Identification and classification of *Lactobacillus acidophilus*, *L. gasseri* and *L. johnsonii* strains by SDS-PAGE and rRNA-targeted oligonucleotide probe hybridization

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Thirty-two strains originally identified as *Lactobacillus acidophilus* and *L. gasseri* were screened for their taxonomic homogeneity by SDS-PAGE of whole-cell proteins. After numerical comparison of the resulting protein electrophoretic fingerprints, two well-delineated clusters were detected. The majority of the strains grouped in one electrophoretic cluster, which contained the type strain of *L. acidophilus* and corresponds to DNA group A1 of Johnson, J. L., Phelps, C. F., Cummins, C. S., London, J. & Gasser, F. (1980; *International Journal of Systematic Bacteriology* 30, 53–68). Another cluster corresponded to DNA group B. It contained two subclusters, which agreed perfectly with DNA subgroups B1 (*L. gasseri*) and B2 (*L. johnsonii*), respectively. The 23S rRNA genes were partially sequenced and 23S-rRNA-targeted oligonucleotide probes were designed for identification of DNA groups A1, B1 and B2. Probe Lbg reacted with all strains of electrophoretic cluster B1 (*L. gasseri*), probe Lbj hybridized with strains of cluster B2 (*L. johnsonii*) and probe Lba with strains of cluster A1 (authentic *L. acidophilus*). The probes were successfully used for the identification of strains belonging to the respective species. The phylogenetic relationship of a representative of *L. johnsonii* was determined by comparative sequence analysis of the 16S rRNA genes. It is very closely related to *L. gasseri*.

Introduction

Identification of bacteria is essential in both basic and applied research. The identification of lactic acid bacteria (LAB) depends mainly on physiological and biochemical criteria. These procedures are not only time-consuming, but owing to an increasing number of LAB species, are also very ambiguous. rRNA sequencing and DNA:DNA hybridization studies have improved our taxonomic knowledge on the generic and suprageneric relationships of LAB (Collins *et al.*, 1989, 1991; Kilpper-Bälz *et al.*, 1982; Ludwig *et al.*, 1985; Martinez-Murcia & Collins, 1990; Schleifer *et al.*, 1985; Schleifer, 1987; Schleifer & Kilpper-Bälz, 1987). Identification to the species level, however, is still time-consuming, requires the back-up of a large database of rRNA sequences and

for very closely related species even rRNA sequence analysis may not be sufficient. In this study, we have applied the complementary use of two molecular screening methods to the improved classification and identification of the industrially important but heterogeneous species *Lactobacillus acidophilus* (Moro, 1900; Holland, 1920). *L. acidophilus* is believed to play an important role in human health and nutrition by its influence on the intestinal flora (Hawley *et al.*, 1959; Fiedler & Kandler, 1964; Mitsuoka, 1969; Mitsuoka *et al.*, 1969; Reuter, 1969). Despite considerable industrial and medical interest, the systematics of this species remained confusing for a long time (Moro, 1900; Orla-Jensen *et al.*, 1936; Hansen & Mocquot, 1970), and a considerable serological heterogeneity has been shown (Sharpe, 1970; Gasser, 1970). The heterogeneity was also reflected by the results of DNA:DNA hybridization studies (Johnson *et al.*, 1980; Lauer *et al.*, 1980). *L. acidophilus* and *L. gasseri* are found in similar habitats and cannot be distinguished by simple phenotypic criteria (Kandler & Weiss, 1986). More recently, the species *L. gallinarum* and *L. johnsonii* were created for the

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Abbreviation: LAB, Lactic acid bacteria.

Table 1. List of strains used

Homologous strain numbers (replicates) are indicated. The Table lists the results of the cluster analysis of the SDS-PAGE patterns of whole-cell proteins, and the 23S rRNA-targeted oligonucleotide probe reactions with probes specific for strains of *L. acidophilus*, *L. gasseri* and *L. johnsonii*.

Name as received	Proposed name	Strain number*	Strain number as received*	DNA similarity group†	SDS-PAGE group	Reaction with probe		
						Lba	Lbg	Lbj
<i>L. acidophilus</i>	<i>L. acidophilus</i>	LAB 84 ^T	DSM 20079 ^T		A1	+	—	—
<i>L. acidophilus</i>	<i>L. acidophilus</i>	LMG 7943 ^T	DSM 20079 ^T	Ia	A1	+	—	—
<i>L. acidophilus</i>	<i>L. acidophilus</i>	LMG 8150 ^T	CCUG 5917 ^T	A1	A1	+	—	—
<i>L. acidophilus</i>	<i>L. acidophilus</i>	LMG 9433 ^T	NCFB 1748 ^T		A1	ND	ND	ND
<i>L. acidophilus</i>	<i>L. acidophilus</i>	LMG 8151	CCUG 12853		A1	+	—	—
<i>L. acidophilus</i>	<i>L. acidophilus</i>	LMG 11428	NCFB 1	A1	A1	+	—	—
<i>L. acidophilus</i>	<i>L. acidophilus</i>	LMG 11429	NCFB 104		A1	+	—	—
<i>L. acidophilus</i>	<i>L. acidophilus</i>	LMG 11430	NCFB 1697		A1	+	—	—
<i>L. acidophilus</i>	<i>L. acidophilus</i>	LMG 11466	ATCC 13651	Ia	A1	+	—	—
<i>L. acidophilus</i>	<i>L. acidophilus</i>	LMG 11467	ATCC 314	A1, Ia	A1	+	—	—
<i>L. acidophilus</i>	<i>L. acidophilus</i>	LMG 11469	ATCC 4355	A1	A1	+	—	—
<i>L. acidophilus</i>	<i>L. acidophilus</i>	LMG 11470	ATCC 4796	A1	A1	+	—	—
<i>L. acidophilus</i>	<i>L. acidophilus</i>	LMG 11472	ATCC 9224	A1	A1	+	—	—
<i>L. acidophilus</i>	<i>L. acidophilus</i>	LAB 64	BMF 3054/62		A1	+	—	—
<i>L. acidophilus</i>	<i>L. acidophilus</i>	LAB 66	BMF 3057/62		A1	+	—	—
<i>L. acidophilus</i>	<i>L. acidophilus</i>	LAB 67	BMF 3060/71		A1	+	—	—
<i>L. acidophilus</i>	<i>L. acidophilus</i>	LAB 69	BMF 3062/62		A1	+	—	—
<i>L. acidophilus</i>	<i>L. acidophilus</i>	LAB 283t1	DSM 20242		A1	+	—	—
<i>L. acidophilus</i>	<i>L. acidophilus</i>	LAB 283t2	DSM 20242		A1	+	—	—
<i>L. gasseri</i>	<i>L. gasseri</i>	LMG 9203 ^T	NCFB 2233 ^T	IIa	B1	—	+	—
<i>L. gasseri</i>	<i>L. gasseri</i>	LAB 88 ^T	DSM 20243 ^T		B1	—	+	—
<i>L. acidophilus</i>	<i>L. gasseri</i>	LMG 11413	NCIMB 8819	B1	B1	—	+	—
<i>L. acidophilus</i>	<i>L. gasseri</i>	LMG 11414	NCIMB 8820	B1	B1	—	+	—
<i>L. gasseri</i>	<i>L. gasseri</i>	LMG 11443	NCFB 1375		B1	—	+	—
<i>L. acidophilus</i>	<i>L. gasseri</i>	LMG 11444	NCFB 3		B1	—	+	—
<i>L. acidophilus</i>	<i>L. gasseri</i>	LMG 11471	ATCC 4962	B1	B1	—	+	—
<i>L. acidophilus</i>	<i>L. johnsonii</i>	LMG 9436 ^T	NCFB 2241 ^T	B2	B2	—	—	+
<i>L. acidophilus</i>	<i>L. johnsonii</i>	LMG 9437	NCFB 1693	B2	B2	—	—	+
<i>L. acidophilus</i>	<i>L. johnsonii</i>	LMG 11468	ATCC 332	B2	B2	—	—	+
<i>L. acidophilus</i>	<i>L. johnsonii</i>	LAB 70	BMF3064/71		B2	—	—	+
<i>L. acidophilus</i>	<i>L. johnsonii</i>	LAB 71	BMF 16Lb6		B2	—	—	+
<i>L. acidophilus</i>	<i>L. johnsonii</i>	LAB 82	BMF 6Lb6		B2	—	—	+
Other Gram-positive bacteria‡						—	—	—

ND, Not determined.

* ATCC, American Type Culture Collection, Rockville, MD, USA; BMF, Bundesanstalt für Milchforschung, Kiel, Germany; CCUG, Culture Collection of the Department of Clinical Bacteriology, University of Göteborg, Göteborg, Sweden; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; LAB, Lactic Acid Bacteria Culture Collection, Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium; LMG, Culture Collection of the Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium; NCFB, National Collection of Food Bacteria, Reading, UK; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, UK.

† Ia and IIa refer to DNA groups described by Lauer *et al.* (1980); other indications refer to Johnson *et al.* (1980).

‡ *Bacillus cereus*, *B. licheniformis*, *B. subtilis*, *Enterococcus faecalis*, *E. faecium*, *L. alimentarius*, *L. amylovorus* (*L. acidophilus* DNA group A3), *L. brevis*, *L. buchneri*, *L. casei* subsp. *casei*, *L. casei* subsp. *rhamnosus*, *L. casei* subsp. *pseudopantarum*, *L. delbrueckii* subsp. *bulgaricus*, *L. crispatus* (*L. acidophilus* DNA group A2), *L. delbrueckii* subsp. *delbrueckii*, *L. delbrueckii* subsp. *lactis*, *L. fermentum*, *L. gallinarum* (*L. acidophilus* DNA group A4), *L. helveticus*, *L. kefir*, *L. plantarum*, *L. plantarum* subsp. *arabinosus*, *Lactococcus lactis* subsp. *cremoris*, *Lc. lactis* subsp. *lactis*, *Leuconostoc lactis*, *Le. mesenteroides*, *Le. mesenteroides* subsp. *cremoris*, *Streptococcus thermophilus*.

L. acidophilus DNA similarity groups A4 and B2, respectively (Fujisawa *et al.*, 1992).

In this paper, we have applied SDS-PAGE, rRNA sequence analysis and oligonucleotide probe hybridization to study the relationships in the heterogeneous *L. acidophilus* complex. Highly standardized SDS-PAGE of whole-cell proteins allows fast screening of large numbers of strains for comparative purposes. Recent data in the literature support the overall correlation between the results of numerical analysis of protein

patterns and DNA:DNA hybridization studies (Costas *et al.*, 1990; Dijkshoorn *et al.*, 1990; Pot *et al.*, 1992; Vauterin *et al.*, 1990; Vandamme *et al.*, 1991). SDS-PAGE can only be used on a relatively large number of cells, derived from a pure culture, whereas rRNA-targeted oligonucleotide probe hybridization is a fast alternative for identification of relatively small numbers of cells or strains from pure and mixed cultures present in complex nutritional environments (e.g. milk). Nucleic acid probes are known to be especially useful for rapid

Table 2. Sequences, specificities, and hybridization and washing temperatures of the oligonucleotide probes

Probe	Sequence	Specificity*	Target†	Temperature (°C) for:	
				Hybridization	Washing
Lba	5' TCTTTCGATGCATCCACA 3'	<i>L. acidophilus</i> (cluster A1)	1159–1180	40	49
Lbg	5' TCCTTTGATATGCATCCA 3'	<i>L. gasseri</i> (cluster B1)	1160–1178	40	50
Lbj	5' ATAATATATGCATCCACAG 3'	<i>L. johnsonii</i> (cluster B2)	1158–1179	40	49

* The cluster indication refers both to the electrophoretic clusters obtained in this paper and to the DNA groups as revealed by DNA similarity studies (Johnson *et al.*, 1980).

† The numbers indicate homologous positions of the *E. coli* 23S rRNA (Brosius *et al.*, 1981).

detection and identification of bacteria and have been used successfully in food products (Schleifer, 1990).

Methods

Strains used. The bacterial strains examined are listed in Table 1. A number of strains were analysed which have been included previously in the DNA hybridization studies of Johnson *et al.* (1980) and Lauer *et al.* (1980). In addition, strains studied were either derived from culture collections or isolated in the framework of the Biotechnology Research for Innovation, Development and Growth in Europe (BRIDGE) T-project on Lactic Acid Bacteria of the Commission of the European Communities.

Maintenance of cultures. Cultures were maintained by weekly transfer in MRS broth (De Man *et al.*, 1960; Oxoid). Cells were incubated in closed screw-capped tubes for 24 h at 30 °C after which they were transferred to room temperature. Purity was checked by plating on MRS agar (Oxoid).

PAGE of proteins. All strains were grown on MRS agar in Roux flasks at 30 °C for 24 h. Roux flasks were inoculated from a 24 h 10 ml MRS broth culture. Whole-cell protein extracts were prepared as described previously (Kiredjian *et al.*, 1986). Registration of the protein electrophoretic patterns, normalization of the densitometric traces, grouping of strains by the Pearson product moment correlation coefficient (r) and UPGMA cluster analysis were performed by the techniques described by Pot *et al.* (1993) using the software package GELCOMP (Version 1.4; L. Vauterin & P. Vauterin, unpublished).

Extraction and purification of DNA. DNA was isolated from lactobacilli according to the method of Lewington *et al.* (1987) with some modifications. Stationary phase culture (1 ml) was harvested and resuspended in 100 µl of 50 mM-Tris/HCl, 50 mM-sodium chloride, 10 mM-EDTA, pH 8. Fifteen microlitres of lysozyme (10 mg ml⁻¹) and of mutanolysin (5000 U ml⁻¹) were added and incubated at 37 °C for 15 min. Forty microlitres of 25% (w/v) SDS were then added, heated to 65 °C for 5 min and the mixture was vortexed vigorously for 5 s. Then 33 µl of 5 M-sodium chloride were added, mixed and left on ice for 1 h. After centrifugation for 15 min, the supernatant fluid was treated once with chloroform-isoamyl alcohol (24:1, v/v). DNA was precipitated with ethanol and resuspended in TE buffer (10 mM-Tris/HCl, 1 mM-EDTA, pH 8). RNAase A was added to a final concentration of 10 µg ml⁻¹ and then incubated at 37 °C for 30 min.

Designing of probes. Variable regions, homologous to positions 457–1703 of the 23S rRNA genes of *Escherichia coli* (Brosius *et al.*, 1981), were amplified from the bulk DNA by PCR (Saiki *et al.*, 1988) in combination with gene-specific primers (5' AGTACCGYGAG-GGAAAG 3' and 5' CCTTCTCCCGAAGTTACGG 3'). The amplified fragments were purified applying the GeneClean kit (Bio 101)

and sequenced directly using the T7-sequencing-kit (Pharmacia). Comparative analysis of the 23S rRNA gene fragment sequences revealed regions that could be used as specific target sites for DNA probes (Table 2). Oligonucleotides complementary to the 23S rRNA were obtained from MWG Biotech (Ebersberg, Germany).

Dot blot hybridization. Labelling of the probes, nucleic acid extractions, and dot blot hybridizations were performed as described previously by Hertel *et al.* (1991).

16S rRNA sequence analysis. *In vitro* amplification and sequencing of 16S rRNA genes was performed as described by Weizenegger *et al.* (1991).

Results and Discussion

The results of the numerical analysis of the SDS-PAGE protein patterns confirmed the heterogeneity of *L. acidophilus*. Fig. 1 shows the clusters formed after numerical analysis of the total protein profile [points 10–320 of the 400 points registered, omitting the stacking gel/separation gel interface (positions 0–9) and the front of the electrophoretic protein profile (positions 321–400)]. SDS-PAGE clearly discriminated between the two DNA similarity groups A and B of Johnson *et al.* (1980) (correlation level $r = 0.60$). The first cluster, homologous to DNA group A1, and therefore labelled cluster A1 (Fig. 1), comprised the majority of the strains, including the type strain of *L. acidophilus*, for which three independently obtained subcultures (Table 1) were investigated (mean correlation $r = 0.94$; Fig. 1). Cluster A1 is delineated at a correlation level of $r = 0.88$, and could clearly be differentiated from the other subgroups of DNA group A (results not shown). The second cluster, homologous to DNA similarity group B, is composed of two subclusters (B1 and B2; Fig. 1), similar to the two DNA subgroups B1 and B2 as defined by Johnson *et al.* (1980). Strains of *L. acidophilus* DNA subgroups B1 (Johnson *et al.*, 1980) and IIa (Lauer *et al.*, 1980) are believed to be synonymous with *L. gasseri* (Lauer & Kandler, 1980a,b; Lauer *et al.*, 1980). We included two different subcultures of the type strain of *L. gasseri* (Table 1) and found them both to belong to the subcluster B1 (mean correlation $r = 0.96$; Fig. 1).

Fig. 1. The mean correlation coefficient (r), represented as a dendrogram calculated by the unweighted average pair grouping method, for all strains investigated. The points 10–320 of the 400 point traces were used for the calculation of similarities between individual pairs of traces.

DNA similarity group B2 was recently named *L. johnsonii* (Fujisawa *et al.*, 1992). The proposed type strain (ATCC 33200) was identical to strain LMG 9436^T, which was obtained from the National Collection of Food Bacteria (Reading, UK) as NCFB 2241^T. From Fig. 1 it is evident that *L. johnsonii* BMF 6Lb6 (LAB 82) is closely related to *L. johnsonii* ATCC 332 (LMG 11468) studied by Fujisawa *et al.* (1992). The phylogenetic affiliation of strain BMF 6Lb6 was determined by comparative sequence analysis of its 16S rRNA genes. (EMBL accession number M99704). When compared to the 16S rRNA sequence of *L. gasseri* (Ribosomal RNA Data Base Project, RDP; Olson *et al.*, 1991) a similarity of 99.4% was found, confirming the very close relationship of *L. johnsonii* and *L. gasseri*.

Partial sequence analysis of 23S rRNA genes of *L. acidophilus* DSM 20079^T, *L. johnsonii* LMG 9436^T, *L. johnsonii* BMF 6Lb6, *L. gasseri* LMG 9203^T and *L. gasseri* LMG 11444 was carried out. Sequence differences were detected within highly variable regions of the genes, which could be used for the design of rRNA-targeted specific oligonucleotide probes. The sequence of the probes, the hybridization and washing temperatures, as well as the target sites are summarized in Table 2. The specificities of the probes are given in Table 1. Probe Lba hybridized exclusively to target nucleic acids of all strains assigned to electrophoretic cluster A1 in the present study. Probe Lbg was proven to be specific for

the strains belonging to electrophoretic subcluster B1 (*L. gasseri*), and probe Lbj reacted only with nucleic acids from representatives of electrophoretic cluster B2 (*L. johnsonii*).

The prescreening of *L. acidophilus*-like strains at a fine taxonomic level by SDS-PAGE of whole-cell proteins has facilitated the subsequent design of three 23S rRNA-based oligonucleotide probes for identification. These probes can be used with a high degree of confidence, and we consider them to be one of the fastest ways for identification of specific LAB species in applied and fundamental research.

The 16S rRNA sequence analysis and the comparison of the whole-cell protein electrophoretic patterns showed that *L. johnsonii* is very closely related to *L. gasseri*.

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