Classification of *Lactobacillus plantarum* by Restriction Endonuclease Analysis of Total Chromosomal DNA Using Conventional Agarose Gel Electrophoresis

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A total of 17 *Lactobacillus plantarum* strains that originated from different environments and 24 reference strains were classified by performing a restriction endonuclease analysis of total chromosomal DNAs digested with *EcoRI*, *HindIII*, and *ClaI*, and the resulting patterns were visualized after the fragments were separated according to size by agarose gel electrophoresis. The patterns were analyzed by using the Pearson product moment correlation coefficient and the unweighted pair group algorithm with arithmetic averages. All but two *L. plantarum* strains formed a cluster that was separated from the reference strains at a similarity level of 29% (cluster 1). The two remaining *L. plantarum* strains (cluster 2) merged with cluster 1 at a level of similarity of 28%. The reference strains formed four additional clusters, and four reference strains were stragglers. Cluster 3 (three *Lactobacillus pentosus* strains) and cluster 4 (including *Pediococcus acidilacti* CCUG 32235^T [T = type strain] and *Lactobacillus fermentum* ATCC 14931^T) merged with cluster 1 at a level of similarity of 25%. Cluster 5 comprised 10 *Lactobacillus reuteri* strains, and cluster 6 contained the type strains of *Lactobacillus amylovorus*, *Lactobacillus gasseri*, and *Lactobacillus vaginalis*. Cluster 1 (*L. plantarum*) was divided into three subclusters, and this subdivision reflected to some extent certain phenotypic features of presumed ecological significance, including the ability to adhere to intestinal mucosa (subcluster 1c) and starch and glycogen degradation (subcluster 1a). A principal-component analysis significantly distinguished the strains belonging to different species. Also, the subcluster 1c strains could be separated from the rest of the *L. plantarum* strains. The results of restriction endonuclease analysis of total chromosomal DNA were found to be reproducible, and this method can be used to (i) differentiate between similar strains belonging to the same *Lactobacillus* species and group strains within a species, (ii) distinguish between strains of different *Lactobacillus* species, and (iii) place strains in specific *Lactobacillus* species.

The genus *Lactobacillus* is heterogeneous, and the relationships among the different species have been studied by reverse transcriptase sequencing of 16S rRNAs (2). Larger evolutionary distances between species have been revealed clearly, but the relationships among more closely related species are muddled. Furthermore, rRNA gene sequencing is useless for classification below the species level; for example, the 16S rRNA gene sequences of *Lactobacillus plantarum* and *Lactobacillus pentosus* are identical, and the same is true for the sequences of *Lactobacillus casei* and *Lactobacillus rhamnosus* (2, 12).

Workers have developed a method in which the genomes of *Lactobacillus* strains are characterized by performing a restriction endonuclease analysis (REA) of chromosomal DNAs and then visualizing the resulting fragment patterns after agarose gel electrophoresis is performed (14). This method has been used to classify *Lactobacillus reuteri* strains (13). The general advantage of REA is that it provides a unique genomic fingerprint of the whole chromosomal DNA for each strain. The presence of multiple band patterns, which are obtained by using restriction endonucleases that are able to split the DNA at many sites, means that a taxonomic analysis can be based on a large number of genomic features (DNA fragment sizes) for each strain. At the strain level, the resolution of REA for *L. plantarum* is better than the resolution of ribotyping (6) or randomly amplified polymorphic DNA analysis (7). Furthermore, it has been suggested that this method can identify strains to the species level (13). Thus, REA might supplement

16S rRNA gene sequencing by providing data for the lower taxonomic levels.

L. plantarum is frequently used as a starter culture in lactic acid-fermented foods (9) and has also been isolated from human intestinal mucosa (10). The only previous major taxonomic study of *L. plantarum* at the interspecies level was performed by Dellaglio et al. (3), who divided strains identified phenotypically into two DNA-DNA homology groups. One of these groups was later designated *L. pentosus* (16).

The aims of this study were to use an improved and technically somewhat simplified REA method to classify *L. plantarum* strains isolated from the mucosa of human intestines and from lactic acid-fermented foods and to compare these strains with strains belonging to other species, including the *L. pentosus* type strain and a set of *L. reuteri* strains.

MATERIALS AND METHODS

Strains and culture conditions. The strains which we used are listed in Table 1. All of the *L. plantarum*, *L. pentosus*, and *L. reuteri* strains had previously been genetically identified to the species level by performing DNA-DNA hybridization experiments with the type strains or by ribotyping (6, 13). The strains were grown and stored as described previously (13).

Preparation of DNA and restriction endonuclease digestion. Chromosomal DNAs were prepared after the strains were grown overnight in stationary cultures at 37°C in 100-ml portions of *Lactobacillus* carrying medium (4) containing 1% glucose. Cells were harvested by centrifugation, washed once in 10 ml of TES (50 mM NaCl, 100 mM Tris, 70 mM disodium EDTA; pH 8.0), and suspended in 3 ml of TES supplemented with 25% (wt/vol) sucrose. Then lysozyme (120 mg; grade VI; Sigma Chemical Co., St. Louis, Mo.) and 140 U of mutanolysin (Sigma) were added. The cells were incubated at 42°C for 2 h, 3 mg of proteinase K (type XI; Sigma) was added, and the preparation was incubated at 37°C for 30 min. After cooling, 1 ml of a 5% NaCl solution, 1 ml of 20% sodium dodecyl sulfate, and 11.4 ml of TES were added to the cell suspension. If necessary, the suspension was gently stirred with a glass rod until it became homogeneous, and then it was heated at 65°C for 15 min. The cell lysate was extracted once with

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TABLE 1. Strains used in this study and their sources of isolation

Strain ^a	Source of isolation	Strain no. ^b
<i>Lactobacillus plantarum</i> ATCC 14917 ^T	Pickled cabbage	1
<i>Lactobacillus plantarum</i> 386 ^{c,d}	Human rectum	2
<i>Lactobacillus plantarum</i> 299 ^{c,d}	Human colon	3
<i>Lactobacillus plantarum</i> 105 ^{c,d}	Human transversum	4
<i>Lactobacillus plantarum</i> 275 ^{c,d}	Human colon	5
<i>Lactobacillus plantarum</i> 299 ^{c,e}	Sour dough, human intestine	6
<i>Lactobacillus plantarum</i> So5 ^{c,f}		7
<i>Lactobacillus plantarum</i> 256 ^{c,g}	Silage	8
<i>Lactobacillus plantarum</i> 36E ^{c,h}	Silage	9
<i>Lactobacillus plantarum</i> 79 ^{c,i}	Nigerian pito	10
<i>Lactobacillus plantarum</i> 107 ^{c,i}	Nigerian ogi	11
<i>Lactobacillus plantarum</i> 98 ^{c,i}	Nigerian ogi	12
<i>Lactobacillus plantarum</i> 95 ^{c,i}	Nigerian ogi	13
<i>Lactobacillus plantarum</i> 53 ^{c,i}	Nigerian ogi	14
<i>Lactobacillus plantarum</i> 97 ^{c,i}	Nigerian ogi	15
<i>Lactobacillus plantarum</i> 101 ^{c,i}	Nigerian ogi	16
<i>Lactobacillus plantarum</i> 120 ^{c,i}	Nigerian ogi	17
<i>Lactobacillus plantarum</i> 44 ^{c,i}	Nigerian ogi	18
<i>Lactobacillus plantarum</i> ATCC 8014 ^c	Corn silage	19
" <i>Lactobacillus plantarum</i> " CH ^k		20
" <i>Lactobacillus plantarum</i> " 442 ^l	Fermented cucumber	21
<i>Lactobacillus pentosus</i> ATCC 8041 ^T	Corn silage	22
<i>Lactobacillus reuteri</i> DSM 20016 ^T		23
<i>Lactobacillus reuteri</i> DSM 20015 ^m		24
<i>Lactobacillus reuteri</i> N2LC ^m	Rat intestine	25
<i>Lactobacillus reuteri</i> N5LC:2 ^m	Rat intestine	26
<i>Lactobacillus reuteri</i> N2J ^m	Rat intestine	27
<i>Lactobacillus reuteri</i> N5D:1 ^m	Rat intestine	28
<i>Lactobacillus reuteri</i> 1044 ^m	Porcine intestine	29
<i>Lactobacillus reuteri</i> 8557:1 ^{d,m}	Human	30
<i>Lactobacillus reuteri</i> 1048 ^m	Porcine intestine	31
<i>Lactobacillus reuteri</i> 8557:3 ^{d,m}	Human	32
<i>Lactobacillus fermentum</i> ATCC 14931 ^T		
<i>Pediococcus acidilacti</i> CCUG 32235 ^T		
<i>Lactobacillus gasseri</i> DSM 20243 ^T		
<i>Lactobacillus vaginalis</i> CCUG 31452 ^T		
<i>Lactobacillus amylovorus</i> DSM 20532 ^T		
<i>Lactobacillus helveticus</i> DSM 20075 ^T		
" <i>Lactobacillus plantarum</i> " ATCC 10776 ⁿ		
<i>Erysipelothrix rhusiopathiae</i> CCUG 221 ^T		
<i>Pediococcus pentosaceus</i> CCUG 32205 ^T		

^a ATCC, American Type Culture Collection, Rockville, Md.; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; CCUG, Culture Collection of the University of Gothenburg, Gothenburg, Sweden.

^b Strain numbers used in Fig. 3 and 4.

^c Genetically identified by DNA-DNA hybridization and affiliated with *L. plantarum* (6).

^d Isolated by Molin et al. (10).

^e Described by Johansson et al. (5).

^f Starter culture obtained from the Swedish Sugar Company, Arlöv, Sweden.

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ⁱ Isolated by Johansson et al. (8).

^j Genetically identified by DNA-DNA hybridization and affiliated with *L. pentosus* (6).

^k Starter culture obtained from Christian Hansen Laboratories, Copenhagen, Denmark.

^l H. Fleming, University of North Carolina, Raleigh.

^m Genetically identified by DNA-DNA hybridization as *L. reuteri* and studied by REA (13).

ⁿ Genetically identified by DNA-DNA hybridization and found to belong to neither *L. plantarum* nor *L. pentosus* (6).

FIG. 1. Agarose gel electrophoresis of *L. plantarum* ATCC 8014 (lanes 2 through 4) and "*L. plantarum*" ATCC 10776 (lanes 5 through 7) DNAs cleaved with *Hind*III (lanes 2 and 5), *Cla*I (lanes 3 and 6), and *Eco*RI (lanes 4 and 7). Lanes 1 and 8 contained a high-molecular-weight DNA marker (Bethesda Research Laboratories) and DNA molecular weight marker VI (Boehringer Mannheim). The bands between the arrows were compared.

redistilled phenol-chloroform (1:1) and once with chloroform-isoamyl alcohol (24:1). The DNA was then precipitated with ethanol at -20°C .

The chromosomal DNA was separated from covalently closed circular plasmid DNA (most of the plasmid DNA) by dye buoyant density centrifugation in a CsCl gradient containing ethidium bromide (1). Some linear and open circular forms of plasmid DNA were still present in the preparation, but they contributed very little to the final results (13) (data not shown). The concentration of chromosomal DNA was monitored with a fluorometer (model TKO 100; Hoefer Scientific Instruments, San Francisco, Calif.).

DNA (0.75 μg) was separately digested at 37°C for 4 h with 10 U of *Hind*III, *Cla*I, or *Eco*RI (Boehringer Mannheim). Ståhl et al. (14) and Ståhl and Molin (13) used *Asp* 718 instead of *Hind*III, but digestion with *Asp* 718 resulted in too many large bands to be optimal for the method which we used.

Electrophoresis and scanning of gels. Gel electrophoresis was carried out by using submerged horizontal 0.9% agarose (ultrapure DNA grade; low electroendosmosis; Bio-Rad Laboratories, Richmond, Calif.) slab gels (150 by 235 mm). A high-molecular-weight DNA marker (0.2 μg) containing DNA fragments having molecular sizes ranging from 8.5 to 48.5 kb (Bethesda Research Laboratories) and 0.5 μg of DNA molecular weight marker VI containing DNA fragments having molecular sizes ranging from 0.2 to 2.2 kb (Boehringer Mannheim) were used as standards. In addition, pH 7.9 digested with *Eco*RI (molecular size, 6.4 kb; Boehringer Mannheim) was added to each well as an internal standard. Minimal band distortion and maximal sharpness were achieved by applying the sample DNA in Ficoll loading buffer (2 g of Ficoll, 8 ml of water, 0.25% bromophenol).

Gels were electrophoresed at a constant voltage of 40 V for 18 h at 8.5°C ; the buffer (89 mM Tris, 23 mM H_2PO_4 , 2 mM sodium EDTA; pH 8.3) was recirculated during electrophoresis. Then the gels were stained for 20 min in ethidium bromide (2 $\mu\text{g}/\text{ml}$), destained in distilled water, visualized at 302 nm with a UV

FIG. 2. Dendrogram of the strains tested based on Pearson product moment correlation coefficients and UPGMA clustering. *Lb.*, *Lactobacillus*; *Pd.*, *Pediococcus*; *E.*, *Erysipelothrix*.

transilluminator (UVP, Inc., San Gabriel, Calif.), and photographed. This method of performing gel electrophoresis gave well-distributed and relatively well-separated bands down to a molecular size of about 1.8 kb (Fig. 1).

Reading band patterns. Band patterns on photographic negatives were scanned with a laser densitometer (UltroScan XL; LKB-Produkter AB, Bromma, Sweden). The level of resolution was 1,000 points per lane, and each lane was about 50 mm long. Data were obtained by using the LKB 2400 GelScan XL software package (LKB-Produkter AB). The band patterns were normalized, and background noise was subtracted by using the rolling-disc algorithm of the GelCompar 3.0 program (Applied Maths, Kortrijk, Belgium). The data from the three experiments (the experiments performed with *Hind*III, *Cla*I, and *Eco*RI) were combined for each strain by using GelCompar 3.0.

Mathematical analyses. The data set was analyzed by using the Pearson product moment correlation coefficient and the unweighted pair group algorithm with arithmetic averages (UPGMA) (11). The Pearson correlation coefficients and the UPGMA values were calculated for the complete data set, and the GelCompar 3.0 program was used to do this. Our analysis also involved a principal-component analysis (PCA) performed with the strains affiliated with *L. plantarum*, *L. reuteri*, and *L. pentosus*, as well as the *L. plantarum* strains alone (using GelCompar 3.0).

Reproducibility. DNAs from the following strains were analyzed in duplicate (the chromosomal DNA of each strain was prepared twice, and the samples were electrophoresed on different gels): *L. plantarum* 53, 97, ATCC 14917^T (T = type strain), 36E, ATCC 8014, 299, 105, 275, and 120. This analysis was followed by a cluster analysis.

Phenotypic analysis. All *L. plantarum* strains and *L. pentosus* ATCC 8041^T were analyzed by using API 50CH kits (API System, Montalieu, Vercieu, France) as described previously (10) at both 30 and 37°C. The profiles obtained at the two temperatures were combined for each strain (in all, 98 characters were examined) and were compared by using the Jaccard coefficient followed by UPGMA clustering.

RESULTS

Reproducibility. The selected strains analyzed in duplicate merged in the cluster analysis, with no strains in between. This indicated that most of the strains in this study could be distinguished by the REA method which we used. In almost all cases the levels of similarity obtained for duplicate runs of the same strain were in the range from of 90 to 95%.

Cluster analysis. The cluster analysis in which we used the Pearson product moment correlation coefficient followed by UPGMA resulted in the dendrogram shown in Fig. 2. Five clusters were defined at a similarity level of 29%. Cluster 1 (*L. plantarum*) comprised 17 *L. plantarum* strains that originated from both human intestines and fermented foods and feeds, including *L. plantarum* ATCC 14917^T and ATCC 8014. Three distinct subclusters were also identified, and these subclusters merged at similarity levels of 54, 52, and 35% (Fig. 2). *L. plantarum* So5 and ATCC 8014 appeared to be stragglers within cluster 1, merging with the other strains at a similarity level of 42%.

Cluster 2 comprised two *L. plantarum* strains. These strains merged with cluster 1 at a similarity level of 28% (Fig. 2).

Cluster 3 (*L. pentosus*) contained one isolate from pickled cucumber, one commercial starter culture, and *L. pentosus* ATCC 8041^T (Fig. 2).

FIG. 3. First- and second-principal-component scores for *L. plantarum* (■), *L. reuteri* (□), and *L. pentosus* (△) strains. The projection was based on the complete data set. The numbers are strain numbers (Table 1).

Cluster 4 included two type strains, *Pediococcus acidilacti* CCUG 32235 and *Lactobacillus fermentum* ATCC 14931 (Fig. 2).

Cluster 5 (*L. reuteri*) comprised 10 *L. reuteri* strains, including *L. reuteri* DSM 20016^T and DSM 20015. Cluster 5 could be divided into three subclusters, subclusters 5a through 5c, which merged at similarity levels of 46, 43, and 34%, respectively (Fig. 2). Subcluster 5b contained strains isolated from rat intestines, and subcluster 5c included strains that originated from humans and pigs (Fig. 2). *L. reuteri* DSM 20016^T and DSM 20015 (subcluster 5a) merged with cluster 5, but at a lower level of similarity (29%).

A sixth cluster, cluster 6, contained three type strains, *Lactobacillus gasseri* DSM 20243, *Lactobacillus vaginalis* CCUG 31452, and *Lactobacillus amylovorus* DSM 20532 (Fig. 2).

Three type strains and one reference strain formed one-member clusters (Fig. 2).

PCA. The PCA and accompanying statistical evaluation of *L. plantarum*, *L. pentosus*, and *L. reuteri* strains revealed that there were three groups (coinciding with the species affiliations) that were significantly separated from each other (Fig. 3) (all data are not shown). Strains that were located peripherally in each group were removed and tested individually to determine their group affiliations. This analysis showed that each strain was more closely related to its assigned species than to any of the other species (data not shown). The *L. plantarum* group of strains (clusters 1 and 2) was more compact than the *L. reuteri* group of strains (Fig. 3) (data not shown).

A separate PCA was performed with the *L. plantarum* strains, and the strains in subcluster 1c formed a compact group that was separated from most of the other strains by both the first and second principal components (Fig. 4). It is also noteworthy that the strains in subcluster 1b (Fig. 2) are the strains that merged closest to cluster 1c in the PCA. According to the PCA plot, *L. plantarum* So5 and 53 are the strains that are most distantly related to subcluster 1c according to the first principal component, while *L. plantarum* 44 and 120 are the strains that are most distantly related according to the second principal component.

Phenotype. We compared the abilities of the different *L.*

FIG. 4. First- and second-principal-component scores for *L. plantarum* strains, based on the complete data set. Symbols: △, *L. plantarum* strains belonging to subcluster 1c; ■, other *L. plantarum* strains. The numbers are strain numbers (Table 1).

plantarum strains (and *L. pentosus* ATCC 8041^T) to ferment different carbohydrates (as determined by API 50CH tests), and the resulting dendrogram is shown in Fig. 5. The clustering in this dendrogram resembles in several respects the clustering obtained with the REA. However, strain ATCC 10776 was misidentified by API 50CH tests as a *L. plantarum* strain, and the phenotypic tests failed to group the strains in subcluster 1c in accordance with their genomic relationships.

DISCUSSION

The REA method described by Ståhl et al. (14) and Ståhl and Molin (13) was modified in this study by changing one of the restriction enzymes (*Asp* 718 to *Hind*III), shortening the running time of the agarose gels, and utilizing the GelCompar program instead of SIMCA for evaluation. These modifications made the REA easier to handle and allowed us to use a larger proportion of the DNA fragments; because of the long time that elapsed at the original setting, fragments that were smaller than 6.4 kb ran out of the gels. Thus, in this study almost the entire chromosomal DNA was used for to compare strains.

In order to evaluate whether our results were comparable to the results obtained by Ståhl and Molin (13), a set of *L. reuteri* strains that previously had been classified by REA (13) was included in the study. In this study, these strains formed a separate cluster at a similarity level of 29% (cluster 5). The

FIG. 5. Dendrogram based on an analysis of combined API 50CH profiles (obtained at both 30 and 37°C) followed by an analysis in which the Jaccard coefficient and UPGMA were used, showing the phenotypic relationships among the *L. plantarum* and *L. pentosus* strains included in this study. *Lb.*, *Lactobacillus*.

formation of subclusters and the relationships among several strains within the subclusters were consistent with the results reported by Ståhl and Molin (13), and this was true despite the fact that one of the restriction enzymes was changed.

All of the *L. plantarum* strains included in cluster 1 have been shown previously either to exhibit high levels of DNA-DNA homology with the type strain of *L. plantarum* or to have the same ribopatterns (as determined with *EcoRI*- and *HindIII*-cleaved DNA) as *L. plantarum* ATCC 14917^T (6). Interestingly, *L. plantarum* 44 and 120, both of which merge with cluster 1 at a similarity level of 28%, were found to have different ribopatterns and to exhibit relatively low levels of DNA-DNA homology with the type strain of *L. plantarum* (6). "*L. plantarum*" ATCC 10776 was singled out from the rest of the *L. plantarum* strains by REA (Fig. 1). This is consistent with the DNA-DNA homology data obtained with the type strain of *L. plantarum* (3, 6). However, on the basis of the results of phenotypic tests "*L. plantarum*" ATCC 10776 can be identified as a member of the species (Fig. 5).

Three distinct subclusters were identified within cluster 1, but the groups did not reflect the sources of isolation. However, we identified some common phenotypic characteristics within subclusters 1a and 1c. Subcluster 1a was made up of three strains that are able to ferment both soluble starch and glycogen, and subcluster 1c (also determined to be a group of closely related strains by PCA) included strains that have the ability to adhere in vitro to human intestinal cell line HT-29 via an α -methyl-mannoside-inhibited binding mechanism (15). Two of the strains in subcluster 1c (*L. plantarum* 299 and 299v) have also been shown to be able to colonize human intestinal mucosa in vivo after oral administration (5). The similarities between strains as well as subclusters of *L. plantarum* were supported by the results obtained by the PCA. We found that phenotypic tests could not be used to group the strains in subcluster 1c (Fig. 5).

REA data can be used to distinguish between very closely related strains. To our knowledge, there are few methods available that are able to identify strains down to such a low level. However, proper gel conditions are necessary i.e., the amount of DNA applied has to be the same, and dots and dirt in the lanes of the gel, as well as normalization errors, must be avoided.

The REA method described in this paper, combined with UPGMA or PCA, has potential for classifying strains at the species level and below. An interesting question is whether this method can be used to estimate levels of relatedness between closely related species. The low levels of similarity between the most distantly related strains belonging to the same species which we observed argue against this possibility. On the other hand, the *L. pentosus* cluster appeared to be closer to the *L. plantarum* clusters than the *L. reuteri* strains did (Fig. 2). However, to answer this question, the data set has to be expanded, and the REA results have to be compared with results obtained by using available methods for phylogenetic studies.

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