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Original article

Rapid identification of dairy *Propionibacterium* species by restriction analysis of the insertion within the 23S rRNA gene

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Abstract — An amplified sequence of approximately 350 bases from the 23S rRNA gene of each bacteria was used to identify 37 strains of dairy propionibacteria. After generation of the fragment by PCR (polymerase chain reaction), it was cut with the restriction endonuclease *Msp*I. The resulting electrophoretic pattern showed a distinct profile for each of the *Propionibacterium* species. Purification of DNA was not necessary and the analyses could also be performed with crude bacterial extract. A collection of anaerobic bacteria commonly found in milk and milk products was also submitted to the analysis. None yielded the same pattern as propionibacteria. The analysis of the propionibacteria was compared to that performed by electrophoresis of the soluble cell-free protein extracts and that of classical microbiological tests. Our method gave identical results and was less time consuming (results could be obtained within a single day and were also easier to read) than both the classical tests and protein electrophoresis. © Inra/Elsevier, Paris.

***Propionibacterium* / identification / PCR / restriction analysis / 23S rRNA**

Résumé — Identification rapide des espèces de bactéries propioniques laitières par analyse de restriction de l'insertion dans le gène de l'ARNr 23S. Une séquence amplifiée d'environ 350 bases du gène de l'ARNr 23S de chaque bactérie a été utilisée pour l'identification de 37 souches de bactéries propioniques laitières. Le fragment a été amplifié par PCR (*polymerase chain reaction*), puis coupé par l'enzyme de restriction *Msp*I. Le profil électrophorétique obtenu a été différent pour chaque espèce de bactérie propionique. Les analyses ont aussi été réalisées avec des extraits bruts de bactéries, sans purification préalable de l'ADN. Toute une série de bactéries anaérobies habituellement présentes dans le lait a

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également été soumise à cette analyse, sans que les profils obtenus aient correspondu à ceux des bactéries propioniques. Cette technique d'analyse a été comparée à celle de l'électrophorèse des extraits solubles des protéines et à celle des tests classiques de la microbiologie. Nos résultats étaient identiques à ceux des deux autres méthodes, mais ils ont été obtenus plus rapidement (en un seul jour) et sont de lecture plus facile. © Inra/Elsevier, Paris.

***Propionibacterium* / identification / PCR / analyse de restriction / ARNr 23S**

1. INTRODUCTION

Propionibacteria were first isolated and described in 1909 by Orla-Jensen [16], and are today separated into eight species. Four were isolated from human skin: *P. acnes*, *P. avidum*, *P. granulosum*, and *P. lymphophilum*. The other four species consist of the dairy propionibacteria: *P. freudenreichii*, *P. jensenii*, *P. thoenii*, and *P. acidipropionici*. Propionibacteria are Gram-positive, non-motile, asporogenous, anaerobic to aerotolerant and generally catalase-positive pleomorphic rods [10]. Dairy propionibacteria are used in the manufacture of Emmental cheese with starters where they ferment lactic acid to propionic, acetic acid and CO_2, which leads to the typical eye formation and flavor of this cheese. They can also cause cheese defects such as brown spots [5] and splitting [13, 15] in Emmental and in other types of Swiss cheese.

P. jensenii, *P. thoenii* and *P. acidipropionici* form a phylogenetical cluster, whereas *P. freudenreichii* is phylogenetically more remote [9].The differentiation of the four dairy species, especially between *P. thoenii* and *P. jensenii* by classical bacteriological methods is apparently difficult. New methods have been proposed to facilitate their differentiation. Baer [2] and Baer and Ryba [3, 4] proposed identification systems based on protein electrophoresis, immunoblotting and serological studies. Riedel and Britz [19] and de Carvalho et al. [12] used ribotyping to differentiate propionibacteria based on the 16S and the 23S rRNA. Riedel et al. [18] used restriction analysis of the 16S rRNA gene. Unfortunately, all of the methods are very time consuming.

Gram-positive bacteria with a high DNA G+C content are characterized by an insertion within their 23S rRNA genes [20]. Propionibacteria have a DNA G+C content of 65–67 mol% [10] and the presence of the insertion was clearly confirmed [20]. In this paper a rapid and efficient method to classify dairy propionibacteria into their four species is described. It is based on the restriction analysis of a fragment of approximately 350 bases centered around the insertion.

2. MATERIALS AND METHODS

2.1. Bacterial strains and culture conditions

All 37 strains of propionibacteria were cultured anaerobically in YEL and on YELA (yeast extract lactate agar) for 5 to 7 days at 30 °C. YELA consists of 24 mL/L sodium lactate (50% v/v), 30 g/L casein peptone, 30 g/L yeast extract, and 15 g/L agar; pH is adjusted to 6.8.

The six strains of *Clostridium* were cultured in RCM broth [1] for 3 days at 37 °C. Enterococci strains were cultured for 2 days at 37 °C in M17 [1] or on M17 agar with 0.05 mol/L lactose. *Lc. lactis* strains were cultured in the

same media as enterococci, but anaerobically for 2 days at 30 °C.

S. thermophilus was grown anaerobically for 2 days at 37 °C in LS5 [8] or on LS5 agar with 0.05 mol/L lactose.

L. delbrueckii was cultured anaerobically for 2 days at 37 °C in MRS [1] or on MRS agar with 0.05 mol/L lactose.

L. casei strains were grown anaerobically for 2 days at 30 °C in MRS with 0.05 mol/L lactose and on FH-agar [14]. *L. rhamnosus, L. zeae* and *L. plantarum* were cultured in the same media as *L. casei.*

An overall view of the analyzed strains of bacteria is given in *tables I* and *II.*

2.2. Preparation of crude DNA extracts

2.2.1. From liquid cultures

400 µL of a liquid bacterial culture were centrifuged for 10 min at 9 990 *g* in a Hettich Mikroliter centrifuge under sterile conditions. The sediment was suspended in 100 µL sterile distilled water. The solution was boiled for 5 min and then centrifuged for 15 min at 9 990 *g*. 2 µL of the supernatant were used for the PCR-reaction.

2.2.2. From colonies

Bacterial material (one colony) was suspended in 100 µL sterile distilled water. The solution was boiled for 5 min and then centrifuged for 15 min at 9990 *g* ; 2 µL of the supernatant were used for the PCR-reaction.

2.3. Polymerase chain reaction and restriction analysis

Taq polymerase, PCR buffer and dNTP mix were purchased from Boehringer Mannheim and the primers from MWG-Biotech. The PCR-reaction was performed in 100 µL containing 10 µL PCR buffer (10 × concentrated), 0.2 mmol/L dNTP mix (10 mmol/L each), 0.1 µmol/L each of primers 5′-MADGCGTAGNCGAWGG-3′ and 5′ GTGWCGGTTTNBGGTA-3′ [20], 2.5 u Taq DNA polymerase and 2 µL crude bacterial extract.

PCR amplification was performed on a thermo-cycler from Inotech as follows: 25 cycles of 1 min at 95 °C, 2 min at 52 °C, and 3 min at 72 °C with a final step of 5 min at 72 °C.

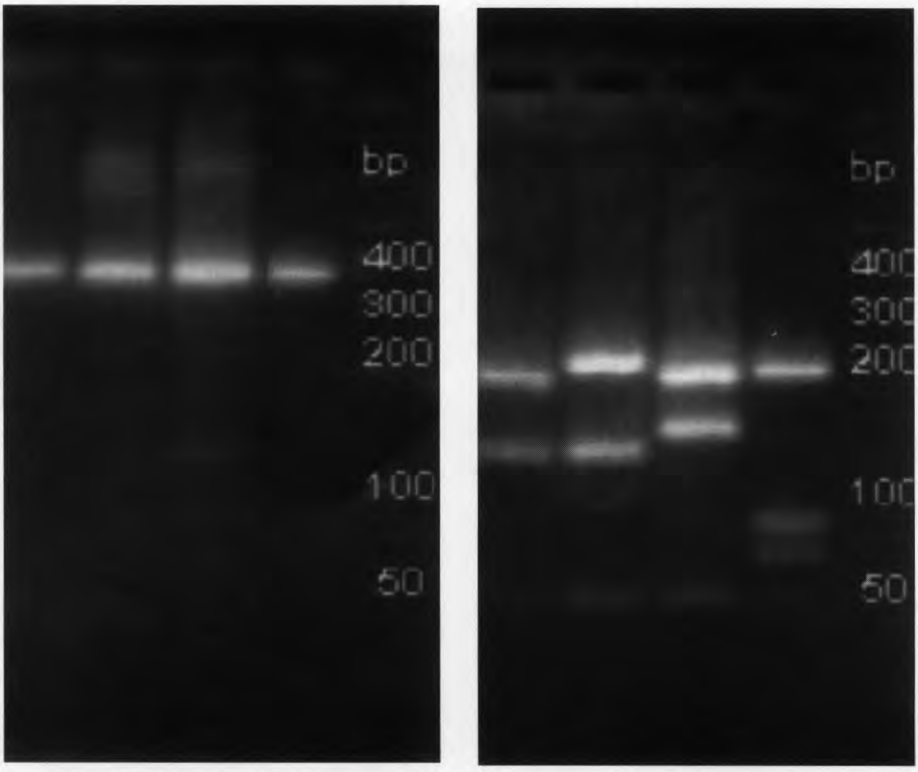
1 µL *Msp*I (10 u/µL, Boehringer Mannheim) and 1 µL BSA (bovine serum albumin, 20 mg/mL, Boehringer Mannheim) were added to 20 µL of the PCR amplification product and incubated for 2 h at 37 °C. Both restricted and untreated PCR amplification products were separated on a 4% agarose gel (NuSieve 3:1 agarose, FMC) and visualized under UV light in the presence of ethidium bromide buffer (10 mmol/L sodium borate, 1 mg/L ethidium bromide, pH 8.0). DNA size standard was 50–2000 bp ladder from BioRad with bands of 2000, 1500, 1000, 700, 500, 400, 300, 200, 100 and 50 bp.

3. RESULTS AND DISCUSSION

PCR with propionibacteria DNA and the chosen primers produced a single amplification product of approximately 350 bases confirming the results previously reported by Roller et al. [20]. Only Gram-positive bacteria with a high G + C content possess a characteristic insertion of about 100 bases within their 23S rRNA genes [20].

Four endonucleases, which were compatible with PCR buffer and a high G+C content, were tested: *Cfo*I and *Mvn*I digested the amplified fragment only partially (results not shown), whereas *Msp*I and *Hpa*II allowed differentiation of the four propionibacteria species. *Msp*I and *Hpa*II generated the same patterns and *Msp*I was chosen for this study.

The patterns generated using the restriction endonuclease *Msp*I were unique for each dairy *Propionibacterium* species. *Figure 1a* shows the amplified fragment of approximately 350 bases of the four propionibacteria type strains and *figure 1b* the patterns generated after restriction of this fragment with *Msp*I. Comparison of the reference strains of propionibacteria in *figure 1b* brings out clearly the diffe-



a. **b.**

Figure 1. a. Amplified fragments of the four *Propionibacterium* type strains. From left to right: 1) ATCC 25562 *P. acidipropionici;* 2) ATCC 4874 *P. thoenii;* 3) ATCC 4868 *P. jensenii;* 4) ATCC 6207 *P. freudenreichii* ssp. *freudenreichii.* **b.** Patterns generated after restriction with *Msp*I of the amplified fragments of the four *Propionibacterium* type strains. From left to right: 1) ATCC 25562 *P. acidipropionici;* 2) ATCC 4874 *P. thoenii;* 3) ATCC 4868 *P. jensenii;* 4) ATCC 6207 *P. freudenreichii* ssp. *freudenreichii;* 5) 50–2000 bp ladder.

Figure 1. a. Fragments amplifiés de quatre souches types de *Propionibacterium.* De gauche à droite : 1) ATCC 25562 P. acidipropionici; 2) ATCC 4874 *P. thoenii*; 3) ATCC 4868 *P. jensenii*; 4) ATCC 6207 *P. freudenreichii* ssp. *freudenreichii.* **b.** Profils obtenus après restriction avec Msp. I du fragment amplifié de quatre souches types de *Propionibacterium.* De gauche à droite : 1) ATCC 25562 *P. acidipropionici*; 2) ATCC 4874 *P. thoenii*; 3) ATCC 4868 *P. jensenii*; 4) ATCC 6207 *P. freudenreichii* ssp. *freudenreichii*; 5) 50–2000 bp ladder.

rences between the patterns of each species.

Restriction analysis of the fragment produced by *P. freudenreichii* gave three bands of approximately 195, 60 and 55 bp (*figure 2*). *P. jensenii* produced two bands of approximately 195 and 135 bp (*figure 3*), *P. thoenii* two bands of approximately 205 and 105 bp (*figure 4*), and *P. acidipropionici* two bands of approximately 190 and 120 bp (*figure 5*). Strains of all species produced an additional band of approximately 40 bp (*figures 1b–5*).

The strains from each species produced the same restriction profile (*figures 2–5*). Dairy propionibacteria could clearly

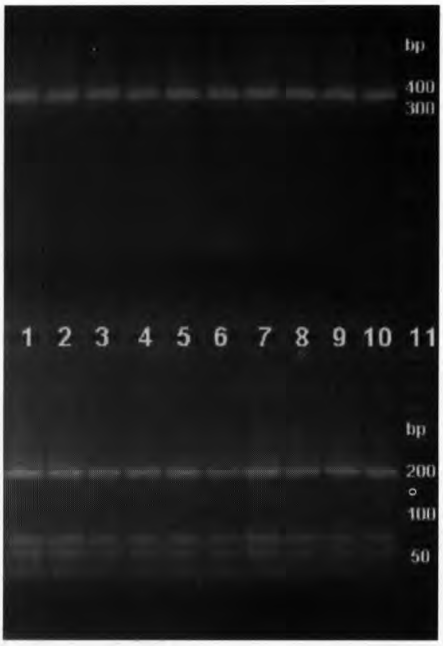


Figure 2. PCR analysis of *P. freudenreichii*, amplified fragments at the top, restriction patterns at the bottom. From left to right: 1) ATCC 6207; 2) ATCC 9614; 3) DSM 20270; 4) P 1409; 5) P 1410; 6) P 1411; 7) P 1412; 8) P 1413; 9) P 1414; 10) P 111; 11) 50–2000 bp ladder.

Figure 2. Analyse de *P. freudenreichii* par PCR; en haut, fragments amplifiés; en bas, fragments de restriction. De gauche à droite : 1) ATCC 6207 ; 2) ATCC 9614 ; 3) DSM 20270 ; 4) P 1409 ; 5) P 1410 ; 6) P 1411 ; 7) P 1412 ; 8) P 1413 ; 9) P 1414 ; 10) P 111 ; 11) 50–2000 bp ladder.

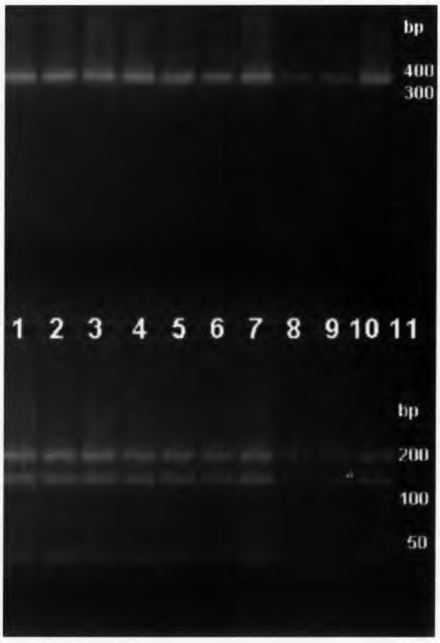


Figure 3. PCR analysis of *P. jensenii,* amplified fragments at the top, restriction patterns at the bottom. From left to right: 1) ATCC 4868; 2) DSM 20274; 3) DSM 20275; 4) DSM 20278; 5) DSM 20279; 6) AG 71.1; 7) BE28.1; 8) FR60.1; 9) SG19.1; 10) ZS44.1; 11) 50–2000 bp ladder.

Figure 3. Analyse de *P. jensenii* par PCR. En haut; fragments amplifiés ; en bas, fragments de restriction. De gauche à droite : 1) ATCC 4868 ; 2) DSM 20274 ; 3) DSM 20275 ; 4) DSM 20278 ; 5) DSM 20279 ; 6) AG 71.1 ; 7) BE28.1 ; 8) FR60.1 ; 9) SG19.1 ; 10) ZS44.1; 11) 50–2000 bp ladder.

be classified into the four existing species *P. freudenreichii, P. jensenii, P. thoenii*, and *P acidipropionici*. In order to facilitate comparison, it is recommended to use reference strains of every species, or at least of *P. jensenii, P. thoenii*, and *P. acidipropionici*, on each gel, since there is only a slight difference between *P. acidipropionici* and *P. thoenii*. The results in *figures 1–5* were obtained with crude bacterial extracts from colonies. Identical results were obtained from extracts from liquid culture and with purified DNA (results not shown).

Table I. Identification of propionibacteria isolates with different methods.
Tableau I. Identification d'isolats de bactéries propioniques par différentes méthodes.

Number	Strain	Source	Identification		
			Biochemical[1]	Proteins	PCR analysis
ATCC[2] 6207	*P. freudenreichii* ssp. *freudenreichii* T[3]	DSM[4]	–	*P. freudenreichii*	*P. freudenreichii*
ATCC 9614	*P. freudenreichii* ssp. *shermanii* T	DSM	–	*P. freudenreichii*	*P. freudenreichii*
DSM 20270	*P. freudenreichii* ssp. *shermanii*	DSM	–	*P. freudenreichii*	*P. freudenreichii*
P 1409	*P. freudenreichii* ssp. *shermanii*	FAM[5]	–	*P. freudenreichii*	*P. freudenreichii*
P 1410	*P. freudenreichii* ssp. *shermanii*	FAM	–	*P. freudenreichii*	*P. freudenreichii*
P 1411	*P. freudenreichii* ssp. *shermanii*	FAM	–	*P. freudenreichii*	*P. freudenreichii*
P 1412	*P. freudenreichii* ssp. *shermanii*	FAM	–	*P. freudenreichii*	*P. freudenreichii*
P 1413	*P. freudenreichii* ssp. *shermanii*	FAM	–	*P. freudenreichii*	*P. freudenreichii*
P 1414	*P. freudenreichii* ssp. *shermanii*	FAM	–	*P. freudenreichii*	*P. freudenreichii*
P 111	*P. freudenreichii* ssp. *shermanii*	FAM	*P. freudenreichii*	*P. freudenreichii*	*P. freudenreichii*
ATCC 4868	*P. jensenii* T	DSM	–	*P. jensenii*	*P. jensenii*
DSM 20274	*P. jensenii (zeae)*	DSM	–	*P. jensenii*	*P. jensenii*
DSM 20275	*P. thoenii (rubrum)*	DSM	–	*P. jensenii*	*P. jensenii*
DSM 20278	*P. jensenii*	DSM	–	*P. jensenii*	*P. jensenii*
DSM 20279	*P. jensenii (peterssonii)*	DSM	–	*P. jensenii*	*P. jensenii*
AG 71.1	*P. isolate*	milk	*P. jensenii*	*P. jensenii*	*P. jensenii*
BE 28.1	*P. isolate*	milk	*P. thoenii*	*P. jensenii*	*P. jensenii*
FR 60.1	*P. isolate*	milk	*P. jensenii*	*P. jensenii*	*P. jensenii*
SG 19.1	*P. isolate*	milk	*P. jensenii*	*P. jensenii*	*P. jensenii*
ZS 44.1	*P. isolate*	milk	*P. jensenii*	*P. jensenii*	*P. jensenii*

Table I. Identification of propionibacteria isolates with different methods.
Tableau I. Identification d'isolats de bactéries propioniques par différentes méthodes.

Number	Strain	Source	Identification		
			Biochemical[1]	Proteins	PCR analysis
ATCC 4874	*P. thoenii* T	DSM	–	*P. thoenii*	*P. thoenii*
DSM 20277	*P. thoenii*	DSM	–	*P. thoenii*	*P. thoenii*
AG 28.1	*P. isolate*	milk	*P. thoenii*	*P. thoenii*	*P. thoenii*
NW 36.1	*P. isolate*	milk	*P. thoenii*	*P. thoenii*	*P. thoenii*
ZS 22.1	*P. isolate*	milk	*P. thoenii*	*P. thoenii*	*P. thoenii*
106.2	*P. isolate*	milk	*P. thoenii*	*P. thoenii*	*P. thoenii*
204.1	*P. isolate*	milk	*P. jensenii*	*P. thoenii*	*P. thoenii*
304.2	*P. isolate*	milk	*P. thoenii*	*P. thoenii*	*P. thoenii*
ATCC 25562	*P. acidipropionici* T	DSM	–	*P. acidipropionici*	*P. acidipropionici*
DSM 20272	*P. acidipropionici (pentosaceum)*	DSM	–	*P. acidipropionici*	*P. acidipropionici*
DSM 20273	*P. acidipropionici (arabinosum)*	DSM	–	*P. acidipropionici*	*P. acidipropionici*
AG 50.1	*P. isolate*	milk	*P. acidipropionici*	*P. acidipropionici*	*P. acidipropionici*
AG 66.1	*P. isolate*	milk	*P. acidipropionici*	*P. acidipropionici*	*P. acidipropionici*
BE 11.1	*P. isolate*	milk	*P. acidipropionici*	*P. acidipropionici*	*P. acidipropionici*
FR 55.2	*P. isolate*	milk	*P. acidipropionici*	not identified	*P. acidipropionici*
TG 17.2	*P. isolate*	milk	*P. acidipropionici*	*P. acidipropionici*	*P. acidipropionici*
ZS 44.2	*P. isolate*	milk	*P. acidipropionici*	*P. acidipropionici*	*P. acidipropionici*

[1]According to fermentation pattern of carbohydrates by Cummins and Johnson [10]. [2]American Type Culture Collection, Rockville, MD. [3]Type strain. [4]Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. [5]Collection of Microorganisms of the Federal Dairy Research Institute, Switzerland.

[1]Selon la fermentation des hydrates de carbone, d'après Cummins et Johnson [10]. [2]American Type Culture Collection, Rockville, MD. [3]Souche type. [4]Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. [5]Collection de microorganismes du Federal Dairy Research Institute, Suisse.

Table II. Bacterial strains (except propionibacteria) analyzed in this study.
Tableau II. Souches bactériennes (autres que les bactéries propioniques) analysées dans cette étude.

Number	Strain and biochemical identification[1]	Source
2492	*L. delbrueckii* ssp. *bulgaricus* T[2]	FAMb[3]
877	*L. delbrueckii* ssp. *lactis* T	FAMb
144	*L. delbrueckii* ssp. *lactis*	FAMb
145	*L. delbrueckii* ssp. *lactis*	FAMb
169	*L. delbrueckii* ssp. *lactis*	FAMb
170	*L. delbrueckii* ssp. *lactis*	FAMb
278	*L. delbrueckii* ssp. *lactis*	FAMb
286	*L. delbrueckii* ssp. *lactis*	FAMb
933	*L. delbrueckii* ssp. *lactis*	FAMb
1050	*L. delbrueckii* ssp. *lactis*	FAMb
1051	*L. delbrueckii* ssp. *lactis*	FAMb
1052	*L. delbrueckii* ssp. *lactis*	FAMb
1086	*L. rhamnosus* T	FAMb
1211	*L. rhamnosus*	FAMb
1212	*L. rhamnosus*	FAMb
1213	*L. rhamnosus*	FAMb
1214	*L. rhamnosus*	FAMb
1215	*L. rhamnosus*	FAMb
1216	*L. rhamnosus*	FAMb
1217	*L. rhamnosus*	FAMb
1218	*L. rhamnosus*	FAMb
1219	*L. rhamnosus*	FAMb
1220/23	*L. rhamnosus*	FAMb
1116	*L. casei* T	FAMb
1227	*L. casei*	FAMb
1228	*L. casei*	FAMb
1229	*L. casei*	FAMb
1087	*L. plantarum* T	FAMb
2051	*L. plantarum*	FAMb
2053	*L. plantarum*	FAMb
2057	*L. plantarum*	FAMb
2062	*L. plantarum*	FAMb
2063	*L. plantarum*	FAMb
2064	*L. plantarum*	FAMb
2065	*L. plantarum*	FAMb
2066	*L. plantarum*	FAMb
2069	*L. plantarum*	FAMb
2071	*L. plantarum*	FAMb
2075	*L. plantarum*	FAMb
2076	*L. plantarum*	FAMb
1084	*L. zeae* T	FAMb

Table II. Bacterial strains (except propionibacteria) analyzed in this study.
Tableau II. Souches bactériennes (autres que les bactéries propioniques) analysées dans cette étude.

Number	Strain and biochemical identification[1]	Source
932	*S. thermophilus* T	FAMb
879	*Enterococcus faecalis* T	FAMb
2645	*Enterococcus faecium* T	FAMb
3271	*Enterococcus* sp.	FAMb
3272	*Enterococcus* sp.	FAMb
3273	*Enterococcus* sp.	FAMb
3274	*Enterococcus* sp.	FAMb
3275	*Enterococcus* sp.	FAMb
3276	*Enterococcus* sp.	FAMb
3277	*Enterococcus* sp.	FAMb
2493	*Lactococcus lactis* T	FAMb
3009	*Lactococcus lactis*	FAMb
3010	*Lactococcus lactis*	FAMb
3011	*Lactococcus lactis*	FAMb
3013	*Lactococcus lactis*	FAMb
3018	*Lactococcus lactis*	FAMb
3233	*Lactococcus lactis*	FAMb
552	*C. butyricum* T	DSM[4]
1322	*C. butyricum*	FAM[5]
795	*C. sporogenes* T	DSM
1755	*C. sporogenes* T	FAM
2637	*C. tyrobutyricum* T	DSM
608	*C. tyrobutyricum*	CNRZ[6]

[1]D'après Cummins et Johnson [10]. [2]Souche type. [3]Biochimie, Federal Dairy Research Institute, Suisse. [4]Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. [5]Collection de microorganismes du Federal Dairy Research Institute, Suisse. [6]Centre national de recherches zootechniques, Jouy-en-Josas, France.

[1]According to Cummins and Johnson [10]. [2]Type strain. [3]Biochemistry, Federal Dairy Research Institute, Switzerland. [4]Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. [5]Collection of Microorganisms of the Federal Dairy Research Institute, Switzerland. [6]Centre national de recherches zootechniques, Jouy-en-Josas, France.

The classification of propionibacteria strains with our method is in agreement with that obtained from protein electrophoresis patterns (*table I*). Strain FR 55.2, which could not be clearly identified by protein electrophoresis, was classified as *P. acidipropionici* by restriction analysis as well as by biochemical methods.

For *P. freudenreichii* and *P. acidipropionici* the results obtained with PCR analysis are also in agreement with those obtained by classical biochemical methods. For *P. jensenii* the correspondence between the two methods is 90% and for *P. thoenii* 87.5% (*table I*). However, it is known, that the identification of dairy *Propionibacterium* species with classical

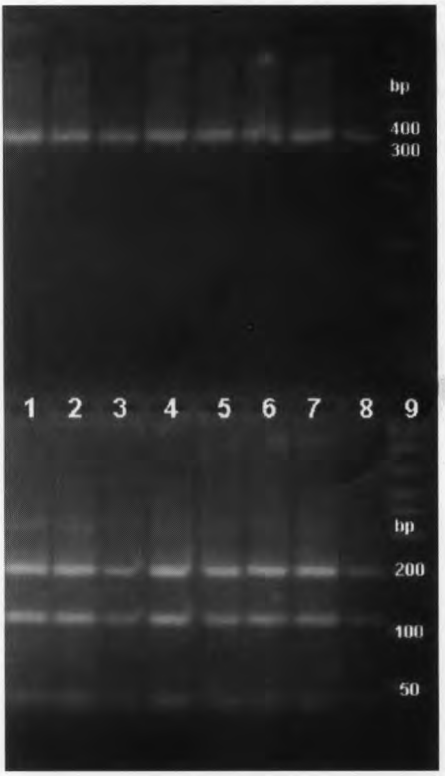


Figure 4. PCR analysis of *P. thoenii,* amplified fragments at the top, restriction patterns at the bottom. From left to right: 1) ATCC 4874; 2) DSM 20277; 3) AG 28.1; 4) NW 36.1; 5) ZS 22.1; 6) 106.2; 7) 204.1; 8) 304.2; 9) 50–2000 bp ladder.

Figure 4. Analyse de *P. thoenii* par PCR. En haut, fragments amplifiés ; en bas, fragments de restriction. De gauche à droite : 1) ATCC 4874 ; 2) DSM 20277 ; 3) AG 28.1 ; 4) NW 36.1 ; 5) ZS 22.1 ; 6) 106.2 ; 7) 204.1 ; 8) 304.2; 9) 50–2000 bp ladder.

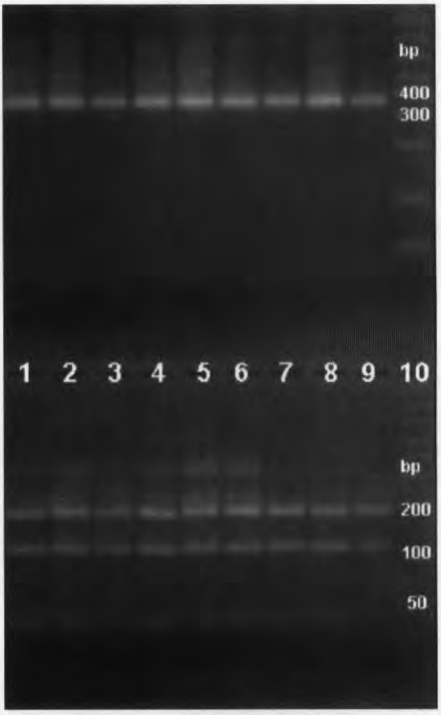


Figure 5. PCR analysis of *P. acidipropionici*; amplified fragments at the top; restriction patterns at the bottom. From left to right: 1) ATCC 25562; 2) DSM 20272; 3) DSM 20273; 4) AG 50.1; 5) AG 66.1; 6) BE 11.1; 7) FR 55.2; 8) TG 17.2; 9) ZS 44.2; 10) 50–2000 bp ladder.

Figure 5. Analyse de *P. acidipropionici* par PCR. En haut, fragments amplifiés ; en bas, fragments de restriction. De gauche à droite : 1) ATCC 25562 ; 2) DSM 20272 ; 3) DSM 20273 ; 4) AG 50.1 ; 5) AG 66.1 ; 6) BE 11.1 ; 7) FR 55.2 ; 8) TG 17.2 ; 9) ZS 44.2; 10) 50–2000 bp ladder.

methods may be ambiguous [6, 11]. The greatest problem seems to be the distinction between *P. jensenii* and *P. thoenii*. Both of these species could clearly be differentiated with the method described here and the earlier *P. rubrum* was classified under *P. jensenii* (*figure 3*). This finding is in agreement with the classification of *P. rubrum* as *P. jensenii* by Britz and Steyn [7], Britz and Riedel [6], de Carvalho et al. [11], Riedel et al. [18] and recently by Riedel and Britz [19].

Figure 6. PCR analysis of other bacteria; amplified fragments at the top; restriction patterns at the bottom. From left to right: 1) 50–2000 bp ladder; 2) 932; 3) 1084; 4) 2076; 5) 2075; 6) 2071; 7) 2069; 8) 2066; 9) 2065; 10) 2064; 11) 2063; 12) 2062; 13) 2057; 14) 2053; 15) 2051; 16) 1087; 17) ATCC 25562 *P. acidipropionici* T; 18) ATCC 4874 *P. thoenii* T; 19) ATCC 4868 *P. jensenii* T; 20) ATCC 6207 *P. freudenreichii* ssp. *freudenreichii* T; 21) 50–2000 bp ladder.

Figure 6. Analyse d'autres bactéries par PCR. En haut, fragments amplifiés ; en bas, fragments de restriction. De gauche à droite : 1) 50–2000 bp ladder ; 2) 932 ; 3) 1084 ; 4) 2076 ; 5) 2075 ; 6) 2071 ; 7) 2069 ; 8) 2066 ; 9) 2065 ; 10) 2064 ; 11) 2063 ; 12) 2062 ; 13) 2057 ; 14) 2053 ; 15) 2051 ; 16) 1087 ; 17) ATCC 25562 *P. acidipropionici* T ; 18) ATCC 4874 *P. thoenii* T ; 19) ATCC 4868 *P. jensenii* T ; 20) ATCC 6207 *P. freudenreichii* ssp. *freudenreichii* T ; 21) 50–2000 bp ladder.

Figure 7. PCR analysis of other bacteria; amplified fragments at the top; restriction patterns at the bottom. From left to right: 1) 50–2000 bp ladder; 2) 3233; 3) 3018; 4) 3013; 5) 3011; 6) 3010; 7) 3009; 8) 2493; 9) 3277; 10) 3276; 11) 3275; 12) 3274; 13) 3273; 14) 3272; 15) 3271; 16) 2645; 17) 879; 18) ATCC 25562 *P. acidipropionici* T; 19) ATCC 4874 *P. thoenii* T; 20) ATCC 4868 *P. jensenii* T; 21) ATCC 6207 *P. freudenreichii* ssp. *freudenreichii* T; 22) 50–2000 bp ladder.

Figure 7. Analyse d'autres bactéries par PCR. En haut, fragments amplifiés ; en bas, fragments de restriction. De gauche à droite : 1) 50–2000 bp ladder ; 2) 3233 ; 3) 3018 ; 4) 3013 ; 5) 3011 ; 6) 3010 ; 7) 3009 ; 8) 2493 ; 9) 3277 ; 10) 3276 ; 11) 3275 ; 12) 3274 ; 13) 3273 ; 14) 3272 ; 15) 3271 ; 16) 2645 ; 17) 879 ; 18) ATCC 25562 *P. acidipropionici* T ; 19) ATCC 4874 *P. thoenii* T ; 20) ATCC 4868 *P. jensenii* T ; 21) ATCC 6207 *P. freudenreichii* ssp. *freudenreichii* T ; 22) 50–2000 bp ladder.

Figure 8. PCR analysis of other bacteria; amplified fragments at the top; restriction patterns at the bottom. From left to right: 1) 50–2000 bp ladder; 2) 1213; 3) 1212; 4) 1211; 5) 1086; 6) 1052; 7) 1051; 8) 1050; 9) 933; 10) 286; 11) 278; 12) 170; 13) 169; 14) 145; 15) 144; 16) 877; 17) 2494; 18) ATCC 25562 *P. acidipropionici* T; 19) ATCC 4874 *P. thoenii* T; 20) ATCC 4868 *P. jensenii* T; 21) ATCC 6207 *P freudenreichii* ssp. *freudenreichii* T; 22) 50–2000 bp ladder.

Figure 8. Analyse d'autres bactéries par PCR. En haut, fragments amplifiés ; en bas, fragments de restriction. De gauche à droite : 1) 50–2000 bp ladder ; 2) 1213 ; 3) 1212 ; 4) 1211 ; 5) 1086 ; 6) 1052 ; 7) 1051 ; 8) 1050 ; 9) 933 ; 10) 286 ; 11) 278 ; 12) 170 ; 13) 169 ; 14) 145 ; 15) 144 ; 16) 877 ; 17) 2494 ; 18) ATCC 25562 *P. acidipropionici* T ; 19) ATCC 4874 *P. thoenii* T ; 20) ATCC 4868 *P. jensenii* T ; 21) ATCC 6207 *P. freudenreichii* ssp. *freudenreichii* T ; 22) 50–2000 bp ladder.

Figure 9. PCR analysis of other bacteria; amplified fragments at the top; restriction patterns at the bottom. From left to right: 1) 50–2000 bp ladder; 2) 608; 3) 2637; 4) 1755; 5) 795; 6) 1322; 7) 552; 8) 1229; 9) 1228; 10) 1227; 11) 1116; 12) 1220/23; 13) 1219; 14) 1218; 15) 1217; 16) 1216; 17) 1215; 18) 1214; 19) ATCC 25562 *P. acidipropionici* T; 20) ATCC 4874 *P. thoenii* T; 21) ATCC 4868 *P. jensenii* T; 22) ATCC 6207 *P. freudenreichii* ssp. *freudenreichii* T; 23) 50–2000 bp ladder.

Figure 9. Analyse d'autres bactéries par PCR. En haut, fragments amplifiés ; en bas, fragments de restriction. De gauche à droite : 1) 50–2000 bp ladder ; 2) 608 ; 3) 2637 ; 4) 1755 ; 5) 795 ; 6) 1322 ; 7) 552 ; 8) 1229 ; 9) 1228 ; 10) 1227 ; 11) 1116 ; 12) 1220/23 ; 13) 1219 ; 14) 1218 ; 15) 1217 ; 16) 1216 ; 17) 1215 ; 18) 1214 ; 19) ATCC 25562 *P. acidipropionici* T ; 20) ATCC 4874 *P. thoenii* T ; 21) ATCC 4868 *P. jensenii* T ; 22) ATCC 6207 *P. freudenreichii* ssp. *freudenreichii* T ; 23) 50–2000 bp ladder.

Protein electrophoresis of propionibacteria yields a more detailed image of the analyzed strains. It produces far more bands than the two or three obtained by restriction analysis. Based on protein profiles propionibacteria strains can not only be classified into their species, but can also be grouped into clusters within the species [17]. However, when it is not necessary to obtain a more detailed analysis of a strain than its species, restriction analysis on the 23S rRNA gene is much faster and the patterns are simpler to read than protein profiles.

PCR with the chosen primers was carried out with different genera (*table II*) of anaerobic bacteria naturally occurring in milk or dairy products, such as lactobacilli, enterococci, clostridia, streptococci, and lactococci. *Figures 6–9* show that only propionibacteria produced a fragment of approximately 350 bases and gave a unique pattern after restriction enzyme analysis with *Msp*I. Many of the bacteria encountered in milk would be expected to produce a smaller fragment than propionibacteria. Indeed most of the other bacteria generated a smaller and weaker fragment of about 250 bases. This fragment was not or only partially cut with the restriction endonuclease *Msp*I. The genus of propionibacteria was clearly distinguishable from the other tested bacteria even without cutting the fragment of about 350 bases (*figures 6–9*). Approximately 100 unidentified strains isolated from milk, growing on lactate, and morphologically as well as microscopically similar to propionibacteria were analyzed. PAGE (polyacrylamide gel electrophoresis) of their proteins demonstrated that they were not propionibacteria. None of these bacteria showed one of the four typical profiles of propionibacteria (results not shown).

The method described here is a rapid, simple and reliable technique to distinguish between the *Propionibacterium* species. It yields reproducible results and can be performed with liquid cultures or bacterial material from colonies, as there is no need to purify DNA. A large number of samples, approximately 40, depending on the thermocycler and the electrophoresis apparatus used, can be analyzed within a single day. This method could in the future help to distinguish other Gram-positive bacteria with a high G+C content.

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