Genus- and Species-Specific PCR-Based Detection of Dairy Propionibacteria in Environmental Samples by Using Primers Targeted to the Genes Encoding 16S rRNA

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PCR assays with primers targeted to the genes encoding 16S rRNA were developed for detection of dairy propionibacteria. *Propionibacterium thoenii* specific oligonucleotide PT3 was selected after partial resequencing. Tests allowed the detection of less than 10 cells per reaction from milk and cheese and 10² cells per reaction from forage and soil.

Dairy or “classical” propionibacteria have great technological importance for Swiss-type cheeses, where they are responsible for “holes” and flavor formation. Strains belonging to the four *Propionibacterium* species *P. freudenreichii*, *P. jensenii*, *P. acidipropionici*, and *P. thoenii*, are frequently present in raw milk, in numbers varying between 10 and 10⁴ CFU/ml (20), as a consequence of environmental contamination. A prevalence of the species *P. freudenreichii* and *P. jensenii* has been observed in milk and derived products after isolation in plates (1, 14, 15, 18, 19). Propionibacteria can also cause defects in cheeses, mainly anomalous blowing (2, 11), and pigmented *P. thoenii* and *P. jensenii* strains cause the “brown spot” of cheese paste (7). To reduce economic losses in dairy productions, the propionibacterium content of raw milk should be better controlled, limiting their diffusion in the dairy environment. Few reports exist regarding the isolation of propionibacteria from substrates like feed or soil coming into contact with raw milk (5, 17, 21). An explanation could be the major inhibiting effects exerted in plates by the typical microflora of such substrates towards propionibacteria. Growth media currently used for propionibacterium isolation are not sufficiently selective, and moreover, typical propionibacterium colonies appear after at least 6 days (20). PCR-based specific assays are a valuable alternative to plating methods, being far more rapid, more specific, and unhindered by the presence of non-target microorganisms. Dasen et al. (6) have developed a multiplex PCR assay using a genus-specific primer, targeted to the genes encoding the 16S rRNA, together with two universal primers (6). Specific DNA fragments, differing slightly in size, were amplified from both dairy and cutaneous species of *Propionibacterium*. The present study regards the selection of a different primer set for each dairy species and PCR detection of propionibacteria from various substrates without previous isolation.

The following type (T) and reference strains plus propionibacterium isolates were used in all the specificity tests: *P. freudenreichii* subsp. *freudenreichii* NCFB 564^T, *P. freudenreichii* subsp. *shermanii* NCFB 853^T, 35 *P. freudenreichii* isolates (19), *P. jensenii* NCFB 572, NCFB 565, NCFB 571, 15 *P. jensenii* isolates (19), *P. acidipropionici* NCFB 563^T, NCFB 570, four *P. acidipropionici* isolates (19), *P. thoenii* NCFB 568^T,

NCFB 569, two *P. thoenii* isolates (19), *Propionibacterium acnes* LMG 16711^T, *Bifidobacterium animalis* LMG 10508^T, *Bifidobacterium longum* LMG 13197^T, *Enterococcus faecalis* LMG 7937^T, *Enterococcus faecium* LMG 11423^T, *Escherichia coli* LMG 2092^T, *Lactobacillus brevis* LMG 7944^T, *Lactobacillus zeae* ATCC 393, *Lactobacillus coryneformis* DSM 20001^T, *Lactobacillus curvatus* LMG 12006, *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901^T, *Lactobacillus fructivorans* LMG 9201^T, *Lactobacillus gasseri* NCFB 2233^T, *Lactobacillus helveticus* ATCC 10797^T, *Lactobacillus hilgardii* LMG 6895^T, *Lactobacillus plantarum* ATCC 14917^T, *Lactobacillus pontis* ATCC 14187^T, *Lactobacillus reuteri* DSM 20016^T, *Lactococcus lactis* subsp. *lactis* LMG 6890^T, *Leuconostoc mesenteroides* subsp. *dextranicum* LMG 6908^T, *Kocuria varians* 55, *Pediococcus acidilactici* LMG 11384^T, and *Streptococcus thermophilus* LMG 6896^T.

Dairy propionibacteria were cultured in sodium lactate (SL) broth (9) in jars for anaerobiosis using the Anaerocult A system (Merck, Darmstadt, Germany) at 30°C for 48 to 72 h. Lactic acid bacteria were grown at 30 or 37°C in anaerobiosis in MRS broth (Oxoid, Basingstoke, United Kingdom) for 24 to 72 h. The other bacteria were grown at 37°C for 24 to 72 h in brain heart infusion broth (Oxoid); *P. acnes* and bifidobacteria were incubated by anaerobiosis. Propionibacterium counts in samples were carried out on SL agar.

Genomic DNA was purified from 1 ml of bacterial culture in late exponential growth phase as previously described (19).

Samples of milk, cheese, forage, and soil used to determine the detection limits had been checked by plate count for absence of dairy propionibacteria and stored frozen until use. Each solid material was homogenized in 1:1 (wt/vol) sterile 1/4-strength Ringer solution. Aliquots of suspensions were artificially inoculated with propionibacterium pure cultures in numbers ranging between 10⁸ and 1 cell/ml.

Prior to nucleic acid extraction, inoculated cheese, soil, and forage suspensions of 1:1 (wt/vol) in 1/4 strength sterile Ringer solution (Oxoid) were filtered under vacuum through 17- to 25-μm-pore-size filter paper (Forlab Carlo Erba, Milan, Italy). One milliliter of filtered suspension or milk was centrifuged at 8,000 rpm for 10 min. For forage and soil samples of unknown *Propionibacterium* content, 50 ml of suspension was centrifuged as described above. The pellet was resuspended in 500 μl of lysis solution (0.1 M NaOH and 1% sodium dodecyl sulfate). After chloroform extraction, nucleic acids were precipitated with 2 volumes of absolute ethanol and washed once with 70%

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TABLE 1. Alignment of the 16S rDNA regions corresponding to *E. coli* nucleotide positions 435 to 478 for dairy propionibacteria

Species	Sequence ^b
<i>P. freudenreichii</i>	<u>CTTTCATCCATGACGAAGCG</u> -----CAAGT
	PF
<i>P. acidipropionici</i>	CTTTCACCAAGGACGAAGGCATT-----CTTTTAGGGTGT
	PA
<i>P. jensenii</i>	CTTTCNCAGGACGAAGTG-----CCTNTCGGGGTGT
	PJ
<i>P. thoenii</i>	CTTTCACCAAGGACGAAGGG-----CCTATCGGGGTAT
	PT1
<i>P. thoenii</i> ^a	CTTTCACCAAGGACAAAAGG-----CCTTTTGGGGTTT
	PT3

^a New sequence determined for *P. thoenii* NCFB 568^T.^b Species-specific primers selected are underlined.

ethanol. In the nucleic acid extraction from forage and soil, 10% polyvinylpyrrolidone (PVP) was added to the 70% ethanol in the final washing step (22). Nucleic acids were finally vacuum dried and dissolved in 20 μ l of Tris-EDTA buffer.

Oligonucleotides reported in Table 1 were designed by comparison of the 16S rDNA sequences available in the EMBL database for dairy and non-dairy propionibacteria (*P. acidipropionici* X53221, *P. freudenreichii* X53217, *P. jensenii* X53219, *P. thoenii* X53220, *P. acnes* X53218, *Propionibacterium cyclohexanicum* D82046, *Propionibacterium propionicus* X53216) (3, 4), some taxonomically related species (*Mycobacterium komosense* X55591, *Eubacterium combesii* L34614, *Corynebacterium xerosis* X84446, *Arthrobacter globiformis* X80739, *Micrococcus luteus* M38242, *Actinomyces israelii* X53228, *Brevibacterium linens* X76566) and microorganisms commonly found in milk and dairy products (*L. helveticus* X61141, *L. delbrueckii* X52654, *L. casei* M23928, *L. brevis* X61134, *S. thermophilus* X68418). Sequence alignment was carried out with the ClustalX software (EMBL, Heidelberg, Germany).

The amplification programs of genus-specific, species-specific, and seminested PCR (10) consisted of denaturation at 94°C for 4 min, 40 cycles of denaturation at 94°C for 30 s, annealing for 15 s at temperatures varying with the upstream primer used, and extension at 72°C for 1 min followed by a final extension at 72°C for 5 min. The 20- μ l reaction mixture contained 100 μ M each dNTP, 0.5 μ M each primer, 1.5 mM MgCl₂, 2 μ l of 10 $\times$ reaction buffer, 1 U of *Taq* DNA polymerase (Boehringer Mannheim, Mannheim, Germany), and 1 μ l of DNA sample. Seminested or double-step PCR was applied to the 1:10 dilutions of the specific PCR mixtures. PCR products were electrophoresed at 100 V on 1.5% (wt/vol) agarose gel stained with 0.5 μ g of ethidium bromide in 0.5 $\times$ TAE buffer (40 mM Tris-acetate, 1 mM EDTA [pH 8.0])/ml.

A PCR product amplified from the 16S rDNA of *P. thoenii* NCFB 568^T (DSM 20276^T) was partially sequenced by the ABI Prism Dye Terminator Cycle Sequencing System (Perkin-

Elmer Co., Norwalk, Conn.) according to the manufacturer's instructions.

Six combinations of genus-specific oligonucleotides selected by 16S rDNA sequence comparison were tested in amplification experiments using purified DNA from all the type and reference strains reported above. Primer pair PB1-PB2 (Table 2) proved specific for dairy propionibacteria and *P. acnes*, providing only one product of the expected size (610 bp). Amplification reactions were carried out with annealing temperatures and time that did not affect product yield. The number of cycles was set to 40, as this determined an increase in product yield from diluted DNA of propionibacteria without appearance of additional PCR products.

Upstream primers for species-specific amplification (Tables 1 and 2) were selected within a region showing the highest variability among the species (*E. coli* positions 435 to 478). Using primer PB2 as the downstream oligonucleotide, amplification products of sizes 868, 867, 865, and 864 bp were expected from *P. acidipropionici*, *P. freudenreichii*, *P. thoenii*, and *P. jensenii*, respectively. Excellent specificity was obtained for *P. freudenreichii*, *P. jensenii* and *P. acidipropionici*. Primer PT1 (Table 1), selected for *P. thoenii* in the same variable region, did not allow amplification from the four *P. thoenii* strains tested while it permitted the amplification of a fragment of the expected size from the three reference strains of *P. jensenii*. Oligonucleotide PT2 (Table 2), which was potentially specific for *P. thoenii*, permitted the amplification of a 1,148-bp DNA fragment from *P. thoenii* strains and from *P. acnes* with equal efficiency, proving not selective.

A primer specific for *P. thoenii*, PT3 (Tables 1 and 2), could be designed by partially sequencing the PT2-PB2 fragment amplified from strain NCFB 568^T (EMBL accession no. AJ132324).

The ability of all primer sets to amplify DNA from other strains belonging to the target species was confirmed without

TABLE 2. Primers used in PCR identification-detection assays for dairy propionibacteria

Label	Sequence	Corresponding <i>E. coli</i> positions	Target species	Annealing temperature (°C)
PB1	5'-AGTGGCGAAGGCGGTTCTCTGGA-3'	720-742	Dairy propionibacteria and <i>P. acnes</i>	70
PB2	5'-TGGGGTCGAGTTGCAGACCCCAAT-3'	1305-1328	Dairy propionibacteria and <i>P. acnes</i>	70
PF	5'-CTTTCATCCATGACGAAGCGCAAG-3'	435-478	<i>P. freudenreichii</i>	69
PJ	5'-GACGAAGTGCTATCGGGGTG-3'	446-478	<i>P. jensenii</i>	68
PA	5'-GACGAAGGCATCTTTTAGGGTGT-3'	445-478	<i>P. acidipropionici</i>	68
PT2	5'-ATATGAGCTCCTGCTGCATNGGT-3'	187-210	<i>P. thoenii</i> , <i>P. acnes</i>	69
PT3	5'-GGACAAAAGGCCTTCGGGGTTT-3'	445-478	<i>P. thoenii</i>	70

FIG. 1. Genus-specific amplification with primer set PB1-PB2 from raw milk inoculated with propionibacteria. Lanes: 1, PCR marker (Amresco, Solon, Ohio); 2, amplification from 20 ng of DNA of *P. jensenii* NCFB 571; 3 to 9, amplifications from initial cell numbers of 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 , and 1.2, respectively, of *P. jensenii* 2S per reaction.

exception in amplifications from previously identified isolates (19).

Primer pairs PB1-PB2, PF-PB2, PJ-PB2, PA-PB2, and PT3-PB2 were used in detection assays from samples of milk, cheese, forage, and soil artificially inoculated with known numbers of propionibacteria. The short procedure described above for nucleic acid purification directly from samples provided better sensitivity compared with the DNA extraction method from pure cultures (19) and other reported procedures (8, 12).

The detection limit was similar for all the specific primer sets and varied with the kind of sample. In milk and cheese, multiples of 10 cells in the PCR were detected after one amplification step. Figures 1 and 2 show examples of amplification with genus-specific primer set PB1-PB2 and with primer set PF-PB2, respectively. A seminested PCR with primers PB1-PB2 from milk and cheese permitted the detection of less than 10 cells of dairy propionibacteria in the reaction mixture. The signal was never obtained from uninoculated aliquots of the same samples used as negative controls.

One-step amplification from forage and soil was considerably less sensitive; cell numbers lower than 10^5 could not be detected and addition of 10% PVP to the 70% ethanol in the final washing step was necessary to allow amplification. The addition of 400 ng of nonacetylated bovine serum albumin/ml in the PCR mixture (12) was also performed, but amplification

FIG. 2. Species-specific amplification with oligonucleotides PF-PB2 from serial dilutions of *P. freudenreichii* C21 in cheese suspension. Lanes: 1, amplification from 20 ng of DNA of *P. freudenreichii* subsp. *freudenreichii* NCFB 564T; 2 to 8, amplifications from initial cell numbers of 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 27, and 2.6, respectively, in the PCR mixture; 9, PCR marker (Amresco).

FIG. 3. Seminested PCR with primers PB1-PB2 after species-specific amplification from strain *P. acidipropionici* NCFB 563T inoculated in soil suspension. Lanes: 1, PCR marker (Amresco, Solon, Ohio); 2, seminested amplification from 15 ng of DNA of the same strain; 3 to 8, seminested amplification from 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , and 23 cells, respectively.

products were not obtained. In case of PVP addition, the seminested PCR assay permitted the amplification of the 610-bp genus-specific fragment from reactions containing multiples of 10^2 propionibacterium cells. Figure 3 shows an example of seminested amplification on species-specific products from soil suspensions inoculated with *P. acidipropionici* NCFB 563T.

The PCR assays were applied to the detection of propionibacteria from six raw milk and 14 forage samples not previously analyzed for their presence. Three samples of milk and two samples of forage were positive after single-step PB1-PB2 amplification. One more forage sample was positive after two-step amplification with the same primers. The single species could be detected by the seminested assay after species-specific PCR; in one milk sample and in two forage samples, positive to one-step PB1-PB2 amplification, none of the dairy species was present. This can be explained with amplification from *P. acnes* or other nondairy propionibacteria. In one PB1-PB2 PCR-positive milk sample, the presence of all four dairy species was demonstrated with the seminested assay. One more milk sample was positive for *P. freudenreichii*, *P. jensenii*, and *P. acidipropionici*; one more forage sample was positive for *P. jensenii* and *P. acidipropionici*.

This study has provided rapid PCR identification-detection assays for *P. acnes* and each dairy species of the genus *Propionibacterium*. Single-species detection can facilitate studies regarding the diffusion and ability to survive in different environments.

The possibility to selectively detect *P. acnes* with primers PT2-PB2 could allow its distinction from the other cutaneous propionibacteria, and, therefore, it should be further investigated.

Sequence comparison shows three different nucleotide positions between oligonucleotide PF and the homologous region reported for the recently proposed species *Propionibacterium cyclohexanicum* (13). The discriminating power of the *P. freudenreichii*-specific assay towards this closely related species must be experimentally evaluated.

The species-specific assays evaluated here can substitute time-consuming phenotypic identification for these slowly growing microorganisms. The preliminary individuation of samples to be further analyzed is an advantage for both practical and research applications, considering the rapidity of the whole procedure of nucleic acid extraction and amplification. The possibility to individuate milk samples containing more

than 10 cells of dairy propionibacteria is useful for the choice of raw milk suitable for particular dairy productions where even initial numbers as low as 10^2 cells/ml are undesired (16).

For forage and soil, the sensitivity of one-step amplification is rather low and must be improved by seminested or double-step amplification. The problem can also be overcome by concentrating cells by centrifugation from larger volumes of sample suspension.

The application of PCR tests here described can give more precise indications on the seasonal variation of propionibacteria in milk and in the dairy environment.

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