Interchange of the active and silent S-layer protein genes of Lactobacillus acidophilus by inversion of the chromosomal slp segment.
Boot, H.J.; Kolen, C.P.A.M.; Pouwels, P.H.

Published in: Molecular Microbiology

DOI: 10.1046/j.1365-2958.1996.401406.x

Citation for published version (APA):
Interchange of the active and silent S-layer protein genes of \textit{Lactobacillus acidophilus} by inversion of the chromosomal \textit{slp} segment

Hein J. Boot,\textsuperscript{1}\textsuperscript{*} Carin P. A. M. Kolen\textsuperscript{1} and Peter H. Pouwels\textsuperscript{1,2}
\textsuperscript{1}BioCentrum Amsterdam, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam, The Netherlands.
\textsuperscript{2}TNO Nutrition and Food Research Institute, PO Box 5815, 2280 HV Rijswijk, The Netherlands.

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. \textit{Lactobacillus acidophilus} harbours two S-protein-encoding genes, \textit{slpA} and \textit{slpB}, only one of which (\textit{slpA}) is expressed. In this study, we show by polymerase chain reaction (PCR) analysis that \textit{slpA} and \textit{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 \textit{slpA} gene seems to be favoured under laboratory growth conditions because 99.7\% of the chromosomes of an \textit{L. acidophilus} ATCC 4356 broth culture had the \textit{slpA} gene present at the \textit{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 Archaeabacteria. 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 \textit{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 \textit{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 \textit{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 (Schnitz 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 \textit{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 (\textit{slpA}; Boot et al., 1993) of the type strain of \textit{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 (\textit{slpB}; Boot et al., 1995) in this species, which has extensive similarity with the expressed \textit{slpA} gene. In this report we show that these two S-protein-encoding genes are present on a chromosomal fragment of 6 kb (the \textit{slp} segment). Inversion of this segment leads to the positioning of the silent \textit{slpB} gene behind the S-promoter, while the active \textit{slpA} gene is stored at the silent position of the \textit{slpB} gene. \textit{L. acidophilus} bacteria grown under laboratory conditions appear to have a selective advantage when they express the \textit{slpA} instead of the \textit{slpB} gene, because 99.7\% of these bacteria had the \textit{slpA} gene present at the \textit{slp} expression site.
**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 halt-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 x 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
Inversion of the sI segment of Lactobacillus acidophilus
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 size, which was visualized by agarose gel electrophoresis (Fig. 5, lane 8). The amplified product was of the expected size (860 bp), as was visualized by agarose gel electrophoresis (Fig. 5, lane 8). The length of some marker (M) DNA fragments (wild-type Lambda DNA digested with BstEII) is indicated on the right-hand side.

**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 BstEII) is indicated on the right-hand side.

**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 x 10^7; lanes 3 and 7, 10^8; lanes 4 and 8, 3 x 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 10^6 copies of chromosomal DNA.
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 suboptimal separation of the 9.2 kbPvu II fragment containing the 5'/39'-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 Pvu II fraction 2 was used in the PCR amplification (Fig. 5, lane 3). The reduced yield of amplified product in Pvu II 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 Sβ-protein expression

Despite this relatively high occurrence, all attempts to detect L. acidophilus bacteria carrying an Sβ-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 Sβ-specific polyclonal antibodies (data not shown). Furthermore, we have used the Sβ-specific antisera in immunofluorescence microscopy analysis to visualize the presence of Sβ-protein on the outside surface of the bacteria. None of the inspected bacterial cells (>100 000) from the −70°C stock reacted with the Sβ-specific antibodies while all the bacteria strongly reacted with antiserum raised against purified Sα-protein (data not shown).

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 recombinalional event, placing the slpB ORF behind the S-promoter.

Immunologic detection of Sβ-protein expression

Despite this relatively high occurrence, all attempts to detect L. acidophilus bacteria carrying an Sβ-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 Sβ-specific polyclonal antibodies (data not shown). Furthermore, we have used the Sβ-specific antisera in immunofluorescence microscopy analysis to visualize the presence of Sβ-protein on the outside surface of the bacteria. None of the inspected bacterial cells (>100 000) from the −70°C stock reacted with the Sβ-specific antibodies while all the bacteria strongly reacted with antiserum raised against purified Sα-protein (data not shown).
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/slprB 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...
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* 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.
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-proteins 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 26bp sequence (six) 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 six. The six 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 DH5α 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 –7°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'-CGTTAACCTTCGTAATTAGC-3'), B-50 (5'-CAGTGTTGGTGTTTTCAACTC-3'), and B-2MR (5'-GGAGTTGAACCAACAACACCTGT-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 (50µl) was: 20 ng template, 0.3 µM 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 µl 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 thereatere 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-Trap PCR 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'-GTCGAATAAAAGTGTGTTGA-TATGCT-3'), specific for the promoter region of slpA, and B-2MR (5'-GGAGTTGAACCAACAACACCTGT-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 50µl PCR mixture was: chromosomal or plasmid DNA (for the amounts see legends to the figures), 0.2µM of each primer, 50µM of each of the dNTPs, 50 mM KCl, 10 mM Tris-HCl (pH9.0), 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 μg = 5.2 × 10^6 chromosomes; Roussel *et al.*, 1993) was digested with *Pvu*II 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.** *Pvu*II fragments of chromosomal DNA of *L. acidophilus* ATCC 4356 were isolated as described above (PCR-II) and mixed with primers B-2EF (5’-ACTTCAAGTTGATGGA-GGC-3’, specific for the coding region of slpB) and A-18 (5’-AATCTAGAGCTTTCGCTCTGCCTTTT-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 MgCl₂ 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 10^6 copies), we used the *Pvu*II fractions (see PCR-II) in this PCR, in combination with oligonucleotides A-7 (5’-GGGTAACAGCTTACCTG-3’, specific for the coding region of slpA) and B-5HF (5’-CTATGAGCGGTAGTCATC-3’, specific for the downstream 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 MgCl₂ 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 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-³²P]-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 NucleotraPCR 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-³²P]-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 S₈₆-protein-expressing bacteria**

Two S₈₆-protein-specific peptides: VTTKNGSSYNTNRI GSC (SP298) and CTNGKEVTPTSVDSVSKS (SP299) were made as described previously (Boersma *et al.*, 1993), and used to generate murine antibodies (Boersma *et al.*, 1992). The antiserum reacted in an enzyme-linked immunosorbent assay (ELISA) with coated free peptide as well as with 1-ethyl-3-(3-dimethyl-aminopropyl)-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 S₈₆-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–S₈₆) was purified as described previously (Boersma *et al.*, 1993). The antiserum 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 S₈₆ and S₈₂-proteins were detected as previously described (Boot *et al.*, 1993), using polyclonal murine S₈₂-protein antiserum (Boot *et al.*, 1993) or antiserum against SP298, SP299 or HisT–S₈₆. 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 SAr or SBr 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 fluorescein (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

Scheirlinck, T., Mahillon, J., Joos, H., Dhaese, P., and Michiels,

© 1996 Blackwell Science Ltd, Molecular Microbiology, 21, 799–809


