Comparative analysis of the genes encoding 23S–5S rRNA intergenic spacer regions of *Lactobacillus casei*-related strains

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In this study, investigations into the 23S–5S rRNA intergenic spacer regions (ISRs) of the *Lactobacillus casei* group were performed. A 1.6 kb fragment, from *Lactobacillus paracasei* strain ATCC 27092, containing part of the 5S rRNA gene (60 bp), the 5S–23S spacer region (198 bp) and part of the 23S rRNA gene (1295 bp) was cloned and sequenced (GenBank no. AF098107). This fragment was used as a probe to determine the rRNA restriction fragment length polymorphism (RFLP) patterns of nine strains belonging to the *Lactobacillus casei* group, along with four other non-*Lactobacillus casei* lactobacilli species. A pair of PCR primers, 23-FI and 5-Ru, was designed and used for PCR amplification of the 23S–5S rRNA ISRs of these strains. The ISR length and sequence polymorphisms provided additional information for the taxonomic study of the *Lactobacillus casei* group. The spacer-length polymorphism of *Lactobacillus rhamnosus* was distinct from those of the other strains and this observation is consistent with the classification of *Lactobacillus rhamnosus* proposed by Mori *et al.* For all *Lactobacillus casei* and *Lactobacillus paracasei* strains, two major bands (approx. 250 and 170 bp in size) were obtained except in the case of *Lactobacillus paracasei* subsp. *tolerans* strain NCIMB 9709^T, which yielded only one amplified product (250 bp). The sequencing data of the PCR products of seven well-characterized *Lactobacillus casei* and *Lactobacillus paracasei* strains revealed the presence of a 76/80 bp insertion/deletion with some random, single-base substitutions between the longer and shorter spacers for each respective strain. A few base variations were also detected within different strains in this group although the overall sequence similarity was very high (95.9–99.5%). The rRNA RFLP and the spacer sequence of *Lactobacillus casei* type strain ATCC 393^T exhibited unique identities in this cluster. On the other hand, *Lactobacillus casei* strain ATCC 334 showed a high level of similarity with the other *Lactobacillus casei* strains tested. These observations lend some support to the request for the reassignment of strain ATCC 334 as the neotype of *Lactobacillus casei* subsp. *casei* in place of strain ATCC 393^T.

Keywords: *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus rhamnosus*, 23S–5S intergenic rRNA spacer, length and sequence polymorphism

Abbreviations: ISR, intergenic spacer region; LISR, long intergenic spacer region; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; SISR, short intergenic spacer region.

The GenBank accession numbers for the *Lactobacillus* nucleotide sequences reported in this paper are as follows: AF098107 (*Lactobacillus paracasei* strain ATCC 27092, cloned fragment); AF098108 (*Lactobacillus paracasei* strain ATCC 27092, SISR); AF097704 (*Lactobacillus paracasei* strain NCIMB 9713, LISR); AF097705 (*Lactobacillus paracasei* strain NCIMB 9713, SISR); AF097702 (*Lactobacillus casei* strain ATCC 334, LISR); AF097703 (*Lactobacillus casei* strain ATCC 334, SISR).

INTRODUCTION

The *Lactobacillus casei*-related group contains a number of well-known lactobacilli found in many habitats (dairy products, wine, silage, sewage and the human intestinal tract and mouth). This species is poorly defined and the classification of the *Lactobacillus casei* group is not yet stable. The debates over the classification of this species have been reviewed recently (Mori *et al.*, 1997). The taxonomic reassignments were based mainly on DNA–DNA hybridization (Dicks *et al.*, 1996; Collins *et al.*, 1989), rRNA sequence analysis (Collins *et al.*, 1991) or rRNA-targeted probes (Ferrero *et al.*, 1996; Hertel *et al.*, 1993) and whole-cell protein analysis (Dellaglio *et al.*, 1991). Recently, ribotyping was used (Zhong *et al.*, 1998) to differentiate between lactobacilli strains recovered from the vaginas of healthy premenopausal women.

In prokaryotes, the rRNA genetic loci contain the genes for all three rRNA subunits (the 16S, 23S and 5S genes) and each rRNA gene is separated by an intergenic spacer region (ISR). These ISRs exhibit a large degree of sequence and length variation at the levels of genus and species (Barry *et al.*, 1991; Gürtler & Stanisich, 1996). Within a single genome there are frequently multiple rRNA genetic loci. Spacer regions found within these loci also show a significant degree of variation in length and sequence. It was suggested (Barry *et al.*, 1991) that the spacer regions should be under minimal selective pressure during evolution and therefore should vary more extensively than sequences within genes that have functional roles. Sequence divergence between different species within a genus has already been reported (Frothingam & Wilson, 1994).

Recently, length and sequence polymorphisms in the spacers within the *rrn* loci have been used to discriminate between species of lactobacilli, as well as to identify different dairy and probiotic lactic acid bacteria (Tilsala-Timisjarvi & Alatossava, 1997; Nakagawa *et al.*, 1994). However, the data available on the ISRs of lactobacilli are not sufficient and all the previous studies targeted the 16S–23S rRNA ISRs. In fact, most of the work on rDNA spacer polymorphisms was done to identify or detect the bacteria between and within species (Jensen *et al.*, 1993; Gürtler & Stanisich, 1996). The aim of this work was to clone, sequence and perform intergroup comparisons of the 5S–23S rRNA spacer genes from *Lactobacillus casei*-related strains. A heterologous probe which carries the 5S, the 5S–23S spacer region and part of the 23S rRNA genes from *Salmonella typhi* (ATCC 167) (Zhu *et al.*, 1996) was used to locate the gene in *Lactobacillus casei*. The clone obtained was sequenced. A pair of primers flanking the spacer region was designed based on the sequences and used for amplification of the 5S–23S spacer regions from nine different well-characterized *Lactobacillus casei*-related strains. The genetic relationship between these strains was investigated by means of ISR sequence alignment and inter- and intraspecific variation analysis. In addition, the use of

the 23S–5S spacer as a genetic marker in taxonomic studies was evaluated.

METHODS

Bacterial strains and culture conditions. The strains used in this study are shown in Table 1. Lactobacilli were cultured in MRS (Man–Rogosa–Sharpe) medium (Oxoid) at 37 °C. All the lactobacilli were phenotypically tested by using the API 50CHL system according to the instructions of the manufacturer. *Escherichia coli* TG1, the host for recombinant plasmids, was cultured in Luria–Bertani (LB) broth at 37 °C with shaking. Stock cultures were maintained in soft agar (0.6% agar) slants at 4 °C. Frozen glycerol cultures were used for long-term storage.

Preparation of DNA. Genomic DNA from *Lactobacillus* cells was prepared as described previously (Stahl *et al.*, 1990), with some modification. Cells were harvested from 250 ml/18 h cultures, washed once, resuspended in 20 ml TES (50 mM Tris/HCl, 50 mM NaCl, 1 mM EDTA, pH 8.0) containing 20 mg lysozyme ml⁻¹ and then incubated for 2 h at 37 °C. For different strains, the incubation time may vary. After the addition of 2.5 ml 5% NaCl, 5 ml 10% SDS and 12.5 ml TES, the cell suspension was incubated at 37 °C for another 30 min. Deproteinization was achieved by extraction twice with an equal volume of phenol followed by removal of phenol, once, with chloroform. DNA was precipitated by adding 0.1 vol. 3 M sodium acetate (pH 5.2) and 2 vols ice-cold ethanol, washing in 90 and 70% ethanol, drying and resuspension in TE (10 mM Tris/HCl, 1 mM EDTA, pH 8.0).

Large-scale preparation of plasmid DNA was carried out using the Wizard Plus Maxiprep DNA purification system (Promega). The rapid plasmid mini-preparation procedure was as described elsewhere (Ish-Horowicz & Burke, 1981). For sequencing purposes, the Wizard Plus Miniprep DNA purification system was used, according to the manufacturer's instructions, to obtain high-purity plasmid DNA.

Restriction endonuclease digestion, gel electrophoresis and hybridization. Chromosomal DNA from *Lactobacillus paracasei* ATCC 27092 was completely cleaved with restriction enzymes (New England Biolabs) and fractionated on 0.8% agarose gel. The DNA fragments were then transferred on to a nylon membrane (Hybond-N⁺; Amersham) by conventional Southern blotting. The probe containing the 5S rRNA gene, the 23S–5S spacer and part of the 23S rRNA gene of *S. typhi* was recovered from low-melting-point gel after digestion of pZQ12 with *Eco*RI. Labelling of the probe and the size marker, hybridization and detection of the result were carried out by using the ECL kit (PRN 3001; Amersham) according to the manufacturer's protocol.

Cloning and transformation. Ligation and transformation of competent *E. coli* TG1 were carried out as described previously (Sambrook *et al.*, 1989). The transformants were screened on LB agar plates containing 50 µg ampicillin ml⁻¹ (Sigma), 40 µg X-Gal ml⁻¹ (Sigma) and 20 µg IPTG ml⁻¹ (Sigma).

Sequencing. A set of overlapping deletion subclones was constructed by using the Erase-a-base system (Promega), according to the manufacturer's instructions. The ligated products were used to transform competent *E. coli* TG1. Only the appropriately sized subclones were sequenced. The insert fragment of the recombinant plasmid was sequenced using Sequenase (version 2.0; USB) with the pUC18 forward

Table 1. Bacterial strains used in this study and their PCR products by 23-FI and 5-Ru

No.	Strain	Source	Spacer length (bp)
1	<i>Lactobacillus casei</i> subsp. <i>casei</i> ATCC 334	Emmental cheese	250 and 170
2	<i>Lactobacillus casei</i> subsp. <i>casei</i> NCIMB 8822	Human saliva	250 and 170
3	<i>Lactobacillus casei</i> subsp. <i>casei</i> ATCC 393 ^T	Cheese; NCIMB 11970	250 and 170
4	<i>Lactobacillus paracasei</i> ATCC 27092	Yakult; DSM 20312	250 and 170
5	<i>Lactobacillus paracasei</i> NCIMB 8001	Unknown; ATCC 10746	250 and 170
6	<i>Lactobacillus paracasei</i> subsp. <i>tolerans</i> NCIMB 9709 ^T	Pasteurized milk; ATCC 25599 ^T	250
7	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i> NCIMB 9713	Milking machine; ATCC 25598	250 and 170
8	<i>Lactobacillus rhamnosus</i> NCIMB 6375 ^T	Unknown; ATCC 7469 ^T	170 and 160
9	<i>Lactobacillus rhamnosus</i> ATCC 53103	Human	170 and 160
10	<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> NCIMB 11778 ^T	Yoghurt; ATCC 11842	125
11	<i>Lactobacillus acidophilus</i> NCIMB 8690 ^T	Human; ATCC 4356 ^T	125
12	<i>Lactobacillus fermentum</i> Coll. mir*	Healthy food	120
13	<i>Lactobacillus fermentum</i> Coll. bl*	Yoghurt	120
14	<i>Lactobacillus plantarum</i> Coll. lyk*	Fermented milk	125
15	<i>Lactobacillus plantarum</i> ATCC 14917 ^T	Pickled cabbage; NCIMB 11974 ^T	125

* Species identification was confirmed by using the API 50 CHL kit. Coll, Collection strains deposited as departmental strains and used for PCR.

	23S rDNA	23-FI
1251	gatgcatgga gcgaccagct actaatcggt cgaggactta accaagtaag	
1301	agtgtgagca ggagcgggta gaaaccggag cataagcggg cctgagcgtg	
1351	atggccgggc ttgtgcccatt gccgtcaggg tccttatgtg caggtttctg	
1401	cgactgcgaa cacgttttga tgacaagtac gttagggtca aggagcaat	
1451	taaacaatga tagccagttt tgagagcgca aagttctcat aagtgtggtg	
1501	gcgatagcaa gaaggataca cctgttccca tgccgaacac agaagttaag	
	5-Ru	5S rDNA

Fig. 1. Sequence of the PCR primers and its corresponding region in the 1.6 kb cloned insert (GenBank no. AF098107). Sequences corresponding to the two primers are underlined. Sequences from the 23S and 5S rRNA genes are shown in bold.

and reverse primers provided with the kit. The reaction conditions were as described in the booklet accompanying the kit. Computer searches of EMBL/GenBank were performed using BLAST network services.

Oligonucleotide primers and PCR. A pair of primers, 23-FI and 5-Ru, which flanks the 5S–23S rRNA spacer region, was designed after comparing the sequence obtained for the *Lactobacillus paracasei* ATCC 27092 rRNA gene with the sequences reported in GenBank. The sequences of the primers were as follows: 5'-CGGTGCGAGGACTTAACC-AAG-3' for 23-FI (nucleotides of 23S rRNA gene) and 5'-GGGAACAGGTGTATCCTTC-3' for 5-Ru (complementary sequence of 5S rRNA gene) (Fig. 1) The PCR was performed by using SuperTaq polymerase (HT Biotechnology) in a DNA thermal cycler (Hybaid OmniGene). A typical reaction mixture (50 µl) for the PCR consisted of

10 mM Tris/HCl, pH 9.0, 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100, 0.01% (w/v) gelatin, 8 µM primer, 200 µM dNTP, 2 µl template DNA and 1 U SuperTaq polymerase. The reaction tubes were overlaid with drops of mineral oil. The amplification was carried out at 95 °C for 30 s, 53 °C for 45 s and then 72 °C for 1 min. This was repeated for 35 cycles. The program also included a preincubation at 95 °C for 5 min before the first cycle and an incubation at 72 °C for 5 min after the last cycle. Ten to twenty microlitres of the PCR mixtures were electrophoresed in 3% agarose gel with ethidium bromide.

Sequencing of PCR-amplified 23S–5S rRNA gene spacer region. PCR products were analysed using agarose gel electrophoresis. If different sizes of DNA fragments were found, the major bands were excised from the low-melting-point gel and purified with ultrapure phenol and dissolved in distilled water or TE. This DNA was used as a template in a second round of the PCR. The amplification product was then purified again and used for sequencing purposes. The spacer-region genes were sequenced directly from both ends, without cloning, on an automated sequencer (model 310, Genetic analyser, ABI) by using the Prism BigDye Terminator Ready Reaction Cycle Sequencing kit (ABI). The nucleotide sequences of the ISRs of lactobacilli strains were aligned by MEGALIGN, using the CLUSTAL method (Higgins & Sharp, 1989) to give maximum homology.

RESULTS AND DISCUSSION

Cloning of the rRNA genes of *Lactobacillus paracasei* ATCC 27092

The rRNA genes in the *Eco*RI-cleaved fragments were located by Southern hybridization with the heterologous rRNA gene probe from pZQ12 (Zhu *et al.*, 1996) which carries the 5S rRNA gene, the 23S–5S spacer and part of the 23S rRNA gene of *S. typhi*. One

Fig. 2. Ribotyping of *L. casei* strain rRNA restriction fragments of genomic DNA digested with *Bam*HI (a) and *Pst*I (b) using the cloned 1.6 kb *Hind*III insert as a probe. Lanes: m, λ *Hind*III marker; 1, *L. paracasei* ATCC 27092; 2, *L. paracasei* NCIMB 8001; 3, *L. casei* NCIMB 8822; 4, *L. paracasei* NCIMB 9713; 5, *L. casei* ATCC 334; 6, *L. casei* ATCC 393^T; 7, *L. casei* subsp. *tolerans* NCIMB 9709^T; 8, *L. rhamnosus* ATCC 53103; 9, *L. rhamnosus* NCIMB 6375^T.

of the hybridizing bands, approx. 6 kb in size, was ligated with *Eco*RI-cleaved dephosphorylated pUC18. It was found that a 1.6 kb *Hind*III fragment within the 6 kb insert yielded the most intense hybridizing band against the previous heterologous probe, when the 6 kb insert was further digested and hybridized with the probe. Thus, the 1.6 kb *Hind*III fragment was isolated and religated with *Hind*III-cleaved, dephosphorylated pUC18. This cloned insert was labelled and used as a probe against *Lactobacillus paracasei* ATCC 27092 genomic DNA completely cleaved with *Hind*III. Two hybridizing bands of the same intensity were obtained: one was 1.6 kb in size, as expected, while the other band was approximately 1.5 kb in size. The cloned insert DNA was therefore confirmed to be from *Lactobacillus paracasei* subsp. *paracasei* ATCC 27092.

The 1.6 kb *Hind*III insert was sequenced and the data were compared with the GenBank database. The

results indicated that the cloned fragment contains part of the 23S rRNA gene (1295 bp), the 23S–5S spacer region (198 bp) and part of the 5S (60 bp) rRNA gene. The partial sequences encoding the 23S and 5S rRNA genes were compared with the rRNA data on other lactobacilli in GenBank. According to the published data from GenBank, the 23S rRNA gene of lactobacilli is 2852–2909 bp in length while 5S is 117 bp long. The partial sequence encoding the 23S rRNA of *Lactobacillus paracasei* ATCC 27092 shares 90% homology with the corresponding parts of the 23S rRNA gene of *Lactobacillus delbrueckii* (2852 bp; GenBank no. X68426) and the *Lactobacillus confusus* gene (2852 bp; GenBank no. X68035). The partial rRNA gene encoding 5S has 96% homology with the first 60 bp of the 5S rRNA of *Lactobacillus brevis* (GenBank no. X02026), 94% homology with *Lactobacillus delbrueckii* (GenBank no. X15425) and 93% homology with *Lactobacillus plantarum* (GenBank no. X12886).

rRNA RFLP by hybridization with the homologous probe

The ribopatterns obtained using the 1.6 kb *Hind*III fragment as a homologous probe against *Lactobacillus casei*-group strain genomic DNA digested by *Bam*HI and *Pst*I are shown in Fig. 2, parts (a) and (b), respectively. These two enzymes generated the same powers of differentiation in the ribotyping of *Lactobacillus casei*-group strains. The ribotyping carried out also demonstrated that the rRNA RFLP of strain ATCC 393^T generated by *Bam*HI and *Pst*I formed patterns divergent from those of the other strains tested, whereas the ribotyping patterns of strain ATCC 334 shared two to four restriction bands with the other strains in this group, indicating their close relationship.

PCR of the 23S–5S rRNA ISR from DNA of *Lactobacillus casei*-related strains

To assess the degree of polymorphism in the 23S–5S spacer region among different lactobacilli, particularly the members of the *Lactobacillus casei* group, a pair of primers, 23-FI and 5-Ru, was designed on the basis of the sequence of the 1.6 kb *Hind*III fragment and used to amplify the spacer region of the 23S–5S rRNA genes from six lactobacilli species, including nine strains belonging to the *Lactobacillus casei* group listed in Table 1. A 250 bp PCR product was expected for *Lactobacillus paracasei* strain ATCC 27092 according to the sequence illustrated in Fig. 1. However, two major amplicons approximately 250 and 170 bp long were obtained (lane 2, Fig. 3). The same PCR patterns were observed for the other five *Lactobacillus casei*-related strains (lanes 3–7, Fig. 3) while *Lactobacillus paracasei* subsp. *tolerans* strain NCIMB 9709^T yielded only the 250 bp product (lane 8, Fig. 3). Unlike the other seven *Lactobacillus casei* and *Lactobacillus paracasei* strains, two *Lactobacillus rhamnosus* strains yielded two smaller bands (165 and 170 bp). Two *Lactobacillus plantarum* strains had three major

Fig. 3. PCR products of amplified 23S–5S rRNA gene spacer regions obtained from different *L. casei*-related strains using primers 23-FI and 5-Ru. Lanes: m, 1 kb DNA ladder (Gibco BRL); 1, negative control; 2, *L. paracasei* ATCC 27092; 3, *L. casei* NCIMB 8822; 4, *L. paracasei* NCIMB 9713; 5, *L. paracasei* NCIMB 8001; 6, *L. casei* ATCC 334; 7, *L. casei* ATCC 393^T; 8, *L. paracasei* subsp. *tolerans* NCIMB 9709^T; 9, *L. rhamnosus* NCIMB 6375^T; 10, *L. rhamnosus* GG; 11, *L. plantarum* Coll. lyk; 12, *L. plantarum* ATCC 14917^T; 13, *L. fermentum* Coll. mir; 14, *L. fermentum* Coll. bl; 15, *L. delbrueckii* subsp. *bulgaricus* NCIMB 11778^T; 16, *L. acidophilus* NCIMB 8690^T. Coll., Collection strains deposited as departmental strains and used for the PCR.

amplicons of 400, 350 and 125 bp (lanes 11 and 12, Fig. 3) and two *Lactobacillus fermentum* strains had only one amplicon of 120 bp (lanes 13 and 14, Fig. 3). The PCR-amplified products of *Lactobacillus acidophilus* and *Lactobacillus delbrueckii* were almost the same length (120 bp; lanes 15 and 16, Fig. 3). The 23S–5S ISR sequences of some other lactobacilli and different genera have also been determined. The 23S–5S ISR of *Lactobacillus helveticus* is 71 bp long (GenBank no. Z75484); *Lactobacillus acidophilus* is 69 bp (GenBank no. Z75474) or 71 bp long (GenBank no. Z75468) and that of *Lactobacillus bulgaricus* is 71 bp long (GenBank no. Z75477). The 23S–5S spacer of *Lactococcus lactis* is 96 bp long (GenBank no. Z75493), the *Leuconostoc mesenteroides* subsp. *dextranicum* spacer is 99 bp long (GenBank no. Z75489), *Leuconostoc mesenteroides* has a 99 bp spacer (GenBank no. Z75486) and *Streptococcus thermophilus* has one that is 83 bp in length (GenBank no. Z83759).

Sequence analysis of the 23S–5S ISRs of *Lactobacillus casei*-related group

PCR products of 250 and 170 bp from the 23S–5S rRNA gene-spacer regions of six *Lactobacillus casei* and *Lactobacillus paracasei* strains and the single 250 bp amplicon of *Lactobacillus paracasei* NCIMB 9709^T were sequenced. Our 23S–5S long spacer-region sequence data for *Lactobacillus casei* ATCC 393^T differed at one position (a C instead of a G at 88 bp) from the published data reported (GenBank no. Z75480). The nucleotide sequences of the long intergenic spacer region (LISR) and the short intergenic spacer region (SISR) were 198 and 122 bp long, respectively, by sequence determination. The sequence

Fig. 4. Alignment of nucleotide sequences obtained from two forms of 23S–5S rRNA intergenic spacer region from certain *L. casei*-related strains, including the published corresponding sequence of *L. curvatus* ARILAIT LC59 (GenBank no. Z75471). Alignment of these sequences required the introduction of multiple gaps (shown as hyphens) to maximize the homology. The bases that are identical to those of the top line are indicated by dots. 27092, *L. paracasei* ATCC 27092; 334, *L. casei* ATCC 334; 8001, *L. paracasei* NCIMB 8001; 8822, *L. casei* NCIMB 8822; 9709, *L. casei* subsp. *tolerans* NCIMB 9709^T; 9713, *L. paracasei* NCIMB 9713; 393, *L. casei* ATCC 393^T; Cur, *L. curvatus* LC59 (GenBank no. Z75471); 1, LISR; s, SISR.

alignment is shown in Fig. 4. The likely ends of the 23S and 5S rRNA subunits were deduced after comparison with the published data from GenBank, particularly in the case of the published sequence for the *Lactobacillus casei* ATCC 393^T 23S–5S spacer (GenBank no. Z75480). When sequenced, the single amplicon from *Lactobacillus paracasei* subsp. *tolerans* NCIMB 9709^T turned out to be 198 bp in length. *Lactobacillus casei* ATCC 393^T was found to be different from the other strains in the group and its unique identity is discussed later.

Comparison of the sequences of either the LISR or the SISR cluster indicated that these sequences are highly conserved. The sequences of SISR of *Lactobacillus casei* subsp. *casei* NCIMB 8822 and *Lactobacillus paracasei* NCIMB 8001 are identical; a similar situation prevails for the LISR of *Lactobacillus casei* subsp. *casei* NCIMB 8822 and the single spacer of *Lactobacillus paracasei* subsp. *tolerans* NCIMB 9709^T. The LISR sequences share 98.0–99.5% identity, while the SISR sequences share 95.9–99.2% identity. The

main difference between the long and short spacers of these strains was the presence of a 76 bp insertion/deletion at position 18 bp together with some single or set base differences. At the junction with the extra segment there are a few base differences between the LISR and the SISR of a particular strain. This observation is also applicable to strain ATCC 393 in spite of its lower level of similarity with the other strains. Some of these point substitutions were unique to a single PCR product, indicating that such sequence alterations were random events and probably due to random mutations or PCR errors. For example, at position 161 bp, the A nucleotide was present in all the strains except for the SISR of *Lactobacillus paracasei* NCIMB 9713 (which contained a G). However, other differences seem representative of subspecies. At position 119 bp, there is an A nucleotide in both the LISR and SISR of *Lactobacillus paracasei* NCIMB 8001 and the LISR of *Lactobacillus paracasei* NCIMB 9713, whereas all the others have a G. A similar change occurs at position 117 bp in both the LISR and the SISR of *Lactobacillus casei* strain ATCC 334. Interestingly, all the substitutions at the junction of the gap between the long and short spacers remained the same for most of the strains. At position 6 bp, a nucleotide C in SISR replaced the A of LISR and CA appeared instead of TG at positions 10/11 bp. After the gap, a C in the short spacers replaced all the T in the long fragments. Given the degree of sequence variation observed in our study, the spacer region may differ between different strains of a species. This is contrary to the observation (Berthier & Ehrlich, 1998) that the 16S–23S spacer sequences of different strains within a species (i.e. *Lactobacillus plantarum*, *Lactobacillus paraplantarum*, *Lactobacillus pentosus*, *Lactobacillus curvatus*, *Lactobacillus graminis* and *Lactobacillus sakei*) are 100% identical.

Sequence differences could be incorporated into different copies of the spacer within the genome, as well as differences between isolates and also possible PCR artefacts. To eliminate the possibility of artefacts, a second round of the PCR was carried out. Reamplification of the purified longer fragment produced only one intense band of the same size, without any smaller bands. Hybridization of the *Hind*III 1.6 kb fragment with the total DNA of ATCC 27092 digested with *Hind*III yielded two bands of about 1.5 and 1.6 kb (there is no *Hind*III site within the 1.6 kb fragment). If there were only one copy of the *rrn* operon, then only one hybridizing band of 1.6 kb should be seen when the 1.6 kb *Hind*III fragment was used as a probe against the complete *Hind*III digest of *Lactobacillus paracasei* ATCC 27092 genomic DNA. These results excluded the possibility that the smaller fragment was generated by an artefact of the PCR amplification in which the extra segment was looped out. A similar approach was used for the 16S–23S spacer of *Enterococcus faecalis* (Hall, 1994). This finding confirmed that at least two forms of rRNA operon are present in this species. It has been reported

that in most bacterial species the rRNA genes are present in more than one copy per bacterial genome (Barry *et al.*, 1991) and that the ISRs often differ in both sequence and length. Heterogeneity in the 23S–5S ISR region within one organism has been observed in *Saccharomonospora azurea* K161^T (Yoon *et al.*, 1997), in which three *rrn* loci were investigated. The results revealed that the 23S–5S sequences of *rrnA* and *rrnC* were identical, whereas the other *rrnA* had a spacer that was 2 bp longer. At present, the exact number of rRNA operons for *Lactobacillus* is still unknown. *Lactobacillus lactis* has been shown to contain six *rrn* operons (Tulloch *et al.*, 1991); *Streptococcus thermophilus*, five to six (Pebay *et al.*, 1992); *Lactobacillus acidophilus*, at least four (Roussel *et al.*, 1987); and *Lactobacillus plantarum*, at least five *rrn* operons (Chevallier *et al.*, 1994). As two ISR amplicons were obtained, we suggest that at least two different sets of rRNA operons are present in this *Lactobacillus casei* species. This phenomenon was also reported for the 16S–23S spacer regions of the other micro-organisms (Yoon *et al.*, 1997; Gürtler & Barrie, 1995; Whiley *et al.*, 1995). From this analysis, we have concluded that the 23S–5S rRNA spacer regions of each of the two sets of *rrn* operons of *Lactobacillus casei*-related strains are very similar in sequence whereas *Lactobacillus casei* strain ATCC 393^T showed unique identity in the cluster. *Lactobacillus paracasei* subsp. *tolerans* strain NCIMB 9709^T may contain a spacer-region sequence that is similar and of the same length within different rRNA operons. This assumption is also based on the fact that the single PCR-amplified band of strain NCIMB 9709^T showed a brighter intensity. Although the PCRs were not carried out with quantitative controls, the same amount of template was used for all reactions.

The spacer sequences of *Lactobacillus casei* strain ATCC 393^T were found to be distinct from the sequences of all the other *Lactobacillus casei*-related strains. These unique features lie in the length and sequence heterogeneity. *Lactobacillus casei* strain ATCC 393^T has spacers 197 and 117 bp in length while the others are 198 and 122 bp long. The sequence of the LISR of strain ATCC 393 had only about 80.2% similarity with the LISR of the other strains including *Lactobacillus paracasei* subsp. *tolerans* NCIMB 9709^T, and 72.4% for SISR with the data from the other strains. The deletion of the LISR is also different in that an 80 bp deletion occurred between positions 24 and 104 bp instead of a 76 bp deletion between positions 17 and 93 bp (as in the other strains). Several workers (Dicks *et al.*, 1996; Dellaglio *et al.*, 1991; Mori *et al.*, 1997) have questioned the status of strain ATCC 393 as the type strain although the request for an opinion by Dellaglio *et al.* (1991) was denied by the judicial commission (Wayne, 1994). Their arguments were based on the low level of DNA homology with other strains of *Lactobacillus casei* subsp. *casei* (8–46%) and strains of *Lactobacillus paracasei* (30–50%), protein profile analysis and random amplified poly-

morphic DNA (RAPD) results. On the other hand, *Lactobacillus casei* ATCC 334 was revealed to be genetically closely related to *Lactobacillus casei* subsp. *casei* (71–90% DNA homology) and *Lactobacillus paracasei* (71–91%) strains, to belong to the same protein profile cluster and to share several DNA amplicons with *Lactobacillus paracasei* strains in RAPD. The 23S–5S spacer sequence of strain ATCC 334 determined in this study showed 98–99.5% homology with the LISRs, 95.9–96.7% with the SISRs of other strains in the group. The *Bam*HI and *Pst*I ribotypes of strain ATCC 393, as previously discussed, were distinct from the others in the group, while strain ATCC 334 shared high levels of similarity with them. These results therefore support the request of Dicks and his colleagues to replace strain ATCC 393 with strain ATCC 334 as the neotype of *Lactobacillus casei* subsp. *casei*.

The 23S–5S spacer sequences obtained in this study provide additional information for the taxonomic study of the *Lactobacillus casei* group. The ISR PCR profiles showed that the length polymorphisms correspond well to the proposed reclassification by Collins *et al.* (1989). *Lactobacillus rhamnosus* (NCIMB 6375^T and ATCC 53103) had two different smaller sized bands; *Lactobacillus paracasei* subsp. *tolerans* type strain NCIMB 9709^T yielded only one long spacer although the sequence was identical to the LISR sequence of strain NCIMB 8822. The length polymorphisms distinctively differentiate *Lactobacillus rhamnosus* and *Lactobacillus casei* subsp. *tolerans* strains from other strains in the group. Previously, the 16S rRNA sequence analysis was commonly used in taxonomy study. The 16S rRNA genes of strains of *Lactobacillus casei* JCM 181^T (strain NCIMB 9713), *Lactobacillus paracasei* JCM 1133^T (strain ATCC 27216), *Lactobacillus paracasei* subsp. *tolerans* JCM 1171^T (strain NCIMB 9709^T) and *Lactobacillus casei* ATCC 334 were reported to be identical (Mori *et al.*, 1997). A pair of primers (R16-2 and R23-1R) flanking the 16S–23S rRNA spacer region reported in a previous work (Nakagawa *et al.*, 1994) was applied to the lactobacilli strains tested in our study. It was found that using primers R16-2 and R23-1R, all nine strains in our study yielded the same PCR profile of three major bands ranging in size from 270 to 490 bp. However, the PCR profiles or sequences of the 23S–5S rRNA spacers presented some interspecific variations. The sequence divergence of the 23S–5S ISR therefore allowed better taxonomic differentiation by PCR-ribotyping among the closely related strains of *Lactobacillus casei*-related species.

In the course of comparisons with the reported data on other 23S–5S spacer sequences of lactobacilli in GenBank, a fortuitous discovery was made. A high similarity of 98% was found between the short 23S–5S spacers of *Lactobacillus casei*-related strains and the 23S–5S ISR of strain *Lactobacillus curvatus* ARILAIT LC59 (GenBank no. Z75471). This sequence homology is consistent with the 16S–23S spacer association

(Berthier & Enrich, 1998). The 16S–23S spacer (GenBank no. Z75469) of this strain had very low similarity with the sequences determined in the *Lactobacillus curvatus* group, small spacer (GenBank no. U97132) (67%) and large spacer (GenBank no. U97139) (75%) regions. However, it shares 94.5–100% similarity with the 16S–23S spacer sequences of *Lactobacillus casei*, *Lactobacillus rhamnosus* and *Lactobacillus paracasei* reported in GenBank (GenBank nos U32964, U32966 and Z75479). It was then suggested that this *Lactobacillus curvatus* strain should be reclassified into the *Lactobacillus casei* group. The high level of 23S–5S ISR sequence homology between strain *Lactobacillus curvatus* LC59 and the sequences of the *Lactobacillus casei* strains in our study supports this suggestion. It is not known if a long spacer similar to that in the *Lactobacillus casei* strains is present in this *Lactobacillus curvatus* LC59 strain. To confirm this proposal, the 23S–5S rRNA ISRs of other *Lactobacillus curvatus* strains could be obtained and compared with the sequence of this strain.

No tRNA-like structures were identified within the 23S–5S regions of the strains tested in this study. The central regions of the 16S–23S ISRs of two groups of lactobacilli (i.e. one composed of *Lactobacillus curvatus*, *Lactobacillus graminis* and *Lactobacillus sakei* and another comprising *Lactobacillus paraplantarum*, *Lactobacillus plantarum* and *Lactobacillus pentosus*) were found to contain the genes of tRNA^{Ile} and tRNA^{Ala} (Nour, 1998). So far, there are no reports of the presence of any tRNA gene in the 23S–5S ISR in any bacterium. We argue that this could be responsible for a higher evolution rate for this non-functional spacer region, which possesses great variability in its sequence and length polymorphisms where deletion/insertion and some random base substitutions occurred. In addition, the 23S–5S ISR sequences are smaller and easily sequenced. The sequences of the 23S–5S spacer provide reliable and easily derived information for inferring the closely related strains, which might not be detected or differentiated by 16S rRNA or even 16S–23S spacer sequences. These data are also very useful in determining the taxonomic status of different strains within a species.

This work has demonstrated a polymorphism in the spacer sequences between the genes for the 23S and 5S rRNAs among bacteria in the *Lactobacillus casei*-related group and has indicated its usefulness in identification. The ISR variabilities lie in the length and sequence polymorphisms, in which deletion/insertion or single- and set-base substitutions occur. This makes it a valuable genetic marker, particularly for identification, for differentiation of closely related strains and for phylogenetic analysis of unknown organisms.

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