A novel species-specific PCR assay for identifying *Lactobacillus fermentum*

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Lactobacillus fermentum is a Gram-positive bacterium that is associated with active caries lesions. Methods for identifying *Lactobacillus* species traditionally have been based upon culture methods coupled with biochemical tests, which are generally unreliable. The aim of this study was to develop a species-specific PCR assay for the direct detection of *L. fermentum* in oral clinical samples. PCR primers specific for *L. fermentum* were identified by alignment of bacterial 16S rRNA genes and selection of sequences specific for *L. fermentum* at their 3' ends. PCR positivity for *L. fermentum* DNA was indicated by amplification of a 337 bp product. The primers were shown to be specific for *L. fermentum* DNA, since no PCR product was obtained when genomic DNA from a wide range of other oral bacteria, including closely related *Lactobacillus* species, were used as test species. The PCR assay was then used in an attempt to identify *L. fermentum* DNA in supragingival plaque samples and in pus aspirates from subjects with acute dento-alveolar abscesses. Four out of 70 (5.7 %) supragingival plaque samples analysed were positive for the presence of *L. fermentum* DNA while none of the 19 pus samples analysed was positive for *L. fermentum* DNA. This PCR assay provides a more rapid, specific and sensitive alternative to conventional culture methods for the identification of *L. fermentum* in clinical specimens.

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INTRODUCTION

Members of the genus *Lactobacillus* are Gram-positive organisms belonging to the general category of lactic acid bacteria. They are found in a wide variety of habitats, including vegetation and the gastrointestinal tracts of animals, and are used in the manufacture of fermented foods (Kandler & Weiss, 1986). *Lactobacillus* species are also involved in the progression of carious lesions and have consistently been shown to be associated with caries in humans (Loesche & Syed, 1973; van Houte, 1980; Botha *et al.*, 1998). *Lactobacillus fermentum* is the major heterofermentative *Lactobacillus* species of the human gut (Fons *et al.*, 1997), but it is also widely employed in industrial fermentation and as a starter culture in the dairy industry. Work carried out by Fitzgerald *et al.* (1981) demonstrated that strains of *L. fermentum* were cariogenic in conventional hamsters receiving high-sucrose diets. The organism has since been isolated frequently from active caries lesions (Botha *et al.*, 1998).

The identification of *Lactobacillus* isolates by phenotypic methods is difficult because in some cases it requires determination of bacterial characteristics beyond those of popular fermentation tests (Kandler & Weiss, 1986). Identification of *L. fermentum* involves the isolation of a Gram-positive, non-spore-forming, catalase-negative, non-motile organism. Commercial kits, such as the API 50 CHL system,

are also available to identify *L. fermentum* by analysis of enzymic and sugar-fermentation profiles. Since traditional methods can be time-consuming and often give rise to ambiguous results, more rapid and accurate identification methods are required. Sequence determination of *Lactobacillus* 16S rRNA genes provides an accurate basis for phylogenetic analysis and identification (Vandamme *et al.*, 1996). The sequence obtained from an isolate can be compared with those of *Lactobacillus* species held in public access sequence databases. Although most of the species-specific sequences are contained in the first half of the 16S rRNA gene, identification is usually more accurate if the whole gene is sequenced (Tannock *et al.*, 1999). Universal probes or primers that will anneal to the genes that code for rRNA of all eubacteria can be designed from conserved regions of the 16S rRNA genes, and species-specific primers or oligonucleotide probes can be designed from highly variable regions of 16S rRNA genes (Drake *et al.*, 1996).

There have been several reports of species-specific probes for different lactic acid bacteria derived from 16S and 23S rRNA gene sequences (Hensiek *et al.*, 1992; Hertel *et al.*, 1993; Ehrmann *et al.*, 1994). However, there are no published works on the development of species-specific PCR primers for the detection of *L. fermentum* in clinical specimens. The objectives of this study were to identify species-specific PCR primers that allowed the direct detection of *L. fermentum* in clinical specimens, and to use the PCR assay in the attempted

detection of *L. fermentum* in supragingival plaque samples from healthy subjects and in pus aspirates from subjects with acute dento-alveolar abscesses.

METHODS

Bacterial culture and genomic DNA purification. *Lactobacillus* reference strains were obtained from the National Centre for Type Cultures (NCTC) and ATCC. Bacteria were cultured on Rogosa SL agar (Becton Dickinson; Rogosa *et al.*, 1951) and incubated at 37 °C for 2–3 days under anaerobic conditions in an atmosphere of 85 % N₂, 10 % CO₂ and 5 % H₂. Bacteria were harvested from the plates and genomic DNA extracted using the PureGene DNA Isolation kit (Novara Flowgen) according to the manufacturer’s instructions.

Sample details. In total, 36 archival supragingival plaque samples from healthy subjects and 34 newly collected supragingival plaque samples from healthy subjects participating in a fluoridated milk study were analysed for the presence of *L. fermentum*. Nineteen archival pus samples that had previously been collected from patients with acute dento-alveolar abscesses were also analysed.

Sample collection and processing. Supragingival plaque samples were collected using a sterile curette into sterile tubes containing 1·0 ml freshly prepared fastidious anaerobe broth (Bioconnections). Samples were vortex mixed for 30 s and 100 µl aliquots lysed with 3 µl achromopeptidase (20 U µl⁻¹ in 10 mM Tris/HCl pH 7·0, 1 mM EDTA). Samples were incubated at 56 °C for 30 min, boiled for 10 min and stored at –70 °C until required.

Aliquots (50 µl) of each pus sample were diluted 10- to 100-fold in PCR diluent (10 mM Tris/HCl pH 8·0, 10 mM NaCl, 1 mM EDTA). Thirty microlitres of 10 % SDS and 3 µl proteinase K (10 mg ml⁻¹) were then added to 300 µl diluted pus and incubated at 55 °C for 3 h. Lysed samples were extracted twice with equal volumes of phenol/chloroform (1 : 1) and once with an equal volume of chloroform. DNA precipitation was performed by the addition of 0·1 vol. 3 M sodium acetate (pH 5·3) and 2 vols 100 % ethanol, followed by mixing and incubation at –70 °C for 30 min. Precipitated DNA was collected by centrifugation and the pellet resuspended in 100 µl sterile, molecular biology-grade water.

PCR primers. The alignment of 16S rRNA gene sequences of *Lactobacillus* species and other oral bacteria (Table 1) permitted the identification of primers specific for *L. fermentum*. Two primers were selected that showed sequence specificity for *L. fermentum* at their 3’ ends: 5’-AATACCGCATTACAACCTTG-3’ (LF1; nt 196–215) and 5’-GGTTAAATACCGTCAACGTA-3’ (LF2; nt 529–510), which gave an expected amplification product of 337 bp.

PCR. All PCR reactions were performed in a total volume of 50 µl, comprising 5 µl bacterial DNA template and 45 µl reaction mixture containing 1× PCR buffer (10 mM Tris/HCl pH 9·0, 50 mM KCl, 1·5 mM MgCl₂, 0·1 % Triton X-100), 1·0 U *Taq* DNA polymerase (Promega), 0·2 mM dNTPs and each primer at a concentration of 0·2 µM. The reaction was carried out in an OmniGene thermal cycler (Hybaid). After an initial denaturation step at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min were carried out, followed by a final extension step at 72 °C for 10 min.

PCR quality control. Stringent anti-contamination measures were employed when carrying out PCR, as described previously (Riggio *et al.*, 2000). Positive and negative control reactions were included with each batch of samples analysed by PCR. A standard PCR reaction mixture containing 10 ng *L. fermentum* genomic DNA instead of sample was used as a positive control and the negative control contained water

Table 1. Bacterial 16S rRNA gene sequences used to create a multiple alignment, from which *L. fermentum*-specific PCR primers were derived

Bacterial species	Strain number	GenBank accession no.
<i>Lactobacillus acidophilus</i>	ATCC 4356	M58802
<i>Lactobacillus casei</i>	ATCC 334	D86517
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	ATCC 11842	AY050171
<i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i>	DSM 20072	M58823
<i>Lactobacillus fermentum</i>	ATCC 14931	M58819
<i>Lactobacillus fructivorans</i>	DSM 20203	M58818
<i>Lactobacillus gasseri</i>	DSM 20243	M58820
<i>Lactobacillus hilgardii</i>	DSM 20176	M58821
<i>Lactobacillus mali</i>	DSM 20444	M58824
<i>Lactobacillus mucosae</i>	CCUG 43179	AF126738
<i>Lactobacillus murinus</i>	DSM 20452	M58826
<i>Lactobacillus oris</i>	NCDO 2160	X61131
<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>	JCM 8130	D79212
<i>Lactobacillus pentosus</i>	JCM 1558	D79211
<i>Lactobacillus plantarum</i>	JCM 1149	D79210
<i>Lactobacillus pontis</i>	LMG 14188	AJ422033
<i>Lactobacillus reuteri</i>	DSM 20016	L23507
<i>Lactobacillus rhamnosus</i>	LR3	AY675253
<i>Lactobacillus sakei</i>	DSM 20017	M58829
<i>Lactobacillus salivarius</i> subsp. <i>salicinius</i>	DSM 20554	M59054
<i>Lactobacillus salivarius</i> subsp. <i>salivarius</i>	ATCC 11741	AF089108
<i>Lactobacillus sanfranciscensis</i>	DSM 20451	M58830
<i>Lactobacillus sharpeae</i>	DSM 20505	M58831
<i>Lactobacillus thermophilus</i>	ATCC 8317	M58832
<i>Lactobacillus vaginalis</i>	NCTC 12197	X61136
<i>Lactobacillus vermiforme</i>	ATCC 13133	M59295
<i>Lactobacillus zeae</i>	ATCC 15820	D86516
<i>Bacteroides forsythus</i>	ATCC 43037	AB035460
<i>Helicobacter pylori</i>	ATCC 43504	U01330
<i>Peptostreptococcus anaerobius</i>	ATCC 27337	D14150
<i>Prevotella intermedia</i>	ATCC 25611	L16468
<i>Prevotella nigrescens</i>	ATCC 33563	L16471
<i>Streptococcus intermedius</i>	NCTC 11324	X58311
<i>Streptococcus mitis</i>	NCTC 12261	D38482

instead of sample. PCR products were electrophoresed on 2 % agarose gels stained with ethidium bromide (0·5 µg ml⁻¹) and visualized with an ImageMaster Video Documentation System (Amersham Pharmacia Biotech).

Bacterial strains used as PCR controls. The following *Lactobacillus* type strains were used as PCR controls when testing the specificity of the *L. fermentum* primers: *L. fermentum* ATCC 14931^T; *Lactobacillus salivarius* subsp. *salivarius* ATCC 11741^T; *Lactobacillus delbrueckii* subsp. *bulgaricus* NCTC 12712^T; *Lactobacillus acidophilus* NCTC 1723; *Lactobacillus vaginalis* NCTC 12197^T; *Lactobacillus plantarum* NCTC 1406; *Lactobacillus casei* ATCC 393^T; *Lactobacillus rhamnosus*

NCTC 6375^T, NCTC 10302; *Lactobacillus oris* ATCC 49062^T; *Lactobacillus pontis* ATCC 51518^T; *Lactobacillus reuteri* ATCC 23272^T. Additionally, the following bacteria were also used as PCR controls: *Helicobacter pylori* ATCC 43504^T; *Escherichia coli* NCTC 10418; *Prevotella intermedia* ATCC 25611^T; *Peptostreptococcus anaerobius* ATCC 27337^T; *Prevotella nigrescens* ATCC 25261; *Bacteroides forsythus* ATCC 43037^T; *Streptococcus mitis* NCTC 12261^T; *Candida albicans* NCTC 3153; *Streptococcus intermedius* NCTC 11324^T.

RESULTS

Sensitivity and specificity of the *L. fermentum* PCR assay

Following 35 cycles of amplification, the lower limit of detection of the PCR assay was approximately 50 bacterial cells (data not shown).

The specificity of the *L. fermentum* PCR assay was confirmed by carrying out PCR using 10 ng genomic DNA from the 12 *Lactobacillus* strains and nine other oral bacteria selected as PCR controls. Only *L. fermentum* DNA produced a positive result with the LF1/LF2 primer pair (data not shown), thus confirming the specificity of the assay for *L. fermentum*.

PCR analysis of clinical samples

L. fermentum DNA was detected in four out of 70 (5.7 %) of the supragingival plaque samples analysed. Some of these PCR-positive samples are shown in Fig. 1. Of these samples, 36 were archival samples from healthy subjects and 34 were from subjects participating in a fluoridated milk study. All four PCR-positive samples were from subjects participating in the fluoridated milk study. None of the 19 pus samples analysed by PCR contained *L. fermentum* DNA. Since there was a low incidence of *L. fermentum* in the clinical samples analysed, a random selection of five plaque and three pus samples were spiked with 10 ng *L. fermentum* ATCC 14931^T DNA in order to rule out the possibility of interference from PCR inhibitors within these samples. This confirmed that there was no inhibition of the PCR assay, since *L. fermentum* was detected in all eight spiked samples (Fig. 2).

PCR analysis of clinical isolates

The *L. fermentum* PCR assay was also used to confirm the identity of 19 clinical isolates that had been previously been identified as *L. fermentum* using a 16S rRNA gene PCR-RFLP method (unpublished data) (Fig. 3). An additional four

Fig. 1. Electrophoresis on a 2 % agarose gel of selected PCR products obtained from supragingival plaque samples following PCR with primers specific for *L. fermentum*. Lanes 1 and 19, 100 bp DNA ladder; lane 2, positive control; lanes 3–18, supragingival plaque samples; lane 20, negative control.

Fig. 2. Electrophoresis on a 2 % agarose gel of PCR products obtained from a selection of plaque and pus samples spiked with 10 ng of *L. fermentum* (*Lf*) DNA following *L. fermentum*-specific PCR. Lane 1, 100 bp DNA ladder; lanes 2 and 3, *L. fermentum* ATCC 14931^T; lane 4, plaque 1; lane 5, plaque 1 + *Lf*; lane 6, plaque 2; lane 7, plaque 2 + *Lf*; lane 8, plaque 3; lane 9, plaque 3 + *Lf*; lane 10, plaque 4; lane 11, plaque 4 + *Lf*; lane 12, plaque 5; lane 13, plaque 5 + *Lf*; lane 14, pus 1; lane 15, pus 1 + *Lf*; lane 16, pus 2; lane 17, pus 2 + *Lf*; lane 18, pus 3; lane 19, pus 3 + *Lf*; lane 20, negative control.

Fig. 3. Electrophoresis on a 2% agarose gel of PCR products obtained from 19 clinical isolates identified as *L. fermentum* using a 16S rRNA gene PCR-RFLP method, and four unidentified isolates, following *L. fermentum*-specific PCR. Lanes 1, 14, 15 and 28, 100 bp DNA ladder; lanes 2–6, 11–13 and 16–26, *L. fermentum* clinical isolates; lanes 7–10, unidentified isolates; lane 27, negative control.

Lactobacillus isolates of unknown identity did not give a positive PCR reaction and were therefore confirmed as species other than *L. fermentum* (Fig. 3).

DISCUSSION

The aims of this study were to develop a novel PCR assay that was specific for *L. fermentum* and apply this to the direct detection of *L. fermentum* DNA in clinical samples. The PCR assay was shown to be specific for *L. fermentum* since other bacterial species, including several species within the closely related *Lactobacillus* genus, were not detected with the PCR primers used. The PCR assay detected *L. fermentum* DNA in four supragingival plaque samples from patients participating in a fluoridated milk study, but not in pus aspirates from subjects with dento-alveolar abscesses. Previous 16S PCR-RFLP analysis of *Lactobacillus* species in our laboratory identified a number of possible *L. fermentum* isolates and the use of this species-specific PCR assay was able to confirm the identification of 19 of the isolates as *L. fermentum*, but not a further four isolates that had very similar RFLP profiles.

The identification of *L. fermentum* and other members of this genus has previously been based on colony morphology, Gram-stain reaction, sugar-fermentation profiles and enzymic activities. The API 50 CHL system, which utilizes the characteristics of bacterial sugar fermentation and enzymic activities, has been used for the identification of *L. fermentum*. However, these conventional methods often lead to ambiguous results and even misidentifications. For example, Song *et al.* (1999) compared the results of API 50 CHL tests with DNA–DNA hybridization and showed that only 30.2% of the *Lactobacillus* strains tested were correctly identified by the API 50 CHL tests.

Another problem with characterizing oral members of the *Lactobacillus* genus is that strains found in saliva are phenotypically close (Richard *et al.*, 2001). Discrimination

between *L. rhamnosus* and *Lactobacillus plantarum* has been recorded as difficult due to the small variation in phenotypic characters. Genotypic methods are becoming the most commonly used taxonomic tools for the characterization of lactobacilli, although very few have been developed to enable more accurate identification of oral lactobacilli. As *L. fermentum* has been frequently identified from active caries lesions, it was timely and useful for our present study to develop a rapid and reliable method that could be used for the identification of this species in various oral clinical specimens.

Other molecular techniques have been applied in an attempt to identify *L. fermentum*, mainly from food products. Vogel *et al.* (1994) utilized whole-cell protein patterns and 16S rRNA gene patterns to demonstrate the different phylogenetic locations of *L. fermentum* and *L. plantarum*. Ribotyping (Rodtong & Tannock, 1993; McCartney *et al.*, 1996; Zhong *et al.*, 1998), PFGE (McCartney *et al.*, 1996) and random amplified polymorphic DNA analysis (Veyrat *et al.*, 1999; Nigatu, 2000) have all been applied to the identification of *L. fermentum*. Species-specific oligonucleotide probes have been used for the successful identification of other important *Lactobacillus* species (Nakagawa *et al.*, 1994; Drake *et al.*, 1996; Tilsala-Timisjärvi & Alatossava, 1997; Yasui *et al.*, 1997; Lucchini *et al.*, 1998; Alander *et al.*, 1999; Chagnaud *et al.*, 2001). However, there are no studies that report the use of specific oligonucleotide primers with direct PCR for identifying *L. fermentum* and their application to clinical samples. Song *et al.* (2000) reported the use of a two-step multiplex PCR assay for identifying isolates of 11 human intestinal lactobacilli, including *L. fermentum*. Although the method identified nine *L. fermentum* isolates among a bank of 84 isolates obtained from human stools, the method was not applied to direct detection of *L. fermentum* in clinical samples, where a mixed microflora is invariably present, as we have done in our current study.

In conclusion, this study reports the use of a novel, species-specific PCR assay that can directly detect *L. fermentum* in clinical samples and which can also be used to confirm the identification of isolates obtained from microbiological culture of clinical samples. The application of this assay could be useful in studies investigating the incidence of *L. fermentum* within the oral cavity, especially within saliva, and its influence on the caries process.

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