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DOI

[10.1046/j.1365-2958.1996.401406.x](https://doi.org/10.1046/j.1365-2958.1996.401406.x)

Publication date

1996

Published in

Molecular Microbiology

[Link to publication](#)

Citation for published version (APA):

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. *Molecular Microbiology*, 21, 799-809. <https://doi.org/10.1046/j.1365-2958.1996.401406.x>

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Interchange of the active and silent S-layer protein genes of *Lactobacillus acidophilus* by inversion of the chromosomal *slp* segment

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Summary

The most-dominant surface-exposed protein in many bacterial species is the S-protein. This protein crystallises into a regular monolayer on the outside surface of the bacteria: the S-layer. *Lactobacillus acidophilus* harbours two S-protein-encoding genes, *slpA* and *slpB*, only one of which (*slpA*) is expressed. In this study, we show by polymerase chain reaction (PCR) analysis that *slpA* and *slpB* are located on a 6 kb chromosomal segment, in opposite orientations. In a small fraction of the bacterial population, this segment is inverted. The inversion leads to interchanging of the expressed and silent S-protein-encoding genes, and places the formerly silent gene behind the S-promoter which is located outside the inverted segment. A 26 bp sequence showing a high degree of similarity with the consensus sequence recognized by the Din family of invertases is present in the region where recombination occurs. Expression of the *slpA* gene seems to be favoured under laboratory growth conditions because 99.7% of the chromosomes of an *L. acidophilus* ATCC 4356 broth culture had the *slpA* gene present at the *slp* expression site.

Introduction

A regular structure on the outside surface of the cell wall has been found for up to 400 different species of Eubacteria and Archaeobacteria. This two-dimensional crystalline layer, known as the S-layer, consists of one species of

(glyco)protein, the S-protein (for reviews, see Beveridge, 1994; Messner and Sleytr, 1992). Several functions or properties have been ascribed to S-layers, i.e. a covering layer masking phage receptors, a molecular sieve, and an attachment structure for high-molecular-weight extracellular proteins. In recent publications concerning the S-layer of *Campylobacter fetus* it was reported that the S-protein of this pathogen is involved in antigenic variation (Tummuru and Blaser, 1993; Blaser *et al.*, 1994). Variation of surface-exposed proteins, such as S-proteins, is commonly found among pathogenic organisms.

Lactobacilli are widely distributed in nature and are found in gastrointestinal and female urogenital tracts. Some *Lactobacillus* strains are thought to be beneficial for the host (Fernandes *et al.*, 1987). The role of the S-protein in the interaction of lactobacilli and the epithelial cells of the host is unclear, as several *Lactobacillus* species which either do or do not possess an S-layer have been recovered from the gastrointestinal and female urogenital tracts of mammalian hosts. In several studies it was found that the S-layer of lactobacilli is involved in adherence to receptors present in epithelial tissues (Schneitz *et al.*, 1993; Mukai and Arihara, 1994; Toba *et al.*, 1995). Greene and Klaenhammer (1994) reported, on the other hand, that the S-layer of the *Lactobacillus acidophilus* type strain is not involved in such interactions.

To obtain more information about the role of the S-layer of lactobacilli in adhesion, we cloned and sequenced the S-protein-encoding gene (*slpA*; Boot *et al.*, 1993) of the type strain of *L. acidophilus*, a strain which was originally isolated from a human pharynx. Besides the expressed gene, we have recently identified a silent S-protein-encoding gene (*slpB*; Boot *et al.*, 1995) in this species, which has extensive similarity with the expressed *slpA* gene. In this report we show that these two S-protein-encoding genes are present on a chromosomal fragment of 6 kb (the *slp* segment). Inversion of this segment leads to the positioning of the silent *slpB* gene behind the S-promoter, while the active *slpA* gene is stored at the silent position of the *slpB* gene. *L. acidophilus* bacteria grown under laboratory conditions appear to have a selective advantage when they express the *slpA* instead of the *slpB* gene, because 99.7% of these bacteria had the *slpA* gene present at the *slp* expression site.

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Results

The *in vivo* activation of a silent gene by its translocation from the silent site to an expression site has been described for several bacteria (Seifert and So, 1988). A homologous region elsewhere on the chromosome or on a plasmid can facilitate translocation either by RecA-dependent homologous recombination or by site-specific recombination (Glasgow *et al.*, 1989b; Robertson and Meyer, 1992). We recently identified a silent S-protein-encoding gene (*slpB*) on the chromosome of *L. acidophilus* ATCC 4356 (Boot *et al.*, 1995). As no plasmids have been detected in the *L. acidophilus* ATCC 4356 strain (Roussel *et al.*, 1993; and our unpublished data), and only one chromosomal locus (*slpA*) exists which shares extensive similarity with the *slpB* region (Boot *et al.*, 1995), we have focused our attention on chromosomal recombination between the two *slp* regions.

Long-range polymerase chain reaction (PCR)

From Southern blot analysis, performed during previous studies, we knew that the two S-protein-encoding genes of *L. acidophilus* ATCC 4356 are relatively close to each other on the chromosome (<125 kb) (Boot *et al.*, 1995) and that the silent *slpB* gene was located downstream from the expressed *slpA* gene (data not shown). To determine the distance between these two genes more precisely and to assess their relative orientation; we have performed a long-range PCR on chromosomal DNA. An oligonucleotide specific for the coding sequences of the *slpA* gene (A-20; see Fig. 1A), was mixed with an oligonucleotide specific for the coding sequence of the *slpB* gene (either B-50 or B-2MR; see Fig. 1A) and used in a PCR analysis with chromosomal DNA as the template. A major amplified DNA fragment of 4.8 kb was found when oligonucleotide A-20 was present in combination with oligonucleotide B-50 (Fig. 2, lane 4). This means that the two genes are in opposite orientation to each other and that they are interspaced with a 3.0 kb region (see Fig. 1A). Additional PCR experiments (PCR-I to IV) were performed using different oligonucleotide and template combinations to examine the possibility of chromosomal recombination involving the *slp* segment.

PCR-I. In PCR-I we used one primer specific for the S-promoter (A-15; Fig. 1A) and another primer specific for

the 5'-region of the *slpB* open reading frame (ORF) (B-2MR; Fig. 1A). As template we used chromosomal DNA from a culture of *L. acidophilus* ATCC 4356, which was prepared after inoculation of a single colony into MRS broth (Difco). A clear amplification product of the expected length (860 bp) was visible in an agarose-gel analysis, signifying the presence of DNA molecules in which the *slpA* promoter and *slpB* ORF are physically combined (Fig. 3, lanes 2–4). A potential pitfall of using PCR analysis to detect a recombination event between homologous regions is a phenomenon called polymerase mediated linkage of primers (PHLOP) (Kim and Smithies, 1988; Pääbo *et al.*, 1989; Frohman and Martin, 1990). A partially extended product is, in this case, switching template in a region which has a high percentage of nucleotide-sequence identity with another region (a PHLOP event). Once such a hybrid product is formed, it will then be amplified in the subsequent rounds of the PCR amplification. The resulting product is exactly the same as the amplified product resulting from a low-frequency recombination event. To determine whether a PHLOP event was occurring under our experimental conditions, we used two plasmids, pBK-13 (containing the S-promoter region) and pBK-101 (containing the 5'-part of the *slpB* ORF). The plasmids were mixed with chromosomal DNA of *Lactobacillus plantarum* 80, which does not contain *slp* genes and which acts as non-specific competitor DNA in this PCR control experiment. While an intense signal was found when 10^8 copies of the *L. acidophilus* ATCC 4356 chromosome were used (Fig. 3, lane 3), only a barely visible signal was present at the same position when 3×10^8 copies of the two plasmids were used as the template (Fig. 3, lane 8). The amplified product of the plasmid template is most likely the result of a PHLOP event, which will happen more frequently at higher template concentrations. PCR amplification is, at least initially, an exponential process. Small differences in any of the reaction components or temperatures in different reaction tubes may greatly influence the final yield. To exclude the possibility that the observed difference in yield of PCR-amplified DNA between *L. acidophilus* ATCC 4356 DNA and plasmid DNA (used as a PHLOP control) in PCR-I is caused by such variations, we performed an additional PCR experiment (PCR-II).

PCR-II. To completely eliminate a PHLOP event in the

Fig. 1. Representation of the *slp* segment of *L. acidophilus* ATCC 4356. The position and orientation of the two *slp* genes (shown as open boxes) is based on the results of long-range PCR (see Fig. 2). The 5'-identity regions (Δ , 280 nt) and 3'-identity regions ($\circ$, 450 nt) and the position and orientation of the oligonucleotides used in the PCR analyses are indicated. All the *PvuII* restriction sites and the *SphI* and *Clal* sites that are unique for the 5'- and 3'-region of identity are indicated. The distances of the restriction sites (between brackets) are given relative to the S-promoter (Boot *et al.*, 1995). The *slp* gene present in the expression site (*slpA* in A) can be replaced by the *slp* gene present in the silent site (*slpB* in A) through inversion, yielding a chromosomal organization of the *slp* region as shown in (B).

Fig. 2. Agarose-gel electrophoresis of amplified DNA fragments generated by long-range PCR. The difference between the five PCR amplifications shown (corresponding to the lane numbers) reflects the oligonucleotides that were used to prime the DNA polymerization. Lane 1, A-20; lane 2, B-50; lane 3, B-2MR; lane 4, A-20 and B-50; lane 5, A-20 and B-2MR. A 4.8 kb amplified DNA fragment of high intensity was visible when oligonucleotide A-20 was used in combination with oligonucleotide B-50 (lane 4). The 4.8 kb band visible in lanes 1 and 5 probably results from a PCR artefact (PHLOP-event; see *PCR-I* of the *Results* section), owing to the presence of the two 3'-identity regions in opposite orientation. The length of some marker (M) DNA fragments (wild-type Lambda DNA digested with *Bst*EII) is indicated on the right-hand side.

PCR analysis, we separated the templates hybridizing with the primers A15 and B-2MR, after digestion of *L. acidophilus* ATCC 4356 chromosomal DNA with *Pvu*II (see the *Experimental procedures*). A recombination event between the S-protein-encoding genes will alter the position of these *Pvu*II sites and will result in the formation of a 1.8 kb *Pvu*II fragment that can hybridize with both primers (Fig. 1B). *Pvu*II-digested chromosomal DNA was separated by agarose-gel electrophoresis and DNA fragments of different length were collected (*Pvu*II fractions). The agarose-gel analysis of the PCR amplification in which these *Pvu*II fractions were used as the template clearly showed a product of the expected size (860 bp) in the *Pvu*II fraction containing fragments of chromosomal DNA with a length between 1.4 and 2.3 kb (Fig. 4, lane 5). No amplified DNA of any size was visible when the other *Pvu*II fractions were used as template. This PCR experiment confirms the conclusion of the PCR-I experiment that some of the isolated *L. acidophilus* ATCC 4356 chromosomes contain the *slpB* ORF instead of the *slpA* ORF behind the S-promoter.

PCR-III. To further analyse the nature of the chromosomal recombination leading to this physical linkage, we used the same *Pvu*II fractions as used in PCR-II in a PCR reaction with oligonucleotides specific for the *slpB* ORF (B-2EF) and for the region downstream of the *slpA* ORF (A-18). If recombination had occurred between the 3'

regions of identity of the two *slp* genes, we would have expected an amplified DNA fragment of 925 bp in the *Pvu*II fraction which contains DNA fragments of 2.6 kb (*Pvu*II fraction 4). However, amplification products were not found in any of the *Pvu*II fractions under conditions which were found to be optimal for this PCR amplification (data not shown). The conclusion of this PCR experiment is that no detectable recombination is occurring between the 3'-identity regions of the *slp* genes. This conclusion excludes the possibility that a gene-duplication event, involving the 5'- and 3'-identity regions, is the mechanism which leads to the physical combination of the S-promoter and the *slpB* ORF.

PCR-IV. To investigate whether an inversion event is responsible for the presence of the *slpB* ORF behind the S-promoter, we used chromosomal DNA as the template and an oligonucleotide specific for the region upstream of *slpB* (B-5HF; see Fig. 1A) and an oligonucleotide specific for the coding region of *slpA* (A-7; see Fig. 1A) as primers in a PCR amplification. We were indeed able to amplify a DNA molecule of the expected size, as was visualized by agarose gel electrophoresis (Fig. 5, lane 8). To exclude the possibility that this amplified product is the result of a PCR artefact, we used, as the template, the same *Pvu*II fractions as were used in PCR-II. In two of these fractions (1 and 2), we found an amplified product of the expected

Fig. 3. Agarose-gel electrophoresis of the fragments resulting from PCR-I. Oligonucleotides A-15 and B-2MR were used in this PCR amplification with different templates. Lanes 1 to 4, chromosomal DNA of *L. acidophilus* ATCC 4356. Lanes 5 to 8, plasmid DNA pBK-13 and pBK-101, mixed with chromosomal DNA of *L. plantarum* 80. The amount of template (copies per 50 µl) was as follows: lanes 1 and 5, 10^7 ; lanes 2 and 6, 3×10^7 ; lanes 3 and 7, 10^8 ; lanes 4 and 8, 3×10^8 ; and lane 9, no template DNA. M, marker DNA (see Fig. 2). The amplified product of *L. acidophilus* ATCC 4356 chromosomal DNA was digested with several restriction enzymes and analysed on an ethidium bromide-containing agarose gel, yielding fragments of the expected length (data not shown). The amount of amplified product in lane 4 is considerably higher than that in lane 3. This means that the yield of product will not reach the plateau level when template concentrations are equal to or below 10^8 copies of chromosomal DNA.

Fig. 4. Agarose-gel electrophoresis of the fragments resulting from PCR-II. DNA fragments of different length, originating from *PvuII*-digested chromosomal DNA of *L. acidophilus* ATCC 4356 were used as a template in separated PCR reactions (PCR-II), utilizing the same primers as used in PCR-I. The resulting amplified DNA was analysed on the agarose gel shown. The length of the template DNA in the different *PvuII* fractions is as follows: fraction 1, 20.1 → 8.5 kb; 2, 8.5 → 4.8 kb; 3, 4.8 → 3.7 kb; 4, 3.7 → 2.3 kb; 5, 2.3 → 1.4 kb; 6, 1.4 → 0.5 kb. Fraction numbers correspond to lanes 1 to 6. Lanes 7 and 8, no DNA (negative controls); M, marker DNA (see Fig. 2).

size (Fig. 5, lanes 2 and 3). Based on the position of the *PvuII* restriction sites (see Fig. 1B), we only expected an amplified DNA product using fraction 1 (lane 2). The sub-optimal separation of the 9.2 kb *PvuII* fragment containing the 5' region of *slpA* and the upstream region of *slpB* from DNA fragments up to 8.5 kb in size (fraction 2) is, in our opinion, the reason that a DNA fragment of the same size was also found when *PvuII* fraction 2 was used in the PCR amplification (Fig. 5, lane 3). The reduced yield of amplified product in *PvuII* fraction 2 compared to that of fraction 1 supports this explanation. The conclusion of this PCR experiment is that some of the isolated chromosomes contain the *slpA* ORF at the silent position which is normally occupied by the *slpB* ORF.

Southern- and Northern blot analysis

To determine whether more strains belonging to the *L. acidophilus* species possess two *slp* loci and whether they are expressing the *slpA* or *slpB* gene when grown in laboratory conditions, we analysed three additional *L. acidophilus* strains from different sources. DNA and RNA from these *L. acidophilus* strains, including the ATCC 4356 strain, were analysed in Southern- and Northern blot experiments. Probes which recognise both *slp* genes, or which are specific for one of the *slp* genes were used. Southern blot analyses show that two *slp* loci are found in all the analysed strains on fragments of the same length, when the endonucleases *EcoRI* (Fig. 6A) or *BclI*, *HindIII* or *PstI* (data not shown) were used. All these strains are expressing the *slpA* gene, as can be concluded from the Northern blot analysis (Fig. 6B).

Competitive PCR

To determine the relative amount of chromosomes in which a recombination event had placed the *slpB* ORF behind the S-promoter, we performed a competitive PCR (Gilliland *et al.*, 1990). The hybrid plasmid pBK-121 containing the S-promoter region, the 5'-part of the *slpB* ORF, and missing the unique *SphI* site (Fig. 1) of this region, was mixed in different ratios with a constant amount of chromosomal DNA. The deleted *SphI* site made it possible to distinguish between amplified product originating from plasmid DNA (where no *SphI* site was present in the amplified product) and chromosomal DNA (the amplified product contained an *SphI* site). From the resulting phosphor-imager data (Fig. 7) of this experiment, it appears that approx. 1 out of 300 chromosomes had undergone a recombinational event, placing the *slpB* ORF behind the S-promoter.

Immunologic detection of *S_B*-protein expression

Despite this relatively high occurrence, all attempts to detect *L. acidophilus* bacteria carrying an *S_B*-layer have been unsuccessful as yet. None of the colonies (>5000) originating from the same culture used to prepare chromosomal DNA was recognized by the *S_B*-specific polyclonal antibodies (data not shown). Furthermore, we have used the *S_B*-specific antisera in immunofluorescence microscopy analysis to visualize the presence of *S_B*-protein on the outside surface of the bacteria. None of the inspected bacterial cells (>100 000) from the -70°C stock reacted with the *S_B*-specific antibodies while all the bacteria strongly reacted with antiserum raised against purified *S_A*-protein (data not shown).

Fig. 5. Agarose-gel electrophoresis of the resulting fragments of PCR-IV. Oligonucleotides which are specific for the coding region of *slpA* and the upstream region of *slpB* were used to detect whether the *slpA* gene is, in some of the chromosomes, present at the silent site of the *slp* segment (PCR-IV). The templates used in the different PCR tubes (which correspond to the lane numbers) are: lane 1, no DNA; lanes 2 to 7, the same *PvuII* fractions (1 to 6, respectively) as described in PCR-II (Fig. 4); lane 8, untreated chromosomal DNA; M, marker DNA (see Fig. 2).

Fig. 6. DNA and total RNA was extracted from four different *L. acidophilus* strains and used in Southern blot (A) and Northern blot (B) analyses. Lanes/panels: 1, ATCC 4356; 2, NCIMB 8607; 3, LMG 11428; 4, LMG 11469. A. Autoradiograph of the Southern blot analysis, using a probe derived from the *slpA* gene which is 77% homologous with the *slpB* gene. B. Autoradiograph of Northern blot analysis using probes specific for either the *slpA* (A) or *slpB* (B) mRNA. The difference in intensity of the signal of the *slpA* probe with the RNA of the four different strains is the result of partial degradation of RNA of two strains (NCIMB 9607 and LMG 11469).

Discussion

Silent genes have been identified on the chromosome and plasmids of several bacterial species. The maintenance of a silent gene during evolution suggests that expression of such a gene gives a selective advantage to the bacterium under certain conditions. We have recently identified and characterized a silent S-protein-encoding gene (*slpB*) on the chromosome of *L. acidophilus* ATCC 4356 that shows extensive similarity with the *slpA* gene, which encodes the expressed S-protein of this strain (Boot *et al.*, 1995). One

of the commonly known ways of activating silent genes is translocation of the gene from its silent site to an expression site (Seifert and So, 1988; Glasgow *et al.*, 1989b). In this study, we describe the inversion of a 6 kb chromosomal fragment containing the *slpA* and *slpB* genes in opposite orientation (*slp* segment), which positions the silent *slpB* ORF behind the active S-promoter. The presence of the *slpB* ORF instead of the *slpA* ORF behind the S-promoter is expected to yield expression of *slpB*, resulting in the production of a different S-protein and hence a different S-layer (S-layer variation).

Although the fraction of *L. acidophilus* bacteria with the physical combination S-promoter/*slpB* ORF is approx. 0.3%, we were unable to detect *S_B*-protein expressing cells. A reason for not being able to detect *L. acidophilus* cells with the *S_B*-protein on the outside of the cell wall might be that, under the growth conditions used, the switch from *S_A*-protein to *S_B*-protein gives a major selective disadvantage to the cells. The incorporation of *S_B*-protein in an already existing S-layer comprising *S_A*-protein could yield such a selective disadvantage. Special growth conditions, such as those found in the human pharynx where this bacterium originates from, might be essential for expression of the *S_B*-protein and for formation of an *S_B*-layer. Through mathematical analysis (Markov chain) it can be proved that the ratio between *S_A*-protein- and *S_B*-protein-expressing bacteria equals the inverse of the ratio between the forward and reverse frequency of the *slp* segment inversion (Hoppensteadt, 1982). The observation that the ratio between the presence of *slpA* and *slpB* genes in the expression site is 300:1 means that there are factors, either external or internal, that influence this ratio. A 1:1 ratio would have been found in the absence of these factors.

Experiments aimed at determining whether *Lactobacillus* can be deprived of its S-layer or express the silent gene, by inactivation of the *slpA*-gene, did not yield the expected results. Inactivation of the S-protein of *L. acidophilus* ATCC 4356 and *Lactobacillus helveticus* CNRZ 32, by disruption of the *slp* gene via homologous recombination, did not yield recombinant bacteria containing the integrated plasmid at the expected position (our unpublished observations; T. R. Klaenhammer, personal communication). No bacteria could be recovered that were deprived of the S-layer or produced an altered S-protein. Therefore, the presence of an S-layer is apparently essential for these bacteria.

Previous experiments from this laboratory have shown that species that are evolutionary related to *L. acidophilus*, such as *Lactobacillus crispatus*, *Lactobacillus amylovorus* and *Lactobacillus gallinarum* also contain S-layers that are antigenically related (Boot *et al.*, 1996). These three organisms harbour two S-protein genes with 5'-identity regions which are strikingly similar to the 5'-identity region

Fig. 7. The ratio of *slpA* and *slpB* genes behind the S-promoter was determined by means of a competitive PCR experiment. Various amounts of hybrid plasmid pBK-101 were mixed with a constant amount of chromosomal DNA, and used as a template for PCR (see the *Experimental procedures*). The ratio of digestable and undigestable PCR product represents the ratio of the *slpA* and *slpB* genes present at the expression site. The ratio of digestable and undigestable DNA is given on a log-scale because PCR amplification is an exponential process. Error bars represent the standard deviation ($n=4$). Less than 10% of the PCR product of the 5'-region is derived from a PHLOP event. This means that the influence of a PHLOP event on the result of the competitive PCR experiment is within the error range of this experiment (see the error bars).

of *L. acidophilus* S-protein genes, suggesting that inversion of an *slp* segment may also occur in these organisms. Considering that two highly homologous *slp* genes, one of which is a silent gene, are conserved in four different organisms, it is tempting to speculate that inversion of the *slp* segment in *L. acidophilus* will cause antigenic variation under conditions that permit S_B-protein-expressing bacteria to survive.

Surface-protein-related antigenic variation is a combination of the frequency of the genetic switch and the selective pressure of the environment for a particular surface protein. The selection pressure for an exposed surface protein of species pathogenic for mammals can be very high if those surface proteins are recognized by the immune system of the host. An example of antigenic variation caused by S-layer variation was described for the pathogenic bacterium *C. fetus*. The immune system of the host provides the selective pressure for variation from the wild-type S-protein to a variant S-protein in this organism (Wang *et al.*, 1993). Lactobacilli are tolerated by the host immune system as inhabitants of the respiratory, gastrointestinal and female urogenital tracts. If our hypothesis that S-layer variation can occur in *L. acidophilus* is correct, it might represent a remnant property of a formerly pathogenic species. Another explanation for S-layer variation might be that *L. acidophilus* bacteria are facing different environmental conditions in nature. These different conditions and the presence of numerous other microorganisms competing for the same substrates, and possibly for the same receptors, could provide the selective pressure for S-layer variation in *L. acidophilus*. Reports concerning the S-layer of different *Bacillus stearothermophilus* strains show that a change in environmental conditions can be the inducing or selecting determinant

for the type of S-layer formed. The wild-type S-protein of these *B. stearothermophilus* strains is replaced by variant S-proteins when different oxygen pressures are used during growth in a fermentor. How the expression of different S-proteins of *B. stearothermophilus* is regulated is not known (Sára *et al.*, 1994; Sára and Sleytr, 1994).

Recently it was shown that antigenic variation in *C. fetus* is accompanied by inversion of a 6.2 kb chromosomal segment containing the S-promoter. This inversion leads to a switch of expression between two S-protein genes flanking this inverted segment (Dworkin and Blaser, 1996). Recombination in *C. fetus* is also taking place at the regions which are, in this case, 654 nucleotides (nt) long.

	*****	**	**	*****
Consensus	TTCT ^C GTAA CCAAGGTTT GGATAA			
<i>gixL</i>	...C.....G.....			
<i>gixR</i>	...C.....G.....			
<i>cixL</i>	T.....A....TG			
<i>cixR</i>	T.....A.....			
<i>pixL</i>	CC.....C..G.G			
<i>pixR</i>	CC.....C...A.T..A..			
<i>hixL</i>	A.....T.....			
<i>hixR</i>	..T..C..TTT GG.....T....			
<i>slxL</i>	A.....CTTT GA.G.....T.C.T			
<i>slxR</i>	A.....CTTT GA.G.....T.C.T			

Fig. 8. Nucleotide sequence of the recognition sites of the Din family of invertases. The recognition sites of the Gin inversion elements from bacteriophage Mu, Cin from phage P1, Pin from ϕ 14 prophage of *E. coli*, and Hin inversion system of *Salmonella* are aligned with the *slx* sequences of *L. acidophilus*. Asterisks at the top indicate positions that are especially important in the *hixL*-site recognition by Hin as indicated by X-ray crystallography data (Feng *et al.*, 1994). The nucleotides underlined in the *hixR* sequence represent base pairs that are contacted by Hin, as indicated by the methylation-interference assay. Dots represent identical nucleotides.

A Chi-like sequence and a (15 nt long) palindrome sequence are present in the 5'-identity region of the *C. fetus* S-protein genes (Tummuru and Blaser, 1993; Dworkin and Blaser, 1996). Six additional, silent S-protein genes are found near the inverted segment. How these silent S-protein genes are activated is currently unknown.

Although the length of the 5'-identity region (280 nt) of *L. acidophilus* ATCC 4356 is considerably shorter than the 3'-region of identity (450 nt), recombination only takes place at the 5'-identity region. This, taken together with the high frequency of recombination (1:300) at the 5'-identity region, suggests a site-specific-recombination mechanism. A 26 bp sequence (*slx*) was found within the 5'-identity regions of *slpA* and *slpB* showing a high degree of similarity with the target site (*hixR*) for the *Salmonella typhimurium* invertase *Hin* (Fig. 8) (Zieg and Simon, 1980). The sequences *hix*, *gix*, *cix*, *pix* and *mix* are all recognized by members of the Din family of invertases (Glasgow *et al.*, 1989b; Sandmeier *et al.*, 1990; Feng *et al.*, 1994). All but one of the *hixR* basepairs that were found to be protected by *Hin* against methylation (Glasgow *et al.*, 1989a) are conserved in *slx*. The *slx* sequences are found near the middle of the 5'-identity regions, within the region that is transcribed into 5'-untranslated mRNA. To what extent the inversion of the *slp* segment resembles the inversion catalysed by the Din family of invertases is presently unclear. Din-family invertases are mostly found to be encoded in the neighbourhood of the segment which is inverted. To determine whether a protein which has sequence identity with the Din family of invertases is encoded in the *slp* segment, we have recently determined the complete nucleotide sequence of this segment (our unpublished results). Two ORFs are encoded in this region, both in the same orientation as the *slpB* gene. Database comparison revealed that no known protein shows a major similarity to the potential polypeptides encoded by these ORFs. Our future research will be aimed at the unravelling of the inversion process of the *slp* segment, including the elucidation of the proteins involved in this process.

Experimental procedures

Strains and plasmids

L. acidophilus strains ATCC 4356, NCIMB 8607, LMG 11428 and LMG 11469 were obtained from the respective culture collections. *L. plantarum* strain 80 was obtained from Dr Scheirlinck (Scheirlinck *et al.*, 1989). *Escherichia coli* strain DH5 α was used as the host for transformation and isolation of recombinant plasmids. Plasmid pBK-13 is a pUC19 derivative and contains the S-promoter and the 5'-part of the *slpA* gene (nt -1100 to nt +618, relative to the *slpA* start codon) (Boot *et al.*, 1993). Plasmid pBK-101 contains the upstream region and 5'-part (nt -1200 to nt +445, relative to the *slpB* start codon) of the *slpB* gene in pUC19 (Boot *et al.*, 1995).

Isolation of chromosomal DNA

A -70°C stock culture of the *L. acidophilus* strains was streaked on a MRS (Difco) plate and incubated overnight at 37°C to obtain single colonies. A 5 ml MRS broth pre-culture, which was inoculated with a single colony, was cultivated for 18 h anaerobically at 37°C. This pre-culture was used to inoculate 250 ml MRS broth, which was incubated under the same conditions as the pre-culture until an OD₆₀₀ of 1.0 was reached. Chromosomal DNA was isolated as described previously (Boot *et al.*, 1995). The same preparation of *L. acidophilus* ATCC 4356 chromosomal DNA was used in all PCR experiments.

Long-range PCR

Chromosomal DNA of *L. acidophilus* ATCC 4356 was used as the template for PCR amplification of the *slp* segment using the oligonucleotides A-20 (5'-CCGTTAAACCTGCTGAATTA-GC-3'), B-50 (5'-CAGGTGTTGTTGTTTCAACTCC-3'), and B-2MR (5'-GGAGTTGAAACAACAACACCTG-3') as primers and a combination of Taq and Pwo (Expand long template; Boehringer Mannheim) as thermostable DNA polymerases. The final composition of the PCR reaction mixtures (50 μ l) in thin-wall 0.5 ml tubes was: 20 ng template, 0.3 μ M of each primer, 50 mM Tris-HCl (pH 9.2), 16 mM (NH₄)₂SO₄, 2.25 mM MgCl₂, 2% (v/v) DMSO, 0.1% (v/v) Tween 20, 0.5 mM dNTPs (each), 0.5 μ l enzyme. The PCR program used for amplification in the PCR apparatus (DNA Thermal Cycler; Perkin-Elmer Cetus) was as follows: (1) 2 min 92°C, (2) 10 sec 92°C, (3) 30 sec 64°C, (4) 12 min 68°C. Steps 2 to 4 were repeated 22 times and the time period of step 4 was increased by 20 sec per cycle after cycle 10. The tubes were held for 7 min at 68°C after the cycling had finished then stored thereafter at 4°C until further analysis. Part of the mixture was directly analysed by agarose gel electrophoresis (Fig. 2), while the remainder was purified using the Nucleo-trapPCR kit (Macherey-Nagel) and digested with endonucleases before being used in agarose-gel electrophoresis (data not shown).

PCR-I. Different amounts of chromosomal DNA were mixed with two primers (A-15 (5'-GTCGACATAAAGTTGTTTGA-TAATGCTC-3'), specific for the promoter region of *slpA*, and B-2MR (5'-GGAGTTGAAACAACAACACCTG-3'), specific for the ORF of *slpB* (Fig. 1)) and subsequently used in a PCR reaction. The final concentration of the different components in the 50 μ l PCR mixture was: chromosomal or plasmid DNA (for the amounts see legends to the figures), 0.2 μ M of each primer, 50 μ M of each of the dNTPs, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.0 mM MgCl₂, and one unit of a thermostable DNA polymerase (SuperTaq; HT Biotechnology). The PCR program used for amplification in the PCR apparatus (Thermal reactor; Hybaid) was as follows: (1) 5 min 94°C, (2) 40 sec 94°C, (3) 60 sec 57°C, (4) 90 sec 75°C, (5) 2 min 75°C. Steps 2 to 4 were repeated 28 times before proceeding to step 5. The 'Hot Start' method was used to avoid non-specific hybridization and extension at low temperatures (Chou *et al.*, 1992). In the PHLOP-control PCR reactions, plasmids pBK-13, which contains the promoter region of the *slpA* gene, and pBK-101, which

contains the 5'-end of the *slpB* ORF, were mixed with chromosomal DNA of *L. plantarum* 80. Plasmid and chromosomal DNA were digested with endonucleases (either *HindIII* or *EcoRI*), prior to the PCR reaction, when they were used simultaneously in a PCR experiment. This digestion was performed to ensure that the template originating from plasmid DNA was approx. the same length (kinetic properties) as the template originating from chromosomal DNA.

PCR-II. Chromosomal DNA of *L. acidophilus* ATCC 4356 ($10\ \mu\text{g} = 5.2 \times 10^9$ chromosomes; Roussel *et al.*, 1993) was digested with *PvuII* and separated on an agarose gel. Agarose blocks containing DNA fragments of different length (see the legend to Fig. 4) were isolated and DNA was extracted from the agarose using GeneCleanII (Bio101). DNA from each fraction (10% of the total yield after GeneCleanII purification) was used as a template in the PCR reaction. The PCR conditions used were the same as those described for PCR-I.

PCR-III. *PvuII* fragments of chromosomal DNA of *L. acidophilus* ATCC 4356 were isolated as described above (PCR-II) and mixed with primers B-2EF (5'-ACTTCAGTTGATGCA-GGC-3', specific for the coding region of *slpB*) and A-18 (5'-AATCTAGAGCTTCGCTCTGCCTTTT-3', specific for the downstream region of *slpA*). The amounts of the PCR components in the final volume (50 μl) are as detailed above (PCR-I) except for the MgCl_2 concentration, which was 1.75 mM. The PCR program used for amplification was as follows: (1) 5 min 94°C, (2) 40 sec 94°C, (3) 60 sec 52°C, (4) 90 sec 75°C, (5) 2 min 75°C. Steps 2 to 4 were repeated 30 times before proceeding to step 5. The amplified product was analysed on an agarose gel.

PCR-IV. Apart from untreated chromosomal DNA of *L. acidophilus* ATCC 4356 (equivalent to 10^8 copies), we used the *PvuII* fractions (see PCR-II) in this PCR, in combination with oligonucleotides A-7 (5'-GGGTAACAGTCTTACCTG-3', specific for the coding region of *slpA*) and B-5HF (5'-CTATGAGCGGTAGTCATC-3', specific for the region upstream of *slpB*) to prime the DNA polymerization. The amounts of the PCR components in the final volume (50 μl) are as described above (see PCR-I) except for the MgCl_2 concentration, which was 3.0 mM. The PCR program used for amplification was as follows: (1) 90 sec 94°C, (2) 40 sec 94°C, (3) 60 sec 55°C, (4) 75 sec 75°C, (5) 2 min 75°C. Steps 2 to 4 were repeated 30 times before proceeding to step 5. The resulting DNA fragments of this PCR were separated by agarose-gel electrophoresis.

Southern and Northern blotting

Restriction-enzyme-digested chromosomal DNA (10 μg) of each *L. acidophilus* strain was separated on a 1% agarose gel and transferred to a Hybond N filter (Amersham) for Southern blot analysis (Southern, 1975). A 335 bp fragment of the *slpA* gene (nt -146 to nt +179, relative to the translation start of the *slpA* gene) was used as a probe. The nucleotide sequence of this probe is, for the first 250 nucleotides, identical to that of the *slpB* gene. Total RNA was extracted and 5 μg of each RNA preparation was separated on a 1% agarose gel

and blotted onto a Hybond N filter for Northern blot analysis. Specific probes were used to detect mRNA derived from either the *slpA* or *slpB* gene as has been described before (Boot *et al.*, 1995). All probes were labelled with [α - ^{32}P]-dATP using random primer labelling (Promega).

Competitive PCR and phosphor-imaging

A competitive PCR was performed using a hybrid plasmid pBK-121 and chromosomal DNA of *L. acidophilus* ATCC 4356. The hybrid plasmid pBK-120 comprises the 1.0 kb *HindIII*-*SphI* fragment of pBK-13 (containing the S-promoter) ligated in the 3.4 kb *HindIII*-*SphI* vector fragment of pBK-101 (5'-part of *slpB* ORF). The unique *SphI* site of pBK-120 was deleted by *SphI* digestion, trimming of cohesive ends with T4 polymerase and religation with T4 ligase, yielding pBK-121. Chromosomal DNA and plasmid DNA were mixed in different ratios and used as the template in PCR experiments. PCR conditions were used as described above (PCR-I). After PCR, the amplified product was purified using the NucleoTrap kit, digested with *SphI*, analysed on an agarose gel and blotted onto a Hybond N filter. The relative amounts of digested and undigested DNA were determined using a random-primed [α - ^{32}P]-dATP labelled, identical PCR fragment (860 bp) and a phosphor-imager apparatus (Molecular Dynamics). Four independent series of PCR experiments were performed, using the same preparation of *L. acidophilus* ATCC 4356 chromosomal DNA.

Immunological detection of *S_B*-protein-expressing bacteria

Two *S_B*-protein-specific peptides: VTTKNGSSYTNGRISGSC (SP298) and CTNGKEVTPSTVDSVSKS (SP299) were made as described previously (Boersma *et al.*, 1993), and used to generate murine antibodies (Boersma *et al.*, 1992). The antisera reacted in an enzyme-linked immunosorbent assay (ELISA) with coated free peptide as well as with 1-ethyl-3-(3-dimethyl-amino-propyl)-carbodiimide (EDC)-peptide conjugates (Deen *et al.*, 1990). Titres of 50% were found at dilutions 1:700 and 1:1.600 for SP298 and SP299, respectively.

A DNA fragment encoding amino acid residues 27 to 182 of the *S_B*-protein (Boot *et al.*, 1995) was cloned into plasmid pQE-32 (QIAexpress; Qiagen). This plasmid was transferred to *E. coli* and the fusion protein (HisT-*S_B*) was purified as recommended by the supplier. Purified fusion protein (100 μg) was used to raise antibodies in Elco rabbits (Harlan) essentially as described before (Boot *et al.*, 1993). Reactivity of the generated antibodies was evaluated in a Western blot analysis with unpurified fusion protein.

Part of the *L. acidophilus* culture that was used to isolate chromosomal DNA was stored as a glycerol stock at -70°C. This glycerol stock was plated on MRS agar plates and cultivated anaerobically overnight at 37°C. The resulting colonies were blotted onto nitrocellulose (Hybaid) and *S_A*- and *S_B*-proteins were detected as previously described (Boot *et al.*, 1993), using polyclonal murine *S_A*-protein antiserum (Boot *et al.*, 1993) or antisera against SP298, SP299 or HisT-*S_B*. An *L. acidophilus* broth culture was inoculated from the same glycerol stock for immunofluorescence

microscopy analysis. Bacteria were spotted on a glass tray, washed with PBS then fixed for 10 min with 2% formaldehyde. The fixed cells were washed with PBS and either S_A- or S_B-protein was detected using the same antisera as was used in the colony blotting, followed by either donkey anti-rabbit fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch Lab.) or donkey anti-mouse dichlorotriazimyl amino fluoris-cein (DTAF) (Jackson) as described previously (Wansink *et al.*, 1993).

Acknowledgements

We thank Bruno Pot for *L. acidophilus* LMG 11428 and 11469, Pieter Postma and Hans van der Spek for stimulating discussions, and Joop de Bree for mathematical analyses.

References

- Beveridge, T.J. (1994) Bacterial S-layers. *Curr Op Struct Biol* **4**: 204–212.
- Blaser, M.J., Wang, E., Tummuru, M.K.R., Washburn, R., Fujimoto, S., and Labigne, A. (1994) High-frequency S-layer protein variation in *Campylobacter fetus* revealed by *sapA* mutagenesis. *Mol Microbiol* **14**: 453–462.
- Boersma, W.J.A., Bogaerts, W.J.A., Bianchi, A.T.J., and Claassen, E. (1992) Adjuvant properties of stable water-in-oil emulsions: evaluation of the experience with Specol. *Res Immunol* **143**: 503–512.
- Boersma, W.J.A., Haaijman, J.J., and Claassen, E. (1993) Use of synthetic peptide determinants for the production of antibodies. In *Immunohistochemistry II*. Cuella A.C. (ed.). Toronto: Wiley and Sons, pp. 1–78.
- Boot, H.J., Kolen, C.P.A.M., and 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., van Noort, J.M., and 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., Pot, B., Kersters, K., and Pouwels, P.H. (1996) The presence of two S-layer protein encoding genes is conserved among species related to *Lactobacillus acidophilus*. *Microbiology*, in press.
- Chou, Q., Russell, M., Birch, D.E., Raymond, J., and Bloch, W. (1992) Prevention of pre-PCR mispriming and primer dimerization improves low-copy-number amplifications. *Nucl Acids Res* **20**: 1717–1723.
- Deen, C., Claassen, E., Gerritse, K., Zegers, N.D., and Boersma, W.J.A. (1990) An improved and modified carbodiimide coupling method for synthetic peptides: Enhanced antipeptide antibody responses. *J Immunol Meth* **129**: 119–125.
- Dworkin, J., and Blaser, J. (1996) Generation of *Campylobacter fetus* S-layer protein diversity utilizes a single promoter on an invertible DNA segment. *Mol Microbiol* **19**: 1241–1253.
- Feng, J.-A., Johnson, R.C., and Dickerson, R.E. (1994) Hin recombinase bound to DNA: the origin of specificity in major and minor groove interactions. *Science* **263**: 348–355.
- Fernandes, C.F., Shahani, K.M., and Amer, M.A. (1987) Therapeutic role of dietary lactobacilli and lactobacillic fermented dairy products. *FEMS Microbiol Rev* **46**: 343–356.
- Frohman, M.A., and Martin, G.R. (1990) Detection of homologous recombinants. In *PCR Protocols*. Innis M.A., Gelfand, D.H., Sninsky J.J., and White T.J. (eds). San Diego: Academic Press, pp. 228–236.
- Gilliland, G., Perrin, S., and Bunn, H.F. (1990) Competitive PCR for quantitation of mRNA. In *PCR Protocols*. Innis M.A., Gelfand D.H., Sninsky J.J., and White T.J. (eds). San Diego: Academic Press, pp. 60–69.
- Glasgow, A.C., Bruist, M.F., and Simon, M.I. (1989a) DNA-binding properties of the Hin recombinase. *J Biol Chem* **264**: 10072–10082.
- Glasgow, A.C., Hughes, K.T., and Simon, M.I. (1989b) Bacterial DNA inversion systems. In *Mobile DNA*. Berg D.E., and Howe M.M. (eds). Washington, DC: American Society for Microbiology, pp. 637–659.
- Greene, J.D., and Klaenhammer, T.R. (1994) Factors involved in adherence of lactobacilli to human Caco-2 cells. *Appl Environ Microbiol* **60**: 4487–4494.
- Hoppensteadt, F.C. (1982) Markov chains. In *Mathematical Methods of Population Biology*. Cambridge: Cambridge University Press, pp. 47–72.
- Kim, H.-S., and Smithies, O. (1988) Recombinant fragment assay for gene targeting based on the polymerase chain reaction. *Nucl Acids Res* **16**: 8887–8903.
- Messner, P., and Sleytr, U.B. (1992) Crystalline bacterial cell-surface layers. *Adv Microb Physiol* **33**: 213–275.
- Mukai, T., and Arihara, K. (1994) Presence of intestinal lectin-binding glycoproteins on the cell surface of *Lactobacillus acidophilus*. *Biosci Biotech Biochem* **58**: 1851–1854.
- Pääbo, S., Higuchi, R.G., and Wilson, A.C. (1989) Ancient DNA and the polymerase chain reaction. The emerging field of molecular archaeology. *J Biol Chem* **264**: 9709–9712.
- Robertson, B.R., and Meyer, T.F. (1992) Genetic variation in pathogenic bacteria. *Trends Genet* **8**: 422–427.
- Roussel, Y., Colmin, C., Simonet, J.M., and Decaris, B. (1993) Strain characterization, genome size and plasmid content in the *Lactobacillus acidophilus* group (Hansen and Mocquot). *J Appl Bacteriol* **74**: 549–556.
- Sandmeier, H., Iida, S., Meyer, J., Hiestand-Nauer, R., and Arber, W. (1990) Site-specific DNA recombination system Min of plasmid p15B: A cluster of overlapping invertible DNA segments. *Proc Natl Acad Sci USA* **87**: 1109–1113.
- Sára, M., and 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.
- Sára, M., Pum, D., Küpcü, S., Messner, P., and Sleytr, U.B. (1994) Isolation of two physiologically induced variant strains of *Bacillus stearothermophilus* NRS 2004/3a and characterization of their S-layer lattices. *J Bacteriol* **176**: 848–860.
- Scheirlinck, T., Mahillon, J., Joos, H., Dhaese, P., and Michiels,

- F. (1989) Integration and expression of α -amylase and endoglucanase genes in the *Lactobacillus plantarum* chromosome. *Appl Environ Microbiol* **55**: 2130–2137.
- Schneitz, C., Nuotio, L., and 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.
- Seifert, H.S., and So, M. (1988) Genetic mechanisms of bacterial antigenic variation. *Microbiol Rev* **52**: 327–336.
- 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., and Korhonen, T.K. (1995) A collagen-binding S-layer protein in *Lactobacillus crispatus*. *Appl Environ Microbiol* **61**: 2467–2471.
- Tummuru, M.K.R., and Blaser, M.J. (1993) Rearrangements of *sapA* homologs with conserved and variable regions in *Campylobacter fetus*. *Proc Natl Acad Sci USA* **90**: 7265–7269.
- Wang, E., Garcia, M.M., Blake, M.S., Pei, Z., and Blaser, M.J. (1993) Shift in S-layer protein expression responsible for antigenic variation in *Campylobacter fetus*. *J Bacteriol* **175**: 4979–4984.
- Wansink, D.G., Schul, W., Vanderkraan, I., Vansteensel, B., Vandriel, R., and Dejong, L. (1993) Fluorescent labelling of nascent RNA reveals transcription by RNA polymerase II in domains scattered throughout the nucleus. *J Cell Biol* **122**: 283–293.
- Zieg, J., and Simon, M. (1980) Analysis of the nucleotide sequence of an invertible controlling element. *Proc Natl Acad Sci USA* **77**: 4196–4200.