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Purification of bacterial genomic DNA in less than 20 min using chelex-100 microwave: examples from strains of lactic acid bacteria isolated from soil samples

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Abstract We established a Chelex 100-Microwave method for the purification of bacterial genomic DNA (gDNA) in less than 20 min with high yield and good quality, useful for multiple purposes. It combines Chelex 100, proteinase K, RNase A and heating in a microwave oven. The resulting gDNA was used directly to identify bacterial species of the Order *Lactobacillales* by means of PCR amplification of their 16S rDNA gene, isolated from sediments on the Yucatan Peninsula, Mexico. This method produced gDNA free of phenolic and protein residual contaminants from 100 of these isolated bacteria. 16S rDNA amplification and sequencing showed *Pediococcus acidilactici* to prevail in inland lagoons, and *Pediococcus pentosaceus*, *Lactobacillus plantarum*, *Lactobacillus sp*., and *Lactobacillus fermentum* to be most abundant in the soils of livestock farms. The combination of Chelex 100, enzymes and microwave heating used in the Chelex 100-Microwave method produced large amounts of highly pure gDNA from Gram-positive and Gram-negative bacteria, in less than 20 min.

Keywords Genomic DNA purification · Lactic acid bacteria · Microbial geography

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Introduction

Lactic acid bacteria (LAB) are widely used in food biotechnology applications due to their high production of lactic acid and bacteriocines, which contribute to flavor, aid in food preservation and provide consumers a probiotic effect (de Vos et al. 2004; De Vuyst and Leroy 2007; Galvez et al. 2007; Liu et al. 2008). The LAB are Gram-positive, with either a coccus or bacillus morphology, do not sporulate, have cytochromes, and despite being considered anaerobic are aerotolerant (Reddy et al. 2008). They belong to the Order *Lactobacillales*, which includes the families *Lactobacillaceae*, *Aerococcaceae*, *Carnobacteriaceae*, *Enterococcaceae*, *Leuconostocaceae*, and

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Streptococcaceae. The main LAB species participating in food fermentation processes are the genera *Lactobacillus*, *Paralactobacillus* and *Pediococcus* genera of the *Lactobacillaceae* family (Reddy et al. 2008).

Typically, LAB are isolated and identified using selective growth media with indicators, as well as biochemical tests (e.g., the API-CHL50 system) based on the fermentation patterns of different carbohydrates (Dimitonova et al. 2008; Tamminen et al. 2004). Identification is made more selective by adding 2,3,5-triphenyltetrazolium chloride (TTC) to the culture medium, because when it is reduced by LAB it generates pink and red colors that differ from undesired white colonies (Zamudio-Maya et al. 2008). Other methods for LAB presence determination and species identification—without involving bacterial population isolation—is metagenome extraction and specific genes-identification, e.g., 16S ribosomal DNA (16S rDNA) (Luna et al. 2006; Mohania et al. 2008; Nocker et al. 2007). Both of these classification systems have been used to confirm the presence of LAB in food, in vertebrate digestive tracts and in sediments (Chen et al. 2005; Kim and Adachi 2007; Olsen et al. 2008; Yanagida et al. 2006; Pfeiler and Klaenhammer 2007).

Taxonomic identification of new LABs using molecular markers such as the 16S rDNA gene is carried out by comparing the nucleotide sequence of a particular species with previously reported sequences for other species (Mohania et al. 2008). Amplifying 16S rDNA using PCR requires a set of primers that must hybridize in the preserved 16S rDNA regions of the strain to be identified (Spiegelman et al. 2005). Therefore, successful amplicon production first requires an effective DNA extraction and purification method that is easy to use and applicable to a large number of strains. Of the many bacterial genomic DNA (gDNA) extraction and purification methods, some of the most frequently used are the cetyltrimethylammonium bromide (CTAB)-phenol-chloroform-isoamyl alcohol method (Ausubel et al. 1997; Cheng and Jiang 2006), the Chelex 100 method (Giraffa et al. 2000), the colony PCR method (Woodman 2008) and the use of microwave ovens (Bollet et al. 1995). All involve long gDNA extraction times and can co-purify contaminants that affect PCR efficiency such as phenol, chloroform or isoamylic alcohol remnants and some bacterial metabolites (Nechvatal et al. 2008). This makes gDNA purification from large quantities of colonies a time-consuming and inefficient process that is not applicable to all species.

In an effort to devise a rapid, efficient LAB identification method using the 16S rDNA gene, we developed a gDNA extraction method by combining and modifying the Chelex 100 and microwave methods, the so-called Chelex 100-Microwave method. This alternative protocol allows extraction of gDNA from one to forty strains of *Lactobacillaes* bacteria, as well as from other Gram-positive and Gram-negative species, in less than 20 min. With this method we established that in the Yucatan Peninsula, the bacteria *P. acidilactici* and *P. pentosaceus* predominate in sediments from two inland wetlands and one coastal wetland, and that four *Lactobacillus* species predominate in sediments from livestock farms.

Materials and methods

Bacterial strains and culture conditions

All sediment bacterial strains used in the study were provided by the Jalisco State Center for Research and Technological and Design Assistance (Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco—CIATEJ, Unidad Sureste) (Table 1), and had been previously isolated using TCC reagent (Zamudio-Maya et al. 2008). The LAB strains were grown in Man, Rogosa and Sharpe medium (MRS, Difco) (De Vuyst and Leroy 2007) for 16 h at 37°C under anaerobic conditions. Other bacterial species were grown in Brain Heart Infusion (BHI-Difco) (De Vuyst and Leroy 2007) for 16 h at 37°C under aerobic conditions. Gram dying and the catalase test were done as preliminary identification tests (Table 1).

LAB genomic DNA extraction

Ten different Gram-positive and Gram-negative bacteria from the ATCC collection were used to design the Chelex 100-Microwave gDNA extraction method. The method was compared with the classic CTAB-phenol-chloroform-isoamylic alcohol method (Ausubel et al. 1997) and with a variant using the microwave oven (Bollet et al. 1995).

Table 1 Strains ATCC used in this study

Strains	Gram	Culture medium	Concentration (CFU/ml)
Lactobacillus plantarum ATCC 8014	Positive	MRS Broth	7.42×10^{10}
Pediococcus acidilactici ATCC 8042	Positive	MRS Broth	9.60×10^{10}
Staphylococcus termophilus ATCC 19258	Positive	MRS Broth	1.00×10^{6}
Enterococcus faecalis ATCC 29212	Positive	BHI Broth	1.45×10^{12}
Listeria innocua ATCC 33090	Positive	BHI Broth	1.00×10^{11}
Staphylococcus aureus ATCC 25923	Postive	BHI Broth	6.65×10^{12}
Bifidobacterium longum DSM 20088	Positive	MRS Broth	2.50×10^{11}
Pseudomonas aeruginosa ATCC 27853	Negative	BHI Broth	1.84×10^{12}
Escherichia coli ATCC 11775	Negaive	BHI Broth	1.00×10^{11}
Salmonella enteritidis ATCC 13076	Negative	BHI Broth	1.00×10^{11}

Geographic distribution of LAB in wetlands and farms of the Yucatan Peninsula

The efficiency of the Chelex 100-Microwave method was assessed in strain identification in biogeography research on 100 strains previously isolated from eight different sediments collected at deferent sites on the Yucatan Peninsula, Mexico (Table 2). After TCC reduction, these strains were identified based on their LAB indicative phenotype (i.e., colors): pale pink; deep pink; pink with red center; and clear with a red or white center (Zamudio-Maya et al. 2008).

Chelex 100-microwave method for genomic DNA purification

Each selected strain was grown in liquid MRS medium overnight at 37°C under anaerobic conditions. 1.5 ml of the bacterial cultures were harvested by centrifugation at 8,000×*g* for 5 min at 4°C. Bacterial pellets were washed with 500 µl TE buffer (10 mM TRIS-Base, pH 7.5; 1 mM EDTA, pH 8.0) and centrifuged again under the same conditions. The cells were resuspended in 50 µl TES lysis buffer (10 mM TRIS-Base, pH 7.5; 1 mM EDTA, pH 8.0; 0.5% SDS), and placed in a microwave oven (Panasonic NN-S655WMRPH) for 10 s at 625 W (Power Level 6). Immediately thereafter, 150 µg proteinase K (Invitrogen, catalogue No. 25530-031) and 20 µg RNase A (Sigma, catalogue No. R5125) were added to the suspension; this was microwaved again under the above conditions. After lysis, the suspension was incubated for 2 min at room temperature, and 150 µl of TE buffer with 25 mg of Chelex 100 (Sigma catalogue No. C7901) were added, and then microwaved again under the above conditions. The sample

Table 2 Distribution of LAB species in lagoons and farms

Species	Lagoons			Farms				
	Sisal[Y,CS]	Las Maravillas[C,I]	Silvituc[C,I]	Tecoh[Y]	X′Tepén[Y]	Chivojá[C]	Chan Laguna[C]	Escárcega[C]
P. acidilactici	0	10	8	2	1	3	0	1
P. pentosaceus	3	0	0	8	13	0	1	6
L. fermentum	0	0	0	0	0	3	4	0
L. pentosus	0	0	0	0	0	0	0	2
L. plantarum	0	0	0	1	0	7	5	0
Lactobacillus sp.	0	0	0	0	0	4	7	3
E. faecium	0	0	2	0	0	0	0	0
W. paramesenteroides	0	0	0	0	0	0	0	1
Margalef index	0	0	0.434	0.834	0.379	1.059	1.059	1.560

C Campeche, *Y* Yucatán, *CS* Coastal, *I* Internal

was then centrifuged at 12,000×*g* for 5 min at 4°C. The supernatant containing the gDNA was recovered and quantified. The purified DNA was then used either in PCR reactions (2 µl per reaction) or concentrated by precipitation with 10% of 3 M sodium acetate and 2.5 volumes of 95% ethanol. Finally, the DNA was rinsed twice with 1 ml 70% ethanol, dried at room temperature and resuspended in 200 µl deionized water.

Genomic DNA purification with the CTAB-phenol-chloroform-isoamyl alcohol method

Strains were grown and collected as described above. Cells were then rinsed with 1 ml TE buffer, centrifuged and lysed with 300 µl TE buffer, 30 µl 10% SDS and 3 µl proteinase K (20 mg/ml). This solution was incubated for 1 h at 37°C, 100 µl 5 M NaCl and 80 µl CTAB/NaCl added, and incubated for 10 min at 65°C. One volume of chloroform:isoamyl alcohol (24:1) was added, the solution mixed for 5 min and centrifuged at 12,000×*g* for 5 min. The aqueous phase was recovered and mixed with one volume of phenol:chloroform:isoamyl alcohol (25:24:1) and it was centrifuged at 12,000×*g* for 5 min. Again the aqueous phase was recovered and the DNA was precipitated with two volumes of absolute ethanol. The DNA was then rinsed twice with 1 ml 70% ethanol and resuspended in 200 µl deionized water.

Genomic DNA purification with the microwave method

The strains were grown and collected as described above and the gDNA was purified by the microwave method as reported previously (Bollet et al. 1995). Cells were rinsed with 1 ml TE buffer, centrifuged and lysed with 100 µl TE buffer and 50 µl 10% SDS. This solution was incubated for 30 min at 65°C, centrifuged and the supernatant removed. The cell pellet was placed in a microwave oven and heated two times for 1 min at 900 W. The pellet was then resuspended in 200 µl TE buffer with one volume of phenol:chloroform:isoamyl alcohol (25:24:1) for 15 min. The aqueous phase was recovered by centrifugation and the DNA precipitated with 95% ethanol and centrifugation at 12,000×*g* for 20 min. The DNA was then rinsed with 1 ml 70% ethanol and resuspended as previous described.

Genomic DNA quantification and integrity

For all three methods, the DNA concentration was calculated by measurements of absorbance at 260 nm, and the purity index was determined by the ratio 260/280 nm. A ratio between 1.8 and 2 were considered as pure DNA, whereas values less than 1.80 was interpreted as DNA with contaminations with polysaccharides and protein impurities, and ratios greater than 2 were taken as polyphenol contaminations (Sambrook and Russell 2001). DNA was viewed by electrophoresis in 0.8% agarose gels dyed with 0.5 µg/µl ethidium bromide. A single band without smear was considered as integral gDNA.

PCR amplification of the 16S rDNA gene

Amplification of the 16S rDNA gene was carried out using two oligonucleotide sets. For all bacterial strains (Table 1), stems 1–46 were amplified with the oligos UPT1 (5′ GAG TTT GAT CCT GGC TCA GGA CG 3′) and LOWT46 (5′GAG GTG ATC CAG CCG CAS STT C 3′). Stems 1–44 were amplified form the 100 strains used in the biogeographic analysis with oligos UPT1 and LOWT36-44 (5′ TGT GTA GCC CAR GTC ATA AGG 3′). The reactions were set in 25 µl 1× reaction buffer, 2 mM $MgCl_2$, 1 µl of each primer, 25 (pMol/µl) 10 mM nucleotide mixture, 2.5 units polymerase DNA Taq, and 2 µl gDNA. Reaction conditions were: denaturation at 94°C for 2 min 30 s; 30 denaturation cycles at 96°C for 15 s; 58°C aligning temperature for 15 s; 72°C extension for 1 min 45 s; and a final extension at 72°C for 5 min. The amplified 16S rDNA gene was viewed in 1.5% agarose gels dyed with ethidium bromide, as described previously. PCR amplicons were column-purified, according to manufacturer instructions (PureLink PCR purification kit; Invitrogen catalogue No. K3100-02). All PCR products were sequenced by MACROGEN, Inc. (Gasan-dong, Seoul, Korea) using the UPT1 primer.

Taxonomic classification of strains and phylogeny

Identification of LAB strains was carried out by BLAST analysis, comparing each sequence with the GenBank and Ribosomal Database (RDB) project databases. Phylogenetic analysis of the strains was done with the CLUSTAL W program (Thompson et al. 1994) using previously described DNA loss

model parameters (Martínez-Perez et al. 2007). The phylograms were edited with the MEGA program (Tamura et al. 2007).

Statistical analysis and the Margalef distribution index

Differences between the means (±SD) were calculated with a one-way ANOVA, and a Bonferroni test applied to compare significantly different means ($P < 0.05$). Diversity was calculated with the Margalef index [DMg = (S − 1)/ln(N)], where S is the number of species and N the total number of individuals (Margalef 1958).

Results

Genomic DNA yields and purity

The gDNA concentration attained with the Chelex 100-Microwave method from the bacterial strains analyzed was higher (2–3 times more DNA) ($P < 0.005$) than those obtained with the CTAB-phenol-chloroform-isoamylic alcohol and Microwave methods (Fig. 1a). Slight variations of gDNA contents were isolated from the different strains with the Chelex 100-Microwave method, except for *L. plantarum,* which rendered about 3 fold more gDNA. This issue needs to be investigated further.

The DNA obtained from Chelex 100-Microwave method showed a higher purity (average 260/280 ratio = 1.9), whereas DNA produced with the CTAB-phenol-chloroform-isoamyl alcohol method showed signs of phenolic contaminants (260/280 ratio > 2), and that obtained with the Microwave method rendered lesser DNA concentrations and contained cellular remains or other contaminants (260/280 ratio < 1.6) (Fig. 1b).

Genomic DNA integrity and 16S rDNA gene amplification

100 ng of gDNA samples extracted with the different methos were analyzed by agarose electrophoresis. *P. acidilactici*, *S. thermophilus*, *S. aureus* and *B. longumse* gDNA samples from CTAB-phenol-chloroform-isoamyl alcohol and Microwave methods showed no DNA or degradation (Fig. 2a and b, lanes 3, 4, 7 and 8). In comparisson with the Microwave method, the CTAB-phenol-chloroform-isoamylic alcohol method appears to be more efficient for the remaining bacterial strains, however both methods co-purify significant amounts of RNA (Fig. 2a and b, lanes 2, 5, 6, 9 and 10). Conversely, with the Chelex 100-Microwave method gDNA was obtained in all bacterial strains, with no significant DNA degradation nor RNA co-purification (Fig. 2c).

The gDNA quality was corroborated by means of 16S rDNA PCR amplification with the UPT1 and LOWT46 oligos. As expected, the gDNA of all bacterial strains rendered amplicons of ca. 1,500 bp (Fig. 2d).

Identification and phylogeny of LAB from sediments

Starting from soils, a total of 100 LAB strains, identified by TTC reduction phenotype, were isolated. All were Gram-positive and catalase negative, suggesting they were LAB. To corroborate their LAB identity, their 16S rDNA gene sequences were analyzed by BLAST (Fig. 3). 33% of a universe of 95 strains—5 duplicates were analyzed with their partners—showed that their 16S rDNA gene was 98–99% homologous to *Lactobacillus plantarum*, *Lactobacillus sp*. and *Lactobacillus fermentum* (distributed in a 1:1:0.5 ratio). 59.5% were homologous to *Pediococcus acidilactici* and *Pediococcus pentosaceus* (distributed in a 1:1 ratio), and the remaining 7.5% corresponded to *Enterococcus faecium*, *Weissella paramesenteroides* and *Lactobacillus pentosus* strains (Supplementary Table 1 shows the Accession Number of each strain and their geographic localization).

The phylogenetic analysis showed that 99% of the identified strains are related to the *Lactobacillaceae* family. Of the *Pediococcus* genus, only *P. acidilactici* was in the same node as *P. pentosaceus*. All the *Lactobacillus* sp. strains were related to some of the nodes of the *Lactobacillus* genus. Ten strains were related to *L. fermentum*, and the remaining five were in the *L. pentosus* or *L. plantarum* nodes, which had the largest number of phylogenetic links between them (Fig. 3).

LAB from sediments: distribution and richness

Figure 4 shows the bacterial isolates from different geographical sites of the Yucatán Peninsula. In inland

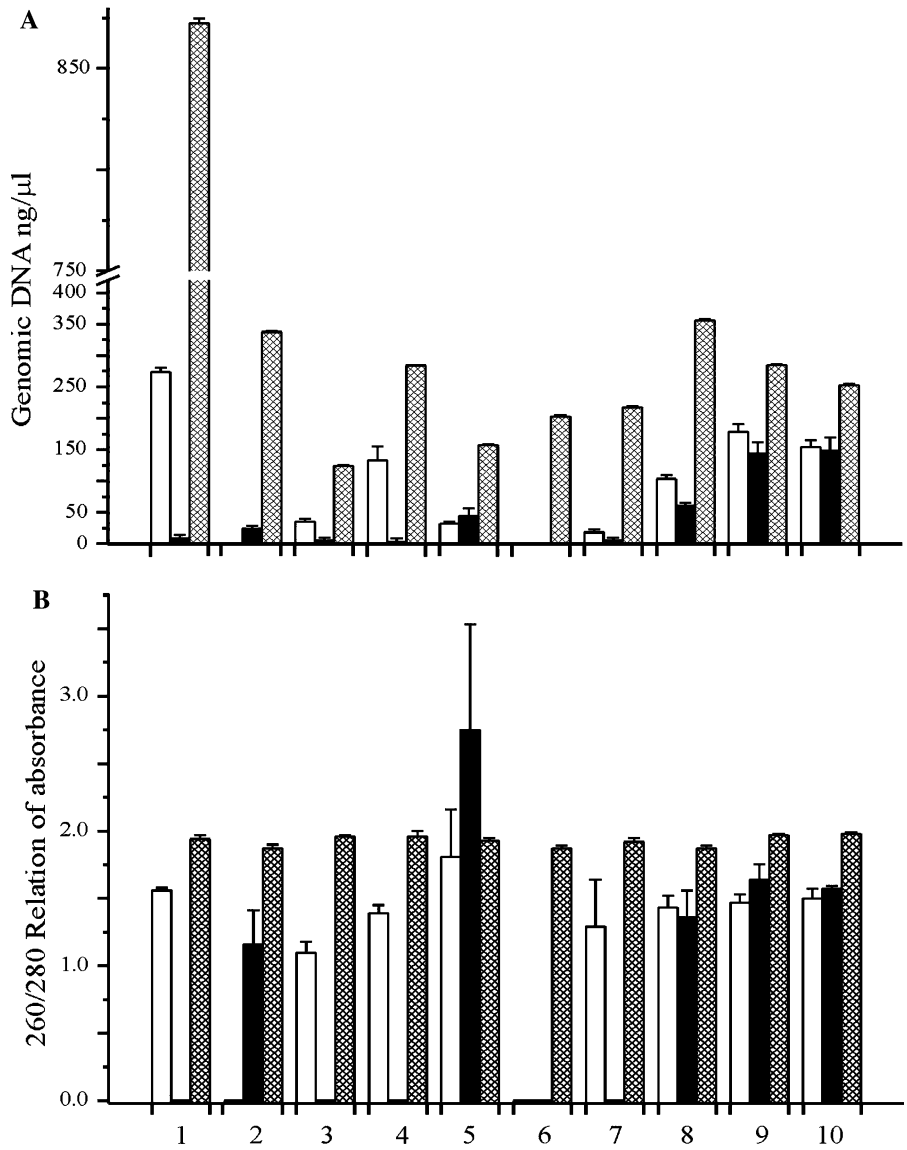

Fig. 1 Different bacterial strains (*Lactobacillus plantarum* ATCC 8014, *lane 1*; *Pediococcus acidilactici* ATCC 8042, *lane 2*; *Staphylococcus termophilus* ATCC 19258, *lane 3*; *Enterococcus faecalis* ATCC 29212, *lane 4*; *Listeria innocua* ATCC 33090, *lane 5*; *Staphylococcus aureus* ATCC 25923, *lane 6*; *Bifidobacterium longum* DSM 20088, *lane 7*; *Pseudomonas aeruginosa* ATCC 27853, *lane 8*; *Escherichia coli* ATCC 11775, *lane 9*; *Salmonella enteritidis* ATCC 13076, *lane 10*) were used to purify genomic DNA with the phenol-chloroform-isoamylic acid (*white columns*), Microwave (*black columns*) or Chelex 100-Microwave (*hatched columns*) methods. **a** The gDNA concentration (ng/µl) for each strain is shown. **b** For each extraction method, the purity of the gDNA was established from the 260/280 nm ratios. The mean (±MSE) was determined from three independent experiments. Significant differences ($P < 0.005$, Bonferroni test) were observed between the three methods

lagoons (Campeche State) and costal lagoons (Yucatan State), the dominant strain was *P. acidilactici*, while *P. pentosaceus* was the most abundant strain at one farm in Campeche, two farms in Yucatan, although it is preset also in the Yucatan coastal lagoons. In contrast, some minor species were isolated from two farms in Campeche, where *L. fermentum*. *L. pentosus L. plantarum, Lactobacillus* sp. and *W. paramesenteroides* were the most abundant. The Margalef diversity index values corroborated this low species richness in the lagoons and farms in Campeche and Yucatan, with values of zero to <0.5 in the lagoons and farms in Yucatan and values >1 at farms in Campeche (Table 2).

Discussion

Worldwide lactic acid consumption ranges from 130 to 150 billion metric tons (MT) annually and is likely to increase in another 200,000 MT by 2011 (Reddy et al. 2008). This high demand has sparked interest in isolating and characterizing new strains of the *Lactobacillaceae* family which can be generated in

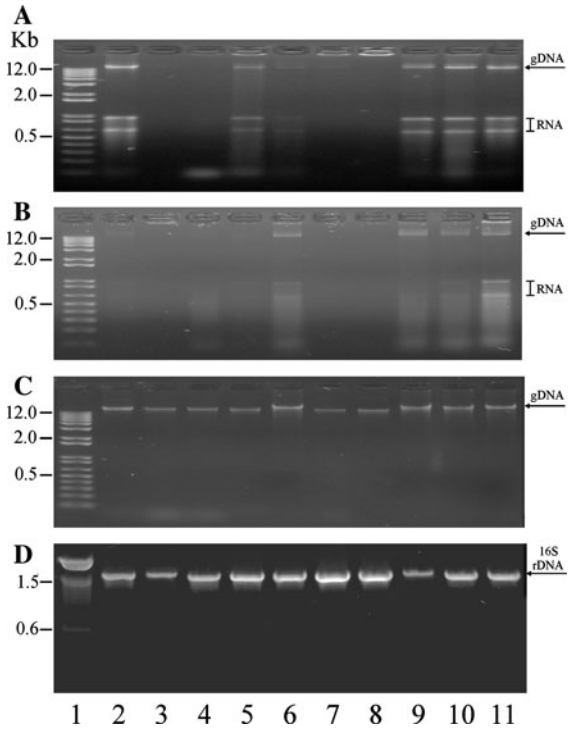

Fig. 2 Agarose gel electrophoresis gels (0.8%) of LAB gDNA (*arrow*) isolated with the phenol-chloroform-isoamylic acid (**a**), the Microwave (**b**), and the Chelex 100-Microwave (**c**) methods; *lane 1*, 1 kb DNA ladder (Invitrogen); *lanes 2–11* as in Fig. 1. Contaminant RNA is indicated with *brackets*. **(d)** PCR amplification of 16S rDNA gene (*arrow*) for the different Gram-positive and Gram-negative strains. Products (30% of the reaction) were fractionated in a 1.5% agarose gel; *lane 1*, 100 bp DNA ladder (Invitrogen); *lanes 2–11* as in Fig. 1

larger quantities and less time. For this purpose, strain libraries are needed including LAB isolated from soils in wild areas as well as from livestock farms. Characterization includes identification of hundreds of strains, which requires rapid, efficient methods that provide correct classifications, be they based on biochemistry or molecular biology (e.g., gene marker comparisons).

The Chelex 100-Microwave method described here is a novel rapid method that extracts high pure genomic DNA without the need for additional purification steps, as proven by the presence of 16S rDNA gene amplicons ready for sequencing. This method combines the Chelex 100 method for extracting genomic DNA from LAB (Giraffa et al. 2000), microwave oven method modifications (Bollet et al. 1995), and addition of proteinase K and RNase A. In comparison to the classic CTAB-phenol-chloroform-isoamyl alcohol and microwave methods, the Chelex 100-Microwave method has three major advantages: (1) the gDNA obtained had no detectable contaminants such as phenol or cellular remnants, and the gDNA concentration was high in *Lactobacillales* as well as in Gram positive and negative strain; (2) the physicochemical characteristics of the Chelex 100 eliminates any peptides and other molecules that might bind to the genomic DNA; (3) the use of the microwave oven results in extraction of genomic DNA in less than 20 min. Indeed, this is the first report of bacterial genomic DNA purification reactions from strains isolated with Chelex 100 and incubated in a microwave oven for 15 s. In other protocols, the Chelex 100 purification step involves incubation at 56 or 100°C for 30–45 min, or at 42°C for over 12 h (Giraffa et al. 2000), or the microwave oven is used to extract DNA from bacteria but involves phenol:chloroform:isoamylic alcohol treatment, therefore increasing extraction times (Bollet et al. 1995). The microwave oven incubation period used in the Chelex 100-Microwave method is what allows purification of gDNA from 1 to 40 strains in less than 20 min, unlike other methods in which purification time is approximately 1.3 h and DNA recovery is three times lower than with Chelex 100-Microwave method (Ausubel et al.1997; Bollet et al.1995; Giraffa et al. 2000).

Proteinase K has been reported as being efficient in the lysis of Gram-positive bacteria, but weak in Gram-negative bacteria (Bollet et al. 1995). In contrast, the Chelex 100-Microwave method is efficient with both types of bacteria. This may be because cellular lysis in this method is caused by the rotation and friction of cell molecules when in the microwave oven (Ling et al. 1991; Kim et al. 2009), and not by enzymatic collision alone (data not shown).

When extracting gDNA to characterize a large number of strains, a cell sample is commonly lysed in the PCR reaction to amplify the gene under study (Nocker et al. 2007; Woodman 2008). This is functional for Gram-negative strains, but not for Gram-positive strains due to the latter's thick cell wall and the presence of metabolites that interfere with the efficiency of the reaction (Nechvatal et al. 2008). Instead of placing a culture aliquot in the PCR reaction, the Chelex 100-Microwave cuts the time needed to extract gDNA pure enough to allow amplification at concentrations of 1.5–2 mM MgCl or in long PCR. However,

Fig. 3 Phylogenetic relationships between LAB strains isolated from Yucatan peninsula. *P. acidilactici* (*filled circles*), *P. pentosaceus* (*empty circles*), *L. fermentum* (*filled triangles*), *L. pentosus* (*filled squares*), *L. plantarum* (*empty triangles*), *Lactobacillus sp., E. faecium,* and *W. paramesenteroides* were identified in the sediments collected from farms (*FR*), coastal lagoons (*CL*), and inland lagoons (*IL*)

the need to increase the amount of $MgCl_2$ in the PCR raises the mutation rate by *Taq* DNA polymerase (Eckert and Kunkel 1991; Ling et al.1991) and to a lesser extent, that of other DNA polymerases such as Deep Vent and *Pfu* (Barnes 1994; Biles and Connolly 2004; Cline et al. 1996). This is critical in strain characterization by PCR since it increases the introduction of unspecific mutations in the resulting amplicon due to method-related effects (Cariello et al. 1991; Huang and Keohavong 1996).

Other advantage of the Chelex 100-Microwave method is their application in environmental and biotechnological studies, because the fast DNA purification gives a simple solution to sequence several strains directly or by microarrays (Ansorge 2009; Mardis 2008; Morozova and Marra 2008; Ohno et al. 1991).

To determine the application of Chelex 100-Microwave method in environmental study to obtain new strains with putative biotechnological potential, we extract gDNA from Order *Lactobacillales* strains collected from eight sediments on the Yucatan Peninsula. These strains were previously identified phenotypically by TCC reduction. Strains of different phenotype colors were selected, but 100% corresponded to species of the Order *Lactobacillales*, corroborating the TCC's specificity in selecting LAB in culture media. Identification of the LAB from the sediments showed for the first time that *P. acidilactici* is present both in inland lagoons and, to a lesser extent, at farms in Yucatan State. In contrast, *P. pentosaceus* was widespread at farms in Yucatan and Campeche, while species of the *Lactobacillus* genera were found only at farms in Campeche. Noteworthy is that *P. acidilactici* and *L. plantarum*, found at the Chivojá farm, were similar to strains identified in the intestines of cattle (Rodriguez-Palacios et al. 2009), pigs, chicken (Collado and Sanz 2007b; Olsen et al. 2008), and lambs and calves (Collado and Sanz 2007a). The Margalef indices showed that LAB diversity in the studied geographic region is low, probably due to phenotypical preselection of strains by TCC reduction. Assuming tat this preselection occurs, and considering that soils are the

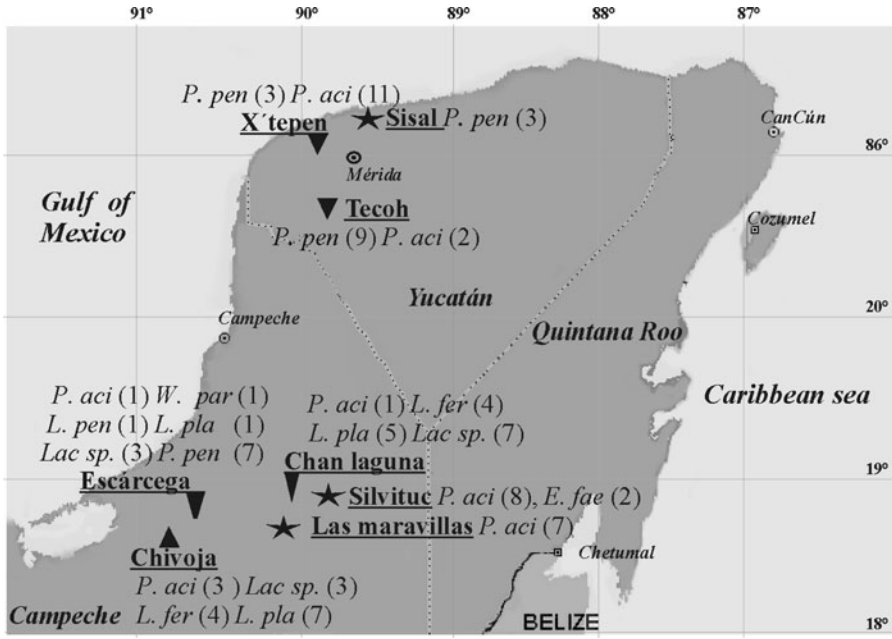

Fig. 4 Location of farms (*open triangles*), inland and coastal lagoon (*stars*) sites on the Yucatan Peninsula where soil samples were collected. The number of LAB species identified at each site appears in parenthesis. For the number of strains per species and the 16S rDNA gene accession numbers refer to Supplementary Table 1

most diverse ecosystems, and that such diversity might be influenced by the isolation methods (Kirk et al. 2004; Nocker et al. 2007), we could expect new *Lactobacillales* strains to be present in the Yucatan peninsula soils. For example in other soils, *Lactobacillales* Order bacteria have been found to exist in grape vineyard soils in Australia (Bae et al. 2006) and Japan (Yanagida et al. 2008), as well as in the rhizosphere of fruit trees and at horse farms in Japan and Taiwan (Chen et al. 2005).

In conclusion, the Chelex 100-Microwave method is a rapid, low-cost and efficient protocol that extracts large quantities of highly pure gDNA that co-purifies no detectable organic solvent remnants.

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