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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

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The bacterial S-layer forms a regular structure, composed of a monolayer of one (glyco)protein, on the surfaces of many prokaryotic species. S-layers are reported to fulfil different functions, such as attachment structures for extracellular enzymes and major virulence determinants for pathogenic species. *Lactobacillus acidophilus* ATCC 4356, which originates from the human pharynx, possesses such an S-layer. No function has yet been assigned to the S-layer of this species. Besides the structural gene (*slpA*) for the S-layer protein (S-protein) which constitutes this S-layer, we have identified a silent gene (*slpB*), which is almost identical to *slpA* in two regions. From the deduced amino acid sequence, it appears that the mature S_B-protein (44,884 Da) is 53% similar to the S_A-protein (43,636 Da) in the N-terminal and middle parts of the proteins. The C-terminal parts of the two proteins are identical except for one amino acid residue. The physical properties of the deduced S-proteins are virtually the same. Northern (RNA) blot analysis shows that only the *slpA* gene is expressed in wild-type cells, in line with the results from sequencing and primer extension analyses, which reveal that only the *slpA* gene harbors a promoter, which is located immediately upstream of the region where the two genes are identical. The occurrence of in vivo chromosomal recombination between the two S-protein-encoding genes will be described elsewhere.

Up to 300 different species of eubacteria and archaebacteria have been reported to be covered with a regular structure, the S-layer. This S-layer is built up entirely of one species of (glyco)protein known as the S-layer protein (S-protein) (for reviews, see references 2 and 23). Despite the common property of forming a regular layer on the outside of the bacteria, not much overall similarity between the primary structures of S-proteins can be identified. No general function seems to be associated with the presence of an S-layer, as S-layers of different species are reported to have different functions, e.g., as a cell shape determinant in *Thermoproteus tenax* (41), a phage receptor-masking layer in *Aquaspirillum serpens* (15), a major determinant for virulence in *Aeromonas salmonicida* (13), and an attachment structure for amylase in *Bacillus stearothermophilus* (11).

Several but not all species of the genus *Lactobacillus* possess an S-layer (21). The S-proteins of *Lactobacillus helveticus* (19) and *Lactobacillus buchneri* and *Lactobacillus plantarum* (24) have been characterized, and the genes of two *Lactobacillus* species, *Lactobacillus brevis* (38) and *Lactobacillus acidophilus* ATCC 4356 (5), have been cloned and sequenced. Lactobacilli are gram-positive bacteria which play a major role in human and animal food production. For many years it has been believed that some *Lactobacillus* strains in the gastrointestinal and female urogenital tracts have a beneficial effect on human and animal health and thus may be used for therapeutic purposes. Several explanations for this effect are possible, e.g., stimulation of production of immunoglobulins G and A (18, 27), acidification of the local environment (44), production of H_2O_2 (22), hypocholesteremic effects (12), binding of mutagenic compounds (26), production of bacteriocins (14) or specific or nonspecific adherence onto mucosal surfaces, which prevents the epithelium from being invaded by pathogenic bacteria such as *Salmonella typhimurium*, *Escherichia coli*, *Yersinia pseudotuberculosis*, *Neisseria gonorrhoeae*, and *Gardnerella vaginalis* (7, 9, 44). Various environmental conditions are faced by the *Lactobacillus* strains, as they are found throughout the whole gastrointestinal and female urogenital tracts of different mammals. It is of interest to know the effect, if any, of the presence of an S-layer on the surface of lactobacilli on adherence and colonization of mucosal surfaces.

Previously we have reported on the properties of the S-protein and on the cloning and sequencing of the corresponding S-protein-encoding gene (*slpA*) of *L. acidophilus* ATCC 4356, the type strain of *L. acidophilus*, which was originally isolated from a human pharynx (5). In this paper we describe the identification, cloning, and sequencing of an open reading frame which is not expressed and which presumably codes for a different S-protein. This gene, named *slpB*, encompasses two regions that have almost complete similarity to the corresponding regions of the *slpA* gene; these regions are interspaced with a region that has reduced similarity. The involvement of the 5′ similarity regions of these genes in chromosomal recombination in vivo will be described in a subsequent paper.

MATERIALS AND METHODS

Strains and plasmids. The neotype strain *L. acidophilus* ATCC 4356 was obtained from the American Type Culture Collection and was cultivated anaerobically in MRS broth (Difco) at 37°C. *E. coli* JM109 or DH5α was used for transformation with derivatives of the pUC19 (43), pBluescript IISK+ (Stratagene), or pGEM-T (Promega) vector. Plasmid pBK-1 is a pUC19 derivative

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containing the whole *slpA* gene on a 4.0-kb fragment, while plasmid pBK-13 is a deletion derivative of pBK-1 containing the coding region for the N-terminal one-third of the S_A-protein and the adjacent 1.0-kb 5′ region (5).

Chromosomal DNA isolation. An exponentially growing culture of *L. acidophilus* ATCC 4356 (500 ml; optical density at 600 nm [OD_{600}] = 0.7 to 1.5) was harvested by centrifugation (10 min at 5,000 × *g*), washed once with 50 ml of 20 mM sodium maleate (pH 6.2), and resuspended in 40 ml of 20 mM sodium maleate (pH 6.2)–0.6 M lactose–20 mM magnesium chloride–80 mg of lysozyme (Boehringer Mannheim). After incubation for 15 min at 37°C, protoplasts were harvested by centrifugation for 10 min at 3,000 × *g* and resuspended in 20 ml of 20 mM Tris (pH 8.2). After addition of 4.4 ml of 0.5 M EDTA, 5.5 ml of a 5% Sarkosyl solution and then 3.3 ml of 5 M NaCl were added. The final suspension was extracted with phenol, phenol-chloroform-isoamyl alcohol (25:24:1), and chloroform-isoamyl alcohol (24:1). Chromosomal DNA was separated from the liquid phase after the addition of 2 volumes of ethanol (20°C) with a glass stirring rod. Chromosomal DNA was solubilized in 5 ml of 0.1× SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate), 0.4 ml of a 10-mg/ml RNase solution was added, and the mixture was incubated for 20 min at 37°C. Subsequently, 0.8 ml of a 20-mg/ml solution of proteinase K (Boehringer Mannheim) was added, and the mixture was incubated for 40 min at 65°C; this was followed by the addition of 0.7 ml of 5 M NaCl, and repeated phenol-chloroform extractions as described above were performed. Chromosomal DNA was isolated by standard ethanol precipitation (31) and dissolved in 2 ml of 0.1× SSC.

Southern blot analysis. Restriction enzyme-digested chromosomal DNA was separated on a 1% agarose gel and transferred to a Hybond filter (Amersham) essentially as described by Southern (36). Specific oligonucleotides for the *slpA* region were used to generate the 5′ region I, 5′ region II, and C-terminal probes with the PCR technique and pBK-1 as a template. The internal and 3′ region probes are specific restriction fragments derived from pBK-1. Both the PCR-based and the restriction fragment-based probes were purified by gel electrophoresis and isolated from the agarose gel with Geneclean II (Bio 101, Inc., La Jolla, Calif.). The probes were labelled with [α-^{32}P]dATP by the nick translation method (31). Prehybridization, hybridization, and washing were all performed at 65°C. As the most stringent washing buffer, 0.5× SSC–0.1% sodium dodecyl sulfate (SDS) was used.

Cloning and sequencing of the *slpB* region. Chromosomal DNA of *L. acidophilus* ATCC 4356 was digested with a combination of the restriction enzymes *Ase*I and *Hin*dIII and separated on an agarose gel. The sizes of the restriction fragments of the *slpB* region which hybridized with probes derived from the *slpA* region were determined by the Southern blot technique. These fragments were purified from the gel with Geneclean II and ligated with *Nde*I- and *Hin*dIII-digested pUC19. Competent *E. coli* cells were transformed with the ligation mixture by electroporation, and recombinant clones with the desired fragments were identified in a colony hybridization experiment with *slpA* probes. Recombinant plasmids pBK-97, containing the 5′ region of the *slpB* gene, and pBK-98, containing the 3′ region of the *slpB* gene, were purified and used for sequence determination on both strands by the dideoxy chain termination method (32). Primers specific for the cloned regions (A-4 and B-2MF [see Fig. 2]) were used to amplify the fragment which was located between the two cloned regions by using chromosomal DNA as template. Two independent PCRs on chromosomal DNA were performed, and the products generated were cloned in the pGEM-T vector, yielding pBK-99.1 and pBK-99.2. The sequence of the intervening *Hin*dIII fragment was determined on both strands for both plasmids, yielding the same sequence. The region 5′ to pBK-97 was cloned by using the inverse PCR technique. Chromosomal DNA of *L. acidophilus* ATCC 4356 was digested which the restriction enzyme *Hin*dIII and separated on an agarose gel. Fragments of the expected size were isolated and ligated, and a PCR with primers A-10 and B-2EF (see Fig. 2) was performed. The extension product was checked by using restriction enzymes, which yielded the expected fragment sizes, and cloned in pBluescript (pBK-100). The nucleotide sequences of the 5′ regions of both the *slpA* and *slpB* genes were determined on both strands by using pBK-13 and pBK-100, respectively, and sequence-specific primers.

Protein analysis. *L. acidophilus* ATCC 4356 cells (500 ml; OD_{600} = 0.7 in MRS broth) were harvested by centrifugation at 5,000 × *g* for 15 min at 4°C. The cells were washed twice with an equal volume of ice-cold water and were resuspended in 10 ml of 4.0 M guanidine hydrochloride (pH 7.0). The bacteria were incubated for 60 min at 37°C in this solution and then centrifuged at 18,000 × *g* for 15 min. The supernatant, in which the S-protein is the predominant protein, was extensively dialyzed against water at 4°C and analyzed on an SDS-polyacrylamide gel. The N-terminal amino acid sequence of the mature S_A-protein (0.8 nmol) was determined with a Procise 494 (Applied Biosystems) protein sequencer. Electrospray ionization mass spectrometry was carried out with a FISONS Platform quadrupole mass spectrometer coupled to a VG Masslynx data system. The S_A-protein (10 μl of a 20-pmol/μl solution) was introduced into the source via a valve-loop system and a Cole Palmer model 74900 syringe pump, using 50% acetonitrile–50% water plus 0.2% formic acid with a flow rate of 5 μl/min.

Isolation of total RNA. Exponentially growing cells (100 ml; OD_{600} = 0.2 to 0.4) of *L. acidophilus* ATCC 4356 were cooled on ice, harvested by centrifugation (5,000 × *g* for 10 min at 4°C), washed with 1 ml of 20 mM Tris (pH 8.0, 0°C), and collected in Eppendorf tubes. The cells were incubated for 5 min at 37°C after being resuspended in 0.25 ml of 20 mM Tris (pH 8.0, 0°C) and after addition of 0.5 ml of 24% polyethylene glycol (molecular weight = 20,000; 0°C) and 0.25 ml of 20 mM Tris (pH 8.0, 0°C) with 5 mg of lysozyme. The Eppendorf tube was cooled on ice and centrifuged for 3 min at 10,000 × *g*. The pellet was resuspended in 0.4 ml of 0.2 M sodium acetate (pH 5.2)–10 mM Na_2EDTA (0°C), and 40 μl of 10% SDS (0°C) was added and mixed. The solution was repeatedly extracted with phenol-chloroform as described under "Chromosomal DNA isolation" above, with intervening periods of incubation on ice. The aqueous phase after the phenol-chloroform extractions was mixed with 40 μl of 3.0 M sodium acetate (pH 5.2) and 1.2 ml of ethanol (−20°C). The RNA was harvested by centrifugation for 15 min (10,000 × *g*, 4°C) and resuspended in 0.2 ml of RNase-free water. Phenol-chloroform extraction and precipitation were repeated until the OD_{260}/OD_{280} ratio of the total RNA solution was over 1.5.

Northern (RNA) blot analysis. Total RNA was separated in a 1× TBE (10× TBE is 108 g of Tris, 55 g of boric acid and 10 g of Na_2EDTA) agarose gel without ethidium bromide. The gel was soaked two times for 20 min each in 0.2 M sodium acetate (pH 5.0) and transferred to a Hybond filter by blotting with the same solution. The RNA was cross-linked to the filter by short-wavelength UV light for 5 min, and the filter was washed with 5× SSC. Prehybridization and hybridization of both the RNA and control DNA filters were performed in 5 ml of 5× SSC–5× Denhardt solution (31) (0.1% SDS–0.1 mg of sonicated salmon sperm DNA per ml) at 65°C. Washing was done two times with 2× SSC–0.1% SDS and three times with 0.1× SSC–0.1% SDS at 65°C. An autoradiogram was made after air drying of the Hybond filters. Probes were labelled with [α-^{32}P]dATP by random primer labelling with hexamer primers (31). Probes A and A/B are restriction fragments from pBK-13 (probe A, nucleotides [nt] 466 to 640; probe A/B, nt 141 to 466 [*slpA* region; see Fig. 3]) purified from agarose gels with Geneclean II. Probe B is an agarose gel-purified PCR fragment obtained by using the oligonucleotides B-2MF (5′TTC.ATC.AGT.TGC.TGG.TG3′) and B-2MR (5′GGA.GTT.GAA.ACA.ACA.ACA.CCT.G3′) and pBK-97 as a template.

Primer extension analysis. The primer extension reaction was carried out with 5 μg of total RNA and 0.25 fmol of primer A-10 (5′CTT.CCC.GGG.GTC.TTT.TCC.TCC3′; complementary to the coding sequence in the region of the *slpA* start codon) in 10 μl of reaction buffer (50 mM Tris [pH 8.3], 125 mM KCl, 5 mM $MgCl_2$). The RNA was denatured by heating the sample at 85°C for 10 min. The sample was gradually cooled to 42°C and held at that temperature for 1 h. Ten units of Superscript (Gibco BRL Life Technologies) was added, and the reaction buffer (20 μl) was adjusted to 50 mM Tris (pH 8.3), 125 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM dGTP, 1 mM dCTP, 1 mM dTTP, and 0.165 mM [α-^{32}P]dATP. The extension reaction was carried out at 37°C for 10 min, and 1 μl of a 20 mM dATP solution was added and incubated at 37°C for another 40 min. The reaction was stopped by adding 1 μl of a 0.5 M Na_2EDTA–1.0% SDS solution, and 5 μl of layer mix (31) was added. The final solution (5 μl) was analyzed on a denaturing 6% polyacrylamide gel next to the four sequence reactions (32) with the same primer and pBK-1 as template.

Pulsed-field gel electrophoresis. Exponentially growing cells of *L. acidophilus* ATCC 4356 (see "Chromosomal DNA isolation" above) were harvested and washed twice in 20 mM Tris-HCl (pH 8.2) to neutralize the acidic environment of the cells before they were embedded in low-melting-point agarose. Lysis of the cells and digestion of chromosomal DNA were done essentially as described by Leblond et al. (17), with incubation for 3 h at 37°C with lysozyme (Sigma). Chromosomal DNA fragments were separated for 24 h by using a contour-clamped homogeneous electric field DRII (Bio-Rad) apparatus under the conditions described by Roussel et al. (30). The *slp* genes were detected after Southern blotting, using the specific probes for the *slpA* and *slpB* regions as described for the Northern blot analysis. The length of the hybridizing fragment was estimated by using the DNA length marker Delta 39 Lambda (Promega) and the results of Roussel et al. (30).

Nucleotide sequence accession number. The EMBL accession number of the *slpB* region is X89376. The sequence of the *slpA* region has been updated as a result of the sequence determination in the 5′ and 3′ untranslated regions and has accession number X89375.

RESULTS

Detection of *slpB* region. On the basis of the restriction map of the *slpA* region of the *L. acidophilus* type strain, we selected five different DNA probes. These probes, which span the whole coding region of *slpA* and up- and downstream regions (Fig. 1a), were used in a Southern blot analysis of the chromosomal DNA of this strain. The result of a Southern blot of chromosomal DNA digested with endonuclease *Eco*RI is shown in Fig. 1b. To our surprise, the hybridization pattern was not the same for all of the probes used. All five probes hybridize with a 4.3-kb fragment, while only the 5′ region II, internal, and C-terminal probes hybridize with a fragment of 6.5 kb. From restriction analysis of a plasmid which contains the *slpA* region (pBK-1), we know that no *Eco*RI restriction site is present in

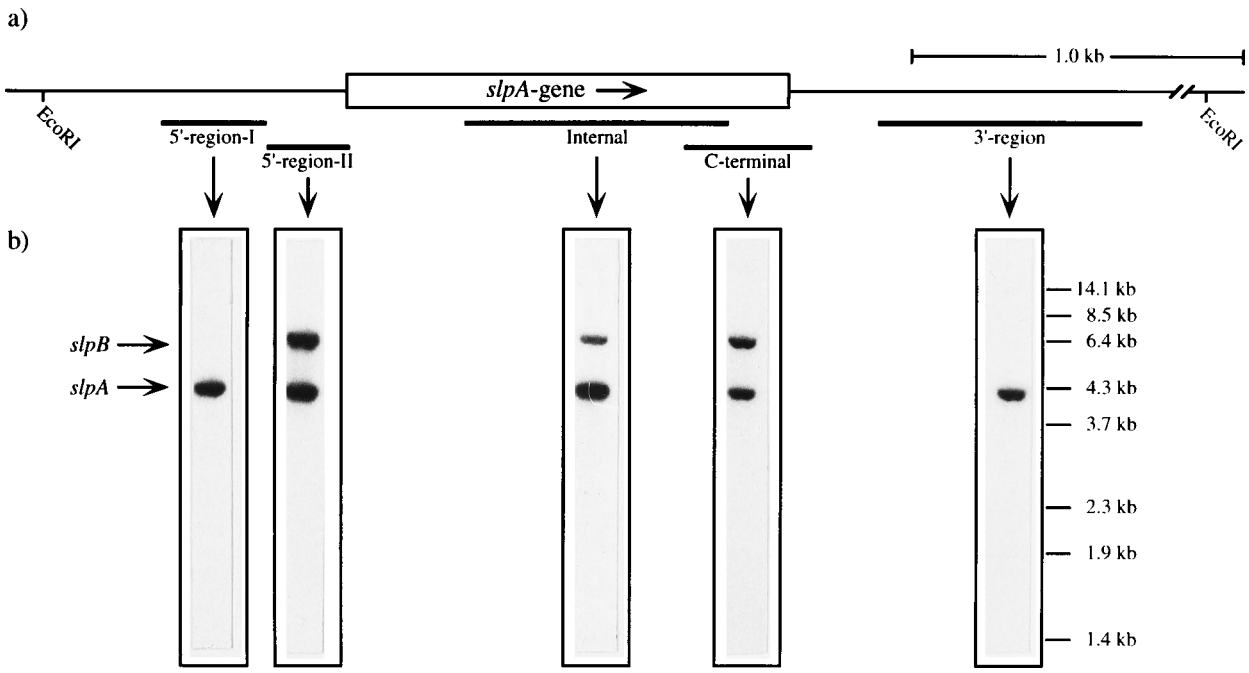

FIG. 1. Detection of the *slp* regions. (a) Schematic drawing of the probes (black bars), which are derived from the *slpA* region. *Eco*RI sites of the *slpA* region are shown, and the direction of transcription of the *slpA* gene is indicated by a horizontal arrow. (b) Autoradiogram of a Southern blot of *Eco*RI-digested chromosomal DNA of *L. acidophilus* ATCC 4356 with the probes indicated in panel a. Sizes of marker DNA fragments (wild-type λ digested with restriction endonuclease *Bst*EII) are indicated on the right.

the region which is spanned by the five probes used (Fig. 1a). We thus expected to find one band of the same size to hybridize with the five probes when we used *Eco*RI-digested chromosomal DNA. The 4.3-kb fragment is the *slpA* region, as could be concluded from Southern blot analysis involving a combination of *Eco*RI with other restriction enzymes which have recognition sites within the *slpA* gene (*Pst*I and *Spe*I; data not shown). On the basis of the hybridization of the 6.5-kb fragment with probes derived from the *slpA* region, we have named this fragment the *slpB* region. The *L. acidophilus* type strain does not contain any plasmids (30), so both *slp* regions must be located on the chromosome. From pulsed-field gel electrophoresis analyses of *Apa*I-digested chromosomal DNA of *L. acidophilus* ATCC 4356, it appeared that the *slpA* and *slpB* genes are located on the same 125-kb chromosomal fragment, which is about 7% of the length of the chromosome (reference 30 and data not shown).

The intensities of the hybridization signals of the *slpA* and *slpB* regions are about the same for the 5′ region II and C-terminal probes, indicating that both sequences are highly similar in these two regions. The intensity of the hybridization signal of the *slpB* region is less than that of the *slpA* region when the internal probe is used, indicating that the two sequences are related but are less similar than the flanking sequences. The absence of a hybridization signal at 6.5 kb when the 5′ region and 3′ region probes are used indicates that there is hardly any similarity between the up- and downstream regions of *slpA* and *slpB*.

Cloning and sequencing of the *slpB* region. To be able to identify the sequence on the *slpB* region showing the high degree of similarity with the *slpA* region, we have cloned this region. Our first approach to clone a DNA fragment which contains the *slpB* region involved the screening of the lambda EMBL3 library of chromosomal DNA of this strain, which had been used to identify and clone the *slpA* region. Although we have performed extensive searches, we have not been able to identify recombinant phages containing sequences derived from the *slpB* region. The second approach to clone the *slpB* region involved the cloning of DNA fragments containing the entire *slpB* region in a plasmid vector. After several attempts which did not yield clones with the desired fragments, we decided to use a third approach, which involved the cloning of two smaller fragments of the *slpB* region (see Materials and Methods), each containing a region which had a high degree of similarity with a part of the *slpA* region. Using this approach, we were able to clone and sequence two specific fragments of the *slpB* region (pBK-97 and pBK-98) (Fig. 2). The presence of 11 direct repeats of at least 10 nucleotides in the sequences of this gene might be the reason for the difficulties in cloning. Cloning in *E. coli* of transcriptionally active S-protein genes from different sources, including *L. acidophilus* (5), *L. brevis* (38), *Bacillus brevis* (42), *B. stearothermophilus* (16), *Bacillus sphaericus* (1), and *A. salmonicida* (8), has been reported to involve structural instability of the plasmids or inviability of transformed *E. coli* cells. For *B. stearothermophilus* and *A. salmonicida*, it was found that the direct repeats in the nucleotide sequences of the structural S-protein genes are involved in rearrangements of the cloned sequence.

A PCR with chromosomal DNA was performed to determine whether the cloned fragments were adjacent. From restriction analysis of this PCR fragment, it appeared that the two fragments were not adjacent on the chromosome, as the fragment contained two *Hin*dIII restriction sites. The sequence of this intervening *Hin*dIII fragment was determined after cloning of the PCR fragment (pBK-99 [Fig. 2]). To be able to determine the nucleotide sequence of the 5′ region of the *slpB* region, which was not present on the cloned fragments, we performed an inverse PCR (25) and determined the nucleotide sequence of the cloned fragment which was generated with this technique (pBK-100 [Fig. 2]).

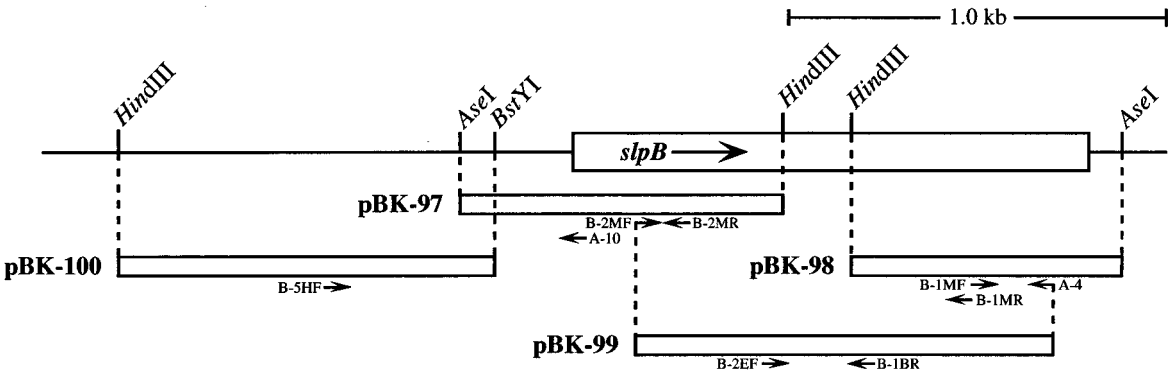

FIG. 2. Schematic drawing of the cloning and sequencing strategy used to determine the nucleotide sequence of the *slpB* region. Specific oligonucleotides used for sequencing and PCR amplification are indicated. Universal sequencing primers (M13) were used when appropriate. The plasmids pBK-99 and pBK-100 were generated by cloning PCR-amplified fragments of the specific regions, using the indicated primers and chromosomal DNA of *L. acidophilus* ATCC 4356 as a template (see Materials and Methods).

Similarity between the *slp* genes and deduced S-proteins. The nucleotide sequence of the *slpB* region is aligned with the sequence of the *slpA* region in Fig. 3. Several regions with different degrees of similarity can been found in this alignment. The far-upstream regions (nt 1 to 100; Fig. 3) show no detectable similarity. The region from nt 100 to 381 (*slpA* region; Fig. 3), which overlaps the start codon of the *slpA* gene, shows almost complete similarity (99%). The region from nt 381 to 1220 (*slpA* gene; Fig. 3) shows on the average 55% similarity when several gaps are introduced. The region from nt 1220 to 1648 (*slpA* gene; Fig. 3), which overlaps the two stop codons of the *slpA* sequence, again shows almost complete similarity (98%). The similarity between the two *slp* sequences in the region where the stem-loop transcription terminator ($\Delta G = -58.6$ kJ/mol) of *slpA* is located is reduced. Despite this reduced similarity, a stem-loop structure ($\Delta G = -49.4$ kJ/mol) can also be identified in the *slpB* sequence at the same position as in the *slpA* sequence. There is no detectable similarity between the two sequences just after this stem-loop structure. The codon in the *slpB* region which aligned with the start codon of the *slpA* gene appeared to be the start codon of an open reading frame (1,371 nt) named *slpB*, which presumably codes for an pre-S-protein (47.7 kDa), named S_B-protein. The deduced amino acid sequences of the *slpA* and *slpB* genes are aligned in Fig. 4.

The first attempt to sequence the mature S_A-protein at the N-terminal site had not been successful (5). The sequence of the mature S_A-protein, as predicted by the Von Heijne rules (39), was different from the amino acid sequences of the mature S-proteins of two *L. helveticus* strains, despite extensive similarity between the proteins in this region (Fig. 5). The molecular mass of the S_A-protein of *L. acidophilus* ATCC 4356 was determined by electrospray ionization mass spectroscopy, yielding a value of 43,639 ± 6 Da (see the legend to Fig. 4). This means that the prediction of the cleavage site of the pre-S_A-protein is not correct and that the actual cleavage site will be between amino acids 30 and 31, yielding a calculated molecular mass for the mature S_A-protein of 43,636 Da. The observed molecular mass is the same as the calculated molecular mass, which means that the N terminus of this protein cannot be modified and that no glycosylated amino acid residues are present, in contrast to what was suggested before (3). Determination of the N-terminal amino acid sequence of the S_A-protein, using another sequencer apparatus, did indeed yield the expected sequence: ATTINAS. The similarity between the amino acid sequences of the N-terminal two-thirds of the two mature S-proteins of *L. acidophilus* ATCC 4356 is 53% (identity, 40%). The sequences in the C-terminal parts (about one-third) of the proteins are identical, except for one residue. The amino acid compositions of the mature S_A- and S_B-proteins are very similar (data not shown). Only the percentage of serine is higher in the S_B-protein (10.8%) than in the S_A-protein (7.5%), while the percentage of threonine is higher in the S_A-protein (12.8%) than in the S_B-protein (9.6%). Other computer-predicted (MacProMass) physical properties, such as molecular mass (S_A-protein, 43,636 Da; S_B-protein, 44,884 Da), isoelectric point (S_A-protein, 10.4; S_B-protein, 10.3), and hydrophilicity pattern (data not shown), are also very similar. The codon usages of the two genes are almost identical (data not shown) and are typical for highly expressed *Lactobacillus* genes (29).

Expression of the *slp* genes. The gene for the S-protein is transcribed into a monocistronic mRNA (4a). To investigate which of the *slp* genes is actively transcribed, we performed a Northern blot analysis. Three different probes were used in this experiment: probe A, which is specific for the *slpA* gene and its mRNA; probe B, which is specific for the *slpB* gene and its mRNA; and probe A/B, which will recognize both the *slpA* and *slpB* genes and their mRNAs (Fig. 6a). A strong signal is found when an *slpA*-specific probe is used (Fig. 6c), proving that the *slpA* gene is indeed the structural gene for the production of the S-protein (S_A-protein) of *L. acidophilus* ATCC 4356. A very faint signal can be seen in the Northern blot when the probe specific for *slpB* mRNA is used. This signal can be explained in several ways (see Discussion).

Determination of transcription start point. A primer extension reaction was performed to determine the transcription start point of the *slpA* gene (Fig. 7a). The result of the primer extension reaction is combined with the nucleotide sequence analysis of the 5′ region of the *slpA* gene in Fig. 7b. A promoter structure with sequences and spacings similar to those of the consensus −35 and −10 hexamers found in other gram-positive bacteria (28) is found upstream of the transcription start point of the *slpA* gene. The alignment of *slpA* and *slpB* shows that the promoter of the *slpA* gene is located immediately upstream of the region where the similarity between the two *slp* genes begins. Computer analysis of the *slpB* region corresponding to the region of the promoter sequences of *slpA* did not show any similarity with known promoter structures.

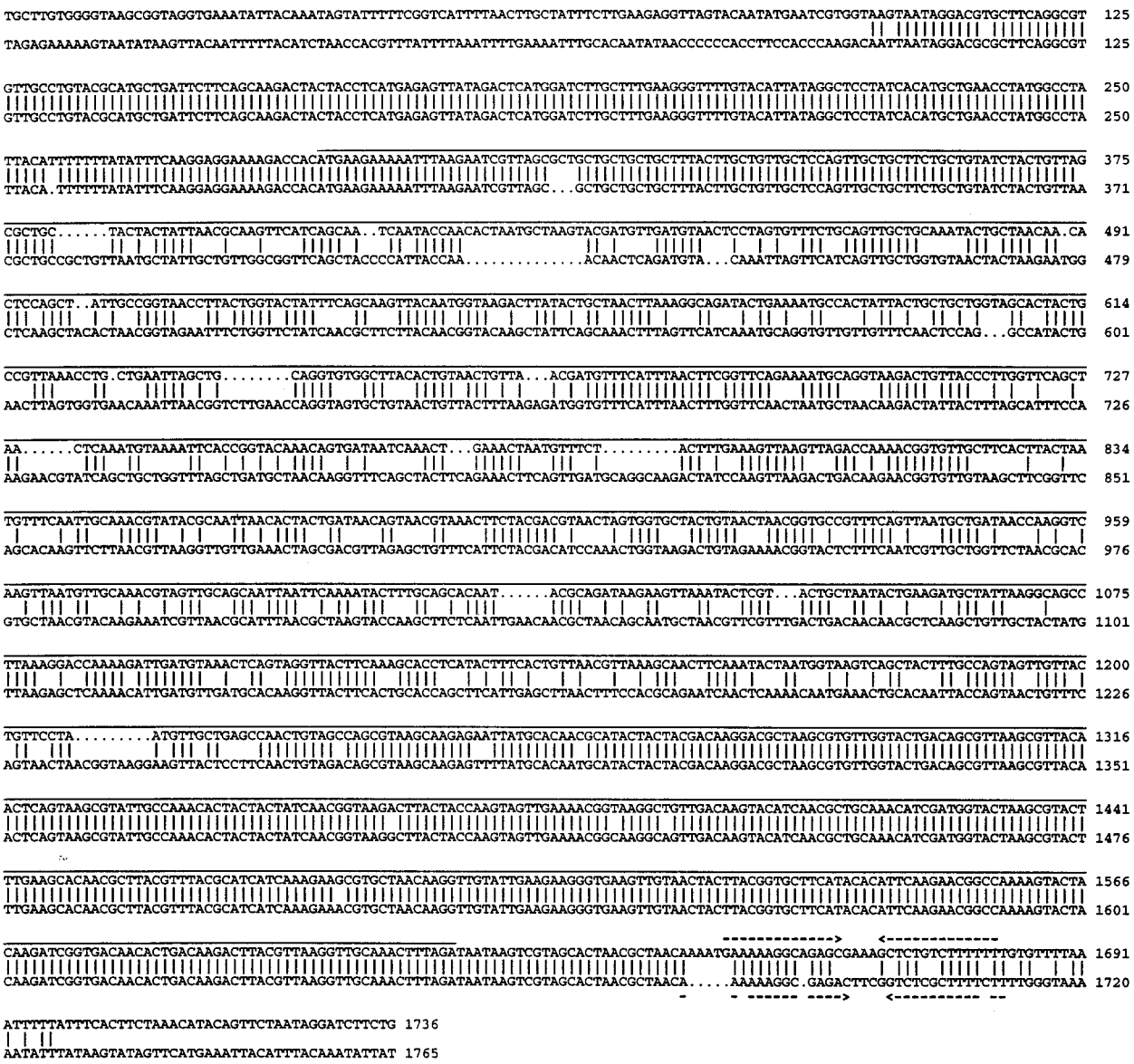

FIG. 3. Aligned (Bestfit; Italgenetics) nucleotide sequence of the *slpA* region (upper sequence) and the *slpB* region (lower sequence). The nucleotide sequence determination of the *slpA* region has been extended in the 5′ and 3′ regions to allow comparison of the sequences of the *slp* regions and to detect the promoter sequence (see Materials and Methods). Identical nucleotides are marked with a vertical line. Several gaps (represented by dots) were introduced to optimize the overall similarity. The horizontal line above the *slpA* region marks the open reading frame which codes for the S_A-protein. The potential stem-loop transcription terminator structures are indicated with arrows in both sequences.

DISCUSSION

This paper describes, for the first time, the occurrence of a silent gene which encodes a surface layer protein in a lactic acid bacterial strain. In a Southern blot analysis of chromosomal DNA of *L. acidophilus* ATCC 4356, probes derived from the *slpA* region yielded not only a signal representing the *slpA* region but also a signal representing another chromosomal locus, named the *slpB* region. Since probes derived from different parts of the *slpA* region hybridized with the *slpB* region with different intensities, it was clear that this region was not just a duplication of the *slpA* region. To be able to address the questions of the function of the *slpB* region and whether it contains an open reading frame which encodes a (S-)protein, we have cloned and sequenced this region.

The C-terminal parts (about one-third) of the two S-proteins are the same except for one amino acid residue (Fig. 4). The protein which is encoded by the *slpB* gene has all of the characteristics of an S-protein, such as almost no sulfur-containing amino acids, a high content of threonine and serine, and a high content of hydrophobic amino acids. The amino acid compositions and physical properties of the deduced mature S_A- and S_B-proteins are very similar. The corresponding genes are almost identical in two areas, which makes it very likely that the *slpB* gene, like the *slpA* gene, indeed encodes an S-protein. It is tempting to speculate that the conserved C-terminal part of the S_A- and S_B-proteins is important for proper functioning, e.g., for attachment of the S-protein to the underlying cell wall or for interaction between individual S-proteins to form the regular structure. The N-terminal and middle parts (about two-thirds) of the S-protein could in this scenario be involved

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MKKNLRIVSAAAAALLAVAPVAASAVSTVSAATTINASSSAINTNTNAKY  50
MKKNLRIVS.AAAALLAVAPVAASAVSTVNAAAVNAIAVGGSATPLPNNS  49

DVDVTPSVSAVAANTANNTPAIAGNLTGTISASYNGKTYTANLKADTENA 100
DVQISSSVAGVT..TKNGSSYTNGRISGSINASYNGTSYSANFSSSNAGV  97

TITAAGST..TAVKPAELAAGVAYTVTVND.VSFNFGSENAGKTVTLGSA 147
VVSTPGHTELSGEQINGLEPGSAVTVTLRDGVSFNFGSTNANKTITLAFP 147

N..SNVKFTGTNSDNQT.ETNV...STLKVKLDQNGVASLTNVSIANVYA 191
KNVSAAGLADANKVSATSETSVDAGKTIQVKTDKNGVVSFGSAQVLNVKV 197

INTTDNSNVNFYDVTSGATVTNGAVSVNADNQGQVNVANVVAAINSKYFA 241
VETSDVRAVSFYDIQTGKTVENGTLSIVAGSNARANVQEIVNAFNAKYQA 247

AQYADKKLNTR...TANTEDAIKAALKDQKIDVNSVGYFKAPHTFTVNVK 288
SQLNNANSNANVRLTDNNAQAVATMLRAQNIDVDAQGYFTAPASLSLTFH 297

ATSNTNGKSATLPVVVTVPN...VAEPTVASVSKRIMHNAYYYDKDAKRV 335
AESTQNNETAQLPVTVSVTNGKEVTPSTVDSVSKSFMHNAYYYDKDAKRV 347

GTDSVKRYNSVSVLPNTTTINGKTYYQVVENGKAVDKYINAANIDGTKRT 385
GTDSVKRYNSVSVLPNTTTINGKAYYQVVENGKAVDKYINAANIDGTKRT 397

LKHNAYVYASSKKRANKVVLKKGEVVTTYGASYTFKNGQKYYKIGDNTDK 435
LKHNAYVYASSKKRANKVVLKKGEVVTTYGASYTFKNGQKYYKIGDNTDK 447

TYVKVANFR 444
TYVKVANFR 456
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FIG. 4. Amino acid sequence alignment (Bestfit) of the pre-S_A- and pre-S_B-proteins. Vertical lines indicate identical amino acid residues. Dots between two amino acid residues in the different sequences indicate residues with similar properties. Dots within a sequence indicate a gap introduced to optimize the overall similarity. The horizontal line above the pre-S_A-protein sequence indicates the secretion leader. The molecular mass of the mature S_A-protein was determined by electrospray ionization mass spectroscopy. This molecular mass determination is based on 10 different *m/z* values, yielding a final value of 43,639 ± 6 Da.

in functions of the S-layer which might be different for the two S-layers, e.g., physical properties of the outside of the bacterium, antigenic variation, or properties related to interaction with intestinal cells.

The *slpA* gene was originally identified by using antibodies raised against purified S-protein. The isolation of the gene by using antibodies makes it very likely that the cloned gene is the gene which is expressed. A Northern blot experiment was performed to analyze the expression of both genes in exponentially growing bacteria. Probes specific for one *slp* gene and its corresponding mRNA (probe A and probe B) and a probe recognizing both genes and mRNAs (probe A/B) were simultaneously used in Northern and Southern blot analyses to detect the expression of the two *slp* genes. Probe A, which is specific for *slpA*, cross-reacts with a very low affinity with the *slpB* gene, as can be seen in the Southern blot with this probe (Fig. 6b). The same is true for probe B, as a faint signal can be seen at the position of the *slpA* gene in the Southern blot. The cross-reaction of probes A and B is probably due to the similarity of short sequences in the two *slp* genes in the regions of these probes. The Northern blot analyses (Fig. 6c) reveal that *slpA* is indeed the actively transcribed gene, as a strong signal was found when the *slpA*-specific probe was used and a very faint signal was found when the *slpB*-specific probe was used. We are not sure whether the faint signal in the Northern blot with probe B really represents mRNA derived from the *slpB* gene, as the probe specific for the *slpB* gene cross-reacts with a low affinity with the *slpA* gene in the Southern blot. Two explanations are possible if this faint signal is really due to *slpB* mRNA. (i) The *slpB* gene could be transcribed at a very low level compared with the *slpA* gene. An argument against this explanation is that the codon usage of the *slpB* gene is virtually the same as that of the *slpA* gene. The codon usages of both *slp* genes are representative of proteins which are expressed at a very high level (29), so it seems unlikely that the *slpB* gene, whose mRNA has the codon usage of a highly expressed protein, is transcribed at a very low level. (ii) The preparation of mRNA could be derived from a mixed culture: in the majority of bacteria, the S_A-protein is expressed at a high level, while a small minority express the S_B-protein at a high level.

The regions upstream of the start codons of the *slpA* and *slpB* genes are almost identical for 186 nt. From Northern blot analysis we conclude that the *slpA* gene is actively transcribed. These arguments make it very unlikely that a promoter sequence is present in this region of high similarity, as this would imply that the same promoter is present in front of the *slpB* gene, which would lead to the production of mRNA from this gene. Primer extension analysis revealed that mRNA for the S-layer protein has an untranslated upstream sequence of 191 nt, which implies that the promoter sequence is indeed located upstream of the high-similarity region. A promoter sequence strongly resembling promoter sequences in other lactobacilli

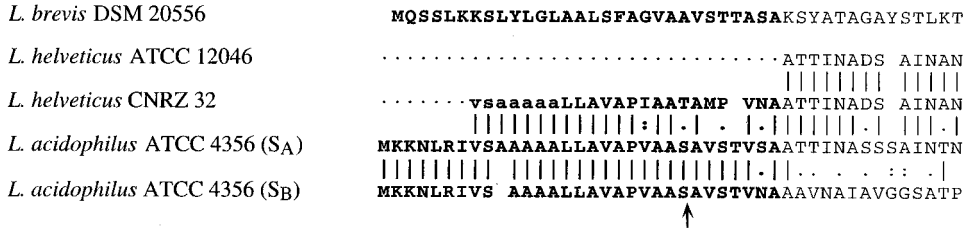

FIG. 5. N-terminal sequences of the (pre-)S-proteins of the *Lactobacillus* strains for which sequence data are available. The secretion leader sequences are given in boldface. Dots between two amino acid residues of different sequences indicate residues with similar properties. The sequences of the mature *L. brevis* DSM 20556 and *L. helveticus* ATCC 12046 S-proteins have been published before (19, 38). The nucleotide sequence of part of the S-protein gene of *L. helveticus* CNRZ 32 was determined after PCR amplification of a part of this gene, using oligonucleotides specific for the *slpA* gene of *L. acidophilus* ATCC 4365. One of the oligonucleotides used is complementary to the sequence encoding part of the secretion leader of the S_A-protein (shown in lowercase letters in the *L. helveticus* CNRZ 32 sequence). The cleavage sites of the secretion leaders of the S_B-protein and the S-protein of *L. helveticus* CNRZ 32 are based upon sequence homology, as the actual cleavage sites are unknown. The cleavage site of the pre-S_A-protein predicted with the Von Heijne rules (indicated with an arrow) differs from the actual cleavage site of the pre-S_A-protein as determined by N-terminal sequence analysis and deduced from the results of the mass spectroscopy analysis.

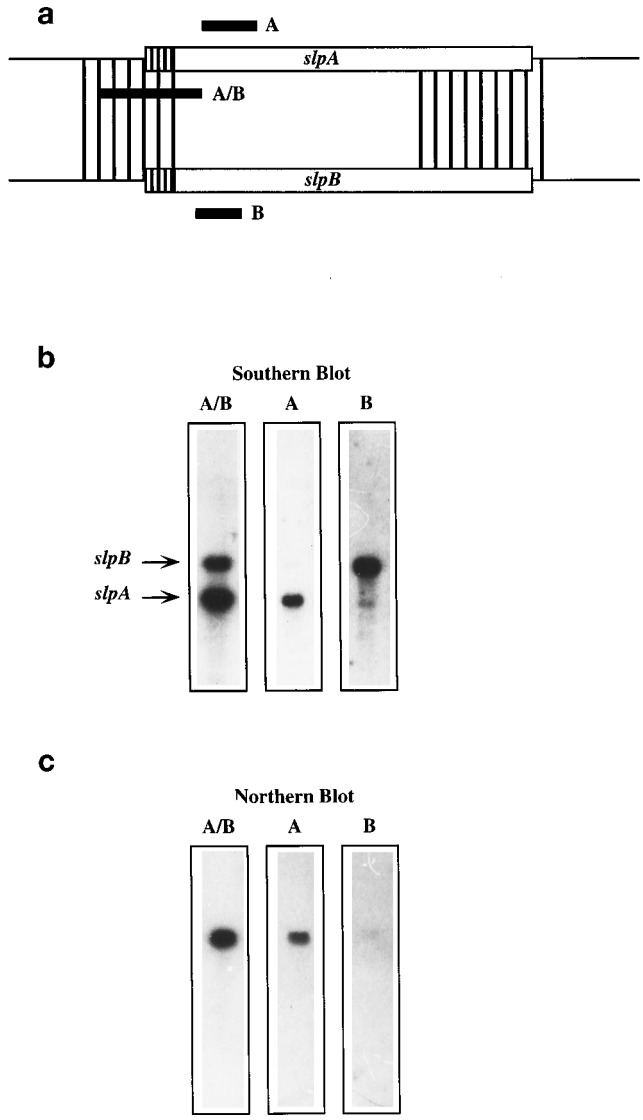

FIG. 6. Detection of the actively transcribed *slp* gene in a Northern blot. (a) Schematic drawing of the probes used. Vertical bars indicated the regions where the two genes are almost identical. (b) Results of Southern blot analysis of *Eco*RI-digested chromosomal DNA with the probes indicated in panel a. (c) Results of a Northern blot analysis of total, nondenatured RNA with the probes indicated in panel a. The Southern and Northern blot analyses were performed simultaneously with the same probes.

(28) is found upstream of the transcription start point in the *slpA* region (Fig. 7b). Four mismatches are present in the 5′ region of high similarity (280 nt). The first two mismatches are found very close to the 5′ end of this region. The third mismatch is found in a nucleotide repeat: there are seven adjacent T's in the *slpA* region and six adjacent T's in the *slpB* region. Nucleotide repeats are known for a greater occurrence of mistakes during DNA replication, leading to a reduced or increased number of nucleotides in such a repeat. It is possible that this difference is the result of slippage during replication of either the *slpA* or *slpB* region in *E. coli* or of slippage replication in *L. acidophilus*. The difference in the length of the T tract is found in the region which corresponds to the 5′ untranslated region of the mRNA. The fourth difference in this high-similarity area is found in the beginning of the coding regions of the two genes. A repeat of five adjacent alanine residues, all encoded by the codon GCT, is present in the alpha-helix part of the secretion leader of the S_A-protein. In the sequence of the *slpB* gene, one of these GCT codons is missing, leading to a secretion leader alpha-helix which is one residue shorter. Only one of the eight differences between the *slpA* and *slpB* genes in the 3′ region of high similarity leads to an altered amino acid residue in the S_B-protein compared with the S_A-protein (S_A-protein Thr-359→S_B-protein Ala-372). Similarity between the two *slp* sequences is absent beyond the last functional genetic structure, i.e., the transcription terminator. The homology between the two stem-loop transcription terminator structures is already reduced, possibly as a result of the absence of a nucleotide-specific selection pressure, as found in the amino acid-encoding regions.

The cleavage site of the pre-S_B-protein is most likely at the same position as that in the pre-S_A-protein, leading to a mature S_B-protein with an N-terminal amino acid sequence different from those of the S_A-protein and the S-protein of *L. helveticus* (Fig. 5). The secretion of the pre-S_B-protein is not likely to be affected by shortening of the alpha-helix sequence of the secretion leader from 13 to 12 residues compared with the secretion leader of the pre-S_A-protein, as an alpha-helix sequence of 8 to 10 residues in the secretion leader is enough to direct secretion in gram-positive bacteria (35).

Recently, a domain (the S-layer homology domain) has been identified in some prokaryotic proteins that are secreted and noncovalently linked to the cell wall, such as pullulanase and endoxylanase (20). Several, but not all, S-proteins of gram-positive bacteria contain a repeat of this S-layer homology domain in either the N- or C-terminal part of the protein. The S-layer homology domain was postulated to be the S-protein determinant for interaction with the underlying peptidoglycan layer. A structure showing similarity to the S-layer homology domain cannot be found in either the S_A-protein, the deduced S_B-protein, or the S-protein of *L. brevis* ATCC 12046.

It has been shown that *B. stearothermophilus* NRS 2004/3a irreversibly changes the expressed S-protein after the oxygen pressure is increased during pH-controlled growth in a fermentor. This change of the S-protein is claimed to be due to chromosomal recombination (33, 34). Besides *L. acidophilus*, two other bacterial species have been reported to possess silent S-protein genes. In addition to the structural gene for the 125-kDa S-protein of *B. sphaericus* WHO 2362, a silent gene encoding a 80-kDa S-protein which has extensive similarity with the structural S-protein gene was discovered (6). Chromosomal recombination between at least two, but presumably more, S-protein genes has been reported for the pathogenic bacterium *Campylobacter fetus*. This chromosomal recombination leads to the expression of different S-proteins, resulting in an S-layer with altered symmetry, which helps this bacterium to circumvent the immune response of the infected host, a phenomenon known as antigenic variation (4, 37, 40). The S-protein genes of *C. fetus* are all located on a 93-kb chromosomal fragment, which is less than 8% of the total length of the chromosome (10). The same organization of the *slp* genes seems to occur in *L. acidophilus*, as the two *slp* regions are located on a fragment which constitutes 7% of the *L. acidophilus* chromosome. Preliminary data concerning PCR analysis of the *slp* regions of *L. acidophilus* reveal that the 5′ similarity regions of the two *slp* regions are, just as found for *C. fetus* strains, involved in in vivo chromosomal recombination, placing the *slpB* gene under control of the promoter of the *slpA* gene.

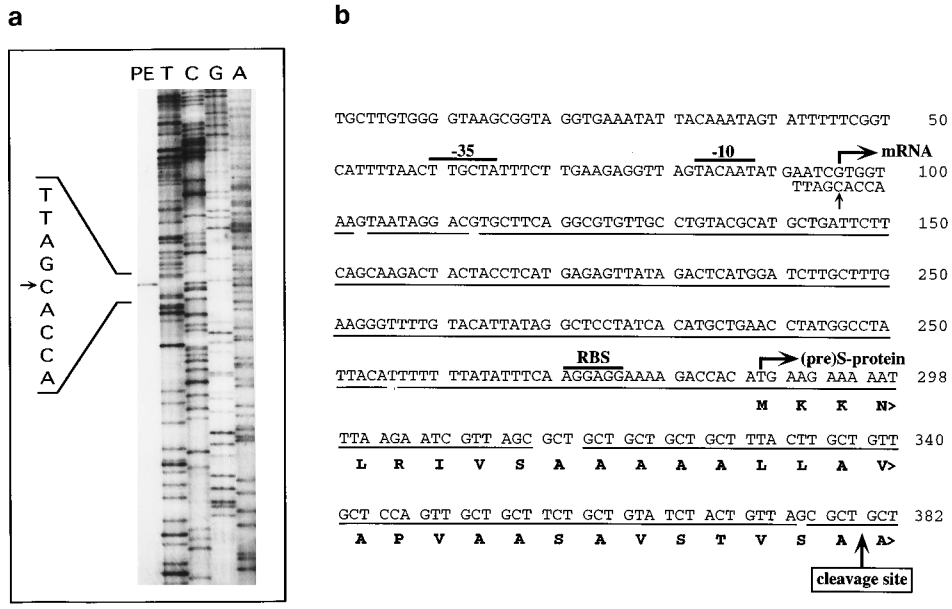

FIG. 7. Determination of the transcription start point of the *slpA* mRNA. (a) Autoradiogram of the result of the primer extension reaction next to the four sequencing reactions. PE, primer extension reaction; T, thymine; C, cytosine, G, guanine; A, adenine. (b) Nucleotide sequence of the upstream region of the *slpA* gene. The start of the mRNA for the S_A-protein on the basis of the primer extension reaction is indicated (double-strand sequence). A putative promoter sequence (−35 and −10 sequences) is present upstream of the transcription start point. The nucleotides which are identical in the *slpA* and *slpB* regions are underlined. RBS, potential ribosome binding site. The deduced amino acids of the N-terminal part of the pre-S_A-protein are given in boldface below the nucleotide sequence.

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