Lait (1999) 79, 71-78

Original article

Classification of propionic acid bacteria and approaches to applied genetics

Léo Meile*, Gottfried Dasen, Susanne Miescher, Mélanie Stierli, Michael Teuber

Laboratory of Food Microbiology, Institute of Food Science, ETH Zürich, ETH-Zentrum, CH-8092 Zurich, Switzerland

Abstract — Strains of the genus *Propionibacterium,* which were recently classified within the new class *Actinobacteria,* are grouped into cutaneous (medical) or dairy (classical) propionic acid bacteria. 16S rDNA sequences of all *Propionibacterium* type strains and some new isolates were completed. A phylogenetic tree was constructed and current trees reexamined. The classical species *P. thoenii, P. jensenii* and *P. acidipropionici* were clustered in a group distinct from both the closely related clusters of *P. freudenreichii* and *P. cyclohexanicum.* Within the cutaneous species, three groups were clustered: *P. granulosum* forms a distinct cluster between the classical groups and a group containing *P. avidum, P. acnes* and *P. propionicum,* whereas *P. lymphophilum* is placed more distantly from any group in this tree. In order to differentiate *Propionibacterium* isolates from other food-isolates a rapid multiplex-PCR (MPCR) method based on 16S rRNA-targeted oligonucleotides and particularly on a 16S rDNA-motif which turned out to be specific for the genus *Propionibacterium* was developed. The MPCR-amplification could be performed within 1 d and reached detection limits of 10^3 colony forming units or 35 pg of DNA. The MPCR-method was used in various *Propionibacterium* screening studies. In the course of screening antifungal and antibacterial activities of propionic acid bacteria, a *Propionibacterium jensenii* strain containing a cryptic 7-kb plasmid was identified. This strain produced the bacteriocin Propionicin SM1. Purification of Propionicin SM1 and cloning of its genetic locus is currently under way. © Inra/Elsevier, Paris.

propionibacteria / classification / PCR-identification / 16S rDNA / genetic

Résumé — Classification des bactéries propioniques et approches de génétique appliquée. Les souches du genre *Propionibacterium* qui ont été récemment classées dans la nouvelle classe *Actinobacteria* sont groupées, selon leur environnement, en bactéries propioniques cutanées (médicales) et laitières (classiques). Nous avons réalisé différentes séquences de l'ARNr 16S de toutes les souches du type *Propionibacterium,* dont certains nouvellement isolées. Nous avons construit un arbre phylogénétique et réexaminé les arbres existants. Les espèces classiques *P. thoenii, P. jensenii et P. acidipropionici* ont été réunis dans un *cluster* distinct des deux groupes *P. freudenreichii* et

---

Oral communication at the 2nd Symposium on Propionibacteria, Cork, Ireland, June 25–27, 1998.
* Correspondence and reprints. leo.meile@ilw.agrl.ethz.ch

P. cyclohexanicum respectivement. Dans les espèces cutanés, trois groupes ont été formés : *P. granulosum* forme un groupe distinct parmi les groupes classiques et une groupe contenant *P. avidum, P. acnes* et *P. propionicum,* tandis que *P. lymphophilum* se trouve le plus éloigné des autres groupes. Dans le but de différencier les *Propionibacterium* isolées d'autres isolats, nous avons développé un multiplex–PCR (MPCR) test rapide basé sur le ciblage du ARNr 16S oligonucleotide et particulièrement sur le motif ARNr 16S, lequel est apparu être spécifique pour le genre *Propionibacterium.* L'amplification MPCR pouvait être réalisée en 1 j et permettait une détection limite de 10^3 UFC ou 35 pg d'ADN. La méthode MPCR a été utilisée pour le *screening* de diverses souches de *Propionibacterium.* Le développement d'un système génétique dans les bactéries propioniques est demandé pour des manipulations biotechnologiques. Des souches de *Propionibacterium* isolées des sources alimentaires ont été *screenées* pour des plasmides et des marqueurs gènes sélectifs. Pendant ce *screening* des activités antifongiques et antimicrobiennes des bactéries propioniques, une souche de *Propionibacterium jensenii,* contenant un plasmide de 7 kb et produisant une bactériocine Propionicine SM1 a été sélectionnée. La purification de cette bactériocine et le clonage de son gène sont actuellement à l'étude. © Inra/Elsevier, Paris.

bactérie propionique / classification / identification-PCR / ARNr 16S / génétique

1. INTRODUCTION

Propionibacteria which belong to the newly described class *Actinobacteria* [18] are divided, according to their habitats, into two main groups, namely the classical and the cutaneous strains [4]. The role of classical strains in dairy products has led to in-depth studies of the physiology and biochemistry of these bacteria. To date the genetics of propionibacteria have not been studied extensively. The classical propionibacteria form a relatively homogenous group as deduced from different classification approaches such as chemotaxonomy [2] or ribotyping [16]. Since comparisons of 16S rRNA genes are reliable taxonomical tools for species differentiation [13], this approach was chosen to construct phylogenetic trees containing some of the species of both groups [3, 12]. The detection of strains by PCR and hybridization techniques has been described for cutaneous strains but not for the classical strains [9, 11].

The aim of our studies was to construct a phylogenetic tree containing all species of the genus *Propionibacterium* and to develop genus specific probes for *Propionibacterium* based on a fast PCR-system. Such a system could then be used to screen propionibacteria to develop tools (strains, plasmids, marker genes) for gene transfer systems necessary for biotechnological applications [8].

2. CLASSIFICATION

16S rDNA nucleotide sequences were not available for all *Propionibacterium* species; therefore, complete 16S rDNA sequences from several type strains were generated. The methodology was recently described elsewhere [5]. The 16S rDNA was obtained by a PCR-approach and sequenced as indicated schematically in *figure 1.* The phylogenetic position of each species (type strain) including the cutaneous strains was calculated by the maximum likelihood method based on the alignment of the corresponding *E. coli* rDNA sequence positions 8 to 1 540. The classical strains formed 2 clusters containing *P. acidipropionici/P. jensenii/P. thoenii* and *P. freudenreichii* subspecies/*P. cyclohexanicum,* respectively (*figure 2A*). *P. cyclohexanicum* is a new species which was recently isolated from orange juice [12]. The cutaneous species can be divided into three clusters:

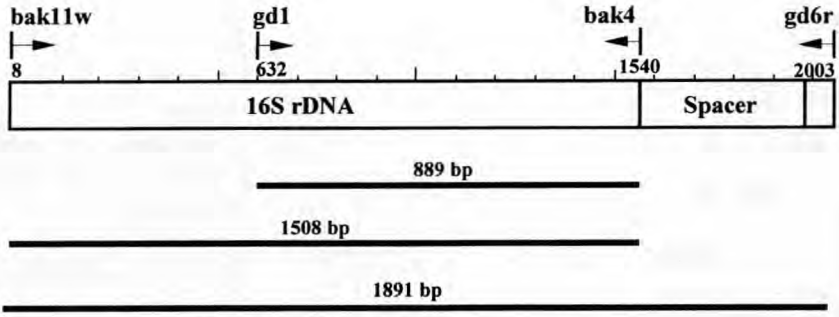

Figure 1. Schematic representation of the multiplex-PCR and sequencing strategy used in the study of *Propionibacterium* ribosomal DNA amplification-products. The positions of oligonucleotides bak11w, gd1, bak4 and gd6r [5] are indicated according to the corresponding *E. coli* numbering of the ribosomal operon. The length of amplified fragments is indicated for the example from *P. freudenreichii* subsp. *shermanii* DSM 4902 (Type strain, Acc. no. Y10819).

Figure 1. Représentation schématique de la multiplex–PCR et stratégie de séquençage utilisée dans l'étude des produits de l'amplification de l'ADN ribosomal. Les positions des oligonucléotides bak11w, gd1, bak4 et gd6r [5] sont indiquées selon leur correspondance avec l'opéron ribosomal d'*E. coli*. La longueur du fragment amplifié est indiquée pour l'exemple de *P. freudenreichii* subsp. *shermanii* DSM 4902 (souche type, Acc. no. Y10819).

P. acnes/P. avidum/P. propionicum are separated from *P. lymphophilum* and *P. granulosum*, respectively (*figure 2A*).

In prokaryotic organisms, the spacer region between the 16S rDNA and the 23S rDNA is usually more variable than the sequences of these two genes. Since it was possible to amplify the spacer region of propionibacteria and to perform nucleotide sequencing (*figure 1* and [5]), the use of the spacer sequences as a tool to roughly classify new isolates was investigated. The phylogenetic tree formed a grouping of the species which was similar to that obtained with the 16S rDNA approach, although the distances were quite different (*figure 2B*). However, all *Propionibacterium* isolates which had been classified by comparative 16S rDNA analysis to the species level before could be correctly grouped (*figure 2B*). Therefore, the spacer approach seems to be reliable for clustering of new *Propionibacterium* isolates, but did not reveal in each case the classification to species level as proposed by Rossi et al. [17].

3. RAPID IDENTIFICATION OF *PROPIONIBACTERIUM*

In order to clearly and rapidly identify propionibacteria, a multiplex-PCR detection system (MPCR) was developed and described recently in detail [5]. Briefly, this PCR-approach (schematically drawn in *figure 1*) is highly dependent on oligonucleotide gd1 (5'-TGCTTTCGAT ACGGGT TGAC) targeted to a 16S rDNA sequence which is unique for *Propionibacterium* sp. In the PCR-system, the primer gd1 in combination with oligonucleotide bak11w (5'-AGTTTGATCMTGGCTCAG) and bak4 (5'-AGGAGGTGATCCARCCGCA) should theoretically generate simultaneously 2 fragments of approximately 900 bp and 1 500 bp, respectively, with *Propionibacterium*-DNA as a template. However, under the PCR conditions selected, only the 900-bp fragment was amplified with *Propionibacterium* DNA as template (*figure 3*). Strains from 20 genera other than *Propionibacterium* generated the 1 500-bp fragment

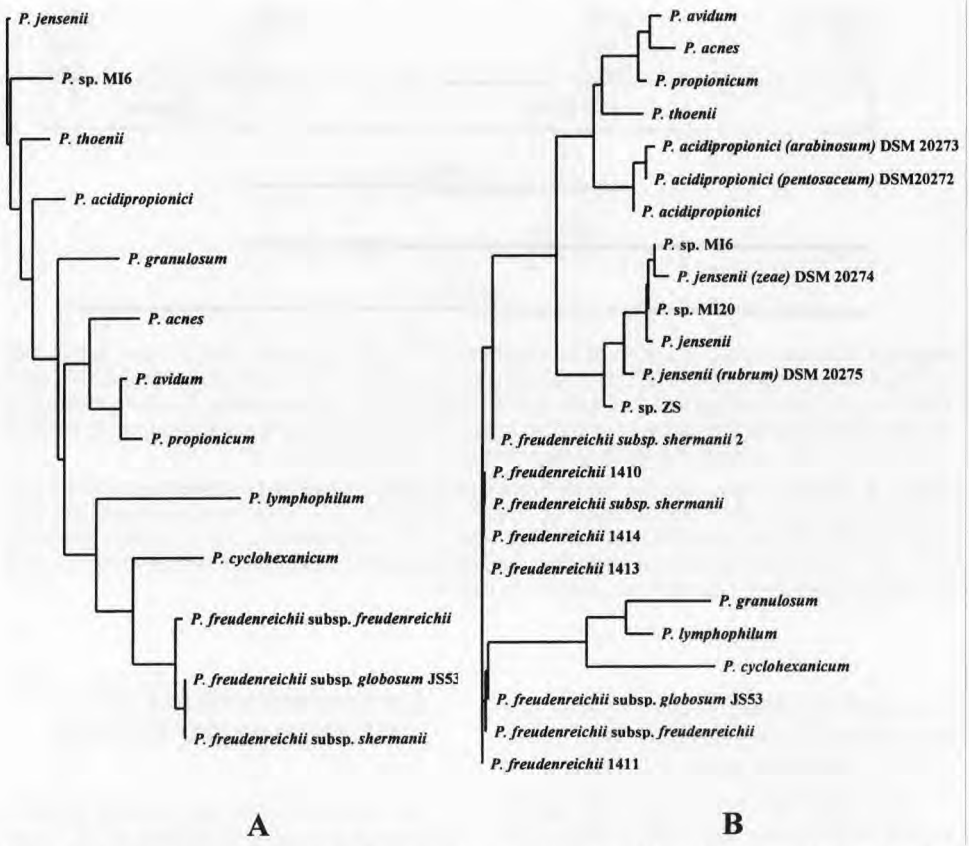

Figure 2. Phylogenetic relationship of propionibacteria based on 16S rDNA (**A**) and 16S-23S ribosomal spacer DNA sequences (**B**). Strains indicated without numbers indicate type-strains obtained from DSM culture collection. The trees were constructed by alignment of all positions in a maximum likelihood algorithm with molecular clock (PHYLIP-package). The bar indicates 10 nucleotide substitutions per 1 000 nucleotides.

Figure 2. Relation phylogénique des bactéries propioniques basée sur l'ADNr 16S (**A**) et de l'ADNr 16S-23S spacer-séquence (**B**). Les souches sans numéros représentent les souches types. Les arbres sont construit par alignement de toutes les positions selon la méthode de *maximum likelihood algorithm* (PHYLIP).

only. These genera contained strains of *Bifidobacterium*, *Corynebacterium*, *Microbacterium*, *Micrococcus*, *Mycobacterium*, *Brevibacterium*, *Streptomyces*, *Actinomyces*, *Arthrobacter* and *Nocardia* which all belong to the *Actinobacteria*-class (data not shown). Other strains giving the 'non-*Propionibacterium*'-MPCR-pattern belonged to the genera *Enteroccoccus*, *Leuconostoc*, *Lactococcus*, *Lactobacillus*, *Staphylococcus*, *Streptococcus*, *Bacillus*, *Clostridium*, *Escherichia* and *Acetobacter*. Representative data showing the 1 500-bp fragment are demonstrated for *Lactococcus lactis* in lane 21 (*figure 3*). This PCR-approach was useful to detect propionibacteria in food rapidly. It could be performed within 1 d and the detection limits were 10^3 colony forming units or 35 pg of DNA.

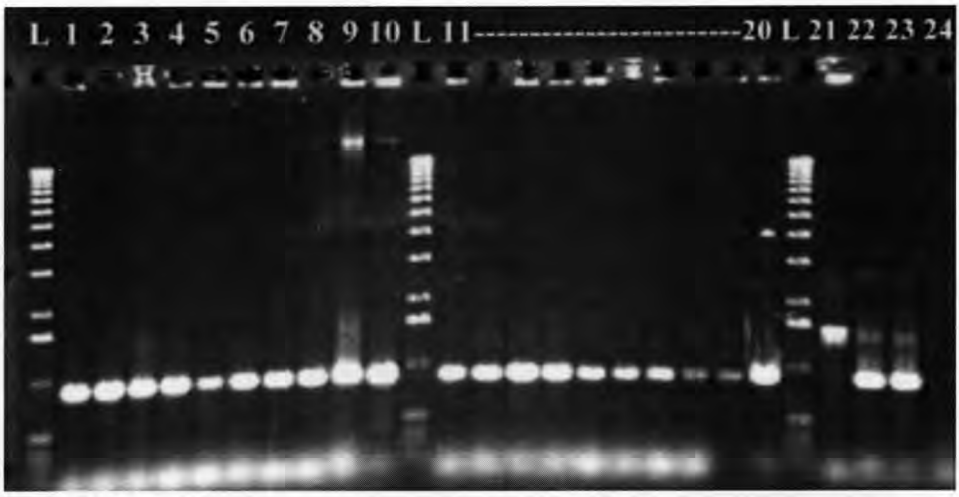

Figure 3. Amplification of a specific 900-bp fragment from *Propionibacterium* strains using the multiplex-PCR with primer bak11w, gd1 and bak4. The amplification conditions and the origin of the strains are described elsewhere [5]. The following strains were used: 1, *P. acidipropionici*; 2, *P. acidipropionici* (*arabinosum*); 3, *P. freudenreichii*; 4, *P. jensenii*; 5, *P. jensenii* (*pentosaceum*); 6, *P. jensenii* (*petersonii*); 7, *P. jensenii* (*rubrum*); 8, *P. shermanii* type strain; 9, *P. shermanii*; 10, *P. thoenii*; 11, *P. jensenii* (*zeae*); 12-17, *P. freudenreichii* FAM1409, 1410, 1411, 1412, 1413, 1414; 18, *P.* sp. DF1; 19, *P.* sp. DF2; 20 *P.* sp. JS53; 21, *Lactococcus lactis* subsp. *cremoris* AC1; 22, *P. shermanii* type strain; 22 *P. jensenii* type strain; 24, blank (no DNA); L, kilobase-ladder.

Figure 3. Amplification d'un fragment de 900 pb spécifique pour *Propionibacterium* en utilisant la multiplex–PCR avec primer bak11w, gd1 et bak4. Les conditions de l'amplification sont décrites par ailleurs [5]. Les souches suivantes ont été utilisées : 1, *P. acidipropionici* ; 2, *P. acidipropionici* (*arabinosum*) ; 3, *P. freudenreichii* ; 4, *P. jensenii* ; 5, *P. jensenii* (*pentosaceum*) ; 6, *P. jensenii* (*petersonii*) ; 7, *P. jensenii* (*rubrum*); 8, *P. shermanii* type strain ; 9, *P. shermanii* ; 10, *P. thoenii* ; 11, *P. jensenii* (*zeae*) ; 12–17, *P. freudenreichii* FAM1409, 1410, 1411, 1412, 1413, 1414 ; 18, *P.* sp. DF1 ; 19, *P.* sp. DF2 ; 20 *P.* sp. JS53 ; 21, *Lactococcus lactis* subsp. *cremoris* AC1 ; 22, *P. shermanii* type strain ; 22 *P. jensenii* type strain ; 24, aucune d'ADN ; L, marqueur de kilobases.

4. PLASMIDS AS GENETIC TOOLS FOR PROPIONIBACTERIA

In order to perform genetic exchange in *Propionibacterium*, several research groups have screened propionibacteria for plasmids suitable for vector construction and described their source, crosshybridization patterns, restriction maps and approximate size which ranged from 3 kb to 40 kb [6, 8, 15]. Potentially suitable *Propionibacterium* plasmids were analyzed for the occurrence of natural antibiotic resistance determinants (by hybridization experiments) and sequencing of DNA from these plasmids was initiated. In this way, information on the genes and the putative noncoding regions where selective marker genes could be integrated was obtained. Plasmid pLME108 (2.05 kb) from *P. freudenreichii* DF2 was sequenced and plasmid pLME106 from *P. freudenreichii* DF1 (7 kb) is currently being sequenced. Both plasmids contain *rep*-homologous sequences. In addition, pLME106 stringently crosshybridizes with pRGO1 (4.4 kb) from *P. acidipropionici* [15].

The conditions for electrotransformation of *Propionibacterium freudenreichii* cells

have been established by transfection with DNA from the *Propionibacterium* phage B22 [7]. Attempts to transform different propionibacteria with plasmids originating from other Gram-positive bacteria such as pAM120, pAM180 or pJIR751 containing tetracycline or erythromycin resistance genes as selectable marker failed. The capacity of theses plasmids to replicate in propionibacteria is unknown. Therefore, the construction of a cloning vector with a *Propionibacterium*-based replicon is required.

5. ANTIMICROBIAL AND ANTIFUNGAL ACTIVITIES OF PROPIONIBACTERIA

Propionibacteria are competitive inhibitors of other microorganisms due to the production of propionic acid and probably other antifungal or antibacterial substances [1, 8] whose production and inhibition mechanisms still have to be studied at the molecular level. The antibacterial agents from propionibacteria best characterized to date are the bacteriocins Propionicin PLG-1 [14] and Jenensin G [10]. The development of genetic tools should lead to the biotechnological application of the antibacterial and antifungal potential of propionibacteria.

The antimicrobial activities of Swiss dairy *Propionibacterium* isolates was screened using an agar spot method. More than 100 examples of their growth inhibitory activity against yeast and moulds and other indicator organisms from the genera *Lactococcus, Lactobacillus* or *Propionibacterium* and several Gram-negative genera was obtained. The yeasts and moulds used were isolates from milk products and were not taxonomically identified further. The *Propionibacterium* indicator organisms used contained all type strains from the DSM culture collection and isolates from our own collection whereas the lactic acid bacteria used included *Lactococcus lactis* subsp. *cremoris* AC1, *L. lactis* subsp. *lactis*, *Lactobacillus plantarum* DSM 20205, *Lb. casei* 160, *Lb. brevis* DSM 20054, *Lb. acidophilus* DSM 20079, *Lb. rhamnosus* DSM 20021, *Lb. delbrueckii* subsp. *bulgaricus* DSM 2008, *Lb. delbrueckii* subsp. *lactis* DSM 20072, *Lb. helveticus* DSM 20075 and *Leuconostoc mesenteroides* subsp. *cremoris* M7-1. The indicator organisms among Gram-positive bacteria were *Enterococcus faecalis* DS5, *Staphylococcus aureus* VF4, *Listeria inocua* L17, *Bacillus subtilis* 168, *Bacillus cereus* DSM31, *Micrococcus luteus* DSM 20030, *Brevibacterium iodinum* DSM 20626 and *Brevibacterium epidermis* DSM 20660, whereas Gram-negative examples were *Serratia liquefaciens* SG64, *Citrobacter freundii* SG84, *Klebsiella pneumoniae* SG89, *Enterococcus cloacae* SG95, *E. coli* SG63 and *E. coli* B.

The more prospective *Propionibacterium* strains are summarized in *figure 4*. *Propionibacterium jensenii* strain DF1 selectively inhibited the growth of both *P. jensenii* strains DSM 20274 and DSM 20535, 15 out of 24 yeasts and 3 out of 4 moulds (*figure 4*). The inhibitory mechanism in strain DF1 which contains plasmid pLME106 (see above) was investigated. An inhibitory activity was assigned to an excreted protein which could be purified from liquid cultures by classical protein purification procedures. The purified and highly stable protein maintained its inhibitory pattern against both propionibacteria, but had lost its inhibitory activity against the yeasts and moulds. This inhibitory protein was named Propionicin SM1. The cloning of the genes for this protein is under investigation.

The level of propionic and acetic acid in the growth medium of DF1 and 11 other yeast-inhibitory *Propionibacterium* strains was similar to that in strains which did not inhibit yeasts. This finding suggests that an agent other than Propionicin SM1or propionate and acetate is inhibiting the yeasts and moulds.

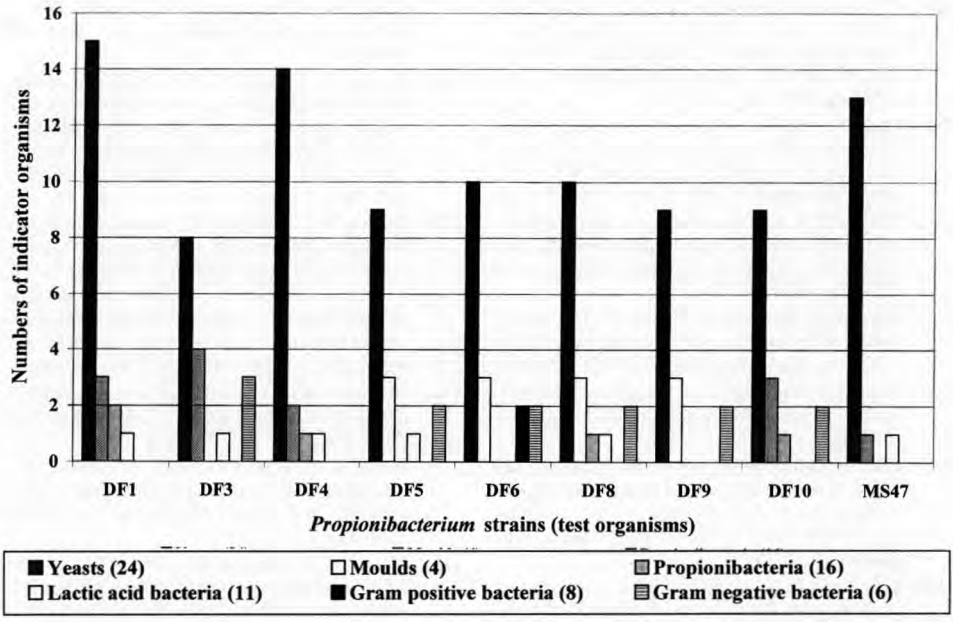

Figure 4. Growth inhibitory activity of 9 different *Propionibacterium* strains against different indicator organisms in the agar spot assay. Propionibacteria were grown for 7 d on sodium lactate-agar-plates which were then overlayed by soft agar containing the indicator organisms in the appropriate medium.

Figure 4. Activité de l'inhibition de la croissance par 9 souches différentes de *Propionibacterium* contre différents microorganismes comme indicateurs. Les bactéries propioniques ont été cultivées 7 j sur un gel d'agar de lactate de sodium recouvert d'une couche d'agar contenant les microorganismes indicateurs avec le milieu approprié.

6. CONCLUSION

To reduce time for the detection of the genus *Propionibacterium* and to verify proper identification and classification within phylogenetic trees, a rapid method based on a multiplex-PCR (MPCR) approach to differentiate the genus *Propionibacterium* from other genera was developed. This method will be helpful in further screening programs for propionibacteria which contain the potential tools for the development of gene transfer systems or which contain biotechnologically interesting proteins like proteinases or antibacterial and antifungal agents.

ACKNOWLEDGEMENT

This work is supported by Grant 5002-041663/1 from the Swiss National Science foundation in the frame of the SPP Biotechnology.

REFERENCES

[1] Al-Zoreky N., Ayres J.W., Sandin E.W., Antimcrobial activity of Microgard™ against food spoilage and pathogenic microorganism, J. Dairy Sci. 74 (1991) 758–763.

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

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

[4] Cummins C.S., Johnson J.L., The genus *Propionibacterium*, in: Balows A., Trüper H.G., Dworkin M., Harder W., Schleifer K.-H. (Eds.), The Prokaryotes, 2nd ed., Springer, New York, 1992, pp. 834–849.

[5] Dasen G., Smutny J., Teuber M., Meile L., Classification and identification of propionibacteria based on ribosomal RNA genes and PCR, Syst. Appl. Microbiol. 21 (1998) 251–259.

[6] Fessler D.S., Characterisation of propionibacteria in Swiss raw milk by biochemical and molecular-biological methods, Ph.D. thesis, ETH Zürich No. 12328, 1997.

[7] Gautier M., Rouault A., Lemée R., Electroporation of *Propionibacterium freudenreichii* TL 110, Lett. Appl. Microbiol. 20 (1995) 125–129.

[8] Glatz B.A., The classical propionibacteria: Their past, present and future as industrial organisms, ASM News 58 (1992) 197–201.

[9] Greisen K., Loeffelholz M., Purohit A., Leong D., PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid, J. Clin. Microbiol. 32 (1994) 335–351.

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

[11] Hykin P.G., Tobal K., McIntyre G., Matheson M.M., Towler H.M.A., Lightman S.L., The diagnosis of delayed post-operative endolphthalmitis by polymerase chain reaction of bacterial DNA in vitreous samples, J. Med. Microbiol. 40 (1994) 408–415.

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

[13] Ludwig W., Schleifer K.-H., Bacterial phylogeny based on 16S and 23S rRNA sequence analysis, FEMS Microbiol. Rev. 15 (1994) 155–173.

[13] Lyon W.J., Glatz B.A., Isolation and purification of Propionicin PLG-1, a bacteriocin produced by a strain of *Propionibacterium thoeni*, Appl. Environ. Microbiol. 59 (1993) 83–88.

[14] Rehberger T.G., Glatz B.A., Characterization of *Propionibacterium* plasmids, Appl. Environ. Microbiol. 56 (1990) 864–871.

[15] Riedel K.-H., Britz T.J., Justification of the classical *Propionibacterium* species concept by ribotyping, Syst. Appl. Microbiol. 19 (1996) 370–380.

[16] Rossi F., Sammartino M., Torriani S., 16S-23S ribosomal spacer polymorphism in dairy propionibacteria, Biotechnol. Techn. 11 (1997) 159–161.

[17] Stackebrandt E., Rainey F.A., Ward-Rainey N.L., Proposal for a new hierarchic classification system *Actinobacteria* classis nov., Int. J. Syst. Bacteriol. 47 (1997) 479–491.