The presence of two S-layer-protein-encoding genes is conserved among species related to *Lactobacillus acidophilus*

Hein J. Boot,^{1†} Carin P. A. M. Kolen,¹ Bruno Pot,² Karel Kersters² and Peter H. Pouwels^{1,3}

Author for correspondence: Hein J. Boot. Tel: +31 320 238881. Fax: +31 320 238668.
e-mail: H.J.Boot@ID.DLO.NL

¹ BioCentrum Amsterdam, University of Amsterdam, Plantage Muidersgracht 12, 1018 TV Amsterdam, The Netherlands

² BCCM/LMG Culture Collection, Laboratory of Microbiology, University of Gent, K. L. Ledeganckstraat 35, B-9000 Gent, Belgium

³ TNO Nutrition and Food Research Institute, PO Box 5815, 2280 HV Rijswijk, The Netherlands

Previously we have shown that the type strain of *Lactobacillus acidophilus* possesses two S-protein-encoding genes, one of which is silent, on a chromosomal segment of 6 kb. The S-protein-encoding gene in the expression site can be exchanged for the silent S-protein-encoding gene by inversion of this *slp* segment. In this study the presence of S-protein and corresponding S-protein-encoding genes of strains belonging to species that are closely related to *L. acidophilus* was determined. All strains investigated were identified by numerical comparison of highly standardized one-dimensional SDS-PAGE whole-cellular-protein patterns. Western blot and Southern blot methods were used to identify the presence of, and homology between, S-proteins and S-protein-encoding genes. From these analyses we conclude that strains of *L. acidophilus*, *L. crispatus*, *L. amylovorus* and *L. gallinarum* possess an S-layer and contain two *slp* genes. Strains of *L. helveticus* possess an S-layer but have only one intact *slp* gene. Strains of *L. gasseri*, *L. johnsonii* and *L. delbrueckii* subsp. *bulgaricus* have neither an S-layer nor S-protein-encoding genes hybridizing with probes derived from the *L. acidophilus* *slpA* or *slpB* region. The presence of a highly conserved 5' region in the *slp* genes of strains of *L. acidophilus*, *L. crispatus*, *L. amylovorus* and *L. gallinarum* suggests that S-layer variation is a common feature for strains of these species.

Keywords: *Lactobacillus*, SDS-PAGE identification, surface layer, S-protein, S-layer variation

INTRODUCTION

Lactic acid bacteria are widespread in nature and are generally used in the production and preservation of food and feed products like cheese, sauerkraut, meat, yoghurt and silage (McKay & Baldwin, 1990). Although some *Lactobacillus* strains can colonize the intestinal tract of the host, living lactobacilli from food or feed preparations are in most cases lost from the gastro-intestinal tract within a few days after the intake has stopped (Lidbeck & Nord, 1993). Those *Lactobacillus* strains which are normal inhabitants of the digestive tract must possess the ability to survive in this environment and must be able to adhere to the exposed surface of the epithelial cells. The

adherence of these colonizing lactobacilli is mediated by fimbrial or afimbrial adhesins, which interact with the epithelial cells (Beachey, 1981; Mukai & Arihara, 1994). The interaction of endogenous lactobacilli with the epithelial surface has attracted the attention of several research groups since *Lactobacillus* species are reported to possess health-promoting properties when present in the gastro-intestinal and (female) urogenital tract of man and animals. Several effects have been reported to be associated with the presence of lactobacilli, e.g. stimulation of immunoglobulin production (Isolaureti *et al.*, 1995; Link-Amster *et al.*, 1994; Perdigon *et al.*, 1993), induction of interferon expression in macrophages (Kitazawa *et al.*, 1992), acidification of the local environment (Zheng *et al.*, 1994), production of H₂O₂ (McGroarty, 1993), hypocholesterolaemic effects (Fernandes *et al.*, 1987), binding of mutagenic compounds (Orrhage *et al.*, 1994), production of bacteriocins

† Present address: Institute for Animal Science and Health (ID-DLO), PO Box 65, NL-8200 AB Lelystad, The Netherlands.

(Klaenhammer, 1993) and prevention of adherence of pathogenic bacteria like *Salmonella typhimurium* and *Neisseria gonorrhoeae* to the epithelial cells (Coconnier *et al.*, 1993; Zheng *et al.*, 1994).

The dominant surface protein of many Eu- and Archaeobacteria, including several *Lactobacillus* species, is the S protein. The S protein is capable of crystallization into a regular structure on the outside of bacteria, the S-layer, which covers the entire cell wall during all stages of growth (for reviews see Beveridge, 1994; Messner & Sleytr, 1992). The S-layer is present on bacteria of several *Lactobacillus* species known to inhabit the gastro-intestinal tract (Johnson *et al.*, 1987; Lortal, 1993; Masuda & Kawata, 1983). Schneitz *et al.* (1993) reported that the *L. acidophilus* S-layer acts as an afimbrial adhesin *in vitro*, and interacts with avian epithelial cells. On the other hand, Greene & Klaenhammer (1994) reported that the chemical removal of the S-layer of *L. acidophilus* did not influence the *in vitro* adhesion of bacteria of this strain to Caco-2 cells. Toba *et al.* (1995) reported that the S-layer of an *L. crispatus* strain was involved in *in vitro* interaction with human intestinal cells, while the S-layer of the *L. acidophilus* type strain did not show such an interaction.

Recently we have reported that the *L. acidophilus* type strain possesses two S-protein-encoding genes (*slpA* and *slpB*) (Boot *et al.*, 1995). This *L. acidophilus* strain is capable of S-layer variation as it can change the position of the *slpB* gene from its silent site to the expression site of the *slpA* gene by inversion of the *slp* segment (Boot *et al.*, 1996). Variation in expression of surface-exposed proteins is known as antigenic variation and is often found for (a)fimbrial adhesins of pathogenic or opportunistic bacteria that are able to adhere to the epithelial cell layer of the gastro-intestinal tract of the host. In this study we have determined whether closely related strains of *L. acidophilus* contain an S protein and corresponding gene(s) that are similar to those of *L. acidophilus*. Our results show that *L. crispatus*, *L. amylovorus* and *L. gallinarum* have two *slp* regions. They all contain two copies of the highly conserved 5' identity region, which in *L. acidophilus* is used for the *in vivo* chromosomal recombination that leads to S-layer variation. *L. helveticus* possesses only one *slp* gene, while *L. gasseri*, *L. johnsonii* and *L. delbrueckii* subsp. *bulgaricus* lack S-protein-encoding genes.

METHODS

Standardized SDS-PAGE of whole-cell proteins. For the isolation of whole-cell proteins for identification, all *Lactobacillus* strains investigated (Table 1) and a number of relevant reference strains (Devuyst *et al.*, 1996; Pot *et al.*, 1993) were cultivated aerobically in MRS broth (Difco) at 37 °C for 24 h. These cultures were used to inoculate two or three Petri dishes of MRS agar (Difco), which were again incubated for 24 h at 37 °C. The bacteria were scraped from the Petri dishes and about 70 mg of wet cells were washed and then lysed by sonication, SDS treatment and boiling with 2-mercaptoethanol, as described before (Pot *et al.*, 1994). Registration of the protein electrophoretic patterns, normalization of the densitometric traces, pattern storage, 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.* (1994), using the software package GelCompar version 3.1 (Applied Maths, Kortrijk, Belgium; Vauterin & Vauterin, 1992).

For numerical analysis of the protein profiles, positions 10–120 and 171–325 of the 400 points registered were taken into account, omitting the stacking gel/separation gel interface (positions 0 to 9), the zone with disturbing high-density protein bands (S-layer proteins; positions 121–170), and the front of the electrophoretic protein profile (positions 326–400).

Protein isolations. For the isolation of their S-layer proteins, *Lactobacillus* strains (Table 1) were cultivated anaerobically in MRS broth at 37 °C for 16 h. Total protein extracts were made by collecting the bacteria from a 4.0 ml culture by centrifugation (5000 *g*, 5 min). The cells were washed with 1.0 ml 20 mM HEPES (pH 7.4), resuspended in 50 µl 20 mM HEPES (pH 7.4) and 0.3 g of glass beads (0.45 mm diameter) was added. This suspension was vortexed for 1 min and centrifuged (5000 *g*, 1 min). The supernatant was used as total-cell extract in the protein analysis. Guanidinium hydrochloride extracts of intact cells were made by collecting bacteria from 10 ml culture by centrifugation (5000 *g*, 5 min). The bacteria were washed with 1.0 ml 20 mM HEPES (pH 7.4) and collected by centrifugation (5000 *g*, 5 min). Part of the cell pellet (30 mg net weight) was resuspended in 0.25 ml 4.0 M guanidine hydrochloride (pH 7.0). This suspension was kept at 37 °C for 60 min and centrifuged (15000 *g*, 5 min). Supernatant was dialysed against water at 4 °C, lyophilized and solubilized in 150 µl water.

Western blotting. Total protein extracts were separated on an SDS-PAGE gel (10–15%) and transferred to nitrocellulose by blotting (Sambrook *et al.*, 1989). Detection of the S-protein antigens was performed as described before (Boot *et al.*, 1993), using the polyclonal antibodies against the S_A protein of *L. acidophilus* ATCC 4356T as the primary antibody and anti-mouse IgG-alkaline phosphatase conjugate (Promega) as the secondary antibody.

Chromosomal DNA isolation. Pre-warmed MRS broth (225 ml) was inoculated with an overnight culture (25 ml) and grown anaerobically for 3.5 h at 37 °C. Bacteria were harvested by centrifugation (10 min at 5000 *g*), washed once with 50 ml 20 mM sodium maleate (pH 6.2) and resuspended in 40 ml 20 mM sodium maleate (pH 6.2), 0.6 M lactose, 20 mM magnesium chloride, 80 mg lysozyme ml⁻¹ (Sigma). After incubation for 10 min at 37 °C protoplasts were harvested by centrifugation (10 min at 3000 *g*) and resuspended in 20 ml 20 mM Tris/HCl (pH 8.2). After addition of 4.4 ml 0.5 M EDTA, 5.5 ml 5% Sarkosyl was added followed by 3.3 ml 5 M NaCl. The final suspension was extracted with phenol, phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) and chloroform/isoamyl alcohol (24:1, by vol.). Chromosomal DNA was separated from the liquid phase after addition of 2 vols of ethanol (20 °C) and stirring with a glass rod. Chromosomal DNA was solubilized in 5 ml 0.1 × SSC (1 × SSC is 0.15 M NaCl with 15 mM sodium citrate); 0.4 ml 10 mg ml⁻¹ RNase solution was added and the mixture incubated for 20 min at 37 °C. Then 0.8 ml of a 20 mg ml⁻¹ solution of proteinase K (Boehringer Mannheim) was added and the mixture incubated for 40 min at 65 °C, followed by addition of 0.7 ml 5 M NaCl and repeated phenol/chloroform extractions as described above. Chromosomal DNA was isolated by standard ethanol precipitation (Sambrook *et al.*, 1989), and then dissolved in 0.1 × SSC and stored at 4 °C for later analysis.

Southern blotting. Chromosomal DNA was digested with either *EcoRI* or *BclI* under conditions recommended by the supplier (Pharmacia), separated on a 1% (w/v) agarose gel and

Table 1. Origin of the *Lactobacillus* strains

Strain*	Place of isolation	Obtained from	Reference
<i>L. acidophilus</i> (A-1)			
ATCC 4356T	Pharynx, human	ATCC	Boot <i>et al.</i> (1993)
LMG 11469	Intestine, rat	LMG	Pot <i>et al.</i> (1993)
<i>L. crispatus</i> (A-2)			
LMG 9479T	Eye, human	LMG	Devuyst <i>et al.</i> (1996)
LMG 12003	Infant faeces	LMG	Devuyst <i>et al.</i> (1996)
<i>L. amylovorus</i> (A-3)			
LMG 9496T	Cattle waste-corn fermentation	LMG	Devuyst <i>et al.</i> (1996)
LMG 13135	Unknown	LMG	Devuyst <i>et al.</i> (1996)
<i>L. gallinarum</i> (A-4)			
LMG 9435T	Crop, chicken	LMG	Devuyst <i>et al.</i> (1996)
T-50	Faeces, chicken	T. Fujisawa	Fujisawa <i>et al.</i> (1992)
<i>L. gasseri</i> (B-1)			
LMG 9203T	Human	LMG	Pot <i>et al.</i> (1993)
NCK 89	Unknown	T. R. Klaenhammer	Muriana & Klaenhammer (1987)
<i>L. johnsonii</i> (B-2)			
LMG 9436T	Blood, human	LMG	Pot <i>et al.</i> (1993)
LMG 11468	Unknown	LMG	Pot <i>et al.</i> (1993)
<i>L. helveticus</i>			
LMG 6413T	Emmental cheese	LMG	Devuyst <i>et al.</i> (1996)
CNRZ 32	Unknown	E. G. Dudley	Dudley & Steele (1994)
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>			
LMG 6901T	Bulgarian yoghurt	LMG	Devuyst <i>et al.</i> (1996)
LAB514	Yoghurt	LMG	Devuyst <i>et al.</i> (1996)

*DNA homology groups according to Johnson *et al.* (1980) are given in parentheses; T, type strain. ATCC, American Type Culture Collection, Rockville, MD, USA; LMG, Culture Collection of the Laboratory of Microbiology Gent, University of Gent, Gent, Belgium; CNRZ, Centre National de Recherches Zootechniques, Jouy-en-Josas, France; LAB, Lactic Acid Bacteria Culture Collection of the Laboratory of Microbiology Gent, Department of Physiology, Biochemistry and Microbiology, Faculty of Sciences, University of Gent, Gent, Belgium.

transferred to Hybond filter (Amersham) essentially as described by Southern (1975). The 5' probe is a restriction fragment of 325 bp derived from the 5' part of the *slpA* gene [*Sph*I (−146) to *Pst*I (+179); numbers are relative to the *slpA* start codon]. The 3' probe is a PCR fragment of 369 bp derived from the 3' end of the *slpA* gene (nt 1017–1386 relative to the *slpA* start codon). The *slpA* probe is a 173 bp restriction fragment of the *slpA* ORF [*Pst*I (+179) to *Pst*I (+352); numbers are relative to the *slpA* start codon]. The *slpB* probe is a 149 bp PCR-amplified part of the *slpB* ORF (nt 175–324 relative to the *slpB* start codon), which is 57% identical with the corresponding region of *slpA* ORF.

All probes were separated by agarose gel electrophoresis, purified from the agar with Glassmilk (Geneclean II; Bio 101), and labelled with [α -³²P]dATP using random-primer labelling (Prime-a-Gene; Promega). Hybridization (6 × SSC/0.1% SDS) and washing (three times in 0.1 × SSC/0.1% SDS) were performed at temperatures indicated in the legends of Figs 5 to 7.

RESULTS

Identification of strains

The type strain and various other strains of *L. acidophilus* (A-1), *L. crispatus* (A-2), *L. amylovorus* (A-3), *L. gallinarum* (A-4), *L. gasseri*, (B-1), *L. johnsonii* (B-2), *L. helveticus* and

L. delbrueckii subsp. *bulgaricus* (Table 1) were analysed in a standardized SDS-PAGE analysis of total cellular proteins. The protein profiles of these strains were compared to a database of normalized protein fingerprints derived from reference strains from almost all known species of lactic acid bacteria. Only relevant reference strains were included to produce the dendrogram presented in Fig. 1. Our SDS-PAGE analysis clearly discriminated between the six different species, which were formerly referred to as *L. acidophilus* (Johnson *et al.*, 1980), as defined by Fujisawa *et al.* (1992). For the type strain of *L. acidophilus*, three different subcultures, independently obtained from different culture collections, were separately processed and compared. Under the above-mentioned conditions, a correlation of $r \geq 0.88$ was calculated between these strains. The two colony variants of the type strains, LMG 9496T and LMG 6901T, of *L. amylovorus* and of *L. delbrueckii* subsp. *bulgaricus* (labelled t1 and t2) were clearly very similar ($r \geq 0.95$; Fig. 1). The first cluster comprised all *L. acidophilus* strains investigated, including the type strain and is equivalent to DNA homology group A-1 (Johnson *et al.*, 1980). Cluster I was delineated at a correlation level of $r \geq 0.81$ and showed a correlation of $r \geq 0.77$ with its closest neighbour, *L. crispatus*, forming cluster II. This

Fig. 1. Dendrogram calculated by the unweighted average pair grouping method of the correlation coefficients obtained between all pairs of one-dimensional SDS-PAGE protein patterns of representative strains of the *L. acidophilus* complex, *L. helveticus* and *L. delbrueckii* subsp. *bulgaricus*, expressed as a percentage (%*r*). For the origin of the strains see Table 1; 'T' indicates type strain; t1 and t2 are two different colony types. Strains used for additional research described in this study are given in bold type.

second cluster, equivalent to DNA similarity group A-2 (Johnson *et al.*, 1980), was delineated above $r = 0.82$ and contained both strains investigated for S-layer proteins. Cluster III, analogous to DNA homology group B-2, contained all *L. johnsonii* strains investigated and was delineated above $r = 0.79$. *L. gasseri* strains (DNA homology group B-1), including the type strain, formed cluster IV at a correlation level of $r = 0.83$. It should be noted that, for example, DNA homology groups A-1 and A-2 showed higher correlation with the DNA homology groups B-1 and B-2 ($r = 0.79$) than with the DNA homology group A-3, which is represented by the *L. amylovorus* strains (cluster V in Fig. 1; $r = 0.73$). This indicates that, although SDS-PAGE of whole-cell proteins is very useful in grouping bacteria at the species level, no phylogenetic conclusions should be drawn from the dendrograms calculated from similarity values. Cluster VI groups all *L. gallinarum* strains (DNA homology group A-4) at a correlation of $r = 0.76$. *L. helveticus*, which is also related to the above-mentioned species of the *L. acidophilus* group, forms cluster VII, and, although well separated from the other species, shows considerable heterogeneity with a correlation level of $r = 0.68$. The

four strains selected from *L. delbrueckii* subsp. *bulgaricus* form cluster VIII at $r = 0.75$.

S-layer protein analysis

The presence of an S-layer on the outside of bacteria belonging to the different *Lactobacillus* species can be deduced from the presence of a dominant protein band with a molecular mass of about 45 kDa in the (surface) protein profile of these bacteria (Boot *et al.*, 1993; Johnson *et al.*, 1987). Guanidinium hydrochloride extracts of surface proteins of intact cells of the type strains of the different *Lactobacillus* species were analysed in a separate SDS-PAGE gel. A dominant protein band of about 45 kDa was present in the extracts of the type strains of *L. acidophilus*, *L. crispatus*, *L. amylovorus*, *L. gallinarum* and *L. helveticus*, showing the presence of S-layer proteins. On the other hand, the type strains of *L. gasseri*, *L. johnsonii* and *L. delbrueckii* subsp. *bulgaricus* did not have such a protein band at the 45 kDa position, indicating the absence of an S-layer (Fig. 2). For each species the guanidinium hydrochloride extract of an additional strain was investigated by SDS-PAGE analysis beside the respective

Fig. 2. Surface proteins of intact cells of *Lactobacillus* strains. The surface proteins of the type strains of *L. acidophilus* (lane 1), *L. crispatus* (lane 2), *L. amylovorus* (lane 3), *L. gallinarum* (lane 4), *L. gasseri* (lane 5), *L. johnsonii* (lane 6), *L. helveticus* (lane 7) and *L. delbrueckii* subsp. *bulgaricus* (lane 8) were extracted with guanidinium hydrochloride and analysed on an SDS-PAGE gel (10–15%). The gel was stained with Coomassie blue after electrophoresis. The molecular masses of the marker proteins (lane M) are given on the right-hand side.

type strain (data not shown). The presence or absence of an S-layer was species dependent for all the investigated *Lactobacillus* strains.

Western blot analysis

To determine the relationship of the S_A -protein of the *L. acidophilus* type strain with the S-proteins of the other strains, we used polyclonal murine antibodies directed against the S_A -protein of the *L. acidophilus* type strain in a Western blot of the total protein extracts from the *Lactobacillus* strains (Fig. 3). From this analysis it appeared that the S protein of the *L. crispatus* strains is not recognized by the antibodies used, while the S-proteins of *L. amylovorus*, *L. gallinarum* and *L. helveticus* show a lower affinity compared with the S proteins of the *L. acidophilus* strains. As expected, no proteins of the *L. gasseri*, *L. johnsonii* and *L. delbrueckii* subsp. *bulgaricus* extracts reacted with the antibodies used.

Southern blot analysis

The *L. acidophilus* type strain shows S-layer variation between two S-proteins (S_A - and S_B -protein) which have 50 % sequence identity in the N-terminal and middle part

Fig. 3. Western blot analysis of total protein extracts of the type strains (T) and non-type strains (N) of *Lactobacillus* species. Abbreviations above the lanes: aci, *L. acidophilus*; cri, *L. crispatus*; amy, *L. amylovorus*; gal, *L. gallinarum*; gas, *L. gasseri*; joh, *L. johnsonii*; hel, *L. helveticus*; bul, *L. delbrueckii* subsp. *bulgaricus*. S protein antigens were detected by polyclonal antibodies against the S_A -protein of *L. acidophilus* type strain (Boot et al., 1993). Molecular mass markers are indicated on the right-hand side.

Fig. 4. Schematic drawing of the chromosomal *slpA* and *slpB* regions of the *L. acidophilus* type strain. The percentage identity between 50 nucleotides of the *slp* regions is represented by shaded boxes between the two sequences. A full-size box means completely identical, while a line means no detectable identity. The probes used in the Southern blot analysis are indicated by black bars above the *slpA* region or below the *slpB* region. The regions which encode the secretion leader sequences are indicated by vertical lines in the ORFs of both *slp* regions.

Fig. 5. Autoradiograph of Southern blot analysis of (a) *Eco*RI- and (b) *Bcl*I-digested chromosomal DNA of *Lactobacillus* strains (see legend to Fig. 3). Hybridization with the 5' probe was performed at 50 °C and washing (0.1 × SSC/0.1% SDS) was performed at 65 °C. The *slpA* and *slpB* regions in the lane of the type strain of *L. acidophilus* are indicated on the left-hand side. DNA length markers (fragments of *Bst*EII-digested wild-type λ phage) are indicated on the right-hand side.

of the mature proteins and have identical sequences at the C terminus (Boot *et al.*, 1995). The two genes encoding these proteins share two regions of identical sequence: a region of 280 bp between the *slp* promoter and the start of the mature proteins and a region of 430 bp encoding the C-terminal part of the S-proteins. Chromosomal DNA of the *Lactobacillus* strains was extracted and analysed by Southern blot analysis using probes derived from the *slpA* and *slpB* regions of the *L. acidophilus* type strain (Fig. 4). In the first Southern blot analysis (Fig. 5) we used a probe surrounding the translation start point of the *slpA* gene of the *L. acidophilus* type strain. This probe was derived from the *slpA* region and 75% of its length is identical to the corresponding *slpB* region. Two bands hybridized with the chromosomal DNAs of the *L. acidophilus*, *L. crispatus*, *L. amylovorus* and *L. gallinarum* strains, digested with either *Eco*RI (Fig. 5a) or *Bcl*I (Fig.

Fig. 6. Autoradiogram of Southern blot analysis of *Eco*RI-digested chromosomal DNA of the *Lactobacillus* strains (see legend to Fig. 3 for lane descriptions). Hybridization with the 3' probe was performed at 50 °C and washing (0.1 × SSC/0.1% SDS) was performed at 65 °C. See legend to Fig. 5 for DNA length markers and arrow descriptions.

5b). These two bands are most likely due to the presence of two chromosomal *slp* loci, as shown for the *L. acidophilus* strains (Boot *et al.*, 1995). Only one band hybridized when chromosomal DNA of *L. helveticus* was used, indicating that the strains of this species contain only one *slp* region. No hybridizing bands were found with chromosomal DNA of *L. gasseri*, *L. johnsonii* or *L. delbrueckii* subsp. *bulgaricus*, suggesting that these strains do not have S-protein-encoding genes.

In the Southern blot of Fig. 6 we used a probe which was also derived from the *slpA* region, but encoding the C-terminal part of the S_A -protein. This probe was 92% identical with the corresponding part of the *slpB* region. The hybridization pattern of this Southern blot was identical with that of Fig. 5(a) for most of the strains, indicating that the two hybridizing bands in these Southern blots were not due to restriction sites within one single *slp* region, but were due to the presence of two separated *slp* regions. One remarkable difference between the hybridization patterns of Fig. 5(a) and Fig. 6 is that two signals were obtained for both *L. helveticus* strains with the 3' probe (Fig. 6), compared to only one signal with the 5' probe (Fig. 5a).

Further characterization of the *slp* regions of the different strains was obtained by Southern blot analysis using probes that are specific for the *slpA* or *slpB* gene of the *L. acidophilus* type strain. No heterologous signals were obtained with the same, stringent, washing conditions used for the 5' and 3' probes (0.1 × SSC/0.1% SDS at 65 °C; data not shown). We therefore repeated the experiment using moderate washing conditions (0.1 × SSC/0.1% SDS at 50 °C) (Fig. 7). Under these conditions,

Fig. 7. Autoradiogram of Southern blot analysis of *EcoRI*-digested chromosomal DNA of the *Lactobacillus* strains (see legend to Fig. 3 for lane descriptions). The *slpA* probe (a) or the *slpB* probe (b) was used to visualize the *slp* regions. Hybridization and washing ($0.1 \times \text{SSC}/0.1\%$ SDS) were both performed at 50°C . See legend to Fig. 5 for DNA length markers and arrow descriptions.

DNA of both strains of *L. amylovorus* and *L. helveticus* as well as the non-type strain of *L. crispatus* contained one dominant hybridizing *slp* region when the *slpA* probe (Fig. 7a) was used. Both *slp* regions of the two *L. amylovorus* and *L. gallinarum* strains and one *slp* region of the type strains of *L. crispatus* and *L. helveticus* yielded faint signals when the *slpB* probe (Fig. 7b) was used. These faint signals are not very specific as the *slpA* region of the *L. acidophilus* type strain yields a signal of about the same intensity, while the identity of this region with the probe used is only 57%. The DNA of the type strain of *L. crispatus* yielded two hybridizing bands when the *slpA* probe was used (Fig. 7a). The lower molecular mass signal (4.2 kb) represents the *slp* region which also hybridizes with the 5' probe (Fig. 5a) and 3' probe (Fig. 6). The nature of the other signal (4.8 kb) is presently unclear. The results obtained with the *slpA*- and *slpB*-specific probes show that the *slpB*-specific sequence is less conserved among the investigated *Lactobacillus* strains than the *slpA*-specific sequence.

DISCUSSION

Recently we found that the type strain of *L. acidophilus*, which has an S-layer, is capable of S-layer variation (Boot

et al., 1996). The present study was initiated to determine whether related species of *L. acidophilus* possess related S proteins and whether these species are also capable of S-layer variation. First we identified the strains using a standardized SDS-PAGE identification method, previously shown to yield groupings similar to those revealed by DNA:DNA hybridization and 16S rRNA probe hybridization (Pot *et al.*, 1993) (Fig. 1). The presence of an S-layer on the outside of the type strain of each species was subsequently determined by extracting the surface proteins of intact cells with 4 M guanidinium hydrochloride and analysing those protein extracts by SDS-PAGE. From our analyses it is clear that the type strain and at least one other strain of the species *L. acidophilus*, *L. crispatus*, *L. amylovorus*, *L. gallinarum* and *L. helveticus* possess an S-layer. On the other hand, the type strain and at least one other strain of the species *L. gasseri*, *L. johnsonii* and *L. delbrueckii* subsp. *bulgaricus* do not possess such an S-layer.

To date, S-layer variation has been described for three different bacterial species: *Campylobacter fetus* (Tummuru & Blaser, 1993), *Bacillus stearothermophilus* (Sára & Sleytr, 1994) and *L. acidophilus* (Boot *et al.*, 1996). For *B. stearothermophilus*, it has been shown that certain growth conditions are inductive or selective for a specific S protein. Nothing is known, however, about the selectivity of growth conditions for the expression of the S-proteins of *L. acidophilus*. The growth conditions used in the present and previous studies seem to be favourable for S_A -protein expression, as expression of the S_B -protein was never detected (Boot *et al.*, 1996). Since the growth conditions favouring the expression of the variant S proteins are not known, we used an indirect approach to determine whether S-layer variation is possible for *Lactobacillus* strains belonging to different species. Western blot analysis using polyclonal antibodies against the S_A -protein of the *L. acidophilus* type strain shows that the S-proteins of *L. amylovorus*, *L. gallinarum* and *L. helveticus* have antigenic determinants in common with the S_A protein. *L. crispatus* on the other hand does not have those common determinants (Fig. 3). Based upon the results of this Western blot analysis, we expected that the genes encoding the S-proteins of different strains would show some sequence identity. This enabled us to determine the number of *slp* loci for the different strains by using probes derived from the two *L. acidophilus* *slp* regions in Southern blot analysis. Only strains that have multiple *slp* regions might be capable of S-layer variation using the same mechanism of chromosomal recombination as we have found for the *L. acidophilus* type strain (Boot *et al.*, 1996).

The results of the Southern blot analysis using chromosomal DNA of the type strain and another strain of *L. acidophilus*, *L. crispatus*, *L. amylovorus*, *L. gallinarum* and *L. helveticus* are summarized in Table 2. The two strains of *L. acidophilus*, *L. crispatus*, *L. amylovorus* and *L. gallinarum* possess two regions which hybridize with both the 5' and the 3' probe (Figs 5 and 6). Apart from these general probes, we also used probes that are specific for the two *slp* genes of *L. acidophilus* (Fig. 4). This analysis shows that there are several strains, including the *L. helveticus* strains,

Table 2. Properties of S proteins and S-protein-encoding genes of *Lactobacillus* strains

Strain*	Western blot†	Hybridization signals‡			
		5' probe	3' probe	<i>slpA</i> probe	<i>slpB</i> probe
<i>L. acidophilus</i>					
ATCC 4356T	+++	2	2	1	1
LMG 11469	+++	2	2	1	1
<i>L. crispatus</i>					
LMG 9479T	—	2	2	—	—
LMG 12003	—	2	2	1	—
<i>L. amylovorus</i>					
LMG 9496T	++	2	2	1	—
LMG 13135	++	2	2	1	—
<i>L. gallinarum</i>					
LMG 9435T	+	2	2	—	—
T-50	+	2	2	—	—
<i>L. helveticus</i>					
LMG 6413T	+	1	2	1	—
CNRZ 32	++	1	2	1	—

* Strains belonging to the species *L. gasseri*, *L. johnsonii* and *L. delbrueckii* subsp. *bulgaricus* have neither an S-layer nor S-protein-encoding regions and are omitted from this table.

† The signal intensity as shown in Fig. 4 is represented by: + + +, strong; + +, moderate; +, weak; and —, no reaction.

‡ Number of independent hybridization signals in a Southern blot analysis, as deduced from the results of Figs 6 to 8; —, no signal.

with a chromosomal region with moderate identity with the *slpA* probe (Fig. 7a). No strains contain regions which hybridize with the *slpB* probe when using the same hybridization and washing conditions as used for the *slpA* probe (Fig. 7b). The gene encoding the S_A-protein is apparently more conserved than the gene encoding the second S protein of each strain studied.

The *L. helveticus* strains show only one band that hybridizes with the 5' probe and the *slpA* probe. However, with the 3' probe, two bands hybridize with *L. helveticus* DNA digested with *EcoRI* (Fig. 6) and with *BclI* (data not shown). One explanation for this difference in hybridization pattern might be that restriction sites are present in the region between the probes. The nucleotide sequences of the S-protein-encoding genes of the *L. helveticus* strains CNRZ 1269 and CNRZ 892 have been elucidated (EMBL accession no.s X92752 and X91199, respectively). These sequences differ by only one nucleotide and no restriction sites are present for either *EcoRI* or *BclI* in these *slp* genes or in the first 150 nt downstream of the *slp* gene of these strains. Although we cannot exclude that the sequence of the *slp* genes of *L. helveticus* strains we have used is different from those of the published *L. helveticus* *slp* genes, it seems unlikely that the recognition sites for two randomly chosen restriction enzymes have changed. A more plausible explanation is that the *L. helveticus* strains which we analysed still have (part of) the 3' identity region of a second *slp* region, while they have lost the 5' identity region. A silent S-protein-encoding gene which lacked the 5' part of the coding region has

already been described for *Bacillus sphaericus* (Bowditch *et al.*, 1989). S-layer variation using the same mechanism as observed with *L. acidophilus* is thus not possible for the *L. helveticus* strains since they lack the 5' region of identity used for inversion of the *slp* segment by *L. acidophilus* ATCC 4356T.

The presence of an S-layer in our study is species-dependent for all the *Lactobacillus* strains tested, including *L. delbrueckii* subsp. *bulgaricus*. To determine whether we could confirm the result of Masuda & Kawata (1983), that at least some *L. delbrueckii* subsp. *bulgaricus* strains (e.g. YIT0045) possess an S-layer, we have analysed 10 different strains of *L. delbrueckii* subsp. *bulgaricus* by SDS-PAGE and Western blotting. Most of the *L. delbrueckii* subsp. *bulgaricus* strains were obtained from the Unilever culture collection, but the strains used by Lortal (1993) [CNRZ 208 (type strain), 369 and 416] and strain YIT0045 were also analysed. None of these *L. delbrueckii* subsp. *bulgaricus* strains, including strain YIT0045 obtained from the Yakult culture collection, contained an S-layer or S protein (data not shown). The strain of *L. delbrueckii* subsp. *bulgaricus* described by Masuda & Kawata (referred to here as M-YIT0045) and provided by K. Masuda contained an S-layer (data not shown). However, this strain appeared not to be a *L. delbrueckii* subsp. *bulgaricus* but a *L. helveticus* strain in our numerical comparison of the SDS-PAGE protein patterns of whole-cell proteins. Several reports, including this one, show that bacteria belonging to the *L. helveticus* species indeed possess an S-layer. Furthermore, Masuda & Kawata (1983) also

reported that *L. fermentum* NCTC 7230 (LMG 8897) possesses an S-layer. In our analysis *L. fermentum* LMG 8897 and two other *L. fermentum* strains (LMG 8896 and 104R; Blomberg *et al.*, 1993) lacked an S-layer (data not shown). In our taxonomic SDS-PAGE analysis, however, the *L. fermentum* strain described by Masuda & Kawata (1983), appeared not to belong to a known species of the genus *Lactobacillus* and definitely is not a *L. fermentum* strain (data not shown). Further taxonomic analysis is needed to reveal the identity of this strain. Strains of *L. delbrueckii* subsp. *bulgaricus* and *L. fermentum* should in our opinion be referred to as non-S-layer possessing strains.

The signal intensity of the *slp* regions of *L. crispatus*, *L. amylovorus*, *L. gallinarum* and *L. helveticus* is less with the 3' probe than with the 5' probe (compare Fig. 5b with Fig. 6). This indicates that the nucleotide sequence in the region of the translation start (5' identity region) is more conserved among the S-protein-encoding regions of lactobacilli, while the region of the 3' probe is less conserved. The highly conserved 5' identity region does not code for a part of the mature protein, in contrast to the 3' identity region (Boot *et al.*, 1995). A reason for conservation of the 5' region might be that a sequence-specific *trans*-acting factor binds to this conserved region to facilitate a high frequency of site-specific chromosomal recombination. Some properties of the different S-proteins are probably conserved, i.e. interaction with the underlying cell wall and crystallization into a regular structure. Other properties, like exposed antigens or interaction with foreign receptors, are presumably different for different S-proteins. The sequence identity near the C terminus and the sequence variation near the N terminus might reflect these opposing constraints.

In this study we have shown that some regions of S-protein-encoding genes of *L. acidophilus*, *L. crispatus*, *L. amylovorus* and *L. gallinarum* have a high degree of sequence identity, while other regions share only a very limited sequence identity. On the basis of the results obtained it seems possible to design a strategy which can rapidly prove or disprove whether a strain belongs to one of these *Lactobacillus* species either by S-protein epitope-specific antibodies or by an S-protein DNA sequence-specific (PCR) method.

ACKNOWLEDGEMENTS

We thank Katrien Vandemeulebroecke for excellent technical assistance and Patricia Conway, Sylvie Lortal, Ed Dudley, Tomohiko Fujisawa, Todd Klaenhammer, Kuniyoshi Masuda and the Unilever and Yakult companies for providing some of the *Lactobacillus* strains used in this study. Part of this study was financially supported by the Foundation for Technical Sciences (STW) of the Netherlands. B. Pot, K. Kersters and P. H. Pouwels are indebted to the Biotechnology (BIOTECH) G-project on Lactic Acid Bacteria of the Commission of the European Communities, contract BIOT-CT94-3055

NOTE ADDED IN PROOF

Lactobacillus strain NCK89 is most likely not a *L. gasseri* but a *L. johnsonii* (T. R. Klaenhammer, North Carolina State University, USA, personal communication).

REFERENCES

- Beachey, E. H. (1981). Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. *J Infect Dis* **143**, 325-345.
- Beveridge, T. J. (1994). Bacterial S-layers. *Curr Opin Struct Biol* **4**, 204-212.
- Blomberg, L., Henriksson, A. & Conway, P. L. (1993). Inhibition of adhesion of *Escherichia coli* K88 to piglet ileal mucus by *Lactobacillus* spp. *Appl Environ Microbiol* **59**, 34-39.
- Boot, H. J., Kolen, C. P. A. M., van Noort, J. M. & Pouwels, P. H. (1993). S-layer protein of *Lactobacillus acidophilus* ATCC 4356: purification, expression in *Escherichia coli*, and nucleotide sequence of the corresponding gene. *J Bacteriol* **175**, 6089-6096.
- Boot, H. J., Kolen, C. P. A. M. & Pouwels, P. H. (1995). Identification, cloning and nucleotide sequence of a silent S-layer protein gene of *Lactobacillus acidophilus* ATCC 4356 which has extensive similarity with the S-layer protein gene of this species. *J Bacteriol* **177**, 7222-7230.
- Boot, H. J., Kolen, C. P. A. M. & Pouwels, P. H. (1996). Interchange of the active and silent S-layer protein genes of *Lactobacillus acidophilus* by inversion of the chromosomal *slp* segment. *Mol Microbiol* (in press).
- Bowditch, R. D., Baumann, P. & Yousten, A. A. (1989). Cloning and sequencing of the gene encoding a 125-kilodalton surface-layer protein from *Bacillus sphaericus* 2362 and of a related cryptic gene. *J Bacteriol* **171**, 4178-4188.
- Coconnier, M.-H., Bernet, M.-F., Kernéis, S., Chauvière, G., Fourniat, J. & Servin, A. L. (1993). Inhibition of adhesion of enteroinvasive pathogens to human intestinal Caco-2 cells by *Lactobacillus acidophilus* strain I.B decreases bacterial invasion. *FEMS Microbiol Lett* **110**, 299-306.
- Devuyst, L., Callewaert, R. & Pot, B. (1996). Characterization of the antagonistic activity of *Lactobacillus amylovorus* DCE 417 and large scale isolation of its bacteriocin amylovorin L471. *Syst Appl Microbiol* VOL. (in press).
- Dudley, E. G. & Steele, J. L. (1994). Nucleotide sequence and distribution of the *pepPN* gene from *Lactobacillus helveticus* CNRZ32. *FEMS Microbiol Lett* **119**, 41-46.
- Fernandes, C. F., Shahani, K. M. & Amer, M. A. (1987). Therapeutic role of dietary lactobacilli and lactobacillic fermented dairy products. *FEMS Microbiol Rev* **46**, 343-356.
- Fujisawa, T., Benno, Y., Yaeshima, T. & Mitsuoka, T. (1992). Taxonomic study of the *Lactobacillus acidophilus* group, with recognition of *Lactobacillus gallinarum* sp. nov. and *Lactobacillus johnsonii* sp. nov. and synonymy of *Lactobacillus acidophilus* group A3 (Johnson *et al.*, 1980) with the type strain of *Lactobacillus amylovorus* (Nakamura 1981). *Int J Syst Bacteriol* **42**, 487-491.
- Greene, J. D. & Klaenhammer, T. R. (1994). Factors involved in adherence of lactobacilli to human Caco 2 cells. *Appl Environ Microbiol* **60**, 4487-4494.
- Isolauri, E., Joensuu, J., Suomalainen, H., Luomala, M. & Vesikari, T. (1995). Improved immunogenicity of oral D × RRV reassortant rotavirus vaccine by *Lactobacillus casei* GG. *Vaccine* **13**, 310-312.
- Johnson, J. L., Phelps, C. F., Cummins, C. S., London, J. & Gasser, F. (1980). Taxonomy of the *Lactobacillus acidophilus* group. *Int J Syst Bacteriol* **30**, 53-68.
- Johnson, M. C., Ray, B. & Bhowmik, T. (1987). Selection of *Lactobacillus acidophilus* strains for use in 'acidophilus products'. *Antonie van Leeuwenhoek* **53**, 215-231.
- Kitazawa, H., Matsumura, K., Itoh, T. & Yamaguchi, T. (1992).

Interferon induction in murine peritoneal macrophage by stimulation with *Lactobacillus acidophilus*. *Microbiol Immunol* **36**, 311–315.

Klaenhammer, T. R. (1993). Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol Rev* **12**, 39–85.

Lidbeck, A. & Nord, C. E. (1993). Lactobacilli and the normal human anaerobic microflora. *Clin Infect Dis* **16**, S181–S187.

Link-Amster, H., Rochat, F., Saudan, K. Y., Mignot, O. & Aeschlimann, J. M. (1994). Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. *FEMS Immunol Med Microbiol* **10**, 55–64.

Lortal, S. (1993). Crystalline surface-layers of the genus *Lactobacillus*. In *Advances in Paracrystalline Bacterial Surface Layers*, pp. 57–65. Edited by T. J. Beveridge & S. F. Koval. New York: Plenum Press.

McGoarty, J. A. (1993). Probiotic use of lactobacilli in the human female urogenital tract. *FEMS Immunol Med Microbiol* **6**, 251–264.

McKay, L. L. & Baldwin, K. A. (1990). Applications for biotechnology: present and future improvements in lactic acid bacteria. *FEMS Microbiol Rev* **87**, 3–14.

Masuda, K. & Kawata, T. (1983). Distribution and chemical characterization of regular arrays in the cell walls of strains of the genus *Lactobacillus*. *FEMS Microbiol Lett* **20**, 145–150.

Messner, P. & Sleytr, U. B. (1992). Crystalline bacterial cell-surface layers. *Adv Microb Physiol* **33**, 213–275.

Mukai, T. & Arihara, K. (1994). Presence of intestinal lectin-binding glycoproteins on the cell surface of *Lactobacillus acidophilus*. *Biosci Biotech Biochem* **58**, 1851–1854.

Muriana, P. M. & Klaenhammer, T. R. (1987). Conjugal transfer of plasmid-encoded determinants for bacteriocin production and immunity in *Lactobacillus acidophilus* 88. *Appl Environ Microbiol* **53**, 553–560.

Orrhage, K., Sillerström, E., Gustafsson, J.-Å., Nord, C. E. & Rafter, J. (1994). Binding of mutagenic heterocyclic amines by intestinal and lactic acid bacteria. *Mutat Res* **311**, 239–248.

Perdigón, G., Medici, M., BibasBonetDeJorrat, M. E., ValverdeDeBudeguer, M. & PesceDeRuizHolgado, A. (1993). Immunomodulating effects of lactic acid bacteria on mucosal and humoral immunity. *Int J Immunother* **9**, 29–52.

Pot, B., Hertel, C., Ludwig, W., Descheemaeker, P., Kersters, K. & Schleifer, K.-H. (1993). Identification and classification of *Lactobacillus acidophilus*, *L. gasseri* and *L. johnsonii* strains by SDS-PAGE and rRNA-targeted oligonucleotide probe hybridization. *J Gen Microbiol* **139**, 513–517.

Pot, B., Vandamme, P. & Kersters, K. (1994). Analysis of electrophoretic whole-organism protein fingerprints. In *Chemical Methods in Prokaryotic Systematics*, pp. 493–521. Edited by M. Goodfellow & A. G. O'Donnell. Chichester, UK: Wiley.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sára, M. & Sleytr, U. B. (1994). Comparative studies of S-layer proteins from *Bacillus stearothermophilus* strains expressed during growth in continuous culture under oxygen-limited and non-oxygen-limited conditions. *J Bacteriol* **176**, 7182–7189.

Schneitz, C., Nuotio, L. & Lounatma, K. (1993). Adhesion of *Lactobacillus acidophilus* to avian intestinal epithelial cells mediated by the crystalline bacterial cell surface layer (S-layer). *J Appl Bacteriol* **74**, 290–294.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98**, 503–517.

Toba, T., Virkola, R., Westerlund, B., Björkman, Y., Sillanpää, J., Vartio, T., Kalkkinen, N. & Korhonen, T. K. (1995). A collagen-binding S-layer protein in *Lactobacillus crispatus*. *Appl Environ Microbiol* **61**, 2467–2471.

Tummuru, M. K. R. & Blaser, M. J. (1993). Rearrangements of *sapA* homologs with conserved and variable regions in *Campylobacter fetus*. *Proc Natl Acad Sci USA* **90**, 7265–7269.

Vauterin, L. & Vauterin, P. (1992). Computer-aided objective comparison of electrophoresis patterns for grouping and identification of microorganisms. *Eur Microbiol* **1**, 37–41.

Zheng, H.-Y., Alcorn, T. M. & Cohen, M. S. (1994). Effects of H₂O₂-producing lactobacilli on *Neisseria gonorrhoeae* growth and catalase activity. *J Infect Dis* **170**, 1209–1215.

Received 1 April 1996; revised 15 May 1996; accepted 17 May 1996.