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

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Thirty-two strains originally identified as \textit{Lactobacillus acidophilus} and \textit{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 \textit{L. acidophilus} and corresponds to DNA group A1 of Johnson, J. L., Phelps, C. F., Cummins, C. S., London, J. & Gasser, F. (1980; \textit{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 (\textit{L. gasseri}) and B2 (\textit{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 (\textit{L. gasseri}), probe Lbj hybridized with strains of cluster B2 (\textit{L. johnsonii}) and probe Lba with strains of cluster A1 (authentic \textit{L. acidophilus}). The probes were successfully used for the identification of strains belonging to the respective species. The phylogenetic relationship of a representative of \textit{L. johnsonii} was determined by comparative sequence analysis of the 16S rRNA genes. It is very closely related to \textit{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 \textit{et al.}, 1989, 1991; Kilpper-Bälz \textit{et al.}, 1982; Ludwig \textit{et al.}, 1985; Martinez-Murcia & Collins, 1990; Schleifer \textit{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 \textit{Lactobacillus acidophilus} (Moro, 1900; Holland, 1920). \textit{L. acidophilus} is believed to play an important role in human health and nutrition by its influence on the intestinal flora (Hawley \textit{et al.}, 1959; Fiedler & Kandler, 1964; Mitsuoka, 1969; Mitsuoka \textit{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 \textit{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 \textit{et al.}, 1980; Lauer \textit{et al.}, 1980). \textit{L. acidophilus} and \textit{L. gasseri} are found in similar habitats and cannot be distinguished by simple phenotypic criteria (Kandler & Weiss, 1986). More recently, the species \textit{L. gallinarum} and \textit{L. johnsonii} were created for the
In this paper, we have applied SDS-PAGE, rRNA sequence analysis and oligonucleotide probe hybridization to study the relationships in the heterogeneous \textit{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

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence</th>
<th>Specificity*</th>
<th>Target†</th>
<th>Temperature (°C) for:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lba</td>
<td>5' TCTTTGATGCATCCACCA 3'</td>
<td><em>L. acidophilus</em> (cluster A1)</td>
<td>1159-1180</td>
<td>40</td>
</tr>
<tr>
<td>Lbg</td>
<td>5' TCTCTTGATAGCATCCACCA 3'</td>
<td><em>L. gasseri</em> (cluster B1)</td>
<td>1160-1178</td>
<td>40</td>
</tr>
<tr>
<td>Lbj</td>
<td>5' ATTAATATCCAGCCACAG 3'</td>
<td><em>L. johnsonii</em> (cluster B2)</td>
<td>1158-1179</td>
<td>40</td>
</tr>
</tbody>
</table>

* The cluster indication refers both to the electrophoretic clusters formed 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).

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 GELOMPAR (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 50 mM-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–isoamylalcohol (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' AGTACCAYGAGGGAAAG 3' and 5' CTTCTCCGAGTACCG 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, nuclease 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 Ha (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).
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_ ATCC 332 (LMG 11468) 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 _Lacticophilus_ 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 A2 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 _Lacticophilus_ 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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