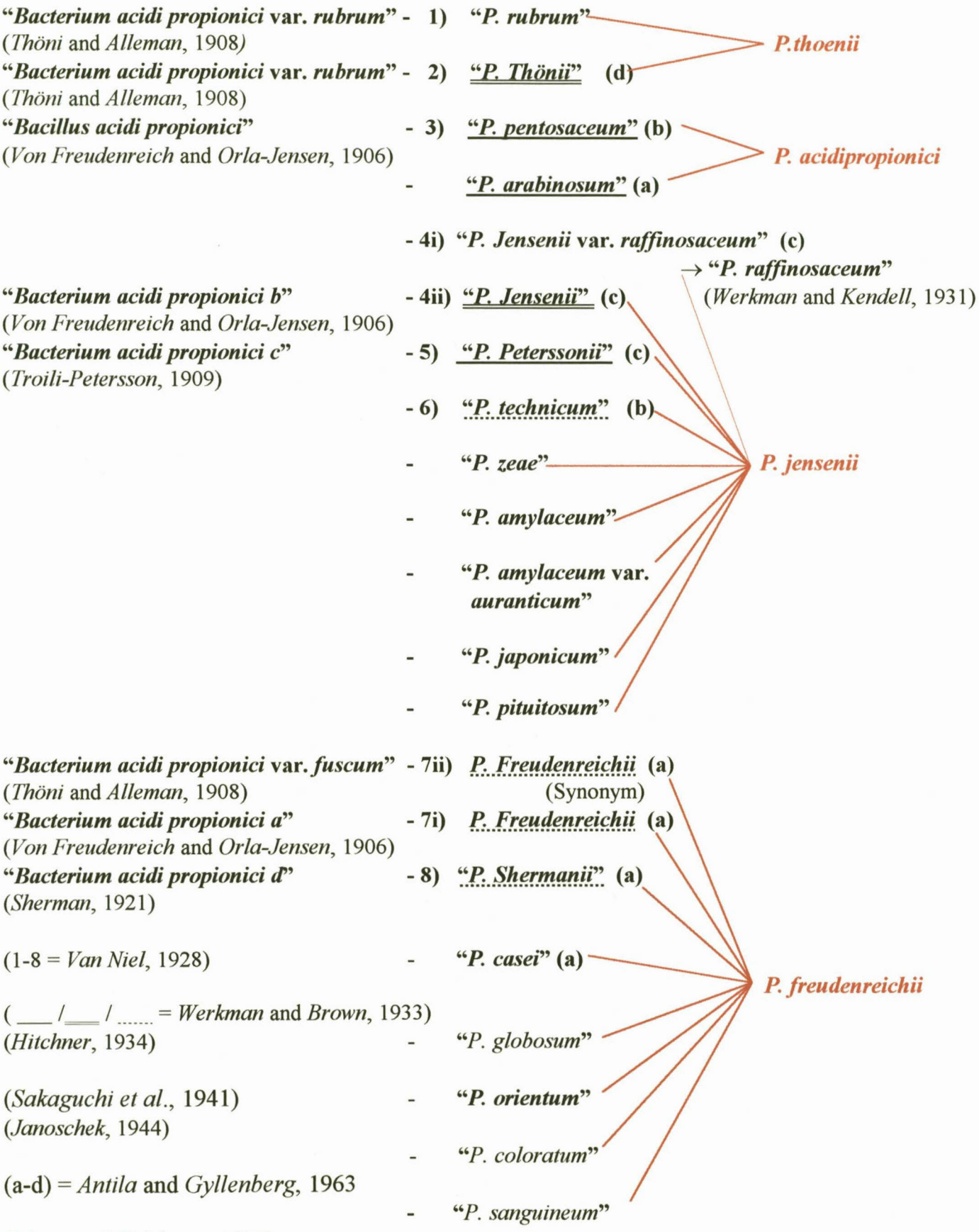


Fig. 7. The four species of the classical *Propionibacterium* group (*Moore* and *Holdeman*, 1974)

antigens. *Propionibacterium avidum, P. granulosum* and *P. lymphophilum* were classified as individual species.

Moore and *Holdeman* (1974) subsequently also proposed a identification key where differentiation between the species was based on the fermentation of certain carbohydrates, the hydrolysis of both gelatine and esculin, and their reduction of nitrate. According to *Reinbold* (1978) the change in the species arrangement within the genus *Propionibacterium*, improved the taxonomy of the cutaneous propionibacteria, but worsened the situation with respect to the identification of the classical species and other propionic acid producing isolates. Despite using several alternative techniques in an attempt to confirm the species groupings of *Moore* and *Holdeman* (1974), *Britz* and *Steyn* (1979a:1979b:1979c) were largely unsuccesfull. Techniques evaluated by them included pyrolysis-gas-liquid-chromatography, cellular fatty acid profiles and deoxyribonucleic acid base composition (mol% G + C). Despite the fact that *Britz* and *Steyn* (1979a) could divide the genus *Propionibacterium* into its two major groups corresponding their ecological differences, they were not able to differentiate the various species of the genus. Using cellular fatty acid profiles (*Britz* and *Steyn*, 1979b), would also not differentiate between the various species of the genus *Propionibacterium*. During an evaluation of the deoxyribonucleic acid base composition (mol% G + C), *Britz* and *Steyn* (1979c) reported that the mol% G + C of the classical group varied from 64-68 mol% whereas that of the cutaneous group varied from 57-63 mol%. Using this technique, *Britz* and *Steyn* (1979c) were, however, again not successful at differentiating the various species.

Even after this major change in the taxonomic status, several reports are to be found in the literature concerning isolates which could not be identified using the classification key of *Moore* and *Holdeman* (1974). In 1975 and 1978, *Britz* reported the isolation of various propionibacteria from South African cheese, which were difficult to identify using the key of *Holdeman* et al. (1973; 1977). In 1983, *Bushel* mentioned a *Propionibacterium denitrificans*, which is not a recognised species since no description of this strain appeared in Bergey's Manual of Systematic Bacteriology (*Cummins* and *Johnson*, 1986). According to *Beerens* et al. (1986), *Samain* (1983) reported the isolation of a *Propionibacterium* sp. from a methanogenic fermentation,

which could not be identified further than the generic level. Unfortunately no further references or literature are available concerning this bacterium. Furthermore, *Dubourgier* et al. (1988) considered *Propionibacterium* as one of the main genera of the microbial conglomerate present in granular sludge, from an anaerobic methanogenic reactor.

Reclassification of "*Arachnia propionica*"

In 1959, *Pine* and *Hardin* isolated an organism morphologically resembling *Actinomyces israelii*, but which produced propionic and acetic acids as major products of glucose fermentation. Based primarily on the major metabolic products produced by this strain, *Buchanan* and *Pine* (1962) classified this organism as *Actinomyces propionicus*. Seven years later, however, *Pine* and *Georg* (1969) discovered the presence of diaminopimelic acid in the cell wall of *Actinomyces propionicus*. Based on these observations, they subsequently reclassified *A. propionicus* by creating a new genus *Arachnia*, with *Arachnia propionica* as the only species.

Arachnia propionica differed from the other *Actinomyces* species in that it produced propionic acid as one of the major end-products of glucose fermentation, it possessed a murein based on the L-DAP and contained major amounts of *iso*- and *anteiso*-methyl branched fatty acids. Most of these characteristics were similar to that of the genus *Propionibacterium*. Based on cell wall composition and deoxyribonucleic acid similarities, *Johnson* and *Cummins* (1972) could, however, not find any homology between *Arachnia propionica*, the classical *Propionibacterium* and the anaerobic coryneforms. The bacterium was subsequently retained as the only species within the genus *Arachnia* by both *Moore* and *Holdeman* (1974) and *Cummins* and *Johnson* (1986).

Using reverse transcriptase sequencing of long regions of the 16S ribosomal ribonucleic acid, *Charfreitag* et al., (1988) found that *A. propionica* was unrelated to members of the genus *Actinomyces*, but formed a distinct group with *P. freudenreichii* and *P. acnes*. *Charfreitag* et al. (1988) subsequently stated that the retention of *Arachnia* as a separate genus was unjustified and proposed that *Arachnia propionica*

be reclassified as *Propionibacterium propionicus*. The placement of *A. propionica* with the propionibacteria on the basis of comparative analysis of 16S rRNA sequences is in accordance with the physiological and chemical similarities of these organisms.

Additional data which supported the transfer of *Arachnia propionica* to the genus *Propionibacterium* were the cellular fatty acid composition as determined by *O'Donnell* et al., (1985) and *Cummins* and *Moss* in 1990. They reported that the cell walls of *Arachnia propionica* contained large amounts of 13-methyltetradecanoic acid (*iso*C15:0) and 12-methyltetradecanoic acid (*antiso*C15:0), resembling the pattern found in propionibacteria. *Cummins* and *Moss* (1990), however, further argued that the name should rather be *Propionibacterium propionicum*, since *Propionibacterium* is a neuter noun.

Systematic position of *"P. coccoides"*

"*Propionibacterium coccoides*" was first described in 1983 by *Vorobjeva* et al. This bacterium was isolated from "Soviet" cheese where it plays a significant role in the aging process of hard cheeses. *Vorobjeva* et al., (1983) designated "*P. coccoides*" as a member of the genus *Propionibacterium* based on the following characteristics:

- the same phenotypic characteristics as for the propionic acid bacteria;
- propionic acid was formed as the major product of lactate fermentation. This propionic acid was produced from methylmalonyl-CoA, formed as a coenzyme B_{12}-dependent isomerization reaction, which is a key systematic characteristic of the propionic acid bacteria;
- the electrophoretic mobility of the enzymes (catalase, superoxide dismutase and peroxidase) were the same as those observed for the propionic acid bacteria;
- the mol% G + C content of their DNA was determined to be 63.4 mol %. That of the other propionic acid bacteria was found to be 65 - 67 mol %; and
- their genome structure (DNA homology) showed closest similarity (49%) to the propionic acid bacterium *P. jensenii*.

According to the definition of the genus *Propionibacterium* as given by *Moore* and *Holdeman* (1974), this propionic acid producing coccus should subsequently be classified in this genus. No mention of this strain or the proposal of *Vorobjeva* et al. (1983) was made in Bergey's Manual of Systematic Bacteriology (*Cummins* and *Johnson*, 1986). Furthermore, "*P. coccoides*" was also not included in Bergey's Manual of Determinative Bacteriology (*Holt* et al., 1994) despite the fact that *Cummins* and *Johnson* (1992) stated that there was sufficient evidence to include this species in the genus *Propionibacterium.*

Reclassification of *Propionibacterium innocuum*

In 1991, *Pitcher* and *Collins* proposed *Propionibacterium innocuum* as a new species within the genus *Propionibacterium* to accommodate strains of coryneform bacteria from human skin with phenotypic characters similar to those of the classical *Propionibacterium* group. The only differences between these strains and the classical *Propionibacterium* group were that they exhibited aerobic respiration and possessed a unique cell wall composition in which LL- diaminopimelic acid and arabinose occurred together. Based on partial 16S rRNA sequence data, *Pitcher* and *Collins* (1991) observed that these strains represented a distinct line within the genus *Propionibacterium*.

In 1994, *Yokota* et al., based on 16S ribosomal DNA analysis, reported that the phylogenetic neighbour of *Propionibacterium innocuum* was *Luteococcus japonicus* and that this pair of organisms branched intermediately between the genus *Propionibacterium* on the one side and both genera *Aeromicrobium* and *Nocardioides* on the other side. The *P. innocuum* strains differed from the species of *Aeromicrobium* and *Nocardioides* by the formation of propionic acid and from the species of *Luteococcus* in their morphology. *Yokota* et al., (1994) subsequently proposed the transfer of *P. innocuum* (*Pitcher* and *Collins*, 1991) to a new genus, *Propioniferax*, as *Propioniferax innocua.*

Reclassification of *"Propionibacterium rubrum"*

Moore and *Holdeman* (1974) consolidated *"P. Thönii"*, *P. rubrum*, and *"P. sanguineum"* to form the species *P. thoenii*. This consolidation was considered valid due to the high DNA homology values observed between these old species (*Johnson* and *Cummins*, 1972) as well as many characteristics in common, including the production of an intense red or reddish brown pigment and being β-hemolytic on blood agar. *Malik* et al. (1968) as well as *Britz* and *Riedel*, (1991:1995), however, reported a low level of phenotypic similarity between the "*P. rubrum*" and *P. thoenii* species. These results were subsequently also confirmed by *Riedel* and *Britz* (1992) who observed a high degree of similarity between the *P. jesenii* and *"P. rubrum"* strains based on electrophoretic protein profiles.

In 1995 *De Carvalho* et al. investigated the application of 16S rRNA sequence analysis in determining the taxonomic relationship of strains previously designated as *"P. rubrum"* to the species *P. thoenii* and *P. jensenii*. They reported that in their comparison of the complete 16S rRNA gene sequence, only twelve bases differed between the old *"P. rubrum"* species and the *P. jensenii* species. *De Carvalho* et al., (1995) considered these two species to be almost identical. Confirming the results of *Malik* et al., (1968), *Britz* and *Riedel* (1991) as well as *Riedel* and *Britz* (1992). *De Carvalho* et al., (1995) thus subsequently proposed that *"P. rubrum"* be reclassified as a β-hemolytic biovar of *P. jensenii*. They further differentiated the genomic species *P. jensenii* and *P. thoenii* by biochemical characteristics such as production of acid from myo-inositol and starch.

The description of *Propionibacterium cyclohexanicum*

The latest addition to the genus *Propionibacterium* was the description of a new species, namely *P. cyclohexanicum*, which was isolated from spoiled orange juice by *Kusano* et al. (1997). The results obtained of a phylogenetic analysis of the 16S rRNA gene indicated that its highest level of homology is with the representative of the classical propionibacteria, *P. freudenreichii* (97.1%). Although this strain was

phenotypically also similar to *P. freudenreichii*, it produced a large amount of lactic acid and had a distinct fatty acid composition, acid tolerance and heat resistance, which differentiated it from *P. freudenreichii* and the other propionic acid-producing bacteria. In their unrooted phylogenetic tree, *Kusano* et al. (1997) reported that the most closely related genera to the genus *Propionibacterium* were *Luteococcus*, *Nocardioides* (*Yokota* et al., 1994) and *Terrabacter*.

The genus *Luteococcus* was first described in 1997 by *Kusano* et al. for a new Gram-positive, non-motile coccus. Isolated from water and soil, *Luteococcus japonicus* has the following chemotaxonomic characteristics: Menaquinone MK-9(H_4); 67mol% G + C content; and L-diaminopimelic acid, alanine, glycine and glutamic acid being produced in a molar ratio of ca. 1:2:1:1. The partial 16S rRNA sequence data further indicated the genus represented a distinct line of descent among the Gram-positive bacteria with a high G + C content.

Characteristics of the genus *Nocardioides*, include the formation of pleomorphic elements to branched vegetative mycelium, which break up into short to elongated rodlike fragments. Members of the genus *Nocardioides* are susceptible to specific phages and contain diphosphatidylglycerol, phosphatidylglycerol and two incompletely characterised lipids. Tetrahydrogenated menaquinones with either eight or nine isoprene units (MK-8(H_4), 9(H_4)) predominate (*Holt* et al., 1994).

Characteristics of the genus *Terrabacter* include a rod-coccus growth cycle that occurs during growth on complex media. Although no aerial mycelium are produced, the long rods show primary branching. They contain diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and some unknown amino-containing phosphoglycolipids. Tetrahydrogenated menaquinone with eight isoprene units saturated at sites II and III (MK-8(II, III-H_4)) are the predominant isoprenolog (*Holt* et al., 1994).

Present work done on propionibacteria

The taxonomic re-evaluation of the groupings amongst the propionibacteria are currently dominated by the application of molecular techniques. In 1989, *Charfreitag* and *Stackebrandt* determined the phylogenetic structure of the genus *Propionibacterium* based on the comparative analysis of the 16S rRNA sequence data. Their results confirmed the relationships within the genus *Propionibacterium* as reported by *Johnson* and *Cummins* (1972), based on total DNA similarities. *Propionibacterium jensenii* and *P. thoenii* were observed to be closely related and formed a tight cluster with *P. acidipropionici*. All three these species contain the LL-isomer of diaminopimelic acid in their peptidoglycan and can ferment sucrose and maltose (*Charfreitag* and *Stackebrandt*, 1989). This phylogenetic cluster was observed to be well separated from the fourth classical species *P. freudenreichii*, which contains *meso*-diaminopimelic acid in their peptidoglycan. *Propionibacterium freudenreichii* was, however, found to be equidistantly related to both *P. propionicus* and *P. acnes* representing the cutaneous *Propionibacterium* group. The data of *Charfreitag* and *Stackebrandt* (1989) subsequently did not support the proposal of Prévot (1976) to accommodate *P. acnes* and related taxa into a separate subgenus *Coryneformis* in the family *Corynebacteriaceae*.

Using two-dimensional gel electrophoresis of ribosomal proteins *Dekio* et al. (1989) could differentiate between the two serological types of *P. acnes*. Differentiation between *P. acnes* and *P. granulosum* was also possible using this technique. The technique is, however, not suitable for the rapid and routine identification of propionibacteria, since it is cumbersome and time consuming.

In 1991, *Britz* and *Riedel* undertook a numerical taxonomic study on propionibacteria obtained from dairy sources. All four classical *Propionibacterium* species could be differentiated. Two distinct groups within the *P. jensenii* species could, however, be identified if pigmentation was used as differential characteristic. In a numerical taxonomic study of propionibacteria isolated from anaerobic digesters, *Riedel* and *Britz* (1993) reported that some isolates could be identified as a specific species using the identification system of *Cummins* and *Johnson* (1986), but based on

overall similarity, many of the strains clustered among members of another species. This was especially obvious in species identified as *P. jensenii* and *P. acidipropionici*. Their explanation for this observation was that the strains were identified as a specific species based on only five biochemical characteristics, and that the major differential characteristic between the species *P. jensenii* and *P. acidipropionici* was the reduction of nitrate, which was observed to be a highly variable phenotypic characteristic.

Riedel and *Britz* (1992) could differentiate all four the classical species based on numerical analysis of electrophoretic protein profiles. Certain clusters were, however, electrophoretically heterogeneous and *P. jensenii* and *P. thoenii* were observed to cluster together. Delineation between the various *P. freudenreichii* subspecies was also not achieved.

Baer and *Ryba* in 1992 evaluated serological methods for the identification of the classical propionibacteria and compared their results with that of *Werkman* and *Brown* (1933). *Werkman* and *Brown* could only distinguish three subgeneric groups containing the 11 species described at that stage. *Baer* and *Ryba* (1992), were, however, able to serologically differentiate the four classical species within the genus *Propionibacterium*. *Werkman* and *Brown* (1933) also succeeded in serologically differentiating *P. freudenreichii* subsp. *freudenreichii* from *P. freudenreichii* subsp. *shermanii*. These results could, however, not be confirmed by *Baer* and *Ryba* (1992). By obtaining these close relationship between the various *P. freudenreichii* subspecies, *Baer* and *Ryba* (1992) also confirmed the DNA-hybridisation results of *Johnson* and *Cummins* (1972) and the SDS-PAGE profiles obtained by both *Baer* and *Ryba* (1988) and *Riedel* and *Britz* (1992).

While studying the complexity of the esterase system of the propionic acid bacteria using PAGE separation, *Dupuis* and *Boyaval* (1993) only observed three different patterns for the five species examined. Strains of *P. freudenreichii* could be differentiated from *P. acidipropionici*, and the *P. jensenii* / *P. thoenii* group, which displayed an identical profile. *Propionibacterium freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii* could not be differentiated, again confirming

the results obtained by *Baer* (1987), *Baer* and *Ryba* (1988) as well as *Riedel* and *Britz* (1992) who evaluated electrophoretic protein profiles.

Plasmid profiling is a simple and expressive technique and the presence of plasmids in propionibacteria has been described by numerous researchers (*Panon*, 1988; *Perez Chaia* et al., 1988; *Rehberger* and *Glatz*, 1990). The occurrence of plasmids in the genus *Propionibacterium* varies between 25 and 38% of all strains. Subsequent studies report that approximately 70% of all propionic acid bacteria either have no plasmids (*Rehberger* and *Glatz*, 1990). The plasmid size is also an arguable issue since it varies from 3kb to over 150kb. The only drawback of this technique is the instability of the profiles obtained primarily due to the acquisition, loss or transfer of plasmids.

With the exception of the ability to ferment lactose, associated with plasmid pRG03 in a strain of *P. freudenreichii* subsp. *globosum* (*Rehberger* and *Glatz*, 1990) and a clumping phenomenon associated with plasmid pRG05 in a strain of *P. jensenii* (*Rehberger* and *Glatz*, 1990), no evidence has been found to indicate plasmid association for bacteriocin production, pigment production, fermentation characteristics, growth characteristics or antibiotic resistance.

In the differentiation of species within the genus *Propionibacterium*, phage typing is generally considered an unsuccessful technique primarily due to the fact that the technique is cumbersome and lacks the precision required for routine strain differentiation. Presently, only bacteriophages active against the cutaneous *P. acnes* species (*Jong* et al., 1975) and the classical *P. freudenreichii* species (*Gautier* et al., 1992b; 1995) have been detected. The application of phagetyping (*Webster* and *Cummins*, 1978) and a combination of phagetyping with biotyping (*Jong* et al., 1975) resulted in the differentiation of the two serotypes of *P. acnes*. *Propionibacterium acnes* I strains were observed to be more susceptible to phage lysis than serotype II strains. Although only 18% of *P. freudenreichii* strains analysed by *Gautier* et al. (1995) were sensitive to phages, bacteriophages that were active against propionibacteria were found in at least 50% of the Swiss cheese examined by them.

In 1992, *Gautier* et al. (1992a) determined that the genome size of the four classical propionibacteria ranged from 2300 to 3200 kb depending on the specific species. A year later, however, *Rehberger* (1993) undertook a genomic analysis of *P. freudenreichii* and determined that the estimates for the genome size ranged from 1600 - 2300 kb.

In 1994 and 1998, *Riedel* et al. reported an analysis of restriction fragment length polymorphisms within the 16S rDNA genes of the classical propionibacteria. Using the restriction endonucleases *Hae*III, *Alu*I and *Hpa*II, all four classical species could be differentiated. A higher degree of similarity was observed between both the old "*P. rubrum*" and "*P. sanguineum*" species and the *P. jensenii* species than between these two species and the *P. thoenii* and *P. freudenreichii* species, respectively.

Later in the same year, *De Carvalho* et al., reported a study where the classical species were evaluated using ribotyping. Using the restriction endonucleases *Bam*HI and *Cla*I, *De Carvalho* et al. (1994) could differentiate between all four the classical species and it was also possible to distinguish *P. freudenreichii* subsp. *freudenreichii* from *P. freudenreichii* subsp. *shermanii*. This was, however, in contrast to the results reported by *Riedel* et al., in 1994, where no differentiation was obtained between the three subspecies *of P. freudenreichii*. *De Carvalho* et al. (1994) reported ribotyping to be an excellent tool for species determination, since different patterns with species specific fragments could be obtained for the four classical *Propionibacterium* species. The ribotype patterns obtained also differed markedly from those obtained for phylogenetically closely related genera (*Bifidobacterium*, *Brevibacterium*, *Corynebacterium* and *Mycobacterium*) as well as for other dairy bacteria (*Lactococcus*, *Lactobacillus* and *Leuconostoc*).

In 1996, *Riedel* and *Britz* also reported the evaluation of ribotyping as a technique for the differentiation of the classical propionibacteria. In contrast to *De Carvalho* et al. (1994), *Riedel* and *Britz* (1996) used the restriction endonucleases *Hae*II and *Sma*I. Sixteen distinct profiles could be observed within the classical propionibacteria. Two profiles were observed within the *P. acidipropionici* species, one profile for *P. freudenreichii*, nine profiles were observed in all strains representing

the *P. jensenii* species and four profiles for the *P. thoenii* species. The ribotyping profiles observed for "*P. rubrum*" and "*P. sanguineum*" were similar to that of the *P. jensenii* species, confirming the RFLP results of *Riedel* et al., (1994).

Gautier et al. (1996) differentiated between various *Propionibacterium* strains by applying DNA fingerprinting using pulsed-field gel electrophoresis. Poor pattern variability was, however, observed amongst the *P. acidipropionici*, *P. jensenii* and *P. thoenii* species and the application of this technique was subsequently limited. A high degree of restriction fragment length polymorphism was observed which confirmed the results obtained by *Rehberger* (1993). The high degree of heterogeneity in the restriction fragment patterns among the majority of *P. freudenreichii* strains was rather unexpected, especially due to the high DNA relatedness among *P. freudenreichii* strains, that has been reported to be 90% (*Johnson* and *Cummins*, 1972).

Despite the application of various conventional and molecular techniques, it is, however, still evident from the literature that many discrepancies are still apparent concerning the isolation, identification and differentiation of the various classical members of the propionic acid producing bacteria. Inspite of the fact that "*P. coccoides*" was originally proposed as a new *Propionibacterium* species by *Vorobjeva* et al. in 1983, no systematic evaluation of "*P. coccoides*" and the propionic acid bacteria has ever been undertaken. Subsequently "*P. coccoides*" has never been officially recognised as a new species within the genus *Propionibacterium*. The exact systematic position of "*P. coccoides*" relative to that of the other *Propionibacterium* species is subsequently still unresolved.

The objectives of this study were thus to phenotypically characterise "*P. coccoides*" and the various classical *Propionibacterium* species in order to determine the relationship between these organisms. The next objective was to determine the relationship of the "*P. coccoides*" strains to known *Propionibacterium* marker strains using various molecular techniques, based on the 16S ribosomal RNA region, as well as DNA:DNA hybridisation.

References

Antila, M., Gyllenberg, H.: Taxometric study of propionic acid bacteria of dairy origin. Milchwiss. 18, 398-400 (1963).

Antipov, V.A., Subbotin, V.M.: Use of probiotics - efficacy and prospects. Dairy Sci. Abstr. 44, 8567 (1980). (As cited by Fessler, 1997).

Baer, A.: Identification and differentiation of propionibacteria by electrophoresis of their proteins. Milchwiss. 42, 431-433 (1987).

Baer, A., Ryba, I.: Identification rapide des bactéries propioniques. Schweiz. Milchw. Forschung. 17, 56-57 (1988).

Baer, A., Ryba, I.: Serological identification of propionibacteria in milk and cheese samples. Int. Dairy J. 2, 299-310 (1992).

Baer, A., Ryba, I., Grand, M.: Study on the appearance of brown-spotting in cheese. Schweiz. Milchw. Forschung. 22, 3-7 (1993).

Beerens, H., Neut, C., Romond, C.: Important properties in the differentiation of Gram positive non-sporing rods in the genera *Propionibacterium*, *Eubacterium*, *Actinomyces* and *Bifidobacterium*, pp. 38-42. In: Anaerobic Bacteria in Habitats Other than Man. (E.M. Barnes, G.C. Mead, eds.) Oxford, Blackwell Scientific Publications 1986.

Beide, S.L., Reinbold, G.W., Hammond, E.G.: Influence of *Lactobacillus bulgaricus* on microbiology and chemistry of Swiss cheese. J. Dairy Sci. 59, 854-858 (1977). (As cited by Fessler, 1997).

Benno, Y., Mitsuoka, T.: Anaerobic bacteria isolated from abscesses in pigs. Jap. J. Vet. Sci. 44, 309-315 (1982).

Bergey, D.H., Breed, R.S., Murray, E.G.D., Hitchens, A.P.: Bergey's Manual of Determinative Bacteriology 5th ed., Baltimore, Williams and Wilkins 1939.

Bergey, D.H.: Manual of Determinative Bacteriology 2nd ed., Baltimore, Williams and Wilkins 1925.

Bergey, D.H.: Manual of Determinative Bacteriology 3rd ed., Baltimore, Williams and Wilkins 1930.

Bergey, D.H.: Manual of Determinative Bacteriology 6th ed., Baltimore, Williams and Wilkins 1948.

Bergey, D.H.: Manual of Determinative Bacteriology, 1st ed., Baltimore, Williams and Wilkins, 1923.

Boyaval, P., Corre, C.: Production of propionic acid. Lait 75, 453-461 (1995).

Breed, R.S., Murray, E.G.D., Smith, N.R.: Genus 1. *Propionibacterium* Orla-Jensen 1909, pp. 569-577. In: Bergey's Manual of Determinative Bacteriology (R.S. Breed, E.G.D. Murray, N.R. Smith, eds.) 7th ed., Bailliére, London, Tindall and Cox 1957.

Britz, T.J.: A reappraisal of the taxonomy of the genus *Propionibacterium* Orla-Jensen 1909. D.Sc.(Agric) Thesis. University of Pretoria, South Africa 1978.

Britz, T.J.: The incidence of propionibacteria in South African dairy products. S. Afr. J. Dairy Technol. 7, 157-161 (1975).

Britz, T.J., Jordaan, H.F.: The rapid presumptive detection of propionibacteria as the causative organisms of defects in Gouda cheese. S. Afr. J. Dairy Technol. 8, 79-83 (1976).

Britz, T.J., Riedel K-H.J.: A numerical taxonomic study of *Propionibacterium* strains from dairy sources. J. Appl. Bacteriol. 71, 407-416 (1991).

Britz, T.J., Riedel K-H.J.: Numerical evaluation of the species grouping among the "classical" propionibacteria. Lait 75, 309-314 (1995).

Britz, T.J., Steyn, P.L.: Identification of propionibacteria by pyrolysis-gas-liquid-chromatography. S. Afr. J. Dairy Technol. 11, 119-122 (1979a).

Britz, T.J., Steyn, P.L.: Cellular fatty acid composition of the clinical and dairy propionibacteria. S. Afr. J. Dairy Technol. 11, 3-6 (1979b).

Britz, T.J., Steyn, P.L.: Deoxyribonucleic acid base composition of dairy and clinical propionibacteria. S. Afr. J. Dairy Technol. 11, 67-72 (1979c).

Brooke, I., Frazier, E.H.: Infections caused by *Propionibacterium* species. Rev. Infect. Dis. 13, 819-822 (1991).

Buchanan, B.B., Pine, L.: Characterisation of a propionic acid producing actinomycete, *Actinomyces propionicus*, sp. nov. J. Gen. Microbiol. 28, 305-323 (1962).

Buchanan, R.E.: General Systematic Bacteriology. Baltimore, Williams and Wilkins 1925.

Buchanan, R.E., Holt, J.G., Lessel, E.F.: Index Bergeyana. Baltimore, Williams and Wilkins 1966.

Bushel, M.E.: Application of the principles of industrial microbiology to biotechnology, p. 24. In: Principles of Biotechnology (A. Wiseman, ed.) New York, Surrey University Press 1983. (As cited by Beerens et al., 1986).

Carcano M., Todesco, R., Lodi, R., Brasca, M.: Propionibacteria in Italian hard cheese. Lait 75, 415-426 (1995). (As cited by Fessler, 1997).

Cerna, B., Cerny, M., Betkova, H., Patricny, P., Soch, M., Opatrna, I.: Effect of the Proma probiotics on calves. Dairy Sci. Abstr. 55, 1735 (1991). (As cited by Fessler, 1997).

Chaia, A.P., De Ruiz Holgado, A.P., Oliver, G.: Peptide hydrolyses of propionibacteria: Effect of pH and temperature. J. Food Protect. 53, 237-240 (1990).

Champagne, C.P., Lange, M.: Analyses sur fromages Mozzarella de type américain ayant montré une production anormale de gaz. Sci. Alim. 10, 43-55 (1990). (As cited by Fessler, 1997).

Charfreitag, O., Collins, M.D., Stackebrandt, E.: Reclassification of *Arachnia propionica* as *Propionibacterium propionicus* comb. nov. Int. J. Syst. Bacteriol. 38, 354-357 (1988).

Charfreitag, O., Stackebrandt, E.: Inter- and intrageneric relationships of the genus *Propionibacterium* as determined by 16S rRNA sequences. J. Gen. Microbiol. 135, 2065-2070 (1989).

Crow, V.L.: Utilisation of lactate isomers by *Propionibacterium freudenreichii* subsp. *shermanii*: regulatory role for intracellular pyruvate. Appl. Environ. Microbiol. 52, 352-358 (1986a).

Crow, V.L.: Metabolism of aspartate by *Propionibacterium freudenreichii* subsp. *shermanii*: effect on lactate fermentation. Appl. Environ. Microbiol. 52, 359-365 (1986b).

Cummins, C.S., Johnson, J.L.: The genus *Propionibacterium*, pp. 1894-1902 In: The Prokaryotes (M. Starr, H. Stolp, H.G. Trüper, A. Balows, H.G. Schlegel, eds.) vol. 2. Berlin, Springer-Verlag 1981.

Cummins, C.S., Johnson, J.L.: Genus *Propionibacterium* Orla-Jensen 1909, pp. 1346-1353. In: Bergey's Manual of Systematic Bacteriology (P.H.A. Sneath, N.S. Mair, M.E. Sharpe, J.G. Holt, eds.) vol. 2. Baltimore, Williams and Wilkins 1986.

Cummins , C.S., Johnson, J.L.: The Genus *Propionibacterium*, pp. 834-849. In: The Prokaryotes (A. Balows, H.G. Truper, M. Dworkin, W. Harder and K-H. Schleifer, eds.) vol. 1. New York, Springer-Verlag 1992.

Cummins, C.S., Moss, C.W.: Fatty acid composition of *Propionibacterium propionicum* (*Arachnia propionica*). Int. J. Syst. Bacteriol. 40, 307-308 (1990).

De Carvalho, A.F., Gautier. M., Grimont, F.: Identification of dairy *Propionibacterium* species by rRNA gene restriction patterns. Res. Microbiol. 145, 667-676 (1994).

De Carvalho, A.F., Guezenec, S., Gautier, M., Grimont, P.A.D.: Reclassification of "*Propionibacterium rubrum*" as *P. jensenii*. Res. Microbiol. 146, 51-58 (1995).

Dekio, S., Hashimoto, K., Makino, M.: Two-dimensional gel electrophoresis of ribosomal proteins from *Propionibacterium acnes* and *granulosum*. Zbl. Bakt. 271, 442-451 (1989).

Delwiche, E.A.: Family *Propionibacteriaceae*. Delwiche. Ithaca, New York: Cornell University 1954.

Delwiche, E.A.: Vitamin requirements of the genus *Propionibacterium*. J. Bacteriol. 58, 395-398 (1949).

De Vries, W., Van Wijk-Kapteyn, W.M.C., Stouthammer, A.H.: Influence of oxygen on growth, cytochrome synthesis and fermentation pattern in propionibacteria. J. Gen. Microbiol. 76, 31-41 (1972).

Douglas, H.C., Gunter, S.: The taxonomic position of *Corynebacterium acnes*. J. Bacteriol. 52, 15-23 (1946).

Dubourgier, H.C., Archer, D.B., Albagnac, G., Prensier, G.: Structure and metabolism of methanogenic microbial conglomerates, pp. 13-23. In: Anaerobic Digestion 1988 (E.R. Hall, P.N. Hobson, eds.) Oxford, Pergamon Press 1988.

Dupuis, C., Boyaval, P.: Esterase activity of dairy *Propionibacterium*. Lait 73, 345-356 (1993).

Elsden, S.R.: Fermentation of carbohydrates in the rumen of sheep. J. Exp. Biol. 22, 51-61 (1945).

Ferguson, D.A., Cummins, C.S.: Nutritional requirements of anaerobic coryneforms. J. Bacteriol. 135, 858-867 (1978).

Fessler, D.S.: Characterisation of propionibacteria in Swiss raw milk by biochemical and molecular-biological methods. D.Sc.(Technical Sciences) Thesis. Swiss Federal Institute of Technology (ETH), Zurich 1997.

Fitz, A.: Über spaltpiltzgährungen. Ber. Deut. Chem. Ges. 11, 1890-1899 (1878). (As cited by Van Niel, 1928).

Fitz, A.: Über spaltpiltzgährungen. Ber. Deut. Chem. Ges. 12, 474-481 (1879). (As cited by Van Niel, 1928).

Fujimura, S., Nakamura, T.: Purification and properties of a bacteriocin-like substance (acnecin) of oral *Propionibacterium acnes*. Antimicrob. Agents Chemother. 14, 893-898 (1978).

Fuller, R.: Probiotics in man and animals. J. Appl. Bacteriol. 66, 365-378 (1989).

Gautier, M., De Carvalho, A., Roualt, A.: DNA fingerprinting of dairy propionibacteria strains by pulsed-field electrophoresis. Curr. Microbiol. 32, 17-24 (1996).

Gautier, M., Mouchel, N., Rounalt, A., Sanséau, P.: Determination of genome size of four *Propionibacterium* species by pulsed-field gel electrophoresis. Lait 72, 421-426 (1992a).

Gautier, M., Roualt, A., Lortal, S., Leloir, Y., Patureau, D.: Characterisation of a phage infecting *Propionibacterium freudenreichii*. Lait 72, 431-435 (1992b).

Gautier, M., Roualt, A., Sommer, P., Briandet, R.: Occurrence of *Propionibacterium freudenreichii* bacteriophages in Swiss cheese. Appl. Environ. Microbiol. 61, 2572-2576 (1995).

Gilchrist, T.C.: A bacteriological and microscopical study of over three hundred vesicular and pustular lesions of the skin, with a research upon the etiology of *Acne vulgaris*. Johns Hopk. Hosp. Rep. 9, 409-430 (1900). (As cited by Zierdt et al., 1968).

Glatz, B.A.: The classical propionibacteria: their past, present and future as industrial organisms. ASM News 58, 197-201 (1992).

Gonzalez-Cancho, F., Nosti-Vega, M., Diez, M.J.F., Buzcu, N.: Especies de *Propionibacterium* relacionadas Con La Zapateria. Microbialogia Española 23, 1-20 (1970).

Goodacre, R., Neal, M.J., Kell, D.B., Greenham, L.W., Noble, W.C., Harvey, R.G.: Rapid identification using pyrolysis mass spectrometry and artificial neural

networks of *Propionibacterium acnes* isolated from dogs. J. Appl. Bacteriol. 76,124-134 (1994).

Goodfellow, M.: Suprageneric classification of actinomycetes, pp. 2333-2339. In: Bergey's Manual of Systematic Bacteriology (S.T. Williams, M.E. Sharpe, J.G. Holt, eds.) vol. 4. Baltimore, Williams and Wilkins 1989.

Gottschalk, G.: Bacterial metabolism, 2nd ed., New York, Springer-Verlag 1986.

Grinstead, D.A., Barefoot, S.F.: Jenseniin G, a heat stable bacteriocin produced by *Propionibacterium jensenii* P126. Appl. Environ. Microbiol. 58, 215-220 (1992).

Gutierrez, J.: Numbers and characteristics of lactate utilising organisms in the rumen of cattle. J. Bact. 66, 123-128 (1953).

Hayashi, S., Furusaka, C.: Studies of *Propionibacterium* isolated from paddy soils. Antonie van Leeuwenhoek 45, 565-574 (1979).

Hettinga, D.H., Reinbold, G.W.: The propionic acid bacteria - A review. I. Growth. J. Milk Food Technol. 35, 295-301 (1972a).

Hettinga, D.H., Reinbold, G.W.: The propionic acid bacteria - A review. II. Metabolism. J. Milk Food Technol. 35, 358-372 (1972b).

Hettinga, D.H., Reinbold, G.W.: The propionic acid bacteria - A review. III. Miscellaneous metabolic activities. J. Milk Food Technol. 35, 358-372 (1972c).

Hettinga, D.H., Reinbold, G.W.: Split defect of Swiss cheese. II. Effect of low temperatures on the metabolic activities of *Propionibacterium*. J. Milk Food Technol. 38, 31-35 (1975).

Hitchner, E.R.: Some physiological characteristics of the propionic acid bacteria. J. Bacteriol. 28, 473-479 (1934).

Holdeman, L.V., Cato, E.P., Moore, W.E.C.: *Arachnia* and *Propionibacterium*. In: Anaerobe Laboratory Manual, 2nd ed., Virginia Polytechnic Institute and State University, Blacksburg, Virginia, Southern Printing Co. 1973.

Holdeman, L.V., Cato, E.P., Moore, W.E.C.: *Arachnia* and *Propionibacterium*, pp. 56-65. In: Anaerobe Laboratory Manual, 4th ed., Virginia Polytechnic Institute and State University, Blacksburg, Virginia, Southern Printing Co. 1977.

Holt, J.G., Krieg, N.R., Sneath, P.H., Staley, J.T., Williams, S.T.: Genus *Propionibacterium*, p. 580. In: Bergey's Manual of Determinative Bacteriology (J.G. Holt, N.R. Krieg, P.H. Sneath, J.T. Staley, S.T. Williams, eds.) 9th ed., Baltimore, Williams and Wilkins 1994.

Horner, S.M., Sturridge, M.F., Swanton, R.H.: *Propionibacterium acnes* causing an aortic root abscess. Br. Heart J. 68, 218-220 (1992).

Janoschek, A.: Zur systematik der Propionsaürebakterien. Zentralbl. Bakteriol. Parasitenk. Infekt. Hyg. Abt. II 106, 321-337 (1944).

Jimeno, J.. Lazaro, M.J., Sollberger, H.: Antagonistic interactions between propionic acid bacteria and non-starter lactic acid bacteria. Lait 75, 401-414 (1995). (As cited by Fessler, 1997).

Johns, A.T.: The mechanism of propionic acid formation by propionibacteria. J. Gen. Microbiol. 5, 337-345 (1951).

Johnson, J.L., Cummins, C.S.: Cell wall composition and deoxyribonucleic acid similarities among the anaerobic coryneforms, classical propionibacteria, and strains of *Arachnia propionica*. J. Bacteriol. 109, 1047-1066 (1972).

Jong, E.C., Ko, H.L., Pulverer, G.: Studies on bacteriophages of *Propionibacterium acnes*. Med. Microbiol. Immun. 161, 263-271 (1975).

Klaenhammer, T.R.: Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. 12, 39-86 (1993).

Kluyver, A.J., Donker, H.J.L.: Thesis, Delft 1925. (As cited by Van Niel, 1928).

Krecek, R.C., Els, H.J. de Wet, S.C., Henton, M.M.: Studies on ultrastructure and cultivation of microorganisms associated with zebra nematodes. Microb. Ecol. 23, 87-95 (1992).

Kusano, K., Yamada, H., Niwa, M., Yamasato, K.: *Propionibacterium cyclohexanicum* sp. nov., a new acid-tolerant ω-cyclohexyl fatty acid-containing *Propionibacterium* isolated from spoiled orange juice. Int. J. Syst. Bacteriol. 47, 825-831 (1997).

Langsrud, T., Reinbold, G.W.: Flavour development and microbiology of Swiss Cheese. A review. II. Starters, manufacturing processes and procedures. J. Milk Food Technol. 36, 531-542 (1973).

Langsrud, T., Reinbold, G.W.: Flavour development and microbiology of Swiss Cheese. A review. IV. Defects. J. Milk Food Technol. 37, 26-41 (1974).

Langsrud, T., Sorhaug, T., Vegarud, G.E.: Protein degradation and amino acid metabolism by propionibacteria. Lait 75, 325-330 (1995). (As cited by Fessler, 1997).

Liu, J.A.P., Moon, N.J.: Commensalistic interaction between *Lactobacillus acidophilus* and *Propionibacterium shermanii*. Appl. Environ. Microbiol. 44, 715-722 (1982).

Lewis, V.P., Yang, S-T.: A novel extractive fermentation process for propionic acid production from whey lactose. Biotechnol. Prog. 8, 104-110 (1992).

Lyon, W.J., Glatz, B.A.: Partial purification and characterisation of a bacteriocin produced by *Propionibacterium thoenii*. Appl. Environ. Microbiol. 57, 701-706 (1991).

Lyon, W.J., Glatz, B.A.: Isolation and purification of propionicin PLG-1, a bacteriocin produced by a strain of *Propionibacterium thoenii*. Appl. Environ. Microbiol. 59, 83-88 (1993).

Malik, A.C., Reinbold, G.W., Vedamuthu, E.R.: Evaluation of the taxonomy of the *Propionibacterium*. Can. J. Microbiol. 14, 1185-1195 (1968).

Mantere-Alhonen, S.: Die Propionibaterien der Molkereiindustrie als Darmkanalmikroben. Meijeritiet. Aikak. 40, 95-96 (1982). (As cited by Fessler, 1997).

Mantere-Alhonen, S.: A new type of sour milk with propionibacteria. Meijeritiet. Aikak. 45, 46-61 (1987). (As cited by Fessler, 1997).

Mantere-Alhonen, S.: Propionibacteria used as probiotics - A review. Lait 75, 447-452 (1995).

Marcoux, V., Beaulieu, Y., Champagne, C.P., Goulet, J.: Production of *Propionibacterium freudenreichii* subsp. *shermanii* in Whey-based media. J. Ferment. Bioeng. 74, 95-99 (1992).

Massa, S., Gardini, F., Sinigaglia, M., Guerzoni, M.E.: *Klebsiella pneumonia* as a spoilage organism in Mozzarella cheese. J. Dairy Sci. 75, 1411-1414 (1992).

Meriläinen, V., Antila, M.: Über die Propionsäurebakterien im finnischen Emmentalerkäse. Meijeritiet. Aikak. 34, 107-115 (1976). (As cited by Fessler, 1997).

Moore, W.E.C., Cato, E.P.: Validity of *Propionibacterium acnes* (Gilchrist) Douglas and Gunter comb. nov. J. Bacteriol. 85, 870-874 (1963).

Moore, W.E.C., Holdeman, L.V.: New names and combinations in certain genera. Int. J. Syst. Bacteriol. 23, 69-74 (1973).

Moore, W.E.C., Holdeman, L.V.: Genus 1. *Propionibacterium* Orla-Jensen 1909, pp. 633-641. In: Bergey's Manual of Determinative Bacteriology (R.E Buchanan, N.E. Gibbons, eds.) 8th ed., Baltimore, Williams and Wilkins 1974.

O'Donnell, A.G., Minnikin, D.E., Goodfellow, M., Parlett, J.H., Schofield, G.M., Schaal, K.P.: Lipid and wall amino acid composition in the classification and identification of *Arachnia propionica*. Zentralbl. Bakteriol. Parasitenk. Infekt. Hyg. Abt. I 260, 300-310 (1985). (As cited by Cummins and Moss, 1990).

Orla-Jensen, S.: Die hauptlinien des natürlichen bakteriensystems. Zentralbl. Bakteriol. Parasitenk. Infekt. Hyg. Abt. II 22, 63-273 (1909). (As cited by Van Niel, 1928).

Orla-Jensen, S.: The main lines of the natural bacterial system. J. Bacteriol. 6, 263-273 (1921). (As cited by van Niel, 1928).

Oterholm, A.: Glycerol ester hydrolases of some lactic and propionic acid bacteria. PhD. Thesis. University of Illinois, Urbana, USA 1967. (As cited by Fessler, 1997).

Panon, G.: Presence of plasmids in propionic acid bacteria. Lait 68, 103-107 (1988).

Paik, H-D., Glatz, B.A.: Propionic acid production by immobilised cells of a propionate-tolerant strain of *Propionibacterium acidipropionici*. Appl. Microbiol. Biotechnol. 42, 22-27 (1994).

Paul, G.E., Booth, S.J.: Properties and characteristics of a bacteriocin-like substance produced by *Propionibacterium acnes* isolated from dental plaque. Can. J. Microbiol. 34, 1344-1347 (1988)

Perez Chaia, A., Pesce de Ruiz Holgado, A.P., Oliver, G.: Interaction between *Lactobacillus helveticus* and *Propionibacterium freudenreichii* subsp. *shermanii*. Microbiol. Alim. Nutr. 5, 325-331 (1982). (As cited by Fessler, 1997).

Perez Chaia, A., Sesma, F., Pesce de Ruiz Holgado, A., Oliver, G.: Screening of plasmids in strains of *Propionibacterium* and mesophilic lactobacilli isolated

from Swiss-type cheeses. Microbiol. Alim. Nutr. 6, 171-174 (1988). (As cited by Fessler, 1997).

Perez Chaia, A., Strasser de Saad, A.M., Pesce de Ruiz Holgado, A., Oliver, G.: Competitive inhibition of *Propionibacterium acidipropionici* by mixed culturing with *Lactobacillus helveticus*. J. Food. Protect. 56, 341-344 (1994). (As cited by Fessler, 1997).

Pine, L., Georg, L.K.: Reclassification of *Actinomyces propionicus*. Int. J. Syst. Bacteriol. 19, 267-272 (1969).

Pine, L., Hardin, H.: *Actinomyces israelii*, a cause of lacrimal canaliculitis in man. J. Bacteriol. 78, 164-168 (1959).

Pitcher, D.G., Collins, M.D.: Phylogenetic analysis of some LL-diaminopimelic acid-containing coryneform bacteria from human skin: description of *Propionibacterium innocuum* sp. nov.. FEMS Microbiol. Lett. 84, 295-300 (1991).

Prévot, A.R.: Nouvelle conception de la position des corynébactéries anaérobies. Comptes Rendus Hebdomadaires des Séances de I' Académie des Sciences, Série D 282, 1079-1081 (1976). (As cited by Britz, 1978).

Pritchard, G.G., Wimponny, J.W.T., Morris, H.A., Lewis, M.W.A., Hughes, D.E.: Effect of oxygen on *Propionibacterium shermanii* grown in continuous culture. J. Gen. Microbiol. 102, 223-233 (1977).

Prottey, C., Ballou, C.E.: Diacyl myoinositol monomannoside from *Propionibacterium shermanii*. J. Biol. Chem. 243, 6196-6201 (1968).

Quesda-Chanto, A., Afschar, A.S., Wagner, F.: Microbial production of propionic acid and vitamin B_{12} using molasses or sugar. Appl. Microbiol. Biotechnol. 41, 378-383 (1994).

Ramos, J.M., Esteban, J., Soriano, F.: Isolation of *Propionibacterium acnes* from central nervous system infections. Anaerobe 1, 17-20 (1995).

Rehberger, T.G.: Genome analysis of *Propionibacterium freudenreichii* by pulsed-field gel electrophoresis. Curr. Microbiol. 27, 21-25 (1993).

Rehberger, T.G., Glatz, B.A.: Characterisation of *Propionibacterium* plasmids. Appl. Environ. Microbiol. 56, 864-871 (1990).

Reinbold, G.W.: The propionibacteria. XXX Int. Dairy Congr. Paris. 3 ST, pp. 41-45. Brussels, International Dairy Federation 1978.

Riedel, K-H.J., Britz, T.J.: Differentiation of "classical" *Propionibacterium* species by numerical analysis of electrophoretic protein profiles. System. Appl. Microbiol. 15, 567-572 (1992).

Riedel, K-H.J., Britz, T.J.: *Propionibacterium* species diversity in anaerobic digesters. Biodiversity and Conservation 2, 400-411 (1993).

Riedel, K-H.J., Wingfield, B.D., Britz, T.J.: Justification of the "classical" *Propionibacterium* species concept by restriction analysis of the 16S ribosomal RNA genes. System. Appl. Microbiol. 17, 536-542 (1994).

Riedel, K-H.J., Wingfield, B.D., Britz, T.J.: rDNA – Restriction fragment length polymorphisms. System. Appl. Microbiol. 21, 419-428 (1998).

Riedel, K-H.J., Britz, T.J.: Justification of the "classical" *Propionibacterium* species concept by ribotyping. System. Appl. Microbiol. 19, 370-380 (1996).

Rippel.: Vorlesungen über theoretische Mikrobiologie. Berlin, Springer 1927. (As cited by Janoschek, 1944).

Rodenkirchen, J.: Die propionsaurebakterien und ihr vorkommen in Tilsiter kase. Milchw. Forschung. 19, 197-202 (1938). (As cited by Janoschek, 1944).

Roszkowski, W., Roszkowski, K., Ko, H.L., Beuth, J., Jeljaszewicz, J.: Immunomodulation by propionibacteria. Zbl. Bakt. 274, 289-298 (1990).

Russel, R.J., Mclnroy, R.J., Wilkinson, P.C., White, R.G.: A lipid chemotactic factor from anaerobic coryneform bacteria including *Corynebacterium parvum* with activity for macrophages and monocytes. Immun. 30, 935-949 (1976).

Sakaguchi, K., Iwasaki, M., Yamada, S.: A study on propionic acid fermentation. J. Agric. Chem. Soc. Jap. 17, 127 (1941).

Samain, P.L.: Métobolisme des composés intermédiaires de la fermentation méthanique. Thése de Doctorat, Université des Sciences et Techniques de Lille, France (1983). (As cited by Beerens et al., 1986).

Sarada, R., Joseph, R.: Characterisation and enumeration of microorganisms associated with anaerobic digestion of tomato-processing waste. Bioresource Technol. 49, 261-265 (1994).

Schlegel, H.G.: General Microbiology, 6th ed., Cambridge, Great Britain, University Press 1992.

Seyfried, P.L.: An approach to the classification of Lactobacilli using computer-aided numerical analysis. Can. J. Microbiol. 14, 313-318 (1968).

Sherman, J.M.: The cause of eyes and characteristic flavour in Emmental or Swiss cheese. J. Bacteriol. 6, 379-392 (1921).

SIGF.: Statistiques pâtes pressées cuites. SIGF, Paris, France 1994. (As cited by Thierry and Madec, 1995).

Skerman, V.B.D., McGowan, V., Sneath, P.H.A.: Approved List of Bacterial Names. Int. J. Syst. Bacteriol. 30, 346-347 (1980).

Society of American Bacteriologists.: Preliminary report on characterisation and classification of bacterial types. J. Bacteriol. 2, 505 (1917).

Sollberger, H.: Einflussfaktoren auf das Verhalten von Propionsäurebakterien in Käse: Situationsanalyse zu Forschungsschwerpunkt 1996 der Sektion Käsetechnolgie. FAM Internal Report 11, 7-17 1996. (As cited by Fessler, 1997).

Sutcliffe, I.C., Shaw, N.: The phospholipids of *Propionibacterium freudenreichii*: absence of phosphatidylinositol mannosides. System. Appl. Microbiol. 16, 9-12 (1993).

Tasman, A. and Brandwijk, A.C.: Experiments on the metabolism of the diphtheria bacillus. J. Infecr. Dis. 63, 10-20 (1938).

Thierry, A., Madec, M.N.: Enumeration of propionibacteria in raw milk using a new selective medium. Lait 75, 315-323 (1995).

Thompson, T.L., Marth, E.H.: Changes in Parmesan cheese during ripening: microflora - coliforms, enterococci, anaerobes, propionibacteria and staphylococci. Milchwiss. 41, 201-205 (1985). (As cited by Fessler, 1997).

Thöni, J., Alleman, O.: Landw. Jahrb. d. Schweiz. 22, 45 (1908). (As cited by Werkman and Brown, 1933).

Tobiassen, R.O., Pripp, A.H., Stepaniak, L., Sorhaug, T.: Purification and characterisation of an endopeptidase from *Propionibacterium freudenreichii*. J. Dairy Sci. 79, 2129-2136 (1996).

Troili-Petersson, G.: Studien über in käse gefundere glyzerin-vergarende und lactavergarende bakterien. Zentralbl. Bakteriol. Parasitenk. Infekt. Hyg. Abt. II 24, 333-342 (1909). (As cited by Van Niel, 1928).

Tuikov, R., Kristova, M., Rizvanov, K., Vladimirov, I.: Early weaning calves using a milk additive and a preparation of freeze-dried bacteria. Dairy Sci. Abstr. 44, 1689 (1980). (As cited by Fessler, 1997).

Unna, P.G.: Histopathology of the diseases of the skin. Brit. Med. J. 1, 1227 (1916). (As cited by Douglas and Gunter, 1946).

Van Niel, C.B.: The propionic acid bacteria. Thesis. Haarlem, Delft, J.W. Boissevain & Co 1928.

Vaughn, R.H.: Lactic acid fermentation of cabbage, cucumbers, olives and other products, pp. 220-224. In: Prescott and Dunn's Industrial Microbiology (G. Reed, ed.) Connecticut Saybrook Press 1981.

Virtanen, A.I.: Über die Propionsäuregärung. Soc. Sci. Fennica, Comment. Physic-Math. 1, 1-23 (1923). (As cited by Hettinga and Reinbold, 1972b).

Vladimirov, I., Rizvanov, K., Vasilev, A., Kenov, P.: Tests on replacement of antibiotics in sucking calf feeding by a freeze-dried bacteria. Dairy Sci. Abstr. 40, 2118 (1977). (As cited by Fessler, 1997).

Von Freudenreich, E., Orla-Jensen, S.: Über die in Emmentalerkäse stattfindende Propionisäuregarung. Zentralbl. Bakteriol. Parasitenk. Infekt. Hyg. Abt. II 17, 529-546 (1906). (As cited by Van Niel, 1928).

Vorobjeva, L.I., Turova, T.P., Kraeva, N.I., Alekseeva, M.A.: Propionic acid cocci and their systematic position. Microbiol. 52, 368-373 (1983).

Vorobjeva, L.I., Cherdinceva, T.A., Abilev, S.K., Vorobjeva, N.V.: Antimutagenicity of propionic acid bacteria. Mutat. Res. 251, 233-239 (1991).

Vorobjeva, L.I., Khodjaev, E.,Y.U., Cherdinceva, T.A.: The study of induced antimutagenesis of propionic acid bacteria. J. Microbiol. Methods 24, 249-258 (1996).

Webster, G.F., Cummins, C.S.: Use of bacteriophage typing to distinguish *Propionibacterium acnes* types I and II. J. Clinical Microbiol. 7, 84-90 (1978).

Werkman C.H., Brown, S.E.: The propionic acid bacteria, II. Classification. J. Bacteriol. 26, 393-417 (1933).

Werkman, C.H., Kendall, R.W.: Propionic acid bacteria I. Iowa State College J. Sci. 6, 17-32 (1931).

Woolford, M.K.: The significance of *Propionibacterium* species and *Micrococcus lactolyticus* to the ensiling process. J. Appl. Bacteriol. 39, 301-306 (1975).

Yokota, A., Tamura, T., Takeuchi, M., Weiss, N., Stackebrandt, E.: Transfer of *Propionibacterium innocuum* Pitcher and Collins 1991 to *Propioniferax* gen.

nov. as *Propioniferax innocua* comb. nov. Int. J. Syst. Bacteriol. 44, 579-582 (1994).

Zierdt, C.H., Webster, C., Rude, W.S.: Study of the corynebacteria. Int. J. Syst. Bacteriol. 1, 33-47 (1968).

Chapter 3

A numerical taxonomic study of "*Propionibacterium coccoides*", other classical *Propionibacterium* species and *Luteococcus japonicus*

Summary

Despite the fact that "*Propionibacterium coccoides*" was originally proposed as a new species within the genus *Propionibacterium* in 1983, the exact systematic position of this bacterium still remains unresolved. The systematic position of "*P. coccoides*" relative to that of the classical *Propionibacterium* species and *Luteococcus japonicus* was subsequently evaluated using numerical taxonomic techniques. Four type, 13 reference and 15 classical *Propionibacterium* strains as well as two "*P. coccoides*" strains were included in this study. The type strain of *Luteococcus japonicus*, which is a phylogenetic neighbour of the genus *Propionibacterium*, was also included as an outgroup. A data set of 75 phenotypic characters, obtained using standardised API systems and conventional methods was analysed. To delineate the clusters, use was made of the Sokal and Michener (S_{SM}) coefficient and the average linkage clustering algorithm (UPGMA). All the classical *Propionibacterium* strains were observed to group into four major clusters, with a final linkage at the 81% S-level. These clusters could be equated with the *P. acidipropionici*, *P. freudenreichii*, *P. jensenii* and *P. thoenii* species using the current classification system. The two "*P. coccoides*" strains, however, grouped in a separate cluster with the *Luteococcus japonicus* type strain. The *L. japonicus* type strain and the two "*P. coccoides*" strains clustered together at the 80% S-level. This cluster was clearly delineated from the various classical *Propionibacterium* species and linked with these clusters at a 72% similarity level. Based on these results it is evident that a higher phenotypic similarity exists between the *L. japonicus* and the "*P. coccoides*" strains than between the latter and the various classical *Propionibacterium* species.

Introduction

The genus *Propionibacterium* consists of two principal groups, the classical or dairy and the cutaneous or clinical strains. During the last 20 years, the taxonomy of the genus *Propionibacterium* has undergone extensive changes. Twenty three classical forms of the genus *Propionibacterium* were isolated and described during the period 1906 to 1944 (*Britz* and *Steyn*, 1980). Of these only 11 were regarded as species by *Breed* et al. (1957). In 1974, *Moore* and *Holdeman* consolidated these 11 species into four classical species, namely: *Propionibacterium acidipropionici*; *P. freudenreichii*; *P. jensenii*; and *P. thoenii*. Recently *Kusano* et al. (1997) described a new classical species namely *P. cyclohexanicum*. Although isolated from spoiled orange juice, results obtained from a phylogenetic analysis of the 16S rRNA genes indicated that its highest level of 16S rRNA similarity was with a representative of the classical propionibacteria, namely *P. freudenreichii*.

Based primarily on the research of *Johnson* and *Cummins* (1972), *Moore* and *Holdeman* (1974) transferred four species from the genus *Corynebacterium* to the genus *Propionibacterium*. These species subsequently constituted the cutaneous members of the genus *Propionibacterium*. These species included: *Propionibacterium acnes*; *P. avidum*; *P. granulosum*; and *P. lymphophilum*. In 1988 *Charfreitag* et al. proposed that *Arachnia propionica* be reclassified as *Propionibacterium propionicum*, confirming the research of *O'Donnell* et al. (1985).

In 1983, *Vorobjeva* et al., described a bacterium which plays a significant role in the aging process of Soviet hard cheeses. Due to the high degree of similarity observed between this bacterium and members of the classical *Propionibacterium*, *Vorobjeva* et al. (1983) proposed that this bacterium be named "*Propionibacterium coccoides*". When compared to the other propionic acid bacteria, this bacterium also produced propionic acid as the major metabolic product of lactate fermentation. Furthermore "*P. coccoides*" also contained the *anteiso* isomer of the C_{15}-saturated fatty acid (12-methyltetradecanoic) as the major type of cellular lipids, which is characteristic of members of the genus *Propionibacterium*. The cellular fatty acid composition of "*P. coccoides*" was also observed to be very similar to that of *P. jensenii* (the old "*P. technicum*" strain). "*Propionibacterium coccoides*" was also

observed to produce considerable quantities of vitamin B_{12}, catalase, superoxide dismutase and peroxide, which are characteristic of the propionic acid bacteria. In addition, the high mol% G + C content of the "*P. coccoides*" strains were similar to that of members of the genus *Propionibacterium*. Furthermore, the highest DNA:DNA homology (49%) was observed between "*P. coccoides*" and *P. jensenii* (*Vorobjeva* et al., 1983).

In spite of this very thorough investigation and the detailed report concerning the systematic position of "*P. coccoides*" (*Vorobjeva* et al., 1983), no further comparative evaluations between "*P. coccoides*" and the classical propionibacteria have ever been undertaken. Although numerous taxonomic studies have attempted to solve the taxonomic dilemma within the classical propionibacteria, no studies have included "*P. coccoides*". Subsequently the correct systematic grouping of this organism still remains unresolved. Furthermore, although this proposal was mentioned by *Cummins* and *Johnson* (1992), no mention of this has ever been made in either the Bergey's Manual of Systematic Bacteriology (*Cummins* and *Johnson*, 1986) or Bergey's Manual of Determinative Bacteriology (*Holt* et al., 1994).

The aim of this study was thus to further characterise "*P. coccoides*" phenotypically and to determine it's systematic position relative to that of the classical *Propionibacterium* species and the phylogenetically related *Luteococcus japonicus*.

Materials and Methods

Bacterial strains, growth conditions and preservation. The various bacterial strains used during this study are presented in Table 1. The 32 *Propionibacterium* strains used in this study comprised of the four type and 13 reference strains from the ATCC (Anon. 1985), NCFB (Anon. 1986) and DSM as well as strains from the dairy industry and anaerobic digesters. The two "*P. coccoides*" strains were kindly supplied by Prof. L.I. Vorobjeva of the Moscow State University, Russia. The type strain of *Luteococcus japonicus* (ATCC(R) 51527) was included as an outgroup during the numerical analysis.

Table 1. List of bacterial strains used in this study. (The names are listed as received).

No.	Lab. No.	Name	Source	Strain	Isolated from	Identification key#
1	78	*"P. arabinosum"*	5	P 50	-	*P. acidipropionici*
2	93	*P. acidipropionici*	7	-	Gouda	*P. acidipropionici*
3	120	*P. acidipropionici*	7	-	Gruyère	*P. acidipropionici*
4	263	*P. acidipropionici*	7	-	Anaerobic digester	*P. acidipropionici*
5	349	*P. acidipropionici*	7	-	Leerdammer	*P. acidipropionici*
6	421	*"P. pentosaceum"*	4	NCFB 570	Emmentaler	*P. acidipropionici*
7	424	*P. acidipropionici*	1	ATCC 25562*	-	*P. acidipropionici*
8	456	*"P. arabinosum"*	5	P 78	-	*P. acidipropionici*
9	73	*P. freudenreichii*	5	P 57	-	*P. freud.* ss. *freudenreichii*
10	131	*P. freudenreichii*	8	-	Emmentaler	*P. freud.* ss. *globosum*
11	308	*P. freudenreichii*	7	-	Anaerobic digester	*P. freud.* ss. *shermanii*
12	348	*P. freudenreichii*	7	-	Leerdammer	*P. freud.* ss. *freudenreichii*
13	423	*P. freudenreichii*	1	ATCC 6207*	Swiss cheese	*P. freud.* ss. *freudenreichii*
14	435	*P. freudenreichii*	6	B 3523	-	*P. freud.* ss. *freudenreichii*
15	453	*"P. shermanii"*	5	P 67	-	*P. freud.* ss. *shermanii*
16	454	*P. freudenreichii*	5	P 73	-	*P. freud.* ss. *freudenreichii*
17	75	*P. jensenii*	7	75	Emmentaler	*P. jensenii*
18	80	*P. jensenii*	2	DSM 20535*	Buttermilk	*P. jensenii*
19	89	*P. jensenii*	7	-	Gouda	*P. jensenii*
20	122	*P. jensenii*	7	-	Emmentaler	*P. jensenii*
21	357	*P. jensenii*	7	-	Leerdammer	*P. jensenii*
22	425	*"P. sanguineum"*	4	NCFB 1080	-	*P. jensenii*
23	427	*"P. pituitosum"*	4	NCFB 1077	-	*P. jensenii*
24	431	*"P. peterssonii"*	4	NCFB 565	Emmentaler	*P. jensenii*
25	97	*P. thoenii*	7	-	Emmentaler	*P. thoenii*
26	260	*P. thoenii*	7	-	Anaerobic digester	*P. thoenii*
27	294	*P. thoenii*	7	-	Anaerobic digester	*P. thoenii*
28	297	*P. thoenii*	7	-	Anaerobic digester	*P. thoenii*
29	307	*P. thoenii*	7	-	Anaerobic digester	*P. thoenii*
30	419	*P. thoenii*	4	NCFB 568*	Emmentaler	*P. thoenii*
31	426	*"P. wentii"*	4	NCFB 1083	Anaerobic digester	*P. thoenii*
32	447	*P. thoenii*	5	P 15	-	*P. thoenii*
33	362	*"P. coccoides"*	3	KM 252	Soviet cheese	-
34	363	*"P. coccoides"*	3	KM 375	Soviet cheese	-
35	364	*L. japonicus*	1	ATCC(R) 51527*	Ground and water	-

*: Type strain
#: Identification key of *Cummins* and *Johnson* (1986).

1: ATCC, American Type Culture Collection, Rockville, Maryland, USA.
2: DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.
3: KM, Katedra Mikrobiologii, Department of Microbiology, Moscow State University, Russia.
4: NCFB, National Collection of Food Bacteria, Shinfield, Reading, UK.
5: G.W. Reinbold, Iowa State University, Iowa, USA.
6: U.S. department of Agriculture, USA.
7: Environmental Microbiology Culture Collection, University of the Orange Free State, Bloemfontein, South Africa.

The bacterial strains were streaked onto yeast extract lactate (YEL) medium, and incubated anaerobically for four days at 30°C in a Forma Scientific (Mallinckrodt, Inc., Ohio, USA) anaerobic cabinet, using an oxygen-free nitrogen gas phase (10% H_2/10% CO_2/balance N_2) as recommended by *Holdeman* et al., (1977). The YEL-medium consisted of (g/l): yeast extract 6.0; sodium lactate (70% v/v) 20.0; peptone 2.0; KH_2PO_4 10.0; hemin 10.0 ml and Tween 80 1.0 ml (*Holdeman* et al., 1977). The pH was adjusted to 7.2 before sterilisation. Culture purity was regularly checked by means of microscopical examination of Gram-stained preparations and by gas chromatographical determination of the major metabolites produced. All the cultures were preserved according to the lyophilisation technique as described by *Joubert* and *Britz*, (1987). Lyophilised cultures were stored at -20°C. Lyophilized discs were used to inoculate YEL-broth to obtain working cultures.

API and conventional tests. Seventy four substrate utilisation and biochemical tests, as previously described (*Britz* and *Riedel*, 1991:1994; *Riedel* and *Britz*, 1993), were determined using the API 20E, API 20NE and API 50CH systems (API System S.A., La Balme le Grottes, 38390 Montalieu Vercieu, France) as well as numerous conventional tests. The conventional tests were undertaken to confirm phenotypic characteristics, which are important in the identification of propionibacteria using the identification key described by *Moore* and *Holdeman* (1974), *Cummins* and *Johnson* (1986) and *Holt* et al. (1994). Phenotypic characteristics of all the *Propionibacterium* strains, the two "*Propionibacterium coccoides*" strains as well as *Luteococcus japonicus* were determined in a Forma Scientific anaerobic cabinet, using oxygen-free nitrogen as gas phase (10% H_2/10% CO_2/balance N_2). Each test was done in duplicate and when different results were obtained the test was repeated. The majority result was taken as being representative.

To determine the optimum growth temperature, growth was determined at 25°C, 30°C, 35°C, 37°C and 40°C by comparison of the optical density every 4 h. Morphological characteristics of the various cultures were determined by bright-field microscopy of Gram-stained preparations. Motility was observed after 48 h in MRS-medium and colony pigmentation after 10 days on YEL plates. Extracellular polysaccharide production was determined using the Indian ink wet method after 10

days incubation in YEL-medium and the formation of endospores was observed using standard methods (*Gerhardt* et al., 1981).

Metabolic end-products were determined using a Hewlett Packard (Avondale, PA, USA) gas chromatograph equipped with a flame ionisation detector and a 30 m x 0.53 mm i.d. Nukol (Supelco, Inc., Avondale, PA, USA) capillary column. The chromatograph was programmed at an initial temperature of 120°C, then increased at a rate of 6°C/min and finally held at 165°C. The detector and inlet temperatures were 250°C and 135°C, respectively. Nitrogen, at a flow rate of 5 ml/min was used as the carrier gas.

A Varian 3300 gas chromatograph, equipped with a thermal conductivity detector and column (2.0 m x 3.4 mm i.d.) packed with Porapak Q (Waters Association, Inc., Milford, MA. USA), 80-100 mesh, was used for the determination of CO_2 in the gas phase. The oven temperature was set at 50°C and hydrogen, at a flow rate of 40 ml/min, was used as the carrier gas. A Varian 3300 gas chromatograph, equipped with a thermal conductivity detector and a stainless steel column (1.2 m x 3.4 mm i.d.) packed with Molsieve 5A (Supelco, Inc., Avondale, PA, USA) was used for the determination of H_2 in the gas phase. The oven temperature was set at 50°C and the detector temperature at 130°C. Nitrogen, at a flow rate of 30 ml/min, was used as carrier gas.

Numerical analyses. Phenotypic and biochemical tests which gave uniform results as well as duplicates of the different API systems, were excluded from the computational analyses. The remaining 75 phenotypic characters were included in a data set and analysed using the simple matching (S_{SM}) coefficient of *Sokal* and *Michener* (1958). The unsorted similarity matrix was rearranged into groups using the average linkage cluster algorithm (UPGMA) (*Lockhart* and *Liston*, 1970). Several strains, randomly chosen, were examined in duplicate to estimate the average probability of error (*P*) using the formula of *Sneath* and *Johnson* (1972).

Results

Strain characteristics. The phenotypic characteristics as determine for the 32 *Propionibacterium* strains, both "*P. coccoides*" strains and those for *Luteococcus japonicus* are presented in Table 2. All 35 bacterial strains examined during this study were Gram-positive, non-motile, non-sporing, anaerobic to aerotolerant cocci to pleomorphic rods which produced propionic acid as major metabolic end-product with lesser amounts of acetic acid and CO_2.

During this study, the phenotypic data obtained for the various *Propionibacterium* strains confirmed the data previously reported by *Britz* and *Riedel* (1994:1995) and *Riedel* and *Britz* (1993). Only two characteristics (lactose and nitrate reduction) were found to differ from those results as reported for "*P. coccoides*" (KM 252) by *Vorobjeva* et al. (1983). With the exception of lactose fermentation which was observed to be negative, the phenotypic data obtained during this study for "*P. coccoides*" (KM 375) was identical to that as reported by *Vorobjeva* et al. (1983). Gelatin liquefaction which was observed to be positive during this study, was found to be the only alteration when the phenotypic data as obtained for *Luteococcus japonicus* (ATCC(R) 51527) during this study was compared with that as reported by *Tamura* et al. (1994). All strains, including the two "*P. coccoides*" and *L. japonicus* strains were lipase positive, produced acid from galactose, D-fructose and were D-xylose and 5 keto-gluconate negative.

Clustering of strains. The average probability of error (*P*) (*Sneath* and *Johnson*, 1972) as calculated for several randomly chosen duplicate strains, was found to be 2.8%. No serious distortion of the taxonomic structure would thus be expected. A dendogram based on the overall similarity of the 32 *Propionibacterium*, the two "*P. coccoides*" strains and the *Luteococcus* type strain based on the S_{SM} similarity coefficient and the UPGMA clustering algorithm is presented in Fig. 1. All thirty-five strains were recovered in five major clusters, which linked at, or above the 72% similarity level.

The first major cluster (cluster A) consisted of the *P. freudenreichii* type, four reference and three *P. freudenreichii* strains with a overall similarity of 86%. Cluster B consisted of the *P. thoenii* type, two reference and five *P. thoenii* strains. The

Table 2: Comparison of the various phenotypic characteristics obtained for the 35 strains evaluated during this study.

Tests	**Organisms** (as listed in Table 1)																																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
Acid from:																																			
Glycerol	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Erythritol	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Arabinose	+	-	+	+	+	+	+	-	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
L-Arabinose	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Ribose	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+
D-Xylose	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
L-Xylose	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adonitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
β-Methyl xyloside	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	-	-	-	-	-	-	-	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Sorbose	-	+	-	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Rhamnose	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Inositol	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+
Mannitol	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+	+	+	-	-	+	+	+	+
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
α-Methyl-D-mannose	+	+	+	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	+	-	-	-
α-Methyl-D-glucoside	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	+
N-Acetyl-glucosamine	+	+	+	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Amygdalin	+	+	+	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-
Arbutin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
Esculin	+	+	+	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	+	+	+	+	+	-	-	-
Salicin	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+
Cellobiose	+	+	+	+	+	+	+	+	-	-	-	+	-	-	-	-	-	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	-	-	+
Maltose	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+
Lactose	+	-	-	+	-	+	-	-	-	+	+	-	-	-	-	-	+	+	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Melibiose	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-

Table 2. Continued

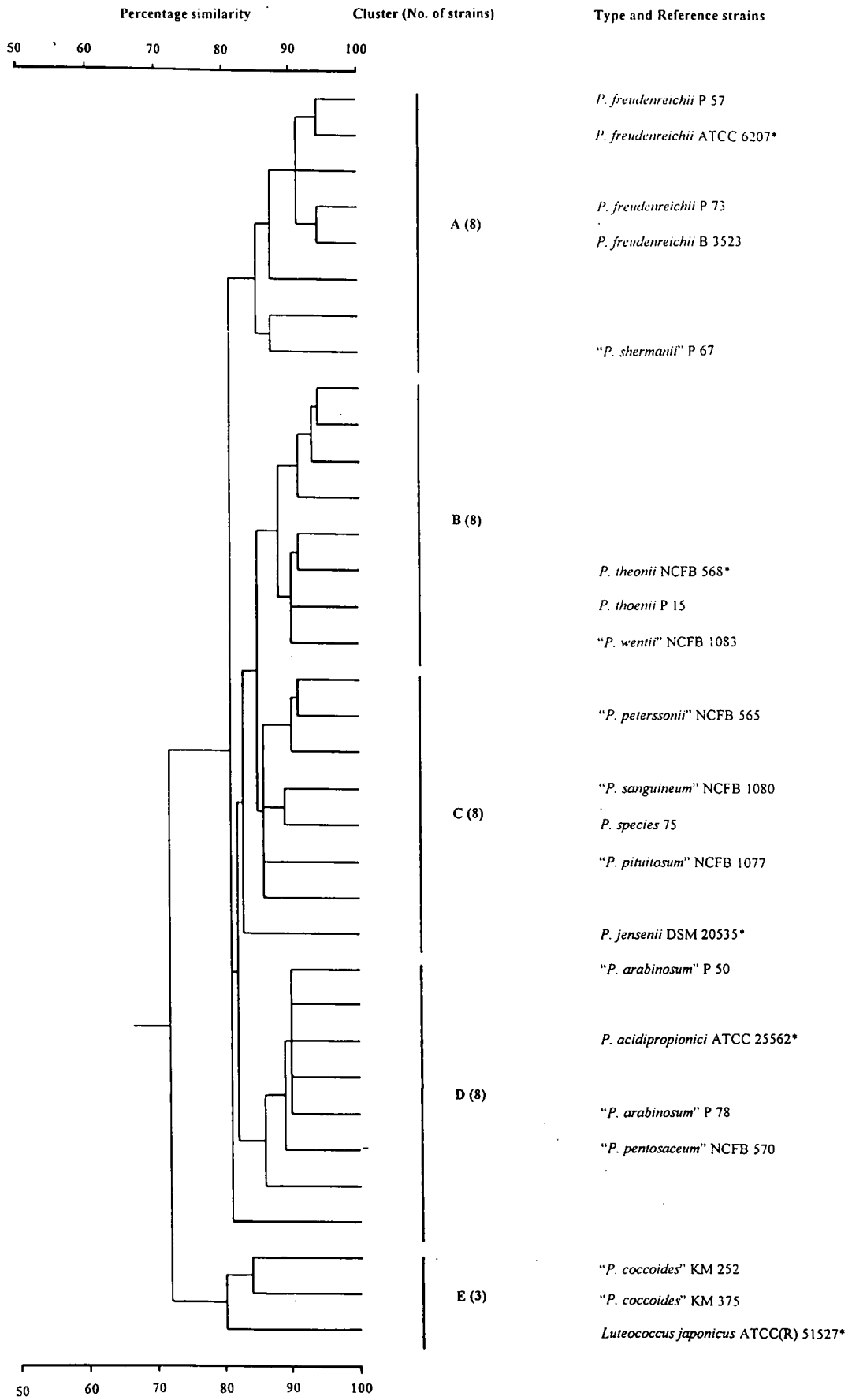
Trehalose	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inulin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
Melezitose	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+
D-Raffinose	+	-	-	+	-	+	-	-	-	-	-	+	-	-	-	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Amidon	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	+	-	+
Glycogene	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
Xylitol	+	+	+	-	+	-	+	+	-	+	-	+	-	-	-	-	-	-	+	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	+
β-Gentiobiose	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-
D-Turanose	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+
D-Lyxose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabitol	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Arabitol	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	+	+	+	+	-	+	+	+	+	-	-	+	+	+	+	+	-	+
Gluconate	-	+	-	+	-	-	+	+	+	-	+	-	-	+	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
2 keto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5 keto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ONPG	-	-	+	+	+	-	+	+	+	-	-	-	-	-	+	-	-	-	+	+	+	-	-	+	+	+	+	+	+	-	-	-	-	-	-
ADH	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
LDC	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
ODC	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
CIT	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
URE	-	+	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
TDA	+	+	+	-	-	+	-	+	-	+	-	-	+	+	+	+	+	-	+	+	-	+	+	+	+	-	-	-	-	+	+	+	-	-	-
IND	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VP	-	-	-	+	+	-	+	-	+	-	+	+	-	+	-	-	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	-	-	-
GEL	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Extracellular Polysacch:																																			
L-DAP	+	+	+	-	+	-	+	-	-	+	-	+	+	+	-	+	-	-	-	-	+	-	-	-	+	+	-	-	+	-	-	-	-	-	-
meso -DAP	+	+	+	N	N	+	+	+	-	-	N	N	-	-	-	-	+	+	+	+	N	+	+	+	+	N	N	N	N	+	+	+	N	N	N
Litmus milk:																																			
acid	-	-	-	N	N	-	-	-	+	+	N	N	+	+	+	+	-	-	-	-	N	-	-	-	-	N	N	N	N	-	-	-	N	N	N
reduction	-	-	-	+	+	+	-	-	+	+	-	-	-	-	+	-	-	-	+	+	+	-	+	-	-	-	-	-	-	-	+	+	-	-	-
stormy	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-

Table 2. Continued

fermentation	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
clot	-	-	-	+	+	+	-	-	+	+	-	-	-	-	+	-	-	-	+	+	+	-	+	-	-	-	-	-	-	-	+	+	-	-	-
Catalase	-	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Pigment:																																			
cream/white	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-
yellow/orange	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
brown/red	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	+	+	+	+	+	+	+	-	-	-
Lipase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Opt. temp.:																																			
30C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
37C	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Morphology:																																			
cocci	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	+	-	+	+	+
rod	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	+	-	-	-
pleomorphic	+	-	+	+	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	+	+	+	-	+	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-

+	Positive
-	Negative
N:	Not determined
ONPG:	Ortho-nitro-phenyl-galactoside
ADH:	Arginine dihydrolase
LDC:	Lysine dihydrolase
ODC:	Ornithine utilisation
CIT:	Citrate utilisation
URE:	Urease
TDA:	Tryrophane desaminase
IND:	Indole
VP:	Acetoin production
GEL:	Gelatin liquefaction
L-DAP:	L-diaminopimelic acid
Meso -DAP:	*Meso* -diaminopimelic acid

Fig. 1. Dendrogram, based on the S_{SM} coefficient and the average linkage clustering algorithm, showing the relationship between the classical *Propionibacterium*, the "*P. coccoides*" and the *L. japonicus* strains. * = Type strain. ATCC, American Type Culture Collection, Rockville, Maryland, USA; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; NCFB, National Collection of Food Bacteria, Shinfield, Reading, UK; KM, Katedra Mikrobiologii, Department of Microbiology, Moscow State University, Russia.


Percentage similarity
Cluster (No. of strains)
Type and Reference strains
50
60
70
80
90
100
A (8)
B (8)
C (8)
D (8)
E (3)
P. freudenreichii P 57
P. freudenreichii ATCC 6207*
P. freudenreichii P 73
P. freudenreichii B 3523
"P. shermanii" P 67
P. theonii NCFB 568*
P. thoenii P 15
"P. wentii" NCFB 1083
"P. peterssonii" NCFB 565
"P. sanguineum" NCFB 1080
P. species 75
"P. pituitosum" NCFB 1077
P. jensenii DSM 20535*
"P. arabinosum" P 50
P. acidipropionici ATCC 25562*
"P. arabinosum" P 78
"P. pentosaceum" NCFB 570
"P. coccoides" KM 252
"P. coccoides" KM 375
Luteococcus japonicus ATCC(R) 51527*

overall similarity of this cluster was 88%. The third cluster (Cluster C) contained the eight strains of *P. jensenii*, namely the type, three reference and four other *P. jensenii* strains. These strains clustered together at an overall similarity level of 83%. The last cluster representing the genus *Propionibacterium* (Cluster D) was comprised of the *P. acidipropionici* type, a single reference as well as six *P. acidipropionici* strains, with a overall similarity of 87%. The two "*P. coccoides*" strains clustered together (84%) with the *L. japonicus* type strain at an overall similarity level of 80% (Cluster E). This cluster could clearly be differentiated from the four clusters representing the various classical *Propionibacterium* species. Cluster E linked with the major clusters representing the various classical *Propionibacterium* species at a 72 % S-level.

Differential phenotypic characteristics of the five major clusters observed during this study are given in Table 3. Nine phenotypic characteristics which could be used to differentiate clusters A, B, C, D and E from each other were identified during this study (Table 4). Differentiation between cluster A and C, representing the *P. freudenreichii* and *P. jensenii* species, respectively was only possible on *P. jensenii*'s inability to ferment both dulcitol and inositol. It was also possible to differentiate both clusters A and C from clusters B, D and E on the basis of the fermentation of trehalose (cluster B), L-arabinose or melezitose (cluster D and E) and D-mannose, sorbitol or yellow / orange pigmentation (cluster E). Based on the results of this study, cluster B representing the *P. thoenii* species could be differentiated from all the other cluster due to the inability of the *P. thoenii* strains to ferment trehalose and D-arabitol. The ability of the "*P. coccoides*" and *L. japonicus* strains within cluster E to ferment D-mannose and sorbitol as well as their characteristic yellow pigmentation facilitated differentiation of this cluster from the other clusters representing the classical *Propionibacterium* species. Bacterial strains within both clusters D and E representing the *P. acidipropionici* and "*P. coccoides*" / *L. japonicus* species, respectively were observed to ferment L-arabinose as well as melezitose, which enabled differentiation of these clusters from the other major clusters.

Although the "*P. coccoides*" strains clustered with the *L. japonicus* strain in cluster E, the "*P. coccoides*" strains could still be differentiated phenotypically from the *L. japonicus* type strain. Differential characteristics included: acid production from ribose; adonitol; L-sorbose; inositol; α-methyl-D-glucoside; N-acetyl-glucosamin;

Table 3. Differential phenotypic characteristics of the various clusters as obtained during this study

Tests	Cluster (No. of strains)				
	A (8)	B (8)	C (8)	D (8)	E (3)
Acid from:					
Erythritol	+	d+	+	+	+
D-Arabinose	-	-	d-	d+	-
L-Arabinose	-	-	-	+	+
Ribose	+	-	+	+	d-
D-Xylose	-	-	-	d-	-
Adonitol	+	+	+	+	d-
β-Methyl xyloside	+	d+	+	+	-
D-Glucose	+	d-	+	+	+
D-Mannose	-	-	-	-	+
L-Sorbose	-	-	-	±	d-
Dulcitol	-	d+	+	+	-
Inositol	-	d-	+	+	d-
Mannitol	d+	-	-	+	+
Sorbitol	-	-	-	-	+
α-Methyl-D-mannose	d+	-	-	+	-
α-Methyl-D-glucoside	d-	-	-	-	d-
N-Acetyl-glucosamine	-	-	-	d+	d-
Amygdalin	d-	-	-	+	-
Arbutin	+	+	+	+	d-
Esculin	d+	-	-	+	-
Salicin	-	-	d-	+	d-
Cellobiose	+	-	±	+	d-
Maltose	d+	d-	d+	+	+
Lactose	-	d-	d+	d-	-
Melibiose	+	d-	+	-	d-
Trehalose	+	-	+	+	+
Inulin	-	-	-	-	d+
Melezitose	-	-	-	+	+
D-Raffinose	-	-	d-	d-	+
Amidon	d-	-	-	+	d+
Glycogene	-	-	-	±	d+
Xylitol	+	d-	d-	+	d-
β-Gentiobiose	d-	-	d-	d+	-
D-Turanose	+	-	d-	+	+
D-Arabitol	+	-	+	+	+
L-Arabitol	d+	d-	+	+	d+
Gluconate	-	d+	d-	±	-
ONPG	d+	d-	±	d+	-
ADH	-	±	-	-	d-
LDC	-	d+	-	-	d-
ODC	-	±	-	-	d-
CIT	-	-	d-	-	-

Table 3. Continued

URE	-	d-	-	-	d-
TDA	±	d+	d+	d+	-
VP	+	±	d+	d-	-
GEL	-	-	-	d-	-
Extracellular Polysacch:					
L-DAP	d-	d+	-	d+	-
meso-DAP	d+	d-	d+	d+	N
Litmus milk:					
acid	d-	d+	-	d-	N
reduction	d-	d-	±	d-	-
stormy	+	+	+	+	-
fermentation	-	-	-	d-	-
clot	d-	d-	±	d-	-
Catalase	+	+	+	d-	+
Pigment:					
cream/white	-	+	d+	+	-
yellow/orange	-	-	-	-	+
brown/red	+	-	d-	-	-
Morphology:					
cocci	d+	d-	-	-	+
rod	d+	d+	+	+	-
pleomorphic	-	d-	-	d+	-
Nitrate reduction	-	d+	-	-	d-

+: > 80% positive;
-: < 20% positive;
d-: 20-50% positive;
d+: 50-80% positive;
±: 50% positive and 50% negative reactions in strains respectively.
N: Not determined

ONPG: Ortho-nitro-phenyl-galactoside
ADH: Arginine dihydrolase
LDC: Lysine dihydrolase
ODC: Ornithine utilisation
CIT: Citrate utilisation
URE: Urease
TDA: Tryrophane desaminase
VP: Acetoin production
GEL: Gelatin liquefaction
L-DAP: L-diaminopimelic acid
meso-DAP: *meso*-diaminopimelic acid

Table 4. Differentiation of the major clusters based on nine differential phenotypic characteristics as obtained during this study

Tests	Cluster (No. of strains)				
	A (8)	B (8)	C (8)	D (8)	E (3)
Acid from:					
L-Arabinose	-	-	-	+	+
D-Mannose	-	-	-	-	+
Dulcitol	-	d+	+	+	-
Inositol	-	d-	+	+	d-
Sorbitol	-	-	-	-	+
Trehalose	+	-	+	+	+
Melezitose	-	-	-	+	+
D-Arabitol	+	-	+	+	+
Pigment:					
cream/white	-	+	d+	+	-
yellow/orange	-	-	-	-	+
brown/red	+	-	d-	-	-

+: > 80% positive;
-: < 20% positive;
d-: 20-50% positive;
d+: 50-80% positive;
±: 50% positive and 50% negative reactions in strains respectively.
N: Not determined

arbutin; salicin; cellobiose; melibiose; inulin; amidon; glycogene; xylitol; L-arabitol; arginine dihydrolase; lysine dihydrolase; ornithine utilisation; urease; and nitrate reduction (Table 5).

Discussion

Five major clusters, four representing the various classical *Propionibacterium* species included in this study, as well as a single cluster containing the *L. japonicus* type strain and the two "*P. coccoides*" strains could be delineated during this study.

All the *P. acidipropionici* strains included in this study and which grouped together in the major cluster D could be characterised and differentiated from the other major clusters in their ability to ferment L-arabinose or melezitose (cluster A, B and C), trehalose or D-arabitol (cluster B) as well as their inability to ferment D-mannose and sorbitol (cluster E). The *P. acidipropionici* strains were further characterised by their ability to hydrolyse esculin, their inability to hydrolyse gelatin as well as their white to creamish and orange yellow pigment. These results obtained during this study subsequently confirm the reports of *Cummins* and *Johnson* (1986), *Britz* and *Riedel* (1995) and *Holt* et al. (1994).

According to *Cummins* and *Johnson* (1986), nitrate reduction can be used as the major differential characteristic between the *P. jensenii* and *P. acidipropionici* species. All the *P. acidipropionici* strains evaluated during this study could not reduce nitrate, confirming the results of *Britz* and *Riedel* (1995). Subsequently, if the identification key of *Cummins* and *Johnson* (1986) was used, these strains would phenotypically have been incorrectly identified as *P. jensenii*.

Although numerous phenotypic characteristics were evaluated, it was, however, not possible to differentiate the *P. freudenreichii* strains which grouped together in cluster A from all the other major clusters, on the basis of one unique characteristic. Furthermore, all the *P. freudenreichii* strains included in this study could ferment glycerol and hydrolyse esculin, confirming the data of *Cummins* and *Johnson* (1986) and *Holt* et al. (1994), as reported in their identification keys. None of the *P. freudenreichii* strains could, however, utilise L-arabinose which is in accordance to the results obtained by *Britz* and *Riedel* (1995), but in contrast to the results as reported by *Holt* et al. (1994).

Table 5. Differential phenotypic characteristics of the two "*P. coccoides*" strains and the *L. japonicus* type strain as obtained during this study.

Tests	**Bacterial strain**		
	"*P. coccoides*" (KM 252)	"*P. coccoides*" (KM 375)	*L. japonicus* (ATCC(R) 51527)
Acid from:			
Ribose	-	-	+
Adonitol	-	-	+
L-Sorbose	-	-	+
Inositol	-	-	+
α-Methyl-D-glucoside	-	-	+
N-acetyl-glucosamine	-	-	+
Arbutin	-	-	+
Salicin	-	-	+
Cellobiose	-	-	+
Melibiose	+	-	-
Inulin	+	-	+
Amidon	+	-	+
Glycogene	+	-	+
Xylitol	-	-	+
L-Arabitol	+	-	+
ADH	+	-	-
LDC	+	-	-
ODC	+	-	-
URE	+	-	-
Nitrate reduction	-	+	-

\+ Positive

\- Negative

ADH: Arginine dihydrolase

LDC: Lysine dihydrolase

ODC: Ornithine utilisation

URE: Urease

According to *Cummins* and *Johnson* (1986) as well as *Holt* et al. (1994), the only differential characteristic between the *P. jensenii* (cluster C) and *P. thoenii* (cluster B) species is β-hemolysis and their pigmentation characteristics. Since β-hemolysis was not evaluated during this study, only the pigmentation of white to pink and orange to red-brown as reported for *P. jensenii* and *P. thoenii*, respectively, could be validated. Except for the hydrolysis of esculin which was observed to be negative for the *P. jensenii* strains and not positive as expected, all the other differential phenotypic characteristics as determined for both *P. jensenii* and *P. thoenii* were in accordance to that as described by *Holt* et al. (1994) as well as *Cummins* and *Johnson* (1986).

Based on the data obtained during this study it is possible to differentiate cluster E (representing the two "*P. coccoides*" strains and the *L. japonicus* type strain) from the *P. acidipropionici*, *P. freudenreichii*, *P. jensenii* and *P. thoenii* major clusters (clusters A, B, C and D) on their ability to ferment D-mannose and sorbitol, as well as their characteristic bright yellow pigmentation.

On the basis of the phenotypic characteristics evaluated during this study, the "*P. coccoides*" strains as described by *Vorobjeva* et al. (1983) could not phenotypically be identified as any of the classical *Propionibacterium* species as currently described in the genus *Propionibacterium* (*Cummins* and *Johnson*, 1986). Furthermore, the "*P. coccoides*" strains grouped in a separate cluster together with the *L. japonicus* type strain. This cluster was clearly delineated from the clusters representing the various classical *Propionibacterium* species.

Based on the results of this study, it is evident that the two "*P. coccoides*" strains could also be phenotypic differentiated from the *Luteococcus japonicus* type strain. Furthermore the two "*P. coccoides*" strains could also be phenotypic differentiated from each other. *L. japonicus* (ATCC(R) 51527), "*P. coccoides*" (KM 252) and "*P. coccoides*" (KM 375) could be differentiated on the basis of: ribose fermentation; the ability to ferment L-arabitol; inulin; glycogen; nitrate reduction; and melibiose fermentation, respectively. It is subsequently apparent that the proposal of *Vorobjeva* et al. (1983) to include "*P. coccoides*" as a new species within the genus *Propionibacterium* could possibly be incorrect. It is evident that a higher degree of phenotypic similarity exists between *Luteococcus japonicus* and the "*P. coccoides*"

strains, than between the latter and members of the genus *Propionibacterium*. In spite of the fact that "*P. coccoides*" was probably incorrectly classified by *Vorobjeva* et al. (1983), it should, however, be taken in consideration that the genus *Luteococcus* was only proposed in 1994 by *Tamura* et al. (1994). This is 11 years after the description of "*P. coccoides*" by *Vorobjeva* et al. (1983). Unfortunately *Vorobjeva* et al. (1983) did not have the advantage of the inclusion of the phylogenetic neighbour of the genus *Luteococcus* in their study. To confirm the relationships observed during this study, alternative techniques such as ribotyping, restriction fragment length polymorphisms, DNA:DNA hybridisation and sequencing of the 16S rRNA regions should, however, also be evaluated. Furthermore, additional "*P. coccoides*" strains should also be characterised phenotypically to confirm the results of this study.

References

Anon.: American Type Culture Collection Catalogue of Bacteria, Phages and rDNA Vectors, pp. 128-129. (R. Gherna, W. Nierman, P. Pienta, eds.) 16th ed., Rockville, ATCC 1985.

Anon.: National Collection of Food Bacteria Catalogue of Strains, p. 161. 3rd ed., Reading, University of Reading 1986.

Breed, R.S., Murray, E.G.D., Smith, N.R.: Genus 1. *Propionibacterium* Orla-Jensen 1909, pp. 569-577. In: Bergey's Manual of Determinative Bacteriology (R.S. Breed, E.G.D. Murray, N.R. Smith, eds.) 7th ed., Bailliére, London, Tindall and Cox 1957.

Britz , T.J. Riedel, K-H.J.: *Propionibacterium* species diversity in Leerdammer cheese. Int. J. Food. Microbiol. 22, 257-267 (1994).

Britz, T.J., Riedel, K-H.J.: Numerical evaluation of the species grouping among the "classical" propionibacteria. Lait 75, 309-314 (1995).

Britz, T.J., Riedel, K-H.J.: A numerical taxonomic study of *Propionibacterium* strains from dairy sources. J. Appl. Bacteriol. 71, 407-416 (1991).

Britz, T.J., Steyn, P.L.: Comparative studies on propionic acid bacteria. Phytophylactica 12, 89-103 (1980).

Charfreitag, O., Collins, M.D., Stackebrandt, E.: Reclassification of *Arachnia propionica* as *Propionibacterium propionicus* comb. nov. Int. J. Syst. Bacteriol. 38, 354-357 (1988).

Cummins, C.S., Johnson, J.L.: Genus *Propionibacterium* Orla-Jensen 1909, pp. 1346-1353. In: Bergey's Manual of Systematic Bacteriology (P.H.A. Sneath, N.S. Mair, M.E. Sharpe, J.G. Holt, eds.) vol. 2. Baltimore, Williams and Wilkins 1986.

Cummins , C.S., Johnson, J.L.: The Genus *Propionibacterium*, pp. 834-849. In: The Prokaryotes (A. Balows, H.G. Truper, M. Dworkin, W. Harder and K-H. Schleifer, eds.) vol. 1. New York, Springer-Verlag 1992.

Cummins, C.S., Moss, C.W.: Fatty acid composition of *Propionibacterium propionicum* (*Arachnia propionica*). Int. J. Syst. Bacteriol. 40, 307-308 (1990).

Gerhardt, P., Murray, R.G.E., Castilow, R.N., Nester, E.W., Wood, W.A., Krieg, N.R., Phillips, G.B.: Manual of Methods for General Bacteriology. American Society for Microbiology, Washington DC 1981.

Holdeman, L. V., Cato, E.P., Moore, W.E.C.: *Arachnia* and *Propionibacterium*, pp. 56-65. In: Anaerobe Laboratory Manual, 4th ed., Virginia Polytechnic Institute and State University, Blacksburg, Virginia, Southern Printing Co. 1977.

Holt, J.G., Krieg, N.R., Sneath, P.H., Staley, J.T., Williams, S.T.: Genus *Propionibacterium*, p. 580. In: Bergey's Manual of Determinative Bacteriology (J.G. Holt, N.R. Krieg, P.H. Sneath, J.T. Staley, S.T. Williams, eds.) 9th ed., Baltimore, Williams and Wilkins 1994.

Johnson, J.L., Cummins, C.S.: Cell wall composition and deoxyribonucleic acid similarities among the anaerobic coryneforms, classical propionibacteria, and strains of *Arachnia propionica*. J. Bacteriol. 109, 1047-1066 (1972).

Joubert, W.A., Britz, T.J.: A simple and inexpensive method for the long-term preservation of microbial cultures. J. Microbiol. Methods 7, 73-76 (1987).

Kusano, K., Yamada, H., Niwa, M., Yamasato, K.: *Propionibacterium cyclohexanicum* sp. nov., a new acid-tolerant ω-cyclohexyl fatty acid-containing *Propionibacterium* isolated from spoiled orange juice. Int. J. Syst. Bacteriol. 47, 825-831 (1997).

Lockhart, W.R., Liston, J.: In: Methods for numerical taxonomy. AmericAN Society for Microbiology, Washington DC 1970.

Moore, W.E.C., Holdeman, L.V.: Genus 1. *Propionibacterium* Orla-Jensen 1909, pp. 633-641. In: Bergey's Manual of Determinative Bacteriology (R.E Buchanan, N.E. Gibbons, eds.) 8th ed., Baltimore, Williams and Wilkins 1974.

O'Donnell, A.G., Minnikin, D.E., Goodfellow, M., Parlett, J.H., Schofield, G.M., Schaal, K.P.: Lipid and wall amino acid composition in the classification and identification of *Arachnia propionica*. Zentralbl. Bakteriol. Parasitenk. Infekt. Hyg. Abt. I 260, 300-310 (1985). (As cited by Cummins and Moss, 1990).

Riedel, K-H.J., Britz, T.J.: *Propionibacterium* species diversity in anaerobic digesters. Biodiversity and Conservation 2, 400-411 (1993).

Sokal, R.R., Michener, C.D.: A statistical method for evaluating systematic relationships. Kansas Univ. Sci. Bull. 38, 1409-1438 (1958).

Sneath, P.H.A., Johnson, R.: The influence on numerical taxonomic similarities of errors in microbiological tests. J. Gen. Microbiol. 72, 377-392 (1972).

Tamura, T., Takeuchi, M., Yokota, A.: *Luteococcus japonicus* gen. nov., sp. nov., a new gram-positive coccus with LL-diaminopimelic acid in the cell wall. Int. J. Syst. Bacteriol. 44, 348-356 (1994).

Vorobjeva, L.I., Turova, T.P., Kraeva, N.I., Alekseeva, M.A.: Propionic acid cocci and their systematic position. Microbiol. 52, 368-373 (1983).

Chapter 4

A molecular and phylogenetic analysis of the relationship between the classical propionibacteria, "*P. coccoides*" and *Luteococcus japonicus*

Summary

Despite the fact that "*P. coccoides*" was originally proposed as a new species within the genus *Propionibacterium* in 1983 it is, however, apparent that a higher degree of phenotypic similarity exists between *L. japonicus* and "*P. coccoides*" than between the latter and members of the genus *Propionibacterium* (Chapter 3). The phylogenetic relationship between "*P. coccoides*", various classical *Propionibacterium* species as well as *L. japonicus* were subsequently evaluated during this study using various molecular techniques. Certain regions of the 16S ribosomal RNA genes of the various bacterial strains were amplified using the polymerase chain reaction. Visual differentiation between the various classical *Propionibacterium* type strains and the two "*P. coccoides*" / *L. japonicus* strains was possible after restriction endonuclease digestion of the PCR products using the restriction endonucleases *Hae*III and *Alu*I. Differentiation between the *L. japonicus* strain and the two "*P. coccoides*" strains was, however, not possible despite the evaluation of various other restriction endonucleases. The two "*P. coccoides*" and *L. japonicus* strains could only be differentiated on the basis of their *Bst*EII rRNA gene restriction endonuclease patterns (ribotyping). Phylogenetic analysis of the 16S ribosomal RNA gene sequence data (PAUP) confirmed the high phenotypic similarity observed between the "*P. coccoides*" and *L. japonicus* strains. Both the "*P. coccoides*" strains and the *L. japonicus* type strain grouped together in a phylogenetically tight cluster which could clearly be differentiated from the various clusters representing the genus *Propionibacterium*. Furthermore, the DNA:DNA probe hybridisation technique resulted in a clear differentiation of the classical propionibacteria from the two "*P. coccoides*" and the *L. japonicus* strains which exhibited a high degree of DNA:DNA homology, confirming the incorrect placement of "*P. coccoides*" within the genus *Propionibacterium*.

Introduction

In 1983, *Vorobjeva* et al. described a certain coccus isolated from Soviet hard cheese with a high mol% G + C content. Since various characteristics were observe to resemble the description of the genus *Propionibacterium* as described by *Moore* and *Holdeman* (1974), *Vorobjeva* et al. (1983) proposed that this bacterium be included in the genus *Propionibacterium* as an independent species under the name "*Propionibacterium coccoides*". Despite the fact that this species constituted a valid description, "*P. coccoides*" was never included in any studies concerning the taxonomy of the genus *Propionibacterium* (*Britz* and *Riedel*, 1991:1994; *Riedel* and *Britz*, 1993; *Riedel* et al., 1992:1994). Furthermore the proposal of *Vorobjeva* et al. (1983) was never mentioned in either the Bergey's Manual of Systematic Bacteriology (*Cummins* and *Johnson*, 1986) or Bergey's Manual of Determinative Bacteriology (*Holt* et al., 1994), although it was mentioned by *Cummins* and *Johnson* in 1992. Subsequently the exact systematic position of this bacterium still remains unresolved.

Based on results obtained during the numerical analysis of phenotypic characteristics of the various classical propionibacteria, the two "*P coccoides*" strains and *L. japonicus*, which was included as an outgroup (Chapter 3), it became evident that a higher degree of phenotypic similarity existed between *L. japonicus* and the "*P. coccoides*" strains than between the latter and members of the genus *Propionibacterium*. It was subsequently evident that the proposal of *Vorobjeva* et al. (1983) to include "*P. coccoides*" as a new species within the genus *Propionibacterium* could possibly be incorrect.

In order to verify the results obtained during the numerical taxonomic study of the phenetic characteristics, alternative molecular techniques such as ribotyping, restriction fragment length polymorphisms, DNA:DNA hybridisation and 16S rRNA gene sequencing data would have to be evaluated. The aim of the present study was thus to characterise the various strains on a molecular level and to further determine the phylogenetic relationship between "*P. coccoides*", *Luteococcus japonicus* and the classical propionibacteria.

Materials and Methods

Bacterial strains and growth conditions. The various bacterial strains evaluated during the analysis of the restriction fragment length polymorphisms section, the ribotyping and the 16S rRNA sequence analysis section of this chapter are listed in Table 1. The bacterial strains analysed during the DNA:DNA probe hybridisation section of this chapter are listed in Table 2. These bacterial strains comprised of various type, reference and other *Propionibacterium* strains, two "*P. coccoides*" strains kindly supplied by Prof L.I. Vorobjeva of Moscow State University, Russia, as well as the type strain of *Luteococcus japonicus*. The bacterial strains were incubated anaerobically in a Forma Scientific (Mallinckrodt, Inc., Ohio, USA) anaerobic cabinet, using oxygen free nitrogen gas phase, as recommended by *Holdeman* et al. (1977). The strains was cultured at 30°C for 4 days in the medium of *Charfreitag* et al. (1988), with the addition of 4% (v/v) mineral salts solution (*Holdeman* et al., 1977). Culture purity was regularly checked by means of microscopical examination of Gram stains and by comparison of morphological characteristics (*Britz* and *Riedel*, 1991:1994; *Riedel* and *Britz*, 1993).

DNA isolation and purification. The various bacterial strains were cultured anaerobically for four days at 30°C in the medium of *Charfreitag et al.* (1988), with the addition of 4% (v/v) mineral salts solution (*Holdeman et al.*, 1977). Genomic DNA was isolated according to the method described by *Sambrook et al.* (1989). The methods used for agarose and polyacrylamide gel electrophoresis were as described by *Perbal* (1988). DNA purity was confirmed using the ratio between the spectrophotometric readings at 260 nm and 280 nm (OD 260/280).

Polymerase chain reaction. Primer design, reaction mixture composition and the optimum conditions for amplification of the small subunit ribosomal RNA region (16S rRNA) using primers 16sP1 (5'-GGGTGACCGGCCACA-3') and 16sP4 (5'-TCGGGTGTTACCGAC-3') were as described by *Riedel* et al. (1992:1994). The reactions were performed using an automated thermal cycler (Techne PHC 2, Techne Incorporated, Brunswick, New Jersey, USA). Polymerase chain reaction fragments

Table 1. List of bacterial strains used in this study. (The names are listed as received).

No.	Lab. No.	Name	Source	Strain	Isolated from	Identification key#
1	424	*P. acidipropionici*	1	ATCC 25562*	-	*P. acidipropionici*
2	423	*P. freudenreichii*	1	ATCC 6207*	Swiss cheese	*P. freud.* ss. *freudenreichii*
3	80	*P. jensenii*	2	DSM 20535*	Buttermilk	*P. jensenii*
4	419	*P. thoenii*	4	NCFB 568*	Emmentaler	*P. thoenii*
5	362	*"P. coccoides"*	3	KM 252	Soviet cheese	-
6	363	*"P. coccoides"*	3	KM 375	Soviet cheese	-
7	364	*L. japonicus*	1	ATCC(R) 51527*	Ground and water	-

*: Type strain
#: Identification key of *Cummins* and *Johnson* (1986).

1: ATCC, American Type Culture Collection, Rockville, Maryland, USA.
2: DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.
3: KM, Katedra Mikrobiologii, Department of Microbiology, Moscow State University, Russia.
4: NCFB, National Collection of Food Bacteria, Shinfield, Reading, UK.

Table 2. List of bacterial strains used during DNA:DNA Hybridisation. (The names are listed as received).

No.	Lab. No.	Name	Source	Strain	Isolated from	Identification key#
1	424	*P. acidipropionici*	1	ATCC 25562*	-	*P. acidipropionici*
2	421	"*P. pentosaceum*"	4	NCFB 570	Emmentaler	*P. acidipropionici*
3	423	*P. freudenreichii*	1	ATCC 6207*	Swiss cheese	*P. freud.* ss. *freudenreichii*
4	453	"*P. shermanii*"	5	P 67	-	*P. freud.* ss. *shermanii*
5	80	*P. jensenii*	2	DSM 20535*	Buttermilk	*P. jensenii*
6	130	"*P. intermedium*"	5	NCIB 8728	-	*P. jensenii*
7	427	"*P. pituitosum*"	4	NCFB 1077	-	*P. jensenii*
8	75	"*P. technicum*"	6	P 74	Emmentaler	*P. jensenii*
9	419	*P. thoenii*	4	NCFB 568*	Emmentaler	*P. thoenii*
10	294	*P. isolate*	7		Anaerobic digester	*P. thoenii*
11	297	*P. isolate*	7		Anaerobic digester	*P. theonii*
12	362	*"P. coccoides"*	3	KM 252	Soviet cheese	
13	363	*"P. coccoides"*	3	KM 375	Soviet cheese	
14	364	*L. japonicus*	1	ATCC(R) 51527	Ground and water	

*: Type strain
#: Identification key of *Cummins* and *Johnson* (1986).

1: ATCC, American Type Culture Collection, Rockville, Maryland, USA.
2: DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.
3: KM, Katedra Mikrobiologii, Department of Microbiology, Moscow State University, Russia.
4: NCFB, National Collection of Food Bacteria, Shinfield, Reading, UK.
5: NCIB, National Collection of Inustrial Bacteria, UK.
6: G.W. Reinbold, Iowa State University, Iowa, USA.
7: Environmental Bacteriology Culture Collection, University of the Orange Free State, South Africa.

were electrophoresed in a 0.8% agarose gel in TBE buffer (0.089 M Tris-borate, 0.089 M Boric acid, 0.002 M EDTA) at a constant voltage of 45 V for 3 h. The fragments were visualisated using Ethidium Bromide (0.5 µl/ml final concentration prepared from a 10 mg/ml stock solution).

Restriction fragment length polymorphisms. The resulting 16S rDNA PCR products were extracted from the PCR reaction mixture using chloroform, purified using Qiagen columns (Diagen, Düsseldorf), precipitated with iso-propanol and redissolved in SABAX water. The PCR products were subsequently digested using the restriction endonucleases *Hae*III and *Sma*I as described by *Riedel* et al. (1994:1998) and *Riedel* (1997). Other restriction endonucleases evaluated during this study included *Ava*II, *Bcl*I, *Bst*EII, *Cfo*I, *Dde*I, *Dra*I, *Hae*II, *Hpa*II, *Ksp*I, *Not*I, *Sac*I and *Ssp*I (Boehringer Mannheim, GmbH, Germany) according to the manufacturing instructions. Restriction fragments were electrophoresed on a 12% Sodium Dodecyl Sulfate Polyacrylamide gel (SDS-PAGE) (Merck) in TBE buffer (0.089 M Tris-borate, 0.089 M Boric acid, 0.002 M EDTA) at a constant voltage of 240 V per 2 gels (Hoefer PS 2500 DC power supply), at 10°C in a water cooled tank (Hoefer SE 600 vertical slab gel unit), for 5 h. DNA molecular markers V and VIII (0.25 µg) were used as reference standards (Boehringer Mannheim, GmbH, Germany). The gels were stained using the silver staining technique as described by *Perbal* (1988). Restriction endonuclease fragment sizes were determined by linear regression analysis, using the molecular weight markers as standards.

Ribotyping. Restriction endonuclease digestion of the genomic DNA of the four *Propionibacterium* type strains, the two "*P. coccoides*" strains and the *Luteococcus japonicus* type strain were performed using the restriction endonucleases *Hae*II, *Sma*I, *Ava*II, *Bcl*I, *Bst*EII, *Cfo*I, *Dde*I, *Dra*I, *Hae*III, *Hpa*II, *Ksp*I, *Not*I, *Sac*I, and *Ssp*I (Boehringer Mannheim, GmbH, Germany). The method used was the same as described by *Riedel* and *Britz*, (1996). The restriction fragments were separated by electrophoresis through a 0.85% molecular grade agarose gel (Promega, USA) at 50 V for 3,5 h in 1 x TBE buffer (0,089 M Tris-base, 0,089 M Boric acid, 0,002 M EDTA, pH 8,0). To determine the sizes of the DNA fragments, molecular weight standard phage Lambda DNA digested with *Eco*RI-*Hind*III was used as standard reference.

After electrophoresis, the DNA was transferred to a positively charged nylon membrane (Hybond N^+, Amersham International plc, Amersham Place, Buckinghamshire, UK), using the Southern blotting technique (*Southern*, 1975). The DNA was cross-linked to the nylon membrane by exposure to a UV transilluminator for 3 min. The membranes were then prehybridised for 5 h at 68°C in hybridisation solution containing 5 x SSC prepared from a 20 x SSC stock solution (0.3 M sodium citrate, 3 M NaCl), 0.1% N-laurylsarcosine, 0.02% sodium dodecyl sulfate (SDS) and 1% blocking reagent (Boehringer Mannheim, GmbH, Germany). Following prehybridisation the membranes were incubated overnight at 68°C in the presence of the hybridisation buffer containing the heat-denatured (100°C for 10 min) DIG-labelled probes (16sP1-16sP4 PCR fragment of *Propionibacterium freudenreichii* subsp. *freudenreichii* (ATCC 6207) and phage Lambda DNA). After hybridisation, the membranes were washed twice for 5 min at room temperature with 2 x SSC-0.1 % SDS and twice for 15 min at 68°C with 0.1 x SSC-0.1 % SDS. The presence of the DIG-labelled DNA probes were determined with an alkaline phosphatase-conjugated antibody as described by the manufacturer of the DIG detection system (Boehringer Mannheim, GmbH, Germany). A similarity matrix was constructed on the basis of the presence or absence of ribotyping bands at a given position over the size range from 1.0 - 8.8 kilobase pairs (kb). Similarity coefficients were calculated using the simple matching coefficient (S_{SM}) (*Sokal* and *Michener*, 1958). The unsorted similarity matrix was rearranged into clusters by single linkage cluster analysis (*Lockhart* and *Liston*, 1970)

Sequencing of the 16S rDNA. In order to reduce the possibility of nucleotide misincorporation errors during the amplification process, the PCR products of several independent amplifications were combined for each sequencing reaction. The PCR products were sequenced either directly using the fmol™ DNA sequencing system (Promega, Madison, USA), according to the manufacturers protocol or via autosequencing using an ABI PRISM™ 377 DNA sequencer. The autosequencing reactions were carried out using a ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Perkin Elmer, Warrington, U.K.) according to the protocol of the manufacturer. Synthetic

oligodeoxyribonucleotide primers 16sP1, 16sP3, 16sP4 (Riedel *et al.*, 1992:1994) as well as the primers: 16sP3R (5'-CCTCATCCCCACCTTCCTCC-3'); 16sP5 (5'-TCGCTTCTCAGCGTCAGGAA-3'); and 16sP5R (5'-TTCCTGACGCTGAGAAGCGA-3') (*Riedel*, 1997) enabled forward and reverse sequence reactions of the amplified PCR products. The primer sequences were selected from the conserved regions of the 16S rRNA genes (*Charfreitag* and *Stackebrandt*, 1989).

The sequences were aligned manually with the published sequences of representatives of the classical and cutaneous propionibacteria (*Charfreitag* and *Stackebrandt*, 1989), the complete 16S rRNA sequence of *P. acnes* (EMBL Accession No. M61903) as well as the partial 16S rRNA sequence data of *Propionibacterium cyclohexanicum* (*Kusano et al.*, 1997), *Propioniferax innocua* (EMBL Accession No. S93388), *Terrabacter tumescens* (EMBL Accessions No. X53215), *Nocardioides jensenii* (EMBL Accession No. X53214) (*Collins* et al., 1989), *Nocardioides luteus* (EMBL Accession No. X53212), *Nocardioides albus* (EMBL Accession No. X53211), *Luteococcus japonicus* (*Tamura* et al., 1994) and *Aeromicrobium erythreus* (EMBL Accession No. M37200). The aligned sequence data was analysed using PAUP version 3.1 (Phylogenetic Analysis Using Parsimony) (*Swofford*, 1993). With PAUP, the heuristic and random sequence addition options were utilized to determine the most parsimonious tree. Bootstrapping (1000 replicates) was performed on the sequence data to determine the confidence intervals of the tree branching points (*Felsenstein*, 1988).

DNA:DNA Probe Hybridisation. The DNA:DNA homology between 14 strains, including the four type strains of the four classical *Propionibacterium* species, seven reference and other *Propionibacterium* strains, two "*P. coccoides*" strains as well as the type strain of *L. japonicus* (Table 2) were evaluated during this study using the DNA:DNA probe hybridisation technique as described by *Viljoen* (1996). The DNA of each bacterial strain was quantified using a TKO 100 Fluorometer (Hoefer Scientific Instruments, San Francisco, California, USA) according to the manufacturers specifications. Lambda DNA (Boehringer Mannheim, South Africa) was used as concentration standard. Hoechst 33258 dye (Sigma-Aldrich, Dorset, UK) was used as fluor at 0.1 µg/ml in 0.1 NaCl, 10 mM Tris-HCl and 1.0 mM EDTA (pH 7.4).

Reference DNA (50 ng) was radiolabelled with ^{32}P using the Random Primed DNA Labeling Kit (Boehringer Mannheim, Randburg, South Africa) according to the manufacturers protocol. Unincorporated ^{32}P was removed by gel-filtration chromatography (*Maniatis* et al., 1989). Aliquots containing the radiolabelled DNA were pooled for hybridisation reactions.

The DNA (300 ng) from the various cultures was denatured using 1.5 M NaOH (final concentration). The denatured DNA was blotted onto a positive charged nylon membrane (Hybond N, Amersham International plc, Amersham Place, Buckinghamshire, UK). For each hybridisation reaction, DNA from the different cultures was placed at 1.5 cm intervals from each other. A set of dot blots was prepared in the same manner for each radiolabelled reference DNA used in a hybridisation reaction. The DNA was covalently linked to the membrane using UV-transillumination for three minutes. The individual membranes were pre-hybridised in hybridisation buffer (5 x SSC (0.075 M NaCitrat, 0.75 M NaCl), pH 7.0), 0.1% N-lauroylsarcosine (w/v), 0.02% SDS, and 1% blocking reagent (Boehringer Mannheim, Randburg, South Africa) for 1 hr at 85°C. After addition of the heat denatured (100°C for 10 min) radiolabelled probe, the membranes were hybridised for 16 hrs at 80°C. Following hybridisation, the membranes were washed twice for 5 min in excess 2 x SSC, 0.1% SDS and twice for 15 min in excess 0.1 SSC, 0.1% SDS at room temperature. The membrane blocks were allowed to dry on paper towels at room temperature.

The membrane blocks were cut into individual squares (1.5 cm^2), each square contained DNA from one culture and the reference radiolabelled DNA that had bound to this DNA. The amount of radiolabelled probe bound to the membrane was determined by Cerenkov counting (*Wilson* and *Goulding*, 1986) using a Beckman LS-6000 scintillation counter (Beckman, South Africa). Hybridised membrane blocks containing no DNA were used to determine the amount of background radiation.

The cpm (counts per minute) from individual hybridisations for each reference DNA were expressed as a percentage, using the reference DNA hybridised to itself as a measure of maximum hybridisation (100%). The degree of hybridisation of other strains simultaneously hybridised by the same reference DNA were then determined as a percentage of this value. The experiment was done in duplicate and repeated twice.

Results

DNA purity. All DNA used during this study was extensively purified and the 260/280 ratio gave values of approximately 1.8.

Polymerase chain reaction. The 16S rRNA genes from the genomic DNA of the four classical *Propionibacterium* type strains, the two "*P. coccoides*" strains and *Luteococcus japonicus* type strain were successfully amplified using PCR. Products of the predicted size (1110 bp), using primers 16sP1-16sP4 were obtained for all the species included in this study (data not shown).

Restriction fragment length polymorphisms. The results obtained from the restriction endonuclease analysis (*Hae*III and *Alu*I) of the PCR products (16sP1-16sP4) are shown in Fig. 1. Visual differentiation between the four classical *Propionibacterium* type strains was possible after restriction endonuclease digestion of the PCR products, using the restriction endonucleases *Hae*III and *Alu*I confirming the results of *Riedel* et al. (1994:1998). Although the two "*P. coccoides*" and the *L. japonicus* strains could be differentiated from the other classical propionibacteria species on the basis of their restriction fragment length polymorphisms it was not possible to differentiate them from each other (Fig. 2). Similar results were observed when the restriction endonucleases *Hae*II, *Sma*I, *Ava*II, *Bcl*I, *Bst*EII, *Cfo*I, *Dde*I, *Dra*I, *Hpa*II, *Ksp*I, *Not*I, *Sac*I, *Sma*I and *Ssp*I were also evaluated.

Differentiation between the *P. acidipropionici* strains and the "*P. coccoides*" / *L. japonicus* strains was possible since the restriction endonuclease pattern obtained with both the restriction endonucleases *Hae*III and *Alu*I differed. Fragment sizes displayed by the *P. acidipropionici* type strain were: *Alu*I - 520, 363 and 220; *Hae*III - 470, 215, 203, 95 and 85, whilst those for "*P. coccoides*" / *L. japonicus* were: *Alu*I - 363, 320, 281 and 220; *Hae*III - 404, 320 and 203 bp. The restriction pattern obtained for the *P. freudenreichii*, *P. jensenii*, *P. thoenii* and the "*P. coccoides*" / *L. japonicus* strains using the restriction endonuclease *Alu*I were identical and differentiation was not possible. Similar results for the various classical *Propionibacterium* species were reported by *Riedel* et al. (1994:1998). Four fragments were obtained with sizes determined to be 363, 320, 281 and 220 base pairs

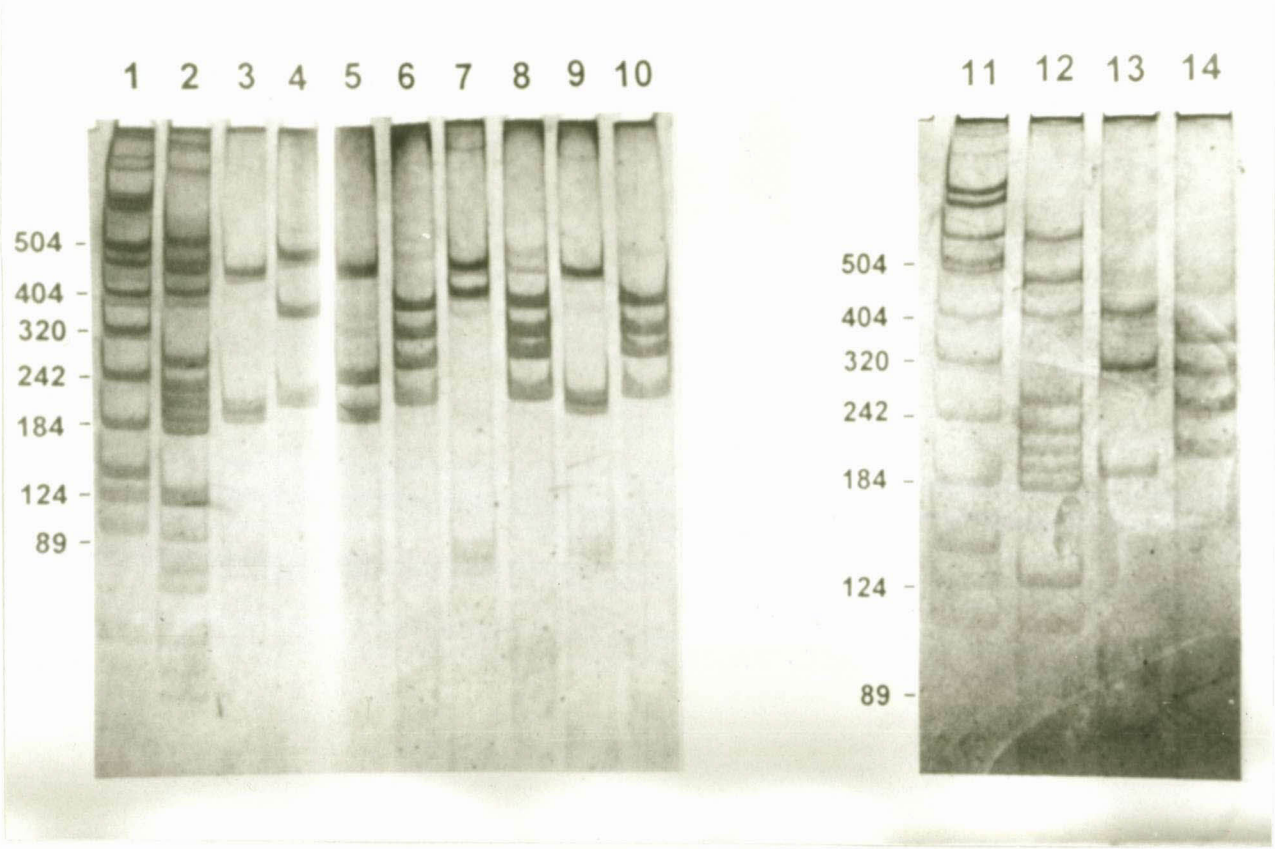


Fig. 1. Silver-stained 12% polyacrylamide gel displaying fragments obtained after restriction endonuclease digestion (*Hae*III and *Alu*I) of the amplified products (1110 bp) obtained using primers 16sP1-16sP4. Lanes: 1, DNA molecular weight marker VIII; 2, DNA molecular weight marker V; 3, *P. acidipropionici* ATCC 25562 - *Hae*III; 4, *P. acidipropionici* ATCC 25562 - *Alu*I; 5, *P. freudenreichii* subsp. *freudenreichii* ATCC 6207 - *Hae*III; 6, *P. freudenreichii* subsp. *freudenreichii* ATCC 6207 - *Alu*I; 7, *P. jensenii* DSM 20535 - *Hae*III; 8, *P. jensenii* DSM 20535 - *Alu*I; 9, *P. thoenii* NCFB 568 - *Hae*III; 10, P. thoenii NCFB 568 - *Alu*I; 11, DNA molecular weight marker VIII; 12, DNA molecular weight marker V; 13, "*P. coccoides*" KM 252 - *Hae*III; 14, "*P. coccoides*" KM 252 - *Alu*I.

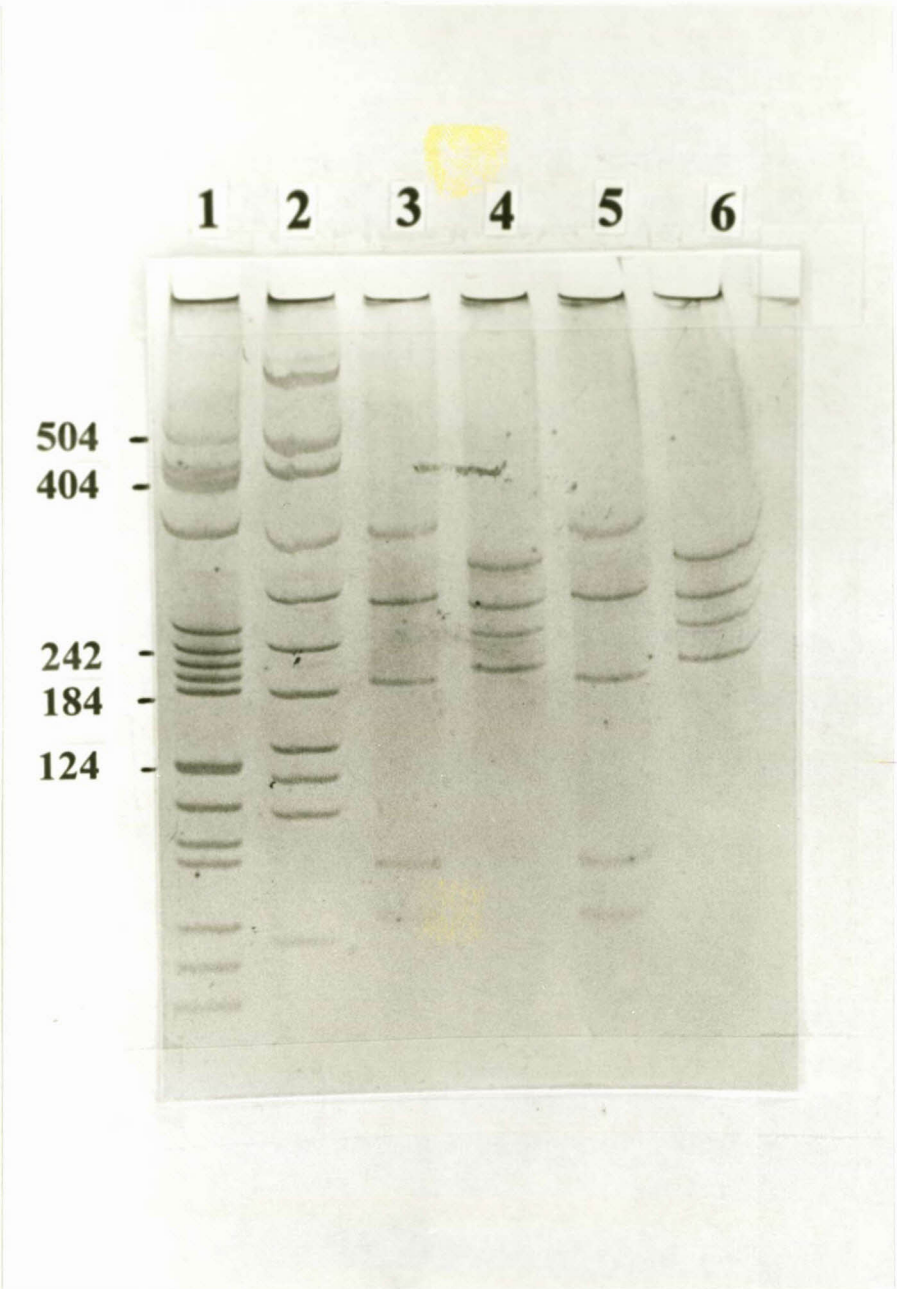


Fig. 2. Silver-stained 12% polyacrylamide gel displaying fragments obtained after restriction endonuclease digestion (*Hae*III and *Alu*I) of the amplified products (1110 bp) obtained using primers 16sP1-16sP4. Lanes: 1, DNA molecular weight marker V; 2, DNA molecular weight marker VIII; 3, "*P. coccoides*" KM 375 - *Hae*III; 4, "*P. coccoides*" KM 375 - *Alu*I; 5, *L. japonicus* ATCC(R) 51527* - HaeIII; 6, *L. japonicus* ATCC(R) 51527* - *Alu*I.

(bp). Differentiation between the *P. freudenreichii*, *P. jensenii*, and *P. thoenii* species as well as the "*P. coccoides*" / *L. japonicus* group was, however, possible based on the *Hae*III restriction endonuclease profiles. For the "*P. coccoides*" / *L. japonicus* profile, three fragments were obtained with sizes of 404, 320 and 203 bp. The fragment sizes observed for *P. freudenreichii* were 448, 255, 203, 85 and 76 bp, for *P. jensenii*, 470, 385, 95 and 85 bp and for *P. thoenii*, 428, 215, 203, 95 and 85 bp, confirming the data as reported by *Riedel* et al. (1994:1998) .

Ribotyping. Both "*P. coccoides*" strains and the *L. japonicus* type strain displayed an identical and unique ribotype profile (profile Q). The ribotype profile obtained using the restriction endonucleases *Hae*II and *Sma*I (Fig. 3) was found to differ significantly from the ribotyping data obtained for all four the classical *Propionibacterium* species (*Riedel* and *Britz*, 1996). The fragment size obtained using the restriction endonuclease *Hae*II (Fig. 3, lane 2) was 4782 (bp). Three different fragments were, however, obtained using the restriction endonuclease *Sma*I (Fig. 3, lane 3). These fragment sizes were: 4260; 3766; and 2227 bp. From the cluster analysis performed on the basis of the ribotype profiles observed for 135 *Propionibacterium* strains as reported by *Riedel* and *Britz* (1996) and the ribotype profile as observed for the two "*P. coccoides*" and *L. japonicus* strains (ribotype profile Q), a dendogram was produced (Fig. 4). Four major clusters, above the 70% similarity level could be delineated in the dendogram. Each of these major clusters represented a classical *Propionibacterium* species. A 82% similarity was observed between the ribotyping profiles obtained for the two "*P. coccoides*" and the *L. japonicus* strains (profile Q) with the various ribotyping profiles obtained for the *P. jensenii* species.

To confirm the differentiation of the two "*P. coccoides*" strains and that of the *L. japonicus* type strain as observed during the numerical analysis of phenotypic characteristics (Chapter 3), numerous other restriction endonucleases (*Ava*II, *Bcl*I, *Bst*EII, *Cfo*I, *Dde*I, *Dra*I, *Hae*III, *Hpa*II, *Ksp*I, *Not*I, *Sac*I and *Ssp*I) were also evaluated. The two "*Propionibacterium coccoides*" strains and the *L. japonicus* type strain could only be differentiated from each other when their genomic DNA was digested with restriction endonuclease *Bst*EII (Fig. 5). The fragment sizes obtained after digestion of the genomic DNA with *Bst*EII of: "*P. coccoides*" (KM 252) were

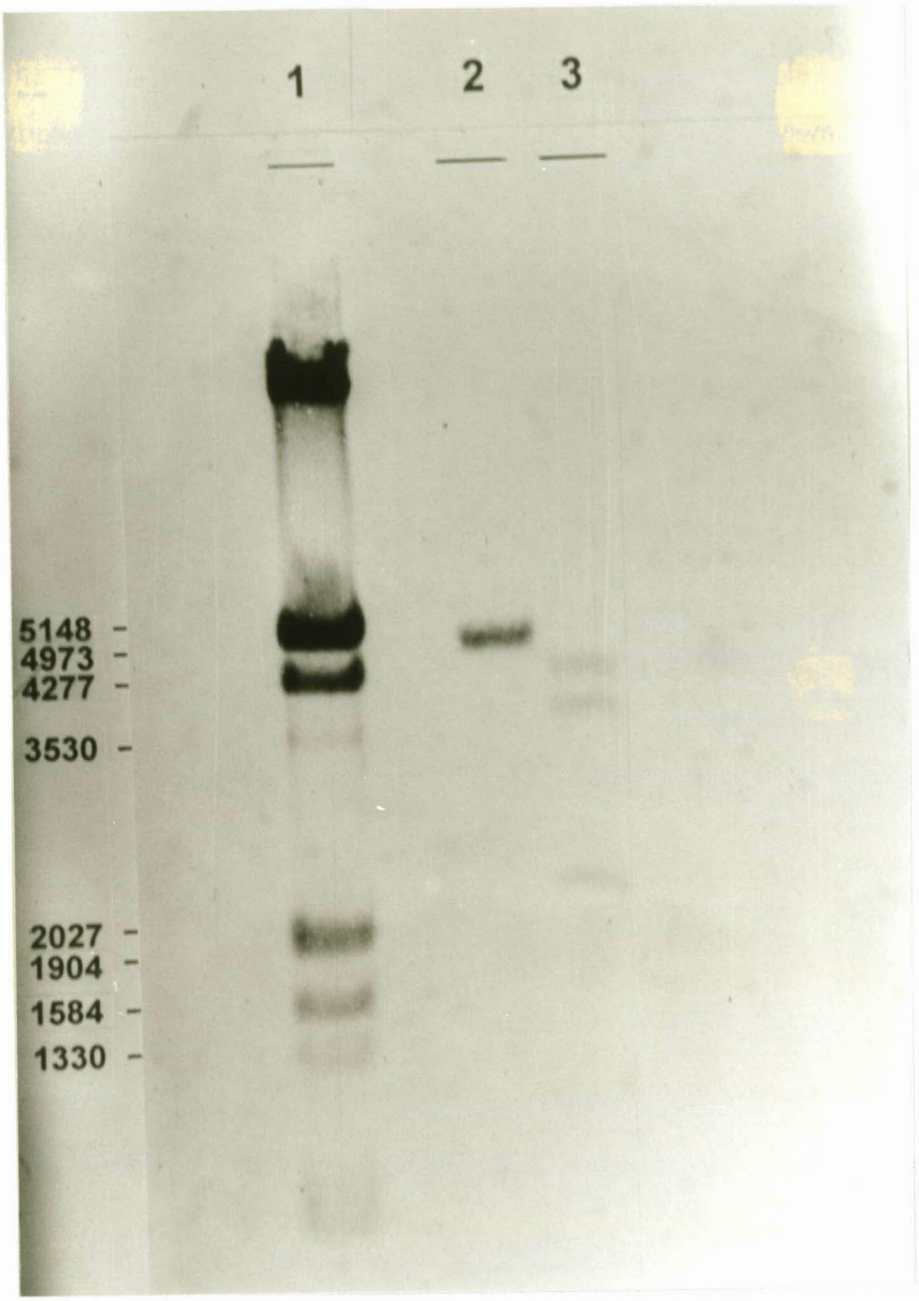
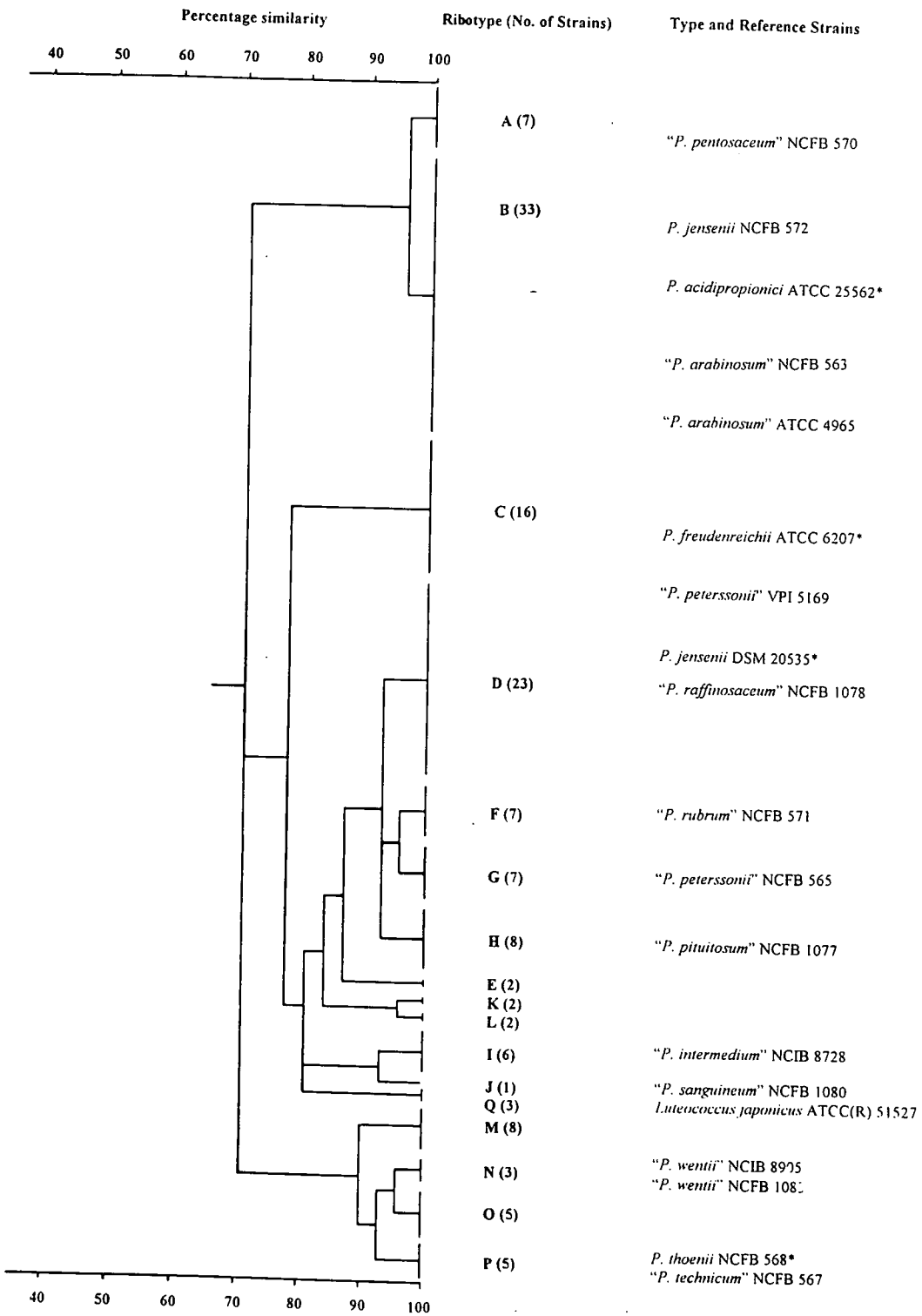


Fig. 3. Representative patterns of the ribotyping profile obtained for the two "*P. coccoides*" and the *L. japonicus* strains after digestion of the genomic DNA with the restriction endonucleases *Hae*II and *Sma*I, separation on an agarose gel and hybridisation with the DIG-labeled 16S rDNA probe of *P. freudenreichii* subspecies *freudenreichii* (ATCC 6207). Lanes: 1, Molecular mass standard (*Eco*RI/*Hind*III digested Phage Lambda DNA); 2, Ribotype Q - *Hae*II; 3, Ribotype Q - *Sma*I.

Fig. 4. Simplified dendrogram showing the grouping of the various ribotypes. Similarity coefficients were calculated using the simple matching (S_{SM}) coefficient. The unsorted similarity matrix was rearranged into groups by single linkage cluster analysis.


Percentage similarity
Ribotype (No. of Strains)
Type and Reference Strains
40
50
60
70
80
90
100
A (7)
"P. pentosaceum" NCFB 570
B (33)
P. jensenii NCFB 572
P. acidipropionici ATCC 25562*
"P. arabinosum" NCFB 563
"P. arabinosum" ATCC 4965
C (16)
P. freudenreichii ATCC 6207*
"P. peterssonii" VPI 5169
P. jensenii DSM 20535*
D (23)
"P. raffinosaceum" NCFB 1078
F (7)
"P. rubrum" NCFB 571
G (7)
"P. peterssonii" NCFB 565
H (8)
"P. pituitosum" NCFB 1077
E (2)
K (2)
L (2)
I (6)
"P. intermedium" NCIB 8728
J (1)
"P. sanguineum" NCFB 1080
Q (3)
Luteococcus japonicus ATCC(R) 51527
M (8)
N (3)
"P. wentii" NCIB 8905
"P. wentii" NCFB 1082
O (5)
P (5)
P. thoenii NCFB 568*
"P. technicum" NCFB 567

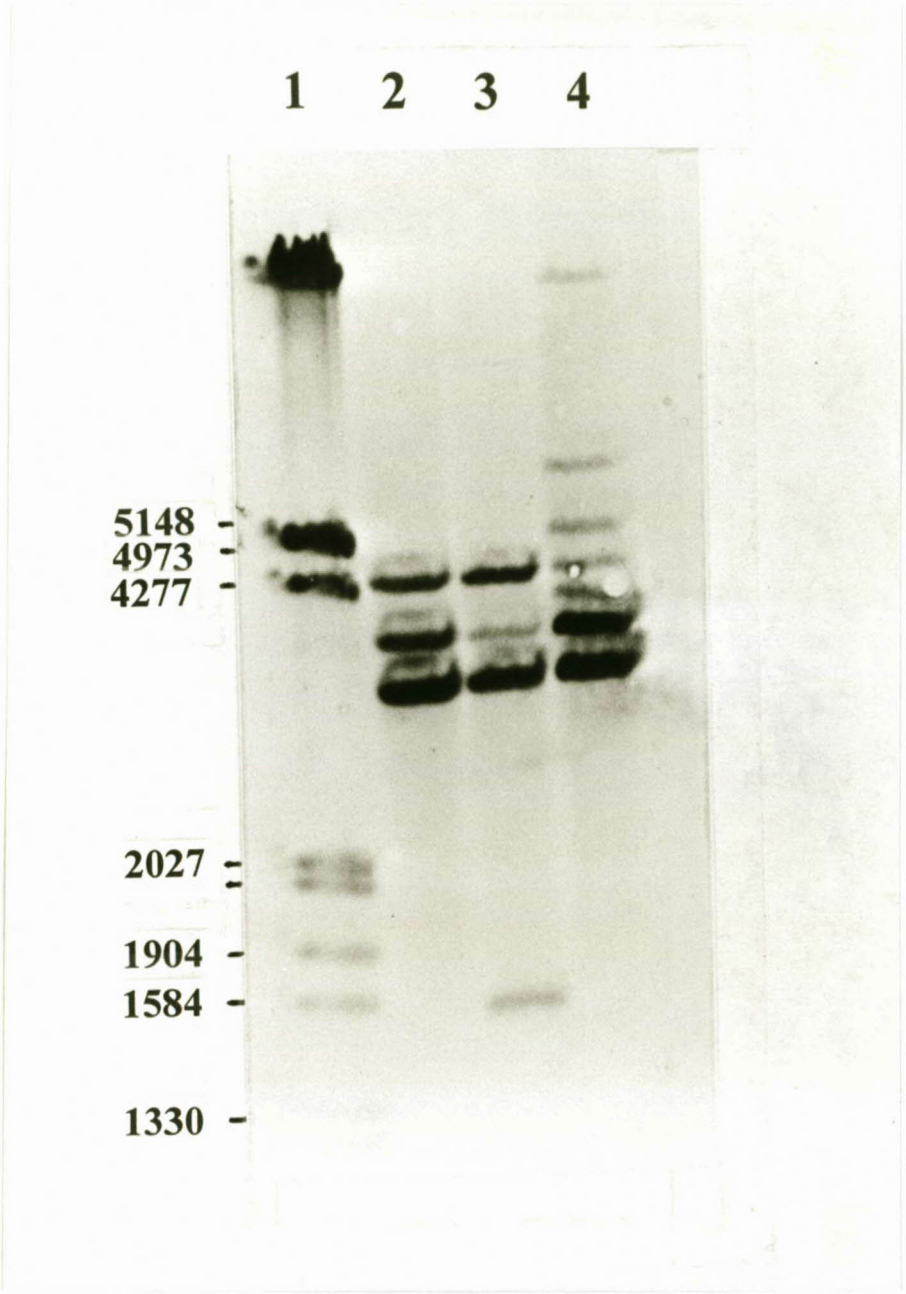


Fig. 5. Representative patterns of the ribotyping profile obtained for the two "*P. coccoides*" strains and the *L. japonicus* type strain after digestion of the genomic DNA with the restriction endonuclease *Bst*EII, separation on an agarose gel and hybridisation with the DIG-labeled 16S rDNA probe of *P. freudenreichii* subspecies *freudenreichii* (ATCC 6207). Lanes: 1, Molecular mass standard (*Eco*RI/*Hind*III digested Phage Lambda DNA); 2, "*P. coccoides*" KM 252 - *Bst*EII; 3, "*P. coccoides*" KM 375 - *Bst*EII; 4, *L. japonicus* ATCC(R) 51527* - *Bst*EII.

3929, 3341 and 2972; (Fig. 5, lane 2), of "*P. coccoides*" (KM 375) were 3929 and 2972; (Fig. 5, lane 3) and of *L. japonicus* (ATCC(R) 51527) were 3413 and 3069 bp. (Fig. 5, lane 4).

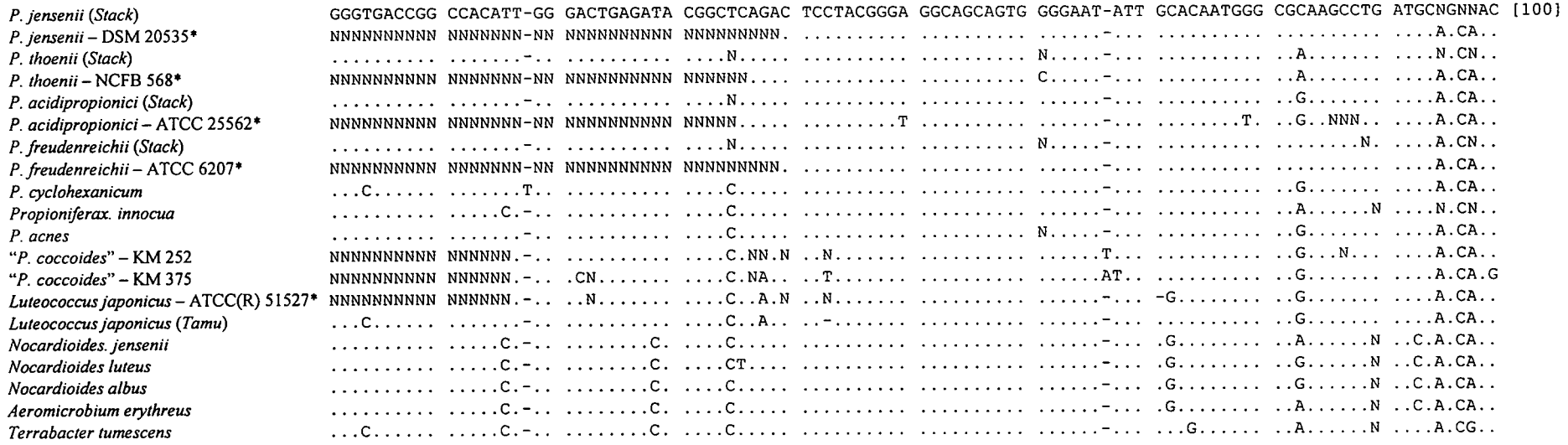
16S rDNA sequencing. An alignment of the 1137 bases of DNA sequence obtained from the amplified 16S rRNA genes of the four classical *Propionibacterium* type strains, as well as that of the two "*P. coccoides*" strains, *P. acnes*, *P. cyclohexanicum*, *Propioniferax innocua*, *Terrabacter tumescens*, *Nocardioides jensenii*, *Nocardioides luteus*, *Nocardioides albus*, *Luteococcus japonicus* (ATCC(R) 51527) and *Aeromicrobium erythreus* is presented in Fig. 6. Regions of DNA that could not be sequenced, especially in the areas of priming were excluded from the PAUP analysis. The phylogenetic analysis was thus subsequently based on a comparative analysis of 921 bases covering the regions 40-274, 297-804, 920-1100 bp (Fig. 6).

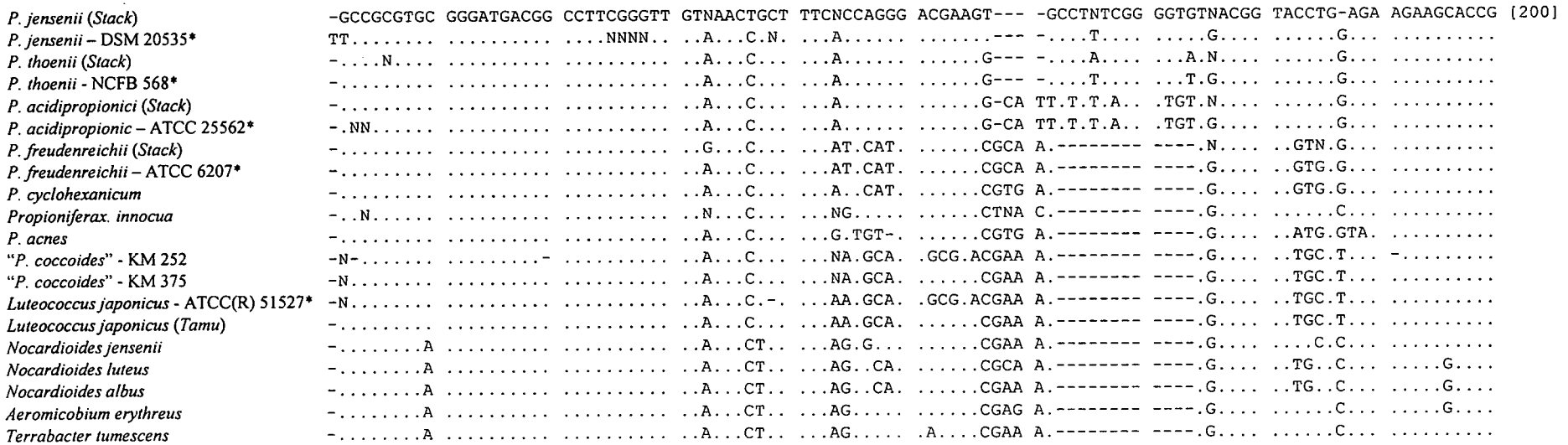
The phylogenetic analysis of the PCR-amplified 16S rRNA gene sequence data of the four *Propionibacterium* type species, *P. acnes*, the two "*P. coccoides*" strains, *Luteococcus japonicus* and the phylogenetically related genera produced one most parsimonious tree. The phylogenetic tree, rooted to *Nocardioides luteus*, is illustrated in Fig. 7. The confidence intervals for the main branches of the tree obtained using bootstrapping analysis (1000 replicates) are also indicated on the figure.

The various *Propionibacterium* species grouped together as a major phylogenetic cluster which was observed to be well separated from the various *Terrabacter*, *Aeromicrobium* and *Nocardioides* outgroups. Two major phylogenetic groups, based on the branching pattern of the phylogram were, however, evident within the classical propionibacteria, confirming the data obtained by *Riedel* (1997). The larger of the two phylogenetic groups, which consisted of the *P. acidipropionici*, *P. jensenii* and *P. thoenii* species, could clearly be separated with a confidence interval of 100% from the smaller *P. freudenreichii* and *P. cyclohexanicum* group.

Comparison of the sequence data for the four type strains of the classical *Propionibacterium* species as obtained during this study and the sequence data as reported by *Charfreitag* and *Stackebrandt* (1989), revealed a 19 bp difference for the *P. thoenii* species, a 13 bp difference for both the *P. acidipropionici* and *P. jensenii* species and a 3 bp difference for the *P. freudenreichii* strains. The sequence data

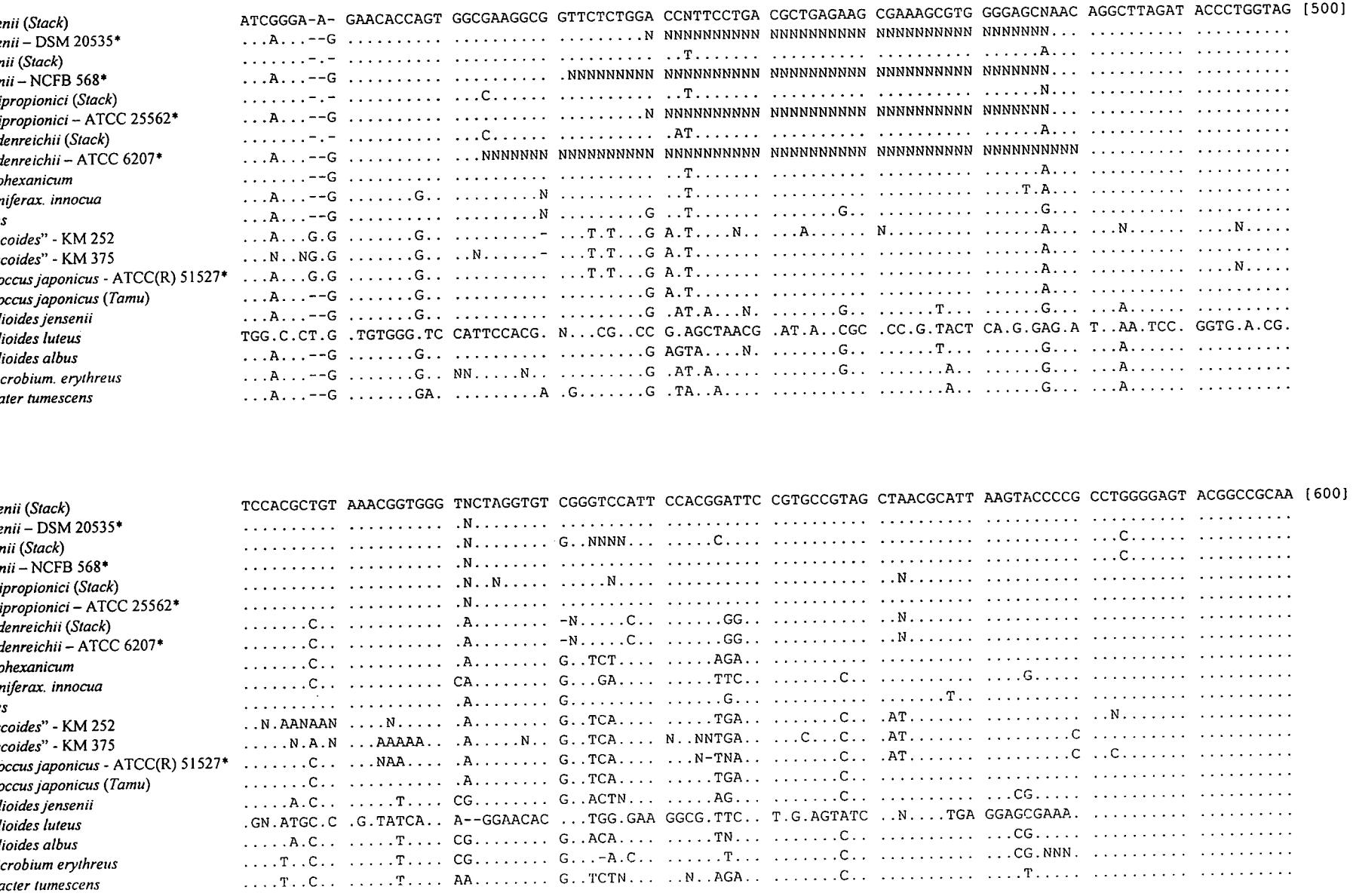
Fig. 6. Aligned DNA sequence (5'-3') of the 16S rDNA region from the two "*P. coccoides*" and the *L. japonicus strains*, the various classical and a cutaneous *Propionibacterium* species, as well as the phylogenetically closely related *Propioniferax innocua*, *Terrabacter tumescens*, *Nocardioides jensenii*, *Nocardioides luteus*, *Nocardioides albus* and *Aeromicrobium erythreus*. (Stack), indicates the sequence data as determined by *Charfreitag* and *Stackebrandt*, (1989); (Tamu), indicates sequence data as determined by *Tamura* et al. (1994); N, indicates a base composition that could not be determined; (-), indicates a gap enabling alignment of the sequence; (.), indicates a base homologous and identical to the corresponding base in *P. jensenii* - (Stack)

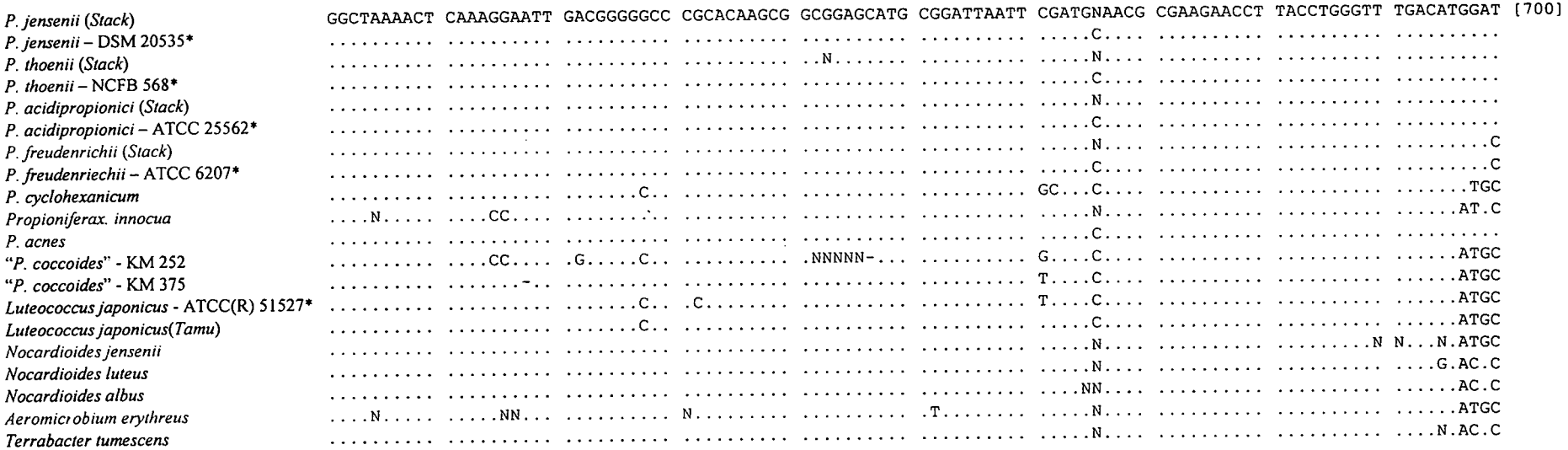

P. jensenii (Stack) GGGTGACCGG CCACATT-GG GACTGAGATA CGGCTCAGAC TCCTACGGGA GGCAGCAGTG GGGAAT-ATT GCACAATGGG CGCAAGCCTG ATGCNGNNAC [100]
P. jensenii – DSM 20535* NNNNNNNNNN NNNNNNN-NN NNNNNNNNNN NNNNNNNNN. .....-... ....A.CA..
P. thoenii (Stack) .....-.. ....N..... N.....-... ..A..... ....N.CN..
P. thoenii – NCFB 568* NNNNNNNNNN NNNNNNN-NN NNNNNNNNNN NNNNNN.... C.....-... ..A..... ....A.CA..
P. acidipropionici (Stack) .....-.. ....N..... .....-... ..G..... ....A.CA..
P. acidipropionici – ATCC 25562* NNNNNNNNNN NNNNNNN-NN NNNNNNNNNN NNNNN..... .....T ....T. ..G..NNN.. ....A.CA..
P. freudenreichii (Stack) .....-.. ....N..... N.....-... .....N. ....A.CN..
P. freudenreichii – ATCC 6207* NNNNNNNNNN NNNNNNN-NN NNNNNNNNNN NNNNNNNNN. .....-... ....A.CA..
P. cyclohexanicum ...C..... .....T.. ....C..... .....-... ..G..... ....A.CA..
Propioniferax. innocua .....C.-.. ....C..... .....-... ..A.....N ....N.CN..
P. acnes .....-.. ....C..... N.....-... ..G..... ....A.CA..
"P. coccoides" – KM 252 NNNNNNNNNN NNNNNN.-.. ....C.NN.N ..N..... .....T... ..G...N... ....A.CA..
"P. coccoides" – KM 375 NNNNNNNNNN NNNNNN.-.. .CN..... ....C.NA.. ..T..... .....AT.. ..G..... ....A.CA.G
Luteococcus japonicus – ATCC(R) 51527* NNNNNNNNNN NNNNNN.-.. ..N..... ....C..A.N ..N..... .....-... -G..... ..G..... ....A.CA..
Luteococcus japonicus (Tamu) ...C..... .....-.. ....C..A.. ..-..... .....-... ..G..... ....A.CA..
Nocardioides. jensenii .....C.-.. .....C. ....C..... .....-... .G..... ..A.....N ..C.A.CA..
Nocardioides luteus .....C.-.. .....C. ....CT.... .....-... .G..... ..G.....N ..C.A.CA..
Nocardioides albus .....C.-.. .....C. ....C..... .....-... .G..... ..G.....N ..C.A.CA..
Aeromicrobium erythreus .....C.-.. .....C. ....C..... .....-... .G..... ..A.....N ..C.A.CA..
Terrabacter tumescens ...C..... .....C.-.. .....C. ....C..... .....-... ...G..... ..A.....N ....A.CG..

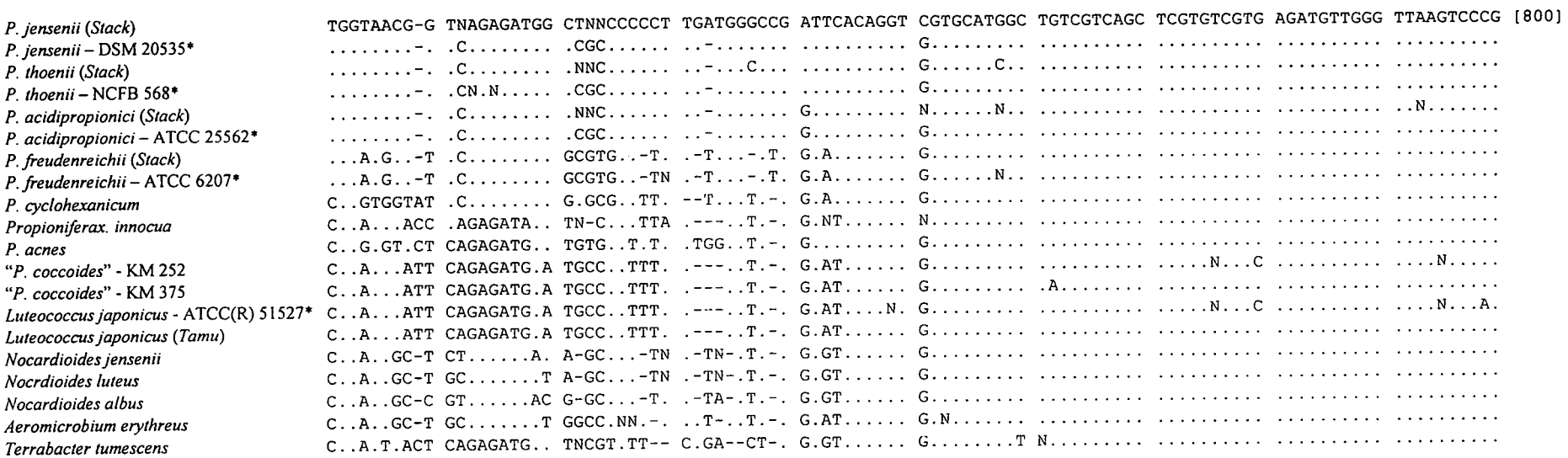

P. jensenii (Stack) -GCCGCGTGC GGGATGACGG CCTTCGGGTT GTNAACTGCT TTCNCCAGGG ACGAAGT--- -GCCTNTCGG GGTGTNACGG TACCTG-AGA AGAAGCACCG [200]
P. jensenii – DSM 20535* TT..... .....NNNN.. ..A...C.N. ...A..... .....--- -....T.... .....G.... ......G... .....
P. thoenii (Stack) -....N.... ..A...C... ...A..... .....G--- -....A.... ...A.N.... ......G... .....
P. thoenii - NCFB 568* -..... ..A...C... ...A..... .....G--- -....T.... ...T.G.... ......G... .....
P. acidipropionici (Stack) -..... ..A...C... ...A..... .....G-CA TT.T.T.A.. .TGT.N.... ......G... .....
P. acidipropionic – ATCC 25562* -.NN..... ..A...C... ...A..... .....G-CA TT.T.T.A.. .TGT.G.... ......G... .....
P. freudenreichii (Stack) -..... ..G...C... ...AT.CAT. .....CGCA A.-------- ----.N.... ..GTN.G... .....
P. freudenreichii – ATCC 6207* -..... ..A...C... ...AT.CAT. .....CGCA A.-------- ----.G.... ..GTG.G... .....
P. cyclohexanicum -..... ..A...C... ...A..CAT. .....CGTG A.-------- ----.G.... ..GTG.G... .....
Propioniferax. innocua -..N..... ..N...C... ...NG..... .....CTNA C.-------- ----.G.... ......C... .....
P. acnes -..... ..A...C... ...G.TGT-. .....CGTG A.-------- ----.G.... ..ATG.GTA. .....
"P. coccoides" - KM 252 -N-..... .....- ..A...C... ...NA.GCA. .GCG.ACGAA A.-------- ----.G.... ..TGC.T... -.....
"P. coccoides" - KM 375 -N..... ..A...C... ...NA.GCA. .....CGAA A.-------- ----.G.... ..TGC.T... .....
Luteococcus japonicus - ATCC(R) 51527* -N..... ..A...C.-. ...AA.GCA. .GCG.ACGAA A.-------- ----.G.... ..TGC.T... .....
Luteococcus japonicus (Tamu) -..... ..A...C... ...AA.GCA. .....CGAA A.-------- ----.G.... ..TGC.T... .....
Nocardioides jensenii -.....A ..A...CT.. ...AG.G... .....CGAA A.-------- ----.G.... ....C.C... .....
Nocardioides luteus -.....A ..A...CT.. ...AG..CA. .....CGCA A.-------- ----.G.... ..TG..C... .....G....
Nocardioides albus -.....A ..A...CT.. ...AG..CA. .....CGAA A.-------- ----.G.... ..TG..C... .....G....
Aeromicobium erythreus -.....A ..A...CT.. ...AG..... .....CGAG A.-------- ----.G.... .....C... .....G....
Terrabacter tumescens -.....A ..A...CT.. ...AG..... .A....CGAA A.-------- ----.G.... .....C... .....

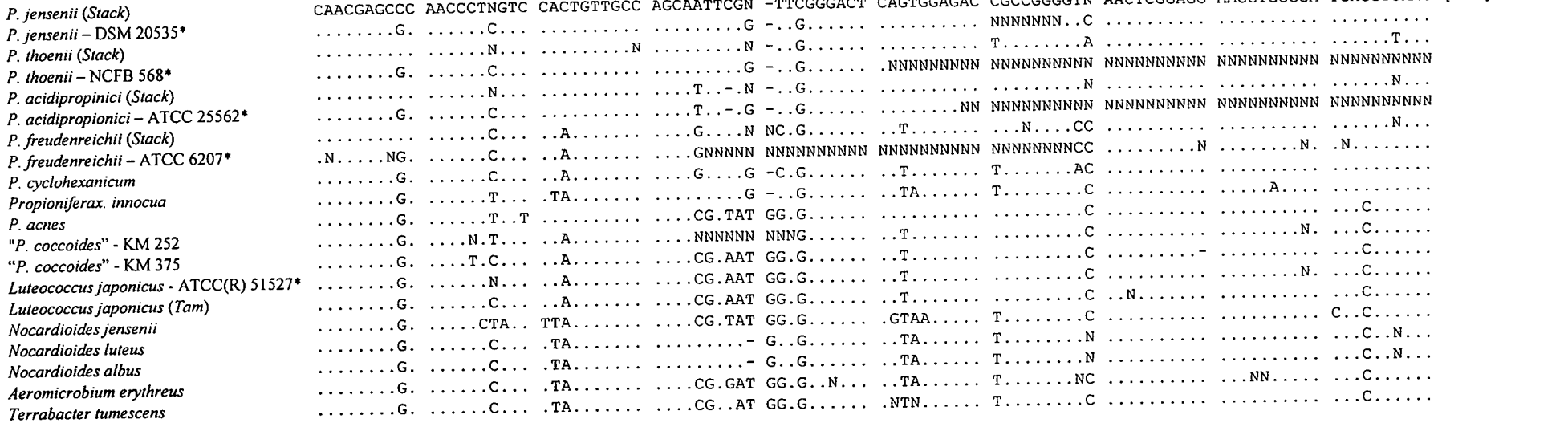
P. jensenii (*Stack*)	GCTAACTACG	TGCCAGCNGC	CGCGGTNATA	CGTAGGGTGC	NAGCGTTGTC	CGGAATTATT	GGGCGTAAAG	AGCTCGTAGG	TGGTTGATCN	CGTCGGAAGT	[300]
P. jensenii – DSM 20535*	....	.......A..	......G...	....	G.........	....T.....	....	....	.........A	....	
P. thoenii (*Stack*)	....	.......N..	......N..N	....	G.........	....	....	....	.........G	....	
P. thoenii – NCFB 568*	....	.......A..	......G...	....	G.........	....T.....	....	....	...NNN...G	....	
P. acidipropionici (*Stack*)	....	.......A..	......N...	....	N.........	....	....	G...N.....	C...N....G	....	
P. acidipropionici – ATCC 25562*	....	.......A..	......G...	....	G.........	....T.....	....	G....NNNNN	NNNNN..NNG	....	
P. freudenreichii (*Stack*)	....	.......A..	......G...	....	G.........	....	....	....N.....	C.........A	....	
P. freudenreichii – ATCC 6207*	....	.......A..	......G...	....	G.........	....	....	....T.....	C.........A	....	
P. cyclohexanicum	..C.......	.......A..	......G...	....	G.........	....	....	....T.....	C.........G	.....A....	
Propioniferax. innocua	..C.......	.......A..	......A...	....	G.........	......C...	....	....T.....	C....TG..A	....	
P. acnes	....	.......A..	......G...	....	G.........	....T.....	....	G.........	.........G	....	
"*P. coccoides*" - KM 252	..C.......	.......A..	......G...	....	G.........	....	A.........	....T.....	C....TG.TG	....A.....	
"*P. coccoides*" - KM 375	..C.......	.......A..	......G...	....	G.........	....	A.........	....T.....	C....TG.TG	....A.....	
Luteococcus japonicus - ATCC(R) 51527*	..C.......	.......A..	......G...	NN........	G.........	....	A.........	..N.T.....	C....TG.TG	....A.....	
Luteococcus japonicus (*Tamu*)	..C.......	.......A..	......G...	....	G.........	....	....	....T.....	C....TG.TG	....A.....	
Nocardioides jensenii	..C.......	.......A..	......A...	........C.	N......A..	....	....	G.........	CC..NTG..A	......G...	
Nocardioides luteus	..C.......	.......A..	......A...	........C.	T...C..A..	....	....	G...T.....	CN..NTG..G	.......N..	
Nocardioides albus	..C.......	.......A..	......A...	........C.	T...C..A..	....	....	G...T.....	CN..NTG..G	......G...	
Aeromicrobium erythreus	..N.......	.......A..	......A...	........C.	N.........	....	......N...	G.........	CN...TG..G	......G...	
Terrabacter tumescens	....	.......N..	......A...	....	N.........	........N.	....	....N.N...	.....NG..G	....T.CT..	

P. jensenii (*Stack*)	CAAAACTTGG	GGCTTAACTC	TGGGCGTGCT	TTCGATCCGG	GTTNACTTGA	GGAAGGTAGG	GGAGAATGGA	ATTCCCGGTG	GAGCGGTGGA	ATGCGCAGAT	[400]
P. jensenii – DSM 20535*	G.........	........C.	....	......A...	...G......	....	....	.....T....	....	....	
P. thoenii (*Stack*)	....	....	....	......A...	...G......	....C.....	..N.......	....	....	....	
P. thoenii – NCFB 568*	G.........	........C.	....	......A...	...G......	....	....	.....T....	....	....	
P. acidipropionici (*Stack*)	....	....N.....	..A.......	......A...	...G......	....	..N.......	....	....	....	
P. acidipropionici – ATCC 25562*	G.........	........C.	..A.......	......A...	...G......	....	....	.....T....	....	....	
P. freudenreichii (*Stack*)	G...TTCCA.	....	.....T....	......A...	...G......	....T.....	....	.C..T.....	....	....	
P. freudenreichii – ATCC 6207*	G...TTCCA.	....	.....T....	......A...	...G......	....T.....	....	.C..T.....	....	....	
P. cyclohexanicum	G...TTCCA.	....	.....T....	......A...	..CG......	....	....	....	....	....	
Propioniferax. innocua	G......CA.	A.........	..A..C....	.C....A...	.C.G...A..	....	......C...	.....T....	....	....	
P. acnes	GT..T.....	........C.	..A.......	......A...	...G......	....	..N.......	.....T....	....	....	
"*P. coccoides*" - KM 252	G...TN.CA.	T.......A.	..A.......	.CT...A...	.CAG...A..	.....T....	......C...	.....T..-.	....	....	
"*P. coccoides*" - KM 375	G...TN.CA.	T.......A.	..A.......	.CT...A...	.CAG...A..	.....T....	......C...	.....T..C.	...N......	....	
Luteococcus japonicus - ATCC(R) 51527*	G...TA.CA.	T.......AN	..A.......	.CT...A...	.CAG...A..	.....T....	......C...	.....TN...	....	....	
Luteococcus japonicus (*Tamu*)	G...T..CA.	T.......A.	..A.......	.CTC..A...	.CAG.G.A..	.....T....	......C...	.....T....	....	........C.	
Nocardioides jensenii	G.....CA..	T.......A.	CTN..C....	.C....A...	.CAG...N..	..T.N.CN..	......C...	.....TN...	T.......A.	....	
Nocardioides luteus	G.....CA..	T.......A.	CTN..C....	......A...	.CAG...N.G	T.TG..G..C	.A.C.GGATT	.GATA.CC..	.TAGTCCAC.	CC.TAA.CG.	
Nocardioides albus	G.....CA..	T.......A.	CTN..C.N..	.C....A...	.CAG...N..	..T.CTC...	....	.....TN...	T.......A.	....	
Aeromicrobium erythreus	G......CA.	....C...C.	..A.N.....	.C....A...	.CAG...A..	..T.T.C...	......C...	.....T....	T.........	....	
Terrabacter tumescens	G....TCC.A	....C...CT	C..A.TN..N	G.G.G.A...	.CAG...N..	.TGT......	.....C....	.....T....	T.........	....	


P. jensenii (Stack) ATCGGGA-A- GAACACCAGT GGCGAAGGCG GTTCTCTGGA CCNTTCCTGA CGCTGAGAAG CGAAAGCGTG GGGAGCNAAC AGGCTTAGAT ACCCTGGTAG [500]
P. jensenii – DSM 20535*
P. thoenii (Stack)
P. thoenii – NCFB 568*
P. acidipropionici (Stack)
P. acidipropionici – ATCC 25562*
P. freudenreichii (Stack)
P. freudenreichii – ATCC 6207*
P. cyclohexanicum
Propioniferax. innocua
P. acnes
"P. coccoides" - KM 252
"P. coccoides" - KM 375
Luteococcus japonicus - ATCC(R) 51527*
Luteococcus japonicus (Tamu)
Nocardioides jensenii
Nocardioides luteus
Nocardioides albus
Aeromicrobium. erythreus
Terrabater tumescens
P. jensenii (Stack) TCCACGCTGT AAACGGTGGG TNCTAGGTGT CGGGTCCATT CCACGGATTC CGTGCCGTAG CTAACGCATT AAGTACCCCG CCTGGGGAGT ACGGCCGCAA [600]
P. jensenii – DSM 20535*
P. thoenii (Stack)
P. thoenii – NCFB 568*
P. acidipropionici (Stack)
P. acidipropionici – ATCC 25562*
P. freudenreichii (Stack)
P. freudenreichii – ATCC 6207*
P. cyclohexanicum
Propioniferax. innocua
P. acnes
"P. coccoides" - KM 252
"P. coccoides" - KM 375
Luteococcus japonicus - ATCC(R) 51527*
Luteococcus japonicus (Tamu)
Nocardioides jensenii
Nocardioides luteus
Nocardioides albus
Aeromicrobium erythreus
Terrabacter tumescens


P. jensenii (Stack) GGCTAAAACT CAAAGGAATT GACGGGGGCC CGCACAAGCG GCGGAGCATG CGGATTAATT CGATGNAACG CGAAGAACCT TACCTGGGTT TGACATGGAT [700]
P. jensenii – DSM 20535*
P. thoenii (Stack)
P. thoenii – NCFB 568*
P. acidipropionici (Stack)
P. acidipropionici – ATCC 25562*
P. freudenrichii (Stack)
P. freudenriechii – ATCC 6207*
P. cyclohexanicum
Propioniferax. innocua
P. acnes
"P. coccoides" - KM 252
"P. coccoides" - KM 375
Luteococcus japonicus - ATCC(R) 51527*
Luteococcus japonicus(Tamu)
Nocardioides jensenii
Nocardioides luteus
Nocardioides albus
Aeromicrobium erythreus
Terrabacter tumescens


P. jensenii (Stack) TGGTAACG-G TNAGAGATGG CTNNCCCCCT TGATGGGCCG ATTCACAGGT CGTGCATGGC TGTCGTCAGC TCGTGTCGTG AGATGTTGGG TTAAGTCCCG [800]
P. jensenii – DSM 20535*
P. thoenii (Stack)
P. thoenii – NCFB 568*
P. acidipropionici (Stack)
P. acidipropionici – ATCC 25562*
P. freudenreichii (Stack)
P. freudenreichii – ATCC 6207*
P. cyclohexanicum
Propioniferax. innocua
P. acnes
"P. coccoides" - KM 252
"P. coccoides" - KM 375
Luteococcus japonicus - ATCC(R) 51527*
Luteococcus japonicus (Tamu)
Nocardioides jensenii
Nocrdioides luteus
Nocardioides albus
Aeromicrobium erythreus
Terrabacter tumescens


P. jensenii (Stack) CAACGAGCCC AACCCTNGTC CACTGTTGCC AGCAATTCGN -TTCGGGACT CAGTGGAGAC CGCCGGGGTN AACTCGGAGG AAGGTGGGGA TGAGGTCAAG [900]
P. jensenii – DSM 20535*
P. thoenii (Stack)
P. thoenii – NCFB 568*
P. acidipropinici (Stack)
P. acidipropionici – ATCC 25562*
P. freudenreichii (Stack)
P. freudenreichii – ATCC 6207*
P. cyclohexanicum
Propioniferax. innocua
P. acnes
"P. coccoides" - KM 252
"P. coccoides" - KM 375
Luteococcus japonicus - ATCC(R) 51527*
Luteococcus japonicus (Tam)
Nocardioides jensenii
Nocardioides luteus
Nocardioides albus
Aeromicrobium erythreus
Terrabacter tumescens
P. jensenii (Stack) TCATNATGCC CCTTATGTCC AGGGCTTCAC GCATGCTACA ATGGCTGGTA CAAAGAGTTG CGACACT-GT GAGGTGGAGC GAATCTCAAA AAGCCAGTCT [1000]
P. jensenii – DSM 20535*
P. thoenii (Stack)
P. thoenii – NCFB 568*
P. acidipropionici (Stack)
P. acidipropionici – ATCC 25562*
P. freudenreichii (Stack)
P. freudenreichii – ATCC 6207*
P. cyclohexanicum
Propioniferax. innocua
P. acnes
"P. coccoides" - KM 252
"P. coccoides" - KM 375
Luteococcus japonicus - ATCC(R) 51527*
Luteococcus japonicus (Tamu)
Nocardioides jensenii
Nocardioides luteus
Nocardioides albus
Aeromicrobium. erythreus
Terrabacter tumescens

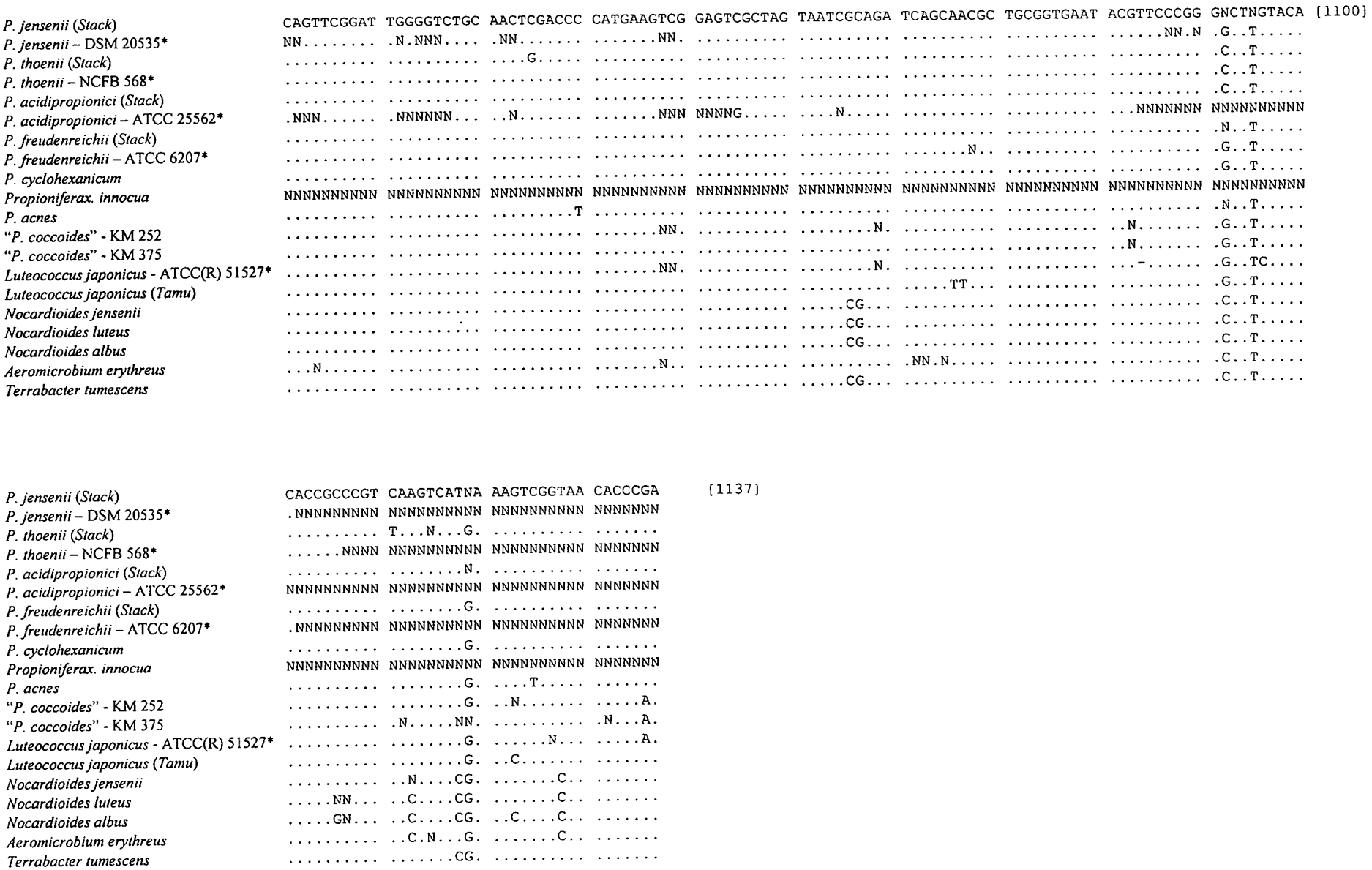
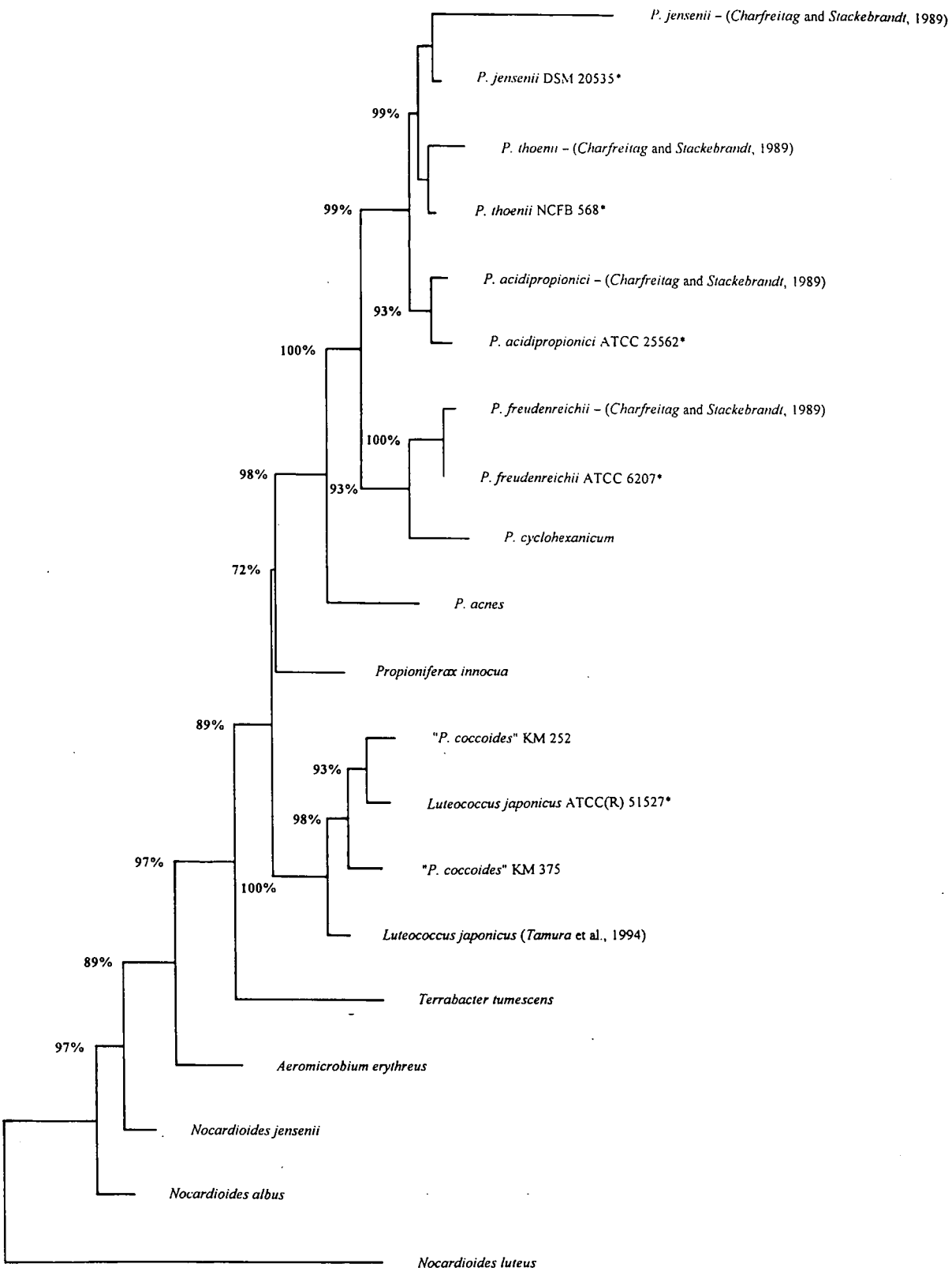

CAGTTCGGAT TGGGGTCTGC AACTCGACCC CATGAAGTCG GAGTCGCTAG TAATCGCAGA TCAGCAACGC TGCGGTGAAT ACGTTCCCGG GNCTNGTACA [1100]
P. jensenii (Stack)
P. jensenii – DSM 20535*
P. thoenii (Stack)
P. thoenii – NCFB 568*
P. acidipropionici (Stack)
P. acidipropionici – ATCC 25562*
P. freudenreichii (Stack)
P. freudenreichii – ATCC 6207*
P. cyclohexanicum
Propioniferax. innocua
P. acnes
"P. coccoides" - KM 252
"P. coccoides" - KM 375
Luteococcus japonicus - ATCC(R) 51527*
Luteococcus japonicus (Tamu)
Nocardioides jensenii
Nocardioides luteus
Nocardioides albus
Aeromicrobium erythreus
Terrabacter tumescens
CACCGCCCGT CAAGTCATNA AAGTCGGTAA CACCCGA [1137]
P. jensenii (Stack)
P. jensenii – DSM 20535*
P. thoenii (Stack)
P. thoenii – NCFB 568*
P. acidipropionici (Stack)
P. acidipropionici – ATCC 25562*
P. freudenreichii (Stack)
P. freudenreichii – ATCC 6207*
P. cyclohexanicum
Propioniferax. innocua
P. acnes
"P. coccoides" - KM 252
"P. coccoides" - KM 375
Luteococcus japonicus - ATCC(R) 51527*
Luteococcus japonicus (Tamu)
Nocardioides jensenii
Nocardioides luteus
Nocardioides albus
Aeromicrobium erythreus
Terrabacter tumescens

Fig. 7. Rooted phylogenetic tree showing the intrageneric relationships of the classical *Propionibacterium* species relative to that of the two "*P. coccoides*" strains, the *L. japonicus* type strain, as well as phylogenetically closely related genera. The percentage values indicated on the tree are the frequencies with which branching occurred in 1000 bootstrap replications.


P. jensenii - (Charfreitag and Stackebrandt, 1989)
P. jensenii DSM 20535*
99%
P. thoenii - (Charfreitag and Stackebrandt, 1989)
99%
P. thoenii NCFB 568*
P. acidipropionici - (Charfreitag and Stackebrandt, 1989)
93%
P. acidipropionici ATCC 25562*
100%
P. freudenreichii - (Charfreitag and Stackebrandt, 1989)
100%
98%
P. freudenreichii ATCC 6207*
93%
P. cyclohexanicum
72%
P. acnes
Propioniferax innocua
89%
"P. coccoides" KM 252
93%
Luteococcus japonicus ATCC(R) 51527*
98%
97%
"P. coccoides" KM 375
100%
Luteococcus japonicus (Tamura et al., 1994)
89%
Terrabacter tumescens
97%
Aeromicrobium erythreus
Nocardioides jensenii
Nocardioides albus
Nocardioides luteus

obtained during this study was identical to that as reported by *Riedel* (1997) and confirmed the disparities reported by *Riedel* (1997) concerning the published sequence data of the classical *Propionibacterium* species. Despite numerous repetitions, the sequence data obtained during this study for the *Luteococcus japonicus* type strain was also observed to differ in 24 bp from the published sequence data of *Tamura* et al. (1994).

The two "*P. coccoides*" strains and the *L. japonicus* type strain evaluated during this study were observed to group together forming a phylogenetic tight cluster which could clearly be differentiated from the members of the genus *Propionibacterium* and the various outgroups included in this study. A tighter phylogenetic relationship was, however, observed between the "*P. coccoides*" (KM 252) strain and the *L. japonicus* type strain than between the latter and "*P. coccoides*" (KM 375), confirming the overall phenotypic similarity observed previously (Chapter 3).

DNA:DNA Probe Hybridisation. Reciprocal hybridisation percentages were averaged to obtain a final percentage of DNA reassociation. These values are presented in Table 3. Despite numerous problems encountered with the application of this technique and the high standard deviation values obtained when two strains were closely related, five major groups could be differentiated on the basis of their high DNA:DNA homology values. Four of the major groups represented the various classical *Propionibacterium* species as described by *Moore* and *Holdeman* (1974) and *Cummins* and *Johnson* (1986). Whilst the fifth group contained the two "*P. coccoides*" strains and the *Luteococcus japonicus* type strain.

The first major group consisted of the two *P. acidipropionici* strains. A 91 ± 21% DNA:DNA homology was observed between these strains. The second major group consisted of the two *P. freudenreichii* strains with a 95 ± 15% DNA:DNA homology. All the strains which were phenotypically and according to their PCR/RFLP as well as ribotyping profiles identified as *P. jensenii* grouped together. The DNA:DNA homology values of this group varied from 60 ± 14 to 88 ± 21%. The three *P. thoenii* strains displayed DNA:DNA homology values ranging from 59 ± 15 to 68 ± 1%. Very high homology values were observed between the two "*P. coccoides*" strains and the *L. japonicus* type strain. Homology calculated between

Table 3. The average normalised percentage hybridisation observed between the different bacterial strains.

Bacterial strains (No.)	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	**100%**													
2	91±21	**100%**												
3	6±2	7±2	**100%**											
4	7±2	6±4	95±15	**100%**										
5	13±5	19±5	7±3	6±2	**100%**									
6	22±1	18±6	12±7	9±2	86±12	**100%**								
7	19±2	19±5	9±0.5	11±7	60±14	88±18	**100%**							
8	14±6	12±5	10±2	4±1	82±17	88±21	75±3	**100%**						
9	19±2	23±5	11±6	9±3	37±0.5	34±1	36±3	39±13	**100%**					
10	19±7	19±12	7±5	5±0.5	20±7	27±0.25	26±15	29±11	66±10	**100%**				
11	19±8	20±13	14±5	20±15	25±9	35±6	30±18	39±21	59±15	68±1	**100%**			
12	8±3	8±1	5±2	5±3	4±0.4	6±1	5±1	4±2	6±2	5±2	7±3	**100%**		
13	6±3	5±1	4±2	8±6	6±3	8±4	6±2	7±3	9±6	9±7	12±10	95±15	**100%**	
14	10±7	6±1	6±3	10±3	9±7	11±7	6±3	7±2	9±6	8±6	12±10	93±21	91±11	**100%**

Bacterial strains (No.)

No.	Lab. No.	Name	Source	Strain	Isolated from	Identification key#
1	424	*P. acidipropionici*	1	ATCC 25562*	-	*P. acidipropionici*
2	421	"*P. pentosaceum*"	4	NCFB 570	Emmentaler	*P. acidipropionici*
3	423	*P. freudenreichii*	1	ATCC 6207*	Swiss cheese	*P. freud.* ss.. *freudenreichii*
4	453	"*P. shermanii*"	5	P 67	-	*P. freud.* ss.. *freudenreichii*
5	80	*P. jensenii*	2	DSM 20535*	Buttermilk	*P. jensenii*
6	130	"*P. intermedium*"	5	NCIB 8728	-	*P. jensenii*
7	427	"*P. pituitosum*"	4	NCFB 1077	-	*P. jensenii*
8	75	"*P. technicum*"	6	P 74	Emmentaler	*P. jensenii*
9	419	*P. thoenii*	4	NCFB 568*	Emmentaler	*P. thoenii*
10	294	*P. thoenii*	7	-	Anaerobic digester	*P. thoenii*
11	297	*P. thoenii*	7	-	Anaerobic digester	*P. thoenii*
12	362	"*P. coccoides*"	3	-	Soviet cheese	-
13	363	"*P. coccoides*"	3	VKM AC 1910	Soviet cheese	-
14	364	*L. japonicus*	1	ATCC(R) 51527*	Ground and water	-

Abbreviations

*: Type strain

#: Identification key of Johnson and Cummins (1986)

1: ATCC, American Type Culture Collection, Rockville Maryland, USA.

2: DSM, Deutsche Sammlung Von Mikroorganismen und Zelkulturen, Braunschweig, Germany.

3: L.I. Vorobjeva, Moscow State University, Russia.

4: NCFB, National Collection of Food Bacteria, Shinfield, Reading, UK.

5: NCIB, National Collection of Industrial Bacteria, UK.

6: G.W. Reinbold, Iowa State University, Iowa, USA.

7: Environmental Microbiology Culture Collection, University of the Orange Free State, Souuth Africa.

the two "*P. coccoides*" strains was 95 ± 15%, between "*P. coccoides*" (KM 252) and *L. japonicus* was 93 ± 21%, and between "*P. coccoides*" (KM 375) and *L. japonicus* was 91 ± 11%. Very low DNA:DNA homology values (ranging from 4 ± 0.4% to 39 ± 21%) were observed between the different classical *Propionibacterium* species and the "*P. coccoides*" / *L. japonicus* strains.

Discussion

Due to the higher degree of overall phenotypic similarity observed between the "*P. coccoides*" strains and the *L. japonicus* type strain, than between the latter and members of the genus *Propionibacterium*, a molecular and phylogenetic analysis of the various strains was undertaken. Using the polymerase chain reaction as described by *Riedel* et al. (1994), the predicted 1110 bp fragment of the 16S rRNA gene was successfully amplified. All four the classical *Propionibacterium* species could be differentiated from each other on the basis of their restriction fragment length polymorphism data using the restriction endonucleases *Hae*II and *Sma*I, confirming the results as reported by *Riedel* et al. (1994:1998). Although the "*P. coccoides*" and *Luteococcus japonicus* strains could be differentiated from the various classical *Propionibacterium* species on the basis of their restriction endonuclease profiles, it was not possible to differentiate them from each other, despite the evaluation of numerous restriction endonucleases. These results subsequently confirm the high degree of overall phenotypic similarity observed between the "*P. coccoides*" strains and the *Luteococcus japonicus* type strain (Chapter 3).

It was only possible to differentiate the "*P. coccoides*" and *L. japonicus* strains via ribotyping on the basis of their *Bst*EII restriction endonuclease profiles. The hybridisation pattern obtained for the two "*P. coccoides*" and *L. japonicus* strains using the 16S rDNA probe of *P. freudenreichii* subsp. *freudenreichii* (ATCC 6207) and the *Bst*EII digested DNA, revealed 3 distinct ribotypes. This data thus confirmed the phenotypic differentiation observed between these three species in chapter 3. An 82% similarity was, however, observed between the *P. jensenii* species and the two "*P. coccoides*" strains as well as the *L. japonicus* type strain when a numerical analysis was performed on the ribotyping data as obtained during this study and that reported by *Riedel* and *Britz* (1996). These results subsequently confirm the high

degree of similarity observed by *Vorobjeva et al.* (1983) between "*P. coccoides*" and *P. jensenii* based on DNA:DNA homology.

Based on the 16S rDNA sequence data and the branching pattern observed in the phylogram, the classical *Propionibacterium* species could be divided into two major phylogenetic groups. The larger major group containing the *P. acidipropionici*, *P. jensenii*, and *P. thoenii* species could be divided into two smaller subdivisions. The *P. jensenii* and *P. thoenii* subdivision, which clustered together with a 99% confidence interval was observed to be the most closely related phylogenetically. Together with the *P. acidipropionici* subdivision, the *P. jensenii* and *P. thoenii* subcluster formed a phylogenetic cluster (99% confidence interval) which embraced the classical propionibacteria containing the LL-isomer of diaminopimelic acid as the diamino acid of their cell wall peptidoglycan. Based on the 16S rDNA relatedness, this major group is phylogenetically well separated from the *P. freudenreichii* and *P. cyclohexanicum* major group with a 93% confidence interval value. *Propionibacterium acnes*, the only member of the cutaneous group evaluated during this study, as well as *Propioniferax innocua* were observed to be phylogenetically closely related to the classical *Propionibacterium* species confirming the report of *Johnson* and *Cummins* (1972). Of the various phylogenetically related genera evaluated during this study, *L. japonicus* was observed to be the most closely related to the classical *Propionibacterium* species, with a 89% confidence interval confirming the report of *Tamura* et al. (1994). Both "*P. coccoides*" strains clustered with the *L. japonicus* type strain, confirming the high degree of phenotypic similarity observed between these strains. The "*P. coccoides*" / *L. japonicus* clade was, however, clearly separated from the cluster representing the various *Propionibacterium* species. With only a 16 bp difference between the sequence data of "*P. coccoides*" (KM 252) and the *L. japonicus* type strain, a tighter phylogenetic relationship with a confidence interval of 93% was observed than between "*P. coccoides*" (KM 375) and *L. japonicus*. The confidence interval value of 98% between these two strains was based on a 23 bp difference in their 16S rRNA sequence data. This divergence 16S rDNA sequence in data subsequently also confirms the variation observed in the phenotypic characteristics of these strains.

Despite numerous precautionary measures, including adaptations during the quantification of the DNA, especially since the lambda phage DNA, which was used

as the concentration standard, had a lower mol% G + C content than the *Propionibacterium* and *Luteococcus* genera and the optimisation of the DNA hybridisation temperatures, numerous difficulties were experience with the application of the DNA:DNA probe hybridisation technique as described by *Viljoen* (1996). Excessive problems were experience with the quantification of the DNA, which could possibly have contributed to the large standard deviation observed. It is subsequently imperative that this technique should be optimised before any conclusive results can be made. Five DNA:DNA homology groups, four resembling the various classical propionibacteria species and one containing the "*P. coccoides*" and *L. japonicus* strains could, however, be differentiated. These results subsequently confirm the phylogenetic analysis of the 16S rDNA sequence data. The *P. jensenii* and *P. thoenii* homology groups showed about 20 to 40% DNA:DNA homology with each other, whilst the *P. acidipropionici* homology group showed from 12 to 23% homology to the *P. jensenii* and *P. thoenii* reference strains, respectively. The *Propionibacterium freudenreichii* homology group showed from 6 - 7, 4 - 12, and 5 - 20% DNA:DNA homology with the *P. acidipropionici*, *P. jensenii* and *P. thoenii* homology groups, respectively. The homology group consisting of the "*P. coccoides*" strains and the *L. japonicus* type strain displayed between 4 - 12% DNA:DNA homology to the four classical *Propionibacterium* homology groups.

Although the four DNA:DNA homology groups, representing the four classical *Propionibacterium* groups as determined by *Johnson* and *Cummins* (1972) could be confirmed during this study, the homology values obtained during this study were approximately 15% lower than those as reported by *Johnson* and *Cummins* (1972). According to *Johnson* and *Cummins* (1972) the *P. jensenii* group showed about 50% homology to the *P. thoenii* group and about 30 to 35% to the *P. acidipropionici* group. During this study, the average DNA:DNA homology between the *P. jensenii* and *P. thoenii* groups was 31%, while the average DNA:DNA homology between the *P. jensenii* and *P. acidipropionici* groups was observed to be 17%. *Johnson* and *Cummins* (1972) also reported that the *P. freudenreichii* strains showed a rather lower level of similarity (8 to 25%) to the other homology groups. Similar results were observed during this study. The statement of *Vorobjeva* et al. (1983) that a high degree of DNA:DNA homology existed between *P. jensenii* and "*P. coccoides*" could, however, not be validated during this study.

It is evident from the results of this study, that a higher degree of molecular and phylogenetic similarity exists between the genus *Luteococcus* and the "*P. coccoides*" strains than between the latter and the genus *Propionibacterium*. The results of this study subsequently also confirm the high degree of phenotypic similarity observed between these strains. During this study the conclusions made during the phenotypic characterisation of "*P. coccoides*" (Chapter 3) could be confirmed. Since no genera of cocci with LL-DAP in their cell wall and with a high mol% G + C content have ever been described, *Tamura* et al. (1994) only compared their data to the genera *Propionibacterium*, *Nocardioides*, *Aeromicrobium* and *Terrabacter*. The genus *Sarcina*, which is the only bacterial coccus containing LL-DAP in their cell wall has a low mol% G + C content in its DNA and due to the fact that it is a strict anaerobe, was not included (*Tamura* et al., 1994). With all the above mentioned high mol% G + C content genera included in this study, it is thus indisputable that the proposal of *Vorobjeva* et al. (1983) to include "*P. coccoides*" as a new species within the genus *Propionibacterium* is incorrect. The "*P. coccoides*" strains should subsequently be transferred to the genus *Luteococcus* as putative subspecies. The genus description of *Luteococcus japonicus* should subsequently also be revised to include the phenetic and molecular description of these strains.

References

Britz, T.J., Riedel, K-H.J.: *Propionibacterium* species diversity in Leerdammer cheese. Int. J. Food. Microbiol. 22, 257-267 (1994).

Britz, T.J., Riedel, K-H.J.: A numerical taxonomic study of *Propionibacterium* strains from dairy sources. J. Appl. Bacteriol. 71, 407-416 (1991).

Charfreitag, O., Stackebrandt, E.: Inter- and intrageneric relationships of the genus *Propionibacterium* as determined by 16S rRNA sequences. J. Gen. Microbiol. 135, 2065-2070 (1989).

Charfreitag, O., Collins, M.D., Stackebrandt, E.: Reclassification of *Arachnia propionica* as *Propionibacterium propionicus* comb. nov. Int. J. Syst. Bacteriol. 38, 354-357 (1988).

Collins, M.D., Dorsch, M., Stackebrandt, E.: Transfer of *Pimelobacter tumescens* to *Terrabacter* gen. nov. as *Terrabacter tumescens* comb. nov. and of *Pimelobacter jensenii* to *Nocardioides* as *Nocardioides jensenii* comb. nov. Int. J. Syst. Bacteriol. 39, 1-6 (1989).

Cummins, C.S., Johnson, J.L.: Genus *Propionibacterium* Orla-Jensen 1909, pp. 1346-1353. In: Bergey's Manual of Systematic Bacteriology (P.H.A. Sneath, N.S. Mair, M.E. Sharpe, J.G. Holt, eds.) vol. 2. Baltimore, Williams and Wilkins 1986.

Cummins , C.S., Johnson, J.L.: The Genus *Propionibacterium*, pp. 834-849. In: The Prokaryotes (A. Balows, H.G. Truper, M. Dworkin, W. Harder and K-H. Schleifer, eds.) vol. 1. New York, Springer-Verlag 1992.

Felsenstein, J.: DNABOOT-Bootstrapping Confidence Intervals on DNA Parsimony 3.1. University of Washington 1988.

Holdeman, L.V., Cato, E.P., Moore, W.E.C.: *Arachnia* and *Propionibacterium*, pp. 56-65. In: Anaerobe Laboratory Manual, 4th ed., Virginia Polytechnic Institute and State University, Blacksburg, Virginia, Southern Printing Co. 1977.

Holt, J.G., Krieg, N.R., Sneath, P.H., Staley, J.T., Williams, S.T.: Genus *Propionibacterium*, p. 580. In: Bergey's Manual of Determinative Bacteriology (J.G. Holt, N.R. Krieg, P.H. Sneath, J.T. Staley, S.T. Williams, eds.) 9th ed., Baltimore, Williams and Wilkins 1994.

Johnson, J.L., Cummins, C.S.: Cell wall composition and deoxyribonucleic acid similarities among the anaerobic coryneforms, classical propionibacteria, and strains of *Arachnia propionica*. J. Bacteriol. 109, 1047-1066 (1972).

Kusano, K., Yamada, H., Niwa, M., Yamasato, K.: *Propionibacterium cyclohexanicum* sp. nov., a new acid-tolerant ω-cyclohexyl fatty acid-containing *Propionibacterium* isolated from spoiled orange juice. Int. J. Syst. Bacteriol. 47, 825-831 (1997).

Lockhart, W.R., Liston, J.: In: Methods for numerical taxonomy. AmericAN Society for Microbiology, Washington DC 1970.

Maniatis, T., Fritsch, E.F., Sambrook, J.: Molecular cloning: A laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA 1989.

Moore, W.E.C., Holdeman, L.V.: Genus 1. *Propionibacterium* Orla-Jensen 1909, pp. 633-641. In: Bergey's Manual of Determinative Bacteriology (R.E Buchanan, N.E. Gibbons, eds.) 8th ed., Baltimore, Williams and Wilkins 1974.

Perbal, B.: A practical guide to molecular cloning, 2nd ed., New York, John Wiley and Sons 1988.

Riedel, K-H.J., Britz, T.J.: Justification of the "classical" *Propionibacterium* species concept by ribotyping. System. Appl. Microbiol. 19, 370-380 (1996).

Riedel, K-H.J., Britz, T.J.: *Propionibacterium* species diversity in anaerobic digesters. Biodiversity and Conservation 2, 400-411 (1993).

Riedel, K-H.J., Wingfield, B.D., Britz, T.J.: Combined influence of magnesium concentration and polymerase chain reaction specificity enhancers. FEMS Microbiol. Lett. 92, 69-72 (1992).

Riedel, K-H.J., Wingfield, B.D., Britz, T.J.: rDNA – Restriction fragment length polymorphisms. System. Appl. Microbiol. 21, 419-428 (1998).

Riedel, K-H.J., Wingfield, B.D., Britz, T.J.: Justification of the "classical" *Propionibacterium* species concept by restriction analysis of the 16S ribosomal RNA genes. System. Appl. Microbiol. 17, 536-542 (1994).

Riedel, K-H.J.: A comparative evaluation and justification of the "classical" *Propionibacterium* species concept. PhD. Thesis. University of the Orange Free State, South Africa 1997.

Sambrook, J., Fritsch, E.F., Maniatis, T.: Molecular cloning: A laboratory Manual, 2nd ed., New York, Cold Spring Harbour Laboratory, Cold Spring Harbour 1989.

Sokal, R.R., Michener, C.D.: A statistical method for evaluating systematic relationships. Kansas Univ. Sci. Bull. 38, 1409-1438 (1958).

Southern, E.M.: Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503-517 (1975).

Swofford, D.L.: PAUP: Phylogenetic Analysis Using Parsimony, Version 3.1.1. Computer programme distributed by Illinois Natural History Survey, Champaign, Illinois 1993.

Tamura, T., Takeuchi, M., Yokota, A.: *Luteococcus japonicus* gen. nov., sp. nov., a new gram-positive coccus with LL-diaminopimelic acid in the cell wall. Int. J. Syst. Bacteriol. 44, 348-356 (1994).

Viljoen, D.C.: A taxonomic study of *Ceratocystis* sensu lato with special reference to species associated with Protea infructescences in southern africa. PhD. Thesis. University of the Orange Free State, South Africa 1996.

Vorobjeva, L.I., Turova, T.P., Kraeva, N.I., Alekseeva, M.A.: Propionic acid cocci and their systematic position. Microbiol. 52, 368-373 (1983).

Wilson, K., Goulding, K.H.: A biologists guide to principles and techniques of practical biochemistry, pp. 333-334. Edward Arnold, UK 1986.

Chapter 5

General discussion and conclusions

Ever since the description of the first propionibacteria by *Von Freudenreich* and *Orla-Jensen* in 1906, this group of organisms has been the centre of many controversies in terms of their identification and taxonomic status. This is not only evident for species already included within the genus *Propionibacterium* but also when newly proposed species are considered. Even after the compilation of classification keys by *Moore* and *Holdeman* (1974) as well as *Cummins* and *Johnson* (1986), several reports can still be found in the literature where difficulties with the identification of isolates were experienced. With the advance of genetically based identification systems, only more questions started arising. In addition to the problems experienced in some publications concerning the identification and the differentiation of the various subspecies within *Propionibacterium freudenreichii* (*Riedel* and *Britz*, 1992; *Bear* and *Ryba*, 1988; *Johnson* and *Cummins* 1972), 16 distinct ribotype profiles were reported by *Riedel* and *Britz* (1996) within the four classical *Propionibacterium* species. Another typical example concerns the bacterium *P. propionicum* which was proposed as a new species within the genus *Propionibacterium* by *Charfreitag* et al. (1988). Despite numerous publications (*O'Donnell* et al., 1985; *Cummins* and *Johnson*, 1992; *Cummins* and *Moss*, 1990) confirming this proposal, *P. propionicum* was retained as *Arachnia propionica* in Bergey's Manual of Determinative Bacteriology (*Holt* et al., 1994). *Reinbold*'s (1978) perspective that the consolidation of various "old" classical species to form the *P. acidipropionici*, *P. freudenreichii*, *P. jensenii* and *P. thoenii* species by *Moore* and *Holdeman* (1974), only worsened the taxonomy of the classical propionibacteria, is thus still very appropriate.

In 1983, *Vorobjeva* et al. described a new species within the genus *Propionibacterium*. Based on the high degree of similarity between this bacterium and members of the classical *Propionibacterium* group, both on a phenotypic as well as on a genetic level, *Vorobjeva* et al. (1983) designated the name "*Propionibacterium*

coccoides" to this newly described Gram-positive coccus. This bacterium not only displayed the same phenotypic similarities, including the formation of propionic acid as the major metabolite of lactate fermentation and the same electrophoretic mobility of the various enzymes (catalase, superoxide dismutase and peroxidase), to those already described for the genus *Propionibacterium*, but it was also found to be genetically related to the genus *Propionibacterium*. The mol% G + C content of the DNA of "*P. coccoides*" was determined to be 63.4 mol%, which was within the range of that published for the genus *Propionibacterium* (*Vorobjeva* et al., 1983). Based on their DAN:DNA homology values, the highest similarity (49%) was observed between "*P. coccoides*" and that of the propionic acid bacterium *P. jensenii*.

According to the definition of the genus *Propionibacterium* as given by *Moore* and *Holdeman* (1974), this propionic acid producing coccus should subsequently be classified within this genus. Although numerous taxonomic studies have been undertaken (*Britz* and *Riedel*, 1991:1994; *Riedel* and *Britz*, 1993; *Riedel* et al., 1992:1994) in an attempt to solve the taxonomic dilemma within the genus *Propionibacterium*, none have ever included "*P. coccoides*". Subsequently the correct systematic position of this organism remained unresolved. Although this proposal of *Vorobjeva* et al. (1983) was mentioned as well as fertified by *Cummins* and *Johnson* (1992), it was never even mentioned in either the Bergey's Manual of Systematic Bacteriology (*Cummins* and *Johnson*, 1986) or Bergey's Manual of Determinative Bacteriology (*Holt* et al., 1994). Based on these reasons, it was thus evident that the taxonomic status of "*P. coccoides*" relative to that of the classical *Propionibacterium* species would have to be re-examined.

The first objective of this study was, therefore, to further characterise "*P. coccoides*" phenotypically and to determine it's systematic position relative to that of the already described classical *Propionibacterium* species. A Gram-positive, high mol% G + C content coccus, namely *Luteococcus japonicus*, which was previously determined to be the closest phylogenetic neighbour of the genus *Propionibacterium* (*Tamura* et al., 1994), was also included during this study as an outgroup. On the basis of the results obtained in chapter 3, it was evident that the "*P. coccoides*" strains as described by *Vorobjeva* et al. (1983) could not be identified as any of the classical

Propionibacterium species as described by *Cummins* and *Johnson* (1986). Furthermore, the "*P. coccoides*" strains clustered together with the *L. japonicus* type strain in a separate cluster, which was clearly delineated from the various clusters representing the classical *Propionibacterium* species. Based on the results obtained during this study (Chapter 3), it was possible to differentiate the two "*P. coccoides*" as well as the *L. japonicus* strains from *P. acidipropionici*, *P. freudenreichii*, *P. jensenii* and *P. thoenii* on their ability to ferment D-mannose and sorbitol, as well as their characteristic bright yellow pigmentation. Although the "*P. coccoides*" / *L. japonicus* cluster coalesced at a 80% similarity level, it was still possible to phenotypically differentiate between *L. japonicus* and the two "*P. coccoides*" strains themselves. Based on the results of the numerical analysis of the phenotypic data, it was apparent that a higher degree of phenotypic similarity existed between *L. japonicus* and "*P. coccoides*" than between the latter and the members of the genus *Propionibacterium*. It was subsequently apparent that the proposal of *Vorobjeva* et al. (1983) to include "*P. coccoides*" as a new species within the genus *Propionibacterium*, could possibly be incorrect.

In order to validate the results observed during the numerical analysis of the phenotypic data, a molecular and phylogenetic analysis of the classical propionibacteria, "*P. coccoides*" and *L. japonicus* was undertaken. The fist objective of this study was to determine the relationship between the "*P. coccoides*" strains and known marker strains using molecular techniques based on the 16S ribosomal RNA genes as well as the flanking regions. Using the rapid yet sensitive restriction fragment length polymorphism technique as described and optimised by *Riedel* et al. (1994:1998), an identical RFLP profile was obtained for both the "*P. coccoides*" strains and the *L. japonicus* type strain. These RFLP patterns were obtained after restriction endonuclease digestion of the PCR products, obtained using primers 16sP1-16sP4, with the restriction endonucleases *Hae*III and *Alu*I. Although it was possible to differentiate between the four classical *Propionibacterium* species, confirming the data obtained by *Riedel* et al. (1994:1998), and the RFLP profile obtained for the two "*P. coccoides*" and the *L. japonicus* strains, differentiation between the latter two species, using various other restriction endonucleases, was, however, not possible.

The next objective of this study was to investigate the relative homogeneity observed within each of the two "*P. coccoides*" strains and the *L. japonicus* type strain based on the RFLP data, which was in total contrast to the phenotypic heterogeneity observed, using ribosomal RNA gene restriction patterns (ribotyping). The combination of the *Hae*II and *Sma*I restriction endonuclease patterns resulted in the generation of a unique ribotype profile (profile Q) which was observed for both the "*P. coccoides*" strains as well as the *L. japonicus* type strain. This ribotyping profile could clearly be differentiated from the 16 distinct ribotype profiles as determined for the classical *Propionibacterium* strains by *Riedel* and *Britz* (1996). Despite the evaluation of 12 other restriction endonucleases using the ribotyping technique, differentiation between the "*P. coccoides*" strains and the *L. japonicus* type strain was only possible using the 16S rDNA probe of the *P. freudenreichii* subsp. *freudenreichii* (ATCC 6207) and the restriction endonuclease *Bst*EII. Although the three distinct ribotypes obtained for the two "*P. coccoides*" strains and the *L. japonicus* type strain confirmed the phenotypic heterogeneity observe between these two species in chapter 3, it is evident that the organisms were very closely related on a molecular level.

Due to the large degree of phenotypic diversity observed between the two "*P. coccoides*" strains and the *L. japonicus* type strain (Chapter 3) and since confirmation of this phenomenon using the RFLP and ribotyping techniques were lacking, the next objective of this study was to determine the intrageneric relationship between these three strains, the type strains of the various classical *Propionibacterium* species and phylogenetically related genera. This phylogenetic relationship was determined by comparative analysis of their 16S ribosomal DNA sequence data. The various *Propionibacterium* species were observed to coalesce in a phylogenetic clade which was clearly delineated from the clade containing the "*P. coccoides*" and *L. japonicus* strains. A 16 and 23 base pair difference was observed to exist in the 16S rDNA sequence data of the *L. japonicus*, "*P. coccoides*" (KM 252) and the "*P. coccoides*" (KM 375) strains, respectively. Four clusters, each representing one of the current classical species (*Cummins* and *Johnson*, 1986) could be delineated in the major *Propionibacterium* clade after PAUP analysis of the sequence data (Chapter 4). Two major groups within the genus *Propionibacterium* were, however, apparent

confirming the results obtained by *Riedel* (1997). The *P. acidipropionici*, *P. jensenii* and *P. thoenii* strains formed a phylogenetic cluster which was clearly separated from the *P. freudenreichii* species.

The last objective of this study was to investigate the heterogeneity observed between various classical *Propionibacterium* species and the two "*P. coccoides*" and *L. japonicus* type strain, using the entire genome structure. DNA:DNA probe hybridisation (*Viljoen*, 1996) was subsequently evaluated as a possible reliable species differentiation technique. Five major DNA:DNA homology groups, four resembling the various classical propionibacteria species and one containing the two "*P. coccoides*" strains and the *L. japonicus* type strain could be differentiated. A very high degree of DNA:DNA homology (>90%) was observed between the *L. japonicus* type and the two "*P. coccoides*" strains. These results subsequently confirm the phylogenetic analysis of the 16S rDNA sequence data, where the *L. japonicus* / "*P. coccoides*"strains clustered together in a clade which was phylogenetically clearly distinct from the clade containing the various *Propionibacterium* species. A low degree of DNA:DNA homology was observed between the various *Propionibacterium* species and the group containing *L. japonicus* and the "*P. coccoides*" strains, confirming the phenotypic, molecular and phylogenetic analyses.

In conclusion, the results of this study confirm the various differentiation techniques as described for the classical *Propionibacterium* species (*Riedel*, 1997). This not only included phenotypic characterisation but also molecular characterisation, which included analysis of the polymerase chain reaction, restriction fragment length polymorphism, ribotyping as well as the 16S rRNA sequencing techniques. The data obtained using these optimised techniques, clearly indicated that the proposal of *Vorobjeva* et al. (1983) to include "*P. coccoides*" as a new species within the genus *Propionibacterium* was incorrect and that this proposition requires some revision. Based on the higher degree of overall phenotypic and molecular similarity between *L. japonicus* and "*P. coccoides*" than between the latter and the various species of the genus *Propionibacterium*, as observed during this study, it is subsequently proposed that the "*P. coccoides*" strains be transferred to the genus *Luteococcus* as putative subspecies. Furthermore it is suggested that the genus

description (*Tamura* et al., 1994), as stated below, should be revised to include the phenetic and molecular description of these strains.

The genus *Luteococcus* was first described in 1994 by *Tamura* et al. This Gram-positive cocci was first known as "*Micrococcus aurantiacus*" IFO 12422 and *Micrococcus* sp. IFO 15385. Originally isolated from soil on Tokara island and from water for brewing "miyamizu", respectively (*Oda*, 1935), they have been maintained in the culture collection of the Institute for Fermentation, Osaka. The taxonomic position of these strains has, however, remained uncertain due to the fact that they contain LL-diaminopimelic acid (LL-DAP) in their cell wall, instead of lysine which is characteristic of other *Micrococcus* species. Since, no Gram-positive cocci with a high mol% G + C content and containing LL-DAP had been described previously, these organisms were compared to the genera *Propionibacterium*, *Aeromicrobium*, *Terrabacter* and *Nocardioides* (*Tamura* et al., 1994). Analysis of the partial 16S rRNA sequence indicated that the genus *Luteococcus* represented a distinct line of descent among the Gram-positive bacteria with a high G + C content. *Luteococcus japonicus* was further revealed to be phylogenetically more closely related to rod-shaped *P. innocuum* than to the other genera of Gram-positive cocci, rods and nocardioforms with high mol% G + C contents.

The description of *Luteococcus japonicus* as described by *Tamura* et al. (1994) is as follows: cells are spherical and 0.7 to 1.0 μm in diameter and occur singly, in pairs, or in tetrads. Endospores are not formed. Gram-positive. Colonies are circular and smooth and may be cream coloured to yellow. Facultative anaerobic. Catalase and oxidase positive. Urease negative. The cells do not reduce nitrate to nitrite. Oxidation-fermentation is fermentative. Acid is produced from D-glucose, D-ribose, D-galactose, D-mannose, D-fructose, sucrose, maltose, trehalose, raffinose, glycerol, mannitol, inositol and L-arabinose but not from D-xylose, D-arabinose or L-rhamnose. Starch is hydrolysed. Tween 20, 40, 60 and 80 are not hydrolysed. Propionic acid is formed from glucose as a major product. Optimum growth temperature is 26 to 28 C.

Cell wall peptidoglycan contains LL-diaminopimelic acid, alanine, glycine and glutamic acid (ca 1:2:1:1). The major menaquinone is MK-9(H_4). Mycolic acid is not

present. The major cellular fatty acid is 16:1 and among the minor components a small amount of 2-OH iso-18:0 is also present. Arabinose is present as a diagnostic sugar in the cell wall. As polar lipids, phosphatidylinositol, diphosphatidylglycerol and phosphatidylglycerol are present. The DNA base composition of the type strain is 67% as determined by HPLC. Distribution: soil and water. The type species is *Luteococcus japonicus* (IFO 12422).

A comparison of the phenotypic characteristics as described by *Tamura* et al. (1994) to those as obtained for "*P. coccoides*" during this study, as well as those published by *Vorobjeva* et al. (1983), revealed that the only difference between *L. japonicus* and the two "*P. coccoides*" strains examined during this study, was the inability of the two "*P. coccoides*" strains to ferment inositol. Nitrate reduction was also determined to be positive for "*P. coccoides*" (KM 375) and yet negative for both "*P. coccoides*" (KM 252) and *L. japonicus* (ATCC(R) 51527).

With the results obtained and presented in this study, it is evident that the "*P. coccoides*" species closely resembles the species description of *Luteococcus japonicus*. Controversy, however, still exists concerning the spelling of the genus *Luteococcus* and according to rule 61 of International Code of Nomenclature of Bacteria the spelling should be changed to *Luteicoccus* (L. adj. *Luteus a um*, yellow; Gr. N. *coccus*, grain, berry; M.L. masc. N. *Luteicoccus*, yellow coccus). Until this controversy is resolved it is, however, proposed that the two "*P. coccoides*" strains examined during this study, namely "*P. coccoides*" (KM 252) and "*P. cocoides*" (KM 375) be reclassified as "*Luteococcus japonicus* subspecies *casei*" and "*Luteococcus japonicus* subspecies *tyrophilus*", respectively. A thorough description of these two subspecies as determined during this study and as described by *Vorobjeva* et al. (1983), is as follows:

Description of "*Luteococcus japonicus* subspecies *casei*" subsp. nov.

Cells are Gram-positive cocci, facultative anaerobic, non-motile, non-spore-forming and are spherical at all stages of growth. Colonies on YEL plates are usually opaque, circular and slightly raised yellow coloured cells. The organism is a facultative anaerobe which ferments lactate with the production of propionate, acetate

and CO_2 as principal end products, apparently by the succinate-methylmalonyl CoA pathway. It exhibits catalase, superoxide dismutase, peroxide, arginine dihydrolase, lysine dihydrolase, ornithine dihydrolase and urease activity. Citrate is not utilised and no ortho-nitro-phenyl-galactoside, tryrophane desaminase, indole, acetoin production, gelatin liquefaction, L-diaminopimelic acid and meso-diaminopimelic acid activity is detectable. Acid is produced from glycerol, erythritol, D-arabinose, galactose, D-glucose, D-fructose, D-mannose, mannitol, sorbitol, maltose, melibiose, trehalose, inulin, melezitose, D-raffinose, amidon, glycogene, D-turanose, D-arabitol and L-arabitol. Acid is not produced from L-arabinose, ribose, D-xylose, L-xylose, adonitol, β-methyl xyloside, L-sorbose, rhamnose, dulcitol, inositol, α-methyl-D-mannose, α-methyl-D-glucoside, N-acetyl-glucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, lactose, xylitol, β-gentiobiose, D-lyxose, D-tagatose, gluconate, 2 keto-gluconate and 5 keto-gluconate. Growth most rapid at 22-30°C, but also down to 8-10°C. This bacterium is also halo-tolerant up to 6.5% NaCl. The designated type species is "*Luteococcus japonicus* subspecies *casei*" (KM 252). The mol% G+C content of the DNA is 63.4 mol% (T_m).

Description of "*Luteococcus japonicus* subspecies *tyrophilus*" subsp. nov.

Cells are Gram-positive cocci, facultative anaerobic, non-motile, non-spore-forming and are spherical at all stages of growth. Colonies on YEL plates are usually opaque, circular and slightly raised yellow coloured cells. The organism was facultative anaerobe which ferments lactate with the production of propionate, acetate and CO_2 as principal end products, apparently by the succinate-methylmalonyl CoA pathway. It exhibits catalase, superoxide dismutase and peroxide activity. Citrate is not utilised and no arginine dihydrolase, lysine dihydrolase, ornithine dihydrolase, urease, ortho-nitro-phenyl-galactoside, tryrophane desaminase, indole, acetoin production, gelatin liquefaction, L-diaminopimelic acid and meso-diaminopimelic acid activity is detectable. Acid is produced from glycerol, erythritol, D-arabinose, galactose, D-glucose, D-fructose, D-mannose, mannitol, sorbitol, maltose, trehalose, melezitose, D-raffinose, D-turanose and D-arabitol. Acid is not produced from L-arabinose, ribose, D-xylose, L-xylose, adonitol, β-methyl xyloside, L-sorbose, rhamnose, dulcitol, inositol, α-methyl-D-mannose, α-methyl-D-glucoside, N-acetyl-

glucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, lactose, melibiose, inulin, amidon, glycogene, xylitol, β-gentiobiose, D-lyxose, D-tagatose, L-arabitol, gluconate, 2 keto-gluconate and 5 keto-gluconate. Growth is most rapid at 22-30°C, but growth also occurs at 8-10°C. This bacterium is halo-tolerant up to 6.5% NaCl. The designated type species is "*Luteococcus japonicus* subspecies *tyrophilus*" The mol% G+C content of the DNA is 63.4 mol% (T_m).

Based on the results as presented during this study it is recommended that future research be conducted in the following directions:

a) More diverse ecological niches be examined for the presence of *Luteococcus japonicus* strains. This will increase the diversity of the strains available for further phenotypic and molecular studies;

b) The phenotypic relatedness between the various *L. japonicus* strains should be re-examined using more phenotypic properties;

c) A more extensive DNA:DNA hybridisation study, encompassing more strains of *Luteococcus japonicus* and the classical *Propionibacterium* species should be undertaken;

d) The significance and distribution of *Luteococcus japonicus* strains in the dairy industry should be investigated; and that

e) Species specific probes, based on the 16S rRNA gene sequence data should be developed. This would enable the rapid identification and enumeration of the genus *Luteococcus*.

References

Baer, A., Ryba, I.: Identification rapide des bactéries propioniques. Schweiz. Milchw. Forschung. 17, 56-57 (1988).

Baer, A., Ryba, I.: Serological identification of propionibacteria in milk and cheese samples. Int. Dairy J. 2, 299-310 (1992).

Britz, T.J., Riedel, K-H.J.: A numerical taxonomic study of *Propionibacterium* strains from dairy sources. J. Appl. Bacteriol. 71, 407-416 (1991).

Britz, T.J., Riedel, K-H.J.: Propionibacterium species diversity in Leerdammer cheese. Int. J. Food. Microbiol. 22, 257-267 (1994).

Charfreitag, O., Collins, M.D., Stackebrandt, E.: Reclassification of *Arachnia propionica* as *Propionibacterium propionicus* comb. nov. Int. J. Syst. Bacteriol. 38, 354-357 (1988).

Cummins, C.S., Johnson, J.L.: Genus *Propionibacterium* Orla-Jensen 1909, pp. 1346-1353. In: Bergey's Manual of Systematic Bacteriology (P.H.A. Sneath, N.S. Mair, M.E. Sharpe, J.G. Holt, eds.) vol. 2. Baltimore, Williams and Wilkins 1986.

Cummins , C.S., Johnson, J.L.: The Genus *Propionibacterium*, pp. 834-849. In: The Prokaryotes (A. Balows, H.G. Truper, M. Dworkin, W. Harder and K-H. Schleifer, eds.) vol. 1. New York, Springer-Verlag 1992.

Cummins, C.S., Moss, C.W.: Fatty acid composition of *Propionibacterium propionicum* (*Arachnia propionica*). Int. J. Syst. Bacteriol. 40, 307-308 (1990).

Holt, J.G., Krieg, N.R., Sneath, P.H., Staley, J.T., Williams, S.T.: Genus *Propionibacterium*, p. 580. In: Bergey's Manual of Determinative Bacteriology (J.G. Holt, N.R. Krieg, P.H. Sneath, J.T. Staley, S.T. Williams, eds.) 9th ed., Baltimore, Williams and Wilkins 1994.

Johnson, J.L., Cummins, C.S.: Cell wall composition and deoxyribonucleic acid similarities among the anaerobic coryneforms, classical propionibacteria, and strains of *Arachnia propionica*. J. Bacteriol. 109, 1047-1066 (1972).

Moore, W.E.C., Holdeman, L.V.: Genus 1. *Propionibacterium* Orla-Jensen 1909, pp. 633-641. In: Bergey's Manual of Determinative Bacteriology (R.E Buchanan, N.E. Gibbons, eds.) 8th ed., Baltimore, Williams and Wilkins 1974.

Oda, M.: Bacteriological studies on water used for brewing sake (part 6). I. Bacteriological studies on "miyamizu." *Micrococcus* and *Actinomyces* isolated from "miyamizu." Jozogaku Zasshi 13, 1202-1228 (1935). (As cited by *Tamura* et al., 1994).

O'Donnell, A.G., Minnikin, D.E., Goodfellow, M., Parlett, J.H., Schofield, G.M., Schaal, K.P.: Lipid and wall amino acid composition in the classification and identification of *Arachnia propionica*. Zentralbl. Bakteriol. Parasitenk. Infekt. Hyg. Abt. I 260, 300-310 (1985). (As cited by *Cummins* and *Moss*, 1990).

Reinbold, G.W.: The propionibacteria. XXX Int. Dairy Congr. Paris. 3 ST, 41-45. Brussels, International Dairy Federation 1978.

Riedel, K-H.J., Britz, T.J.: Differentiation of "classical" *Propionibacterium* species by numerical analysis of electrophoretic protein profiles. System. Appl. Microbiol. 15, 567-572 (1992).

Riedel, K-H.J., Britz, T.J.: Justification of the "classical" *Propionibacterium* species concept by ribotyping. System. Appl. Microbiol. 19, 370-380 (1996).

Riedel, K-H.J., Britz, T.J.: *Propionibacterium* species diversity in anaerobic digesters. Biodiversity and Conservation 2,400-411 (1993).

Riedel, K-H.J., Wingfield, B.D., Britz, T.J.: Combined influence of magnesium concentration and polymerase chain reaction specificity enhancers. FEMS Microbiol. Lett. 92, 69-72 (1992).

Riedel, K-H.J., Wingfield, B.D., Britz, T.J.: Justification of the "classical" *Propionibacterium* species concept by restriction analysis of the 16S ribosomal RNA genes. System. Appl. Microbiol. 17, 536-542 (1994).

Riedel, K-H.J., Wingfield, B.D., Britz, T.J.: rDNA – Restriction fragment length polymorphisms. System. Appl. Microbiol. 21, 419-428 (1998).

Riedel, K-H.J.: A comparative evaluation and justification of the "classical" *Propionibacterium* species concept. PhD. Thesis. University of the Orange Free State, South Africa 1997.

Tamura, T., Takeuchi, M., Yokota, A.: Luteococcus japonicus gen. nov., sp. nov., a new gram-positive coccus with LL-diaminopimelic acid in the cell wall. Int. J. Syst. Bacteriol. 44, 348-356 (1994).

Van Niel, C.B.: The propionic acid bacteria. Thesis. Haarlem, Delft, J.W. Boissevain & Co 1928.

Viljoen, D.C.: A taxonomic study of *Ceratocystis* sensu lato with special reference to species associated with Protea infructescences in southern africa. PhD. Thesis. University of the Orange Free State, South Africa 1996.

Von Freudenreich, E., Orla-Jensen, S.: Über die in Emmentalerkäse stattfindende Propionisäuregarung. Zentralbl. Bakteriol. Parasitenk. Infekt. Hyg. Abt. II 17, 529-546 (1906). (As cited by *Van Niel*, 1928)

Vorobjeva, L.I., Turova, T.P., Kraeva, N.I., Alekseeva, M.A.: Propionic acid cocci and their systematic position. Microbiol. 52, 368-373 (1983).

Chapter 6

Summary

A study was undertaken to re-evaluate the systematic position of "*Propionibacterium coccoides*" relative to that of other classical *Propionibacterium* species. Two "*P. coccoides*" strains were evaluated by numerically relating them to the type and reference strains of the genus *Propionibacterium* as well as the type strain of the genus *Luteococcus* (as outgroup) by comparison of a wide range of phenotypic characters. Numerical clustering revealed five major groups, four of which corresponded to the existing classical species, while the fifth cluster grouped the two "*P. coccoides*" strains with the type strain of the species *Luteococcus japonicus*. These results clearly indicated that a higher degree of overall phenotypic similarity existed between *L. japonicus* and the "*P. coccoides*" strains than between the latter and the genus *Propionibacterium*.

Using the polymerase chain reaction, the various 16S ribosomal RNA genes of the four *Propionibacterium* type strains, the two "*P. coccoides*" strains and the type strain of *L. japonicus* were successfully amplified. Visual differentiation between the four classical *Propionibacterium* type strains was possible after restriction endonuclease digestion of the PCR products obtained using primers 16sP1-16sP4 with the restriction endonucleases *Hae*III and *Alu*I. Although a unique pattern was obtained for the two "*P. coccoides*" and the *L. japonicus* strains when compared to those as obtained for the four classical *Propionibacterium* type strains, differentiation between the two "*P. coccoides*" and the *L. japonicus* strains, even after the evaluation of numerous restriction endonucleases, was essentially still problematic.

Due to the success obtained during the application of ribotyping as a technique to enable species differentiation within the genus *Propionibacterium*, this technique was also utilised during this study. The combination of the *Hae*II and *Sma*I restriction endonuclease patterns resulted in the elucidation of the four classical *Propionibacterium* type strains as well as the two "*P. coccoides*" and the *L. japonicus* strains. A unique yet identical ribotyping profile was obtained for the two "*P. coccoides*" strains and the *L. japonicus* type strain. In an attempt to justify the

phenotypic heterogeneity observed between the two "*P. coccoides*" strains and the *L. japonicus* type strain, numerous other restriction endonucleases were also evaluated using ribotyping. The two "*P. coccoides*" strains and the *L. japonicus* type strain could only be differentiated after digestion of the genomic DNA with the restriction endonuclease *Bst*EII.

The intrageneric phylogenetic relationship of the four classical *Propionibacterium* type strains, the two "*P. coccoides*" strains as well as the *L. japonicus* type strain and various other phylogenetic related genera was determined by comparative analysis of their 16S ribosomal RNA gene sequence data. The "*P. coccoides*" strains were observed to cluster together with the type strain of the genus *Luteococcus* in a clade which was phylogenetically clearly delineated from the cluster containing the various *Propionibacterium* species. This data subsequently confirmed the results obtained during the numerical analysis of the phenotypic characteristics.

Finally DNA:DNA probe hybridisation was evaluated as a possible reliable species differentiation technique. Five major DNA:DNA homology groups could be distinguished. Four of these homology groups resembled the various classical *Propionibacterium* species, while the fifth DNA:DNA homology group consisted out of the "*P. coccoides*" strains and the *L. japonicus* type strain. The high degree of DNA:DNA homology between the "*P. coccoides*" strains and the *L. japonicus* type strain subsequently confirmed the phylogenetic analysis of the 16S rDNA sequence data.

The results of this study subsequently indicate that the proposal by Vorobjeva et al. (1983) to include "*P. coccoides*" as a new species within the genus Propionibacterium is incorrect due to the higher degree of phenetic, molecular and phylogenetic similarity between *Luteococcus japonicus* and "*P. coccoides*" than between the latter and the genus Propionibacterium. It is subsequently proposed that the "*P. coccoides*" strains be putatively reclassified as *Luteococcus japonicus* subspecies *casei* and *Luteococcus japonicus* subspecies *tyrophilus* and that the genus description of *Luteococcus* be revised to include the phenetic and molecular description of these strains.

Hoofstuk 6

Samevatting

'n Studie was onderneem om die sistematiese posisie van "*Propionibacterium coccoides*" te herevalueer relatief tot dié van ander klassieke *Propionibacterium* spesies. Twee "*P. coccoides*" stamme was geevalueer deur hulle numeriese verwantskappe aan die tipe- en verwysingsstamme van die genus *Propionibacterium* sowel as the tipe stam van die genus *Luteococcus* (as buite-groep) te bepaal, deur 'n verskeidenheid fenotipiese karakters te vergelyk. Numeriese groepering het op vyf hoofgroepe gedui, waarvan vier ooreenstem met die bestaande klassieke spesies en die vyfde groepering wat die twee "*P. coccoides*" stamme saamgroepeer met die tipe stam van die spesie *Luteococcus japonicus*. Hierdie resultate dui duidelik aan dat 'n hoër graad van algehele ooreenkoms tussen *L. japonicus* en die "*P. coccoides*" stamme bestaan, as tussen laasgenoemde en die genus *Propionibacterium*.

Deur gebruik te maak van die polimerase ketting reaksie (PKR), was die verskeie 16S ribosomale RNA gene van die vier *Propionibacterium* tipe stamme, die twee "*P. coccoides*" stamme en die tipe stam van *L. japonicus* suksesvol vermeerder. Visuele differensiasie tussen die vier klassieke *Propionbacterium* tipe stamme was moontlik na vertering van die PKR produkte verkry, deur gebruik te maak van die voorvoerders 16sP1-16sP4, met die endonukleases *Hae*III en *Alu*I. Alhoewel 'n unieke profiel verkry is vir die twee "*P. coccoides*" en die *L. japonicus* stamme, as dit vergelyk word met dié verkry vir die vier klassieke *Propionibacterium* tipe stamme, was die differensiasie tussen die twee "*P. coccoides*" en die *L. japonicus* stamme, selfs na evaluasie van verskeie restruksie endonukleases, essensieel steeds problematies.

As gevolg van die sukses behaal gedurende die aanwending van ribotipering as tegniek om spesies differensiasie in staat te stel binne die genus *Propionibacterium*, was hierdie tegniek ook aangewend gedurende hierdie studie. Die kombinasie van die *Hae*II en *Sma*I restruksie endonuklease pofiele het die verduideliking van die vier klassieke *Propionibacterium* tipe stamme asook die twee "*P. coccoides*" en die *L. japonicus* stamme tot gevolg gehad. 'n Unieke tog identiese ribotiperings profiel was verkry vir die twee "*P. coccoides*" stamme en die *L. japonicus* tipe stam. In 'n

poging om die fenotipiese heterogenitiet waargeneem tussen die twee "*P. coccoides*" stamme en die *L. japonicus* tipe stam te verdedig, was talryke ander restruksie endonukleases ook geevalueer deur die gebruik van ribotipering. Die twee "*P. coccoides*" stamme en die *L. japonicus* tipe stam was slegs differensieerbaar na snyding van die genomiese DNA met die restruksie endonuklease *Bst*EII.

Die intrageneriese filogenetiese verwantskap van die vier klassieke *Propionibacterium* tipe stamme, die twee "*P. coccoides*" stamme asook die *L. japonicus* tipe stam en verskeie ander filogeneties verwante genera is bepaal deur vergelykende analise van hulle 16S ribosomale RNA geenopeenvolging. Die "*P. coccoides*" stamme is bepaal om saam te groepeer met die tipe stam van die genus *Luteococcus* in 'n groep wat filogeneties duidelik afbeeldbaar was van die groepering wat die verskeie *Propionibacterium* spesies bevat. Hierdie data bevestig vervolgens die resultate verkry gedurende die numeriese analise van die fenotipiese karaktertrekke.

Ten slotte is DNA:DNA kruising ondersoek as 'n moontlike betroubare spesies differensieerbare tegniek. Vyf hoof DNA:DNA homologie groepe was onderskeibaar. Vier van hierdie groepe kom met die verskeie klassieke *Propionibacterium* spesies ooreen, terwyl die vyfde DNA:DNA homologie groep uit die "*P. coccoides*" stamme en die *L. japonicus* tipe stam bestaan. Die hoë graad van DNA:DNA homologie tussen die "*P. coccoides*" stamme en die *L. japonicus* tipe stam bevestig vervolgens die filogenetiese analise van die 16S rDNA geenopeenvolging data.

Die resultate van hierdie studie dui eerstens aan dat die voorstel van *Vorobjeva* et al. (1983) om "*P. coccoides*" in te sluit as 'n nuwe spesie binne die genus *Propionibacterium* verkeerd is as gevolg van die hoë graad van fenetiese, molekulêre en filogenetiese ooreenkomste tussen *Luteococcus japonicus* en "*P. coccoides*" as tussen laasgenoemde en die genus *Propionibacterium*. Gevolglik word dus voorgestel dat die "*P. coccoides*" stamme putatief heringedeel word as *Luteococcus japonicus* subspecies *casei* en *Luteococcus japonicus* subspecies *tyrophilus* en dat die genus beskrywing van *Luteococcus* hersien moet word om die fenetiese en molekulêre beskrywing van hierdie stamme in te sluit.