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PCR detection and 16S rRNA sequence-based phylogeny of a novel *Propionibacterium acidipropionici* applicable for enhanced fermentation of high moisture corn

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ABSTRACT

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Aims: The aims of this study were to develop a sensitive and more rapid detection of *Propionibacterium acidipropionici* DH42 in silage and rumen fluid samples, and to explore its 16S rRNA sequence-based phylogeny.

Methods and Results: Nested polymerase chain reaction (PCR) was used with DH42-specific primers dhb1 and dhb2 for the secondary amplification of a 1267-bp fragment of 16S rRNA encoding gene. Using the established protocols for PCR amplification, as low as 10^2 and 10^3 CFU ml^{-1} of strain DH42 in silage extracts and rumen fluid, respectively, were detected. To determine phylogenetic relationships between DH42 and other representatives of *Propionibacterineae*, a 1529-bp fragment of its 16S rRNA was amplified by PCR and sequenced. The propionibacterium DH42 formed a cluster with *Eubacterium combesii*, *P. acidipropionici* and *P. microaerophilus*.

Conclusions: 16S rRNA-based PCR detection technique was developed for DH42 in silage and rumen fluid samples. The 16S rRNA sequence confirmed the earlier identification of strain DH42 as *P. acidipropionici*. However, variable nucleotide positions were revealed.

Significance and Impact of the Study: Variability of 16S rRNA sequence within the species *P. acidipropionici*, determined in this study, poses the need of re-sequencing for some species of the suborder *Propionibacterineae* for a more reliable classification.

Keywords: 16S rRNA sequence, PCR detection, phylogeny, *Propionibacterium acidipropionici* DH42.

INTRODUCTION

A propionibacterium designated DH42, isolated from fermented, high moisture corn, was previously evaluated as a starter culture to enhance bunk stability of high moisture corn (Dawson 1994; Dawson *et al.* 1998). DH42 was shown to improve bunk stability and significantly reduce the number of yeasts and moulds in the final product (Bato *et al.* 1999). The organism also demonstrated effectiveness in using as a daily dietary amendment to lessen the effects of lactic acid accumulation in cattle fed high grain diets (Aviles *et al.* 1999).

Detection of propionibacteria in the environment is complicated because the media currently being used for their isolation are not sufficiently selective and colonies often appear only after several days of incubation (Thierry and Madec 1995). In addition, many strains of propionibacteria are resistant to lysozyme (Johnson and Cummins 1972), so DNA recovery from a given sample can be limited. PCR techniques have been used to identify and distinguish different propionibacteria species (Riedel *et al.* 1994, 1998; Rossi *et al.* 1997, 1999), and to distinguish them from other genera (Dasen *et al.* 1998; Meile *et al.* 1999). Rossi *et al.* (1998, 1999) observed that with forage and soil samples, propionibacteria

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numbers lower than 10^5 could not be detected and they recommended a double-step amplification or semi-nested amplification to improve sensitivity. Nested PCR can eliminate unwanted amplification products while at the same time dramatically increasing sensitivity (Mullis and Faloona 1987; Porter-Jordan *et al.* 1990; Zhang and Ehrlich 1994).

With the potential commercial application of strain DH42 as a bioinoculant for enhanced fermentation of high moisture corn, a method of detection and monitoring is needed to evaluate its persistence and efficacy. This study was conducted to develop a sensitive and more rapid detection of DH42 in silage and rumen fluid samples, and to explore its 16S rRNA sequence-based phylogeny relative to other propionibacteria representatives.

MATERIALS AND METHODS

Bacterial strains and culture conditions

A total of 16 strains including DH42, eight propionibacteria, two lactobacilli and five other bacterial strains were used in this study (Table 1). Propionibacteria and *Eu. combesii* were cultured using Reinforced Clostridial Medium (Oxoid, Unipath Ltd., Basingstoke, UK) under CO_2 atmosphere and incubated at 30°C. The lactobacilli strains were cultured in Lactobacilli MRS broth (Difco, Detroit, MI, USA) and incubated at 37°C. For propionibacteria strains, culture purity was initially checked by plating the cultures in Purple Broth Base (Difco) agar supplemented with 1% (w/v) i-erythritol (Sigma, St Louis, MO, USA). Some propionibacteria have the ability to ferment erythritol (Hetinga and Reinbold 1972) and colonies are pigmented yellow in colour. Rogosa SL agar (Difco) was used to check the lactobacilli strains.

Silage and rumen fluid sampling and inoculation

Corn silage was collected from the Michigan State University (MSU) Beef Cattle Teaching and Research Center. Equal amounts (w/v) of silage (250 g) and 0·9% (w/v) sterile saline solution (250 ml) were mixed for *ca* 10 min using a Stomacher (Tekmar, Cincinnati, OH, USA). The mixture was filtered using four layers of cheesecloth. The recovered homogenate was measured into five 50-ml centrifuge tubes. Each tube was artificially inoculated with DH42 culture at the levels of 10^2, 10^3, 10^4, and 10^5 CFU ml^{-1} silage extract. One tube was kept uninoculated. The silage homogenates were kept in ice until DNA extraction was performed.

Rumen fluid samples were collected from ruminally fistulated cows fed a high concentrate diet at the MSU Dairy Cattle Research Center. Rumen fluid was strained using four layers of cheesecloth. Homogenate samples were

Table 1 Bacterial cultures used in the study

Organism	Source and designation
DH42	ATCC* 55737; DH-42
P. acidipropionici	ATCC 25562; VPI 0399 [14X]
P. freudenreichii subsp. *shermanii* CDC 3094	Laboratory stock
P. pentosaceum P11	Laboratory stock
P. freudenreichii subsp. *shermanii* P92	Laboratory stock
P. freudenreichii subsp. shermanii	ATCC 13673; 33 [VPI 0409]
P. freudenreichii subsp. freudenreichii	ATCC 6207; [Hitchner No. 33; VPI 394]
P. thoenii P15	Laboratory stock
Propionibacterium sp. P42	Laporte Biochemical International, Milwaukee, IN, USA
Lactobacillus sakei	ATCC 15521; T.S [K. Kitahara 37]
L. delbrueckii subsp. *lactis*	ATCC 7830; 313 [BUCSAV 244; NCDO 302; NCIB 8118]
Weissella confusa	ATCC 27646; NCIB 4037 [NCDO 233; NCTC4037]
Bacillus subtilis	Laboratory stock
Bacillus sp.	Laboratory stock
B. subtilis	ATCC 6633; NRS 231 [BUCSAV 425; Boots 218; CCM 1999; CIP 52·62; DSM 347; IAM 1069; JCM 2499; NCIB 8054; NCTC 10400; NCTC 6752; PCI 219; WHO 9]
Eu. combesii	ATCC 25545; VPI 0136 [A13D]

*American Type Culture Collection (http://www.atcc.org/home.cfm).

placed into 50-ml centrifuge tubes. Each tube was inoculated with DH42 as described above for the silage samples and one tube was kept uninoculated. The samples were kept in ice until DNA extraction was performed. Aliquots of the corn silage and rumen fluid samples were frozen (−20°C).

DNA extraction

DNA was extracted from pure bacterial cultures and from corn silage and rumen fluid samples using UltraClean™ Soil DNA Isolation Kit (Mo Bio Laboratories Inc., Solana Beach, CA, USA). Approximately 100 μl of sample was used for extraction. With the pure bacterial cultures, *ca* 0·5 ml cultures incubated overnight were used in DNA extraction. DNA extracts were stored at −20°C until use.

DNA was quantified using a Beckman DU-600 spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA) as described by Maniatis *et al.* (1982). DNA integrity and verification of spectrophotometric determination was checked by gel electrophoresis in a 0·8% (w/v) agarose TAE (40 mM l^{-1} Tris-acetate, 1 mM l^{-1} EDTA, pH 8·2) gel containing 0·5 μg ml^{-1} ethidium bromide. Approximately 8 μl of DNA from each extract was mixed with 2 μl loading dye and electrophoresed at *ca* 80 V for *ca* 2–3 h.

Primer design and synthesis

Primers dhb1 and dhb2 (Tables 2 and 3) were designed using the ARB program (Strunk and Ludwig 1996) from the alignment of the 16S rRNA of DH42 (accession number AY360222) with other sequences in the program's database (as of July 1999). Regions apparently unique to DH42 were selected and primers complementary to these regions were designed. The universal primers bak11 and bak4 (Dasen *et al.* 1998) corresponding to *E. coli* 16S rRNA positions 8–25 and 1522–1540, respectively, were also used (Table 3). The relative position of four primers is shown on Fig. 1. Primers were synthesized by the MSU Macromolecular Structure Facility and stored at −20°C until use.

PCR and its optimization

Nested PCR reaction was performed as described by Herman *et al.* (1995) and Rossi *et al.* (1999). Initially, the first round of PCR was performed in a 50-μl reaction mixture containing 5 μl of 10X PCR buffer (Gibco BRL, Gaithersburg, MD, USA), 2·0 μl of 50 mM l^{-1} $MgCl_2$ (Gibco BRL), 1 μl of 1·25 mM l^{-1} dNTPs mixture (Gibco BRL), 2·5 U of *Taq* DNA polymerase (Gibco BRL), 20 pmol μl^{-1} of each primer (bak4 and bak11) and 1 μl template DNA (100 ng). Distilled water (Gibco BRL) was added to make up a volume of 50 μl. PCR was performed using a Perkin Elmer 9600 thermal cycler (Norwalk, CT, USA) as follows: 3 min of denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 1 min, with a final extension step at 72°C for 10 min. For the second round of PCR performed with primers dhb1 and dhb2 under the above conditions and at desired annealing temperature, an aliquot of the dilution of the primary PCR product was used as template.

In addition, optimum annealing temperature and concentrations of Mg^{++}, primers, dNTPs and *Taq* DNA polymerase were determined. The calculated annealing temperature for primers dhb1 and dhb2 (54°C) was used as the starting temperature for the optimization. Different concentrations of $MgCl_2$ (0·5, 0·75, 1·0, 1·25 and 1·5 mM l^{-1}), primers (7·5, 10 and 20 pmol μl^{-1}), dNTPs (12·5 and 25 μM), and *Taq* DNA polymerase (2·0 and 2·5 U) were tested. The optimum temperature and concentration were determined which yielded the strongest band staining after u.v. illumination of the gel without the appearance of other bands. The target DNA in the secondary PCR product obtained with primers dhb1 and dhb2 was a 1267-bp band when DH42 was present in the sample.

Table 2 Alignment of 16S rRNA of propionibacteria and other bacterial species with primers dhb1 and dhb2

Species	Primer dhb1 (5′ → 3′) CCGGATATGAGCTCCTG	Primer dhb2 (3′ → 5′) TTGTGCAAGACGCACCC
DH42	CCGGATATGAGCTCCTG	AACACGTTCTGCGTGGG
Eu. combesii	CCG**N**ATA.GAGCT**TTCA**	AACACGTTCTGCGTGGG
P. acidipropionici	CCGGATAT**N**AGCT**TTCA**	AACACGT**N**CTGCGTG.G
P. thoenii	CCGGATATGAGCTCCTG	**TTCC**C.T**NG**T..G.GGG
P. jensenii	CCGGATATGAGCTC**TNA**	**N**AC.C.T**NT**TG.G.GGG
P. granulosum	C**T**GGATATG**T**GCTCCTG	AACAC.TT**TT**T.GTGGG
P. cyclohexanicum	C**T**GGATATGA**A**CT**GGGC**	A**T**C.C**C**TT**G**T..G.GGG
P. propionicus	CCGGATA**GACAT**CCTTG	AAC.C..T**G**TG..TGGG
Actinomyces israelii	CCGGATA**G**GAGCT**N**CT**N**	**N**AC.CGTT..G..TGGG
Faecalibacterium prausnitzii	CCGGATA**G**GAGCTCCT**N**	**N**AC.CGTT..G..TGGG

Ambiguous or nonmatching positions are bold.

Table 3 Synthetic oligonucleotide sequences, positions and calculated melting temperature (T_m)

Primer	Sequence (5′ → 3′)	*E. coli* position	T_m (°C)*
dhb1	CCGGATATGAGCTCCTG	172–188 F	54
dhb2	CCCACGCAGAACGTGTT	1429–1445R	54
bak11	AGGAGGTGATCCARCCGCA	8–25 F	50
bak4	AGTTTGATCMTGGCTCAG	1522–1540 R	58

*Calculated based on the formula: $T_m = 4$ (G + C) + 2 (A + T).

A two-step PCR amplification was also performed to compare it with sensitivity of the nested PCR in detecting the presence of DH42 in rumen fluid samples. The first round of initial two-step PCR was similar to that of nested PCR except for primers (dhb1 and dhb2), $MgCl_2$ concentration (1·5 mM l^{-1}), and annealing temperature (64°C). Another round of amplification was performed using the same primers and 5 μl of the 1 : 10 dilution of the primary PCR products. The annealing temperature was maintained at 64°C but $MgCl_2$ concentration was reduced to 1·25 mM l^{-1} to allow amplification of the target band in the DH42-inoculated samples.

PCR products were run in 0·8% (w/v) agarose gel. Approximately 2 μl 10X Blue Juice™ gel loading buffer (Gibco BRL) was added to 8 μl of PCR product and electrophoresed at 80 V for *ca* 2–3 h. A 1-kbp DNA ladder (Gibco BRL) was used as a marker.

Primer specificity evaluation

DNA extracts from pure bacterial cultures and from rumen fluid and corn silage samples were used as templates. Nested and double-step PCR reactions were performed as described above using the optimized conditions for the primers. Specificity was determined by the appearance of the 1267-bp band in the secondary PCR products.

16S rRNA sequencing

Pure culture of strain DH42 was used for 16S rRNA sequencing at Midilabs Inc. (Newark, DE, USA) (deposited in GenBank database under accession number AY360222). A partial sequencing of the 1267-bp fragment amplified with primers dhb1 and dhb2 was also performed in the Molecular Pathogenesis Laboratory (MSU Department of Animal Science). For this purpose, secondary PCR product amplification from rumen fluid sample was verified by gel electrophoresis in a 0·8% (w/v) agarose gel. The remaining PCR product was then purified using Wizard® PCR Preps DNA Purification System (Promega, Madison, WI, USA). Sequencing was performed by employing ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Applied Biosystems Division, Foster City, CA, USA) with primers dhb1 and dhb2 as the forward and reverse primers, respectively. The ABI 373 Automated Sequencer (Perkin Elmer, Applied Biosystems Division) was used for in-house sequencing.

Phylogenetic analysis

Using the DH42 16S rRNA sequence AY360222 as a query sequence, a local alignment was performed by means of the Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST/).

Using the computer software package Windows 32 MegAlign 5.06 © 1993–2003 (DNASTAR Inc., Madison, WI, USA), multiple alignment of 16S rRNA sequences of DH42, other *Propionibacterineae* species and *E. coli* (as a nonrelated organism) was performed and their phylogenetic relationships were estimated. The alignments of the sequences were performed using the ClustalW method as described in Thompson *et al.* (1994), and were deposited in the EMBL-Align database (http://www.ebi.ac.uk/embl/submission/alignment.html) under accession numbers ALIGN_000594, ALIGN_000647-ALIGN_000649, and ALIGN_000667. Following the multiple alignment, a phylogenetic tree using Neighbour-Joining method (Saitou and Nei 1987) was reconstructed on which branch distances (lengths) corresponded to sequence divergence. The phylogenetic tree represented either a phenogram with balanced branches that averaged the distances between ancestors in the tree or a cladogram with unbalanced branches that forced branch distances to correspond to sequence divergence.

RESULTS

Nested PCR optimization

With DNA extracted from DH42, the optimum conditions of the primary PCR were 66–68°C annealing temperature, using 1·5 mM l^{-1} $MgCl_2$, 10 or 20 pmol μl^{-1} of each primer, and 2 U *Taq* DNA polymerase. With various pure bacterial cultures, use of 1 μl of a 1 : 1000 dilution of the primary PCR product as template for secondary PCR, at 68°C annealing temperature and 1·5 mM l^{-1} $MgCl_2$, prevented the nonspecific amplification of other nonpropionibacteria and most propionibacterial species tested. Among the propionibacteria strains used, only *P. acidipropionici* (ATCC 25562) had the 1267-bp fragment after secondary PCR under these conditions (Fig. 2). The PCR optimization was then aimed at preventing the amplification of the 1267 bp band with the *P. acidipropionici* (ATCC 25562) samples. This was performed by adjusting the $MgCl_2$ concentration to 1·0 mM l^{-1} and using

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1       TGGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGTAAG    70

              bak11→

71      GCCCTTTCGGGGGTACACGAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCACTTCTTCGGGAT   140

141     AACGCTAGGAAACTGGTGCTAATACCGGATATGAGCTCCTGCCGCATGGTGGGGGTTGGAAAGTGTTTGT   210

                                  dhb1→

211     GGTGGTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAGTGGCTCACCAAGGCGGTGACGGGTAG   280

281     CCGGCCTGAGAGGGTGACCGGCCACATTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTG   350

351     GGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCAACGCCGCGTGCGGGATGACGGCCTTCGGGTTGT   420

421     AAACCGCTTTCACCAGGGGCGAAGGCATYCTTTTGGGGTGTTGACGGTACCTGGAGAAGAAGCACCGGCT   490

491     AACTACGTGCCAGCAGCCGCGGTGATACGTAGGGTGCGAGCGTTGTCCGGATTTATTGGGCGTAAAGGGC   560

561     TCGTAGGCGGTTGATCGCGTCGGAAGTGAAAACTTGGGGCTTAACCCTGAGCGTGCTTTCGATACGGGTT   630

630     GACTTGAGGAAGGTAGGGGAGAATGGAATTCCTGGTGGAGCGGTGGAATGCGCAGATATCAGGAGGAACA   700

701     CCAGTGGCGAAGGCGGTTCTCTGGACCTTTCCTGACGCTGAGGAGCGAAAGCGTGGGGAGCAAACAGGCT   770

771     TAGATACCCTGGTAGTCCACGCTGTAAACGGTGGGTACTAGGTGTGGGGTCCATTCCACGGATTCCGTGC   840

841     CGTAGCTAACGCATTAAGTACCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGG   910

911     GGCCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACA   980

981     TGGATTGGTAACGGTCAGAGATGGCCGCCCCCCTTGTGGGCCGGTTCACAGGTGGTGCATGGCTGTCGTC   1050

1051    AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTCCACTGTTGCCAGCATTT   1120

1121    GGTTGGGGACTCAGTGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGC   1190

1191    CCCTTATGTCCAGGGCTTCACGCATGCTACAATGGCCGGTACAAAGAGTGGCGACATCGTGAGGTGGAGC   1260

1261    GAATCTCAGAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAG   1330

1331    TAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGGCTTGTACACACCGCCCGTCAAGTCATGA   1400

1401    AAGTCGGTAACACCCGAAGCCGGTGGCCCAACACGTTCTGCGTGGGGGAGTCGTCGAAGGTGGGACTGGT   1470

                                          ←dhb2

1471    AATTAGGACTAAGTCGTAACAAGGTAGCCGTACCGGAAGGTGCGGYTGGATCACCTCCTT        1529

                                                  ←bak4
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Fig. 1 A 1529-bp 16S rRNA sequence of the DH42 strain (AY360222) and the target sites of the four PCR primers used

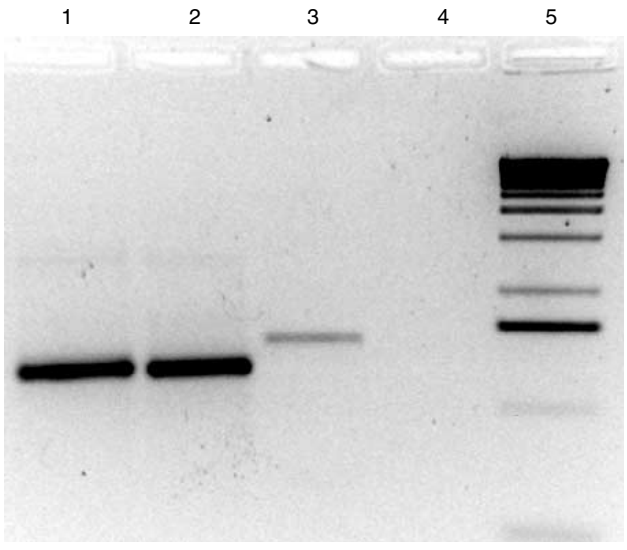

Fig. 2 Secondary nested PCR amplification products: lane 1, 1267-bp fragment in DH42; lane 2, *Propionibacterium acidipropionici* (ATCC 25563); lane 3, 1·5-kbp fragment in *Eubacterium combesii*; lane 4, negative control and lane 5, 1-kbp DNA ladder

10 pmol μl^{-1} of each primer that resulted in no amplification of *P. acidipropionici* (ATCC 25562) and other bacterial species (Fig. 3).

Primer specificity

Based on the alignment of the 16S rRNA of DH42 with the ARB (Strunk and Ludwig 1996) program's database, *Eu. combesii* was found to have the closest match differing by only four nucleotides in the primer dhb1 sequence (Table 2). During the secondary amplification using 68°C annealing temperature, the estimated 1267-bp fragment was not detected using *Eu. combesii* DNA; instead, it formed a fragment size that is *ca* 1·5 kbp (Fig. 2). A similar 1·5-kbp fragment size was observed in other bacteria when the amount of template for secondary amplification was high (data not shown).

The detection limit of the nested PCR assay was also determined using rumen fluid and corn silage extracts. The estimated 1·5-kbp fragment size was observed in all the samples, although there was a smearing of the bands in the rumen fluid samples that can indicate the abundance of bacterial DNA in the samples. For the rumen fluid samples, stringency of the secondary amplification was sufficient at 66°C annealing temperature and 1·5 mM l^{-1} $MgCl_2$. Under these PCR conditions, it was observed that the lowest inoculation number that can be detected was 10^3 CFU ml^{-1}. Lower annealing temperature for secondary PCR was used when compared with that found in the PCR optimization protocol (66°C vs 68°C) because it was observed that at

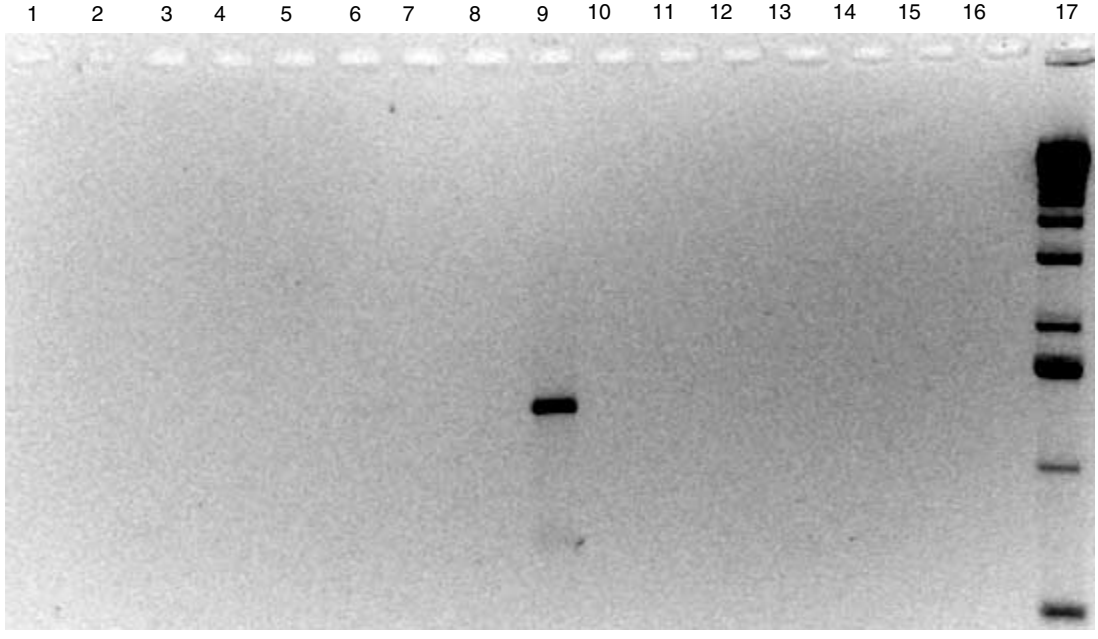

Fig. 3 Secondary nested PCR that targets the 1267-bp fragment amplification using primers dhb1 and dhb2 with the following bacterial strains: lane 1, *Propionibacterium freudenreichii* subsp. *freudenreichii*; lane 2, *P. freudenreichii* subsp. *shermanii* CDC3094; lane 3, *P. freudenreichii* subsp. *shermanii* P92; lane 4, *P. thoenii* P15; lane 5, *P. pentosaceum* P11; lane 6, *P. acidipropionici* (ATCC 25562); lane 7, *P. freudenreichii* subsp. *shermanii*; lane 8, *Propionibacterium* sp. P42; lane 9, DH42; lane 10, *L. sakei*; lane 11, *Weissella confusa*; lane 12, *L. delbrueckii* subsp. *lactis*; lane 13, *B. subtilis*; lane 14, *Bacillus* sp.; lane 15, *B. subtilis* (ATCC 6633); lane 16, *Eu. combesii*; lane 17, 1-kbp DNA ladder

higher annealing temperature, the 10^4 CFU ml^{-1} was the lowest inoculation number that could be detected. With the silage extracts and the same annealing temperature applied, lower $MgCl_2$ concentration (1·0 mM l^{-1}) was used to prevent the nonspecific amplification of the uninoculated samples. Under these conditions, as low as 10^2 CFU ml^{-1} of DH42 can be detected in the silage extracts.

A double-step PCR amplification for the rumen fluid samples was tried to check if selective amplification of DH42 and better detection could be achieved. In the first round of double-step PCR, slightly lower annealing temperature (64°C) was used to allow better amplification since at higher temperature (66–68°C), no primary amplification product was observed in the samples with lower inoculation levels. In the second step, while the temperature was maintained at 64°C, $MgCl_2$ level was reduced to 1·25 mM l^{-1} to provide a more stringent condition and prevent amplification of non-DH42 DNA. In the primary amplification, the uninoculated sample was characterized by the presence of the 1267-bp band (data not shown). This is not unusual as a less stringent condition would allow amplification of nonspecific hybridization products. The presence of other *P. acidipropionici* species in the rumen could have produced this band. In the second amplification, while the decreased amount of $MgCl_2$ prevented the amplification in the uninoculated samples, the 10^3 CFU ml^{-1} of DH42 was the lowest inoculation number that could be detected.

DNA sequencing

The generated DH42 16S rRNA sequence AY360222 was 1529 bp long. The in-house sequencing of the rumen fluid sample PCR product yielded 1267 bp. The alignment of both sequences starting from *E. coli* position 196–767 showed that of the 580 nucleotides six positions differed between DH42 DNA sequence and the fragment amplified from the rumen fluid sample. Differences in the sequences of the DH42 and that of the rumen fluid sample fragment could be due to the sequencing quality and differences in the primer synthesis. Method of primer synthesis and approach to primer purification can affect the quality of the sequencing data obtained in the dye terminator cycle sequencing reactions (Perkin Elmer). BLAST search was performed using the 580-bp sequence of the fragment, and Table 4 shows the top 10 of the 184 BLAST hits on the query sequence. The highest BLAST scores (1048–1066; *E*-value 0·0) and 97–98% sequence identity with DH42 were found for three species: *P. microaerophilus*, *Eu. combesii* and *P. acidipropionici*.

Phylogenetic analysis

On the base of the entire 1529-nucleotide sequence obtained for DH42, BLAST search was also accomplished that generated 244 hits, all of them having *E*-value 0·0. The highest BLAST scores (2387–2778) were found for the same three species: *P. microaerophilus*, *Eu. combesii* and *P.*

Sequences producing significant alignments	Score (bits)	*E*-value
gi|7288855|gb|AF234623·1|AF234623 *Propionibacterium microae*…	1066	0·0
gi|762797|gb|L34614·1|EUBRRDH *Eubacterium combesii* 16S ribo…	1058	0·0
gi|45311|emb|X53221·1|PACP116S *Propionibacterium acidipropionici*…	1048	0·0
gi|45898|emb|X53220·1|PTH16S *Propionibacterium thoenii* part…	897	0·0
gi|45549|emb|X53219·1|PJ16S *Propionibacterium jensenii* part…	847	0·0
gi|25989868|gb|AY154602·1| Uncultured earthworm cast bacter…	623	e-176
gi|7109919|gb|AF131485·1|AF131485 *Saccharopolyspora* sp. IM-…	601	e-169
gi|2644979|emb|AJ003058·1|PPAJ3058 *Propionibacterium propio*…	587	e-165
gi|2644976|emb|AJ003055·1|PAAJ3055 *Propionibacterium avidum*…	587	e-165
gi|6688987|emb|Y17821·1|PRSP17821 *Propionibacterium* sp. V07…	574	e-161

Table 4 Distribution of top 10 BLAST hits on the 580-bp query sequence of the DH42 16S rRNA

Sequences producing significant alignments	Score (bits)	*E*-value
gi|7288855|gb|AF234623·1|AF234623 *Propionibacterium microae*…	2778	0·0
gi|762797|gb|L34614·1|EUBRRDH *Eubacterium combesii* 16S ribo…	2680	0·0
gi|45311|emb|X53221·1|PACP116S *Propionibacterium acidipropionici*…	2387	0·0
gi|45898|emb|X53220·1|PTH16S *Propionibacterium thoenii* part…	2240	0·0
gi|45549|emb|X53219·1|PJ16S *Propionibacterium jensenii* part…	2065	0·0
gi|2644979|emb|AJ003058·1|PPAJ3058 *Propionibacterium propio*…	1764	0·0
gi|2644976|emb|AJ003055·1|PAAJ3055 *Propionibacterium avidum*…	1756	0·0
gi|34106052|gb|AY354921·1| *Propionibacterium* sp. LG 16S rib…	1696	0·0

Table 5 Distribution of top 10 BLAST hits on the 1529-bp query sequence of the DH42 16S rRNA (AY360222)

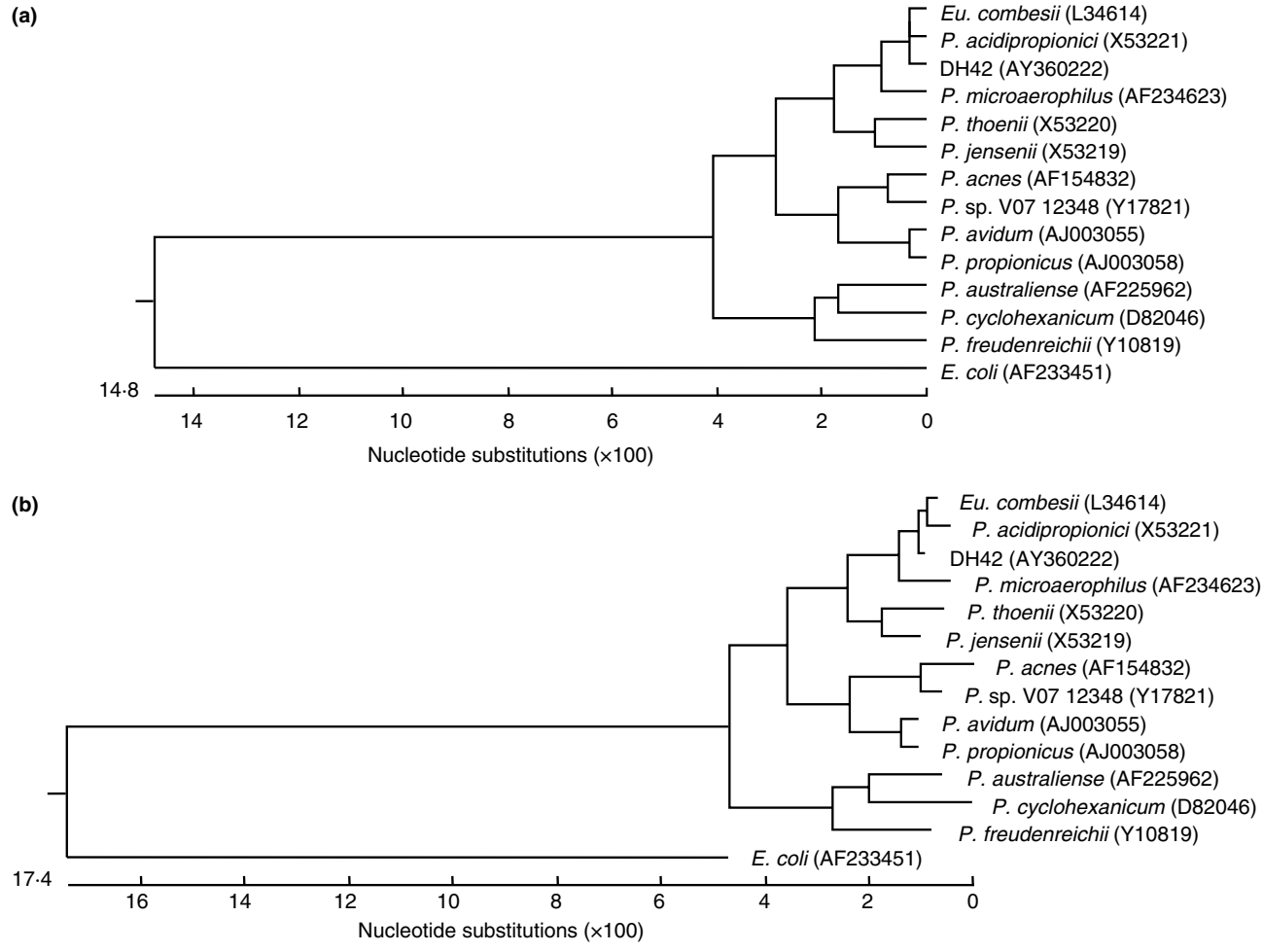

Fig. 4 A neighbour-joining phylogenetic tree of 13 propionibacteria based on the ClustalW alignment analysis of 16S rRNA sequences using Windows 32 MegAlign 5.06 © 1993–2003 (DNASTAR Inc.): (a) a phenogram with balanced branches that averaged the distances between ancestors in the tree; (b) a cladogram with unbalanced branches that forced branch distances to correspond to sequence divergence. The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events

acidipropionici (Table 5), which agree with the above BLAST results for the 580-bp DH42 sequence. This shows that the three organisms are the ones closely related to DH42 based on their 16S rRNA sequences.

For the phylogenetic analysis, total of 12 classified propionibacteria produced by the BLAST search plus DH42 were used. As a result, the phylogenetic relationships were compatible with multiple sequence alignment. DH42 formed a subcluster with the *P. acidipropionici* and *Eu. combesii*, demonstrating high similarity with them (Fig. 4). DH42 was also within the larger cluster of *P. microaerophilus*, *P. jensenii* and *P. thoenii*.

DISCUSSION

Dawson (1994) observed that the metabolic profile of *P. acidipropionici* (ATCC 25562) closely matched that of DH42. This confirms the identity of this strain as *P. acidipropionici*. In the initial identification of DH42, Dawson (1994) had used *Eu. combesii* as a reference and had also observed the similarity of its metabolic profile with DH42. This indicates that the two micro-organisms are closely related, which is not surprising as *Eu. combesii* is considered as an unclassified *Propionibacterineae* representative that is phylogenetically close to the *Propionibacterium* species (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=39481). In the alignment of the propionibacteria-specific primer gd1, Dasen *et al.* (1998) observed that *Eu. combesii* showed 100% similarity with the target sequence. Moreover, they also observed it clustered with *P. thoenii* (92·3%) and had only 78% similarity with other *Eubacterium* species. They concluded the need for re-evaluation of these strains or sequencing problems.

Based on our previous HPLC analysis of *Eu. combesii* overnight culture medium (Dawson 1994), it produced iso-acids but did not produce significant amounts of either propionic acid or acetic acids, which would indicate that it is not a propionibacterium. Moreover, it did not form yellow colonies using Purple Base Agar with i-erythritol while all the propionibacteria species used in this study did (Dawson 1994). During PCR, *Eu. combesii* DNA did not form the 1267-bp fragment with primers dhb1 and dhb2 (Fig. 2). However, the selective nucleotide positions in the primer dhb1 sequence, being located at the 3′ end of the primer (Table 2), are also extremely relevant for the success of amplification in *P. acidipropionici* (ATCC 25562). If such a difference was real, no amplification of the 1267-bp specific fragment should have arisen, as instead happened in almost stringent conditions (*i.e.* annealing at 66–68°C). These observations for *Eu. combesii* and *P. acidipropionici* (ATCC 25562) indicate that their reported sequences may contain errors and, thus, should be checked with newer sequencing methods.

The difference in the detection limit in rumen fluid and corn silage samples could be due to their differences in their microbial load and how these affect amplification efficiency and the presence of contaminants that interfere with PCR. While it can be assumed that the amplification efficiency is the same for all 16S rRNA in the rumen fluid samples, the use of universal primers contains degeneracy that may influence the formation of primer–template hybrids (Wintzingerode *et al.* 1997). Moreover, varying molecular percentage G + C composition of 16S rRNA genes can also cause differential amplification. Templates with lower G + C content will have more efficient strand separation, thus preferential amplification may result (Wintzingerode *et al.* 1997). With the abundance of DNA in the rumen fluid samples, higher competition with non-DH42 DNA could have reduced the amplification of DH42 16S rRNA during primary nested PCR. Consequently, the initial template for secondary amplification may have been less in the rumen fluid samples as compared to the corn silage extracts. Increasing the amount of template for secondary PCR or using less stringent condition such as higher $MgCl_2$ concentration did not improve the sensitivity of the assay. It is also possible that certain amounts of PCR inhibitors are present in the rumen fluid samples making amplification less efficient as compared to the silage extracts. While the DNA extraction kit included a solution to remove PCR inhibitors, it might have been insufficient to remove all the inhibitors in the rumen fluid samples. The presence of humic acids and humic substances in environmental samples and their negative effect in lysis efficiency and PCR amplification had been documented (Wintzingerode *et al.* 1997).

The sequence variability we found between DH42 silage and rumen fluid samples and *P. acidipropionici* (ATCC 25562) could indicate the existence of a subgroup of *P. acidipropionici* strains or even a new species. The propionibacterium DH42 is likely a representative of a taxon that is included in or is strictly related to *P. acidipropionici*. Also, more diverse *P. acidipropionici* strains should be analysed to assert a DH42 strain-specificity of the PCR assay described here. Our data were derived from a single silage sample and a single rumen fluid sample and, therefore, the PCR test developed should be considered, at this stage, as specific for a group of *P. acidipropionici*-related strains. It is possible that other strains more similar to DH42 were absent in our specific experiments but they could be present in other instances, thus giving false positive results in DH42 detection. This must be confirmed or excluded by experiments of detection on more samples.

The phylogenies obtained in this study are concordant with the previous observations (Johnson and Cummins 1972; Charfreitag and Stackebrandt 1989; Pitcher and Collins 1991; Kusano *et al.* 1997; Dasen *et al.* 1998; Meile *et al.* 1999). Phylogenetic identification based on rRNA analysis and exact spatiotemporal quantification of micro-organisms are considered as prerequisites for high quality studies in microbial ecology just as good taxonomy and solid quantification have always been for macroecology (Amann and Ludwig 2000). However, there is a necessity to further clarify the taxonomy of propionibacteria and *Eu. combesii* and, possibly, double check their 16S rRNA sequences in the future studies.

In conclusion, the present study showed that as low as 10^2 and 10^3 CFU ml^{-1} of *P. acidipropionici* DH42 in corn silage and rumen fluid, respectively, can be detected. While the PCR assay is not as sensitive in the rumen fluid, when compared with the silage samples, it appears sufficient considering the current suggested inoculation number of at least 10^5 CFU g^{-1} material in silage. Improvement in the detection of DH42 in rumen fluid samples might be achieved with a different DNA extraction method, use of PCR enhancers or application of more advanced PCR techniques. Judging from the metabolic profile, DH42 closely matched *P. acidipropionici* that is confirmed by its 16S rRNA sequence-based affinity to this species. However, a re-sequencing of 16S rRNA for representative bacteria belonging to the closely related species may be needed to make the classification of DH42 more precise.

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