Classification of *Lactobacillus reuteri* by Restriction Endonuclease Analysis of Chromosomal DNA

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A total of 14 field strains originally labeled *Lactobacillus reuteri*, 4 field strains of *Lactobacillus fermentum*, and 12 *Lactobacillus* type strains, as well as four additional reference strains, were classified by performing a restriction endonuclease analysis of chromosomal DNAs digested with *Asp* 718, *Cla*I, and *Eco*RI. The patterns were analyzed by using (i) Jaccard coefficients and the unweighted pair group algorithm with arithmetic averages and (ii) principal-component analysis (PCA) and soft independent modeling of class analogy (SIMCA). Grouping by using the Jaccard coefficients and the unweighted pair group algorithm with arithmetic averages resulted in six clusters, defined at a similarity level of 69%. Cluster 1 comprised 10 field strains of *L. reuteri*, *Lactobacillus* sp. strain DSM 20056, and *L. reuteri* DSM 20016^T (T = type strain) and could be divided into four subclusters. Cluster 2 included two *L. reuteri* field strains. Cluster 3 included one field strain labeled *L. reuteri* and one field strain labeled *L. fermentum*. Cluster 4 could be divided into three subclusters and included two field strains labeled *L. fermentum*, one reference strain and the type strain of *L. fermentum*, and four additional type strains. Cluster 5 contained two *Lactobacillus plantarum* strains. Cluster 6 included two type strains. The two numerical methods gave the same general results, but the PCA-SIMCA method resulted in a more complete view of the relationships. The SIMCA analysis grouped *L. reuteri* DSM 20016^T, *Lactobacillus* sp. strain DSM 20056, and, with one exception, all of the *L. reuteri* field strains as a "class." *Lactobacillus helveticus* DSM 20075^T and *Lactobacillus casei* subsp. *pseudoplatantarum* DSM 20008^T were the type strains most closely related to the *L. reuteri* SIMCA class. The *L. reuteri* strains isolated from rats could be separated from the *L. reuteri* strains isolated from humans and pigs by the PCA. DNA-DNA-hybridization showed that strains classified as *L. reuteri* on the basis of the results of the restriction enzyme analysis also exhibited >70% DNA-DNA-homology to the type strain of *L. reuteri*. The phenotype of *L. reuteri* (ability to ferment carbohydrates) coincided in most cases with the genotype; this seemed not to be the case for *L. fermentum* strains.

The genus *Lactobacillus*, which contains more than 70 species, is by tradition phenotypically founded, and the different species are grouped together on the basis of their metabolic pathways (12). However, from a genetic perspective the genus is heterogeneous because the G+C contents range from 30 to 55 mol%, and comparisons of 16S rRNAs have revealed relationships between different species which are not consistent with the traditional metabolic groups (8). Several of the traditional phenotypic traits used for species characterization in the genus *Lactobacillus* are unstable and uncertain (1a, 5). Thus, a genotypically based classification of *Lactobacillus* strains, both above and below the species level, is urgently needed. One approach is to use restriction endonuclease analysis (REA) and chromosomal DNAs (17), which is feasible since REA gels can be numerically analyzed for similarity. This can be done either by a more traditional numerical analysis or by principal-component analysis (PCA).

Lactobacillus reuteri, which is an obligatory heterofermentative bacterium, was first described by Kandler et al. (11). Isolates having the *L. reuteri* phenotype are frequently found associated with the gastrointestinal tracts of humans (12, 13) and animals (2, 14). Industrial interest in *L. reuteri* is increasing as it has been proved that some strains can produce an antimicrobial compound called reuterin (4, 7). A commercial milk product based on *L. reuteri* ("BRA-milk") was introduced in Sweden in 1991. Furthermore, a *Lacto-*

bacillus strain that was isolated from rats and was identified phenotypically as *L. reuteri* (strain R2LC) (14) hindered acetic acid-induced colitis in rats (10).

The aims of this study were to use REA for the classification of strains that had been phenotypically identified as *L. reuteri* and to compare these strains with other heterofermentative field strains having a similar phenotype (*Lactobacillus fermentum*) and with some type strains of organisms belonging to different catabolic categories. The classification obtained was compared with the results of a DNA-DNA homology study.

MATERIALS AND METHODS

Strains and culture conditions. The strains which we tested are listed in Table 1. In addition, *L. reuteri* N4I (representing 20 identical isolates) (14) was also tested. The field strains isolated from humans, pigs, and rats were all phenotypically identified as either *L. reuteri* or *L. fermentum* on the basis of the results of API 50 CH tests (API Systems S.A., Montalieu Vercieu, France). The strains were grown at 37°C in *Lactobacillus* carrying medium (9) containing 0.3 to 1% glucose. Stock cultures were stored at -80°C in freezing buffer (1a).

Preparation and agarose gel electrophoresis of plasmid DNAs. Plasmid DNAs were prepared from strains 2DS20057:R, 8704:3, 1044, 8557:1, 1068, 8557:3, 8581:1, and 8580:3 (6) and were electrophoresed in 0.7% agarose gels together with *Escherichia coli* V517 reference plasmids (Plasmid Reference Center, Stanford, Calif.). The gels were electrophoresed for 4 h at 120 V in TB electrophoresis buffer (89 mM boric acid, 23 mM H₃PO₄, 2.5 mM EDTA; pH 8.3) after an initial run at 12 mA.

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TABLE 1. Clusters of *Lactobacillus* strains obtained at an S_J similarity level of 69% by using the UPGMA method^a

Cluster	Similarity level (%) ^b	Strain	Source of isolation	Culture no. ^c	Similarity distance from ^d :		
					Strain 1073	Strain R2J	Strain DSM 20016 ^T
1a	77	<i>L. reuteri</i> 1073	Pig ^e	1	0	26	24
1a		<i>L. reuteri</i> N5D:1	Rat ^f	11	16	23	30
1a		<i>L. reuteri</i> 1068	Pig ^e	2	18	28	30
1a		<i>L. reuteri</i> 8557:3 (108) ^g	Human ^h	5	21	32	33
1a		<i>L. reuteri</i> 1063	Pig ^e	3	16	30	29
1b	76	<i>L. reuteri</i> 1048	Pig ^e	14	24	27	28
1b		<i>Lactobacillus</i> sp. strain DSM 20056		34	28	32	35
1c	76	<i>L. reuteri</i> N2J ⁱ	Rat ^f	7	26	0	36
1c		<i>L. reuteri</i> N2D ^j	Rat ^f	8	22	17	30
1c		<i>L. reuteri</i> N5LC:2 ^k	Rat ^f	12	26	26	35
1c		<i>L. reuteri</i> R2LC ^l	Rat ^f	9	23	26	31
1d	69	<i>L. reuteri</i> DSM 20016 ^T		19	24	36	0
None	68 ^m	<i>L. reuteri</i> DSM 20015	— ^{f,h}	20	29	30	40
None		<i>L. helveticus</i> DSM 20075 ^T		33	36	31	37
2	91	<i>L. reuteri</i> 8557:1	Human ⁿ	4	28	43	39
2		<i>L. reuteri</i> 1044	Pig ^e	13	29	42	36
3	85	<i>L. reuteri</i> 8580:3 (133)	Human ^h	6	33	44	40
3		<i>L. fermentum</i> 8581:1 (134)	Human ^h	15			
None		<i>L. fermentum</i> 8607:2 (142)	Human ^h	16			
4a	79	<i>L. fermentum</i> 8690:1	Human ⁿ	17			
4a		<i>L. casei</i> subsp. <i>pseudoplantarum</i> DSM 20008 ^T		29			
4b	72	<i>Lactobacillus brevis</i> DSM 20054 ^T		26			
4b		<i>L. casei</i> subsp. <i>casei</i> DSM 20011 ^T		30			
4b		<i>L. fermentum</i> ATCC 14931 ^T		32			
4b		<i>L. rhamnosus</i> DSM 20021 ^T		31			
4c	95	<i>L. fermentum</i> 8704:3 (245)	— ^h	18			
4c		<i>L. fermentum</i> 2DS20057:R (16) ^o	— ^h	23			
5	72	<i>L. plantarum</i> ATCC 14917 ^T		21			
5		<i>L. plantarum</i> ATCC 8014	— ^h	22			
None		<i>Lactobacillus crispatus</i> DSM 20584 ^T		25			
6	69	<i>Lactobacillus hamsteri</i> ATCC 43851 ^T		24			
6		<i>Lactobacillus amylovorus</i> DSM 20531 ^T		27			
None		<i>Lactobacillus alimentarius</i> DSM 20249 ^T		28			

^a DSM, Deutsche Sammlung von Mikroorganismen, Göttingen, Germany; ATCC, American Type Culture Collection, Rockville, Md.^b The similarity level at which the strains of a cluster or subcluster merge.^c Culture numbers used in Fig. 1 and 2 for indicating strains.^d Similarity distances (D_J) between strains were calculated by using the following formula: $D_J = 100(1 - S_J)$.^e Isolated and described by Axelsson and Lindgren (3).^f Phenotypically characterized by Molin et al. (14).^g The numbers in parentheses are the designations used by Molin et al. (13).^h Phenotypically characterized by Molin et al. (13) (cluster 13, which was labeled *Lactobacillus buchneri*).ⁱ Represents four isolates having identical REA patterns.^j Represents three isolates having identical REA patterns.^k Represents two isolates having identical REA patterns.^l Represents 12 isolates having identical REA patterns.^m *L. reuteri* DSM 20015 merges with cluster 1 at a similarity level of 68%.ⁿ Phenotypically characterized by Molin et al. (13), but the phenotype is not shown in the results.^o *L. fermentum* 2DS20057:R was phenotypically characterized by Molin et al. (13) and was assumed to be *Lactobacillus buchneri* DSM 20057^T; strain 2DS20057:R was later assigned to *L. fermentum* by workers in our laboratory on the basis of a partially identical 16S rRNA sequence and >70% DNA-DNA-homology to *L. fermentum* ATCC 14931^T (1).

Restriction endonuclease digestions. Chromosomal DNAs were prepared and cleaved by restriction endonucleases as previously described (17). Aliquots (750 ng) of DNA were digested with *Asp* 718, *Eco*RI, and *Cla*I and were electrophoresed in agarose gels. The DNA concentrations were

determined with a fluorometer (model TKO 100; Hoefer Scientific Instruments, San Francisco, Calif.).

Electrophoresis and scanning of gels. Agarose gel electrophoresis of chromosomal DNA was performed as described by Ståhl et al. (17). The band patterns of the REA gels were

FIG. 1. First and second principal-component scores obtained for the projection based on the complete data set (except the data for *L. reuteri* N4I). The strains used and their culture numbers are listed in Table 1. The axes are in arbitrary units.

scanned with a laser densitometer (UltraScan XL; Pharmacia LKB Biotechnology AB, Uppsala, Sweden), and the resulting absorbance curves were transformed into data sets by using the method of Ståhl et al. (17). Each lane was scanned three times, and the mean value for the relative absorbance in each interval was computed. The absorbance curves obtained with *Cla*I and *Eco*RI digests were each divided into 20 intervals, and the curves obtained with *Asp* 718 digests were each divided into 40 intervals. The recorded band patterns gave a data set consisting of 34 objects (strains) and 80 variables (total relative peak areas of absorbance intervals). In some cases plasmid bands interfered with the recording of the chromosomal band patterns, and the variable was then defined as missing. The DNA of strain N4I was resistant to *Cla*I digestion, and the data for all of the variables from *Cla*I digestion of the DNA of *L. reuteri* N4I were considered missing data.

Mathematical analyses. The data set was numerically analyzed by using the Jaccard coefficient (S_J) (16), and clustering was accomplished by using the unweighted pair group method with arithmetic averages (UPGMA) (15). The UPGMA values were calculated for the complete data set (except the data for strain N4I), and the CLUSTAN 2 program (release 3.2; D. Wishart, Program Library Unit, Edinburgh University) was used. The values used for variables for which there were no data were the mean values of the variables for all strains. Similarity distances (D_J) between strains were calculated [$D_J = 100(1 - S_J)$].

The SIMCA-3B program (version TMP; Sepanova AB, Enskede, Sweden) was used for PCA and soft independent modeling of class analogy (SIMCA) of the data set. A PCA was performed with the complete data set (except the data for strain N4I) without direct reference to class separation (Fig. 1). The values used for variables for which there were no data were the mean values of the variables for all strains.

SIMCA was used to classify the isolates and to investigate the distances between different type strains belonging to a select group (labeled "class" in the SIMCA manual) of *L. reuteri*. A class model (Table 2) was constructed with the 12 isolates that clustered together with the *L. reuteri* type strain

TABLE 2. Residual standard deviation to the class model of *L. reuteri*^a

Strain	Residual standard deviation
<i>L. reuteri</i> R2LC	0.478 ^b
<i>L. reuteri</i> 1044	0.591 ^b
<i>L. reuteri</i> 1068	0.676 ^b
<i>L. reuteri</i> 8557:1	0.751 ^b
<i>L. reuteri</i> N2J	0.752 ^b
<i>L. reuteri</i> 1073	0.808 ^b
<i>L. reuteri</i> 8557:3	0.851 ^b
<i>L. reuteri</i> 1048	0.918 ^b
<i>L. reuteri</i> N5LC:2	0.975 ^b
<i>L. reuteri</i> 1063	1.01 ^b
<i>L. reuteri</i> N5D:1	1.02 ^b
<i>L. reuteri</i> N2D	1.10 ^b
<i>L. reuteri</i> N4I	1.06 ^c
<i>L. reuteri</i> DSM 20016 ^T	1.33 ^c
<i>Lactobacillus</i> sp. strain DSM 20056	1.34 ^c
<i>L. helveticus</i> DSM 20075 ^T	1.47
<i>L. casei</i> subsp. <i>pseudoplantarum</i> DSM 20008 ^T	1.50
<i>L. rhamnosus</i> DSM 20021 ^T	1.53
<i>L. fermentum</i> 8690:1	1.54
<i>L. casei</i> subsp. <i>casei</i> DSM 20011 ^T	1.57
<i>L. plantarum</i> ATCC 14917 ^T	1.63
<i>L. fermentum</i> 8581:1	1.72
<i>Lactobacillus brevis</i> DSM 20054 ^T	1.83
<i>Lactobacillus crispatus</i> DSM 20584 ^T	1.83
" <i>L. reuteri</i> " 8580:3	1.84
<i>L. fermentum</i> 8607:2	1.84
<i>Lactobacillus hamsteri</i> ATCC 43851 ^T	1.85
<i>L. fermentum</i> 8704:3	1.88
<i>L. fermentum</i> ATCC 14931 ^T	1.91
<i>L. fermentum</i> 2DS20057:R	2.12
<i>L. plantarum</i> ATCC 8014	2.18
<i>L. reuteri</i> DSM 20015	2.31
<i>Lactobacillus amylovorus</i> DSM 20531 ^T	2.64
<i>Lactobacillus alimentarius</i> DSM 20249 ^T	2.82

^a The pooled RSD of the class is 1.14.

^b Strains used for construction of the class model. The values were corrected for loss of degrees of freedom.

^c Strains belonging to the class with a 99% confidence limit according to the F-test.

in the initial PCA, and this set of isolates exhibited 80 variables. A total of 15 variables were technically unreliable or missing and were defined as "missing values" (these values were representative of the mean values of the variables for strains in the class). All of the strains were subjected to an F-test, in which the residual variance of each strain in the whole group was compared with the pooled residual variances of the strains in the class model; the square of the pooled residual variance was defined as the sum of all squared variances divided by the number of objects. When strains were tested with the class model, no missing data had to be filled in with approximate values since missing values do not interfere with SIMCA.

An additional PCA was performed with the strains used in the class model, together with *L. reuteri* DSM 20016^T (T = type strain), *L. reuteri* DSM 20015, and *Lactobacillus* sp. strain DSM 20056 (Fig. 2). The values used for the missing variables were the mean values of the variables for all of the strains.

Nonradioactive labeling of DNA. Portions (1 µg) of chromosomal DNAs from *L. reuteri* DSM 20016^T, 1073, and N2J were sheared by sonication (model 250 Sonifier; Branson Ultrasonics Corp., Danbury, Conn.) for eight 30-s periods.

FIG. 2. First and second principal-component scores obtained for the projection based on the 12 strains used for the *L. reuteri* class model together with *L. reuteri* DSM 20016^T, *Lactobacillus* sp. strain DSM 20056, and *L. reuteri* DSM 20015. The strains used and their culture numbers are listed in Table 1. The axes are in arbitrary units.

The fragment sizes were determined to be approximately 0.4 kb by gel electrophoresis. Each sheared DNA was labeled with digoxigenin (DIG-DNA labeling and detection kit; Boehringer Mannheim Scandinavia AB, Bromma, Sweden) for 18 h according to the instructions of the manufacturer.

DNA-DNA hybridization. Hybridization reactions and washing and detection steps were performed by using the reagents in the DIG-DNA labeling and detection kit according to the instructions of the manufacturer (Boehringer Mannheim Scandinavia AB). Chromosomal DNAs from 10 strains were diluted in TE (10 mM Tris, 1 mM EDTA; pH 8.0). The DNAs were immobilized on a positively charged nylon membrane (Boehringer Mannheim Scandinavia AB) by filtration in a Minifold apparatus (type SRC 96; Schleicher & Schuell, Inc., Keene, N. H.) and subsequent baking at 120°C for 20 min. Aliquots (75 µl) of each DNA concentration (50, 25, 12.5, 6.25, and 3.125 ng/75 µl) from each strain were filtered, and the wells were then washed once with 150 µl of TE. The DNA was only loaded in every second well in order to avoid the hybridization signals from neighboring wells interfering with each other. Four parallel membranes containing immobilized DNA were prepared. Two membranes were hybridized with a digoxigenin-labeled *L. reuteri* DSM 20016^T DNA probe, and the other two membranes were hybridized with digoxigenin-labeled DNAs from strains 1073 and N2J (one membrane was hybridized with strain 1073 DNA and one membrane was hybridized with strain N2J DNA). The membranes were hybridized for 18 h at 64°C. The digoxigenin-labeled probes were detected with anti-digoxigenin antibody Fab fragments conjugated to alkaline phosphatase. The dephosphorylation of the chemiluminescent substrate Lumigen PPD generated light, which was documented on Kodak X-OMAT AR film during 2 min of exposure to the membranes.

The X-ray films were scanned two dimensionally with a laser densitometer (UltraScan XL; Pharmacia LKB Biotechnology AB), and the data were processed by using the LKB 2400 GelScan XL program (Pharmacia LKB Biotechnology AB). The total density of each spot was recorded.

TABLE 3. Filter hybridization at the "optimal" temperature with nonradioactivity labeled DNA

DNA on filter	% DNA-DNA homology with labeled DNA from:		
	Strain DSM 20016 ^T	Strain 1073	Strain N2J
<i>L. reuteri</i> DSM 20016 ^T	100	86 ± 10 ^a	80 ± 5
<i>L. reuteri</i> DSM 20015	93 ± 5	77 ± 5	65 ± 5
<i>Lactobacillus</i> sp. strain DSM 20056	75 ± 5	56 ± 10	58 ± 5
<i>L. reuteri</i> 1044	77 ± 5	84 ± 5	68 ± 5
<i>L. reuteri</i> 1073	84 ± 5	100	80 ± 5
<i>L. reuteri</i> 1063	70 ± 5	90 ± 5	74 ± 5
<i>L. reuteri</i> N2J	89 ± 5	83 ± 10	100
<i>L. reuteri</i> R2LC	80 ± 5	81 ± 5	89 ± 5
<i>L. reuteri</i> N5D:1	80 ± 10	80 ± 5	84 ± 5
<i>L. fermentum</i> ATCC 14931 ^T	34 ± 10	25 ± 10	29 ± 10
<i>L. casei</i> subsp. <i>pseudoplantarum</i>	5 ± 5		

^a Percentage of hybridization ± estimated margin of error based on the range of variation obtained in replicate hybridizations of homologous DNA (*L. reuteri* DSM 20016^T).

The levels of DNA-DNA-homology were calculated from direct plots of absorption versus DNA concentration. The final level of reassociation (or homology) was defined as the ratio of the maximum density of a tested DNA to the saturation density of the probe DNA. A plot of the homology values (including reference values) versus logarithmic values for the initial reassociation ratios (defined as the initial slope from the saturation plots) resulted, in most cases, in a linear relationship. When the calculated levels of homology were not proportional to the initial reassociation values, the homology value was defined as the average of the results of the two calculations (these homology values were assigned a larger margin of error) (Table 3). Deviations were assumed to result from the facts that (i) at lower levels of homology final saturation densities were not reached at the corresponding DNA concentrations, and (ii) different regions of the chromosomal DNA might exhibit different levels of homology. The experimental margin of error was estimated from the results of two independent experiments by using DNA from *L. reuteri* DSM 20016^T as a probe.

RESULTS

S_J-based UPGMA. The S_J-based cluster analysis identified six clusters, which were defined at a similarity level of 69% (Table 1).

Cluster 1 comprised 10 field isolates (all originally identified as *L. reuteri*), *Lactobacillus* sp. strain DSM 20056, and *L. reuteri* DSM 20016^T and could be divided into four subclusters. The type strain formed a one-member cluster that merged with the other strains at a similarity level of 69% (Table 1). The straggling strain *L. reuteri* DSM 20015 merged with cluster 1 at a similarity level of 68%.

Cluster 2 included two field strains phenotypically identified as *L. reuteri* (Table 1).

Cluster 3 included two field strains phenotypically identified as *L. reuteri* and *L. fermentum* (Table 1).

Cluster 4 comprised two field strains, one reference strain, and five different type strains and could be divided into three subclusters (Table 1). Subcluster 4a included one field strain labeled *L. fermentum* and *Lactobacillus casei* subsp. *pseudoplantarum* DSM 200008^T; subcluster 4b included the

type strain of *L. fermentum* and three additional type strains; and subcluster 4c consisted of one field strain phenotypically identified as *L. fermentum* and *L. fermentum* 2DS20057:R.

Cluster 5 contained two *Lactobacillus plantarum* strains, one of which was the type strain (Table 1).

Cluster 6 included two type strains (Table 1).

PCA-SIMCA results. The PCA of the complete data set (except the data for strain N4I) resulted in the a loading plot of the first and second principal components (Fig. 1). The loading plot showed that all of the phenotypically identified *L. reuteri* strains (except strain 8580:3) formed a group with *L. reuteri* DSM 20016^T (plus *L. reuteri* DSM 20015 and *Lactobacillus* sp. strain DSM 20056). This group was well separated from all other strains (Fig. 1).

L. fermentum 8704:3 and 2DS20057:R were closely related, differing only in one band on the REA gels (Fig. 1).

All of the strains were tested with a class model constructed by the SIMCA method from the *L. reuteri* labeled field strains shown in Fig. 1 (Table 2). *L. reuteri* N4I and DSM 20016^T and *Lactobacillus* sp. strain DSM 20056 belonged to the class with a confidence limit of 99%, but all of the other strains outside the class reference set, as well as *L. reuteri* DSM 20015, were excluded by the model (Table 2). With the exception of *L. reuteri* R2LC, the different strains belonging to the class reference set could be omitted during class construction (one by one), but they still fitted the class (data not shown). *L. reuteri* R2LC only fitted the class when it had been included in the reference set.

According to the SIMCA class model, the type strains most closely related to *L. reuteri* were *Lactobacillus helveticus* DSM 20075, *L. casei* subsp. *pseudoplatantarum* DSM 20008, *Lactobacillus rhamnosus* DSM 20021, and *Lactobacillus casei* subsp. *casei* DSM 20011 (Table 2).

The results of a second PCA, which included only the strains belonging to the class model (Table 2) and *L. reuteri* DSM 20016^T, *L. reuteri* DSM 20015, and *Lactobacillus* sp. strain DSM 20056, are shown in Fig. 2. The first principal component separated the strains isolated from rats from the other isolates. The second principal component singled out *L. reuteri* DSM 20015.

Plasmid profiles. Eight strains produced four pairs of almost identical REA patterns in which the members of each pair differed in only one to three bands. Plasmid profiles were obtained for these eight strains. The pair consisting of *L. fermentum* 2DS20057:R and 8704:3 appeared to be plasmid free. *L. reuteri* 1044 and 8557:1 harbored three plasmids whose sizes were the same in the two strains (5.4 ± 0.3 , 7.1 ± 0.4 , and 37 ± 2 MDa). *L. fermentum* 8581:1 and "*L. reuteri*" 8580:3 contained two plasmids whose sizes were the same in the two strains (1.2 ± 0.06 and 5.4 ± 0.3 MDa). And *L. reuteri* 1068 and 8557:3 contained five plasmids whose sizes were the same in the two strains (2.6 ± 0.1 , 5.4 ± 0.3 , 7.1 ± 0.4 , 25.3 ± 1.3 , and 32 ± 2 MDa), but in addition *L. reuteri* 1068 contained two other plasmids (5.1 ± 0.3 and 18.2 ± 1 MDa).

DNA-DNA hybridization. The results of hybridization of chromosomal DNAs from some of the strains to DNAs from *L. reuteri* DSM 20016^T, 1073, and N2J are shown in Table 3. The type strain exhibited the highest level of similarity to *L. reuteri* DSM 20015 and a similarity level of 70% or more to all of the field strains tested. *L. reuteri* 1073 exhibited the highest levels of homology to the type strain and the two field strains isolated from pigs tested and also exhibited relatively high levels of homology to the other *L. reuteri* strains (Table 3). *L. reuteri* N2J exhibited the highest levels of homology to the field strains isolated from rats and a

relatively low level of homology to strain 1044 (68%). The levels of homology to *L. fermentum* ATCC 14931^T were low (Table 3).

DISCUSSION

The phenotypic approach to *Lactobacillus* taxonomy has been challenged (1a, 5). However, in the case of *L. reuteri*, our data indicate that the genotype reflects the phenotype (that is, the ability to ferment different carbohydrates in API 50CH tests), even if there is one striking exception, "*L. reuteri*" 8580:3 (Table 1 and Fig. 1).

The two different numerical models used for comparisons of the restriction patterns gave similar results. However, the PCA-SIMCA model (Fig. 1 and 2) resulted in a better understanding of the relationships among the strains. For example, when the UPGMA model was used, *L. reuteri* 1044 and 8557:1 were members of a single cluster (Table 1, cluster 2), widely separated from the major *L. reuteri* cluster (cluster 1). Even if these two strains were also separated by the PCA, the SIMCA class model clearly demonstrated that they were very similar to the other strains of *L. reuteri* (Table 2). This similarity was also confirmed by the DNA-DNA homology data (Table 3).

Another advantage of SIMCA is the ability to handle missing values. The values for *Cla*I digests were missing from the *L. reuteri* N4I data set. Therefore, the strain could not be processed by the UPGMA method but could be included in the SIMCA analysis (Table 2).

Dye-buoyant density centrifugation separates covalently closed circular plasmid DNA from chromosomal DNA, but open circular and linear forms of large plasmids may contaminate the preparation and obstruct the interpretation of the REA band patterns. However, with the PCA-SIMCA method it is possible to spot bands which may be of plasmid origin (17). It can also be pointed out that the plasmid profiles were similar for the four pairs of closely related strains found in this study, which may suggest that the few distinguishing REA bands in these cases were not of plasmid origin.

The REA classification determined in this study might, at first glance, give the impression that there is a relatively heterogeneous group of *L. reuteri* strains (Table 1 and Fig. 1 and 2). However, the *L. reuteri* strains were separated from other strains, and DNA-DNA-hybridizations showed that the *L. reuteri* strains exhibited levels of homology of 70% or more to the type strain (Table 3). Thus, the REA and DNA-DNA homology data are in general agreement.

The only major discrepancy between the DNA-DNA homology data and the REA data was observed with *L. reuteri* DSM 20015. In the REA this strain was excluded from *L. reuteri* (Tables 1 and 2), while its level of DNA-DNA homology to *L. reuteri* DSM 20016^T was 93% (Table 3), which is consistent with the data of Vescovo et al. (18).

It may seem anomalous that the type strain of *L. fermentum* exhibits 34% DNA-DNA homology to the type strain of *L. reuteri* (Table 3) when the G+C contents of these strains differ by about 10 mol%. However, this level of homology may be due to the high G+C content (52 to 54 mol% [thermal denaturation method]) of *L. fermentum* and to the fact that the hybridization reaction was performed at 64°C. REA data place *L. fermentum* ATCC 14931^T far from the *L. reuteri* strains in the multidimensional space diagram.

Lactobacillus sp. strain DSM 20056 exhibited 75% DNA-DNA homology to the type strain of *L. reuteri* but was previously reported to have a DNA-DNA homology level of

less than 70% (18); the discrepancy in these values might be because the former value was obtained in an experiment that was not performed at the "stringent" temperature.

REA combined with numerical analyses has a potential for effective assessment of relationships on the species level. Furthermore, REA groups biovariants and detects individual strains at a level far below the species level. However, a crucial question is to what extent the relationships between the *L. reuteri* cluster and the most closely related surrounding strains should be trusted. For example, is *L. reuteri* more closely related to *L. helveticus* and *L. casei* subsp. *pseudoplantarium* than to the other type strains tested (Fig. 1 and Table 2)?

Can REA be used to determine relationships above the species level? This question may be answered by using SIMCA with an expanded data set in which each species is described by its "own" class model. Then the distances between the species (class models) can be calculated. However, the question can be answered only by comparing REA results with the results of other methods. Nevertheless, it should be pointed out that the type strains of the related taxa *L. casei* subsp. *pseudoplantarium*, *L. rhamnosus* (formerly *L. casei* subsp. *rhamnosus*), and *L. casei* subsp. *casei* merged in the SIMCA class model (Table 3).

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