Genomic subpopulations within the species *Pediococcus acidilactici* detected by multilocus typing analysis: relationships between pediocin AcH/PA-1 producing and non-producing strains

Diego Mora,1 Maria Grazia Fortina,1 Carlo Parini,1 Daniele Daffonchio2 and Pier Luigi Manachini1

Author for correspondence: Diego Mora. Tel: +39 2 23955849. Fax: +39 2 70630829. e-mail: diego.mora@unimi.it

Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, sezione Microbiologia Industriale1 and sezione Microbiologia Agraria, Alimentare e Ecologica2, Università di Milano, via Celoria 2, 20133 Milano, Italy

A high degree of genetic polymorphism among *P. acidilactici* strains was highlighted by a multilocus typing approach analysing several housekeeping genes and by sampling the whole genome using random amplified polymorphic DNA (RAPD) fingerprint analysis performed by using a single primer *pedA* gene targeted in low-stringency amplification conditions. Restriction fragment length polymorphism of the *rpoC, ldhD/L* and *mle* genes, and a modified RAPD analysis, permitted the grouping of *Pediococcus acidilactici* strains in seven genotypes (I–VII). Genotypic results obtained by analysing housekeeping genes involved in the transcription/translation machinery and in primary metabolism were supported by phylogenetic analysis based on the partial 16S rDNA sequencing of a reference strain of each of the seven clusters obtained. Three of the seven genotypes detected showed relationships with pediocin AcH/PA-1 production and carbohydrate fermentation patterns: all pediocin-producing and sucrose-positive strains were grouped in genotype VII, melibiose-, sucrose- and raffinose-positive strains in genotype VI, and arabinose-positive strains in genotype V.

Keywords: *Pediococcus acidilactici*, pediocin AcH/PA-1, multilocus typing, intraspecific genetic variability

INTRODUCTION

The importance of *Pediococcus acidilactici* strains in the food industry is related to their use as starter cultures in fermented meat and vegetable products. This species, like most other lactic acid bacteria (LAB), is involved in extending the shelf life and improving the hygienic quality of various fermented products, via the production of lactic acid and/or the secretion of antimicrobial compounds like bacteriocins (Stiles, 1996). The activity of the bacteriocin AcH/PA-1, produced by some *P. acidilactici* strains, against a broad spectrum of Gram-positive bacteria, including *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium perfringens* and *Clostridium botulinum*, is well known (Bhunia et al., 1988, 1991; Motlagh et al., 1994; Okereke & Montville, 1991; Pucci et al., 1988). Because of this important feature, *P. acidilactici* has been studied for its pediocin production and its possible utilization in biopreservation processes (Schoeman et al., 1999; Stiles, 1996). In the last few years several studies have been carried out to investigate the mode of action of pediocin AcH/PA-1 and to obtain its expression in other species of LAB more suitable than *P. acidilactici* for the preparation of fermented dairy products (Chen et al., 1997; Chikindas et al., 1995; Finland et al., 1996; Horn et al., 1998; Miller et al., 1998; Motlagh et al., 1992).

Despite the commercial importance of *P. acidilactici*, few attempts have been made to investigate the genetic variability in this species and to understand the molecular relationship between pediocin producer and non-
producer strains. *P. acidilactici* has actually been studied only in relation to the DNA G + C content, the level of DNA–DNA homology (Garvie, 1986), and the phylogenetic position by analysis of the sequence of the 16S rRNA gene (Collins et al., 1990). Pediocin ACh/PA-1 producer strains have been studied using whole-genome restriction fragment length polymorphism (RFLP) analysis and by determining the sequence of the plasmid which carries the pediocin operon (Luchansky et al., 1992; Marugg et al., 1992; Motlagh et al., 1994).

The use of different PCR-based techniques addresses the issue of investigating genetic variability, since many methods have been developed for typing DNAs at different levels. Random amplified polymorphic DNA (RAPD) (Welsh & McClelland, 1990) may be used for typing the whole genome while RFLP analysis of PCR products is useful for the typing of discrete genes. As recently noted by Maiden et al. (1998), the use of multiple loci is essential to achieve the resolution required for providing meaningful relationships among strains, since clones diversify as a consequence of mutational and recombinational events, whose rates are different at different genetic loci; strains might be typed incorrectly if only a single locus is examined.

The aim of this study was to investigate the molecular variability of *P. acidilactici* strains, including pediocin ACh/PA-1 producer and non-producer strains, obtained from culture collections and food samples. The molecular genetic characterization of the species *P. acidilactici*, including pediocin producer and non-producer strains, should expand the knowledge on their phylogeny, and should lead to a simple, rapid and reliable identification of strains which may be important in food biotechnology processes. Moreover, the understanding of the molecular relationship between pediocin producer and non-producer strains could help in the assessment of the natural spread of pediocin ACh/PA-1 to other strains of *P. acidilactici*. The issue of the spread of pediocin ACh/PA-1 is particularly interesting because, to our knowledge, only a few strains of *P. acidilactici* producing the pediocin have been isolated worldwide (Ray, 1996).

**METHODS**

**Strains and laboratory media.** The strains of LAB used in this work, their origin and some relevant characteristics are shown in Table 1. Strains were routinely maintained at 4 °C after growth at 37 °C for 12 or 24 h in MRS broth (Difco). For longer-term maintenance, stock cultures were stored in 20 % (v/v) glycerol, 80 % (v/v) MRS at −20 °C and −80 °C. Carbohydrate fermentation was carried out using BSM medium containing chlorophenol red at a final concentration of 4 % (w/v) and the filter-sterilized carbohydrate at a final concentration of 0.2 % (w/v), as described by de Mann et al. (1960).

**DNA preparation.** For the PCR reaction, 100 μl of an overnight culture in MRS broth was added to 400 μl 1× TE buffer (10 mM Tris/Cl, 1 mM Na₂EDTA, pH 8) containing 0.45 mg lysozyme ml⁻¹ (Sigma). This suspension was incubated for 30 min at 37 °C and then SDS and proteinase K (Sigma) were added at a final concentration of 0.6 % (w/v) and 7 U ml⁻¹ respectively. After incubation for 30 min, the solution was extracted with an equal volume of phenol saturated with 1× TE. The DNA was then precipitated by adding 0.1 vol. sodium acetate and 2 vols 95 % ethanol. The DNA pellet was air-dried, dissolved in 50 μl sterilized water (HPLC grade) and stored at −20 °C.

**Primer selection, PCR conditions and restriction analysis.** The sequences of the primers and their description are shown in Table 2. Before the use of the primers in PCR experiments their sequences were checked in the EMBL prokaryotes database using the EBI sequence homology searches, FASTA (Pearson & Lipman, 1988; Pearson, 1990), to ensure that no significant matches with other genes were present. The amplifications of the 16S rDNA and the rpoC, ldhB, ldhD, mleD (mlf) fragments were carried out in a volume of 100 μl containing: 3 μl bacterial DNA solution obtained as above; 0.1 vol. 10X reaction buffer (Amersham Pharmacia Biotech); 200 μM of each dNTP; 2.5 mM MgCl₂; 1 μM each primer (Amersham Pharmacia Biotech); and 0.02 U Taq polymerase ml⁻¹ (Amersham Pharmacia Biotech). The temperature profile used consisted of the following: primary DNA denaturation step at 94 °C for 2 min followed by 5 cycles of 45 s at 94 °C, 45 s at 50 °C and 1 min at 72 °C; 30 additional cycles were carried out increasing the annealing temperature to 55 °C. The amplifications of lpbD, mlf were carried out using the reaction mixture described above and the following temperature profile: primary DNA denaturation step at 94 °C for 2 min followed by 35 cycles of 45 s at 94 °C, 45 s at 60 °C and 45 s at 72 °C. The amplification of the pedA, pedB, pedC and pedD genes was carried out using primers and experimental conditions as previously described (Mora et al., 1998).

The RAPD experiments with pedAF primer were performed in 25 μl final volume using the following temperature profile with a modified ramp at 50 %: primary DNA denaturation step at 94 °C for 2 min followed by 5 cycles of 45 s at 94 °C, 45 s at 50 °C and 1 min at 72 °C; 30 additional cycles were carried out increasing the annealing temperature to 55 °C. For all the amplification cycles the final extension was continued for 7 min at 72 °C. The reproducibility of RAPD patterns was tested by repeating the analysis three times on the same DNA template and also using DNA extracted at different times.

All amplification reactions were performed in a Gene Amp PCR System 2400 (Perkin-Elmer). Following amplification, 5 μl product was electrophoresed at 5 V cm⁻¹ (1.5 % agarose gel, 0.2 μg ethidium bromide ml⁻¹) in TAE buffer.

Restriction digestion of the amplified fragments was carried out for 3 h at 37 °C or at 65 °C in 20 μl reaction mixture containing 7 μl of the PCR product solution, 2–4 μl incubation buffer and 5–10 U of one of the following restriction enzymes: AluI, HaeIII, HpaII, Rsal, TagI, HindIII (Amersham Pharmacia Biotech). Restriction digests were subsequently analysed by agarose electrophoresis (3 %, w/v, agarose gel, containing 0.2 μg ethidium bromide ml⁻¹, TAE buffer) or by polyacrylamide electrophoresis (6 %, acrylamide: bisacrylamide, 29:1, w/w; TBE buffer). The gels were run at 5 V cm⁻¹ in the appropriate buffer, stained in a solution containing 0.5 μg ethidium bromide ml⁻¹ and photographed in UV light.

16S rDNA cloning, sequence determination and phylogenetic analysis. After amplification, 16S rDNA products were cloned.
### Table 1. Tested strains, origin and relevant characteristics

<table>
<thead>
<tr>
<th>Strain</th>
<th>Bacteriocin phenotype</th>
<th>Origin and relevant characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pdi11*</td>
<td>Bac− S</td>
<td>MAAE; sour dough (Mora et al., 1998)</td>
</tr>
<tr>
<td>PG*</td>
<td>Bac− S</td>
<td>MAAE; sour dough (Mora et al., 1998)</td>
</tr>
<tr>
<td>Psp2*</td>
<td>Bac+ R</td>
<td>MAAE; fermented Italian sausages; pediocin AcH/PA-1 producer strain (Mora et al., 1998)</td>
</tr>
<tr>
<td>F†</td>
<td>Bac+ R</td>
<td>Fermented sausages (Ray, 1996); pediocin AcH/PA-1 producer strain</td>
</tr>
<tr>
<td>JD1-23‡</td>
<td>Bac+ R</td>
<td>Commercial meat starter (Ray, 1996); pediocin JD producer strain</td>
</tr>
<tr>
<td>UL5§</td>
<td>Bac+ R</td>
<td>Cheddar cheese (Daba et al., 1991); pediocin S producer strain</td>
</tr>
<tr>
<td>PAC1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAC750F¶</td>
<td>Bac+ R</td>
<td>PAC1.0 cured of the plasmid carrying the pediocin AcH/PA-1 operon (Mora et al., 1998)</td>
</tr>
<tr>
<td>LMG 17674**</td>
<td>Bac− S</td>
<td>Chili bo (Leisner et al., 1999)</td>
</tr>
<tr>
<td>LMG 17680**</td>
<td>Bac− S</td>
<td>Chili bo (Leisner et al., 1999)</td>
</tr>
<tr>
<td>LMG 17677**</td>
<td>Bac− S</td>
<td>Chili bo (Leisner et al., 1999)</td>
</tr>
<tr>
<td>LMG 17692**</td>
<td>Bac− S</td>
<td>Chili bo (Leisner et al., 1999)</td>
</tr>
<tr>
<td>DSMZ 20284†</td>
<td>Bac− S</td>
<td>Barley (Back &amp; Stackebrandt, 1978)</td>
</tr>
<tr>
<td>DSMZ 20238</td>
<td>Bac− S</td>
<td>Mash (Nakagawa &amp; Kithara, 1959)</td>
</tr>
<tr>
<td>ATCC 8042</td>
<td>Bac− S</td>
<td>Mash (Nakagawa &amp; Kithara, 1959)</td>
</tr>
<tr>
<td>ATCC 12697</td>
<td>Bac− S</td>
<td>Origin not known</td>
</tr>
<tr>
<td>ATCC 25740</td>
<td>Bac− S</td>
<td>Plants (Dellaglio et al., 1981)</td>
</tr>
<tr>
<td><em>Lactobacillus</em> sp.</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>NCK 537</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Strains kindly provided by Professor A. Galli Volonterio, Department of Food Science and Microbiology, Agricultural, Food and Ecological Microbiology section (MAAE), University of Milan, Italy.
† Strain kindly provided by Professor Bibek Ray, Department of Animal Science, Food Microbiology Laboratory, University of Wyoming, USA.
‡ Strain kindly provided by Professor Bob Hutkins, Department of Food Science and Technology, University of Nebraska-Lincoln, Lincoln, USA.
§ Strain kindly provided by Dr Eric Emond, Stela Research Centre, Department of Food Science and Nutrition, Laval University, Quebec, Canada.
|| Strains kindly provided by Dr T. R. Klaenhammer, Department of Food Science, College of Agriculture and Life Sciences, North Carolina State University, obtained by Dr G. Giraffa, Istituto Sperimentale Lattiero Caseario, Lodi, Italy.
¶ Strain kindly provided by Dr G. Giraffa, Istituto Sperimentale Lattiero Caseario, Lodi, Italy.
** Belgian Co-ordinated Collections of Microorganisms (BCCM), Laboratorium voor Microbiologie Universiteit Gent (LMG); strains kindly provided by Dr J. Leisner, Department of Veterinary Microbiology, Royal Veterinary and Agricultural University, Frederiksberg, Denmark.
Lactobacillus sp. NCK 537. The absence of zones of growth inhibition around P. acidilactici strains was detected by overlaying colonies with agar containing the pediocin operon was used as the template for the amplification of pedA, pedB, pedC and pedDF fragments as described previously (Mora et al., 1998). The amplified fragments obtained were then DIG-dUTP labelled by random priming with a labelling and detection kit (Boehringer) and used as probes in hybridization experiments. Hybridization was performed according to the manufacturer’s recommendations with prehybridization and hybridization steps in 50% formamide at 42 °C and stringency washed in 0.1× SSC at 55 °C.

**Computation of strain similarities.** A computer similarity analysis was done by means of the Jaccard coefficient (Sneath & Sokal, 1973) and clustering of strains was based on the unweighted pair group method with arithmetic averages (UPGMA). The ntsys-pc computer program (version 1.30) (Rohlf, 1987) was used in the data analysis.

**Antibacterial activity test.** Antibacterial activity from *P. acidilactici* was detected by overlaying colonies with agar containing *Lactobacillus* sp. strain NCK 537 and examining overlay lawns for zones of growth inhibition. *P. acidilactici* strains were grown overnight in MRS liquid medium and then the cells were serially diluted into fresh medium. One millilitre of selected dilutions containing 10–50 cells was added to 9 ml MRS soft agar, and the mixtures were poured into Petri plates containing 10 ml solid MRS agar. The plates were incubated at 37 °C for 24 h until colonies were formed. Ten millilitres of soft MRS agar containing 100 µl of an overnight culture of *Lactobacillus* sp. NCK 537 was subsequently overlaid on each plate. The plates were incubated overnight at 37 °C and examined for zones of growth inhibition around *P. acidilactici* colonies. Detection of pediocin AcH/PA-1 immunity was monitored, as described above, by overlaying *P. acidilactici* PAC1.0 producer colonies with agar containing the *P. acidilactici* strains, instead of *Lactobacillus* sp. NCK 537. The absence of zones of growth inhibition around the PAC1.0 colonies was interpreted as evidence that the strain tested was immune to pediocin AcH/PA-1.

**Determination of the amount of D- and L-lactic acid.** Fifty microlitres of an overnight cell culture in MRS broth were used to inoculate 50 ml of the same medium. After 48 h at 37 °C the cells were collected by centrifugation to determine the dry weight. A 1 ml sample of supernatant, diluted 1:30 (v/v) in distilled water, was used for the determination of D- and l-lactic acid, which was done using the Boehringer kit according to the manufacturer’s recommendations. The amount of D- and l-lactic acid was expressed as g lactic acid per g cell dry weight.

**RESULTS**

**Antibacterial activity test**

Pediocin production was evaluated for all the strains of *P. acidilactici* using *Lactobacillus* sp. NCK 537 as bacteriocin-sensitive test strain as described in Methods. Only strains PAC1.0, F, Psp2, JD1-23 and UL5 showed colonies with zones of growth inhibition. When *P. acidilactici* strains were used as test strains against colonies of strain PAC1.0, zones of growth inhibition were observed for 12 strains (strains DSMZ 20284, DSMZ 20238, ATCC 8042, ATCC 12697, ATCC 25740, PG, Pdi11, LMG 17674, LMG 17680, LMG 17687, LMG 17689 and LMG 17692) while the remaining six strains (PAC1.0, F, Psp2, JD1-23, UL5 and the PAC1.0 cured strain PAC750F) were immune to pediocin AcH/PA-1. The results for strain PAC750F confirmed that this strain had acquired resistance to the pediocin despite the loss of the plasmid-encoded genetic determinant of immunity, as suggested by Venema et al. (1995). Bacteriocin phenotypes of the tested strains are summarized in Table 1.

**Table 2.** Sequence and description of the primers, and plasmid used in PCR experiments

<table>
<thead>
<tr>
<th>Primer/ plasmid</th>
<th>Sequence (5’→3’)</th>
<th>Description*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16F</td>
<td>AGAGTTTGATCCTGGCTCAG</td>
<td>16S rRNA, 1500</td>
<td>Lane (1991)</td>
</tr>
<tr>
<td>16R</td>
<td>CTACCGCTACCTTGTACGA</td>
<td>Lane (1991)</td>
<td></td>
</tr>
<tr>
<td>rpoC</td>
<td>TTGATCGATATATAAATTGAA</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>rpoR</td>
<td>GGAAGGTGTTGATAATCAGCTAC</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>ldhF</td>
<td>ATGTCGAATTCCAAATCATCA</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>ldhL</td>
<td>TTATTTGCTTTTTCAGCGAG</td>
<td>ldhL, 972</td>
<td>This study</td>
</tr>
<tr>
<td>ldhR</td>
<td>GGACCTTGAACGTAACCCGC</td>
<td>Mora et al. (1997)</td>
<td></td>
</tr>
<tr>
<td>ldhF</td>
<td>GTTCCGTCTTGGATTGGCC</td>
<td>ldhD, 449</td>
<td>Mora et al. (1997)</td>
</tr>
<tr>
<td>mleF</td>
<td>ACCATGCGGTACGATCGA</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>mleR</td>
<td>CAGCTCAAAGTCTTCAAGT</td>
<td>mle, 441</td>
<td>This study</td>
</tr>
<tr>
<td>pedAF</td>
<td>ATACTACGGAATGGGTGTT</td>
<td>pedA</td>
<td>This study</td>
</tr>
<tr>
<td>PSRQ220†</td>
<td>Plasmid containing the pediocin PA-1 operon</td>
<td>J. Kok †</td>
<td></td>
</tr>
</tbody>
</table>

* Gene target and size (bp) of the amplified fragment.
† Plasmid kindly provided by Dr Jan Kok, Department of Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, The Netherlands.
Metabolic profiles

The *P. acidilactici* strains were analysed for their ability to ferment several carbohydrates. All the tested strains were able to acidify BSM medium in the presence of trehalose, D-xylose, D-glucose, D-fructose, D-mannose and galactose as sole carbon source, after 24 h incubation at 37 °C. Weak acidification was detected for all the strains in the presence of rhamnose after more than 48 h incubation. Strains Pdi11 and PG acidified the medium in the presence of 1-arabinose; strains PAC1.0, Psp2, F, JD1-23, UL5, PAC750F, LMG 17680 and LMG 17689 fermented sucrose. Strains LMG 17680 and LMG 17692 fermented melibiose and raffinose. None of the strains fermented inositol, sorbose, maltose, lactose or D-melezitose.

**RAPD analysis and amplification of the pediocin AcH/PA-1 operon genes**

In order to type *P. acidilactici* strains and to differentiate the pediocin producer strains, RAPD analysis was carried out using, in low-stringency conditions, a primer targeted to the plasmid gene (*pedA*) encoding pediocin AcH/PA-1 (Marugg *et al.*, 1992; Motlagh *et al.*, 1994). The reproducibility of RAPD analysis was tested by comparing the amplified patterns of different DNA extractions and of several amplification reactions. As shown in Fig. 1, the tested strains showed six amplification pattern profiles. One profile, typical of strains DSMZ 20284, DSMZ 20238, ATCC 12697 and ATCC 25740, showed three main amplified fragments of about 450, 950 and 2200 bp. The 450 bp fragment was also present in the remaining strains, with the exception of ATCC 25740. An analogous pattern was observed from strains LMG 17674, LMG 17687 and LMG 17689, which showed the 450 and 950 bp fragments. A marked 700 bp amplified fragment and a 1350 bp band permitted grouping of all the pediocin producer strains (PAC1.0, Psp2, F, JD1-23, UL5) and the PAC1.0 cured strain PAC750F. Identical RAPD profiles were observed among the pediocin producers and the PAC1.0 cured strain, suggesting that the plasmid harbouring the pediocin determinant was not involved in the amplification. Moreover Southern hybridization performed on the RAPD patterns profiles using *pedAf*, *pedBi*, *pedCf* and *pedDf* fragments as probes, showed that none of the RAPD fragments originated from the pediocin AcH/PA-1 operon despite the fact that a primer targeted at the *pedA* gene was used (data not shown).

Identical RAPD pattern profiles were observed among strains PAC1.0, F and Psp2, which were known to carry the plasmid harbouring the pediocin AcH/PA-1 operon, and strains JD1-23 and UL5, suggesting that these strains are highly related. PCR experiments performed with primers targeted to the four genes involved in pediocin AcH/PA-1 production, as previously described (Mora *et al.*, 1998), confirmed that strains JD1-23 and UL5 also had the genetic determinants related to pediocin AcH/PA-1 production. Analogous PCR experiments performed on all the other non-producing strains gave negative results.

**Primary metabolism housekeeping genes: restriction analysis of the ldhD and ldhl genes**

To investigate if the variability highlighted by RAPD analysis was the consequence of a wide and consolidated genetic diversity among the tested strains rather than the effect of the presence of minimal sequence variation fortuitously detected by the *pedAF* primer, several housekeeping genes were analysed. Restriction analysis of the 449 bp *ldhDF* amplified fragment, previously used for the specific PCR identification of the *P. acidilactici* strains (Mora *et al.*, 1997), was carried out using five four-cutting restriction enzymes (*AluI*, *HaeIII*, *HhaI*, *HpaII*, *RsaI*). While restriction analysis using *HaeIII* and *HpaII* did not reveal any sequence variation in the *ldhDF* fragments, restriction analysis of the *HhaI* fragments permitted the differentiation of strains Psp2, F, JD1-23, UL5 and PAC1.0 into three main profiles: two profiles (M and 1–5) and one profile (6–8).

**Fig. 1.** RAPD patterns of *P. acidilactici* strains, using the primer pedAf. Lanes 1–5, strains DSMZ 20284, DSMZ 20238, ATCC 8042, ATCC 12697 and ATCC 25740; lanes 6–8, strains LMG 17674, LMG 17674 and LMG 17689; lanes 9 and 10, strains Pdi11 and PG; lanes 11–15, strains PAC1.0, Psp2, UL5 and JD-23; lanes 16 and 17, strains LMG 17680 and LMG 17692; M, molecular mass marker 100 bp ladder (Amersham-Pharmacia Biotech).
**Fig. 2.** (a) Agarose gel electrophoresis showing the restriction profiles of amplified *ldhD* fragment of *P. acidilactici* strains obtained using *Hha* (lanes 1–6) and the double digestion *Alu* + *Rsa* (lanes 7–12). Lane 1, strain DSMZ 20284;
fragments, an unexpected polymorphism was detected using the remaining three enzymes. Based on the results obtained (Fig. 2a, Table 3) the strains were clustered in four distinct sequence groups. The first group was constituted by strains DSMZ 20238, ATCC 8042 and ATCC 12697; the second sequence cluster included all the pediocin producer strains (PAC1.0, Psp2, F, JD1-23, UL5), two chili bo isolates (LMG 17680, LMG 17692) and the PAC1.0 cured strain; the third group included strains DSMZ 20238, ATCC 8042, and one typical of strains Pdi11, PG, PAC1.0, Psp2, F, JD1-23, UL5, LMG 17674, LMG 17680, LMG 17692 and the PAC1.0 cured strain; the fourth group was constituted by strains Pdi11 and PG. It is interesting to note that the sizes of the restriction fragments of the amplified \(ldh\text{Df}\) were in agreement with the theoretical analysis of the published sequence (Garmyn et al., 1995a) only for the pediocin producer strains.

A lower degree of variability was observed when the \(ldhL\) gene was analysed. The entire 972 bp coding sequence of the \(ldhL\) gene (\(ldh\text{Lf}\)) was amplified using the primers (Table 2) selected from published sequence (Garmyn et al., 1995b). Restriction analysis was performed using the above-mentioned five restriction enzymes. Restriction analysis using HhaI and HaeIII highlighted three different \(ldhL\) alleles among the 17 strains analysed, while no differences were detected using AluI, HpaII and Rsal. One sequence was characteristic of strain DSMZ 20284\(^7\), one typical of strains DSMZ 20238, ATCC 8042, ATCC 12697 and ATCC 25740, and one typical of strains Pdi11, PG, PAC1.0, Psp2, F, JD1-23, UL5, LMG 17674, LMG 17680, LMG 17687, LMG 17689, LMG 17692 and the PAC1.0 cured strain PAC750F. The sizes of the restriction fragments

\begin{table}[h]
\centering
\caption{PCR and restriction patterns of the tested strains}
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textbf{P. acidilactici strain} & \textbf{PCR and RFLP pattern*} & \textbf{Bacterial genotype†} \\
& \textbf{rpoC\text{I} restriction} & \textbf{LdhL\text{f} restriction} & \textbf{ldhD\text{f} restriction} & \textbf{mlef restriction} & \textbf{RAPD} \\
\hline
DSMZ 20284\(^7\) & C & D & E & F & G & I \\
DSMZ 20238 & C & D1 & E1 & F & G & II \\
ATCC 8042 & C & D1 & E1 & F & G & III \\
ATCC 12697 & C & D1 & E1 & F & G & \\
ATCC 25740 & C & D1 & E & F & G1 \\
LMG 17674 & C & D2 & E & F1 & G2 & IV \\
LMG 17687 & C & D2 & E & F1 & G2 & \\
LMG 17689 & C & D2 & E & F1 & G2 & \\
Pdi11 & C1 & D2 & E2 & F2 & G3 & \\
PG & C1 & D2 & E2 & F2 & G3 & \\
LMG 17680 & C2 & D2 & E3 & F1 & G4 & VI \\
LMG 17692 & C2 & D2 & E3 & F1 & G4 & \\
PAC1.0 & C1 & D2 & E3 & F2 & G5 & \\
PAC750F & C1 & D2 & E3 & F2 & G5 & \\
Ped F & C1 & D2 & E3 & F2 & G5 & \\
JD1-23 & C1 & D2 & E3 & F2 & G5 & \\
UL5 & C1 & D2 & E3 & F2 & G5 & \\
Psp2 & C1 & D2 & E3 & F2 & G5 & \\
\hline
\end{tabular}

* Letter/number codes indicate different DNA haplotypes.
† Bacterial genotypes obtained by UPGMA computer analysis.
\end{table}
obtained were in agreement with the theoretical analysis of the sequence only for the \(ldhL\) fragments amplified from the group of strains comprising the pediocin producers (Fig. 2b, Table 3).

**Primary metabolism non-housekeeping genes: restriction analysis of the \(mle\) gene**

Restriction analysis of the first 441 bp of the gene encoding malolactic enzyme (\(mle\)) was carried out using \(AluI\), \(HhaI\) and \(RsaI\). \(AluI\) digestion did not permit discrimination among the strains tested, while \(RsaI\) and \(TaqI\) highlighted three different \(mle\) alleles: one characteristic of strains DSMZ 20284\(^T\), DSMZ 20238, ATCC 8042, ATCC 12697 and ATCC 25740, one characteristic of pediocin-producing strains and of PG and Pdi11 strains, and another typical of all chili bo isolates. The sizes of the restriction fragments obtained were in agreement with the theoretical analysis of the sequence published (Groisillier & Lonvaud-Funel, 1999) only for the \(mle\) fragments amplified from the group of strains obtained from DSMZ and ATCC (Fig. 2c, Table 3).

**Housekeeping gene involved in transcription and translation machinery: \(rpoC\)**

Further genetic investigation was then carried out on the \(rpoC\) gene to investigate if the high degree of variability highlighted by the previous analysis was also present in this more conserved chromosomal region. When the first 2000 bp of the coding region of the \(rpoC\) gene (\(rpoCf\)) was analysed, the degree of sequence variation observed was similar to that detected by analysing the \(ldb\) genes: three \(rpoC\) alleles were observed using \(AluI\), \(RsaI\), \(HpaI\) and \(HindIII\). One sequence was characteristic of strains DSMZ 20284\(^T\), DSMZ 20238, ATCC 8042, ATCC 12697, ATCC 25740, one characteristic of pediocin-producing strains and of PG and Pdi11 strains, and another typical of all chili bo isolates. The sizes of the restriction fragments obtained were in agreement with the theoretical analysis of the published sequence (Morse et al., 1996) only for the \(rpoCf\) fragments amplified from strains DSMZ 20284\(^T\), DSMZ 20238, ATCC 8042, ATCC 12697 and ATCC 25740 and for strains LMG 17674, LMG 17680 and LMG 17682. The sizes of the restriction fragments obtained were in agreement with the theoretical analysis of the published sequence (Morse et al., 1996) only for the \(rpoCf\) fragments amplified from strains DSMZ 20284\(^T\), DSMZ 20238, ATCC 8042, ATCC 12697 and ATCC 25740 and for strains LMG 17674, LMG 17680 and LMG 17682.

**Numerical analysis of PCR and restriction profiles**

A computer similarity and clustering analysis of \(P\). \(acidilactici\) strains was performed on the data from all the PCR and restriction experiments. A UPGMA dendrogram derived from similarity coefficients calculated by the Jaccard method (\(S_J\), shown on the scale at the top), showing the relationship among \(P\). \(acidilactici\) strains analysed by RAPD and by restriction analysis of \(mle\), \(ldhD\), \(ldhL\) and \(rpoCf\) fragments. Roman numerals refer to the bacterial genotypes obtained. The shaded circle highlights the pediocin producer strains.

**16S rDNA partial sequencing and phylogenetic analysis**

Two strains for genotype VII (PAC\(1.0\) and Psp2) and one strain for each of genotypes I to VI (DSMZ 20284\(^T\), DSMZ 20238, ATCC 25740, LMG 17674, Pdi11 and LMG 17680) were selected for 16S rDNA sequencing. Sequences were obtained for the region 8–524 (\(E\). \(coli\) numbering) of the 16S rDNA, encompassing the variable regions V1, V2 and V3. The sequences of each of the eight strains were used to search the GenBank and EMBL databases with the aim of finding the closest LAB
Table 4. 16S rDNA signature sequences of *P. acidilactici* strains

GenBank accession numbers for the sequences used in this table and in the construction of the phylogenetic tree (Fig. 4) are: *P. acidilactici* DSMZ 20284\(^\dagger\), M58833 (Collins *et al*., 1990); DSMZ 20284\(^\dagger\), AJ249535 (this work); DSMZ 20238, AJ249539; ATCC 25740; AJ249537; LMG 17680, AJ249891; LMG 17687, AJ249538; PAC1.0, AJ249893; Psp2, AJ249894; Pdi11, AJ249892; LA3, AB018213; LA35, AB018214; *P. pentosaceus* DSMZ 20336, M58834; LS5, AB018215). Bold type indicates differences from the sequence for DSMZ 20284\(^\dagger\) determined in this work (top line).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype(^\ast)</th>
<th>Position(^\dagger)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. acidilactici</em> DSMZ 20284(^\dagger)</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td><em>P. acidilactici</em> DSMZ 20283</td>
<td>III</td>
<td></td>
</tr>
<tr>
<td><em>P. acidilactici</em> ATCC 25740</td>
<td>IV</td>
<td></td>
</tr>
<tr>
<td><em>P. acidilactici</em> LMG 17680</td>
<td>V</td>
<td></td>
</tr>
<tr>
<td><em>P. acidilactici</em> LMG 17687</td>
<td>VI</td>
<td></td>
</tr>
<tr>
<td><em>P. acidilactici</em> Pdi11</td>
<td>VII</td>
<td></td>
</tr>
<tr>
<td><em>P. acidilactici</em> LA3</td>
<td>VIII</td>
<td></td>
</tr>
<tr>
<td><em>P. acidilactici</em> LA35</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. pentosaceus</em> DSMZ 2036Q</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(\ast\) Roman numerals refer to the bacterial genotypes obtained by UPGMA analysis (Fig. 3).

\(\dagger\) *E. coli* numbering.

\‡ Sequence reported by Collins *et al.* (1990).

\§ Sequence reported by Cai *et al.* (1999).

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**Fig. 4.** Phylogenetic relationship of *P. acidilactici* strains based on the neighbour-joining method. Roman numerals, in parentheses, refer to the bacterial genotypes obtained by UPGMA analysis (Fig. 3). \*Sequence published by Collins *et al.* (1990).

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species. The sequences of strains PAC1.0 and Psp2 were identical. The region 8–524 (*E. coli* numbering) was aligned and compared with that published for *P. acidilactici* DSMZ 20284\(^\dagger\), LA3 and LA35 and with that of the closest relatives *P. pentosaceus* DSMZ 20336 and LS5. For all the eight tested sequences high similarity values (97.2–99.5%) were found with the 16S rDNA of *P. acidilactici* DSMZ 20284\(^\dagger\), LA3 and LA35, while lower values ranging from 94.7% to 96.2% were found with *P. pentosaceus* DSMZ 20336 and LS5. Signature sequences typical of each representative strain analysed are shown in Table 4. The phylogenetic tree based on neighbour-joining is shown in Fig. 4.

**Variation among strains in production of D- and L-lactic acid**

To investigate if the remarkable intraspecific variability highlighted by analysing the *ldh* genes was also reflected in the phenotype of the strains studied, the amount of D-
and l-lactic acid produced was evaluated for one or two strains of each ldhD/L sequence type previously detected. The amount of d-lactic acid produced ranged from 0-8 to 2-4 g of lactic acid (g dry weight)−1, while l-lactic acid ranged between 2 and 3-7 g (g dry weight)−1. For all the analysed strains the production of l-lactic acid was higher (by 60–72 %) than that of d-lactic acid. Despite the degree of variability detected among the strains the variability did not reflect the genetic polymorphism previously observed.

**DISCUSSION**

In this study we used several DNA fingerprinting techniques to characterize 17 strains of *P. acidilactici* isolated from different environments such as vegetables, sour dough, fermented sausages and cheese. PCR experiments on strains JD1-23 and ULS, known to produce the pediocins JD and 5 respectively, revealed that they had the genetic determinants related to pediocin AcH/PA-1 production. These results strongly suggest that the three pediocins (AcH/PA-1, JD1-23, 5) are probably identical. The practice of premature naming, before knowing the amino acid sequence of bacteriocins, has often resulted in assigning two or more names to the same bacteriocin (Ray, 1996).

With the aim of seeking a genomic/phylogenetic relationship among *P. acidilactici* strains and to verify if the molecular characteristics were correlated with their ecological origin, all the strains were analysed using a RAPD protocol, by the restriction analysis of several housekeeping genes, and by partial sequencing of the 16S rRNA gene.

A modified RAPD protocol using a single primer targeted to the gene encoding the pediocin AcH/PA-1 was able to group together all the pediocin producer strains, and detect another six distinct pattern types. Despite the use of a primer targeted at the pedA gene and the generation of an identical amplification profile among the pediocin producer strains, none of the RAPD fragments was generated from the pediocin operon, as demonstrated by hybridization experiments and by the fact that PAC1.0 and the cured strain PAC750F derived from it showed the same amplification pattern. The genetic variability detected by RAPD analysis was also reflected in several housekeeping genes, showing that a wide and consolidated genetic diversity among the tested strains was present.

A high degree of variability and an analogous clustering of the strains were observed by the restriction analysis of an amplified fragment originating from the ldhD gene, while the same analysis on the ldhl gene revealed a lower degree of intraspecific variability. These results agree with the fact that the nucleotide sequence of the ldhl gene is more conserved among different species of lactic acid bacteria than that of the ldhD gene (Garmyn et al., 1995a, b). All the pediocin producer strains were grouped together by analysis of the ldhD gene, while they were grouped with strains Pdi11 and PG by analysis of the ldhL gene. Analysis of part of the mle coding sequence confirmed the close relation among pediocin producer strains and strains Pdi11 and PG. Furthermore all the chili bo strains were clearly clustered together and separated from the other strains of different isolation source.

The high degree of variability discovered in the ldh genes was phenotypically tested by the evaluation of the amount of d- and l-lactic acid produced by *P. acidilactici* strains. All the tested strains exhibited a non-racemic production of the two lactic acid enantiomeric forms, with proportionately more l-lactic acid. Despite a certain degree of variability present among the strains, it was not possible to find any direct correlation between the phenotypic and the genotypic data. Intraspecific variability in the ldh gene sequences was also observed in *Lactococcus lactis* strains and it was found consistent with the 16S rRNA genotype but not with the phenotype (Urbach et al., 1997).

The results obtained by analysing the rpoC gene were similar to those obtained with the ldh genes; three different rpoC alleles were detected. The DNA-dependent RNA polymerase has a fundamental role in cell biology that justifies the hypothesis of its ancient origin in evolutionary terms and is in accordance with the fact that it appears to be maintained throughout most species of bacteria (Morse et al., 1996). Nevertheless it was possible to detect a certain degree of variability among the analysed strains. This result was unexpected but could be an indication of a marked intraspecific variability in the species *P. acidilactici*.

The dendrogram summarizing the data obtained showed two main branches in the species, the first including strains obtained from DSMZ and ATCC (most of which were isolated from vegetables) and three chili bo isolates (LMG 17674, LMG 17687 and LMG 17689), and the second branch including sour-dough isolates, pediocin producer strains and the remaining chili bo isolates. In this second main branch, sour-dough strains, pediocin producer strains and chili bo isolates were well separated and represent three distinct clusters that seem directly related to the metabolic traits of the strains and partially related to their ecological origin. Specifically, sucrose-positive and pediocin-producing strains (most of them isolated from meat products) represent genotype VII, arabino-negative pediocin-producing strains isolated from sourdough samples represent the nearest genotype V, while sucrose-, melibiose- and raffinose-positive strains (LMG 17680 and LMG 17692) represent genotype VI. The remaining chili bo strains (LMG 17674, LMG 17687 and LMG 17689) were clustered in genotype IV. It was not possible to detect phenotypic evidence for genotypes I, II, III and IV, except that all these strains were arabino-, sucrose-, melibiose- and raffinose-negative. Relationships among genotypic analysis and acidification in presence of carbohydrates were also recently observed in *Pediococcus acidilactici* and *P. pentosaceus* strains (Cai et al., 1999) and in *Lactobacillus garvieae* strains (Eldar et al., 1999).
With the exception of strain ATCC 12697, whose isolation source is not known, the four genotypes I, II, III, IV seem to be related to the ecological origin of the strains: strain DSMZ 20284T was isolated from barley, strains DSMZ 20238 and ATCC 8042 from ‘mash’, strain ATCC 25740 from ‘plants’, while strains LMG 17674, LMG 17687 and LMG 17689 were all isolated from chili bo.

The variability revealed by analysis of the protein-coding housekeeping and non-housekeeping genes (rpoC, ldhD/L and mle) was confirmed by the partial 16S rDNA sequencing of representative strains of each genotype obtained. The phylogenetic analysis substantiates the hypothesis that each genotype obtained could represent a genomic and/or evolutionary subpopulation within the species P. acidilactici. The 16S rDNA sequencing was also consistent with the fact that genotypes I, II, III and IV were different genotypes. Our findings seem to agree with the suggestion by Palys et al. (1997) that while 16S rRNA sequence data are useful for distinguishing moderately divergent populations into separate sequence clusters, protein-coding genes provide a better opportunity for distinguishing very closely related ecological populations. These authors also suggested that the inability of 16S rRNA sequences to distinguish some taxa is a consequence of the low evolutionary rate of the 16S rRNA genes. In this context we could suppose that each genetic subpopulation detected in P. acidilactici could be relatively ancient in evolutionary terms, because few but significant substitutions were detected among the 16S rDNA sequences of the analysed strains. Some discrepancies were observed in strain DSMZ 20284T between the published 16S rDNA sequence and that determined in this study. Some of these differences were due to the presence of ambiguities in the published sequence, while the others could be the consequence of an intra-chromosomal rearrangement.

A wide selection of different environments of isolation of P. acidilactici were considered and seven different genotypes or genomic subpopulations were detected. Three of these genomic subpopulations were found to be consistent with the phenotype. We could speculate that the strains able to utilize some carbohydrates and/or to produce pediocin have a strong competitive advantage that should have allowed the evolution of the genome in a different way compared with strains which do not have these metabolic features or that colonize other ecological niches. In this context, chili bo isolates, despite having the same isolation source, were clustered in two different genetic subpopulations. Pediocin AcH/PA-1-producing strains were isolated from meat products and from cheddar cheese, and despite the fact that no Ped+ strains from the same products were included in this study (we were unable to obtain, or find in the literature, any examples of such strains), our data strongly suggested that they represent a homogeneous subpopulation in the species.

In conclusion, the multilocus typing approach, as carried out in this study, should be considered as a useful tool for the determination of the population structure in the species P. acidilactici, and the data obtained from this kind of approach should be more accurate and precise than those obtained by the analysis of a single locus.

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REFERENCES


