

Genomic Diversity within the Genus *Pediococcus* as Revealed by Randomly Amplified Polymorphic DNA PCR and Pulsed-Field Gel Electrophoresis

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The genomic diversity of 33 previously assigned strains from six species within the genus *Pediococcus* was assessed by randomly amplified polymorphic DNA (RAPD) PCR and pulsed-field-gel electrophoresis (PFGE). The RAPD PCR patterns produced by two separate random primers, termed P1 (ACGCGCCCT) and P2 (ATGTAACGCC), were compared by the Pearson correlation coefficient and the unweighted pair group method with arithmetic averages clustering algorithm. Pattern variations between repeat samples set a strain discrimination threshold of less than 70% similarity. P1 and P2 primers alone and in combination produced 14, 21, and 28 distinct patterns, respectively. When each strain was assigned with a type strain with which it shared the highest level of similarity, both primers grouped 17 of the 27 strains to their proposed species. PFGE following genomic digestion with the restriction enzymes *ApaI*, *NotI*, and *AscI* produced 30, 32, and 28 distinct macrorestriction patterns, respectively. Specific DNA fragments within the *NotI* and *AscI* macrorestriction patterns for each strain were observed that allowed 27 of the 33 strains to be assigned to their proposed species. For example, following digestion with *AscI*, all *Pediococcus parvulus* strains were characterized by two DNA fragments, one of approximately 220 kb and another between 700 and 800 kb. The exceptions correlated with those observed with both RAPD PCR primers and included three *P. damnosus* and two *P. pentosaceus* strains that grew at temperatures regarded as nonpermissive for their proposed species but not for those with which they grouped.

Pediococci are gram-positive, facultatively anaerobic cocci belonging to the group of lactic acid bacteria. The genus consists of eight species, *Pediococcus acidilactici*, *P. pentosaceus*, *P. parvulus*, *P. dextrinicus*, *P. damnosus*, *P. inopinatus*, *P. halophilus*, and *P. urinaeequi*, although the taxonomic status of the latter two species remains uncertain (10, 13, 16). Lactic acid bacteria have a GRAS (generally regarded as safe) status, and two *Pediococcus* species, *P. acidilactici* and *P. pentosaceus*, have been widely used in the fermentation of vegetables (20), meats (24, 25), dough (32), fruit juices (20), dairy products (5, 22), and silage (7, 14). Both species have been recovered from the gastrointestinal tracts of poultry (19), ducks (21), and other animals (17), and several commercial probiotic feeds containing either species are currently available (39, 42). *Pediocins*, inhibitory to a range of food pathogens, have been isolated from both species and *P. damnosus* (8, 24, 25, 31). Within the brewing industry, *pediococci*, principally *P. damnosus*, occur as primary contaminants in pitching yeast and are among the most prevalent spoilage microbes (35).

Various molecular genetic methods have been used to discriminate between strains in the genus *Pediococcus*. These include the use of specific DNA target probes (23, 27, 28, 29, 37), ribotyping (2, 18, 38), total DNA-DNA hybridization (1, 13), 16S rRNA gene sequencing (2, 10, 21, 33), randomly

amplified polymorphic DNA (RAPD) PCR (21, 29, 30, 32) and pulsed-field gel electrophoresis (PFGE) (3, 12, 24).

RAPD PCR uses low-stringency hybridization conditions with a single random oligonucleotide primer and PCR to amplify DNA sequences from the bacterial genome (43, 45). To date, RAPD PCR studies of *pediococci* have been based on single random primers. An 18-mer primer originally designed to target a *pediocin*-encoding gene on a plasmid from *P. acidilactici* was only effective at discriminating strains of *P. acidilactici*, with 19 strains producing seven distinct RAPD patterns (29, 30). In another study, a 17-mer primer discriminated nine *pediococci* isolated from the gastrointestinal tracts of ducks into four groups and partial 16S ribosomal sequencing indicated that each group represented a single species (21). A 9-mer primer was used to assign proposed *Pediococcus* isolates from fermented cereals to one of six species of the genus (32). Although clear clusters involving all but the *P. dextrinicus* and *P. damnosus* type strains were found, species classification was not confirmed by any other means and more than 40% of the isolates failed to cluster. It remains unclear if RAPD PCR can be used effectively to discriminate strains and species from across the genus.

PFGE, unlike conventional electrophoresis, can resolve DNA fragments of greater than 30 kb and separate a bacterial genome digested with a rarely cutting restriction enzyme into a discrete number of DNA fragments termed a macrorestriction pattern (26, 40). The use of PFGE to discriminate among *pediococci* appears to have been limited to *P. acidilactici* and *P. pentosaceus* strains (3, 12, 24), and in only one case was a large

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number of isolates examined (3). For these two species, PFGE discriminated between both strains and species, with the latter discrimination being based on a difference in cutting frequency with the restriction enzyme *Sma*I (3). However, it is not known how effectively PFGE can discriminate strains and species from across the genus.

The objective of this study was to examine the genomic diversity of a range of strains previously assigned to species within the genus *Pediococcus* by using RAPD PCR with two separate random primers and PFGE with a number of restriction enzymes. From this analysis, we were able to determine the degree of strain diversity evident with both techniques and identify genomic fingerprints specific for each species.

MATERIALS AND METHODS

Strains, media, and growth conditions. The strains used in this study are listed in Table 1. Cultures were grown under anaerobic conditions (anaerobic jars with Anaerocult A gas packs [Merck, Darmstadt, Germany]) in modified MRS (mMRS), comprising Lactobacilli MRS medium (Difco, Detroit, Mich.) supplemented with 0.05% (wt/vol) cysteine hydrochloride at temperatures recommended by each strain supplier (Table 1).

Growth at nonpermissive temperatures. Strains of *P. parvulus* and *P. damnosus* were cultured in broth at 25°C, serially diluted in mMRS agar, and incubated at either 25 or 37°C for 3 and 2 days, respectively. Growth was expressed as the number of CFU per milliliter of the broth culture. Strains of *P. acidilactici* and *P. pentosaceus* were cultured overnight at 37°C in mMRS broth, inoculated at 0.1% (vol/vol), and incubated at 37 or 50°C for 24 h. Increases in culture turbidity were determined with a Hitachi U-1100 spectrophotometer (Hitachi, Tokyo, Japan) set at a wavelength of 550 nm.

RAPD PCR. From a stationary-phase culture, determined by spectrophotometer readings, 1.5 ml of cells was centrifuged at 5,000 × g for 5 min and the DNA was extracted by the method detailed in reference 9 as follows. The resulting cell pellet was resuspended in 200 µl of a solution containing 2% (vol/vol) Triton X-100, 1% (wt/vol) sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 200 µl of phenol-chloroform-isoamyl alcohol. Acid-washed glass beads (0.3 g; Sigma-Aldrich, Dublin, Ireland) were added, mixed with a Sorvall vortex mixer for 2 min, and centrifuged at 10,000 × g for 5 min. The upper layer was removed, and to it 0.1 volume of 3 M sodium acetate and 3 volumes of absolute ethanol at -20°C were added. The mixture was centrifuged at 10,000 × g for 10 min, and the resulting pellet was washed in 70% (vol/vol) ethanol. Centrifugation was repeated, and the pellet was dried at 37°C for 30 min and dissolved in 50 µl of sterile water (9). Two microliters of extracted DNA was used per PCR amplification in a total volume of 50 µl containing 0.25 µM each deoxynucleoside triphosphate (supplied as a 50× deoxynucleoside triphosphate Mastermix; Bioline USA, Inc.), 10 mM (NH₄)₂SO₄, 67 mM Tris-HCl (pH 8.8), 0.1% (vol/vol) Tween 20 (supplied as a 10× NH₄ Reaction Buffer; Bioline), and 5 mM MgCl₂ (supplied as a 50 mM stock; Bioline). Two random primers, termed P1 (5' ACGCGCCCT 3') and P2 (5' ATGTAACGCC 3') (both synthesized by Sigma-Genosys Ltd.), were previously found to yield approximately 10 distinct DNA fragments of 100 to 1,200 bp for a range of lactic acid bacteria (15). The primers were used in separate RAPD reactions at a concentration of 1 µM. The final mixtures were denatured for 6 min at 94°C in a DNA Thermal Cycler (Hybaid Ltd.), paused for the addition of 1 U of BIOTAQ DNA polymerase (Bioline), and subjected to 35 amplification cycles, each consisting of three 1-min stages of 94, 46, and 72°C. PCR products from a 10-µl sample were separated in a 1.5% agarose gel by using a 100-bp ladder (Amersham-Pharmacia-Biotech, Uppsala, Sweden) as a molecular size standard.

Computer comparisons of RAPD PCR patterns. From a negative digitized image of each RAPD PCR pattern, the strains were compared by the Pearson product moment correlation coefficient (*r*) and cluster analysis by the unweighted pair group method with arithmetic averages (21, 32, 34) using the GelCompar version 4 program (Applied Maths, Krottrijk, Belgium). Reproducibility tests were performed by using triplicate DNA preparations for each type strain and a series of independent PCR amplifications. The level of similarity observed between repeats, when included within the dendrogram for all strains, established a discrimination threshold below which RAPD PCR patterns were considered to be different. To determine the ability of RAPD PCR to discriminate *Pediococcus* species, each strain was assigned to the type strain with which it shared the highest level of similarity.

TABLE 1. *Pediococcus* strains used in this study

Species and strain ^a	Source	Growth temp (°C) ^b
<i>P. acidilactici</i>		
NCIMB 6990		37
NCIMB 700993	Summer sausage	30
NCIMB 701851	Silage	30
NCIMB 701860		30
NCFB 2767 ^T	Barley	37
CNCM MA18/5M		37
CNCM MA28/6B		37
<i>P. pentosaceus</i>		
NCIMB 7837	Dairy products	37
NCIMB 12009	Sake mash	37
NCIMB 8106		30
NCIMB 8127		25
NCIMB 8952		25
NCIMB 700814	Pickled cucumber	30
NCDO 1850	Silage	30
NCFB 990 ^T	Dried beer yeast	37
<i>P. inopinatus</i>		
ATCC 49902 ^T	Brewery yeast	30
<i>P. damnosus</i>		
NCIMB 8519	Beer	25
NCIMB 10563	Cider	25
NCIMB 1782	Marinated herring	30
NCIMB 701834		22
NCIMB 701835		22
NCIMB 701864	Japanese beer	22
NCIMB 701866		22
ATCC 29358 ^T	Lager beer yeast	26
<i>P. parvulus</i>		
NCIMB 701107	Cheese	30
NCIMB 701248		30
NCIMB 701558	Hay	30
NCIMB 701559	Cow shed air	30
NCIMB 701852	Silage	30
ATCC 19371 ^T	Silage	30
<i>P. dextrinicus</i>		
DSMZ 20293	Beer	30
DSMZ 20334	Silage	30
DSMZ 20335 ^T	Silage	30

^a Culture collections: NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom; ATCC, American Type Culture Collection, Manassas, Va.; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; NCDO, National Collection of Dairy Organisms (now NCIMB); NCFB, National Collection of Food Bacteria (now NCIMB); CNCM, National Collection of Cultures of Microorganisms, Paris, France. A superscript capital T indicates a type strain.

^b Recommended growth temperature.

PFGE. High-molecular-weight DNA was isolated from 1 ml of a stationary-phase culture, washed once in 1 M NaCl-10 mM Tris-HCl (pH 7.6), and resuspended in 300 µl of the same. The cell suspension was mixed with an equal volume of 2% (wt/vol) low-melting-point agarose (Bio-Rad Laboratories, Richmond, Calif.) in 0.125 M EDTA (pH 7.6), dispensed into molds 10 mm long by 5 mm wide by 1 mm deep, and allowed to solidify at 4°C. The agarose-cell mixture set within each mold was referred to as a plug. Up to three plugs per strain were added to 1 ml of 1 M NaCl-6 mM Tris-HCl-100 mM EDTA-1% (wt/vol) Sarkosyl (Sigma-Aldrich), pH 7.6, containing 10 mg of lysozyme/ml (L-7651; Sigma-Aldrich) and incubated overnight at 37°C. The lysozyme solution was replaced with 1 ml of 0.5 M EDTA-1% (wt/vol) Sarkosyl, pH 8.0, containing 0.5 mg of proteinase K/ml (P-6556; Sigma-Aldrich) and incubated at 37°C overnight. This step was repeated with a fresh proteinase K solution. After two 1-h washes with 1 mM phenylmethylsulfonyl fluoride in 10 mM Tris-HCl-1 mM EDTA (pH 8) at 37°C, the plugs were stored in 10 mM Tris-HCl-100 mM EDTA (pH 8.0) at 4°C. Prior to incubation with selected restriction enzymes, a 1-mm slice (1

mm long by 5 mm wide by 1 mm deep) of the plug was removed and washed three times for 15 min each time in 1 ml of 10 mM Tris-HCl-0.1 mM EDTA (pH 8.0) at room temperature. Each slice was preincubated with 100 μ l of the restriction buffer recommended for the enzyme (all supplied by New England Biolabs, Beverly, Mass.) for 30 min at 4°C and then replaced with 100 μ l of fresh buffer containing 20 U of the restriction enzyme *Apa*I, *Sma*I, *Asc*I, *Not*I, or *Sfi*I. Restriction digestion was performed overnight at temperatures recommended by the supplier. Plug slices were loaded directly into the wells of a 1% (wt/vol) pulsed-field grade agarose (Bio-Rad Laboratories) gel and sealed with 1% agarose. DNA fragments were resolved with a contour-clamped homogeneous electric field DRIII pulsed-field system (Bio-Rad Laboratories) at 6 V/cm for 18 to 24 h with 0.5 \times Tris base-borate-EDTA running buffer maintained at 14°C. Linear ramped pulse times were selected depending on the sizes of the DNA fragments to be resolved, 1 to 15 s for fragments of less than 250 kb (resulting from *Apa*I, *Sma*I, and selected *Not*I digestions), 5 to 50 s for fragments of up to 600 kb (resulting from selected *Asc*I digestion), and 10 to 100 s for fragments of up to 1,500 kb (resulting from *Sfi*I and selected *Asc*I and *Not*I digestions). Gels were stained in distilled water containing 0.5 μ g of ethidium bromide/ml for 30 min and destained for 60 min.

Gel photography and analysis. Both RAPD PCR and PFGE gels were visualized by UV transillumination and photographed with Polaroid film (type 677). Photographs were digitized on a Scanjet 4P scanner with Deskscan II software (Hewlett-Packard). The sizes of PFGE fragments were estimated by using two sets of standards ranging from 2 to 600 kb (low-range PFGE marker no. N0350S) and 225 to 1,900 kb (yeast chromosome marker no. N0345S), both supplied by New England Biolabs.

RESULTS

RAPD PCR strain discrimination. Under the conditions stipulated, RAPD PCR using either the P1 (Fig. 1) or the P2 (Fig. 2) primer produced an average of 9 or 13 DNA fragments per strain, respectively. Fragments ranged from 100 to 2,000 bp, with fragments of greater than 1,000 bp mainly confined to the P2 primer. Repeated RAPD reactions produced patterns with 70 to 95% similarity, and therefore, discrimination was only considered when strains were less than 70% similar. The P1 and P2 primers discriminated 14 and 21 RAPD patterns, respectively. However, five of the P2 patterns were considered borderline, with similarities to other pattern groups ranging from 67 to 69%. Cross-referencing of the RAPD groupings for both primers discriminated the strains further, with a number of strains grouped by one primer being segregated by the other. Overall, 28 distinct RAPD groupings were observed, with only two cases considered borderline.

RAPD PCR species assignment. Both primers assigned 17 of the 27 strains to their proposed species type strain, with 15 of the strains being common to both primers (Fig. 1 and 2). Both primers grouped *P. damnosus* NCIMB 10563, NCIMB 1782, and NCIMB 701834 with *P. parvulus* and *P. pentosaceus* NCIMB 8127 and NCIMB 8952 and *P. dextrinicus* DSMZ 20293 with *P. acidilactici*. *P. acidilactici* NCIMB 6990, NCIMB 70093, and NCIMB 701851 did not group with their proposed type strain but were assigned to different species by each primer.

PFGE strain discrimination. PFGE, following restriction digestion with *Apa*I, produced 30 distinct macrorestriction patterns. The enzyme produced a high number of DNA fragments of generally less than 150 kb (data not shown). Although not all fragments could be fully resolved, due to the large number of fragments, three pairs of strains, *P. acidilactici* CNCM MA18/5M and MA28/6B, *P. pentosaceus* NCIMB 8106 and NCIMB 8952, and *P. pentosaceus* NCIMB 8127 and NCIMB 8952, appeared to have the same macrorestriction patterns.

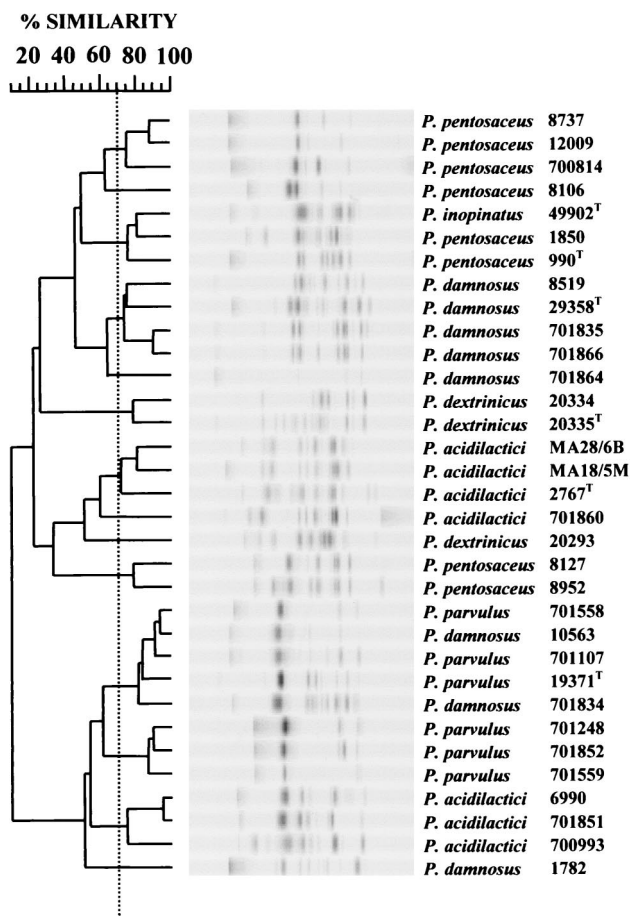


FIG. 1. Dendrogram based on computer comparisons of RAPD PCR patterns produced by random primer P1 with *Pediococcus* strains previously assigned to species within the genus (Table 1). The dendrogram was constructed by using the Pearson product moment correlation coefficient (r) (similarity, $100 \times r$) and the unweighted pair group algorithm method with arithmetic averages. The strain discrimination threshold (dashed line) was set at a similarity value of 70%. A superscript capital T denotes a type strain.

Restriction digestion with either *Not*I (Fig. 3) or *Asc*I (Fig. 4) produced 32 and 28 distinct macrorestriction patterns, respectively. Only strains CNCM MA18/5M and MA28/6B were not discriminated by *Not*I (Fig. 3A, lanes 7 and 8), and additional digestion with *Sma*I and *Sfi*I also failed to distinguish these two strains (data not shown). It seems highly likely that these two isolates are the same strain.

Species discrimination by PFGE. The *Not*I and *Asc*I macrorestriction patterns for the majority of strains appeared to characterize each species, and based on PFGE data presented below, 27 of the 33 strains were assigned to their proposed species.

For *P. acidilactici*, a high frequency of cutting with either *Not*I (Fig. 3A) or *Asc*I (Fig. 4A) distinguished the species from *P. pentosaceus*, *P. parvulus*, *P. damnosus*, *P. dextrinicus*, and *P. inopinatus*. With RAPD PCR for both primers, the *P. acidilactici* strains fell into two categories. Although PFGE clearly distinguished all of the strains as *P. acidilactici*, with *Asc*I, the two categories were again apparent, with strains CNCM MA18/5M, MA28/6B, NCFB 2767 (type strain), and NCIMB

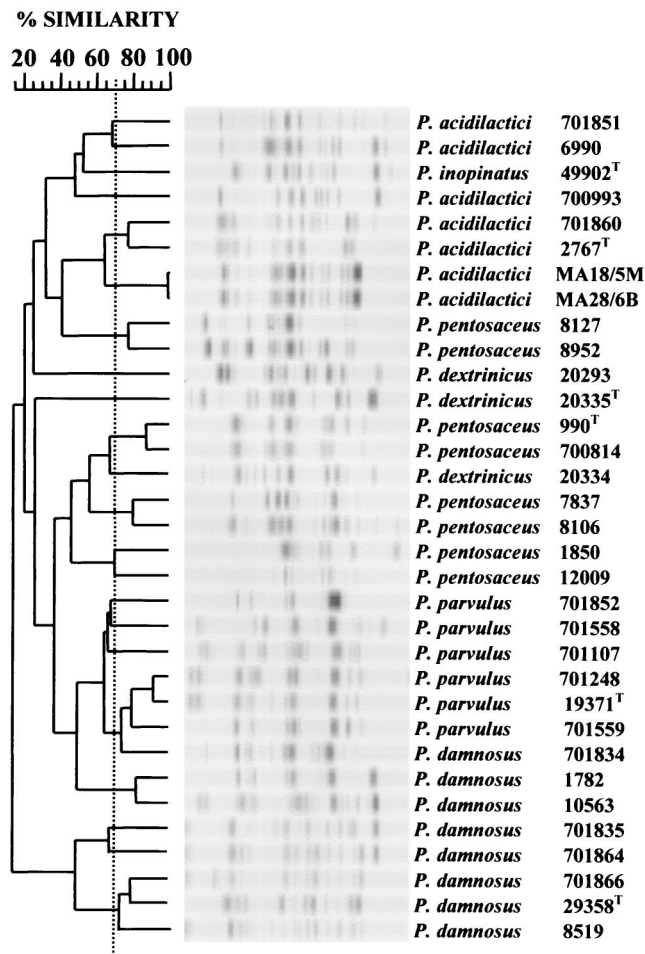


FIG. 2. Dendrogram based on computer comparisons of RAPD PCR patterns produced by random primer P2 with *Pediococcus* strains previously assigned to species within the genus (Table 1). The dendrogram was constructed by using the Pearson product moment correlation coefficient (r) (similarity, $100 \times r$) and the unweighted pair group algorithm method with arithmetic averages. The strain discrimination threshold (dashed line) was set at a similarity value of 70%. A superscript capital T denotes a type strain.

701860 (Fig. 4A, lanes 4 to 7) sharing DNA fragments of approximately 80, 130, and 170 kb. Therefore, both approaches suggest that the species can be divided into at least two genotypic groups and this diversity within the species is in agreement with recent studies (29, 30). Interestingly, two *P. pentosaceus* strains, NCIMB 8127 and NCIMB 8952, which shared the *P. acidilactici* PFGE patterns for *NotI* (Fig. 3A, lanes 9 and 10, and B, lane 5) and *AscI* (Fig. 4A, lanes 2 and 3, and B, lane 6), were also assigned to *P. acidilactici* by both RAPD PCR primers (Fig. 1 and 2). In addition, broth cultures of all *P. acidilactici* strains and *P. pentosaceus* NCIMB 8127 and NCIMB 8952, but not the remaining *P. pentosaceus* strains, grew at 50°C, a permissive temperature for *P. acidilactici* only (16).

The general *AscI* macrorestriction patterns for *P. pentosaceus* (Fig. 4B, lanes 3 to 9) appeared to be characteristic for the species. Only two or three fragments were evident, with one between 340 and 400 kb and another greater than 1,100 kb. Additional marker fragments were found with *NotI* (Fig. 3B,

lanes 2 to 8) at approximately 110 kb and between 720 and 800 kb.

All *P. parvulus* strains digested with *NotI* (Fig. 3C, lanes 11 to 16) produced DNA fragments of approximately 80 and 125 kb and between 280 and 350 kb. Following *AscI* digestion (Fig. 4C, lanes 9 to 14), they shared fragments of approximately 220 kb and 700 to 800 kb.

The general *AscI* macrorestriction patterns for *P. damnosus* (Fig. 4C, lanes 2 and 6 to 8) were characteristic for the species, with very few DNA fragments produced. However, strains NCIMB 1782, NCIMB 701834, and NCIMB 10563 (Fig. 4C, lanes 3 to 5) did not concur with the general *P. damnosus* pattern. Similarly, digestion with *NotI* (Fig. 3C, lanes 3, 6, and 10) produced a fragment of approximately 100 kb and two others of 160 to 220 kb for members of the species, with the exception of strains NCIMB 1782, NCIMB 701834, and NCIMB 10563 (Fig. 3C, lanes 4 to 6). With both restriction enzymes, these three strains actually shared fragments associated with *P. parvulus* and, interestingly, all grouped to *P. parvulus* when RAPD PCR was used. The two species can be distinguished by growth at 37°C, which is nonpermissive for *P. damnosus* but not *P. parvulus* (16). All of the *P. parvulus* strains tested and strains NCIMB 1782, NCIMB 701834, and NCIMB 10563, but not the remaining *P. damnosus* strains, grew at 37°C (Table 2).

Digestion of *P. dextrinicus* DNA with either *NotI* (Fig. 3D, lanes 2 to 4) or *AscI* (Fig. 4C, lanes 15 to 18) failed to identify DNA fragments that were common to only the three *P. dextrinicus* strains. However, if strain DSMZ 20293 was excluded, the remaining two strains shared fragments of approximately 20, 125, and 160 kb with *NotI*. When *AscI* was used, fragments of approximately 45 kb and three of 150 to 250 kb were again common to the two strains but additional strains from the species are required to confirm the species specificity of these fragments.

Only the type strain of *P. inopinatus* was included in the study. Although it did not conform to any of the other species-specific PFGE data, more strains are needed to clarify if PFGE can be used to identify strains of this species.

Species identification based on either *NotI* or *AscI* alone would have resulted in two cases of ambiguous assignment. *P. dextrinicus* DSMZ 20335 had the characteristic *NotI* PFGE features (Fig. 3D, lane 1) of *P. parvulus* and *P. dextrinicus*. Similarly *P. damnosus* NCIMB 10563, which appeared to be *P. parvulus* based on PFGE, RAPD PCR, and growth at selected temperatures, also had the characteristic *AscI* PFGE features of *P. dextrinicus* (Fig. 4C, lane 4). However, in both cases, reference to the second restriction enzyme PFGE patterns confirmed their proposed classification.

DISCUSSION

Both RAPD PCR and PFGE revealed a considerable degree of genomic diversity throughout the genus *Pediococcus*, with most strains being discriminated by either method.

With RAPD PCR, the level of strain discrimination achieved on combination of the data for both primers was higher than that achieved with each primer alone or those previously reported for single primers (21, 29, 30, 32). From the PFGE data, it was apparent that additional genomic differences between

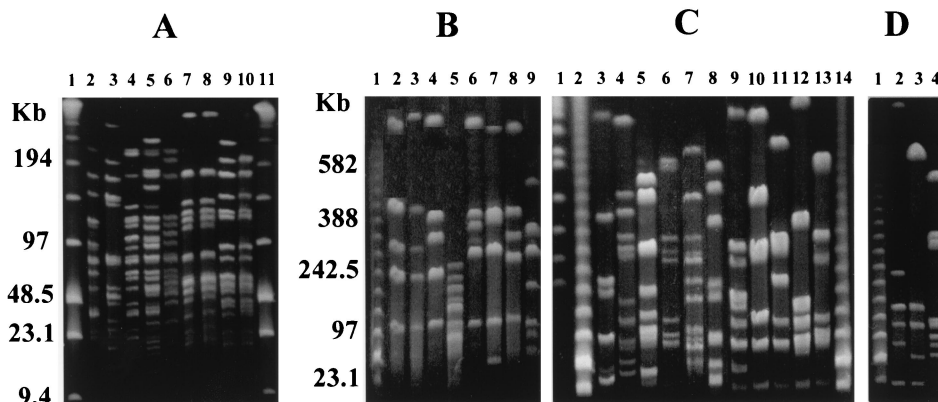


FIG. 3. PFGE macrorestriction patterns for the restriction enzyme *NotI*. Pulse times were 1 to 15 s for the gel shown in panel A and 10 to 100 s for the gels shown in panels B to D. Unless stated otherwise, all strains were NCIMB strains. A superscript capital T denotes a type strain. For species-specific DNA fragments, see Results. Panel A, lanes 2 to 10, *P. acidilactici* 6990, 700993, 701851, 701860, NCFB 2767^T, CNCM MA18/5M, and MA28/6B and *P. pentosaceus* 8127 and 8952. Panel B, lanes 2 to 9, *P. pentosaceus* 7837, 8106, 12009, 8127, 700814, NCDO 1850, and NCFB 990^T and *P. inopinatus* ATCC 49902^T. Panel C, lanes 3 to 16, *P. damnosus* 8519, 10563, 1782, 701834, 701835, 701866, 701864, and ATCC 29358^T and *P. parvulus* 701107, 701248, 701558, 701559, 701852, and ATCC 19371^T. Panel D, lanes 2 to 4, *P. dextrinicus* DSMZ 20335^T, 20334, and 20293. Panel A, lanes 1 and 11, panel B, lane 1, panel C, lanes 2 and 17, and panel D, lane 1, contained molecular size markers (lambda *HindIII* fragments with lambda concatemers). Panel C, lane 1, contained *Saccharomyces cerevisiae* chromosomes.

strains existed and the inclusion of additional primers might have increased the level of strain discrimination still further. Strain discrimination was only considered when RAPD patterns were less than 70% similar, and this was consistent with an earlier study (21). Such experimental variation is well documented for RAPD PCR (11) and limits both the degree of strain discrimination and the establishment of reference patterns that can be used to verify and track specific strains.

Under the conditions used in the present study, PFGE was found to discriminate more strains than RAPD PCR and this agrees with the findings presented for a number of other genera (4, 6, 41). The level of discrimination achieved across the genus agreed well with that previously reported for 67 *P. aci-*

lactici and *P. pentosaceus* strains isolated from human clinical samples that produced 59 distinct macrorestriction patterns following digestion of genomic DNA with either *SmaI* or *NotI* (3). In addition, the high degree of reproducibility observed with PFGE allowed the establishment of reference patterns for the potential tracking of strains. For example, of the 19 *P. acidilactici NotI* macrorestriction patterns presented for isolates recovered from human clinical samples (3), two isolates, 3144-90 and 562-89, exhibited the same *NotI* macrorestriction patterns as *P. acidilactici* NCIMB 700993 and NCIMB 701851, respectively (Fig. 3A, lanes 3 and 4). The *AscI* macrorestriction pattern for NCIMB 700993 (Fig. 4A, lane 9) also matched four *P. acidilactici* strains isolated from fermented sausages (24).

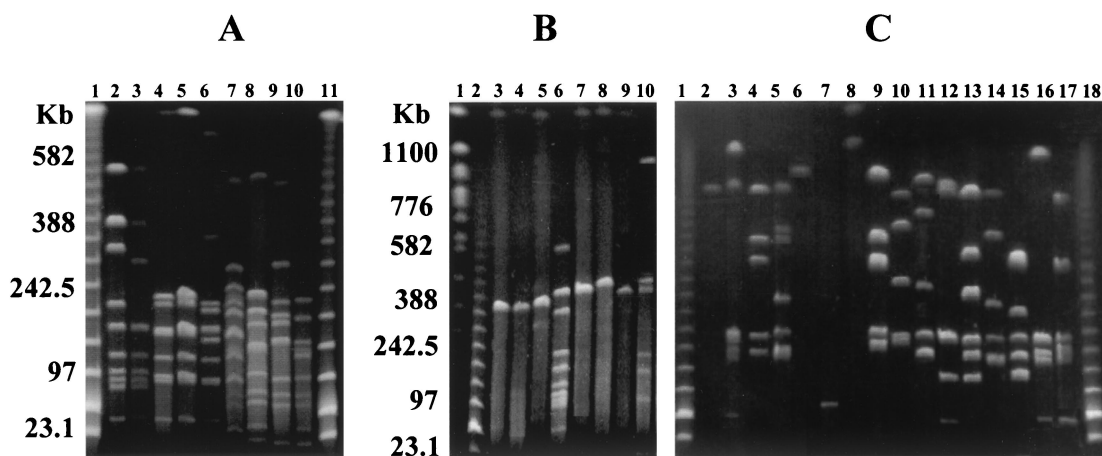


FIG. 4. PFGE macrorestriction patterns for *AscI*. Pulse times were 5 to 50 s for the gel shown in panel A and 10 to 100 s for gels shown in panels B to D. Unless stated otherwise, all strains were NCIMB strains. A superscript capital T denotes a type strain. For species-specific DNA fragments, see Results. Panel A, lanes 2 to 10, *P. pentosaceus* 8127 and 8952 and *P. acidilactici* CNCM MA18/5M, MA28/6B, NCFB 2767^T, 701860, 701551, 700993, and 6990. Panel B, lanes 2 to 9, *P. pentosaceus* 7837, 8106, 12009, 8127, 700814, NCDO 1850, and NCFB 990^T and *P. inopinatus* ATCC 49902^T. Panel C, lanes 2 to 17, *P. damnosus* 8519, 10563, 1782, 701834, 701835, 701864, and ATCC 29358^T, *P. parvulus* 701107, 701248, 701558, 701559, 701852, and ATCC 19371^T, and *P. dextrinicus* DSMZ 20293, 20334, and 20335^T. Panel A, lanes 1 and 10, panel B, lane 1, and panel C, lanes 1 and 18 contained molecular size markers (lambda *HindIII* fragments with lambda concatemers). Panel B, lane 1, contained *S. cerevisiae* chromosomes.

TABLE 2. Colony counts of *P. parvulus* and *P. damnosus* cultures plated at selected temperatures

Strain	No. of CFU/ml at:	
	25°C	37°C
<i>P. parvulus</i> 701248	2.4×10^9	1.9×10^9
<i>P. parvulus</i> 701107	4.2×10^9	2.7×10^9
<i>P. parvulus</i> 19371	2.4×10^9	2.2×10^9
<i>P. damnosus</i> 701864	10^{10}	10^{4a}
<i>P. damnosus</i> 29358	6.0×10^9	10^{4a}
<i>P. damnosus</i> 701835	6.3×10^9	10^{4a}
<i>P. damnosus</i> 1782	3.5×10^9	3.6×10^9
<i>P. damnosus</i> 701834	2.0×10^9	1.8×10^9
<i>P. damnosus</i> 10563	2.9×10^9	3.0×10^9

^a No colonies at the lowest dilution of 10^4 .

Since NCIMB 700993 was also isolated from fermented sausage, they may represent the same strain. Indeed, in another study, two of the four isolates were considered to be identical after ribotyping and genomic restriction followed by conventional gel electrophoresis (18). Although pediococci remain uncommon as etiological agents of disease, their natural vancomycin resistance makes them a growing concern as opportunistic agents associated with human infections (3, 36).

In a previous study, proposed pediococci isolated from fermented cereals were assigned at the species level by using RAPD PCR and a primer identical to P1 (32). Grouping was based on a minimum similarity threshold of 33% and resulted in approximately 60% of the isolates being assigned. By using the same similarity threshold, we found that all strains could be assigned to a type strain. This difference may reflect either (i) a novel or non-*Pediococcus* component within the proposed cereal isolates that were genotypically distinct from the six type strains or (ii) differences in the method of DNA extraction and/or PCR conditions. In addition, from the present study, it was evident that with either primer, approximately 40% of the strains were misidentified. Approximately half of these strains were assigned to the same incorrect species by both primers. This, together with their PFGE fingerprints and growth at selected temperatures, discussed later, implies that they may have been originally misidentified. The remaining strains were assigned to different species by each primer and show that misidentification can arise through assignment by RAPD PCR.

PFGE was recently used to distinguish two *Pediococcus* species, *P. acidilactici* and *P. pentosaceus*, based on a higher frequency of cuts with *Sma*I (3). We similarly found a higher frequency of cuts for *P. acidilactici* with *Not*I and *Asc*I, but reasons for this finding are unclear since the three enzymes do not have a nucleotide sequence in common and the reported G+C contents of the *Pediococcus* species appear to be very similar (16).

In the present study, we have extended the application of PFGE macrorestriction patterns to identify 21 of the 27 strains assigned to *P. acidilactici*, *P. pentosaceus*, *P. parvulus*, *P. damnosus*, or *P. dextrinicus*. The six strains not assigned correctly were grouped to the same incorrect species by RAPD PCR, and it was considered that these strains may have been originally misidentified, but additional classification, by 16S ribosomal sequencing or DNA-DNA hybridization, is required to clarify their taxonomic status. These strains are considered below.

Two *P. pentosaceus* strains grouped with *P. acidilactici* and also grew at 50°C, a permissive temperature for *P. acidilactici* only (16). Problems associated with classification across the two species resulted in the original *P. acidilactici* type strain being reclassified as *P. pentosaceus* (1, 13, 16), and in two recent studies, *P. acidilactici* strains were considered to be *P. pentosaceus* based on gene-specific PCR and RAPD PCR (30) or whole-cell protein profiles (3).

Three *P. damnosus* strains grouped with *P. parvulus* and also grew at 37°C, a nonpermissive temperature for *P. damnosus*. From a number of recent studies using ribotyping (2, 38) and monoclonal antibody reactions (44), it appears that the assignment of *Pediococcus* isolates to *P. damnosus* based on their brewery habitat and carbohydrate fermentation profiles (16, 35) can lead to misidentification. Although none of these involved reclassification as *P. parvulus*, data from total DNA-DNA hybridization (1) and 16S rRNA sequencing (10) have shown a higher level of genetic relatedness between the two species than with any other species of the genus.

One *P. dextrinicus* strain, DSMZ 20293, failed to group with any other *Pediococcus* species by PFGE and was only weakly associated with *P. acidilactici* by RAPD PCR. Unlike the other *P. dextrinicus* strains, which were isolated from silage, DSMZ 20293 was recovered from beer, and in a recent ribotyping study, the strain was considered to be a potentially new *Pediococcus* species (38).

Data from 16S rRNA sequence comparisons and DNA-DNA hybridization reactions between the various type strains of the genus led to the proposal of a three-branched phylogenetic tree comprising *P. damnosus* with *P. parvulus* and *P. inopinatus*, *P. acidilactici* with *P. pentosaceus*, and *P. dextrinicus* alone (10, 13). However, the RAPD PCR and PFGE data for the type strains showed distinct differences between the species. For example, the *P. damnosus* and *P. parvulus* type strains produced very different PFGE patterns and were not associated by RAPD PCR but had the highest degree of interspecies relatedness noted in the genus (1, 10).

In summary, the genomic diversity within the genus *Pediococcus* was determined by using RAPD PCR and PFGE and each technique could prove useful for the rapid classification of pediococci isolated from a variety of sources, including food, feed, silage, beer, and human clinical samples.

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