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Molecular Identification and Applied Genetics of Propionibacteria

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SUMMARY

Propionibacteria have been found so far mainly in dairy products and on the skin of humans and animals. Due to the absence of real selective media, the isolation and identification of propionibacteria is difficult and time consuming. Because both groups of propionibacteria are of a certain importance, either as dairy starter cultures or as potential pathogens, a faster identification process is necessary. In addition, correct identification is needed as a basis for further molecular studies with propionibacteria.

The taxonomic situation of the genus *Propionibacterium* was reevaluated by the construction of a phylogenetic tree based on 16S rDNA sequences of all type strains. When necessary, the complete 16S rDNA sequences were determined in this thesis (two classical and four medically relevant species) or existing database entries were completed.

A rapid molecular method based on multiplex PCR (MPCR) was developed to determine whether unknown bacteria belong to the genus *Propionibacterium* or not. Briefly, DNA from a pure bacterial culture is isolated using a short method (mediated by SDS and Proteinase K) and amplified by MPCR. A specific gene probe for propionibacteria, gd1 (5'-TGCTTTCGATACGGGTTGAC-3') is used in the MPCR assay. This probe hybridizes only with the 16S rDNA (*E. coli* positions 632-651, V4 variable region) of strains belonging to the genus *Propionibacterium*. Propionibacteria are present in an isolate when a specific 900-bp (basepairs) sized DNA fragment is amplified in the MPCR assay. When "foreign" bacteria (not belonging to the genus *Propionibacterium*) occur in the sample, a 1500-bp sized fragment is amplified. This fragment is used as an internal control; as a proof that the MPCR analysis was performed correctly, but that no detectable amounts of propionibacteria were present in a sample. For pure cultures of propionibacteria, the detection limit was at 2 x 10^3 cfu per ml in a broth culture.

The MPCR method can be further used for a rapid screening for propionibacteria in samples, e.g. in raw milk or in clinical specimens. Due to the simple sample preparation, a large number of new habitats can now be investigated rapidly for the presence (or absence) of propionibacteria.

After determining the genus of isolates, the next step is the identification to species level. In this thesis, a molecular approach using partial sequencing of the 16S rDNA genes of propionibacteria was selected. *Propionibacterium* isolates were classified to their corresponding species by comparing the obtained 16S rDNA sequences to existing database entries (e.g. GenEmbl). The whole analysis was performed within two days.

In addition, a method for the identification of *P. freudenreichii*, the most relevant classical species, was developed. By using the PCR-amplified 16S-23S rDNA intergenic spacer region of the *P. freudenreichii* type strain as hybridization probe, all strains of our collection belonging to this species were detected. The same principle is proposed in this thesis for the identification of all other strains of the genus but this was not further investigated.
The *P. freudenreichii* species has been separated by certain researchers into two subspecies (*freudenreichii* and *shermanii*). Neither the 16S nor the 23S rDNA sequences of the type strains of both subspecies differed significantly in their base compositions. Therefore the separation of *P. freudenreichii* into subspecies should be abandoned.
For the genetic analysis of propionibacteria, plasmids suitable as cloning vectors were isolated. These plasmids are needed for the development of a genetic system with propionibacteria. Two plasmids, pLME101 and pLME108 were selected and further analyzed.
From *P. freudenreichii* JS53 plasmid pLME101 (40-kb sized) was isolated and a restriction map was constructed. Partial sequences (approximately 3 kb) of pLME101 were obtained using shotgun cloning. For one sequence fragment, an amino acid sequence identity of 53.2% with a hypothetical protein from *Synechocystis* sp. was found.
The complete nucleotide sequence of plasmid pLME108 (2051 bp), isolated from *P. freudenreichii* DF2, was determined. Two putative protein coding regions, *orf2* and *rep*, were identified. *Orf2* has no significant homologies with already known protein sequences. The *rep* region shows 42.1% amino acid identity with the Rep protein of plasmid pAP1 from *Arcanobacterium pyogenes*. Specific amino acid motifs needed for a replication by the rolling circle (RC) mechanism were found on pLME108 and led to the conclusion that this plasmid also replicates by the RC mode.
A genetic system with propionibacteria requires the investigation of DNA transfer methods like electroporation and conjugation. Both techniques were tested for propionibacteria, but in no experiment transformants were obtained. Either the conditions and plasmids used in this thesis were not suitable or complete new approaches for the genetic modification of propionibacteria have to be established.

ZUSAMMENFASSUNG

Propionibakterien wurden vor allem aus Milchprodukten und von der Haut von Menschen und Tieren isoliert. Das Fehlen eines echten Selektivmediums erschwert und verzögert die Isolierung und Identifizierung von Propionibakterien. Von Bedeutung sind Propionibakterien entweder als Starterkulturen in der Milchtechnologie oder als potentielle Pathogene. Zu ihrer raschen Identifikation wird eine Methode benötigt, die im weiteren auch als Basis für molekulargenetische Arbeiten mit Propionibakterien dienen soll.

Die taxonomische Situation innerhalb des Genus *Propionibacterium* wurde durch die Konstruktion eines phylogenetischen Stammbaums reevaluiert, der auf den 16S rDNS Sequenzen aller Typstämme basiert. Falls notwendig wurden in dieser Arbeit komplette 16S rDNS Sequenzen bestimmt (für zwei Spezies der klassischen Propionibakterien, sowie für vier medizinisch relevante Spezies) oder bestehende Sequenzen komplettiert.

Es wurde eine molekularbiologische Schnellmethode basierend auf „multiplex PCR“ (MPCR) entwickelt, die es ermöglicht, die Zugehörigkeit unbekannter Bakterien zum Genus *Propionibacterium* zu bestimmen. DNS aus einer Reinkultur wird mit einer Schnellmethode isoliert (unter Verwendung von SDS und Proteinase K) und mittels MPCR vermehrt. Eine spezifische Gensonde für Propionibakterien, gd1 (5‘-TGCTTTCGATA-CGGGTTGAC-3‘) findet in dieser MPCR Methode Verwendung. Diese Sonde hybridisiert nur mit der 16S rDNS von Stämmen die zum Genus *Propionibacterium* gehören (*E. coli* Positionen 632-651 der V4-variablen Region). Propionibakterien sind in einer Probe vorhanden, wenn ein spezifisches DNS-Fragment einer Länge von 900 Basenpaaren (Bp) mit der MPCR Methode amplifiziert wird. Treten Fremdkeime, die nicht zum Genus *Propionibacterium* gehören, in einer Probe auf, so wird nur ein DNS-Fragment mit der Länge von 1500 Bp gebildet. Dieses Fragment dient der internen Kontrolle der Methode und gilt als Beweis dafür, dass der MPCR-Nachweis korrekt durchgeführt wurde, aber eben keine Propionibakterien in der Probe vorhanden waren. Die Nachweisgrenze für Reinkulturen von Propionibakterien lag bei 2 x 10^3 koloniebildenden Einheiten pro ml einer Flüssigkultur.

Im weiteren kann die MPCR-Methode für ein schnelles „Probenscreening“ eingesetzt werden, z.B. in Rohmilch oder in klinischen Isolaten. Da die Probenaufarbeitung sehr einfach ist, können viele verschiedene neue Habitate nun auf das Vorhandensein von Propionibakterien untersucht werden.

Der nächste Schritt nach der Bestimmung des Genus eines Isolates ist die Identifikation

bis auf Spezies-Ebene. In dieser Arbeit wurde ein molekularbiologisches Vorgehen ausgewählt, das auf der partiellen Sequenzierung der 16S rDNS Gene von Propionibakterien basiert. Durch Vergleichen der gewonnen 16S rDNS Sequenzen mit existierenden Datenbankeinträgen (z.B. GenEmbl) wurden *Propionibacterium* Isolate den ihnen entsprechenden Spezies zugeordnet. Diese gesamte Analyse kann innerhalb zweier Tage durchgeführt werden.

Zusätzlich wurde eine Methode zur Identifikation des wichtigsten Vertreters der klassischen Spezies, *P. freudenreichii,* entwickelt. Durch Verwendung der mittels PCR vermehrten 16S-23S rDNS „Spacer-Region" des Typstamms von *P. freudenreichii* als Hybridisierungs-Sonde konnten alle Stämme dieser Spezies aus unserer Laborstammsammlung korrekt identifiziert werden. Für die Identifikation aller anderen Stämme des Genus kann möglicherweise das gleiche Prinzip angewendet werden, dies wurde aber nicht weiter verfolgt.

Die Spezies *P. freudenreichii* wird von verschiedenen Forschern in zwei Subspezies aufgeteilt (*freudenreichii* und *shermanii*). Weder die 16S noch die 23S rDNS Sequenzen der Typstämme beider Subspezies unterschieden sich wesentlich in ihrer Basenzusammensetzung. Darum wird in dieser Arbeit vorgeschlagen, dass die Trennung von *P. freudenreichii* in zwei Subspezies fallen gelassen wird.

Um ein genetisches Arbeiten mit Propionibakterien zu ermöglichen, wurden als Erstes Plasmide zur Konstruktion von Klonierungsvektoren isoliert. Diese Plasmide werden zur Entwicklung eines genetischen Systems für Propionibakterien benötigt. Zwei Plasmide, pLME101 und pLME108 wurden in der Folge ausgewählt und näher untersucht.

Aus *P. freudenreichii* JS53 wurde das Plasmid pLME101 (40 kb) isoliert und eine Restriktionskarte erstellt. Teilsequenzen von pLME101 (ca. 3 kb) wurden durch „Shotgun Cloning" gewonnen. Die Sequenz eines Fragments zeigte auf Aminosäureebene eine Identität von 53.2% mit einem hypothetischen Protein von *Synechocystis* sp.

Für das Plasmids pLME108 (2051 bp), isoliert aus *P. freudenreichii* DF2, wurde die vollständige Nukleotidsequenz bestimmt. Zwei möglicherweise für Proteine kodierende Regionen, *orf2* und *rep,* wurden identifiziert. *Orf2* weist keine grösseren Ähnlichkeiten mit bereits bekannte Proteinsequenzen auf, während die *rep* Region 42.1% Aminosäure-Identität mit dem Rep Protein des Plasmids pAP1 aus *Arcanobacterium pyogenes* aufweist. Spezielle Aminosäuremuster, die für die Replikation durch den „rolling circle" (RC) Mechanismus benötigt werden, konnten innerhalb der *rep* Region des Plasmids pLME108

identifiziert werden. Dies führte zum Schluss, dass dieses Plasmid ebenfalls den RC-Mechanismus für seine Replikation verwendet.
Die Entwicklung eines genetischen Systems für Propionibakterien setzt im Weiteren die Untersuchung von DNS Transfermethoden wie Elektroporation und Konjugation voraus. Beide Techniken wurden mit Propionibakterien getestet, aber Transformanten konnten in keinem Experiment erzielt werden. Entweder waren die verwendeten Bedingungen und Plasmide in dieser Arbeit nicht geeignet oder vollständig neue Ansätze zur genetischen Modifikation von Propionibakterien müssen entwickelt werden.

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1. INTRODUCTION

1.1 A short history of propionibacteria

In 1906, von Freudenreich and Orla-Jensen (1906) isolated bacteria from Emmental cheese which they named *Bacterium acidi-propionici* and *Bacillus acidi-propionici*. These organisms were characterized by their ability to produce propionic acid and the authors suspected them to be responsible for the typical eye formation of that kind of cheese. The genus *Propionibacterium* was first proposed by Orla-Jensen (1909) for these bacteria.

The inclusion of *Corynebacterium acnes* into the genus *Propionibacterium* as *Propionibacterium acnes* was first proposed by Douglas and Gunter (1946) and reconfirmed by Moore and Cato (1963).

1.2 Characteristics and occurrence of propionibacteria

Propionibacteria are Gram-positive and rodshaped ("coryneform") bacteria which prefer microaerophilic to anaerobic growth conditions. The G+C content of their genome ranges from 53 to 67 mol% (Cummins and Johnson, 1986 & Kusano et al., 1997) which places them in the high G+C group of bacteria (Olsen et al., 1994).

The most characteristic and common property of the propionibacteria is the production of propionic and acetic acid as main metabolites from lactic acid as substrate according to the formula: 3 lactate = 2 propionate + acetate + CO_2 + H_2O.

1.2.1 Classical propionibacteria

The classical or dairy propionibacteria are found mainly in raw milk and in dairy products like buttermilk and especially in Swiss-type cheeses, where they are important in the ripening process (Cummins and Johnson, 1992). In modern cheese making, propionibacteria are of great importance as wild types strains or as starter cultures, utilized to obtain a full flavored and well textured Swiss-type cheese. They are responsible for the typical eyes that are characteristic for that kind of cheese. Propionibacteria contribute also to the flavor of these products, mainly by the production of propionic and acetic acid. Propionibacteria were found in honey and maize and as spoilage organisms in olives (Plastourgos and Vaughn, 1957) and orange juice (Kusano et al., 1997). The latter contained *Propionibacterium cyclohexanicum*, an organism that is

highly resistant to heating (survival of 10 minutes at 90°C). Further, classical propionibacteria are found in silage, where they are added sometimes also as preservants (Weinberg et al., 1995). Vegetable products like "sauerkraut" or red beet juice (Babuchowski et al., 1998.), sekete (Adegoke et al., 1995), a Nigerian beverage made from maize, have been improved or made with propionibacteria. Propionibacteria are also discussed for the industrial production of vitamin B_{12} and propionic acid (Cummins and Johnson, 1992; Glatz 1992).

The ability of certain propionibacteria to produce substances inhibitory to the growth of other bacteria or yeasts and moulds has also been investigated (Al-Zoreky et al., 1991; Hsieh et al., 1996a, 1996b; Grinstead and Barefoot, 1992). The study of substances like bacteriocins is currently one of the major research areas concerning propionibacteria.

In a study, Sidorchuk and Bondarenko (1984) inoculated milk with a mutant strain of *P. freudenreichii*, which was multiresistant to antibiotics and produced high amounts of vitamins B_1, B_2, B_{12} and C. This fermented milk was administered together with an antibiotic treatment to children (age of 4 to 18 months) with symptoms of enteral dysbacteriosis. Reconvalescence time for children supplied with this milk was shorter than for children of the control group.

1.2.2 "Cutaneous" propionibacteria

The first isolates of the cutaneous propionibacteria were obtained from the human skin. They have also been found in feces, in the mouth and on moist parts of the skin of humans and animals (Cummins and Johnson, 1992). The cutaneous strains seem to be involved in certain human diseases (for example acne, endophthalmitis, endocarditis). Because these organisms are part of the natural skin flora, it is often difficult to determine whether they are the cause of an infection or have been isolated as contaminants during the sampling procedure. As an example, *Propionibacterium acnes* has been found at densities of 10^5-10^6 cfu/cm^2 in body areas rich with sebaceous glands (scalp, face) and at 10^2 cfu/cm^2 on other parts of the skin (Leyden et al., 1998). Recent publications (Funke et al., 1997, Jakab et al., 1996) indicate that propionibacteria are indeed the cause of certain infections. In the case of acne, which is the most common and best known disease connected with propionibacteria, *P. acnes* is clearly involved but only in combination with other factors (Leyden et al., 1998). Mainly in cases where an invasive treatment is carried out, propionibacteria can cause serious infections in the human body (Jakab et al., 1996; Tunney et al., 1998).

Strains of *P. acnes* (often named *Corynebacterium parvum* in this context, a name no longer valid), or of *P. avidum* (e.g. Pottkämper et al., 1996; Randerath et al., 1997) are used as immunostimulants (for animals and humans). In these studies, cells were heat and formaldehyde inactivated, washed, lyophilized and orally administered to the test persons to achieve an activation of their immune systems.
In addition bacteriocin production has been described for *P. acnes* by Fujimura and Nakamura (1978) and by Paul and Booth (1988).

1.3 Taxonomy and its relevance in food microbiology

Microorganisms play an important role in food microbiology because they are involved in various productive and destructive processes in food. As spoilage organisms they make food unsuitable for the consumption and are responsible for substantial financial losses. An even worse scenario is the occurrence of pathogenic microorganisms that contaminate food and endanger the health of consumers. On the other side, microorganisms are used to preserve or produce food. Products like bread, wine, beer and cheese would not exist without them.
The clear identification of microorganisms from and in food is necessary to asses the risk-potential for the consumer, to achieve minimal losses during the production process and to ensure an optimal product quality. The use of defined and well known strains for fermentation processes includes also methods for the identification of these strains, which has become an important task of food microbiology.
The basis to identification is the taxonomic position of a microorganism. Organisms that are related have more common characteristics than non-related ones. In the classical taxonomy, phenotypic features like cell morphology, carbohydrate fermentation patterns and the production of primary and secondary metabolites were used as a key for the classification of an organism. Theses features are highly dependent on the environment and can change for example when bacteria are under stress, leading to faulty identification of isolates. In modern taxonomy, information about the genotype, especially data obtained from the analysis of conserved DNA regions like the genes for 16S or 23S rDNA of bacteria, is also taken into consideration (Ludwig and Schleifer, 1994).

1.4 Taxonomy of the propionibacteria

Propionibacteria form a relatively homogeneous taxonomic cluster (Britz and Riedel, 1991) within the class *Actinobacteria* which was recently defined by Stackebrandt et al. (1997). This class consists mainly of organisms previously referred to as coryneform bacteria and characterized mainly by their shape (club shaped, branched) and a high G+C content of their DNA. The proposal for this class is mainly based on comparisons of 16S rDNA sequences. Propionibacteria are positioned in the suborder *Propionibacterineae* in the family of *Propionibacteriaceae*. Their closest taxonomic relatives are members of the genera *Luteococcus*, *Microlunatus* and *Propioniferax* which belong also to the family *Propionibacteriaceae*.

Various approaches to classify propionibacteria have been carried out. Recently the new species *Propionibacterium cyclohexanicum*, isolated from orange juice, has been proposed (Kusano et al., 1997), strains have been reclassified within the genus (de Carvalho et al., 1995) or were transferred from other genera (*Arachnia propionica* to *Propionibacterium propionicus*; Charfreitag et al., 1988).

The currently known and accepted species of the genus *Propionibacterium* are listed in table 1. The propionibacteria have been divided by their source of isolation (habitat) into two main groups: the dairy (or classical) and the medically relevant (or cutaneous) species. In this thesis the new species *P. cyclohexanicum* is referred to as belonging to the "classical" propionibacteria because it has been isolated from food and seems to be non-pathogenic.

Table 1: Species classified currently in the genus *Propionibacterium*

Classical propionibacteria	Cutaneous propionibacteria
P. acidipropionici	*P. acnes*
P. cyclohexanicum	*P. avidum*
P. freudenreichii	*P. granulosum*
P. jensenii	*P. lymphophilum*
P. thoenii	*P. propionicum*[1]

[1] Correct latin form of the name.

The species *P. freudenreichii* is further divided by certain authors into 2 (or 3) subspecies: *P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii* and sometimes *P. freudenreichii* subsp. *globosum* (Cummins and Johnson, 1992; Holt, 1984). This separation is based mainly on the ability (subsp. *shermanii*) or lack (subsp. *freudenreichii*) of a strain to produce acid from lactose (Cummins and Johnson, 1986).

1.5 Isolation and identification of propionibacteria

1.5.1 "Selective" media

Several media for the isolation of propionibacteria (mainly from cheese) have been proposed, the most popular being the YEL medium (yeast extract lactate) proposed by Malik et al. (1968). This medium is based on lactic acid as carbohydrate source and lacks selectivity. Because propionibacteria are relatively slow growing organisms (1 day to 14 days), contaminants will almost always grow faster on this medium. Drinan and Cogan (1992) suggest the addition of 4 µg/ml of the antibiotic cloxacillin (MIC for propionibacteria < 8 µg/ml) to the medium to inhibit the growth of the lactic acid bacteria commonly used as starter cultures in dairy products. A selective solid medium for the enumeration of propionibacteria from dairy products named PAL (Standa industries, Rouen, France) is patented and sold, but data on its selectivity in other systems (like clinical isolates) or the nature of the selective compounds are not available. In addition, PAL is rather expensive (sFr. 1.50 per plate) and the identification of propionibacteria is based on a color reaction in the medium (pink to yellow). This can lead to errors since the detection of single colony contaminants is no longer possible at high colony counts.
For the isolation of the cutaneous strains, no selective media are in use, the isolates are incubated on standard media under anaerobic conditions and identification is performed by using classical methods (von Graevenitz and Funke, 1996).

1.5.2 Identification using "classical" methods

Methods based on chemotaxonomy and colony morphology are the most commonly used procedures to identify propionibacteria (Cummins and Johnson, 1992). Care has to be taken of the fact that propionibacteria can appear Gram-variable under certain growth conditions (Beveridge, 1990). A loss of pigmentation has also been observed (Rehberger et al., 1994). The characteristic production of propionic and acetic acid is the simplest way to determine this genus. Britz and Riedel (1994) grouped propionibacteria into five

clusters using chemotaxonomy, but strains of *P. avidum* and *P. granulosum* could not be included into these clusters. Methods based on comparative protein profiles (Baer, 1987), serology and immunology have been described (Baer and Ryba, 1991 & 1992) to differentiate between the classical *Propionibacterium* species. A method using simple antibiotic resistance patterns to classify anaerobes to genus level has been proposed by Essers (1982). A method for the detection of *P. acnes* by pyrolysis mass spectrometry and artificial neural networks has been developed by Goodacre et al. (1994, 1996).

1.5.3 Identification by means of DNA analysis

Reliable and fast detection of production strains as well as wild type strains of propionibacteria is not easy to perform and can be shortened and simplified by using DNA probe techniques. In raw milk, these techniques could prove the occurrence of wild type strains that could replace the starter cultures and cause undesired side effects (for example red spots) during the ripening of cheese.

In addition, a method that allows a fast screening procedure of clinical samples to detect medically relevant strains would help to shorten the time for diagnosis.

The size of the chromosome of propionibacteria was determined using PFGE (pulsed field gel electrophoresis) of chromosomal DNA (Rehberger, 1993; Gautier et al., 1996). This method has also been used to identify some classical propionibacteria to species level.

Using ribotyping, Riedel and Britz (1996) and de Carvalho et al. (1994) achieved differentiation between classical species. Further, the use of RAPD (random amplified polymorphic DNA) has also been investigated to achieve species discrimination (Fessler, 1997), although RAPD has been shown to be a rather irreproducible method (Jones et al., 1997).

Methods based on the amplification of target genes with PCR and RFLP (restriction fragment length polymorphisms) of the resulting fragments have been designed: restriction analysis of the 16S-23S rDNA intergenic spacer region was proposed by Rossi et al. (1997). Fessler et al. (1998) used restriction of the 23S rDNA insertion region typically found in the "high G+C Gram-positives" (Roller et al., 1992) The restriction analysis of the 16S rDNA gene was investigated by Riedel et al. (1994 & 1998). All these methods were successful for the classical species.

Methods based on specific gene probes targeted on the 16S rDNA for use in hybridization or PCR experiments have been developed for classical propionibacteria by Rossi et al.

(1998) and for medically relevant propionibacteria by Greisen et al. (1994) (patent application) and by Hykin et al. (1994).

All these techniques have been used to detect and identify either cutaneous or classical strains of the genus *Propionibacterium.* A method that allows the detection of the whole genus has yet not been described. One of the aims of this thesis is therefore the development of a simple identification method for the genus *Propionibacterium.*

1.6 Molecular genetics of propionibacteria

1.6.1 Plasmids and phages

Plasmids in dairy propionibacteria have been isolated by various researchers and characterized by restriction analysis (Rehberger and Glatz, 1990; Pérez-Chaia et al., 1988; Frère, 1994), no sequences of plasmids exist so far. Different functional characteristics were screened for (e.g. carbohydrate metabolism, proteolytic activity, production of inhibitory substances, drug resistance, inorganic ion resistance) to determine plasmid-encoded properties (Gautier and Rouault, 1990). The function of most plasmids could not be elucidated at the molecular level. There is only weak evidence for plasmid-associated cell aggregation, and only the ability to ferment lactose has been described (Rehberger and Glatz, 1987 & 1990) so far.

The plasmid pRGO1 (Rehberger and Glatz, 1990), isolated from *Propionibacterium acidipropionici* ATCC 4875, was used in this thesis as a reference for the isolation and characterization of plasmids from propionibacteria, its characteristics are shown in table 4.

A small plasmid, pTLPO1 (6.5 kb), is proposed for the construction of a cloning vector to transform propionibacteria (Gautier and Rouault, 1990). Plasmid pTY1, like pRGO1 also isolated from *Propionibacterium acidipropionici* ATCC 4875, is claimed in a patent application as a vector that could be used for the transformation of propionibacteria (Patent no. EP400931, december 1990). This patent may be obsolete since the publication of Rehberger and Glatz (1990) dates from april 1990.

Gautier et al. (1995) described the occurrence of *P. freudenreichii* bacteriophages in Swiss cheese.

1.6.2 Transformation of propionibacteria

Dairy propionibacteria have been transformed using electrotransfection (Gautier et al., 1995). Electroporation and PEG induced transformation of protoplasts was attempted by Woskow and Glatz (1990). Transformation of the plasmids pT181, pC194, pE194 and pUB110 from *Staphylococcus aureus* and pAMβ-1 from *Enterococcus faecalis* into protoplasts of *Propionibacterium* strains was attempted by Pai and Glatz (1987). In the latter experiment, erythromycin-resistant strains with pE194-DNA were obtained, although the intact plasmid was never identified in the presumptive recipients. The *Staphylococcus aureus* plasmid pC194 was electroporated into several *Propionibacterium* strains but the presence of an autonomous plasmid could not be confirmed (Zirnstein and Rehberger, 1991).

Conjugation, as another possible way of DNA transfer, between or involving propionibacteria as donors or recipients has so far not been described.

1.6.3 Genome size and sequenced genes of propionibacteria

The genome size of some classical propionibacteria was determined by Gautier et al. (1992) and ranges from 2.3 to 3.2 megabases (Mb). The genome sizes of the following species were estimated by pulsed field electrophoresis: *P. acidipropionici* (3.05 Mb), *P. freudenreichii* (2.5 Mb), *P. jensenii* (2.41 Mb) and *P. thoenii* (2.44 Mb). For 23 strains of *P. freudenreichii*, Rehberger and Glatz (1993) determined a genome size ranging from 1.6 to 2.3 Mb.

The most often sequenced genes of propionibacteria are the genes for the 16S rDNA. Parts of these genes have been determined for at least the type strains of each species. For the cutaneous species, especially for *P. avidum*, *P. granulosum* and *P. lymphophilum*, the known sequences of the 16S rDNA are relatively short (approx. 350 bp each), a fact that limits the determination of the exact phylogenetic position of these species.

Genes involved in the production of vitamin B_{12} are important as well and often cited in patent applications. A selection of complete or partially sequenced genes from propionibacteria is shown in table 2.

Table 2: A selection of sequenced genes of propionibacteria (GenEmbl-database, September 1998)

Gene for	Strain	Acc.No.	Year
δ-Aminolevulinic acid dehydratase Ferrochelatase Glutamate 1-semialdehyde 2,1-aminomutase *hemB; hemH; hemL; hemR; hemX; hemY* Porphyrinogen synthase Protoporphyrinogen oxidase	*P. freudenreichii*	D85417	1997
Biotin carboxyl carrier protein	*P. freudenreichii*	A03401	1993
Ferrochelatase (*hemH*) Aminolevulinic acid dehydratase (*hemB*) Membrane-bound transport protein Repressor protein	*P. freudenreichii*	U51164	1996
Glucokinase	*P. freudenreichii*	S29735	1993
Glutamate 1-semialdehyde aminomutase (*hemL*)[1]	*P. freudenreichii*	D12643	1993
Hyaluronidase	*P. acnes*	U15927	1994
Membrane associated ATPase (*cbiO*) uroporphyrinogen III methyltransferase (*cobA*)	*P. freudenreichii*	U13043	1995
Methylmalonyl-CoA carboxyltransferase 5S subunit	*P. freudenreichii*	L06488	1995
Methylmalonyl-CoA carboxyltransferase 12S subunit	*P. freudenreichii*	L04196	1993
mutA and *mutB* genes for methylmalonyl-CoA mutase	*P. freudenreichii*	X14965	1993
Proline iminopeptidase (*pip*)	*P. freudenreichii*	A41525	1997
Pyrophosphate-fructose 6-phosphate 1-phosphotrans-ferase	*P. freudenreichii*	M67447	1993
Superoxide dismutase (*sodA*)	*P. freudenreichii*	X91650	1996
Transcarboxylase 1.3S biotin-subunit[2]	*P. freudenreichii*	M11738	1990
Triacylglycerol lipase	*P. acnes* P.37	X99255	1997
Uroporphyrinogen-III-methyltransferase[3]	*P. freudenreichii*	E10849	1997

[1] Patent application no JP06113861, 1994.

[2] Patent applications no WO9014431, 1990; US5252466, 1993; J4267880, 1992.

[3] Patent application no WO942688, 1994.

1.6.4 Patent applications claiming transformation of propionibacteria

There exist only a few patents involving the transformation of propionibacteria and propionibacteria are cited there mainly because they are commercially used as starter cultures and the owner of the patent wants the vector to be protected also for the case that the system is found to be working for propionibacteria.

None of the mentioned vectors originates from the genus *Propionibacterium*: A food-grade cloning vector derived from plasmid pWV01 (2.3 kb) for the stable integration into the *Propionibacterium* chromosome (patent no: EP487159, 1992) is claimed. The host of pWV01 is a strain of *Lactococcus lactis* subsp. *cremoris*.

The plasmid pVS40 (approximately 11 kb) is mentioned as a potential cloning vector for *Propionibacterium* sp. (WO9109132, 1991). Host strain of pVS40 is *Lactococcus lactis* subsp. *lactis*, the plasmid carries a nisin-resistance gene as a selectable marker.

In another application *Propionibacterium* sp. are referred to as potential bacterial hosts for the plasmid pC1750, isolated from a strain of *Lactococcus lactis* (EP246909, 1987). Also *Propionibacterium* sp. as potential bacterial hosts for plasmids pDP211, pDP222, pDP228, pDP301 or pBM45, enabling integrative gene expression, are claimed (EP564965, 1993). Vitamin B_{12} production by vector expression in "a propionic acid bacterium" (original for *Propionibacterium*) is covered by an other patent (JP08056673, 1996).

The only plasmid originating form propionibacteria claimed in a patent application is pTY1, isolated from *Propionibacterium acidipropionici* ATCC 4875 (Patent no. EP400931, 1990).

1.6.5 Mutagenesis of *Propionibacterium* strains

Several strains of the genus *Propionibacterium* were treated with various mutagenic agents (e.g. ethyl methanesulfonate, dimethyl sulfate; *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine; X-rays; UV) to obtain stable mutants. The strains were selected mainly for an increased production of vitamin B_{12}, other strains were selected for auxotrophy (Voorhees and Glatz, 1987), cold-sensitivity (Hofherr et al., 1983) and aspartase deficiency (Glatz and Anderson, 1988). The mutant strains are involved in various patent applications, especially for the production of vitamin B_{12}.

1.7 Aim of this thesis

This thesis aims to contribute to the following 3 points:

- Since a selective procedure for the isolation of propionibacteria from different sources has not been described so far, the development of a method that allows the identification of unknown bacteria as belonging to the genus *Propionibacterium* is needed. The method should allow a fast screening procedure for *Propionibacterium* species in samples from different origins and should be based on molecular data.

- In order to clarify the taxonomic situation within the genus the selection of a sequencing strategy and acquisition of additional data for the 16S or 23S rDNA of propionibacteria is necessary. Especially, data for the phylogenetic positions of the medically relevant species are needed. Based on these data, a simple method that allows the detection of *Propionibacterium* species using a hybridization technique should be developed.

- To simplify further molecular studies with propionibacteria, the prerequisites for a genetic system have to be established. Especially the search for a suitable vector, stable genetic markers for strains and methods for the DNA-transfer by conjugation and electroporation have to be performed.

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2. MATERIAL AND METHODS

2.1 Bacterial strains and plasmids

Table 3: Bacterial strains used in this study

no.	*Propionibacterium* strains	Origin[a]	no.	Non-*Propionibacterium* strains	Origin[a]
1	*P. acidipropionici*[T]	DSM 4900	43	*Acetobacter diazotrophicus*[T]	DSM 5601
2	*P. acidipropionici* (*arabinosum*)	DSM 20273	44	*Actinomyces georgiae*[T]	DSM 6843
3	*P. acidipropionici* (*pentosaceum*)	DSM 20272	45	*Arthrobacter citreus*[T]	DSM 20133
4	*P. acnes*[T]	DSM 1897	46	*Bacillus subtilis*	Wiesby 168
5	*P. acnes*	DSM 20458	47	*Bacillus thuringiensis*[T]	DSM 2046
6	*P. avidum*[T]	DSM 4901	48	*Bifidobacterium lactis*[T]	DSM 10140
7	*P. cyclohexanicum*[T]	IAM 14535	49	*Bifidobacterium minimum*[T]	DSM 20102
8	*P. freudenreichii*[T]	DSM 20271	50	*Brevibacterium linens*[T]	DSM 20425
9	*P. freudenreichii* (*shermanii*[T])	DSM 4902	51	*Cellulomonas uda*[T]	DSM 20107
10	*P. freudenreichii* (*shermanii*)	DSM 20270	52	*Corynebacterium variabile*[T]	DSM 20132
11	*P. freudenreichii*	FAM 1409	53	*Dermacoccus nishinomiyaensis*[T]	DSM 20448
12	*P. freudenreichii*	FAM 1410	54	*Enterobacter* sp.	LME
13	*P. freudenreichii*	FAM 1411	55	*Enterococcus faecalis* FO1	LME
14	*P. freudenreichii*	FAM 1412	56	*Enterococcus faecalis* JH2-2	LME
15	*P. freudenreichii*	FAM 1413	57	*Enterococcus faecalis* RE25	LME
16	*P. freudenreichii*	FAM 1414	58	*Enterococcus* sp. RE21/1	LME
17	*P. freudenreichii* ARE1	LME	59	*Enterococcus* sp. RE39	LME
18	*P. freudenreichii* ARE2	LME	60	*Escherichia coli* S17-1	LME
19	*P. freudenreichii* ARE3	LME	61	*Escherichia coli* XL1-Blue	LME
20	*P. freudenreichii* DF2	LME	62	*Gardnerella vaginalis*[T]	DSM 4944
21	*P. freudenreichii* JS53	LME	63	*Lactobacillus acidophilus*	Wiesby 145
22	*P. granulosum*[T]	DSM 20700	64	*Lactobacillus casei*	Wiesby 160
23	*P. "intermedium"*	NCIMB 8728	65	*Lactobacillus rhamnosus*	Wiesby 744
24	*P. jensenii*[T]	DSM 20535	66	*Lactococcus lactis* subsp. *cremoris*	LME
25	*P. jensenii*	DSM 20278		AC1	
26	*P. jensenii* (*petersonii*)	DSM 20279	67	*Lactococcus lactis* subsp. *lactis*	LME
27	*P. jensenii* (*pituitosum*)	NCIMB 8899		BU2-60	
28	*P. jensenii* (*raffinosaceum*)	NCIMB 8900	68	*Lactococcus lactis* subsp. *lactis*	LME
29	*P. jensenii* (*rubrum*)	DSM 20275		K214	
30	*P. jensenii* (*technicum*)	NCIMB 5965	69	*Leuconostoc mesenteroides* GD14	LME
31	*P. jensenii* (*zeae*)	DSM 20274	70	*Microbacterium arborescens*[T]	DSM 20754
32	*P. jensenii* DF1	LME	71	*Microbacterium lacticum*[T]	DSM 20427
33	*P. jensenii* DF3	LME	72	*Micromonospora* sp.	DSM 1043
34	*P. lymphophilum*[T]	DSM 4903	73	*Mycobacterium chlorophenolicum*	DSM 43826
35	*P. propionicum*[T]	DSM 43307	74	*Nocardia* sp.	DSM 43280
36	*P. propionicum*	DSM 43015	75	*Streptococcus thermophilus*	LME
37	*P.* sp. (*animalis*)	ATCC 49745		55n R/S	
38	*P.* sp. (*wentii*)	NCIMB 8905	76	*Streptococcus thermophilus* S 203	LME
39	*P.* sp. Z/S	LME	77	*Streptomyces azureus*[T]	DSM 40106
40	*P. thoenii*[T]	DSM 20276			
41	*P. thoenii*	DSM 20277		plus additional 164 strains of	LME
42	*P. thoenii* (*sanguineum*)	NCIMB 8902		*Propionibacterium* species	

[a] ATCC: American Type Culture Collection, Manassas, USA.
DSM: Deutsche Sammlumg für Mikroorganismen und Zellkulturen, Braunschweig, Germany.
FAM: Forschungsanstalt für Milchwirtschaft, Liebefeld-Bern, Switzerland.
IAM: Institute of Molecular and Cellular Biosciences, University of Tokyo, Japan.
LME: Laboratory of Food Microbiology, ETH, Zürich, Switzerland.
NCIMB: National Collections of Industrial and Marine Bacteria Ltd., Aberdeen, UK.
Wiesby: Wiesby GmbH & Co. KG, Niebüll, Germany.

[T] Type strain.

( ): former strain designation, used in this study to differentiate the various strains. (e.g. "*P. zeae*").

Strains and plasmids used in this study are listed in table 3 and table 4. Single colonies of strains received from culture collections were transferred three times on agar plates before they were stored in 30% glycerol at –72°C.

Table 4: Plasmids used in this work and their characteristics

Plasmid	Size	Host	Origin	Properties	References
pAMβ1	26.5 kb	*Enterococcus* RE21/1	-	MLS^R	Clewell et al., 1974
pAM120	21.4 kb	*E. coli* S17-1	-	Amp^R, Tet^R	Gawron-Burke and Clewell, 1984
pAM180	41 kb	*E. faecalis* JH2-2	-	Tet^R	Bertram and Duerre, 1989
pCL1921	4.6 kb	*E. coli* XL1-Blue	-	Spc^R, Str^R, lacZ'	Lerner and Inouye, 1990
pFO1	~18 kb	*E. faecalis* FO1	cheese	Tn*FO1*, Tet^R	Perreten, 1995
pGEM-T	3 kb	-	-	Amp^R, lacZ'	Promega, Madison, USA
pJIR750	6.5 kb	*C. perfringens*	-	Cm^R	Bannam and Rood, 1993
pJIR751	5.9 kb	*C. perfringens*	-	Em^R	Bannam and Rood, 1993
pK214	~30 kb	*L. lactis* K214	cheese	Cm^R, Tet^R	Perreten et al, 1997
pLME101	40.3 kb	*P. freudenreichii* JS53	cheese		Smutny, 1997
pLME106	~7 kb	*P. jensenii* DF1	raw milk		Stierli, 1998
pLME108	2 kb	*P. freudenreichii* DF2	raw milk		this study
pLME116	4.7 kb	*E. coli* XL1-Blue	pLME108[1]	pLME108::pUC18	this study
pLME117	4.7 kb	*E. coli* XL1-Blue	pLME108[1]	pLME108::pUC18	this study
pLME118	4.2 kb	*E. coli* XL1-Blue	pLME108[2]	pLME108::pUC18	this study
pLME119	4.8 kb	*E. coli* XL1-Blue	pLME108[3]	pLME108::pGEM-T	this study
pRE25	50 kb	*E. faecalis* RE25	sausage	Cm^R, Em^R	Perreten, 1995
pRE39	26.5 kb	*Enterococcus* RE39	minced meat	MLS^R	Wirsching, 1995
pRGO1	6.7 kb	*P. acidipropionici*	DSM20272	reference strain	Rehberger and Glatz, 1990
pSI22	5.42 kb	*E. coli* XL1-Blue	-	Cm^R, Em^R, NisI	Engelke et al., 1994
pUC18	2.7 kb	*E. coli* XL1-Blue	-	Amp^R, lacZ'	Yanisch-Perron et al., 1985

[1] Complete pLME108, *Nhe*I digested, cloned into the *Xba*I site of pUC18.
[2] 1.5 kb fragment of *Pvu*II digested pLME108, cloned into the *Hinc*II site of pUC18.
[3] 1.8 kb PCR-fragment of pLME108 (primer pr108fw and pr108rev), cloned into pGEM-T Easy.

2.2 Strain maintenance and control

2.2.1 Growth Conditions

The strains that were used in this study are listed in table 3. Propionibacteria were inoculated aerobically and grown under anaerobic conditions either in adapted YEL-medium (Malik et al., 1968) or in NL-medium (Grinstead and Barefoot, 1992). 1 liter of YEL medium contained 10 g yeast extract (Becton Dickinson), 10 g DL-sodium lactate (Sigma), 0.25 g K_2HPO_4 (Fluka), 0.005 g $MnSO_4$ (Fluka), 0.5 g cysteine-HCl (Fluka), 1 ml Tween 80 (Merck), 10 g casein peptone (pankreatic digested, Merck) and 1 mg resazurin (Fluka), the pH was adjusted with NaOH (Merck) to 7.0. The composition of NL-medium was the following: 1% trypticase soy broth without dextrose (Becton

Dickinson), 1% yeast extract (Becton Dickinson) and 1% DL-sodium lactate (Sigma), no pH adjustment. For the preparation of solid media. 1.5% Agar-agar (Oxoid) were added. When required, other media like MRS, PYG (DSM catalogue of strains) or blood agar for the cutaneous propionibacteria were used. All media were prepared with distilled water and sterilized by autoclaving at 121°C for 15 minutes.

Propionibacteria were maintained and transferred under aerobic conditions, but growth was achieved using an anaerobic atmosphere. Anaerobic growth conditions were achieved by using gas-tight 20-ml McCartney tubes (broth media) or anaerobic jars (Oxoid) containing the Anaerocult A system (Merck). Dairy propionibacteria were grown at 30°C, cutaneous strains at 37°C for 2 to 7 days.

Strains were stored either at 4°C or at -72°C in 30% (v/v) glycerol. Strain purity was checked regularly by microscopy, colony morphology, Gram-test and catalase activity. All other strains were cultivated as recommended in the DSM catalogue of strains.

2.2.2 Catalase activity

To determine the catalase activity, bacteria were placed on a glass slide after they had been in contact with oxygen for at least 30 minutes. A few drops of 30% H_2O_2 were added. When gas production was observed within 10 minutes, the strain was considered as catalase-positive.

2.2.3 KOH test

Instead of classical Gram-staining, bacteria were treated with KOH to determine their Gram-grouping (Bamarouf et al., 1996). Cells were placed on glass slides, 3% KOH was added and the suspension mixed for 30 seconds with a platin loop. For most Gram-negative bacteria the solution becomes very viscous and mucoid whereas for most Gram-positive organisms no reaction occurs.

2.2.4 Nitrate reduction

Nitrate medium, consisting of 0.3% meat extract (Merck), 0.5% bacto peptone (Difco), 0.1% KNO_3 (Merck), 0.17% agar-agar (Oxoid), was inoculated with the test organisms and incubated anaerobically. Gas production was measured using Durham tubes submersed in the medium. Samples were taken after incubation for 1, 2, 3, 6 and 11 days and nitrate reduction was determined as described by Gerhardt et al. (1981).

2.2.5 Carbohydrate fermentation patterns using the API-CH system (BioMérieux)

Propionibacteria (mid-logarithmic growth phase) were suspended in 10 ml 0.85% NaCl-solution, centrifuged (10 minutes, room temperature, 3'000g) and the pellet was resuspended in 2 ml sterile bidistilled water. To obtain a standardized inoculum, the volume of the cell suspension needed to obtain an optical density of 0.2 at 600 nm in 5 ml water was determined. Twice this volume was added to 10 ml of modified API CH50 medium (de Carvalho et al., 1995). The API 50 CH system (BioMérieux) was inoculated with the propionibacteria as described by the manufacturer. The system was incubated anaerobically (sealed "sous vide" bags with Anaerocult A, Merck) at the optimal growth temperature. Carbohydrate fermentation patterns were determined after 24, 48, 72 hours and 1 week of incubation. The resulting patterns were compared with published data (Britz and Riedel, 1991; Cummins and Johnson, 1986 & 1992).

2.3 General DNA isolation and analysis procedures

2.3.1 Isolation of total chromosomal and plasmid DNA

Total DNA from propionibacteria and other organisms was isolated as described by Leenhouts et al. (1989) by using an enzymatic lysis procedure with lysozyme, mutanolysin and Proteinase K for the disruption of the cell walls.

2.3.2 Isolation of PCR-grade DNA

To obtain template DNA suitable for PCR from different organisms, the simple DNA extraction method of Goldenberger et al. (1995) mediated by SDS and Proteinase K was used.

For propionibacteria an even simpler approach proved to be sufficient: with a sterile toothpick, cells were selected from agar-plates and directly used as templates for the PCR reactions (or 1-10 µl of a liquid culture were added).

2.3.3 Small scale plasmid DNA preparation

Plasmid DNA was isolated from propionibacteria cells, harvested after incubation for 2 to 3 days, using the method of Frère (1993) or as described by Anderson and McKay (1983). From *E. coli*, plasmid DNA was extracted using the boiling procedure of Holmes and Quigley (1981).

2.3.4 Large scale plasmid DNA isolation from *E. coli*

Plasmid DNA up to 10 kb in size and suitable for automated sequencing was isolated from *E. coli* strains using the Wizard™ *Plus* Midipreps DNA Purification System (Promega).

2.3.5 Large scale plasmid DNA isolation from propionibacteria

Plasmid DNA was isolated using a modified method of Frère (1993): 50-100 ml of a liquid culture, optical density (600 nm) of 1 to 1.5, were harvested by centrifugation (20 minutes, 4°C, 5'000 g). The sediment was suspended in 3 ml of solution 1 (50 mM Tris; 10 mM EDTA, pH 7,5; 200 µg/ml RNase A) and incubated for 5 minutes at room temperature. 5 g sterile glass beads (diameter of 0.1-.11 mm, Merck) were added and the cells were broken by vortexing for 1 minute at highest speed. 3 ml of solution 2 (1% SDS; 0,2 M NaOH) were added. After 5 minutes incubation at room temperature, 3 ml of solution 3 (2,55 M potassium acetate; pH 4,88) were added. After centrifugation (20 minutes, 4°C, 10'000 g) the supernatant was cleared by filtration through a folded paper filter. The further purification of the filtrate was performed using the Wizard™ *Plus* Midipreps DNA Purification System (Promega) as described by the manufacturer. Alternately (for plasmids >10 kb) an equal volume of phenol-methylenechloride-isoamylic alcohol (25:24:1) was added to the filtrate and mixed by inverting the tube 10 times. After centrifugation (5 minutes, room temperature, 10'000 g) the supernatant was precipitated for 1 hour at –72°C with 2 volumes of ethanol (Fluka, 96%, -20°C). The plasmid-DNA was pelleted by centrifugation (20 minutes, 4°C, 15'000 g) and dried for 10 minutes in a vacuum concentrator (SpeedVac, Savant). The plasmid DNA was dissolved in 500 µl of TE-buffer (10 mM Tris; 1 mM EDTA; pH 7.5)

2.3.6 Agarose gel electrophoresis

DNA was mixed with 6x glycerol-loading buffer (Sambrook et al., 1989) and separated in agarose gels using 1x TAE or TBE buffers (Sambrook et al., 1989). For the separation of smaller fragments and the analysis of PCR-products 1-2% agarose (FMC) and 1x TBE buffer was used. Routine electrophoresis was performed with 0.8% agarose in 1x TAE buffer. For linear DNA samples, a 1-kb DNA ladder (Gibco) or *Hind*III digested λ–DNA (Gibco) were used as size-reference. Circular DNA standards were plasmid DNA from *Lactococcus lactis* subsp. *cremoris* AC1 (Neve et al., 1984) and a supercoiled DNA ladder (Promega). After electrophoresis (Gel Electrophoresis Apparatus GNA 100,

Pharmacia), the agarose gels were stained in a 5 µl/ml ethidium bromide (Sigma) solution for 10 minutes and washed for 10 minutes in distilled water. The DNA fragments were visualized under ultraviolet light (302 nm), photographed and digitized with "The Imager" system (Appligene).

2.3.7 Quantification of DNA

DNA was quantified by gel electrophoresis of the sample together with defined amounts of λ-DNA (Gibco) followed by densitometric analysis with the Wincam software (Cybertech, Berlin, Germany).

2.3.8 Restriction endonuclease digests of DNA

All endonuclease digests were performed using the OPA-buffer (Pharmacia) with the conditions recommended by this supplier. Enzymes from other suppliers were used with the same buffer system. Digests were analyzed by gel electrophoresis or using pulsed field gel electrophoresis for better separation of larger fragments.

2.3.9 Pulsed field gel electrophoresis

Since the resolution of classical gel electrophoresis for the separation of larger plasmid DNA fragments was not satisfactory, pulsed field gel electrophoresis (PFGE) was selected to achieve a better separation of larger DNA fragments (>12 kb). Plasmid-DNA was isolated as described by Anderson and McKay (1983) and purified using $CsCl_2$ density gradient centrifugation (Sambrook et al., 1989). Restriction endonuclease digests of the plasmid-DNA were mixed with loading dye (Sambrook et al., 1989) and applied onto the agarose gel.

The CHEF-DR™ II System (BioRad) was used with the following parameters: 1.5% TBE agarose gel, 0.5% TBE running buffer, 4°C, 200 V, 1-20 seconds pulse ramping time, 18 hours running time. Preparation of the apparatus and electrophoresis were performed according to the manufacturers instructions.

2.4 Polymerase chain reactions

2.4.1 PCR-primers

All oligonucleotide primers were synthesized by Microsynth (Balgach, Switzerland) and are listed in table 5. A stock solution (0.1 mM) of each primer was prepared in sterile bi-distilled water, irradiated with ultraviolet light (302 nm) for 5 minutes and stored at -20°C.

Table 5: Synthetic oligonucleotides, position and origin used for PCR or MPCR

Name	Sequence (5' → 3')	position	target gene	reference
bak11w	AGTTTGATCMTGGCTCAG	8-25[1]	universal[5] 16S rDNA	Goldenberger, 1997
bak4	AGGAGGTGATCCARCCGCA	1540-1522[1]	universal[5] 16S rDNA	Greisen et al., 1994
eubac338	ACTCCTACGGGAGGCAGC	338-355	universal[5] 16S rDNA	Amann et al., 1995
gd1	TGCTTTCGATACGGGTTGAC	632-651[1]	Genus *Propionibacterium*	this study
gd5srev	CGGCGGYGWCCTACTCTCCCA	5102-5072[1]	High GC 5S rDNA	this study
gd6r	GTGCCWAGGCATCCACCG	2020-2003[1]	propionibacteria 23S rDNA	this study
lm30	CGTGTCGTGAGATGTTGG	1033-1050[1]	Gram-positive 16S rDNA	Meile, 1998
lm38	TTAACCTTCCAGCACCG	3835-3819[1]	Gram-positive 23S rDNA	Meile, 1998
m13rev	CAGGAAACAGCTATGAC	465-481[4]	M13 / pUC18	Pharmacia, Sweden
pr108fw	GCCCGCCACACCCGAACCGGT	1845-1865[2]	pLME108	this study
pr108rev	CAACGTCCGCAGGAAGATCAT	1771-1751[2]	pLME108	this study
prampfw	GCAAGCAGCAGATTACGCGC	1418-1437[4]	*amp*^{R} of pUC18	this study
pramprev	AAGGGCCTCGTGATACGCCT	2681-2662[4]	*amp*^{R} of pUC18	this study
TM1	TGATAGCGGGAACAAATA	307-334[3]	*tetM* (pAM120)	Perreten, 1995
TM2	ATACAGGACACAATATCC	2570-2553[3]	*tetM* (pAM120)	Perreten, 1995
vp1	GAYACGCCAGGMCATATGGA	738-758[3]	*tetM* (pAM120)	Perreten, 1995
vp2	TYGGRCGRCGGGGYTGGCAA	2392-2373[3]	*tetM* (pAM120)	Perreten, 1995

[1] *E. coli* positions according to Brosius et al., 1978.
[2] Position according to pLME108 (EMBL accession no AJ006662).
[3] Position according to *tetM* (EMBL accession no M85225).
[4] Position according to pUC18 (EMBL accession no L09136).
[5] Universal for most of the known bacterial 16S rDNA sequences.

2.4.2 Standard PCR conditions

Target DNA was amplified in 0.2 ml thin-walled tubes using thermocyclers equipped with heated lids (Personal Cycler, Biometra; Genius, Techne). A standard 50 µl reaction mixture contained 0.2 mM each of dATP, dCTP, dGTP, dTTP (Pharmacia), 1 Unit *Taq* DNA-Polymerase (Pharmacia), 5 µl 10x standard PCR-buffer (Pharmacia), sterile bidistilled water, 1 µM each of forward and reverse primers and template DNA. For high quantities of probes, a mastermix that contained all components (no template DNA) was prepared and distributed to the reaction tubes.

The standard PCR program is shown in figure 1. Annealing temperatures (T_A) were calculated using a simple formula (T_A = [no. of GC]x 4°C + [no. of AT]x 2°C) and further optimized empirically. For one kb of amplicon size, one minute was added to the polymerization time. The resulting PCR programs are shown in table 8.

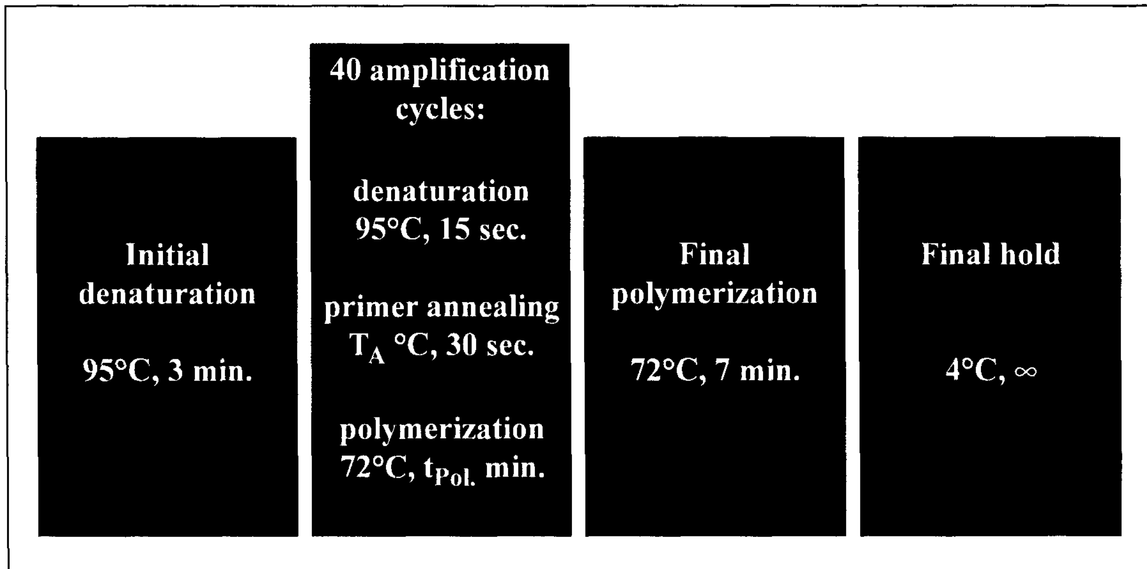

Figure 1: Standard PCR protocol. The annealing temperatures (T_A) and polymerization times ($t_{Pol.}$) for the different amplification protocols are shown in table 6.

Table 6: PCR primer pairs and amplicons

Primer pair	Amplicon	T_A	$t_{Pol.}$	Application
bak11w-bak4	16S rDNA, ~1500 bp	56°C	2 min.	Sequencing
bak11w-gd6r	16S rDNA + ITS, ~1900 bp	56°C	2 min.	Sequencing
bak11w-gd5sr	16S → 5S rDNA, ~5000 bp	50°C	5 min.	Sequencing
bak4-gd6r	ITS, ~400 bp	56°C	1 min.	Hybridization probe
lm30-lm38	16S → 23S rDNA, ~2800 bp	50°C	3 min.	Sequencing
pr108fw-pr108rev	partial pLME108, 1977 bp	56°C	3 min.	Cloning
prampfw-pramprev	complete *amp*^{R}, 1263 bp	50°C	4 min.	Cloning
prampfw-m13rev	partial pLME116, 3800 bp	44°C	5 min.	Cloning
vp1-vp2	partial *tetM*, 1654 bp	56°C	2 min.	Hybridization probe
TM1-TM2	complete *tetM*, 2246 bp	44°C	3 min.	Cloning
(bak11w, gd1)-bak4	16S rDNA, 900 +1500 bp	56°C	2 min.	MPCR

T_A: Annealing temperature; $t_{Pol.}$: Polymerization time.
ITS: 16S-23S rDNA intergenic spacer region.

2.4.3 Multiplex-PCR (MPCR)

To bind SDS (inhibitory for DNA-polymerases) used in the sample preparation, 2% Tween 20 (Merck) were added to the 50 µl standard reaction mixture. 1 µM forward primers bak11w, gd1 and 1 µM reverse primer bak4 (table 5) were used. Annealing temperature was set to 56°C, with the polymerization steps at 72°C for 2 minutes.

2.4.4 Long PCR conditions

To generate PCR products longer than 3 kb, the "*Taq*Plus Long™ PCR System" (Stratagene) was used according to the manufacturers instructions.

2.5 Labeling of DNA probes

2.5.1 Labeling of oligonucleotides with [γ-^{32}P]ATP

100 pmol of the respective oligonucleotide was labeled with radioactive [γ-^{32}P]ATP (DuPont) using the T4-Kinase technique as described by Sambrook et al. (1989). The probe was purified using NAP-columns (Pharmacia) to remove non-incorporated radioactivity. Prior to use, the probe was denatured for 5 minutes at 95°C and kept on ice before it was added to the hybridization solution.

2.5.2 Random primed labeling of DNA-fragments with [α-^{32}P]dATP

In a final volume of 25 µl, the linearized DNA-probe was labeled with [α-^{32}P]dATP (DuPont) using the random priming technique of Feinberg and Vogelstein (1983). To remove surplus radioactivity, the probe was purified using a NICK-column (Pharmacia) according to the manufacturers instructions. Prior to use, the probe was linearized for 5 minutes at 95°C and kept on ice.

2.5.3 Terminal labeling of oligonucleotide-probes with DIG-ddUTP

Oligonucleotide probes were labeled with digoxigenin-ddUTP using the DIG 3'-end labeling kit (Boehringer) as recommended by the manufacturer.

2.5.4 Labeling of DNA-fragments with DIG-dUTP

DNA fragments were labeled using the "DIG DNA labeling mix" (Boehringer) and the random primer incorporating method as described by the manufacturer.

2.6 Hybridization techniques

2.6.1 Southern blotting

DNA transfer from agarose gels to Zeta-blot membranes (BioRad) was achieved with the Southern blotting technique using 0.4 N NaOH as transfer buffer and a vacuum blotting equipment (Biometra).

2.6.2 Colony hybridization

Nylon membranes (Amersham) were sterilized by UV-irradiation (302 nm, 10 minutes each side) and placed on petri-dishes with suitable growth medium. Using sterile toothpicks, single colonies were transferred to the membrane. After incubation, the membrane was placed with the colony side upwards on a 3MM paper (Whatman) soaked with lysis-solution (10 mM Tris, pH 7.5; 0.25 M sucrose; 5 mg/ml lysozyme) and incubated for 1 hour at 37°C. For propionibacteria, 30 units/ml mutanolysin were added to the lysis-solution. After the cell-lysis step the membrane was placed on a 3MM paper soaked with denaturation solution (0.5 M NaOH; 1 M NaCl). After 4 minutes at room temperature, the membrane was transferred to a fresh 3MM paper soaked with 1 M Tris (pH 8.0) for 4 minutes. A fresh filter paper was soaked with 2x SSC (pH 7.0) and the membrane placed there for 4 minutes. The membrane was air-dried and kept for 2 hours at 80°C to fix the released DNA. Finally membranes were stored air-tight and light-protected at 4°C.

2.6.3 Hybridizations with DNA probes

Membranes were incubated in a Micro-4 oven (Hybaid) at hybridization temperature (large DNA-fragments: 65°C; primer gd1: 56°C) with 30 ml prehybridization solution (5x SSC; 5x Denhardts; 0.25 mg/ml sssDNA; 0.05 M sodiumphosphate buffer, pH 6.5). After 3 hours, the prehybridization solution was discarded and 30 ml of hybridization solution (5x SSC; 1x Denhardts; 0.5 mg/ml sssDNA; 0.04 M sodiumphosphate buffer pH 6.5) containing the labeled probe were added.

When DIG-labeled probes were used, washing steps and colorimetric detection of the DNA-DNA or DNA-RNA hybrids were performed as described by the manufacturer in the "DIG nucleic acid detection kit" (Boehringer).

Membranes with radioactive probes were washed, detected and reprobed as described by Sambrook et al. (1989).

2.7 Analysis of propionibacteria isolated from dairy products

2.7.1 Isolation of propionibacteria from cheese

10 g samples of each cheese were dissolved in 90 ml YEL broth and grinded for 5 minutes in a stomacher (Colworth). Serial dilutions were prepared in YEL and streaked out on YEL agar. After anaerobic incubation for up to 1 week, "typical" colonies (50 colonies per plate, 200 colonies per isolation) were selected and prepared for colony-hybridization. After hybridization with the genus-specific probe gd1 and the universal oligonucleotide probe eubac338 (table 5), colonies that gave a positive signal both with the universal and specific probes were further investigated. To obtain pure cultures, single colonies of the isolates were transferred to fresh agar for 3 times. These strains were Gram-stained (KOH test), catalase activity was measured and for strains with uncertain identification, carbohydrate fermentation patterns were determined. The affiliation of all propionibacteria-isolates to the genus *Propionibacterium* was reconfirmed by the MPCR method developed in this study. In addition, for strains of special interest, 16S rRNA genes were sequenced using the cycle sequencing method described in this study.

2.7.2 Species determination

As described by Rossi et al. (1997), dairy *Propionibacterium* species can be easily determined using a simple restriction analysis of PCR-amplified 16S-23S rDNA intergenic spacer regions. In addition, partial sequencing of 16S rDNA and the 16S-23S rDNA intergenic spacer region was used in this study to classify isolates to species level. To differentiate between the *P. freudenreichii* subspecies, the identification scheme of Cummins and Johnson (1986) was used.

2.7.3 Screening for plasmids and plasmid-encoded properties

In all *Propionibacterium*-isolates the presence of plasmids was investigated. For strains that contained plasmids, antibiotic resistance patterns were determined and curing of these strains was attempted.

2.7.4 Determination of antibiotic resistance patterns

Propionibacteria were precultured in broth culture under optimal conditions and harvested by centrifugation (5 minutes, room temperature, 6'000 g). A solution of the cells, corresponding to the McFarland standard 2, was prepared in peptone-water (0.1%

peptone, 0.8% NaCl). Using a sterile swap, this solution was spread on MuellerHinton agar plates. Etest-strips (Etest® System, AB Biodisk) with different antibiotics were placed on the plates and results were determined after anaerobic incubation for 3-4 days at 30°C. The reading of the minimal inhibitory concentrations (MIC) of each antibiotic was performed as recommended by the manufacturer.

The following antibiotics were investigated: ampicillin, bacitracin, benzylpenicillin, cefotaxim, cephalothin, chloramphenicol, ciprofloxacin, erythromycin, fusidic acid, gentamicin, kanamycin, methicillin, nalidixic acid, ofloxacin, rifampicin, streptomycin, tetracycline and vancomycin.

2.7.5 Curing experiments with plasmid containing *Propionibacterium* strains

Curing of propionibacteria was performed by incubation at suboptimal growth temperatures (Smutny, 1997). Briefly, the highest possible growth temperature (T_{max}) for a strain was determined and the strain was incubated at this temperature for 2 weeks. A serial dilution was prepared and plated on solid NL-medium, incubated at T_{max}. Cells were selected and colony hybridizations with plasmid-DNA as probe were performed as described in chapter 2.6.2. Colonies that gave no signal with this probe were selected for further analysis to confirm the absence of the original plasmids (plasmid-DNA isolation, MPCR confirmation for propionibacteria).

2.8 DNA sequencing and analysis techniques

2.8.1 Cloning of DNA fragments using pUC18 and *E. coli* XL1-Blue

Vector DNA (pUC18) and DNA fragments to be inserted were digested separately with the appropriate enzymes, if necessary dephosphorylated using alkaline phosphatase (CIP, Pharmacia) and analyzed with gel-electrophoresis. The desired fragments were excised from the gels and purified using the Quiaex II System (Quiagen). Ligation, electro-transformation and selection of recombinant *E. coli* was performed according to Sambrook et al. (1989). The origin of the plasmid insertions from the resulting clones were confirmed using hybridization and PCR techniques as described previously.

2.8.2 Cloning of PCR fragments

PCR fragments were cloned in *E. coli* XL1-Blue using the pGEM®-T Easy Vector system (Promega).

2.8.3 Purification of PCR-products for sequencing reactions

100 µl of each PCR product were purified with the Nucleospin-Extract kit (Macherey & Nagel) and eluated with 50 µl of 10 mM Tris, pH 8.5 (Merck).

2.8.4 Cycle sequencing

PCR products (200-300 ng) or cloned DNA fragments were amplified with the "Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza dGTP" (Amersham) as recommended by the manufacturer. Sequencing primers were synthesized (Microsynth, Balgach, Switzerland) and labeled with cyanine (Cy5, Amersham) and are listed in table 7. The amplification program consisted of 25 cycles with 30 seconds at 95°C, 30 seconds 50 °C and 1 minute at 72°C. Samples were cooled to 4°C and 5 µl stop solution (Amersham) was added. Finally the products were analyzed on an ALF-Express automatic DNA-sequencer (Pharmacia) and the generated DNA sequences were further analyzed as described in the following chapter.

Table 7: Cy5-labeled oligonucleotide primers used for automated sequencing

Name	Sequence (5' → 3')	*E. coli* position	target gene	reference
Primers used for the sequencing of DNA fragments cloned in pUC18				
alfm13rev	CAGGAAACAGCTATGAC	-	pUC18	Pharmacia, Sweden
alfm13uni	CGACGTTGTAAAACGACGGCCAGT	-	pUC18	Pharmacia, Sweden
Primers used for 23S rDNA sequencing				
b0	CCTTTCCCTCACGGTACT	3974-3957, fw	*Proteobacteria* 23S rDNA	Boesch, 1998
b1	ATAGCTGGTTCTCCCCGAAA	4321-4302, fw	universal 23S rDNA	Boesch, 1998
b1rev	TTTCGGGGAGAACCAGCTAT	4302-4321, rev	universal 23S rDNA	Boesch, 1998
b2	GTTGGCTTAGAAGCAGCCATC	4559-4579, fw	universal 23S rDNA	Boesch, 1998
b2rev	GATGGCTGCTTCTAAGCCAAC	4579-4559, rev	universal 23S rDNA	Boesch, 1998
b3	CCCCTAAGGCGAGGCCGAAAG	4848-4868, fw	universal 23S rDNA	Boesch, 1998
b4	AGAGAATACCAAGGCGCTTG	5130-5194, fw	*Proteobacteria* 23S rDNA	Boesch, 1998
b5	AAGTTCCGACCTGCACGAAT	5452-5471, fw	universal 23S rDNA	Boesch, 1998
b6	GTAACGGAGGCGCGCGAT	5757-5774, fw	α-*Proteobacteria* 23S rDNA	Boesch, 1998
b7	AGAACGTCGTGAGACAGTTC	6027-6080, fw	α-*Proteobacteria* 23S rDNA	Boesch, 1998
gd6r	GTGCCWAGGCATCCACCG	2003-2020, rev	propionibacteria, 23S rDNA	this study
Primers used for 16S rDNA sequencing				
eubac338	ACTCCTACGGGAGGCAGC	338-355, fw	universal 16S rDNA	Amann et al., 1995
ms350r	CTGCTGCCTCCCGTA	343-357, rev	universal 16S rDNA	Lane, 1991
uni515	ACCGCGGCTGCTGGCAC	515-531, rev	universal 16S rDNA	Lane, 1991
uni785	GGMTTAGATACCCTGGTAGTCC	785-806, fw	universal 16S rDNA	Amann et al., 1995
uni1088	GGTTAAGTCCCGCAACGAGC	1088-1107, fw	universal 16S rDNA	Amann et al., 1995
uni1392	GTACACACCGCCCGTCA	1392-1408, fw	universal 16S rDNA	Lane, 1991
uni1392r	TGACGGGCGGTGTGTAC	1392-1408, rev	universal 16S rDNA	Lane, 1991

fw= forward; rev= reverse; *E. coli* positions according to Brosius et al., 1978.
Universal: universal for all known bacterial sequences.

2.8.5 Sequence data analysis and phylogenetic calculations

DNA sequences were analyzed with the programs provided in the GCG-package version 8.0 (Genetics Computer Group, Madison, Wisconsin, USA), the EGCG programs (Peter Rice, The Sanger Centre, Cambridge, GB) and the PHYLIP software tools (Felsenstein, 1982). DNA sequences were compared with a local GenEmbl database copy (Genbank and EMBL). The use of this programs is indicated in the respective parts of this work. Graphic representations of plasmids were drawn using the Plasmid Map Enhancer 3 program (sci.-ed software, Durham, USA) or the Omiga software (version 1.1, Rainbow technologies, USA).

2.9 Conjugation between propionibacteria and other genera

2.9.1 Selective supplements for the identification of transconjugants

The main problem in conjugation experiments is the selection of the transformants: the separation of donor and recipient organisms from the real transconjugants. To achieve mainly the inhibition of donor cells, the addition of different supplements to the medium was tested. All test strains were grown on BHI-agar before inoculation of the test-medium. After anaerobic incubation at 30°C for 7 days growth was evaluated visually. The following "selection enhancers" were tested: the use of a pH-indicator (bromcresol green, 0.02 g/l medium), the addition of end products of the "propionibacteria-pathway" (acetic and propionic acids), of sodium chloride and of the antibiotic cloxacillin.

2.9.2 Adaptation of propionibacteria to high concentrations of antibiotics

In order to obtain propionibacteria suitable as recipient strains in conjugation experiments, an easy selectable marker had to be introduced in these strains. High antibiotic resistant isolates were found by incubating the strains in media containing increasing concentrations of fusidic acid, and rifampicin in a second phase. The resulting strains were resistant to 100 μg/ml fusidic acid and 50 μg/ml of rifampicin.

2.9.3 Conjugation with the filter-mating technique

Donor and recipient strains were precultured in broth media with the respective antibiotic supplements. The cells were then concentrated on a 0.45 μm cellulose membrane (Millipore), with a ratio donor to recipient cells of 3:1 based on the optical density (OD_{600} ~2.0 for donor cells), using the filter-mating procedure described by Wirsching (1995). After incubation for 1-3 days on unselective medium, the cells were washed from the filters and transformants were selected by incubation of serial dilutions on the respective selective medium. The strains were then incubated at 30°C for up to 4 weeks and checked for growth daily. Growth of donor and recipient strains (positive control) was checked on media supplemented with the appropriate antibiotics. As negative controls, donor and recipient were as well plated and incubated on the selective media. Transformants were analyzed by hybridization of the clones with the transferred plasmid DNA or the antibiotic resistance gene as probe.

2.10 Electrotransformation of propionibacteria

Competent cells of propionibacteria were obtained using 3 different methods: the method described for *E. coli* (Sambrook et al., 1989) using 10% glycerol as buffer, a procedure described by Gautier et al. (1995) and an adapted method using 30% PEG (polyethylenglycole 10'000, Merck) (Zirnstein and Rehberger, 1991). For all methods, propionibacteria from a 500-ml culture were harvested (mid-logarithmic growth stage) by centrifugation (15 minutes, 4°C, 4'000g). The pellet was resuspended in the selected buffer system and concentrated by further washes with decreasing amounts of buffer until a volume of 2-3 ml was achieved. Competent cells were stored at –72°C and kept on ice before electroporation. Viability of the competent cells was determined by plating out cells that were electroporated without DNA. A list of the competent cells used in this study and the respective competence method is shown in table 8.

Table 8: Prepared competent *Propionibacterium*-strains

"Competent" strains	Growth medium[1]	Buffer[2]
P. acidipropionici (arabinosum)	NL or NLG	10% glycerol
P. cyclohexanicum^T	NL or NLG	10% glycerol
P. freudenreichii (shermanii)	NL	10% glycerol
P. freudenreichii (shermanii^T*)*	YELG	GS
P. freudenreichii ARE1	NL or NLG	30% PEG or 10% glycerol
P. freudenreichii FAM1409	NL or NLG	10% glycerol
P. freudenreichii FAM1410	NL or NLG	10% glycerol
P. freudenreichii FAM1411	NL or NLG	10% glycerol or 30% PEG
P. freudenreichii FAM1412	NL	10% glycerol
P. freudenreichii FAM1413	NL or NLG	10% glycerol or 30% PEG
P. freudenreichii FAM1414	NL or NLG	10% glycerol
P. freudenreichii JS53	NL or NLG	30% PEG or 10% glycerol
P. freudenreichii^T	YELG	GS
P. "intermedium"	NL or NLG	30% PEG
P. jensenii (petersonii)	NL or NLG	10% glycerol
P. jensenii (pituitosum)	NL or NLG	10% glycerol
P. jensenii (raffinosaceum)	NL or NLG	30% PEG
P. jensenii (technicum)	NL or NLG	10% glycerol
P. jensenii	MRS	10% glycerol or 2.5x PEB
P. jensenii^T	NL or NLG	10% glycerol
P. sp. *(wentii)*	NL or NLG	30% PEG
P. sp. Z/S	NL or NLG	10% glycerol
P. thoenii^T	NL or NLG	30% PEG

[1] NL: NL-broth; NLG: NL-broth containing 2.5% (w/vol) glycine.
MRS: MRS broth; YELG: YEL-broth with 2.5% (w/vol) glycine.

[2] PEG: Polyethylenglycole 10'000 (Zirnstein and Rehberger, 1991).
PEB: PEB-buffer (Luchansky et al., 1988).
GS: 10% glycerol and 0.5 M sucrose (Gautier et al., 1995).

40 µl of competent cells (~10^{12} cfu) were used for the electroporation experiments. Competent cells and approximately 1 µg of desalted DNA were mixed in new electroporation cuvettes (Equibio) and kept on ice for 1 minute. Then, a 3-6 ms pulse (Gene Pulser, Bio-Rad: 2.5 kV, 25 µF, 200 Ω, 2 mm gap cuvettes) was applied. After electroporation, the cells were kept for 2-3 hours at 30°C in regeneration medium (Gautier et al., 1995).
0.1 ml of the regenerated cells were directly plated on selective medium. The rest of the cells was harvested by centrifugation (5 minutes, room temperature, 5'000 g), resuspended in 0.1 ml of NL-broth and plated on the appropriate selective medium. The regenerated cells were the incubated for 14 days or until growth of the negative control was observed. As negative controls (no growth should occur), competent cells were electroporated without DNA and incubated on the selective medium.
Recombinant propionibacteria were analyzed by hybridization of the clones with the inserted vector DNA as probe. For further confirmation, plasmid isolation was performed on putative transformants.
Contamination by non-propionibacteria was routinely checked using the MPCR method developed in this thesis.

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3. RESULTS

3.1 Strain maintenance and control

Propionibacteria are relatively slow growing organisms (up to 14 days). In order to maintain pure cultures and to avoid contaminations, all cultures were routinely checked by microscopy for the absence of cocci and examined using simple tests (KOH, slime production, colony color and catalase activity).

In table 9, these characteristics are shown. For certain strains, complete fermentation patterns were determined (data not shown).

Table 9: Phenotypic characteristics used for a simple "strain control" of propionibacteria

Characteristic	Classical propionibacteria						
	P. acidi-propionici	*P. cyclo-hexanicum*	*P. freuden-reichii*	*"P. sher-manii"*	*P. jensenii*	*P. thoenii*	*P. freuden-reichii* JS53[2]
KOH[1]	G+	G+	G+	G+	G+	G+	G+
Slime	d	-	+	+	d	d	+
Color	milky white	light brown	light brown	light brown	cream or red	red	light brown
Catalase	+[3]	-	++	++	+	+	++
Nitrate reduction	+	-[4]	-	-	-	-	+
Fermentation of:							
Lactose	+	nd	-	+	+	+	+
L-Arabinose	+	nd	-	-	-	-	-
Esculin	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+
Maltose	+	nd	-	-	+	+	-

Characteristic	Cutaneous propionibacteria				
	P. acnes	*P. avidum*	*P. granulo-sum*	*P. lympho-philum*	*P. propioni-cum*
KOH[1]	G+	G+	G+	G+	G+
Hemolysis	+	+	-	-	+
Catalase	+	+	+	weak	-
Nitrate reduction	+	-	-	+	+
Esculin	-	+	-	-	-

+: Positive reaction; ++: very strong reaction; -: negative; d: detected for some of the strains. nd: no data.

1 G+: no reaction occurred, the strain is Gram-positive. An interpretation can be difficult, when slime production occurs.

2 Data from Smutny, 1997.

3 The type strain is characterized by very weak activity.

4 Data from Kusano et al., 1997.

3.2 Identification of propionibacteria using molecular methods

3.2.1 Sequencing of 16S rRNA genes of propionibacteria

Due to existing large 16S rDNA libraries, the determination of corresponding sequences for propionibacteria should allow the construction of a correct evolutionary tree for the genus *Propionibacterium*. In addition, the taxonomic position of propionibacteria within the group of high G+C Gram-positive bacteria could be confirmed.

To avoid isolation of the 16S rRNA gene from the genome and cloning, PCR amplification and cycle sequencing of the 16S rDNA was selected. To test the applied cycle sequencing strategy, the 16S rRNA gene sequences of the type strains of the genus *Propionibacterium* were elaborated and analyzed (table 10). A total of 10883 nucleotides were analyzed. The data were in a good agreement with sequences already published.

Table 10: 16S rDNA sequences of propionibacteria

Strain	Acc. no. (GenEmbl)	length[1]
Partial[2] 16S rDNA sequences determined in this study:		
P. avidum^T	AJ003055	1504 bp
P. freudenreichii subsp. shermanii^T	Y10819	1510 bp
P. freudenreichii subsp. globosum JS53	AJ009989	1505 bp
P. jensenii DF1	-	1502 bp
P. granulosum^T	AJ003057	1514 bp
P. lymphophilum^T	AJ003056	1502 bp
P. propionicum^T	AJ003058	1506 bp
16S rDNA sequences that were completed:		
P. acidipropionici^T	X53221	1351 + 134 bp
P. freudenreichii^T	X53217	1462 + 26 bp
P. jensenii^T	X53219	1364 + 126 bp
P. thoenii^T	X53220	1467 + 54 bp
16S rDNA sequences used without change:		
P. acnes^T	M61903	1530 + 0 bp
P. cyclohexanicum^T	D82046	1471 + 0 bp

^{T} Type strain.
[1] Complete 16S rDNA sequences or missing parts (+) that were sequenced in this study.
[2] Only sequences longer than 1500 bp are shown.

In addition to the newly determined 16S rDNA sequences, incomplete or poor DNA sequences of the database were updated. These sequence updates mainly concerned the medically relevant *Propionibacterium* species where only sequence stretches of

approximately 350 base pairs were known for *P. avidum*, *P. granulosum* and *P. lymphophilum* whereas the known sequence of *P. propionicum* contained a gap of 120 undefined nucleotides.

The analysis of these sequences was used to construct the first complete phylogenetic tree of the genus *Propionibacterium* containing all type strains belonging to this genus, including the medically relevant propionibacteria (figure 2).

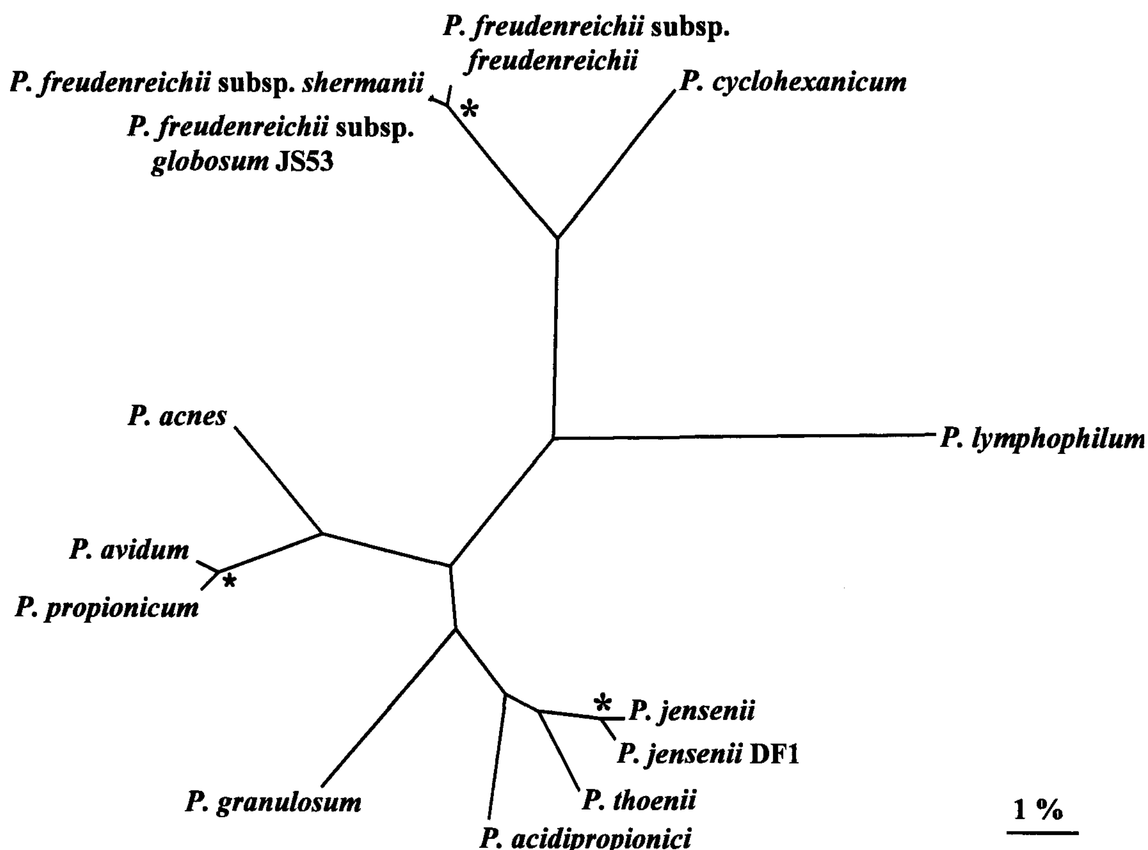

Figure 2: Unrooted tree revealing the phylogenetic relationships among the genus *Propionibacterium*. The tree is based on an alignment of 1511 basepairs of the 16S rRNA genes of propionibacteria (table 10). The alignment was constructed using the eclustalw program (GCG). The tree topology was calculated using the dnamlk algorithm (PHYLIP, maximum likelihood method with molecular clock). Terminal branchings marked with * indicate that the 16S rDNA sequences are so closely related that the shown distance reveals not the correct phylogenetic distance between these strains. The bar indicates 1% base differences.

The phylogenetic tree contains the information on almost the entire 16S rDNA regions of the selected strains. There is only a lack of approximately the first 8 nucleotides and the primer sequences (37 nucleotides, 2 ambiguities) used to generate the PCR product. A

clear distinction between classical and medically relevant species is evident. None of the medically relevant species clusters directly with the classical propionibacteria, *P. lymphophilum* is the most distantly related species of the whole genus.

The analysis of the 16S rDNA relationships within the genus *Propionibacterium* showed a clear distinction between the different species. A distance matrix with the determined homology values between the species is shown in table 11.

Table 11: Homology values (%) of the 16S rDNA genes of propionibacteria

	***P. jensenii* DF1**	***P. acidipropionici***	***P. acnes***	***P. avidum***	***P. cyclohexanicum***	***P. freudenreichii***	***P. "globosum"* JS53**	***P. granulosum***	***P. jensenii***	***P. lymphophilum***	***P. propionicum***	***P. "shermanii"***	***P. thoenii***
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
1	100	97.2	93.6	94.5	91.4	92.5	92.4	95.1	99.4	90.8	94.3	92.4	97.4
2	97.2	100	93.7	94.8	90.9	92.2	92.0	95.8	97.3	90.2	94.5	92.0	96.4
3	93.6	93.7	100	96.4	91.4	92.2	91.9	94.3	94.1	90.1	96.4	91.8	93.7
4	94.5	94.8	96.4	100	91.5	92.7	92.4	94.4	95.1	90.1	99.4	92.4	94.1
5	91.4	90.9	91.4	91.5	100	95.0	95.3	90.2	91.5	90.6	91.3	95.3	91.4
6	92.5	92.2	92.2	92.7	95.0	100	99.5	91.4	92.5	90.3	92.5	99.4	92.5
7	92.4	92.0	91.9	92.4	95.3	99.5	100	91.4	92.3	90.4	92.2	99.9	91.9
8	95.1	95.8	94.3	94.4	90.2	91.4	91.4	100	95.3	91.0	94.4	91.4	94.5
9	99.4	97.3	94.1	95.1	91.5	92.5	92.3	95.3	100	90.9	94.9	92.3	98.0
10	90.8	90.2	90.1	90.1	90.6	90.3	90.4	91.0	90.9	100	90.1	90.4	90.4
11	94.3	94.5	96.4	99.4	91.3	92.5	92.2	94.4	94.9	90.1	100	92.3	94.0
12	92.4	92.0	91.8	92.4	95.3	99.4	99.9	91.4	92.3	90.4	92.3	100	91.9
13	97.4	96.4	93.7	94.1	91.4	92.5	91.9	94.5	98.0	90.4	94.0	91.9	100

The homologies were calculated using an alignment (eclustalw, GCG) of the sequences listed in table 10. Based on this alignment, the matrix was calculated with the dnadist program (PHYLIP, method using a maximum likelihood algorithm).

The highest DNA homology values are observed for the three *P. freudenreichii* strains (99.4 - 99.9%), the two *P. jensenii* strains (99.4%) and between the two different species *P. avidum* and *P. propionicum* a homology of 99.4% exists. Towards *P. lymphophilum* none of the other species shows a higher homology than 91% (*P. granulosum*) which clearly locates this species in a border- or outgroup.

The new species *P. cyclohexanicum* is most closely related to the *P. freudenreichii* group (95% sequence homology), a fact also observed by Kusano et al. (1997).

3.2.2 Classification of isolates from dairy products

To investigate a large number of isolates, the possibility of a classification of isolates based on partial sequencing of the 16S rRNA genes was tested. Isolates of dairy origin (and other sources) were investigated. As shown in table 12, this "partial sequencing" strategy can be used for the identification of propionibacteria (and certain other Gram-positive species). A total of 16698 nucleotides were analyzed.

Table 12: Identification of bacterial strains based on partial 16S rDNA sequences

Strain or isolate	Classified as	Sequence-Identity[1]	In accordance with[4]
Strains identified as belonging to the genus *Propionibacterium*:			
"*P. arabinosum*" DSM	*P. acidipropionici*[2]	92.3% (765 bp)	supplier (DSM)
P. freudenreichii FAM1409	*P. freudenreichii*	97.1% (373 bp)	supplier (FAM)
P. freudenreichii FAM1410	*P. freudenreichii*	96.9% (770 bp)	supplier (FAM)
P. freudenreichii FAM1411	*P. freudenreichii*	93.3% (624 bp)	supplier (FAM)
P. freudenreichii FAM1412	*P. freudenreichii*	96.9% (779 bp)	supplier (FAM)
P. freudenreichii FAM1413	*P. freudenreichii*	77.2% (136 bp)	supplier (FAM)
P. freudenreichii FAM1414	*P. freudenreichii*	94.7% (357 bp)	supplier (FAM)
"*P. pentosaceum*" DSM	*P. acidipropionici*[2]	94.9% (783 bp)	supplier (DSM)
"*P. petersonii*" DSM	*P. thoenii*	92.9% (397 bp)	supplier (DSM)
"*P. rubrum*" DSM	*P. thoenii*[3]	93.0% (739 bp)	de Carvalho et al., 1995
"*P. shermanii*" DSM	*P. freudenreichii*	92.1% (607 bp)	supplier (DSM)
P. sp. DF1	*P. jensenii*	94.4% (1453 bp)	Fessler, 1997
P. sp. DF3	*P. jensenii*	92.4% (1270 bp)	Fessler, 1997
P. sp. DF15	*P. freudenreichii*	97.1% (582 bp)	Fessler, 1997
P. sp. DF16	*P. freudenreichii*	97.8% (495 bp)	Fessler, 1997
P. sp. JS53	*P. freudenreichii*	99.6% (1508 bp)	Chemotaxonomy
P. sp. JS62	*P. freudenreichii*	95.4% (153 bp)	Chemotaxonomy
P. sp. JS127	*P. freudenreichii*	98.6% (645 bp)	Chemotaxonomy
P. sp. ZS	*P. jensenii / thoenii*	90.3% (627 bp)	Chemotaxonomy
Identification of strains belonging to other genera:			
AC1	*Lactococcus lactis*	97.4% (684 bp)	Chemotaxonomy
BU2-60	*Lactococcus lactis*	97.1% (456 bp)	Chemotaxonomy
JS38	*Lactobacillus casei*	97.9% (628 bp)	Chemotaxonomy
JS63	*Lactobacillus casei*	97.1% (449 bp)	Chemotaxonomy
JS65	*Lactobacillus casei*	98.6% (580 bp)	Chemotaxonomy
JS84	*Lactobacillus casei*	98.0% (646 bp)	Chemotaxonomy
JS85	*Lactobacillus casei*	99.1% (534 bp)	Chemotaxonomy
JS86	*Lactobacillus casei*	98.1% (698 bp)	Chemotaxonomy
JS108	*Lactobacillus casei*	97.1% (419 bp)	Chemotaxonomy
K214	*Lactococcus lactis*	96.2% (522 bp)	Chemotaxonomy
PA2	*Staph. epidermidis*	97.8% (980 bp)	Chemotaxonomy

[1] Highest identity value over xy basepairs found using the FASTA algorithm.
[2] Best sequence identity found for *Eubacterium combesii*, an obviously wrong database entry !
[3] Correct would be: *P. jensenii* (92.6% in 739 bp).
[4] Identification by 16S rDNA sequencing confirms the findings of other authors or is in accordance with chemotaxonomical properties of the respective strains as determined in this thesis.

Database queries (FASTA algorithm) with the newly determined sequences revealed the most closely related organism which usually was the correct identification. Errors occurred due to wrong database entries or when 16S rDNA sequences of limited length were obtained (smaller than 400 bp). For the discrimination of the species *P. thoenii* and *P. jensenii*, especially long stretches of the 16S rDNA had to be used (at least 1200 bp are needed). All identifications based on the sequencing data were compared to other data (supplier, chemotaxonomy or morphology) and could be confirmed. In table 12, the identification of some isolates based on 16S rDNA sequences is shown. All *Propionibacterium* strains received from other sources could be correctly classified to species level.

The analysis of the 16S rDNA gene turned out to be a fast and reliable tool for the identification of unknown isolates to species level. Especially for propionibacteria, the method developed in this study allowed the fast identification of strains within two days.

3.2.3 Construction of a specific gene probe for the genus *Propionibacterium*

Based on the 16S rDNA sequences determined before (chapter 3.2.1), an oligonucleotide probe (gd1, table 5) specific for the genus *Propionibacterium* was constructed.

Table 13: FASTA-comparison of the *Propionibacterium* genus-specific probe gd1 with the GenEmbl database

Species	Sequence (16S rDNA, 5' → 3')	% Identity	Acc. no.
Primer gd1	TGCTTTCGATACGGGTTGAC	-	-
P. acidipropionici	.TGCTTTCGATACGGGTTGAC.	100%, 20 bp	X53221
P. acnes	.TGCTTTCGATACGGGTTGAC.	100%, 20 bp	M61903
P. acnes	.TGCTTTCGATACGGGTTGAC.	100%, 20 bp	Y12288
P. acnes	.TGCTTTCGATACGGGTTGAC.	100%, 20 bp	X53218
P. avidum	.TGCTTTCGATACGGGTTGAC.	100%, 20 bp	AJ003055
P. freudenreichii	.TGCTTTCGATACGGGTTGAC.	100%, 20 bp	AJ009989
P. freudenreichii	.TGCTTTCGATACGGGTTGAC.	100%, 20 bp	X53217
P. freudenreichii	.TGCTTTCGATACGGGTTGAC.	100%, 20 bp	Y10819
P. granulosum	.TGCTTTCGATACGGGTTGAC.	100%, 20 bp	AJ003057
P. jensenii[1]	.TGCTTTCGATACGGGTTGAC.	100%, 20 bp	X53219
P. lymphophilum	.TGCTTTCGATACGGGTTGAC.	100%, 20 bp	AJ003056
P. propionicus	.TGCTTTCGATACGGGTTGAC.	100%, 20 bp	AJ003058
P. thoenii	.TGCTTTCGATACGGGTTGAC.	100%, 20 bp	X53220
Actinomyces israelii[2]	.TGCTTTCGATACGGGTTGAC.	100%, 20 bp	X53228
Unknown bacterial species	.TGCTTTCGATACGGGTTGAC.	100%, 20 bp	Z73442
Bacterial species	.TGCTTTCGATACGGGTTGAC.	100%, 20 bp	Z69317
M. chitae[2]	.TGCTTTCGATACGGGTTGAC.	100%, 20 bp	M29560
Unknown bacterial species	.TGCTTTCGATNCGGGTTGAC.	95%, 20 bp	AF059764
Eubacterium combesii[2]	.TGCTTTCGATACGNGTTGAC.	95%, 20 bp	L34614
Unknown bacterial species	.CGCTTTCGATACGGGTTGAC.	100%, 19 bp	Z73456
P. cyclohexanicum	.TGCTTTCGATACGGGTCGAC.	95%, 20 bp	D82046
Actinomycete species	.TGCTTTCGATATGGGTTGAC.	95%, 20 bp	Z73375
Unknown bacterial species	.TGCTTTCGATACGGGCTGAC.	95%, 20 bp	AB011304
Unknown bacterial species	.TGCTTTCGATACGGGCTGAC.	95%, 20 bp	AB011303
Nocardioides	.TGCTTTCGATACGGGCAGAC.	90%, 20 bp	X86620
Nocardioides simplex	.TGCTTTCGATACGGGCAGAC.	90%, 20 bp	AF005012
Nocardioides luteus	.TGCTTTCGATACGGGCAGAC.	90%, 20 bp	X53212

[1] Corrected sequence for this region (determined in this study).

[2] Doubtful database entry: a higher homology exists with propionibacteria-sequences than with sequences from the correct genus (up to 20% sequence-differences).

In table 13 the best hits of a FASTA comparison of gd1 versus 3'295'425 DNA-sequences (GenEmbl; November 3, 1998) are shown. Differences between the sequences are marked with black boxes. The highest 50 ranking matches of gd1 with the database were all sequences of 16S rRNA genes. Only propionibacteria and not further characterized

isolates showed a 100% identity in all 20 bases of the primer sequence. From all known propionibacterial 16S rDNA sequences, only the sequence of *P. cyclohexanicum* shows one base-mismatch with the specific probe gd1.

Certain 16S rDNA sequences of the database that belong to other genera showed 100% identity with the sequence of gd1, they are marked in table 13 (*Actinomyces israelii*, *Mycobacterium chitae* and *Eubacterium combesii*). The whole sequences were compared (FASTA-algorithm) with other 16S rDNA sequences from the same genera and from propionibacteria. A higher degree of homology, up to 20% more, with sequences from the genus *Propionibacterium* than with the sequences of their "own" genera was obvious. This indicates either sequencing, strain purification or identification problems with these organisms and thus, their 16S rDNA sequence-entries were arbitrarily regarded as wrong and were, therefore, not further taken into consideration in this study.

3.2.4 Multiplex PCR (MPCR)

To reduce time for the detection of the genus *Propionibacterium* and to verify the identification of isolates, a rapid method to differentiate the genus *Propionibacterium* from other genera based on a multiplex-PCR (MPCR) approach was developed (Dasen et al., 1998). The method is based on the above mentioned genus-specific gene probe gd1, which is targeted on the 16S rRNA gene of propionibacteria (table 5).

Using this specific primer and two universal bacterial 16S rDNA primers bak11w and bak4 (also targeted on the 16S rDNA), fragments of the 16S rDNA were amplified by means of multiplex PCR. As shown in figure 3, propionibacteria are characterized by the presence of a specific 900-bp sized fragment (the size of the fragment varies from 889 to 915 base pairs, depending of the *Propionibacterium* species) whereas for other genera only an universal 1500 bp PCR-fragment ("housekeeping" gene) is expected to be amplified (bak11w - bak4).

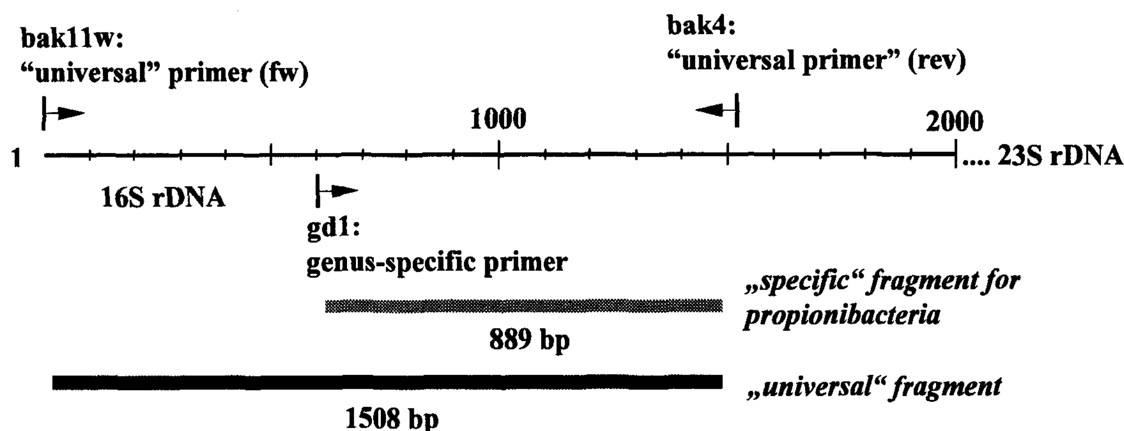

Figure 3: Graphical representation of oligonucleotide-positions and amplification products shown for the 16S rDNA sequence of *P. freudenreichii* subsp. *shermanii* (GenEmbl Accession no Y10819). Multiplex PCR reaction targeted on 16S rDNA resulting in a 889 (primers bak11w, gd1) and a 1508-bp sized (primers bak11w, bak4) fragment.

A total of 150 propionibacteria isolates and reference strains from culture collections were tested with the MPCR assay and the desired 900-bp PCR-fragment was always obtained. In addition, various species related to propionibacteria or involved in dairy fermentations were tested as negative controls and no false positive signals were detected (figure 4). The detection limit for propionibacteria was at 2 x 10^3 cfu (colony forming units).

As shown in figure 4, only propionibacteria (figure 4a and b; up to strain no. 39) are characterized by the formation of the specific 900-bp sized fragment. For the negative controls (figure 4b, c, and d; starting with strain no. 43), only the housekeeping gene (1,5-kb fragment) was amplified, which proved the absence of inhibitory substances in the PCR assay. The negative control (lane -) where no DNA was added to the MPCR reaction confirms that no contaminating DNA was introduced into the assay system.

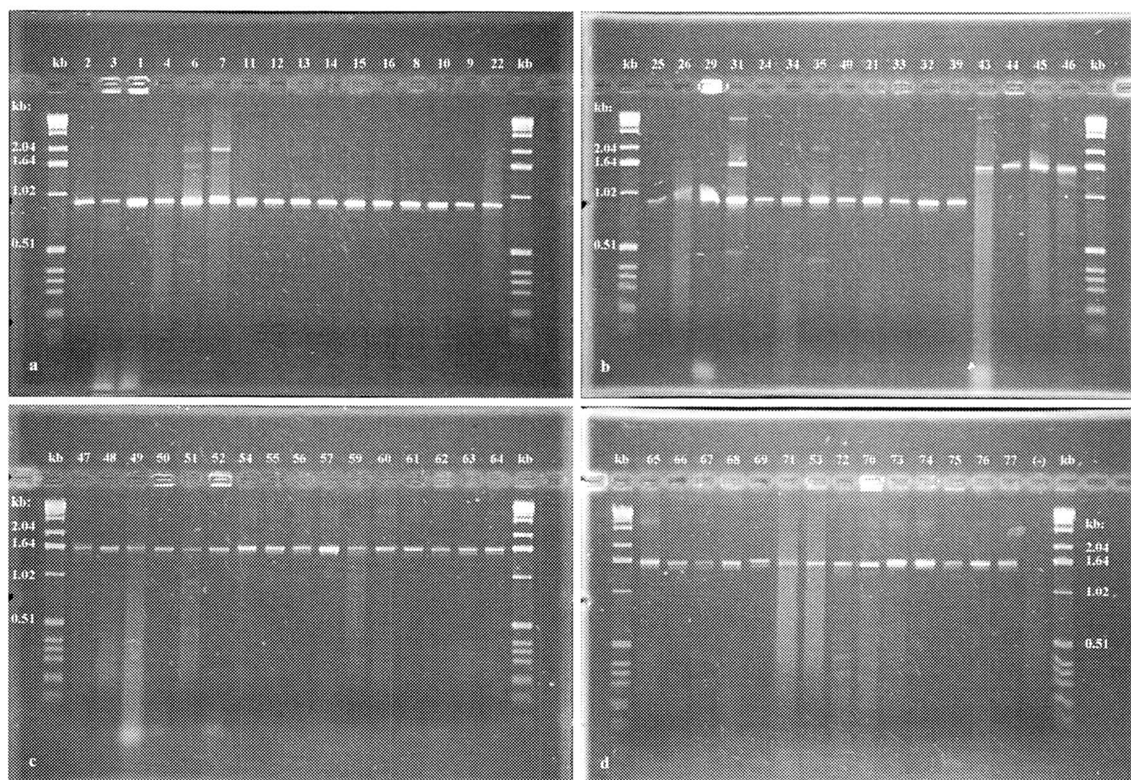

Figure 4: Detection of *Propionibacterium* strains using Multiplex PCR (primers bak11w, gd1 and bak4). DNA from propionibacteria and other organisms was analyzed using the MPCR protocol, stained with ethidium-bromide and examined on a 2% TBE agarose gel. The numbering of the lanes corresponds to the strains listed in table 3. Lane "kb": kb-ladder (Gibco). (-) stands for the negative control (MPCR without DNA)

3.2.5 Differentiation and identification of propionibacteria based on the 16S-23S rDNA spacer region

Using the MPCR method developed in this thesis, the fast and simple identification of isolates to genus level is possible for propionibacteria. In a next step, a method allowing the identification of *Propionibacterium* isolates down to species level was investigated. As shown by Rossi et al. (1997), the 16S-23S rDNA intergenic spacer regions of propionibacteria can be used to differentiate between the species. By means of PCR the spacer regions were amplified and then sequenced. The DNA sequences of the spacers of 25 strains, including all type strains were determined. Based on these data, a phylogenetic tree was constructed (figure 5).

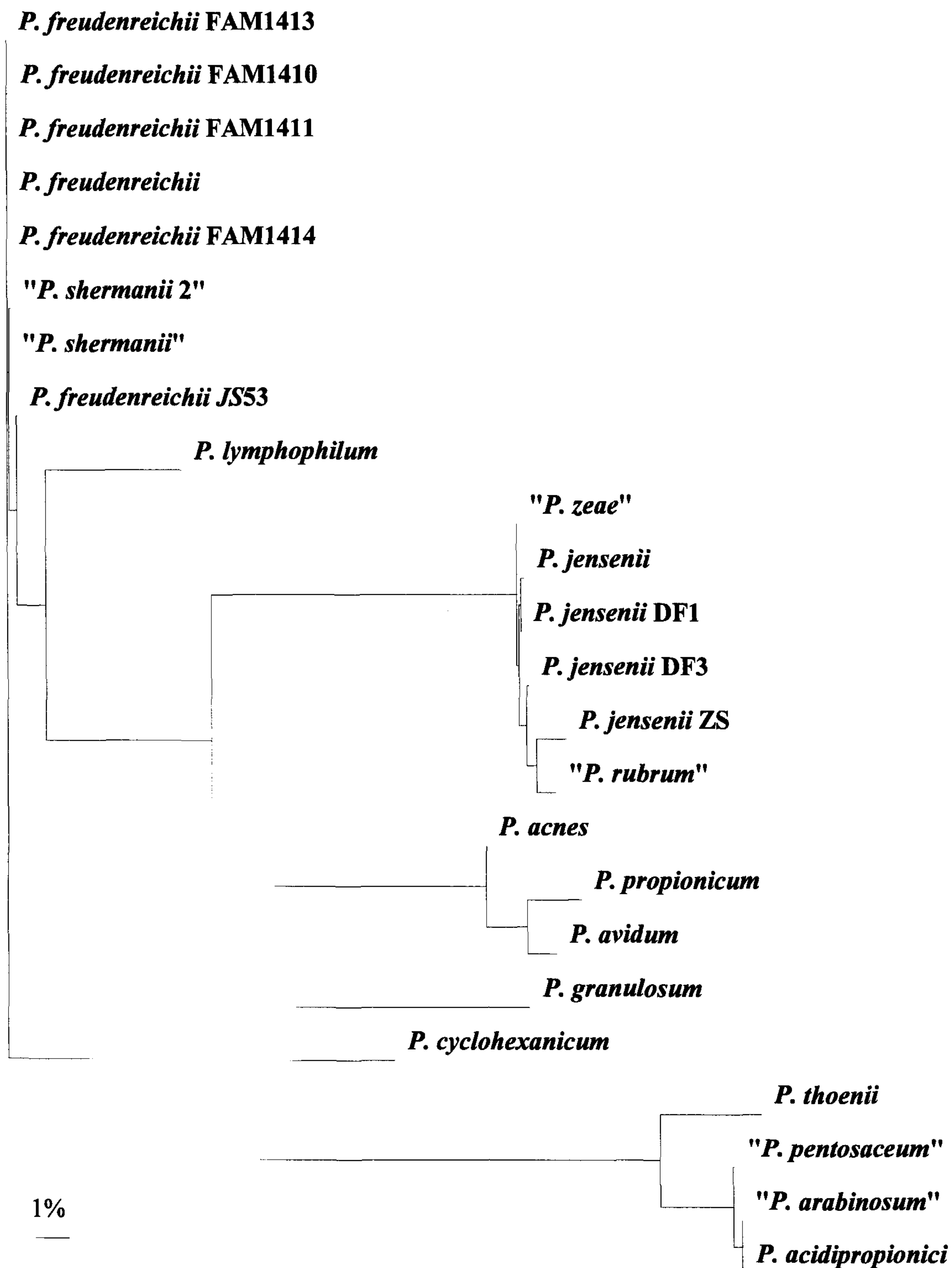

Figure 5: Phylogenetic tree of the genus *Propionibacterium* based on sequences of 16S-23S rDNA intergenic spacer regions (eclustalw-alignment of 265 nucleotides). " ": old strain designations which are no longer correct (used to simplify strain separation). The bar indicates 1% base differences. The alignment was constructed using the eclustalw program (GCG) and the tree topology was calculated using the dnamlk algorithm (PHYLIP, maximum likelihood method with molecular clock).

Based on the analysis of the intergenic spacer region it is possible to assign strains to the correct species as shown for example in figure 5 for the strains combined in the *P. jensenii* cluster or all the isolates belonging to the *P. freudenreichii* group. But contrary to the tree shown in figure 2, the species are grouped differently in figure 5, e.g. the *P. jensenii* cluster does not appear near the *P. thoenii* / *P. acidipropionici* branches of the tree.

In figure 6, the alignment of the 16S-23S rDNA intergenic spacer regions of selected propionibacteria, used for the construction of the phylogenetic tree of figure 5, is shown.

```
                                          Start 16S-23S rDNA ITS region
                                          |
                                          |              Start of alignment for tree constrution
                       1                  ↓              ↓                                      80
P. freud. FAM1413      GGCTG.GNTCACCTCCTTTCT.AAGGNGCCTTTTCGCCATCGTGCGTTTCGTGGGCTGTG............TCCTGCG.
P. freud. FAM1410      GGCTG.GATCACCTCCTTTCT.AAGGAGCCTTTTCGCCATCGTGCGTTTCGTGGGCTGTG............TCCTGCG.
P. freud. FAM1411      GGCTG.GTTCACCTCCTTTCT.AAGGNGCCTTTTCGCCATCGTGCGTTTCGTGGGCTGTG............TCCTGCG.
P. freudenreichii           ...................................CGTGCGNNN.GTNGGCTGTNN...........TCCTGCNN
P. freud. FAM1414      GGCTG.GNTCACCTCCTTTCT.AAGGNGCCTTTTCGCCATCGTGCGTTTCGTGGGCTGTG............TCCTGCG.
"P. shermanii 2"       GGCTG.GATCACCTCCTTTCT.AAGGAGCCTTTTCGCCATCGTGCGTTTCGTGGGCTGTG............TCCTGCG.
"P. shermanii"         GGCTG.GATCACCTCCTTTCT.AAGGAGCCTTTTCGCCATCGTGCGTTTCGTGGGCTGTG............TCCTGCG.
P. freud. JS53         .....................................NATCGTGCGGNNNGTNGGCTGTN............TCCTGCNN
P. lymphophilum        GGCTG.GATCACCTCCTTTCT.AAGGAGCCATT.........T.....TCAACCGTT.....................
"P. zeae"              GGCTG.GATCACCTCCTTTCT.AAGGAGCTGT.....GACTGTGTGGGTCGTCG.TTGTG............GCGGTCTG
P. jensenii            GGCTG.GNTCACCTCCTTTCT.AAGGAGCTGT.....GACTGTNTGGGTCGTCG.TTGTG............GCGGTCTG
P. jensenii DF1        GGCTG.GNTCACCTCCTTTCT.AAGGNGCTGT.....GACTGTNTGGGTCGTCG.TTGTG............GCGGTCTG
P. jensenii DF3        GGCTG.GNTCACCTCCTTTCT.AAGGNGCTGT.....GACTGTNTGGGTCGTCG.TTGTG............GCGGTCTG
P. sp. ZS              GGCTG.GATCACCTCCCTTCN.AAAGAGCTGT.....GACTGTNTNGGTCNTCN.TTNTGN...........GCCGTCTA
"P. rubrum"            NGCTT.GATCACCTCCTTTCNNAAGGAGCCGT.....GACNGTNTTGGTCGTCN.NTGTGA...........GCCGTCTG
P. acnes               GGCTG.GATCACCTCCTTTCT.AAGGAG..........TTTTGTGA..................................
P. propionicum         GGCTG.GATCACCTCCTTTCT.AAGGAGCACTT...CATTGTCAGGACTCATCATTGCTGTGTG.......GCTCGTTGT
P. avidum              GGCTGNGATCACCTCCTTTCT.AAGGNGCAAGT...NTTGATTGTGGC..ATTGTTCGAGNTCG.......TCCCGT.GT
P. granulosum          GGCTG.GATCACCTCCTTTCT.AAGGAGCAAGTGGCGCACTGTTGTGTGTTCNTGTNGTGN..................A
P. cyclohexanicum      GGCTG.GATCACCTCCTTTCT.AAGGTGCCTGT..GGCTCGGTGCGGCTGGTTGGTCGTGGCTGGGTCCATTGTCTGCCA
P. thoenii             GGCTG.GATCACCTCCTTTCT.AAGGAGC.TTTCATGGGCCCTGTGCTCACCGTGTGNTGGGCG........GGGGGTTN
"P. pentosaceum"       GGCTG.GATCACCTCCTTTCT.AAGGNGC.TTTCTGGAAGCCGGCCGTCGCCCGANTGTGGTGA........TGGTGCGG
"P. arabinosum"        GGCTG.GNTCACCTCCTTTCT.AAGGNGC.TTTCTGGAAGCNGGCCGTCGCCCGANTGTGGTGA........TGGTTCGG
P. acidipropionici     GGCTG.GATCACCTCCTTTCT.AAGGAGC.TTTCTGGAAGCCGGCCGTCGCCCGAGTGTGGTGA........TGGTTCGG
Primer bak4            GGNTG-GATCACCTCCT

                       81                                                                            160
P. freud. FAM1413      GCGTTGGTGGTGGAGGT...........TGTGGAGC......ATTGANCG.........TAGATTGTCGGCTNN.TTTCT
P. freud. FAM1410      GCGTTGGTGGTGGAGGT...........TGTGGAGC......ATTGACCG.........TAGATTGTCGGCTGG.NTTCT
P. freud. FAM1411      GCGTTGGTGGTGGAGGT...........TGTGGAGC......ATTGACCG.........TAGATTGTNGGCTNN.TTTCT
P. freudenreichii      GCGTTGGTGGTGGAGGT...........TGTGGAGC......ATTGACCG.........TAGATTGTNGGCTGG.NTTCN
P. freud. FAM1414      GCGTTGGTGGTGGAGGT...........TGTGGAGC......ATTGACCG.........TAGATTGTCGGCTNN.NTTCT
"P. shermanii 2"       GCGTTGGTGGTGGAGGT...........TGTGGAGC......ATTGACCG.........TAGATTGTCGGCNNN.TTTCT
"P. shermanii"         GCGTTGGTGGTGGAGGT...........TGTGGAGC......ATTGACCG.........TAGATTGTCGGCTGNGTTTCT
P. freud. JS53         GCGTTGGTGGTGGAGGT...........TGTGGAGC......ATTGACCG.........TAGATTGTNGGCTGN.NTCTG
P. lymphophilum        ...TGGTTGGTGGTTTT............GTGGAAC......ATTGACTTT........TGACTGATCAGGTTT.TTCTG
"P. zeae"              GCATGGTCCATTGGGTCG.GGCGCCTCCTGTGTGGG..GTGTGTG.GCCTGC..TTCTGTGGAATGT.TGACTGGATGTA
P. jensenii            GCATGGTCCATNGGGTCG.GGCGCCTCCTGTGTGGG..GTGTGTG.GCCTGC..TTCTGTNGNATGT.TGACTGGATGTA
P. jensenii DF1        GNATGGTCCATTGGGTCG.GGCGCCTCCTGTGTGGG..GTGTGTG.GCCTGC..TTCTGNNGAATGT.TGACTGGATGTA
P. jensenii DF3        GCATGGTCCATTGGGTCG.GGCACTNCCTGTNTGGG..GTGTGTG.GCCTGC..TTCTGTGGAATGT.TGACTGGATGTA
P. sp. ZS              GCATNGNCCATTGGGNCG.GGNACNCCCTNTNTNGG..GTGTGTN.GCCTGC..TTCTGNGGNATGT.TNACTGGATGTA
"P. rubrum"            GCANGGTNCATTGGGNCG.GGCACTCNCTGTGTAGGA.GTGTGTANGCCTGCNCTCTNNGNGNATGTGTGACTGGATGTA
P. acnes               .............................GTGGAAT......GTTGGCGTCCTGC...CTGTNATGGGTGG.GGTTGAGN
P. propionicum         GAGTCGTTGGTGGATGGGTTA.GCTTCTTGTGGAAC......ATTGGCGTGTNNC...ATCGTGTGGTGGACGGTCGGGG
P. avidum              GGGGTGATGGTAATGGTGTCGTGNNTCTTGTGGNAN......ATTGGCGTCCTGN...NNTTAGTGATGG..GGTTGGGG
P. granulosum          ATGTTGGCTGTGGTCAC....CGTTTGTGGTGGGGCACGTTGTTGGGGTTCTGGG...GTATCACGCCGGCTGN.GTGTG
P. cyclohexanicum      GTGTGGGTGTCGGGGTTGTCTCGGTGCTTGTGGTGCTNNTGGGCTGGCTGAGGNT...GTGGATCATTGACTGG.GTGGT
P. thoenii             NTGCTGCTGCCGGGGTTTCCTGG....TGGTGGTGCTTCT..GTGGA...................ATG....TNGACTG
"P. pentosaceum"       TTCAGGCTGTCGGGTCCTTGCGGGT.CCGGTGGTGCTTCTGAGTGGAATGTTGGCTATGGACGCTCCTGGTCTTCGGGTC
"P. arabinosum"        TTCAGGCTGTCGGGTCCTTGCGGGT.CCGGTGGTGCTTCTGAGTGGN.TNTTGGCTATGGACGCTTCTGGTCTTCGGGTC
P. acidipropionici     TTCAGGCTGTCGGGTCCTTGCGGGT.CCGGTGGTGCTTCTGAGTGGAATGTTGGCTATGGACGCTTCTGGTCTTCGGGTC
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                       161                                                                            240
P. freud. FAM1413      GTTTGTNAGTACTGCTGGTGCCC.TGTTGGGG...TGTTGGTGTNNNACGCGGGT..GGGGGTTGGTTGGTG..GTCGTG
P. freud. FAM1410      GTTTGTTAGTACTGCTGGTGCCC.TGTTGGGG...TGTTGGTGTGGT.CGCGGGT..GGGGGTTGGTTGGTG..GTCGTG
P. freud. FAM1411      GTNTGTNAGTACTGCTGGTGCCC.TGTTGGGG...TGTTGGTGTNNN.CGCGGGT..GGGGGTTGGTTGGTG..GTCGTG
P. freudenreichii      NTNTGTNAGTACTGCTGGTGCCC.TGTNGGGG...TGTTGGTGTGGANCGCNGGT..NGGGGTTGGTTGGTG..GTCGTG
P. freud. FAM1414      GTTTGTTAGTACTGCTGGTGCCC.TGTTGGGG...TGTTGGTGTNNAACGCGGGT..GGGGGTTGGTTGGTG..GTCGTG
"P. shermanii 2"       GTTTGTTAGTACTGCTGGTGCCC.TTTTGGGG...TGTTGGTGNGAA.CGCGGGTN.GGGGGTTGGTTGGTG..GTCGTN
"P. shermanii"         GTTTGTTAGTACTGCTGGTGCCC.TTTTGGGG...TGTTGGTGTGGANCGCGGGTN.GGGGGTTGGTTGGTG..GTCGTG
P. freud. JS53         TT.TGTNAGTACTGCTGGTGCCC.TTTTGGGG...TGTTGGTGTGGAACGCGGGT..GGGGGTTGGTTGGTG..GTCGTG
P. lymphophilum        GTTGTTTAGTACTGCTTGC.....TTTTGNGN.......AGTGTGGAACGCGCTG.....GTTTGGTTTG.G..TTGGTT
"P. zeae"              TTTTTCTGCCTGG.TCTGGTGTCCTCCTNNNT..GGGGGTGTCGGGTGGGTANNGGA.TAGCACTGTTGGGGGTTCTGGG
P. jensenii            TTTTTCTGCCTGGGTCTGGTGTCCTCCTGTNT..GGGGGTGTCGGGTGNGNTNNNN..TC..ACTGTTGGGG.TTCTGGG
P. jensenii DF1        TTTTTCTGCCTGG.TCTGGTGTCCTCCTGTNT..GGGGGTGTCGGGTGGGTNGGGG..TGC.ACTGTTGGGG.TTCTGGG
P. jensenii DF3        TTTTTCTGCCTGG.TCTGGTGTCCTCCTGTGT..GGGGGTGTCGGNGTGGTTGTGG..TNTNACTGTTGGGG.TTCTGGG
P. sp. ZS              NTTTTCNNNCCNGGNCNGGGGNCCNCCNGGGGN.GGGGGTGTCNGGTTGGTNNTTNA.TANNAATGNTGGGGNTCNGGGG
"P. rubrum"            ATTTTCTGCCTGG.NCNGGTGNCCTNCTNNNT..NGGGGTGTCNGGTTGNNACTNNCACTNNTCTGNTGGGG.NTCTNGG
P. acnes               GCATGCTGTTGGG.TTGTGGGG..TATCACA.......TGTGTGNT.GGCCTG.TGCGGTGCTGTGTTGCG..TGCGT.
P. propionicum         GCATGCTGTTGGGGTTCTGGGG..TATCACAC...TGG.TGTGTGGT.GGCCTGGTGCGGTGATGCTGCGTC..TGCT..
P. avidum              GCATGCTGTTGGGGTTCTGGGG..TATCACAC...TG..TGTGTGGT.GGCCTGGTGCGGTGATGCGTCATC..TGTCTG
P. granulosum          GTTTCCTGGATGTGCGAACGC...CGTGGCTG...CATGCGTTNGGTGTGTGTCAGTGGGTGTCTCGATGTG..GTGGTT
P. cyclohexanicum      TNGTGCTGGNNGTTCTGGTGCGAGTACAGCCG...TGCTCCTTTGTGGGGTGTGGA.GTGGGAAGTACTGGG..ATGGTG
P. thoenii             AAATTTTGGGCGCACTGTTGGGGTTCTGGGGT.ATCACGCACGNNNGTG.TGGT...GTCTGGCCGGCTCC......TCG
"P. pentosaceum"       GGGGGTGGGATGCACTGNNGGGGTTCTGGGGT.ATCACCTGCACAGGTGGTGGCCTGGTGTGGCGGACATCG..CTGNCG
"P. arabinosum"        GGGGGTGGGATGCANTGTTGGGGTTCTGGGGT.ATCACCTGCACAGGTGGTGGCCTGGTGTGGCGGACATCG..CTGCCG
P. acidipropionici     GGGGGTGGNATGCANTGTTGGGGTTCTGGGGGNANCACCTGCACNGGTGGTGGCCTGGTGTGGCGGACATCG..CTGCCG

                                                                             End of "tree-alignment"
                       241                                                                ↓        320
P. freud. FAM1413      GGAGGCACGTTGTTG.GGTCCTGAGGGATCGGCCAT..GTTTGTGGCTGGTTTTT...CGGTGTGCCAGGTGGTTGTTC
P. freud. FAM1410      GGAGGCACGTTGTTG.GGTCCTGAGGGATCGGCCAT..GTTTGTGGCTGGTTTTT...CGGTGTGCCAGGTGGTTGTTC
P. freud. FAM1411      GGAGGCACGTTGTTG.GGTCCTGAGGGATCGGCCAT..GTTTGTNGCTGGTTTTT...CGGTGTGCCAGGTGGTTGTTC
P. freudenreichii      GGAGGCACGTTGTTG.GGTCCTGAGGGATCGGCCAT..GTTTGTNGCTGGTTTTT...CGGTGTGCCAGGTGGTTGTNC
P. freud. FAM1414      GGAGGCACGTTGTTG.GGTCCTGAGGGATCGGCCAT..GTTTGTGGCTGGTTTTT...CGGTGTGCCAGGTGGTTGTTC
"P. shermanii 2"       GGAGGCACGTTGTTG.GGTCCTGAGGGATCGGCCAT..GTTTGTGGNTGGNTTTT...CGGTGTGCCAGGTGGNTGNTC
"P. shermanii"         GGAGGCACGTTGTTG.GGTCCTGAGGGATCGGCCAT..GTTTGTGGCTGGTTTTT...CGGTGTGCCAGGTGGTTGTTC
P. freud. JS53         GGAGGCACGTTGTTG.GGTCCTGAGGGATCGGCCAT..GTTTGTNGCTGGTTTTT...CGGTGTGCCAGGTGGTTGTTC
P. lymphophilum        TTTTGCACGCTGTNG.GGTTTTGAAGCCTCG......GATTTTGGCT.........TCGTGCGTCAAGCGCGCATGG
"P. zeae"              GGTATCACCTTGTGGGTGTCCTGGCGAATGCCGCTG..CTGCGGGTCCTGTTGTG...GGGCTTGG...GTGGGTGTCT
P. jensenii            G.TATCACCTTGTGG.TGTCCTGGCGAATNCCGCTG..CTGCGGGTCCTGTTGTG...GGGCCTGGN..GTGGGTGTCT
P. jensenii DF1        G.TATCACCTTGTGG.TGTCCTGGCGAATNNCGCTG..CTGCGGGTCCTGTTGTG...GGGCTTGT...GTGGGTGTCT
P. jensenii DF3        G.TATCACCTTGTGG.TGTCCTGGCGAATNNCGCTG..CTGCGGGTCCTGTTGTG...GGGCTTG.............
P. sp. ZS              GANNNCACCNTNNGGGGNCCCNGGNGAATNCCGCTTNCTTGCGGGNCCCNGTTTGTNGGGGCNTTTTNGTGGGGTGTCN
"P. rubrum"            G.TATCACCNTGTGGGTGTCCTGGCGNATNCCGCTN..CTNCGGGTNCTGNTGTN...GGGCNTTTT..GTTGGTGTCT
P. acnes               GTGTGCGTGGTGTGG.TGTT....CGTGTGGTG.....GTTGAGAACTGTATAGT...GGATGCGGTATCTTTATTGTT
P. propionicum         GTGTGGTGGGTGTGG.TGTTGATTCGTGTGGTG.....GTTGAGAACTGTATAGT...GGATGCGGCATCTTAATTTTT
P. avidum              TTGTGGACGGTGGTG.TGTTGATTCGTGTGGTG.....GTTGAGAACTGTATAGT...GGATGCGGCATCTTAATTTTT
P. granulosum          TAGAACTGCATANNG.GATGCGAGCATTTTTGTTTT..GTGATTTTGTTGTTTGT...TCGTGTGCAAGCTACTAAGTN
P. cyclohexanicum      GTGGTGATCGTGAGG.CACACTGTTGGGTCCTGAAG..GGCCNNTTGTNGNNNTT...TCGTGCGTCTGGTGTCTGGTT
P. thoenii             GCTGCGCNGGTCTTC.NGGTTCGTGT.....GGTGG..GTGTCNGNNNNGTNGNT...GAGAACTTACAGTGGATATG.
"P. pentosaceum"       GCTGGCCTGGTTGTC.GGGTTGGNGATAGNNCNTAN..NGCCNCGTGTGGTGGTT...GAGAACTTACAGTGGATATG.
"P. arabinosum"        GCTGGCCTGGTTGTC.GGGTTGGTTGN....GATTG..TGTCTCGTGTGGTGGTT...GAGAACTTNCAGTGGATATGG
P. acidipropionici     GCTGGCCTGGTTGTC.GGGTTGGNNG.....GNNNG..TGTCTCGTGTGGTGGTT...GAGAACTTACAGTGGATAT..
```

Figure 6: Alignment of sequences of the 16S-23S intergenic spacer regions from propionibacteria. The alignment was constructed using the eclustalw program (GCG). The strains correspond to the strains shown in figure 5 and are listed in table 3 (*P. freud.* = *P. freudenreichii*).

The sequencing of the intergenic spacer regions allows the simple designation of isolates to species level. Even sequences containing ambiguities could be assigned to the correct species. As an example, in figure 5 and figure 6, "*P. rubrum*" clusters in the *P. jensenii* group and not with strains of *P. thoenii*, a fact that has been recently discovered by de Carvalho et al. (1995) by the use of 16S rDNA analysis. Based on the alignment of the "spacer sequences" of propionibacteria, a simple method to identify *Propionibacterium* species using DNA hybridization techniques with PCR-amplified spacer regions of propionibacteria as gene probes was developed.

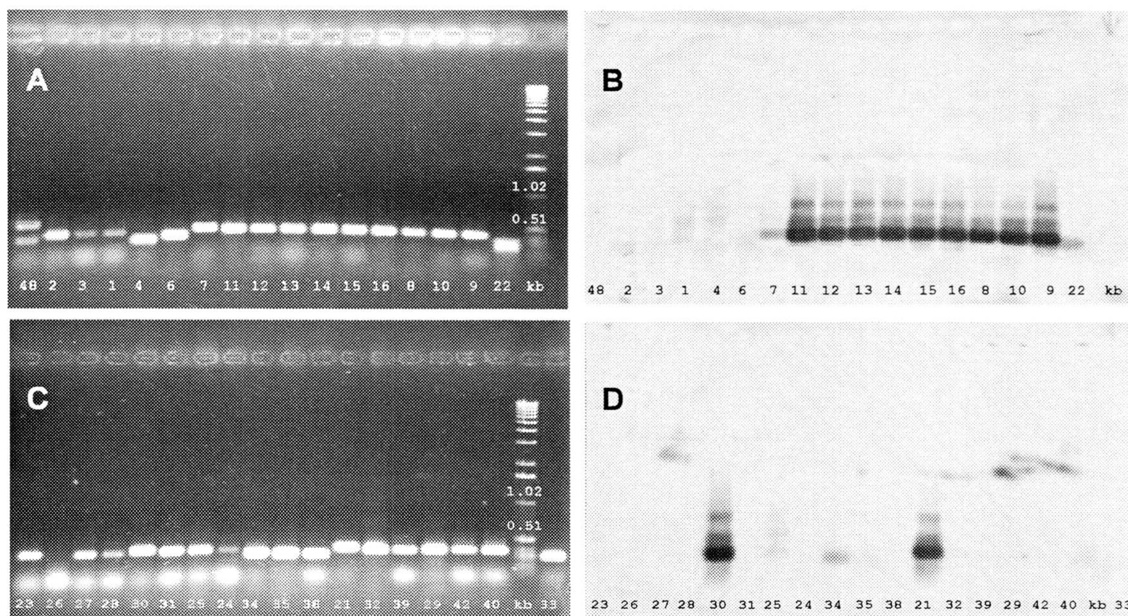

Figure 7: Detection of strains of *Propionibacterium freudenreichii* by hybridization with a DIG-labeled PCR-fragment of the spacer-region from *P. freudenreichii* subsp. *shermanii*^{T}. Intergenic spacer regions from propionibacteria and *Bifidobacterium lactis* were amplified (PCR, primers bak4 - gd6r), analyzed on a 2% TBE agarose gel and transferred to a nylon membrane by the Southern-blot technique.
figure 7A and C: 2.0% TBE-agarose gel, stained with ethidium-bromide.
figure 7B and D: Colorimetric detection (Boehringer) of the hybridization products. The numbering of the lanes corresponds to the strains listed in table 3.
"kb": kb-ladder (Gibco).

As shown in figure 7 all strains belonging to the *P. freudenreichii* cluster (strains 7 to 9 in figure 7A and B) gave a positive signal when probed with the spacer region of one strain, only one strain, "*P. technicum*" (no. 30 in figure 7C and D), gave a false positive result. Weak positive signals (interpreted as negative) were observed for *P. granulosum*, *P. lymphophilum* and *P. cyclohexanicum* (figure 7, no. 22, 34 and 7).

For one strain, *P. jensenii* (*petersonii*), no amplification product was obtained in the experiment shown above (figure 7C, no. 26). The spacer region of this strains could be amplified successfully in other experiments and did also not hybridize with the probe for *P. freudenreichii* (data not shown).

3.2.6 Discrimination between the *P. freudenreichii* subspecies

The differentiation between the subspecies of *P. freudenreichii*, (subsp. *freudenreichii* and *shermanii*, eventually subsp. *globosum*) is based on chemotaxonomic features. To find a molecular method (e.g. specific oligonucleotide probes) capable to distinguish the subspecies, the 16S and 23S rRNA genes were if needed sequenced and analyzed. The alignments of these sequences are shown in figure 8 and in figure 9.

```
P.freud.    .......AGTTNGATCCTGGCTCAGGACGAACGCTNGCGGCGTGCTTAACACATGCAAGTCGGACGGTNAGGCCTTTNNN   80
P.freud.2   AACGGAGAGTTNGATCCTGGCTCAGGACGAACGCTNGCGGCGTGCTTAACACATGCAAGTCGGACGGTNAGGCCTTTNN.
P.sherm.    .......AGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGGACGGTAAGGCCCCTTTC
P.globo.    .......AGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGGACGGTAAGGCCC.TTTC

P.freud.    NGGGGGTNCTCGAGTGGCGAACGGGTGAGTAACACGTGAGGAACGTGCCCTTGACTTCGGGATAGCTCCAGGAAACTGGT  160
P.freud.2   .GGGGGTNCTCGAGTGGCGAACGGGTGAGTAACACGTGAGGAACGTGCCCTTGACTTCGGGATAGCTCCAGGAAACTGGT
P.sherm.    GGGGGGTACACGAGTGGCGAACGGGTGAGTAACACGTGAGGAACGTGCCCTTGACTTCGGTATAGCTCCAGGAAACTGGT
P.globo.    GGGGGGTACACGAGTGGCGAACGGGTGAGTAACACGTGAGGAACGTGCCCTTGACTTCGGTATAGCTCCAGGAAACTGGT

P.freud.    GGTAATCCCGAATATGAGCCTNGCCTGCATNGGTCGGGTTGGAAAGCTTTATGCGGTNAGGGATCGTCTCGCGGCCTATC  240
P.freud.2   GGTAATCCCGAATATGAGCCTGGCCTGCATNGGTCGGGTTGGAAAGCTTTATGCGGTNAGGGATCGTCTCGCGGCCTATC
P.sherm.    GGTAATCCCGAATATGAGCCTGGCCTGCATGGGTTGGGTTGGAAAGCTTTATGCGGTCAGGGATCGTCTCGCGGCCTATC
P.globo.    GGTAATCCCGAATATGAGCCTGGCCTGCATGGGTTGGGTTGGAAAGCTTTATGCGGTCAGGGATCGTCTCGCGGCCTATC

P.freud.    AGCTNGTTGGTGGGGTAATGGCCTACCAAGGCAGCGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACATTGGGACTGA  320
P.freud.2   AGCTNGTTGGTGGGGTAATGGCCTACCAAGGCAGCGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACATTGGGACTGA
P.sherm.    AGCTTGTTGGTGGGGTAATGGCCTACCAAGGCAGCGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACATTGGGACTGA
P.globo.    AGCTTGTTGGTGGGGTAATGGCCTACCAAGGCAGCGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACATTGGGACTGA

P.freud.    GATACGGCNCAGACTCCTACGGGAGGCAGCAGTGNGGAATATTGCACAATGGGCGCAAGCCNGATGCAGCNACGCCGCGT  400
P.freud.2   GATACGGCNCAGACTCCTACGGGAGGCAGCAGTGNGGAATATTGCACAATGGGCGCAAGCCNGATGCAGCNACGCCGCGT
P.sherm.    GATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCAACGCCGCGT
P.globo.    GATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCAACGCCGCGT

P.freud.    GCGGGATGACGGCCTTCGGGTTGTGAACCGCTTTCATCCATGACGAAGCGCAAGTNACGGTAGTNGGAGAAGAAGCACCG  480
P.freud.2   GCGGGATGACGGCCTTCGGGTTGTGAACCGCTTTCATCCATGACGAAGCGCAAGTNACGGTAGTNGGAGAAGAAGCACCG
P.sherm.    GCGGGATGACGGCCTTCGGGTTGTAAACCGCTTTCATCCATGACGAAGCGCAAGTGACGGTAGTGGGAGAAGAAGCACCG
P.globo.    GCGGGATGACGGCCTTCGGGTTGTAAACCGCTTTCATCCATGACGAAGCGCAAGTGACGGTAGTGGGAGAAGAAGCACCG

P.freud.    GCTAACTACGTGCCAGCAGCCGCGGTGATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTNGTAGG  560
P.freud.2   GCTAACTACGTGCCAGCAGCCGCGGTGATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTNGTAGG
P.sherm.    GCTAACTACGTGCCAGCAGCCGCGGTGATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGG
P.globo.    GCTAACTACGTGCCAGCAGCCGCGGTGATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGG

P.freud.    CGGTTG.TCACGTCGGAAGTGAAATTCCAGGGCTTAACTCTGGGCTTGCTTTCGATACGGGTTGACTTGAGGAATGTAGG  640
P.freud.2   CGGTTGATCACGTCGGAAGTCAAATTCCAGGGCTTAACTCTGGGCTTGCTTTCGATACGGGTTGACTTGAGGAATGTAGG
P.sherm.    CGGTTGATCACGTCGGAAGTGAAATTCCAGGGCTTAACTCTGGGCTTGCTTTCGATACGGGTTGACTTGAGGAATGTAGG
P.globo.    CGGTTGATCACGTCGGAAGTGAAATTCCAGGGCTTAACTCTGGGCTTGCTTTCGATACGGGTTGACTTGAGGAATGTAGG

P.freud.    GGAGAATGGAACTCTCGGTGGAGCGGTGGAATGCGCAGATATCGGGAAGAACACCAGTGGCGAAGGCGGTTCTCTGGACA  720
P.freud.2   GGAGAATGGAACTCTCGGTGGAGCGGTGGAATGCGCAGATATCGGGAAGAACACCAGTGGCGAAGGCGGTTCTCTGGACA
P.sherm.    GGAGAATGGAACTCTCGGTGGAGCGGTGGAATGCGCAGATATCGGGAAGAACACCAGTGGCGAAGGCGGTTCTCTGGACA
P.globo.    GGAGAATGGAACTCTCGGTGGAGCGGTGGAATGCGCAGATATCGGGAAGAACACCAGTGGCGAAGGCGGTTCTCTGGACA

P.freud.    TTTCCTGACGCTGAGAAGCGAAAGCGT..GGGGAGCAAACAGGCTTAGATACCCTGG.TAGTCCACGCCGTAAACGGTGG  800
P.freud.2   TTTCCTGACGCTGAGAAGCGAAAGCGT..GGGGAGCAAACAGGCTTAGATACCCTGG.TAGTCCACGCCGTAAACGGTGG
P.sherm.    TTTCCTGACGCTGAGAAGCGAAAGCGT..GGGGAGCAAACAGGCTTAGATACCCTGG.TAGTCCACGCCGTAAACGGTGG
P.globo.    TTTCCTGACGCTGAGAAGCGAAAGCGTTGGGGGAGCAAACAGGCTTAGATACCCTGGGTAGTCCACGCCGTAAACGGTGG

P.freud.    GTACTAGGTGTNGGTCCCTTCCACGGGGTCCGTGCCGTAGCTNACGCATTAAGTACCCCGCCTGGGGAGTACGGCCGCAA  880
P.freud.2   GTACTAGGTGTNGGTCCCTTCCACGGGGTCCGTGCCGTAGCTNACGCATTAAGTACCCCGCCTGGGGAGTACGGCCGCAA
P.sherm.    GTACTAGGTGTGGGTCCCTTCCACGGGGTCCGTGCCGTAGCTAACGCATTAAGTACCCCGCCTGGGGAGTACGGCCGCAA
P.globo.    GTACTAGGTGTGGGTCC.TTCCACGGGGTCCGTGCCGTAGCTAACGCATTAAGTACCCCGCCTGGGGAGTACGGCCGCAA

P.freud.    GGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGNAACGCGAAGAACCT  960
P.freud.2   GGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGNAACGCGAAGAACCT
P.sherm.    GGCTAAAACTCAAAGGAATTGACGGGGCCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCT
P.globo.    GG.TAAAACTCAAAGGAATTGACGGGGCCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCT
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P.freud.   TACCTGGGTTTGACATGTACTGGAAGCGTTCAGAGATGGGCGTGCCTTTTTGGCTGGTACACAGGTGGTGCATGGCTGTC 1040
P.freud.2  TACCTGGGTTTGACATGGACTGGAAGCGTTCAGAGATGGGCGTGCCTTTTTGGCTGGTACACAGGTGGTGCATGGCTGTC
P.sherm.   TACCTGGGTTTGACATGTACTGGAAGCGTTCAGAGATGGGCGTGCCTTTTTGGCTGGTACACAGGTGGTGCATGGCTGTC
P.globo.   TACCTGGGTTTGACATGTACTGGAAGCGTTCAGAGATGGGCGTGCCTTTTTGGCTGGTACACAGGTGGTGCATGGCTGTC

P.freud.   GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTCCAATGTTGCCAGCAGTTCGGCTGG 1120
P.freud.2  GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTCCAATGTTGCCAGCAGTTCG.CTGG
P.sherm.   GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTCCAATGTTGCCAGCAGTTCGGCTGG
P.globo.   GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTCCAATGTTGCCAGCAGTTCGGCTGG

P.freud.   GGACTCATTGGAGACCGCNGGGGCCAACTCGGAGGAAGGTGGGGATGAGGTNAAGTCATCATGCCCCTTATGTCCAGGGC 1200
P.freud.2  GGACTCATTGGAGACCGCNGGGGCCAACTCGGAGGAAGGTGGGGATGAGGTNAAGTCATCATGCCCCTTATGTCCAGGGC
P.sherm.   GGACTCATTGGAGACCGCCGGGGCCAACTCGGAGGAAGGTGGGGATGAGGTCAAGTCATCATGCCCCTTATGTCCAGGGC
P.globo.   GGACTCATTGGAGACCGCCGGGGCCAACTCGGAGGAAGGTGGGGATGAGGTCAAGTCATCATGCCCCTTATGTCCAGGGC

P.freud.   TTCACGCATGCTACAATGGCCGGTACAAAGAGTTGCGAGCCTGTGAGGGTGAGCGAATCTCAGAAAGCCGGTCTCAGTTC 1280
P.freud.2  TTCACGCATGCTACAATGGCCGGTACAAAGAGTTGCGAGCCTGTGAGGGTGAGCGAATCTCAGAAAGCCGGTCTCAGTTC
P.sherm.   TTCACGCATGCTACAATGGCCGGTACAAAGAGTTGCGAGCCTGTGAGGGTGAGCGAATCTCAGAAAGCCGGTCTCAGTTC
P.globo.   TTCACGCATGCTACAATGGCCGGTACAAAGAGTTGCGAGCCTGTGAGGGTGAGCGAATCTCAGAAAGCCGGTCTCAGTTC

P.freud.   GGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTC 1360
P.freud.2  GGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTC
P.sherm.   GGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTC
P.globo.   GGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTC

P.freud.   CCGGGNCTTGTACACACCGCCCGTCAAGTCATGAAAGTCGGTAACACCCGAA.CCGGTGGCCCAACCTNTTGGGGGGAGC 1440
P.freud.2  CCGGGNCTTGTACACACCGCCCGTCAAGTCATGAAAGTCGGTAACACCCGAA.CCGGTGGCCCAACCTNTTGGGGGGAGC
P.sherm.   CCGGGGCTTGTACACACCGCCCGTCAAGTCATGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCTTTTGGGGGGAGC
P.globo.   CCGGGGCTTGTACACACCGCCCGTCAAGTCATGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCTTTTGGGGGGAGC

P.freud.   CGTCGAAGGTGGGACTGGTGATTAGGACTAACGTCGTAACCANGTAGCCGTACCGGAAGGTGNGGCTGGNTCACCTCCTT 1520
P.freud.2  CGTCGAAGGTGGGACTGGTGAT..........................................................
P.sherm.   CGTCGAAGGTGGGACTGGTGATTAGGACTAA.GTCGTAACAAGGTAGCCGTACCGGAAGGTGCGGCTGGATCACCTCCTT
P.globo.   CGTCGAAGGTGGGACTGGTGATTAGGACTAA.GTCGTAACAAGGTAGCCGTACCGGAAGGTGCGGCTGGATCACCT....
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Figure 8: Alignement of 16S rRNA genes of *Propionibacterium freudenreichii* strains. Sequences were aligned using the eclustalw algorithm (GCG). Black boxes mark differences between the sequences (ambiguous bases were not considered as valid). *P. freud.*: *P. freudenreichii* subsp. *freudenreichii*^{T}, GenEmbl X53217; *P. freud.* 2: *P. freudenreichii* subsp. *freudenreichii*^{T} (Ribosomal database project, Michigan State University, USA); *P. sherm.*: *P. freudenreichii* subsp. *shermanii*^{T} (GenEmbl Y10819); *P. globo.*: *P. freudenreichii* subsp. *globosum* (GenEmbl AJ009989). The latter two sequences were determined in this study. Using the FASTA algorithm, the first two sequence showed 99.5% and the last two 99.6% identity with each other. Comparison of the two first and the last two sequences are showed 97.5% identity.

The construction of specific oligonucleotide probes requires at least a one- but better a two-base difference between DNA sequences. In the 16S rDNA alignment shown in figure 8 only one region (positions 74-81) shows sufficient discrepancies between the four sequences. A probe specific for *P. freudenreichii* subsp. *globosum* seems possible around the position 668, where the sequence of this strain is differentiated by a TG insert from the other three sequences.

Because of the close relationship between the subspecies of *P. freudenreichii* shown in the 16S rDNA alignment of figure 8 only few differences between the sequences exist. Base shifts may even occur between the same subspecies (e.g. position 580 of the

alignment). Due to these problems with the 16S rRNA genes, the 23S rDNA was investigated. The 23S rDNA sequence of *P. freudenreichii* subsp. *shermanii* was determined in this study (1st complete 23S DNA sequence in the database of the whole genus *Propionibacterium*) and compared with the sequence of *P. freudenreichii* subsp. *freudenreichii* (not published, but kindly provided by W. Ludwig, TU Munich).

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P. freud.  TGACAAGCTACTAAGTGCGATCGGTGGATGCCTAGGCACCAAGAGCCGATGAAGGACGTTGTAACCTGCGATAAGCCCTG   80
P. sherm.  TGACAAGCTACTAAGTGCGATCGGTGGATGCCTAGGCACCAAGAGCCGATGAAGGACGTTGTAACCTGCGATAAGCCCTG

P. freud.  GGGAGCTGGTAAACGAGCTTTGATCCGGGGATGTCCGAATGGGAAACCTCGAAGGTGACCAGTTTAGCTACTGGCGACC  160
P. sherm.  GGGAGCTGGTAAACGAGCTTTGATCCGGGGATGTCCGAATGGGAAACCTCGAAGGTGACCAGTTTAGCTACTGGCGACC

P. freud.  GCCGCCTGAATGTATAGGGCGGTTGGAGGGAACGTGGGGAAGTGAAACATCTTAGTACCCACAGGAAGAGAAAACAACCG  240
P. sherm.  GCCGCCTGAATGTATAGGGCGGTTGGAGGGAACGTGGGGAAGTGAAACATCTTAGTACCCACAGGAAGAGNAAACAACCG

P. freud.  TGATTCCGTGAGTAGTGGCGAGCGAAAGCGGAAGAGGCCAAACCGTATGTGTGTGATAGCCGGCAGGTGTTGCATGTGCG  320
P. sherm.  TGANTCCGTGAATATTGGCGAGCGAAAGCGGAAGAGGCCAAACCGGA.GTGTGTGATAGCCGGCAGGTGTTGCATGTGCG

P. freud.  GGGTTGTGGGAAGCGTTTTGACTGAACTGCCGTGAGGTCGGAGAGTGATAAAGGATTGATGAAGCAGAAGCGTCTGGGAA  400
P. sherm.  GGGTTGTGGGAAGCGTTTTGACTGAACTGCCGTGAGGTCGGAGAGTGATAAAGGATTGATGAAGCAGAAGCGTCTGGGAA

P. freud.  GGCGCGGCATAGATGGTGATACCCCTGTATGCGTAAGTTGATCTCTCTCTTAATGTTTTCCCAAGTAGTACGGAACCCCT  480
P. sherm.  GGCGCGGCATAGATGGTGATACCCCTGTATGCGTAAGTTGATCTCTCTCTTAATGTTTTCCCAAGTAGTACGGAACCCCT

P. freud.  GAAATTCCGTACGAATCTGGCGGGACCACCCGTTAAGCCTAAATACTCCTTGGTGACCGATAGCGGACAAGTACC.GTGA  560
P. sherm.  GAAATNCCGTACGAATCTGGCGG.ACCACCCGTTAAGCCTAAATACNCCTTGGTGACCGATNGCGGACAAGTACCCGTGA

P. freud.  GGGAAAGGTGAAAAGTA...CCCCGGGAGGGGAGTGAAATAGTACCTGAAACCGATCGCATACAATCCGTCGGAGCCTGC  640
P. sherm.  GGGAAAGGGTGAAAATGTACCCCCGGGAGGGGAGTGAAATAGTNCCTGAAACCGATCGCATACAATCCGTCGGAGCCTGC

P. freud.  CCTTGTGGTGGGTGACGGCGTGCCTTTTG.AAGAATGAGCCTGCGAGTTAGTGGTGTGTGGCGAGGTTAACCCGTGTGGG  720
P. sherm.  CCTTGTGGTGGGTGACGGCGTGCCTTTTGNAAGAATGAGCCTGCGAGTTAGTGGTGTGTGGCGAGGTTAACCCGTGTGGG

P. freud.  GAAGCCGTAGCGAAAGCGAGTCCGAATAGGGCGTTTGAGTCGCATGCTCTAGACCCGAAGCGGTGTGATCTATCCATGGC  800
P. sherm.  GAAGCCGTAGCGAAAGCGAGTCCGAATAGGGCGTTTGAGTCGCATGCTCTAGACCCGAAGCGGTGTGATCTATCCATGGC

P. freud.  CAGGGTGAAGCGACGGTAAGACGTCGTGGAGGCCCGAACCCACCAGGGTTGCAAACCTGGGGGATGAGCTGTGGATAGGG  880
P. sherm.  CAGGGTGAAGCGACGGTAAGACGTCGTGGAGGCCCGAACCCACCAGGGTTGCAAACCTGGGGGATGAGCTGTGGATAGGG

P. freud.  GTGAAAGGCCAATCAAACACCGTGATAGCTGGTTCTCCCCGAAATGCATTTAGGTGCAGCGTCATGTGTTTCTTGTCGGA  960
P. sherm.  GTGAAAGGCCAATCAAACACCGTGATAGCTGGTTCTCCCCGAAATGCATTTAGGTGCAGCGTCATGTGTTTCTTGTCGGA

P. freud.  GGTAGAGCACTGGATGGTCTAGGGGGCTTACCAGCTTACCGAAATCAGCCAAACTCCGAATGCCGACAAGTGAGAGCATG 1040
P. sherm.  GGTAGAGCACTGGATGGTCTAGGGGGCTTACCAGCTTACCGAAATCAGCCAAACTCCGAATGCCGACAAGTGAGAGCATG

P. freud.  GCAGTGAGACGGCGGGGGATAAGCTTCGTCGTCGAGAGGGAAACAGCCCAGATCATCAGCTAAGGCCCCTAAGTGGTGAC 1120
P. sherm.  GCAGTGAGACGGCGGGGGATAAGCTTCGTCGTCGAGAGGGAAACAGCCCAGATCATCAGCTAAGGCCCCTAAGTGGTGAC

P. freud.  TAAGTGGAAAAGGACGTGGAGTTGCGGAGACAACCAGGAGGTTGGCTTGGAAGCAGCCATCCTTGAAAGAGTGCGTAATA 1200
P. sherm.  TAAGTGGAAAAGGACGTGGAGTTGCGGAGACAACCAGGAGGTTGGCTTGGAAGCAGCCATCCTTGAAAGAGTGCGTAATA

P. freud.  GCTCACTGGTCAAGTGATTCTGCACCGACAATTTAGCGGGGCTCAAGTCATCCGCCGAAGCTGTGGCATCTACGCGTGTA 1280
P. sherm.  GCTCACTGGTCAAGTGATTCTGCACCGACAATTTAGCGGGGCTCAAGTCATCCGCCGAAGCTGTGGCATCTACGCGTGTA

P. freud.  TCCGGCATCCTTTGGGGTGTCCAGGTGCGTGGATGGGTAGGGGAGCGTTGTGTGTGCGTTGAAGCGGCGGGGTGACCCGG 1360
P. sherm.  TCCGGCATCCTTTGGGGTGTCCAGGTGCGTGGATGGGTAGGGGAGCGTTGTGTGTGCGTTGAAGCGGCGGGGTGACCCGG

P. freud.  TCGTGGAGTGCACGCAAGTGAGAATGCAGGCATGAGTAGCGTATGACGGGTGAGAAACCCGTCCGCCGAATATCCAAGGG 1440
P. sherm.  TCGTGGAGTGCACGCAAGTGAGAATGCAGGCATGAGTAGCGTATGACGGGTGAGAAACCCGTCCGCCGAATATCCAAGGG

P. freud.  TTCCAGGGTCAAGCTAATCTGCCCTGGGTGAGTCGGGTCCTAAGGCGAGGCCGACAGGCGTAGTCGATGGACAACGGGTT 1520
P. sherm.  TTCCAGGGTCAAGCTAATCTGCCCTGGGTGAGTCGGGTCCTAAGGCGAGGCCGACAGGCGTAGTCGATGGACAACGGGTT

P. freud.  GATATTCCCGTACCGGCGCGAGAACGATCCTGCCGAGGCGAGTGATGCTAAGCATGCAAGGCGGTCGTGGGGGCTTCGGT 1600
P. sherm.  GATATTCCCGTACCGGCGCGAGAACGATCCTGCCGAGGTGAGTGATGCTAAGCATGCAAGGCGGTCGTGGGGGCTTCGGT

P. freud.  TCCCTTGATCGTTGAGTCTGTAACCCGATCTTGTAGTAGGCAAGCTGCGGAGGGACGCAGGAAGGTAGTCTGGCACCGTA 1680
P. sherm.  TCCCT.GATCGTTGAGTCTGTAACCCGATCTTGTAGTAGGCAAGCTGCGGAGGGACGCAGGAAGGTAGTCTGGCACCGTA

P. freud.  TTGGTTTGCGGTGTTAAGCCTGTAGGGTGTCTGGCCAGGTAAATCCGGTCGGACGTGTGCCTGAGAGGTGATGAGTGGTG 1760
P. sherm.  TTGGTTTGCGGTGTTAAGCCTGTAGGGTGTCTGGCCAGGTAAATCCGGTCGGACGTGTGCCTGAGAGGTGATGAGTGGTG

P. freud.  CCACTTTTGTGGTACGTATCCGGATGATCCTATGCTGCCTAGAAAATCTTCGTGAGCGAGTTCTCGAGCTGCCCGTACCC 1840
P. sherm.  CCACTTTTGTGGTACGTATCCGGATGATCCTATGCTGCCTAGAAAATCTTCGTGAGCGAGTTCTCGAGCTGCCCGTACCC

P. freud.  CAAACCGACACTGGTGGATAGGTAGAGAATACCAAGGCGATCGAGATAATCATGGTGAAGGAACTCGGCAAAATCCTCCC 1920
P. sherm.  CAAACCGACACTGGTGGATAGGTAGAGAATACCAAGGCGATCGAGATAATCATGGTGAAGGAACTCGGCAAAATCCTCCC
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P. freud.  GTAACTTCGGGATAAGGGAGACTGGAGGCGTGACGGCAGTTTACTTGTCGGTGCGTCGATAGTCGCAGAGAATAGGCCCA 2000
P. sherm.  GTAACTTCGGAATAAGGGAGACTGGAGGCGTGACGGCAGTTTACTTGTCGGTGCGTCGATAGTCGCAGAGAATAGGCCCA

P. freud.  AGCGACTGCTTACTAAAAGCACAGGTCCGTGCTAAGTCGAAAGACGATGTATACGGACTGACTCCTGCCCGGTGCTG.GA 2080
P. sherm.  AGCGACTGCTTACTAAAAGCACAGGTCCGTGCTAAGTCGAAAGACGATGTATACGGACTGACTCCTGCCCGGTGCTCNGA

P. freud.  AGGTTAAGGGGACGTGTTAGCACTTTTGTGCGAGGCACTGAACTTAAGCCCCAGTAAACGGCGGTGGTAACTATAACCAT 2160
P. sherm.  AGGTTAAGGGGACGTGTTAGCACTTTTGTGCGAGGCACTGAACTTAAGCCCCAGTAAACGGCGGTGGTAACTATAACCAT

P. freud.  CCTAAGGTAGCGAAATTCCTTGTCGGGTAAGTTCCGACCTGCACGAATGGAGTAACGACTTGGGCGCTGTCTCCACCATG 2240
P. sherm.  CCTAAGGNAGCGAAATTCCTTGTCGGGNAAGTTCCGNCCTGNACGAATGGAGTAACGACTTGGGCGCTGTCTCCACCATG

P. freud.  AACTCGGCGAAATTGCATTACGAGTAAAGATGCTCGTTACGCGCAGCAGGACGGAAAGACCCCGGGACCTTTACTATAGT 2320
P. sherm.  AACTCGGCGAAATTGCATTACGAGTAAAGATGCTCGTTACGCGCACAGGGACGGAAAGNNCCCGGGACCTTTACTATAGT

P. freud.  TTGGTATTGGTGATCGGTACGACTTGTGTAGGATAGGTGGGAGACTTTGAAGCGGTCACGCTAGTGATTGTGGAGTCATT 2400
P. sherm.  TTGGTATTGGTGATCGGTACGACTTGTGTAGGATAGGTGGGAGACTTTGAAGCGGTCACGCTAGTGATTGTGGAGTCATT

P. freud.  GTTGAAATACCACTCTGGTCGTTCTGGTTATCTAACCTAGGTCCGTGATCCGGATCAGGGACAGTGCCTGATGGGTAGTT 2480
P. sherm.  GTTGAAATACCACTCTGGTCGTTCTGGTTATCTAACCTAGGTCCGTGATCCGGATCAGGGACAGTGCCTGATGGGTAGTT

P. freud.  TGACTGGGGCGGTCGCCTCCCAAAAGGTAACGGAGGCGCCCAAAGGTTCCCTCAGCCTGGTTGGTAATCAGGTGTTGAGT 2560
P. sherm.  TGACTGGGGCGGTCGCCTCCNAAAAGGTAACGGAGGCGCCCAAAGGTTCCCTCAGCCTGGTTGGTAATCAGGTGTTGAGT

P. freud.  GTAAGTGCACAAGGGAGCTTGACTGTGAGACAGACATGTCGAGCAGGGACGAAAGTCGGGACTAGTGATCTTCTGGTGGA 2640
P. sherm.  GTAAGTGCACAAGGGAGCTTGACTGTGAGACAGACATGTCGAGCAGGGACGAAAGTCGGGACTAGTGATCNTCTGGTGGA

P. freud.  TTGTGGAATCGCCAGAACTCAACGGATAAAAGGTACCCCGGGGATAACAGGCTGATCTTTCCCGAGCGCTCACAGCGACG 2720
P. sherm.  TTGTGGAATCGCCAGAACTCAACGGATAAAAGGTACCCCGGGGATAACAGGCTGATCTTTCCCGAGCGCTCACAGCGACG

P. freud.  GAATGGTTTGGCACCTCGATGTCGGCTCGTCGCATCCTGGGGCTGGAGTCGGTCCCAAGGGTTGGGCTGTTCGCCCATTA 2800
P. sherm.  GAATGGNTTGGCACCTCGATGTCGGCTCGTCGCATCCTGGGGCTGGAGTCGGTCCCAAGGGTTGGGCTGTTCGCCCATTA

P. freud.  AAGCGGCACGCGA.GCTGGGT.TAAGAACGTCGTGAGACAGTTC.GGTCCCTAT.CCGCTGCGCGCGTAGGAATCTTGAG 2880
P. sherm.  AAGCGGCACGCGANGCTGGGGNTAAGAACGTCGTGAGACAGTTCCGGNCCCTATACCGCTGCNCGTN...GNATCTTGAG

P. freud.  AAGGGCTGTCCTTAGTACGCAAGGACCGGGACGGACCAACCTCTGGTGTGCCAGTTGTTCCACCAGGAGCATGGCTGGTT 2960
P. sherm.  A.GGGCTGTCCTTAGTACGCAAGGACCGGGACGGACCAACCTCTGGTGTGCCAGTTGTTCCACCAGGAGCATGGCTGGTT

P. freud.  GGCTACGTTGGGGAGTGATAACCGCTGAAAGCATCTAAGTGGGAAGCACGCTTCAAGATGAGGGTTCCTGCACAGTTAAT 3040
P. sherm.  GGCTACGTTGGGGAGTGATAACCGCTGAAAGCATCTAAGTGGGAAGCACGCTTCAAGATGAGGGTTCCTGCACAGTTAAT

P. freud.  GTGGTAAGGCCCCCGGTAGACCACCGGGTGATAGGTCGGATGTGGAAGCATGGTGACATGTGGAGCTGACCGATACTAAG 3120
P. sherm.  GTGGTAAGGCCCCCGGTAGACCACCGGGTGATAGGTCGGATGTGGAAGCATGGTGACATGTGGAGCTGACCGATACTAAG
```

Figure 9: Alignment of 23S rRNA genes of *P. freudenreichii* subsp. *freudenreichii*^{T} (sequence kindly provided by W. Ludwig, TU Munich, Germany) and *P. freudenreichii* subsp. *shermanii*^{T} (this study, GenEmbl Y10819). The sequences were aligned using the eclustalw algorithm (GCG). Differences between the sequences are marked with black boxes (ambiguities were not taken into account). The identity of both sequences determined with the FASTA algorithm is 98.5%.

The evaluation of these alignments revealed regions where the construction of specific probes would be possible (e.g. alignment positions 569-583 of figure 9), but additional sequencing data from other strains should be obtained first.

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The construction of a genetic system for propionibacteria requires first the construction of suitable vectors before transformation experiments with these organisms can be performed. The isolation and analysis of plasmids from propionibacteria is therefore presented in this thesis before conjugation or electroporation experiments are shown.

In addition, the fast identification methods for propionibacteria that were developed previously (chapter 3.2) are needed to ensure that the "correct" strains are used for the experiments.

3.3 Analysis of *Propionibacterium* plasmids

3.3.1 Screening for plasmids in propionibacteria

As a preliminary to find suitable plasmid vectors all defined *Propionibacterium* strains of our collection and all other isolates were screened for the presence of plasmids (table 14). Three plasmids were selected and further characterized: pLME101 (40 kb; Smutny, 1997), pLME106 (7 kb; Stierli, 1998) and pLME108 (2 kb; this study).

Table 14: Plasmids isolated from propionibacteria

source	no of strains investigated	no of strains with plasmids	References
cheese, raw milk	200	14	this study
cheese	130	15	Smutny, 1997
Culture collections[1]	27	2	this study
cheese	30	8	Pérez-Chaia et al., 1988
"industrial"strains	50	6	Rehberger and Glatz, 1985
raw milk	446	135[2]	Fessler, 1997
"industrial"strains	50	4	Gautier and Rouault, 1990

[1] As listed in table 3, cutaneous species were not investigated for the presence of plasmids.
[2] 30 strains were kindly provided by the FAM for further analysis in this thesis.

As shown in table 14, plasmids have been found only in small numbers in strains of classical propionibacteria. The medically relevant species have so far not been investigated for the presence of plasmids.

3.3.2 Antibiotics and propionibacteria

Antibiotics were used as markers in gene transfer experiments to simplify the isolation of transformed cells. In table 15 antibiotic resistance patterns of propionibacteria are shown.

Table 15: Antibiotic resistance patterns of medically and classical propionibacteria

Antibiotic	Cutaneous propionibacteria[1]					Dairy propionibacteria[2]					
	P. acnes	*P. avidum*	*P. granulosum*	*P. propionicum*	Cutaneous *P.* species	*P. acidipropionici*	*P. jensenii*	*P. jensenii* DF3	*P. freudenreichii*	*P. freudenreichii* JS53	*P. thoenii*
Ampicillin	S	S	S	S	S	S	S	S	S	S	S
Bacitracin	S	S	S	nd	nd	S	S	nd	S	nd	S
Benzylpenicillin	S	S	S	nd	nd	nd	nd	nd	nd	nd	S
Cefotaxime	S	nd	nd	nd	nd	I	nd	R	nd	S	V
Cephalothin	S	S	S	S	S	S	S	nd	V	nd	S
Chloramphenicol	S	S	S	S	S	S	V	S	S	S	V
Ciprofloxacin	I	nd	nd	nd	S	nd	nd	nd	nd	nd	R
Clindamycin	S	S	S	S	S	S	nd	S	nd	S	I
Cloxacillin	nd	nd	nd	nd	nd	S	S	nd	S	nd	S
Dicloxacillin	S	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Erythromycin	S[4]	S	S	S	S	S	S	S	S	S	S[4]
Fusidic acid	S	I	I	nd	nd	S	nd	S	S	S	I
Gentamicin	V	I	S	nd	nd	I	nd	R	R	R	R
Kanamycin	R	R	R	nd	nd	R	R	R	R	R	R
Lincomycin	S	S	S	nd	nd	V	S	I	S	R	V
Methicillin	S	nd	nd	nd	nd	R	R	R	R	R	R
Metronidazole	R	nd	nd	R	R	R	nd	R	R	R	R
Nalidixic acid	nd	nd	nd	nd	nd	R	R	R	R	R	R
Neomycin	R	R	R	nd	nd	V	V	S	R	R	S
Penicillin G	S	nd	nd	S	S	S	S	S	S	S	S
Polymyxin B	nd	nd	nd	nd	nd	V	V	I	V	S	V
Rifampicin	nd	nd	nd	nd	nd	S	nd	S	nd	S	S[4]
Streptomycin	I	I	S	nd	nd	I	I	I	S	R	I
Sulfonamides	nd	nd	nd	nd	nd	R	R	R	R	R	R
Tetracycline	S[4]	I	nd	R	R	V	S	S	S	S	S[4]
Tobramycin	R[3]	R	S	nd	nd	R	nd	R	nd	R	nd
Trimethoprim	S	S	I	nd	nd	nd	nd	nd	S	nd	nd
Trimet./Sulfa.	S	S	S	nd	nd	I	nd	R	R	R	S
Vancomycin	S	nd	S	nd	S	S	S	S	S	S	S

nd: not determined or no data available. R: resistant; S: sensitive; I: intermediate; V: variable.

1 Combined resistance patterns: Denys et al., 1983; Dubreuil et al., 1996; Ednie et al., 1998; Essers, 1982; Hoeffler et al., 1976; Hoellmann et al., 1998; Laforest et al., 1988; Lorian, 1996; Sutter and Finegold, 1973; Tunney et al., 1998; Wang et al., 1977.

2 This study; Guldimann, 1998; Smutny, 1997; Reddy et al., 1973a & 1973c.

3 Strains with intermediate MIC exist (Hoeffler et al, 1976).

4 High resistant strains exist: this study; Ross et al., 1998; Ross et al., 1997; Tunney et al., 1998.

The data obtained in this study were combined with published data. When published data were contrary, the most recent values were selected and figures obtained by methods like agar- or broth-dilution and Etest were ranked higher than disk-test values. Interpretation of the selected MIC-values (minimal inhibitory concentration in µg/ml for 90% of the investigated strains) was performed as recommended by Lorian (1996).

Propionibacteria are usually sensitive to the following antibiotics: ampicillin, bacitracin, benzylpenicillin, cephalothin, chloramphenicol, clindamycin, cloxacillin, erythromycin, fusidic acid, lincomycin, penicillin G, rifampicin and vancomycin. Most strains are sensitive to trimethoprim and tetracycline.

Resistance against gentamicin, kanamycin, metronidazole, nalidixic acid and sulfonamides is observed. Most strains are also resistant to tobramycin, neomycin (most aminoglycoside antibiotics) and methicillin.

Variable resistance patterns between propionibacteria were found for cefotaxime, ciprofloxacin, polymyxin B, streptomycin and trimethoprim/sulfamethoxazole.

No data concerning antibiotic resistance patterns were obtained for *P. lymphophilum* and *P. cyclohexanicum*.

3.3.3 DNA-DNA hybridization assays within *Propionibacterium* plasmids

A Southern blot with DNA of the *Propionibacterium* plasmids pLME101, pLME106, pLME108 and pRGO1 (used as reference plasmid) and chromosomal DNA of the respective host strains was hybridized, stripped and reprobed with each plasmid DNA ([α-^{32}P]dATP labeled) as probe.

As shown in table 16, only the plasmids pRGO1 and pLME106, both of roughly the same size (approximately 7 kb) but with different restriction endonuclease digestion patterns, showed strong cross-hybridization signals with each other. The plasmid-DNA probes did not hybridize with chromosomal DNA of the host strains. In figure 10 the hybridization results with pRGO1 as probe are shown as an example.

Table 16: DNA-DNA hybridization analysis of *Propionibacterium* plasmids

Probe Target DNA	pLME101	pLME106	pLME108	pRGO1
Plasmid DNA:				
pLME101	+	-	-	-
pLME106	-	+	-	+
pLME108	-	-	+	-
pRGO1	-	+	-	+
Chromosomal DNA:				
P. freudenreichii JS53[1]	-	-	-	-
P. jensenii DF1[2]	-	-	-	-
P. freudenreichii DF2[3]	-	-	-	-
P. acidipropionici[4]	-	-	-	-

[1] host strain of pLME101.
[2] host of pLME106.
[3] host of pLME108.
[4] host of pRGO1.
+: positive hybridization signal detected; -: no signal detected.

The relationship between the plasmid pLME106 and pRGO1 is shown in figure 10B where the selected plasmid DNA is probed with [α-P^{32}]dATP labeled DNA from pRGO1. DNA from plasmid pLME106, digested (lane 4) and undigested (lane 5) hybridizes with this probe. In the lanes 10 and 11 of figure 10B, the positive control is shown: pRGO1 hybridizes with itself as well with the digested (lane 11) as with the undigested DNA fragments (lane 10). Neither lane 3 or 9, which contain the chromosomal DNA of the respective host strains hybridized with pRGO1 as probe.

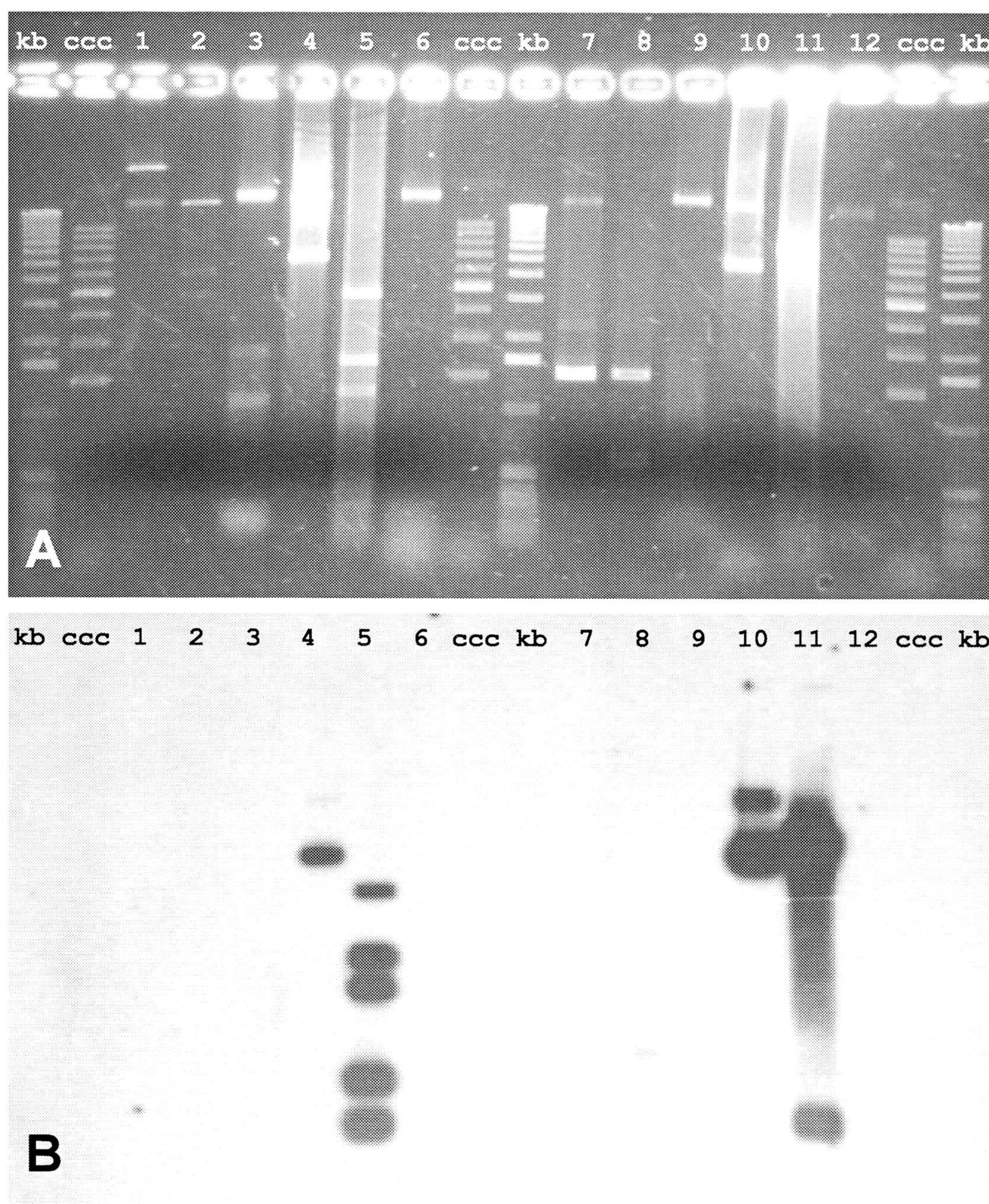

Figure 10: Southern hybridization analysis of *Propionibacterium* plasmids. (A) 1% agarose gel stained with ethidium bromide. (B) Autoradiography of membrane-bound DNA that hybridized with the [α-P^{32}]dATP labeled plasmid pRGO1. Lane 1: Plasmid-DNA of pLME101; lane 2: Plasmid-DNA of pLME101, digested with *Kpn*I; lane 3: Chromosomal DNA from *P. freudenreichii* JS53 (host strain of pLME101). lane 4: pLME106; lane 5: *Hinc*II-digest of pLME106; lane 6: Chr. DNA from *P. jensenii* DF1; lane 7: pLME108; lane 8: *Hinc*II-digest of pLME108; lane 9: Chr. DNA from *P. freudenreichii* DF2; lane 10: pRGO1; lane 11: pRGO1 digested with *Sal*I; 12: Chr. DNA from *P. acidipropionici* DSM 20272. kb: kb-ladder (Gibco); ccc: supercoiled DNA-ladder (2-10 kb in 1 kb steps, Promega).

3.3.4 Characterization of plasmid pLME101

Because "natural" plasmid transfer by conjugation usually requires larger plasmids, the plasmid pLME101 was selected and further characterized in this study. Based on DNA restriction endonuclease analysis (an example is shown in figure 11) the size of the plasmid pLME101 was estimated to 40.3 kb by adding of the fragment sizes of *Sst*I digested pLME101 (Smutny, 1997).

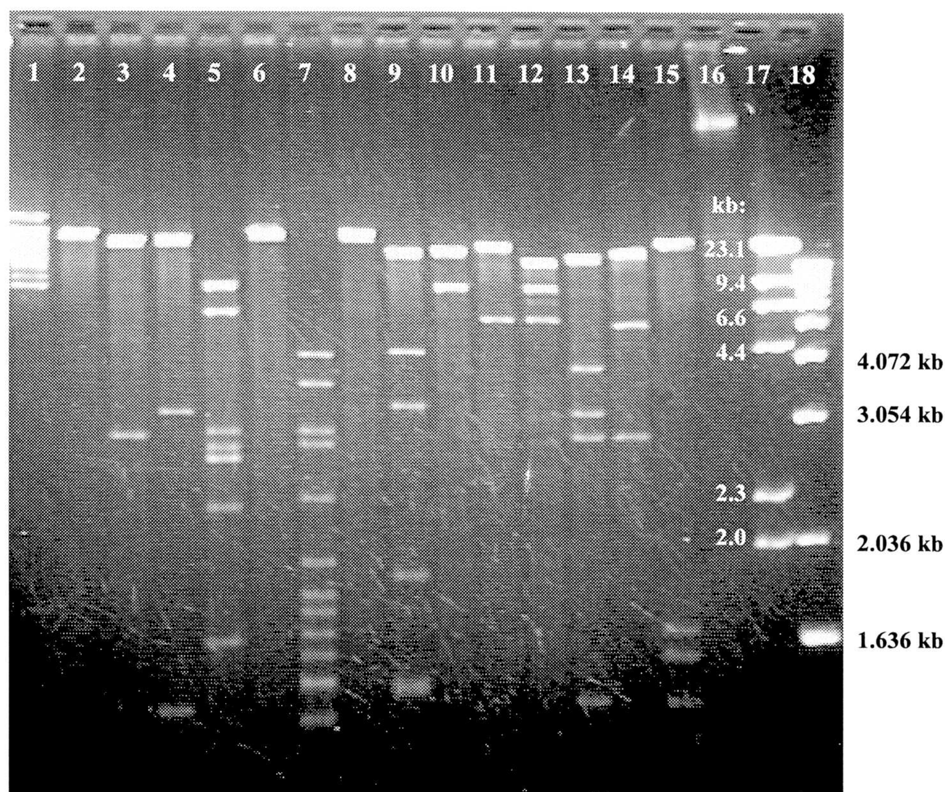

Figure 11: Restriction endonuclease analysis of plasmid pLME101. Pulsed field gel electrophoresis of pLME101 digested with different enzymes. Lanes 1: 8-48 kb ladder (BioRad); Lanes 2-15: digests of pLME101. 2: *Hin*dIII digest; 3: *Bam*HI; 4: *Eco*RV; 5: *Sst*I; 6: *Pst*I; 7: *Hinc*II; 8: *Sph*I; 9: KpnI; 10: *Hin*dIII / *Sph*I digest; 11: *Hin*dIII / *Pst*I; 12: *Hin*dIII / *Sph*I / *Pst*I; 13: *Bam*HI / *Eco*RV; 14: *Bam*HI / *Hin*dIII; 15: *Eco*RV / *Hin*dIII; 16: undigested pLME101; 17: *Hin*dIII digest of λ DNA (Gibco); 18: kb-ladder (Gibco).

Based on data of single and double digests of pLME101 with restriction enzymes, a simple restriction map of pLME101 was constructed and is shown in figure 12. Note the relatively few sites of often used restriction enzymes between bp 20'000 and 40'000.

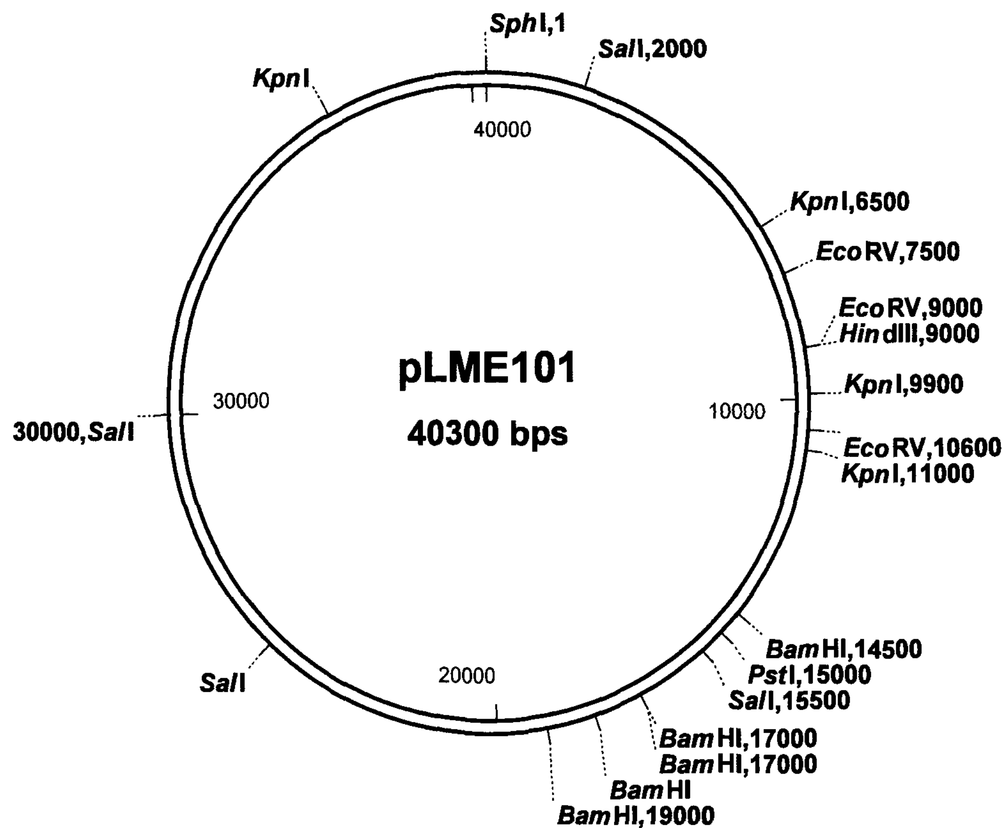

Figure 12: Restriction map of plasmid pLME101, a plasmid isolated from *P. freudenreichii* JS53 (this study and Smutny, 1997).

Shotgun-cloning of pLME101 in pUC18 (Smutny, 1997; Guldimann, 1998) produced 18 plasmids with 0.3 to 1.0-kb sized insertions of *Sal*I-digested pLME101 and 86 clones with 0.3 to 3.0-kb insertions of a *Sst*I digest. 10 clones were selected in this thesis and a total of 3815 basepairs from these plasmids were sequenced. However, these sequences don't cover one single connecting fragment of pLME101.

With one exception, the sequencing and analysis of cloned DNA revealed no similarities to already known DNA sequences. The FASTA comparison of the amino acid sequence from the plasmid of one clone (566 bp, 188 aa, neither start nor stop codon) with the Swissprot database led to a relatively high degree of relatedness with a known sequence (hypothetical 70.2 kDa protein from *Synechocystis* sp.). The sequence alignment of both hypothetical proteins is shown in figure 13.

```
                                         .         .         .         .         .
pLME101             14 PTGVATDEYxPQVQFWTSRGFGVLSVNYSGSAGFGRAYRERLRGQWGIAD 63
                       ||:.|.:..  .:|:||||||: :.|||:||.|:||.||:||.|||||.|
Synechocystis sp. 417 PTAAAGNSLSLKIQYWTSRGFAYVDVNYGGSTGYGRDYRQRLNGQWGIVD 466
                                         .         .         .         .         .
pLME101             64 VDDCIDAAESLLSADLADQSKIAIMGGSAGGFTVLAALTRSSVFSAGICR 113
                       |.||::||  | ..:|.|...:|| ||||||:|.|||||  .||.|| :
Synechocystis sp. 467 VADCVNAARYLADQGLVDGEQLAISGGSAGGYTTLAALTFHNVFKAGASY 516
                                         .         .         .         .         .
pLME101            114 YGIADLVAMQEGGTHKFEATYNDGLLGPWPQARKVYEERSPIHHLDQLHA 163
                       ||:.||.|: ..:|||||| | |||:||:|: :.:|| |||::| ||| .
Synechocystis sp. 517 YGVSDLTAL.ATDTHKFEARYLDGLIGPYPERKDLYERRSPVNHADQLTC 565
                                         .         .
pLME101            164 PMLILQGLDDAVVPPQQADELG 185
                       |::::|||:| ||||.|.: :.
Synechocystis sp. 566 PVIFFQGLEDKVVPPNQTEMMV 587
```

Figure 13: Amino acid sequence alignment (bestfit algorithm) of a part of pLME101 with a hypothetical 70.2 kDa protein from *Synechocystis* sp. (Swissprot Q55413).

In a 171-amino acid overlap region a similarity of 71.9% and an identity of 53.2% between the protein sequences of figure 13 was calculated (bestfit algorithm).

Curing experiments with *P. freudenreichii* JS53, the host strain of pLME101, followed by hybridization with pLME101 of the potential cured strains gave no clear result (Smutny, 1997), 8 strains did not hybridize with the probe and were selected for further analysis. After plasmid analysis, two of the strains contained no or smaller plasmids: *P. freudenreichii* ARE1 harbored no plasmid whereas in *P. freudenreichii* ARE2 a smaller plasmid was found. Carbohydrate fermentation patterns (API CH50, BioMérieux) revealed no differences between the original strain JS53 (with pLME101) and ARE1 (no plasmid). Differences were observed for strain ARE2 for the fermentation of maltose and sucrose. Antibiotic resistance patterns of ARE1 and JS53 were the same, whereas strain ARE2 had strangely acquired additional resistances to clindamycin and penicillin G. Additional analysis of ARE1 revealed, that pLME101 was under certain circumstances still present in this strain. Due to this problems, no plasmid-encoded properties could be detected for pLME101.

3.3.5 Characterization of plasmid pLME108

Small sized plasmids are commonly used as cloning vectors, mainly for electroporation experiments. Therefore, the 2-kb sized plasmid pLME108 was selected for further analysis. The whole sequence of the plasmid pLME108 (2051 bp) was determined in this study using shotgun cloning, cycle sequencing of the plasmids of the resulting clones (table 17) and an automated DNA sequencer (ALF Express, Pharmacia Biotech, Uppsala, Sweden). The sequence was submitted to the GenEmbl database (Accession no. PFR6662) and is the first published complete nucleotide sequence of a *Propionibacterium* plasmid. A physical map of the plasmid pLME108 was constructed and is shown in figure 14.

Table 17: Cloning of pLME108 using different vectors

pLME108 digest	Ligated in	Size of inserted DNA fragments
*Ava*I digest	*Ava*I digested pUC18	1400 bp
*Hinc*II digest	*Hinc*II digested pUC18	500 – 1200 bp
*Pvu*II digest	*Hinc*II digested pUC18	800 - 1500 bp
*Hinc*II digest	*Sma*I digested pCL1921	600 –1000 bp
*Pvu*II digest	*Sma*I digested pCL1921	400 bp
NheI digest	*Xba*I digested pUC18	2051 bp (complete)
PCR product[1]	pGEM-T Easy	1800 bp

[1] PCR amplification of missing part of pLME108 (primers pr108fw - pr108rev)

The mapping of pLME108 revealed single sites for restriction endonucleases that were not found previously. Especially the sites for *Nhe*I, *Xho*I and *Eco*47III are located in a "attractive" region that is not part of the putative *rep* gene shown in figure 14. Digests of pLME108 with these enzymes were successful, indicating that the sequence of pLME108 was correct. The sites for the enzymes used to clone pLME108 in pUC18, *Hinc*II, *Ava*I and *Pvu*II, were all found in the sequence.

In addition, no sites for enzymes that did not cut pLME108 in previous experiments were found when the sequence was analyzed. Sites for common enzymes like *Eco*RI, *Eco*RV, *Bam*HI or *Hin*dIII are not present in the DNA of pLME108.

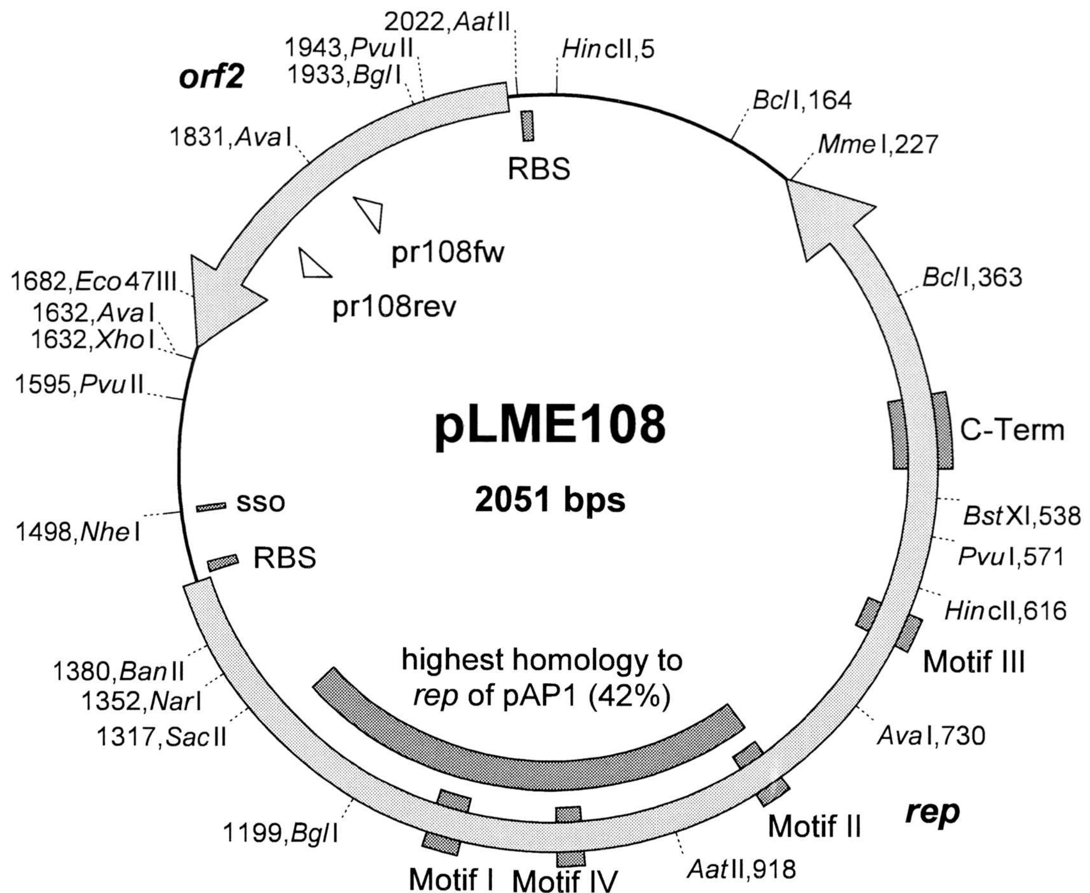

Figure 14: Physical map of plasmid pLME108, a plasmid isolated from *P. jensenii* DF2. Two putative open reading frames *rep* (223-1434) and *orf2* (1642-2013) are marked. The putative ribosome binding sites (RBS), the putative single strand replication origin (sso) and the positions of characteristic motifs for rolling circle replication (motif I to IV, C-Term) are shown. The zero position for pLME108 was selected randomly. The binding sites for the primers pr108fw and pr108rev used for completing the sequence of pLME108 by means of PCR are also shown.

The analysis of pLME108 shown in figure 14 revealed only two restriction sites where the introduction of a foreign gene seems possible: the cutting site for *Eco*47III (at position 1682), an enzyme that produces blunt ends and for *Xho*I (at position 1632) an enzyme that forms "sticky" ends. Both enzymes cut pLME108 only once, are commercially available and can be ligated with products of a digest with other common enzymes. In addition, both *Eco*47III and *Xho*I do cut outside the putative *rep* gene.

Table 18: The most closely related "plasmidic" Rep protein sequences of the putative Rep protein of pLME108

rep of	Length	Aligned[1]	Gaps	Similarity	Identity	GenEmbl	Isolated from[2]
pAP1	460 aa	474 aa	14	57.0%	36.3%	U83788	*Arcanobacterium pyogenes*
pIJ101	456 aa	398 aa	12	54.8%	31.0%	M21778	*Streptomyces* sp.
pJV1	528 aa	476 aa	21	53.6%	26.6%	L06019	*Streptomyces* sp.
pSG5	481 aa	361 aa	12	51.3%	28.5%	X80774	*Streptomyces* sp.
pAB49	394 aa	440 aa	12	51.2%	27.2%	L77992	*Acinetobacter baumannii*
pCA2.4	336 aa	394 aa	15	49.1%	22.4%	L13739	*Synechocystis* sp.
pTD1	335 aa	413 aa	15	48.6%	19.6%	M87856	*Treponema denticola*

[1] GCG-bestfit alignment with the complete putative Rep protein of pLME108 (445 aa).

[2] pAP1 (Billington et al., 1998), pIJ101 (Kendall and Cohen, 1988), pJV1 (Servín-Gonzãlez et al., 1995), pSG5 (Muth et al., 1995), pCA2.4 (Yang and McFadden, 1993), pTD1 (MacDougall et al., 1992), pAB49 (no reference, only accession number is given).

By comparison of 23 possible open reading frames (orf's) of pLME108 with the GenEmbl and Swissprot databases, a putative *rep* gene ranging from bases 223 to 1434 was identified. An overlap of 159 amino acids shows 63.5% similarity and 42.1% identity with the putative Rep protein of plasmid pAP1 from *Arcanobacterium pyogenes* (GenEmbl U83788), the localization of this overlap region is shown in figure 14. The alignment of the two putative Rep proteins is shown in figure 15.

The second open reading frame of pLME108, orf2, shows no homology to known sequences of the database. *Orf2* was selected based on the following criteria: methionine as start-codon, ribosome binding site (RBS) at its start and *orf2* is processed in the same frame as the putative *rep*.

In table 18 the overall similarities and identities of the closest related Rep proteins of plasmids with pLME108 are shown.

```
                             .         .         .         .         .
pLME108 (Rep)     89 AADKRKHRFSVRYWLWRHTSLKRVAFCGRVAASAVASVGVRCSDGRAGFA 138
                     | .:|..|:.:|  |   ..:..|  ||||:..::.|: .::.:  ||::
pAP1 (Rep)        89 AKSRRSERYELRDGLAEISTIESVRKCGRVPVAPLVSLRAKSDGKGAGYG 138
                             .         .         .         .         .
pLME108 (Rep)    139 GLQSCGSVWACPVCNAKIMARRGLELGAAVETWTKHGGRVAFMTFTVRHS 188
                     ||:.||||||||||.||| |||  :|...|:  .|||  |.::|:| ||
pAP1 (Rep)       139 GLHTCGSVWACPVCSAKIAARRKTDLQQVVDHAVKHGMTVSMLTLTQRHH 188
                             .         .         .         .         .
pLME108 (Rep)    189 RKDSLTAVWDGVASGWRRVTSGKGWTSDQLRHGVEGFVRVVEVTHGRNGW 238
                     : ::|. :||::..:|.|||||: |.. . . |: |:||. |:|||::||
pAP1 (Rep)       189 KGQGLKHLWDALSTAWNRVTSGRRWIEFKEQFGLVGYVRANEITHGKHGW 238

pLME108 (Rep)    239 HVHLHVLVF 247
                     ||| |||::
pAP1 (Rep)       239 HVHSHVLII 247
```

Figure 15: Amino acid sequence alignment (bestfit algorithm) of parts of the putative Rep proteins of the plasmids pLME108 and pAP1 (GenEmbl no. U83788).

As assumed by Billington et al. (1998), the plasmid pAP1, which has the most similar Rep protein to the one of pLME108, uses the rolling circle replication mode. By comparison with other plasmids, known or suspected to use the same replication mechanism, typical motifs were found on the replicons of all these plasmids. The sso (single strand origin) of replication, characterized by the sequence TAGCGT, is present also in pLME108. The alignments of the motifs are shown in figure 16.

The localization of the motifs I to IV and for the C-term motif identified for pLME108 are indicated in figure 14. Amino acids conserved for at least 50% of the positions in the alignment are marked with black boxes. The distances in amino acids between the motifs for the plasmids of the alignment are indicated as well. Almost all motifs appear at approximately the same distances in all plasmids. Only the C-Term motif of pAP1 differs: it occurs before motif III and is indicated in figure 16 with a (-).

The plasmids originate from the following species: *Arcanobacterium pyogenes* (pAP1), *Streptomyces* sp. (pIJ101, pJV1, pSG5), *Acinetobacter baumannii* (pAB49), *Synechocystis* sp. (pCA2.4) and *Treponema denticola* pTD1.

		Motif IV	Δaa		Motif I	Δaa
pLME108	143	CGSVWACPVC	..37	180	FMTFTVRH	..57
pAP1	143	CGSVWACPVC	..37	180	MLTLTQRH	..57
pAB49	92	CGSIWSCPVC	..38	130	LLTLTFSH	..57
pCA2.4	91	FCRVRHCPVC	..39	130	FLTLTVRN	..51
pIJ101	25	CGRIWLCPVC	..37	62	LVTFTARH	..84
pJV1	43	CGRIWFCPEC	..37	80	VVVLTARH	..66
pSG5	15	CNNVHTCPWC	..37	52	LGTNTLRH	..68
pTD1	61	MYCQLRCPVC	..38	99	FITLTVKN	..56

		Motif II	Δaa		Motif III	Δaa
pLME108	237	GWHVHLHVLVF	..60	297	LARYLSKAQF	..52
pAP1	237	GWHVHSHVLII	..77	314	IGNYVSKMQT	..-48
pAB49	187	GWHPHHHVLLL	..55	242	ADQYVSKWGL	..50
pCA2.4	181	SAHPHFHCLLM	-		-	-
pIJ101	146	GWHPHIHAIVL	..79	225	LAEYIAKTQD	..64
pJV1	146	GYHPHLNLLVF	..126	272	LIEYLTKNQD	..81
pSG5	120	GWHLHWHTLWV	..78	198	QARYLYKDGD	..62
pTD1	155	EYHPHYHILAA	-		-	-

		C-Term Motif
pLME108	349	WFEWEKGSRGRRQIGWSAGL R
pAP1	266	WKEYEKASFGRRALTWSKGL R
pAB49	292	FQEFAISMKGARQLVWSRGL K
pIJ101	289	WHEYERATRGRRAIEWTRYL R
pJV1	353	WAQYEEALAGRRAIEWTRGL R
pSG5	260	YREREFGVFQVRKHYRSQNLNR

Figure 16: Alignment of the putative Rep protein of pLME108 against other replication proteins of rolling circle replication plasmids. With Δaa the distance in amino acids between the patterns in the respective sequences is shown. A negative distance indicates that the following motif is located upstream of the actual motif. Amino acids conserved for at least 50% of the sequences are marked with black boxes. The plasmids used are listed in table 18.

3.3.6 Electroporation of *E. coli* XL1-Blue with derivatives of pLME108

The single restriction sites for *Nhe*I and *Xho*I (figure 14, positions 1498 and 1632) found by mapping of the sequence of pLME108, were used to fuse the complete pLME108 with pUC18. Only for *Nhe*I, the electroporation was successful, resulting in the plasmids pLME116 and pLME117.

3.4 Gene transfer

3.4.1 Test of selective supplements for the isolation of propionibacteria

The main problem in all conjugation experiments was the selection of the transformants: the separation of donor and recipient cells from the real transconjugants. To achieve mainly the inhibition of donor cells, the addition of different supplements to the medium was tested. The results are shown in table 19.

Table 19: Growth of propionibacteria and other species on various media with or without selective supplements

Isolation-medium and respective "selective" supplement	*Enterococcus faecalis* JH2-2	*E. coli* XL1-Blue	*Lactobacillus bulgaricus*	"*Propionibacterium shermanii*^{T}"
YEL	+	+	+	+
PAL[1]	+[4]	-	-	+
YEL + indicator[2]	+	+	-	+
YEL + cloxacillin[3]	+	+	-	+
YEL + 0.1% propionate	+	+	-	+
YEL + 0.05% propionate	+	+	-	+
YEL + 2% acetate	+	+	-	+
YEL + 3% NaCl	+	+	-	+
YEL + 6% NaCl	+	-	-	weak
YEL + 10% NaCl	-	-	-	-
MRS	+	-	+	+
BHI	+	+	+	+

[1] Pal Propiobac (Standa industrie, Caën, France).
[2] 0.02 g/l bromcresol green.
[3] 4 µg/ml cloxacillin.
[4] Growth, but no "propionibacteria-typical" color change of the medium.

As shown in table 19, all tested strains grew well on YEL-medium, usually the medium recommended for the isolation of propionibacteria from dairy products, no inhibition was observed. The inhibition of *Lactobacillus bulgaricus* and *E. coli* proved to be relatively easy: growth on PAL, the addition of cloxacillin to the medium or the use of MRS were sufficient to inhibit one or both species. *Enterococcus faecalis* grew under all conditions, only the addition of 10% salt to the medium was too much for this strain. Unfortunately, also *P. shermanii* did not grow at this high salt concentration. The commercial PAL did not inhibit *E. faecalis*, but *E. faecalis* was detectable because a color reaction from violet to yellow occurs when propionibacteria grow on this medium.

3.4.2 Conjugation experiments between other genera and propionibacteria

The ability of propionibacteria to acquire foreign DNA using a "natural" way was tested with various conjugation experiments. Broad host range plasmids were "presented" to propionibacteria using a filter mating technique. All donor plasmids carried antibiotic resistance genes that were used as selective markers. Recipient strains (propionibacteria) were also characterized by the presence of features allowing simple and specific selection of transconjugants. The experiments and results are shown in table 20. In none of the experiments transconjugants could be selected.

Table 20: Conjugation experiments using the filter mating technique and propionibacteria as recipient strains.

Donor **x Recipient**	**Plasmid**	**Selection[2]**	**Result[3]**
***E. faecium* FO1** **x *P. thoenii*^{T}**	pFO1[1]	YEL-Agar, Tet15 red colonies	**no TC after 2 weeks**
Negative controls: *E. faecium* FO1 *P. thoenii*^{T}		YEL-Agar, Tet15	 growth, no red cfu no growth
***Enterococcus* sp. RE39** **x *P. freudenreichii*^{RF}**	pRE39	MRS-Agar, Rif50, Fus100, Em10	**no TC after 3 weeks**
Negative controls: *E. faecium* RE39 *P. freudenreichii*^{RF}		MRS-Agar, Rif50, Fus100, Em10	 no growth **growth**
***Enterococcus* sp. RE39** **x *P. shermanii*^{RF}**	pRE39	MRS-Agar, Rif50, Fus100, Em10	**no TC after 3 weeks**
Negative controls: *E. faecium* RE39 *P. shermanii*^{RF}		MRS-Agar, Rif50, Fus100, Em10	 no growth **growth**
***Enterococcus* sp. RE39** **x *P. thoenii*^{RF}**	pRE39	MRS-Agar, Rif50, Fus100, Em10	**no TC after 3 weeks**
Negative controls: *E. faecium* RE39 *P. thoenii*^{RF}		MRS-Agar, Rif50, Fus100, Em10	 no growth **growth**

Table 20 continued..

Donor x Recipient	Plasmid	Selection[2]	Result[3]
***Lactococcus lactis* K214** **x *P. shermanii*[RF]**	pK214	MRS-Agar, Cm20, Tet10, Fus100, Rif50	**no TC after 4 weeks**
Negative controls: *L. lactis* K214 *P. shermanii*[RF]		MRS-Agar, Cm20, Tet10, Fus100, Rif50	no growth
***Lactococcus lactis* K214** **x *P. thoenii*[RF]**	pK214	MRS-Agar, Cm20, Tet10, Fus100, Rif50	**no TC after 4 weeks**
Negative controls: *L. lactis* K214 *P. thoenii*[RF]		MRS-Agar, Cm20, Tet10, Fus100, Rif50	no growth

[1] Containing transposon Tn*FO1*.
[2] Abbreviations: TC: Transconjugants; [RF]: strain has been made resistant to Rifampicin and Fusidic acid (50 resp. 100 µg/ml); Cm: Chloramphenicol; Em: Erythromycin; Fus: Fusidic acid, Rif: Rifampicin; Tet: Tetracycline; numbers after antibiotics indicate their concentrations in the medium (e.g. Tet10: 10 µg/ml of Tetracycline).
[3] Experimental data from Guldimann (1998) were included in this table.

The main problem in the conjugation experiments shown in table 20, was the growth of the recipients *Propionibacterium* strains on the media used for the selection of the transconjugants. Especially when erythromycin was used for the selection, growth of the negative controls, possibly due to spontaneous mutations, was observed.

It is presumed, that conjugation may have occurred but the number of transconjugants was significantly smaller than the number of spontaneous mutants (e.g. one real transconjugant for 500 spontaneous mutants). Due to this fact no transconjugants could be isolated under the selected conjugation conditions.

3.4.3 Electroporation of propionibacteria

As a base to the introduction of foreign genes into propionibacteria, electroporation conditions had to be established. Plasmids with a broad host range and plasmids containing parts of pLME108 were used to test the DNA-uptake capability of propionibacteria.

In a first step, the optimal electroporation conditions for the different buffer systems used were determined experimentally and are shown in table 21. A resistance of 200 Ω was finally selected for all experiments.

Table 21: Determination of optimal electroporation conditions using different buffer systems (2.5 kV, 25 μF, 2 mm gap cuvettes, 40 μl of each buffer)

Resistance	10% glycerol	GS	30% PEG
1000 Ω	22.2 ms	22.8 ms	"Short circuit"
800 Ω	18.3 ms	15.5 ms	11.5 ms
600 Ω	13.9 ms	14.0 ms	13.0 ms
400 Ω	9.4 ms	9.4 ms	9.1 ms
200 Ω	**4.8 ms**	**4.8 ms**	**4.6 ms**
100 Ω	2.4 ms	2.4 ms	2.4 ms

PEG: Polyethylenglycole 10'000 (Zirnstein and Rehberger, 1991).
GS: 10% glycerol and 0.5 M sucrose (Gautier et al., 1995).

In table 22 the conditions and strains for electroporation experiments are listed. No transformants were observed under the selected conditions and with the investigated strains and plasmids. The tetracycline resistance gene (*tetM*) was amplified using PCR and ligated with pLME108 (*Nhe*I site). Since the TetM resistance mechanism does not function well in *E. coli* (Levy et al., 1989), an attempt to introduce the pLME108::*tetM* vector directly in propionibacteria was selected. The same was attempted using the whole plasmid pLME101 (40 kb) fused with plasmid pJIR751. This plasmid would not replicate in *E. coli* and therefore a problematic direct cloning attempt was tried. Mainly due to very small amounts of DNA present when no *E. coli* passage is performed this approach is difficult.

The use of ampicillin as selectable marker for the electroporation of the plasmids pLME116 to pLME119 in propionibacteria did not lead to success. Since this ampicillin resistance gene is mainly active in Gram-negative bacteria, this fact is not surprising.

All "transformants" shown with "+" in table 22, resulted after selection with erythromycin. In none of these transformants the transferred plasmid DNA was found. DNA integration into the chromosome was also not observed. Growth of these strains on the selective media was due to the acquisition of erythromycin-resistance as observed in the conjugation experiments in chapter 3.4.2.

Table 22: Electrotransformation of competent *Propionibacterium* cells

Plasmid	Strain[1]	Selection[2]	Result[3]
pAM120	*P. cyclohexanicum*	Tet15, 28 days	-
pAM120[4]	*P. cyclohexanicum*	Tet15, 28 days	-
pAM120	*P. freudenreichii*^{T}	Tet15, Tet30, 28 days	-
pAM120	"*P. shermanii*"	Tet30, 28 days	-
pAM120	FAM1409	Tet15, Tet30, 21 days	-
pAM120	*P. jensenii*	Tet15, Tet30, 21 days	-
pAM180	*P. cyclohexanicum*	Tet15, 28 days	-
pAM180[4]	*P. cyclohexanicum*	Tet15, 28 days	-
pAM180	*P. freudenreichii*^{T}	Tet15, Tet30, 28 days	-
pAM180	"*P. shermanii*"	Tet30, 28 days	-
pAM180	*P. jensenii*	Tet30, 28 days	-
pJIR750	*P. freudenreichii*^{T}	Cm10, Cm20, 28 days	-
pJIR750	"*P. shermanii*"	Cm10, Cm20, 28 days	-
pJIR750	*P. jensenii*	Cm10, Cm20, 28 days	-
pJIR751	*P. freudenreichii*^{T}	Em5, Em15, Em40, 28 d	+
pJIR751	"*P. shermanii*"	Em5, Em15, Em40, 28 d	+
pJIR751	*P. jensenii*	Em15, Em40, 28 days	-
pLME116	*P. cyclohexanicum*	Amp50, 28 days	-
pLME116	*P. freudenreichii*^{T}	Amp50, 28 days	-
pLME116	ARE1	Amp50, 28 days	-
pLME116	FAM1413	Amp50, 28 days	-
pLME116	JS53	Amp50, 28 days	-
pLME116	"*P. intermedium*"	Amp50, 28 days	-
pLME116	*P. jensenii*	Amp50, 28 days	-
pLME118	*P. jensenii*^{T}	Amp50, 28 days	-
pLME119	*P. jensenii*^{T}	Amp50, 28 days	-
pSI22	"*P. shermanii*"	Cm10, 28 days	-
pSI22	*P. freudenreichii*^{T}	Cm10, 28 days	-
pLME101::pJIR751 (*Eco*RV)	*P. freudenreichii*^{T}	Em10, 10 days	+
pLME101::pJIR751 (*Eco*RV)	"*P. shermanii*"	Em10, 10 days	+
pLME101::pJIR751 (*Hin*dIII)	*P. freudenreichii*^{T}	Em10, 10 days	+
pLME101::pJIR751 (*Hin*dIII)	"*P. shermanii*"	Em10, 10 days	+
pLME101::pJIR751 (*Pst*I)	*P. freudenreichii*^{T}	Em10, 10 days	+
pLME101::pJIR751 (*Pst*I)	"*P. shermanii*"	Em10, 10 days	+
pLME108::*tetM*	FAM1413	Tet15, Tet30, 21 days	-
pLME108::*tetM*	JS53	Tet15, Tet30, 21 days	-
pLME108::*tetM*	"*P. intermedium*"	Tet15, Tet30, 21 days	-

[1] Made competent as listed in table 8.
[2] NL medium with selective supplement (concentrations in µg/ml of Cm: Chloramphenicol; Em: Erythromycin and Tet: Tetracycline).
[3] +: growth on selective medium, potential transformants; -: no transformants observed. Parts of the data were included from Guldimann (1998).
[4] Treatment of cells for 10 minutes at 48°C prior to electroporation.

4. DISCUSSION

4.1 Isolation of propionibacteria

4.1.1 "Selective" media and identification tests

The main problem encountered when propionibacteria are isolated is the lack of suitable selective media. Depending on the other organisms present in samples, propionibacteria can be either isolated relatively easily or with great difficulties. Samples like hard or semi-hard cheeses, where mainly lactic acid bacteria occur as competitive flora for the propionibacteria are relatively easy to process. Using the method of Drinan and Cogan (1992), with cloxacillin added to the medium (YEL-like) to inhibit growth of the lactic acid bacteria, almost pure cultures of propionibacteria can be isolated from cheese. Problems arise mainly when the competing microflora consists of bacteria that grow much faster than propionibacteria (and almost all do that !). Especially enterococci grow well on the same media as propionibacteria and are also found in dairy products made from raw milk (Giraffa et al., 1997). The addition of antibiotics or other inhibitory substances to the medium is usually not possible, since propionibacteria are more sensitive to these substances than enterococci (table 19).

Another problem that arises when propionibacteria are cultured is the often unclear interpretation of simple test reactions like Gram staining, the determination of catalase activity or microscopy. The morphology of propionibacteria is highly dependent on the growth medium and the age of the cells. Propionibacteria have been even called "Gram-variable" by certain authors (Beveridge, 1990). The use of the KOH-test (Bamarouf et al., 1996) instead of Gram-staining is helpful, but strains of the classical propionibacteria produce slime. Consequently, they could be interpreted using this test as Gram-negative. Cell shapes may vary from very short, almost coccoid rods to the "typical" coryneform morphology. Tests like the catalase activity can also be misleading, because for certain strains, a positive reaction is observed only after incubating the cells for 10 to 30 minutes and only after exposition of the cells to oxygen.

The commercially available medium, PAL Propiobac (Standa industrie, France) is tested mainly in use for dairy species and no data are available how cutaneous strains or still unknown *Propionibacterium* species will grow on this medium. As already mentioned, PAL is quite expensive (sFr. 1.50 per plate) and therefore not the medium of choice for routine analysis.

The use of numerical taxonomy, mainly based on the determination of carbohydrate fermentation patterns as described by Britz and Riedel (1991) is still a good approach to characterize propionibacteria. Commercial identification kits, e.g. APICoryne (version 2) and API20A (BioMérieux), often include only information for medically relevant propionibacteria in their databases.

Since no real selective medium for the isolation of propionibacteria from different sources was found, a molecular approach was selected for the identification.

4.2 Identification of propionibacteria using molecular methods

Recently, screening techniques based on molecular methods revealed the presence of propionibacteria in other habitats. These techniques, mainly based on the amplification of 16S rDNA genes with universal primers, followed by cloning and sequencing analysis, allow the detection of small amounts of DNA, theoretically down to one single DNA molecule in a sample.

16S rDNA sequences closely related with those of propionibacteria were found in the rumen of red deer (Jarvis et al., 1998). These sequences showed 99.6% sequence similarity to *P. acnes*. Boivin-Jahns et al. (1996) found propionibacteria 16S rDNA sequences in a deep-surface clay sediment (97% similarity with *P. granulosum* and 98% similarity with *P. acnes*). Other sequences, all related to cutaneous propionibacteria were amplified by Sekiguchi et al. (1998) from sludge. A new species, *P. cyclohexanicum,* was isolated from spoilt orange juice by Kusano et al. (1997).

4.2.1 Identification to genus level

Different researchers have already described specific primers for the detection of propionibacteria. The primer RDR514 (*E. coli* position 1376-1400) was tested and proposed as hybridization probe to detect *P. acnes, P. avidum, P. lymphophilum* and *P. granulosum* (Greisen et al., 1994). However, this primer was not tested with the classical strains, and an alignment of these 16S rDNA sequences shows certain mismatches (Dasen et al., 1998). Since this primer is relatively long (25 bp), its suitability for PCR may not be optimal. Primers for the amplification of *Propionibacterium* 16S rDNA fragments have been used (Riedel et al., 1994), but these primers are not specific for the genus. The genus specific probe, gd1 (table 5) and the MPCR strategy developed in this study are further discussed in chapter 4.2.5. The use of gd1 as a hybridization probe

allowed the confirmation of propionibacteria isolated from cheese samples. When used for direct colony hybridization experiments, gd1 gave no satisfactory results. It was supposed that the target 16S rRNA region for gd1 (*E. coli* position 632) exhibits a strong secondary structure which prevents hybridization of the probe with the target or that slime produced by propionibacteria inhibited cell lysis.

4.2.2 Identification to species level

To identify mainly dairy *Propionibacterium* species and in some cases also the medically relevant species, methods like ribotyping or the restriction analysis of PCR products of the 16S rDNA, of the 23S rDNA and of the intergenic 16S-23S rDNA region (ITS) have been described by several authors (Riedel et al., 1994 & 1998; Fessler et al., 1998; Rossi et al., 1997).

PCR primer pairs for the detection of *P. acnes* have been reported by Hykin et al. (1994). Rossi et al. (1998) proposed specific oligonucleotide primers for the PCR detection of each of the dairy propionibacteria species (*P. acidipropionici*, *P. freudenreichii*, *P. jensenii* and *P. thoenii*).

The hybridization method developed in my thesis, using the ITS regions of propionibacteria as probes, has so far been tested only for the detection of strains of *P. freudenreichii* (figure 7). But this method should also allow the detection of other species as well, by simply using the ITS regions of these species as probes. The ITS sequence is short enough (300-400 bp) in all *Propionibacterium* species to allow enough specificity for the probe in such a hybridization.

The main advantage of a method based on a hybridization assay is its potential for a direct counting of bacterial colonies. By replica-plating of colonies grown from a dilution series of a sample, followed by a transfer of the colonies to nylon membranes and cell lysis, a quantification for bifidobacteria by hybridization is possible (Kaufmann et al., 1997). This direct approach was not tested in this study, but as a first step, DNA of *Propionibacterium* isolates (200 strains) isolated by using the protocol of Goldenberger et al. (1995) was transferred to a nylon membrane and probed with the ITS region of *P. freudenreichii*. All strains of *P. freudenreichii* tested could be detected with this method (data not shown).

Other methods for the identification of dairy *Propionibacterium* species based on the comparative analysis of protein profiles (Baer, 1987; Fessler, 1997) have been developed

with good results. But this method requires, like some of the other methods the use of pure *Propionibacterium* cultures prior to identification. Only the use of PCR or hybridization-based techniques would allow the detection of mixed cultures of different species of propionibacteria. Since quantitative PCR requires a lot of experience and expensive equipment (Brochure, Perkin Elmer) the simpler hybridization technique should perform better in the practical use and is therefore proposed in this study.

4.2.3 Identification of the *P. freudenreichii* subspecies

Since *P. freudenreichii* is very important as a starter culture in cheese production and certain strains are not able to ferment lactose, a division of this species in two subspecies is currently still in use. This separation into the two subspecies *P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii* (the first does not ferment lactose), is based on no other features (Cummins and Johnson, 1986). By using a variety of methods mentioned before (protein profiling, RAPD, ribotyping, RFLP), most researchers were unable to find other significant differences between the two subspecies. In this thesis, four 16S rDNA and two 23S rDNA sequences of strains of *P. freudenreichii* were analyzed (figure 8 and figure 9). The differences found between these sequences were small: at least 97.4% identity for the 16S rDNA, 98.5% for the 23S rDNA. In addition, the sequences for the *P. freudenreichii* strains contain 23 and 26 ambiguous bases (N) that were not included in the calculation of these identities. Especially the analysis of the 16S rDNA sequences shown in figure 8 reveals that differences occur even between the two sequences of *P. freudenreichii* subsp. *freudenreichii*. In the case of the 23S rDNA sequences, where only two sequences exist so far, these differences between the same subspecies could also occur. It is a known fact that different strains of the same species may differ in up to 24 bases (Pettersson et al, 1998) over their whole 16S rDNA sequence. Propionibacteria harbor one to three copies of the rRNA genes on their chromosome (de Carvalho et al., 1994 & Ross et al., 1997), which can also be different from each other, a fact discovered for other bacteria (Nübel et al., 1996).

The sequence analysis of the 16S-23S rDNA intergenic spacer regions of the *P. freudenreichii* strains did not result in a clear grouping into 2 subspecies. The strains received from the FAM (Forschungsanstalt für Milchwirtschaft, Liebefeld, Switzerland), claimed to be members of "*P. shermanii*" and used as starter cultures for cheese grouped with both of the type strains of the subspecies.

Rehberger and Glatz (1987 & 1990) reported the presence of a plasmid-linked lactose utilization in propionibacteria, an observation which renders the single clear distinctive factor obsolete. A differentiation between a *P. freudenreichii* subsp. *freudenreichii* strain, with a plasmid encoded lactose-utilization, and a *P. freudenreichii* subsp. *shermanii* strain (lactose fermentation chromosomally encoded) is no longer possible.
Based on these experimental facts, the proposal to abandon the separation of *P. freudenreichii* into two subspecies (Fessler, 1997) is also recommended.

4.2.4 Identification of strains

The use of molecular methods allows the differentiation between strains of the same species. PFGE (pulsed field electrophoresis) is the most popular method. It is based on the distribution of rare recognition sites of a particular restriction enzyme on a genome and is highly reproducible. RAPD is also used for strain identification. In contrast to PFGE, it is based on PCR which in the worst cases with non stringent primers, will lead to a high variability of the results. RAPD should only be used as an "internal" method or in the absence of other ideas, because the results of these analysis are fairly irreproducible between different laboratories (Jones et al., 1997). PFGE has been used by Rehberger (1993) to compare strains of *P. freudenreichii*. RAPD analysis has been performed for *P. freudenreichii* strains and other dairy propionibacteria and allowed the differentiation between these strains in a single laboratory study (Fessler, 1997).
In my thesis, however, the sequence analysis of the 16S-23S rDNA intergenic spacer region (ITS) was found to be another possible approach to differentiate between *Propionibacterium* strains. The use of the intergenic spacer region as a tool to species identification among bifidobacteria has been reported by Leblond-Bourget et al. (1996). As shown in figure 5 and figure 6, different strains of *P. freudenreichii* as well as *P. jensenii*, *P. thoenii* and *P. acidipropionici* could be identified. Because not enough strains of the cutaneous propionibacteria were available, the use of this method for such strains was not further investigated.

4.2.5 Multiplex PCR: a fast and reliable approach to identify propionibacteria

Using a standard PCR protocol with primers gd1 and bak4 alone, a single fragment of 900 bp approximately (889-915 bp, dependent on the species) is amplified for propionibacteria. Template DNA from other organisms resulted in no detectable

amplification products. Since it was not possible to determine whether the absence of a signal was due to no amplification or limited DNA extraction it was necessary to run a second PCR analysis using the universal primers bak11w and bak4. In order to simplify the method I developed a multiplex-PCR like approach. Multiplex PCR (MPCR) is commonly used to amplify 2 or more loci simultaneously. The method has already been applied to detect pathogens in food samples (Deng and Fratamico, 1996; Witham et al., 1996). The difference between "classical" MPCR and the method developed in this thesis is that the same gene, the 16S rDNA, is the target for all primers.

Propionibacteria could be distinguished from other organisms by amplification of a specific 900 bp fragment amplified with primers gd1 and bak4. All other organisms showed only a "universal" fragment of 1500 bp resulting of amplification with primers bak11w and bak4. This fragment is used as an internal standard. When the protocol was applied to pure cultures, the detection limit for propionibacteria was at 2×10^3 colony forming units (cfu).

In my MPCR approach using *Propionibacterium* DNA, the oligonucleotides gd1, bak11w and bak4 (figure 3), theoretically 2 fragments of approximately 900 bp and 1500 bp should be formed. Under the selected conditions and DNA-concentrations, only the specific fragment of 900 bp was amplified in detectable amounts. Depending on DNA-concentrations and annealing temperatures, both fragments were amplified for propionibacteria, but the protocol was further optimized until the "universal" fragment was no longer amplified in propionibacteria.

In all strains of propionibacteria tested, the specific fragment was amplified and no false positive signals were found to occur for other tested organisms (figure 4).

The alignment of the primer gd1 (*E. coli* position 632-651) is shown in table 13. In a FASTA comparison of this primer with all sequences of the GenEmbl database (November 1998) only 24 sequences showed at least 95% homology (in all 20 nucleotides) with the target sequence. Of these sequences, 14 belonged to propionibacteria, 7 belonged to organisms that were not further identified to genus level. Of the three remaining, one sequence of *Actinomyces israelii* serotype 1 (Accession no. X53228), *Mycobacterium chitae* (Accession no. M29560) and *Eubacterium combesii* (Accession no. L34614) showed 100% identity with gd1. These sequences show lower similarity to other sequences from the same genera than to propionibacteria: *A. israelii* has a similarity of 96.3% to *P. acnes* with only 84% similarity to other species from the genus

Actinomyces. *M. chitae* is more closely related to *P. acnes* (98.3%) than to other *Mycobacterium* species (87%). The questionable sequence of *E. combesii* is related to *P. thoenii* (92.3%) and shows only 78% similarity with other *Eubacterium* species. That indicates either the need of reclassification and re-evaluation of these strains or sequencing problems. When cells of *M. chitae* were submitted to the MPCR protocol no typical *Propionibacterium* banding pattern was amplified for this species, hardening the suspicion that the 16S rDNA sequence entry (Accession no. M29560) for this species is wrong.

The 16S rDNA of the new classified strain *P. cyclohexanicum* shows one mismatch with our primer sequence. Since this strain is relatively new (Kusano et al., 1997) its occurrence in other habitats than orange juice has not been investigated. The strain could be detected using the MPCR technique by lowering the annealing-temperature from 60°C to 56°C resulting in a slightly lower specificity for the detection and, in certain cases, the formation of a third fragment of approximately 2 kb (figure 4, *P. avidum* and *P. cyclohexanicum*) due to unspecific binding of the oligonucleotides at the lower annealing temperature. This problem could possibly be solved by using degenerated primers with a mixture of A and C at position 18 of the oligonucleotide gd1.

Since PCR is theoretically sensitive enough to detect one single molecule of DNA in a reaction tube, great care has to be taken in every PCR amplification to avoid even the smallest amounts of contaminating DNA. The combination of the MPCR method with the DNA isolation method of Goldenberger et al. (1995) using only SDS and Proteinase K allowed to minimize the number of handling steps needed to obtain "PCR grade" DNA. Probes could be treated in one single cap, with only minimal pipetting necessary. This method was successful with all species tested. The use of this method for propionibacteria would not be necessary (the simple addition of propionibacteria cells to the MPCR would guarantee suitable results) but especially the lysis of lactobacilli is otherwise difficult. The method was tested with milk samples in which propionibacteria have been cultivated. Without any purification, the milk was submitted to the protocol and it was possible to detect the propionibacteria.

In all the PCR experiments, a negative control that contained no DNA but that was treated exactly like DNA containing samples, allowed the detection of contaminants.

4.3 Phylogenetic analysis of the genus *Propionibacterium*

The 16S rDNA sequences of all type strains were completed or resequenced as far as possible. This was necessary to obtain as many nucleotides as possible to compute an optimal alignment (Ludwig and Schleifer, 1994). A phylogenetic tree (figure 2) of the complete genus including the medically relevant strains was calculated (maximum likelihood method) based on the alignment from *E. coli* positions 28 to 1521. The species could be grouped into different clusters: The classical species *P. acidipropionici, P. jensenii* and *P. thoenii* form one and *P. freudenreichii* (both subspecies) and *P. cyclohexanicum* another cluster. These data are in accordance with the phylogenetic trees of Kusano et al. (1997) and Charfreitag and Stackebrandt (1989). The cutaneous species separate into a cluster containing *P. acnes, P. avidum* and *P. propionicum*. *P. granulosum* forms a separate cluster between the classical and cutaneous groups whereas *P. lymphophilum* is the most distant relative of the whole genus. In fact, the 16S rRNA sequence of *P. lymphophilum* shows more similarity (91.1%) with *Luteococcus japonicus* than with other propionibacteria (90.7% with *P. freudenreichii*).
As shown by Charfreitag and Stackebrandt (1989) and Britz and Riedel (1991) the propionibacteria form a very homogeneous group, with no other species intermixed. Unlike other genera of the *Actinobacteria* like *Arthrobacter* or *Corynebacterium* (Kollöffel et al., 1997) the taxonomic situation of the propionibacteria has changed little over the last 30 years, a fact that may be due to the well known and investigated habitats of the known species: the skin and dairy products. A new species was included into the genus when Charfreitag et al. (1988) proposed that *Arachnia propionica* should be renamed as *P. propionicum*. Recently a complete new species *P. cyclohexanicum* was proposed by Kusano et al. (1997). In the catalogue of strains of the American type culture collection (ATCC) strains like "*P. intermedium*", "*P. wentii*" and "*P. animalis*" are listed, these strains have so far not been clearly classified within the genus. Using the MPCR approach, the first two were identified as propionibacteria, "*P. animalis*" was not tested. Most other strains like "*P. rubrum*" have been shown to be variants of the already existing species, "*P. rubrum*" has been shown to belong to the *P. jensenii* group (de Carvalho et al., 1995), a fact that was confirmed in my study by the analysis of the ITS region. It is not impossible that new *Propionibacterium* species will be discovered in the future when the occurrence of propionibacteria in other habitats is investigated using molecular methods. The MPCR method developed in this thesis is a promising tool that can be used

for screening experiments to reveal the presence of propionibacteria in environmental or clinical samples.

4.3.1 Sequencing strategy

The sequencing strategy using PCR-products instead of cloned fragments allows the amplification of parts of the 16S rDNA of unknown organisms in a short time. Within two days an organism can be identified according to the similarity of its 16S rRNA with known organisms of the database. This method is now more often used than the cumbersome cloning of either PCR products or "gel-excised" DNA fragments.

As shown in this study, almost the complete 16S rRNA gene of the selected organisms could be sequenced, except the bases corresponding to the *E. coli* positions 1-8, since the universal primer bak11w binds behind this region. The sequences of the universal primers used for PCR (bak11w and bak4, table 5), forming the beginning and the end of the 16S rDNA, were not included in the submitted sequences because both primers contain ambiguous bases in their sequences.

To avoid mistakes due to the *Taq* polymerase, different PCR products were used as templates for the sequencing reactions. Control-sequencing of already known sequences showed a good agreement of the data I obtained. It is also possible to use "proof reading" polymerases like *Pfu* (Stratagene) to minimize amplification errors. As mentioned before, propionibacteria posses two copies of the rRNA operon (de Carvalho et al., 1994), which may be slightly different. A preference for one or the other operon by the polymerase was not observed, but cannot be excluded. The 16S rDNA sequence of *P. jensenii*, already published in the database, showed differences when resequenced for the region where the specific primer gd1 binds (table 13).

In my study, the same strategy was applied to obtain a 23S rDNA sequence of *P. freudenreichii* subsp. *shermanii*. A PCR product using primers gd1 and gd5sr ranging from the start of the 16S rDNA to the middle of the 5S rDNA was used as template (table 5 and table 6). Sequencing primers were selected from conserved regions of the 23S rRNA. The obtained sequence shows a high similarity with the 23S rRNA sequence of *P. freudenreichii* subsp. *freudenreichii* (W. Ludwig, personal communication).

4.4 Genetic system

In several studies, the development of a system allowing genetic analysis and engineering of propionibacteria was attempted. The genetic engineering of bacteria requires the detection of vector systems (e.g. phages or plasmids) that could be used to transport "foreign" DNA into a host cell. In addition, selectable markers for the recognition of a successful DNA transfer that are functional in the host cells are needed.

Phages have been found in dairy propionibacteria (Gautier et al., 1995a) and their DNA was used in electrotransfection experiments (Gautier et al., 1995b). Plasmids as the main DNA transport vehicles were found in propionibacteria (Pérez-Chaia et al., 1988; Rehberger and Glatz, 1990) but no sequences have previously been published.

Selectable markers, mainly antibiotic resistance genes, have so far not been described for propionibacteria. Antibiotic resistances have been found for propionibacteria, but data exist mainly for the medically relevant species (Lorian, 1996; Tunney et al., 1998). The last study concerning the antibiotic susceptibilities of dairy propionibacteria was performed by Reddy et al. (1973).

4.4.1 Plasmids pLME101 and pLME108

All strains of propionibacteria strains either isolated during this study or received from culture collections were screened for the presence of plasmids (table 14). Finally, two plasmids were selected for further analysis: a small plasmid of 2.051 kb, pLME108, that might be used for electroporation experiments and a large plasmid of 40 kb, pLME101, intended to be used in conjugation experiments.

The large plasmid pLME101, isolated from *P. freudenreichii* JS53 (Smutny, 1997), was characterized by restriction endonuclease mapping and partial sequencing of roughly 3 kb. For one sequence fragment, an amino acid sequence identity of 53.2% with a hypothetical protein from *Synechocystis* sp. was found. In curing experiments with the host of pLME101, a plasmid free strain was obtained, but no differences concerning carbohydrate fermentations and antibiotic resistance patterns were found between the two strains.

Plasmid pLME108, isolated from *P. freudenreichii* DF2, was completely sequenced in this study and a putative replicase gene was identified (*rep*). This *rep* gene shows relatively high identity (42%) to the *rep* gene of plasmid pAP1, isolated from *Arcanobacterium pyogenes*. pAP1 is a plasmid that uses the rolling circle (RC) mechanism for its replication (Billington et al., 1998). Briefly, this mechanism uses a

single stranded intermediate form of the plasmid for its replication and is characterized by a two step "copy" mechanism for the plasmid: first a leading strand of the plasmid is replicated, followed by DNA-synthesis at the lagging strand (del Solar et al., 1998; Khan, 1997). Plasmids belonging to the family of pIJ101/pJV1 RC plasmids, like pAP1 and supposedly pLME108, are characterized by 5 common motifs occurring in their Rep proteins. In figure 16, an alignment of these motifs for pLME108 with other plasmids of the same family is shown. The conserved amino acids show clearly the relatedness of pAP1 and pLME108. In addition two special regions characterize this family of RC plasmids: a conserved DNA sequence at the single stranded replication origin (sso) and at the origin for double stranded replication (dso). The sequence characteristic for the sso (TAGCGT) was found on the pLME108 sequence (shown in figure 14) and correctly outside (Zaman et al., 1993) of the putative *rep* gene. This region is in addition usually characterized by high secondary structures, with the sso region included in one of the loops that form this structures (Fernandez-Gonzalez et al., 1994). For pLME108, secondary structures around the sso site were found, but none of these structures contained the sso in its loop region. The dso region usually contains the "nick-site" for the break of the DNA double strand. This nick-site is characterized by the occurrence of two following G (Billington et al., 1998). Such a typical dso region for pLME108 was not identified as it has also been observed for other plasmids (Yang and McFadden, 1993).
Plasmid pAP1 has been shown to be able to replicate in *Escherichia coli* (Billington et al., 1998): by adding a kanamycin resistance gene into pAP1, the plasmid was successfully introduced in *E. coli* by electroporation. A fact that is promising for the case of pLME108. In a first attempt, the ampicillin resistance gene of pUC18 was amplified by means of PCR, ligated into the *Nhe*I site of pLME108 and electroporated into *E. coli* XL1-Blue. No transformants were found in this experiment, possibly because *Nhe*I cuts at the sequence GCTAGC, which is also part (underlined) of the sso sequence needed for replication of this plasmid. When the complete pLME108, digested with *Nhe*I or *Xho*I was ligated to pUC18 only the *Nhe*I digest was successfully cloned into pUC18 (plasmid pLME116). Both *Xho*I and *Nhe*I were selected because they cut pLME108 only once and not in the *rep* region. The fact that pLME108 digested with *Xho*I could not be added to pUC18 and cloned is possibly due to a competition between two functional *rep* genes on the same plasmid. Even pLME116 did not replicate well in *E. coli*, indicating that the *rep* gene of pLME108 had perhaps maintained part of its activity.

4.4.2 Electrotransformation of propionibacteria

Electroporation of propionibacteria has been reported first by Pai and Glatz (1987), the plasmid pE194 from *Staphylococcus aureus* was transferred to protoplasts of *P. freudenreichii*. Selection with erythromycin was used to obtain transformants, but no pE194 DNA was found in these presumptive recipients.

Luchansky et al. (1988) proposed an electroporation protocol for several Gram-positive bacteria using the plasmid pGK12 isolated from lactococci, which contains erythromycin and chloramphenicol resistances as selectable markers. This plasmid was transferred to several genera, including propionibacteria. They were transformed at a rate of 3.2×10^1 transformants per µg DNA. The results are poorly documented and the protocol described in that study is difficult to reproduce; as an example, the buffer used to obtain competent cells, PEB, "was used in a concentration of 0.5 to 2.5x". In addition, the resulting cells were not analyzed for the presence of the plasmid or of the gene responsible for erythromycin or chloramphenicol resistance. Zirnstein and Rehberger (1991) reported the transfer of plasmid pC194 from *Staphylococcus aureus* to propionibacteria using also erythromycin resistance as marker gene. No plasmids were found in the resistant cells, but the gene was detected in the genome of these strains by hybridization analysis. Both the findings of Pai and Glatz (1987) and Zirnstein and Rehberger (1991) were published only as poster-presentations with no further following publication.

In this study, several plasmids from different hosts were used in electroporation experiments, mainly according to the strategy of Gautier et al. (1995b). Other methods for the "production" of competent cells as well as different electroporation conditions were tested. In no experiment (table 22) plasmid transfer into the 23 tested strains was detected. Only when erythromycin was used for the selection of transformants growth of resistant propionibacteria was observed after transformation, but in all cases neither plasmid DNA nor the erythromycin resistance gene was found in these strains. In addition, cells electroporated without DNA as negative controls, grew on the selective media containing erythromycin when incubated for a longer time. As discussed later, in chapter 4.5, propionibacteria can acquire erythromycin resistance by modification of their 23S rRNA sequences. Since in most other studies erythromycin is used as a selectable marker, this phenomenon may have occurred before.

Since the use of "foreign" plasmids may have been another reason for the failure of the electroporation experiments, plasmids pLME108 and pLME101 were used as potential

vectors. In both cases either resistance genes (*beta-lactamase:* ampicillin or *tetM*) or other plasmids were fused with the propionibacteria plasmids and electroporated into the putative competent propionibacteria. No transfer was observed. Since the *tetM* gene was introduced at the *Nhe*I site of pLME108 the problem described in chapter 4.4.1 may have been the reason for this result.

Briefly the following facts may have inhibited the electroporation of propionibacteria:

- Lack of competence of the investigated propionibacteria, either due to the methods used or a natural absence of this ability in the strains.
- No suitable plasmid was tested.
- No suitable selectable marker was used.
- A restriction system in the recipient strains "destroys" foreign DNA.

Since the traditional methods have been shown in my thesis not to work, completely new transformation strategies and protocols must be developed.

4.4.3 Conjugation experiments

Plasmid DNA transfer by means of conjugation between species of different genera has been observed also for species of dairy origin (Wirsching, 1995; Perreten et al., 1998). Especially enterococci are important as a reservoir for such plasmids (Perreten et al., 1997) and were selected as donor strains in my study. In addition, *Lactococcus lactis* K214, was used as donor strain. Using enterococci, the main problem became the selection of transformed propionibacteria with a total inhibition of the donor *Enterococcus* strains. Several media, media supplements and growth conditions were tested, but enterococci were at least as resistant as were the propionibacteria (table 19). Only when propionibacteria were adapted to high concentrations of rifampicin and fusidic acid, a sufficient selectivity was achieved.

In none of the experiments, a DNA transfer by conjugation was observed. When erythromycin was used, resistant mutants of propionibacteria appeared in the negative controls and no transferred DNA was found in the putative transconjugants. Even with plasmids like pFO1, harboring the transposon Tn*FO1* which can integrate chromosomally and commits tetracycline resistance, no DNA transfer was observed.

Since propionibacteria can produce extracellular slime (Reddy et al., 1973) the contact between donor and recipient cells could be inhibited by this slime. The lack of indigenous plasmids from propionibacteria with selectable markers to be tested may also be a reason for the failure of the conjugation experiments.

4.5 Selectable and transferable markers

Both electroporation and conjugation experiments revealed the need for markers that cam be used for the selection of propionibacteria. In addition, a marker that can be transferred into propionibacteria and is functional in these organisms has to be used.

4.5.1 Selectable markers for conjugation experiments

A selectable marker used in conjugation experiments must allow the simple identification of transformants. Such a marker is usually encoded on the chromosome of the recipient strain, for example the ability to use lactose as carbon source. In this thesis, recipient propionibacteria strains were adapted to high concentrations of rifampicin and fusidic acid. All donor strains were inhibited by these antibiotics.

Naturally occurring resistance of propionibacteria (against gentamicin, kanamycin, metronidazole, methicillin, nalidixic acid, neomycin, sulfonamides and tobramycin) could be used for the selection of recipient cells for conjugation experiments with other genera. These antibiotics could also be used in a selective medium for the isolation of propionibacteria.

Conjugation experiments within the genus *Propionibacterium* require the use of resistance markers for recipient cells that are strain specific, for example streptomycin resistance. The best solution would be the use strains of adapted to high concentrations of antibiotics.

4.5.2 Transferable markers for conjugation and electroporation

To detect a successful gene transfer the markers used must be functional in propionibacteria. Ideal would be therefore a marker which originates from propionibacteria, for example a bacteriocin resistance gene. The development of antibiotic resistance patterns has been observed mainly for medically relevant propionibacteria: after intake of antibiotics used for the treatment of acne vulgaris, antibiotic resistant strains of *P. acnes* were found (Eady, 1998).

Good candidates for transferable markers are antibiotics for which *Propionibacterium* strains show varying resistance patterns, indicating that resistance mechanisms are available and fully functional. The following antibiotics are such candidates: cefotaxime, ciprofloxacin, chloramphenicol, erythromycin, polymyxin B, streptomycin and tetracycline.

The uselessness of erythromycin as a marker because of a to high spontaneous mutation rate was shown in this thesis. Ross et al. (1997) showed that a single mutation in 23S rDNA led to resistance to erythromycin in cutaneous propionibacteria. The same problem occurs for tetracycline, where mutations in the 16S rDNA caused resistance in cutaneous propionibacteria (Ross et al., 1998). The mutation rate for tetracycline resistance seems to be lower than for erythromycin since no growth of the negative controls was observed in the experiments of this thesis.

Chloramphenicol seems to have a good potential as resistance marker: resistance is observed for *P. thoenii* and *P. jensenii* strains whereas the other species are sensitive. Resistance genes for chloramphenicol are described for Gram-positive organisms and commonly used in vector systems, for example the plasmid pJIR750, a *Clostridium perfringens* - *E. coli* shuttle vector (Bannam and Rood, 1993). This plasmid was used without success in electroporation experiments in this thesis, but this is not necessarily due to an unsuitable resistance gene, other factors could have inhibited a successful transfer.

Propionibacteria are sensitive to ampicillin and other antibiotics of the penicillin group. A resistance is mainly observed in Gram-negative organisms, since the β-lactamase is excreted in the periplasmatic gap which exists only in these organisms (Lorian, 1996). This classical mechanism does not function in propionibacteria and can be the reason why no transformants were found in this thesis when an ampicillin resistance transfer was attempted.

4.6 Conclusions

Using the multiplex PCR method developed in this study a simple, reliable and fast detection of strains belonging to the genus *Propionibacterium* is possible. The method can also be used for rapid screening of various habitats for the presence of propionibacteria. Species specific gene probes for a hybridization assay can be obtained by using PCR amplified 16S-23S rDNA intergenic spacer regions. This ITS regions can be labeled for example with digoxigenin and provide stable probes. Even if the system has so far only be tested for *P. freudenreichii*, for all 10 species currently classified in the genus *Propionibacterium* such probes should be possible. The probes could also be used for the quantitative detection of *Propionibacterium* species by hybridization.

Both for conjugation and electroporation experiments, more conditions, strains and plasmids must be tested. Especially a suitable selectable marker has to be found, preferably originating from the classical propionibacteria or other dairy relevant genera. Prior to conjugation experiments, electroporation conditions have to be established and a suitable plasmid must be selected and introduced into propionibacteria. This plasmid could then further be used in conjugation experiments.

Plasmid pLME108 should be further investigated and the introduction of a selectable marker into this plasmid is necessary for further electroporation experiments. Preferentially this selectable marker should "work" in propionibacteria as well as in *E. coli*.

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