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The *Lactobacillus acidophilus* S-Layer Protein Gene Expression Site Comprises Two Consensus Promoter Sequences, One of Which Directs Transcription of Stable mRNA

HEIN J. BOOT,1* CARIN P. A. M. KOLEN,1 FOTINI J. ANDREADAKI,1 ROB J. LEER,2 AND PETER H. POUWELS1,2

BioCentrum Amsterdam, University of Amsterdam, 1018 TV, Amsterdam, and TNO Nutrition and Food Research Institute, 2280 HV Rijswijk,2 The Netherlands

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S-proteins are proteins which form a regular structure (S-layer) on the outside of the cell walls of many bacteria. Two S-protein-encoding genes are located in opposite directions on a 6.0-kb segment of the chromosome of *Lactobacillus acidophilus* ATCC 4356 bacteria. Inversion of this chromosomal segment occurs through recombination between two regions with identical sequences, thereby interchanging the expressed and the silent genes. In this study, we show that the region involved in recombination also has a function in efficient S-protein production. Two promoter sequences are present in the S-protein gene expression site, although only the most downstream promoter (P-1) is used to direct mRNA synthesis. S-protein mRNA directed by this promoter has a half-life of 15 min. Its untranslated leader can form a stable secondary structure in which the 5' end is base paired, whereas the ribosome-binding site is exposed. Truncation of this leader sequence results in a reduction in protein production, as shown by reporter gene analysis of *Lactobacillus casei*. The results obtained indicate that the untranslated leader sequence of S-protein mRNA is involved in efficient S-protein production.

The cell walls of many bacterial species are covered by a crystalline structure known as the S-layer (for reviews, see references 3 and 26). The monomolecular S-layer is composed of a single species of protein, the S-protein, which is capable of crystallizing into a regular array. S-layers have been found on eu- and archaebacteria living in all kinds of environmental conditions and are considered membrane-like structures on the outside of the cell. Several specific functions have been reported for S-layers of different species; however, no general function is known. For some archaebacteria, it was found that the S-layer is involved in maintenance of the cell shape (33). The S-layer plays an important role in the overall virulence of several pathogenic bacteria (16). An S-layer can also act as a shield to cover phage receptors present in the underlying cell wall (16, 29) or prevent invasion by *Bdellovibrio bacteriovorus* (19). Furthermore, S-layers can serve as an attachment structure for extracellular proteins (25). For both pathogenic (11) and nonpathogenic (42) bacteria colonizing the gastrointestinal tracts of mammals, it was found that the S-layer interacts with components of the epithelial cell layer.

*Lactobacilli* are found widely distributed throughout nature and have been used for a long time in food and feed production. Several reports have appeared ascribing probiotic properties to certain *Lactobacillus* species which are naturally found in the gastrointestinal tracts of humans and animals (e.g., *Lactobacillus acidophilus* and *Lactobacillus gasseri*), or species which are used in the production of standard yogurt (i.e., *Lactobacillus delbrueckii* subsp. *bulgaricus*) or health promoting yogurt variants (e.g., *Lactobacillus casei*). Effects reported to be induced by these lactobacilli are lowering of levels of cholesterol in serum (12), binding of mutagenic compounds (28), and stimulation of immunoglobulin production (20). In addition, protection of the host against invasion by pathogenic bacteria is reported to be partially due to the presence of lactobacilli.

The type strain of the *L. acidophilus* species was originally isolated from the human pharynx and is considered to be probiotic (18). The *slpA* gene, encoding the S<sub>A</sub>-protein which forms the wild-type S-layer of this species, has been cloned and sequenced previously (7). Near this expressed gene, we have identified a silent gene (*slpB*) encoding the S<sub>B</sub>-protein (5). The *slpA* gene is interchanged with the *slpB* gene through inversion of a chromosomal fragment in a fraction of an *L. acidophilus* culture (0.3% of the cells grown under laboratory conditions). Such a recombination event is expected to lead to the production of a partially different S-protein and S-layer (S-layer variation) (6). It is estimated that the S-protein represents 10 to 15% of the total cellular protein of the bacterial cell. This implies that the single S-protein gene must be transcribed at a high level and/or give rise to a stable mRNA. Why the S-protein genes of some species are preceded by a multiple promoter structure, whereas the S-protein genes of other species are preceded by a single promoter is presently unclear. To gain further insight into the regulation of the expression of the *L. acidophilus* S-protein gene(s), we have analyzed the expression directed by the S-promoter. We found that although two putative promoter sequences are present, only one is used under all of the tested growth conditions. The long untranslated leader sequence present in the mRNA appears to be involved in efficient production of the S-protein.

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**MATERIALS AND METHODS**

*Strains and plasmids.* *L. acidophilus* ATCC 4356 and *L. casei* ATCC 393, which were obtained from the American Type Culture Collection, were cultivated anaerobically in MRS (Difco) broth at 37°C. Plasmids pBK-1 and pBK-2
are multisopy plasmids containing the entire slpA gene and flanking sequences (7). Escherichia coli DH5α was used as a host for recombinant plasmids. Isolation of chromosomal DNA and total RNA. Chromosomal DNA and total mRNA of L. acidophilus ATCC 4356 were isolated essentially as described before (5).

Northern (RNA) and Southern (DNA) blot analysis. Total RNA was separated in a 1.0× or a 1.0× TBE (10× TBE is 10× g of Tris, 55 g of boric acid, and 10 g of Na2EDTA) agarose gel without ethidium bromide. The gel was soaked twice for 20 min in 0.2 M sodium acetate (pH 5.0) and transferred to a Hybond membrane (Amersham) by blotting with the same solution. Southern analysis was performed essentially as described by Souther et al. (40). Restriction enzyme-digested chromosomal DNA was separated on a 1% agarose gel and transferred to a Hybond filter. RNA and DNA were cross-linked to the filter byshortwave UVlight for 5 min. Prehybridization and hybridization filters were performed in 10 ml of 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-5× Denhardt solution (35)-0.1% sodium dodecyl sulfate (SDS)-1.0 mg of sonicated salmon sperm DNA at 65°C. The filters were washed twice with 2× SSC-0.1% SDS and three times with 0.5× SSC-0.1% SDS at 65°C. Probe SI was a restriction fragment of plasmid pBK-2 and was located entirely within the coding sequence of the slpA gene (nt +353 to +1135, relative to the start of the coding sequence [7]). Probes SP-1 and SP-2 were generated by PCR using the specific oligonucleotides as indicated in Fig. 1. Probe SI was a 5′-32P-labeled part of the DNA encoding the S-protein which was used for Northern blot analysis. The hybridization and wash conditions were the same for both Northern and Southern blots.

RNA from cells in different growth phases. Premature MRS broth (1 liter) was inoculated with an overnight preculture (10 ml) of L. acidophilus ATCC 4356, Samples of this culture were taken at three different time points (RNA-1, RNA-2, and RNA-3) by centrifugation (5,000 × g for 10 min at 4°C), washed with 10 ml of 0.5 M lithium chloride (15 min at 0°C). After centrifugation (10,000 × g for 30 min at 4°C), the supernatant was used as a template for the PCR-based method and the results were used as a control. The RNA samples were electrophoresed on a 0.1% agarose gel for 1.5 h at 100 V. Southern blotting was performed on nylon membranes (Hybond N membrane) using the HybWriter system. The membranes were hybridized with 32P-labeled probe SI. Autoradiography was performed with an intensifying screen at −70°C. laboratories. After autoradiography, the membranes were stripped and hybridized with another probe.

RESULTS

Mapping of the transcription start. Two putative promoter sequences (Fig. 1 [P-1 and P-2]) were present in the 500-bp region upstream of the slpA ORF of wild-type L. acidophilus ATCC 4356 cells. Total RNA of an exponentially growing L. acidophilus ATCC 4356 culture was extracted. This RNA was precipitated in an agarose gel with ethanol and the conditions which allowed (1.0× TBE) or prevent secondary-structure formation (0.1× TBE) (14) and was used for Northern blot analysis. mRNA encoding the S-protein was detected by using a radioactively labelled part of the slpA gene (probe SI; see Materials and Methods). A sharp band was visible on the autoradiogram of the Northern blot when secondary-structure formation was not prevented at a position below that of the 16S rRNA (Fig. 2A). When conditions were used which resulted in the loss of secondary structure of the RNA, a broad band was visible at the position corresponding to the position of 16S rRNA (Fig. 2A). These results indicate that the size of the slpA mRNA is approximately the same as that of 16S rRNA (1,500 nt). A transcription terminator-like hairpin structure is found behind the slpA gene, which ends 63 nt downstream from the translational stop codons (7). Since the poly(A) tract generates 19 additional acid residues, this implies that the stop gene is monocistronic and that the start point of the mRNA is located approximately 100 nt upstream from the translation start codon. The exact position of the transcription start point was identified by a primer extension analysis with the same RNA preparation. A single

To determine the time which corresponds to 50% of the signal intensity (half-life).

Promoter-reporter plasmids. The erythromycin gene of pE194 (15) was cloned between the PstI and BgIII sites of promoter screening vector pBR329 (39), yielding plasmid pBK-50. A Sau3AI fragment of 513 nt containing the promoter region and the first 22 coding nt of the lidh gene of L. casei ATCC 393 (17) was ligated in the unique BamHI site of pBK-50, and the resulting plasmids were used to transform E. coli DH5α. Plasmid DNA of ampicillin-resistant colonies was prepared with endonuclease digestion. A plasmid harboring this 513-bp fragment in the same orientation toward the chloramphenicol acetyl transferase (CAT) reporter gene as that originally toward the wild-type lidh gene was selected (pBK-60). The promoter region of the slpA gene of L. acidophilus ATCC 4356 was isolated with a PstI-SmaI fragment from the above plasmid at approximately 1.0 kb before the start of the slpA open reading frame (ORF) (unpublished data) and an artificially introduced SmaI site 1 nt before the start of the slpA ORF. This fragment was ligated between the PstI and SmaI restriction sites before the CAT reporter gene of pBK-50. The resulting plasmids were used to transform E. coli DH5α, and plasmid DNAs of ampicillin-resistant colonies were analyzed with endonucleases. A plasmid with the expected restriction pattern of the correct orientation was selected (pBK-61). Part of the untranslated leader sequence (~147 to ~1, relative to the transcription start point of the S-proromer region was deleted by digesting pBK-61 with SphiI and KpnI.

The single-stranded ends were blunted with T4 polymerase, and the resulting fragment was self-ligated and transformed to E. coli DE35a, yielding plasmid pBK-62. Plasmid DNA was used to transform L. casei ATCC 393 by electroporation, and recombinant bacteria were selected by plating the transformation mixture on MRS plates containing 5 µg of erythromycin per ml. Determination of CAT activity. L. casei ATCC 393 bacteria which harbor pBK-62 derivatives of this plasmid were grown on solid MRS containing overnight anarmonically in MRS broth at 37°C in the presence of 5 µg of erythromycin per ml. Fresh MRS was inoculated (1:100) with this preculture, and the mixture was incubated until an optical density at 600 nm of between 0.5 and 1.0 (OD600 40 min). The reaction was stopped by centrifugation (5,000 × g, 5 min). The cell pellet was washed with 1.0 ml of 20 mM HEPES (pH 7.4) and resuspended in 50 µl of 20 mM N-2-hydroxypropyazino-N′-2-ethanesulfonic acid (HEPES; pH 7.4) and 0.5 % of glass beads (0.20 to 0.45 mm) was added. This suspension was vortexed for 1 min and centrifuged (5,000 × g, 1 min). The supernatant was used as a total-cell extract for determination of the CAT activities. The CAT activities of three independent cultures for each plasmid were determined twice spectrophotometrically with the 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) assay described by Shaw (38), with a Cobas Bio automatic analyzer (Hoffmann-La Roche). Specific CAT activities were calculated after determination of protein contents of the total-cell extracts by the biocinonic acid (Sigma) method (39). A Southern blot experiment with total DNA of the L. casei ATCC 393 recombinant strains indicated that the copy numbers of all pBK50-based plasmids are approximately the same (data not shown).
signal at position -191 nt relative to the start of the coding sequence (Fig. 2B) and 8 nt downstream of promoter P-1 was detected (Fig. 1). No extension signal is present at the position which corresponds to slpA mRNA directed by the putative promoter P-2, indicating that this promoter is not active during exponential growth under the applied growth conditions.

Promoter activity during different growth stages. Only promoter P-1 was found to be used for transcription of S-protein mRNA in all RNA samples derived from bacteria in different phases of growth (Fig. 3). The first RNA sample (RNA-1) was obtained from cells in the mid-exponential growth phase, the second sample (RNA-2) was derived from cells at the end of the exponential growth phase, and the third sample (RNA-3) was derived from cells at the beginning of the stationary growth phase.
phase (Fig. 3A). To determine whether specific induction of S-protein mRNA will occur in *L. acidophilus* bacteria deprived of the S-layer (e.g., autoregulation), we isolated RNA from an exponentially growing culture which was treated with lithium chloride and cultivated for an additional 30 min in fresh MRS broth (RNA-L; see Materials and Methods). The lithium chloride treatment specifically extracts the S-protein from the cell wall without being deleterious (21). RNAs isolated from these culture samples were used for Northern blotting, and specific mRNAs were detected with different probes. Three probes derived from different parts of the *slpA* region were used to detect mRNA directed by the S-promoter. Probe S I is entirely located within the coding region of the *slpA* gene, and probe S P-1 overlaps the transcription start of mRNA derived from both promoters P-1 and P-2, whereas probe S P-2 overlaps only the transcription start of mRNA derived from promoter P-2 (Fig. 1 and legend). Comparison of the three probes for the *slpA* mRNA shows that no hybridization signal is found when the S P-2 probe is used, while probes S I and S P-1 show a hybridization signal for all RNA samples. Probes S P-1 and S P-2 are equally labelled, which can be concluded from the intensity of the signals in the Southern blot control experiment (Fig. 3C).

Furthermore, we used a probe directed against mRNA for the EF-Tu protein as a control for mRNA detection in the different samples. The amount of mRNA for the EF-Tu protein is, at least for *E. coli*, known to vary in direct proportion to the growth rate (43). The hybridization signals obtained for *L. acidophilus* mRNA by using an EF-Tu probe are consistent with this idea (Fig. 3B). Primer extension analysis confirmed that only promoter P-1 is used to direct the synthesis of *slpA* mRNA. Only signals representing *slpA* mRNA directed by promoter P-1 were found when RNAs from the different stages of growth were used (data not shown).

**Stability of S-protein mRNA.** Computer analysis shows that the untranslated leader sequence of the mRNA which is directed by promoter P-1 can fold into a large stem-loop structure (Fig. 4) with an energy of −191 kJ/mol (Mfold; Genetics Computer Group). A stably folded 5’ end can protect mRNA from 5’→3’ degradation, thereby increasing the half-life. The half-life of the mRNA for the S X-protein of *L. acidophilus* ATCC 4356 was determined by adding rifampin to an exponentially growing culture, thereby blocking further RNA synthesis. Total RNA was isolated at 5-min time intervals from samples of this culture and analyzed in a Northern blot (Fig. 5A) by using a probe which comprises the entire coding region of the *slpA* gene (probe S II; see Materials and Methods). The PhosphorImager data of this experiment are presented in Fig. 5B. From these data, it can be deduced that mRNA of the S X-protein has a half-life of 15 min.

**S-promoter activity in *L. casei* ATCC 393.** The intact untranslated leader of the S-protein mRNA gives rise to a reporter protein level higher than that of a truncated leader sequence (Fig. 6). The S-promoter and the untranslated leader sequences were cloned before a CAT reporter gene of a broad-host-range plasmid (see Materials and Methods), yielding pBK-61. To determine whether the untranslated leader sequence of the transcript directed by the S-promoter influences the reporter protein level, we deleted more than half of the DNA sequence (147 nt [Fig. 4; see Materials and Methods]) encoding this region to construct the S AUL-promoter (pBK-62). This truncated untranslated leader sequence is no longer able to fold into a stable stem-loop structure. Furthermore, we used the reporter plasmid without any promoter (pBK-50) and with the promoter of the lactate dehydrogenase gene (LDH-promoter, pBK-60) of *L. casei* ATCC 393 (17). The reporter protein levels of the above-mentioned plasmids were analyzed.
in *L. casei*, a species which is related to *L. acidophilus* but which possesses no S-protein-encoding genes and is therefore not expected to have trans-acting regulatory factors specific for the S-promoter. Total-cell extracts of exponentially growing cultures harboring these plasmids were analyzed for CAT activity. The CAT activity of *L. casei* cells transformed with the S*α*-promoter plasmid appeared to be approximately half of the CAT activity of *L. casei* cells containing the plasmid with the complete untranslated region behind the S-promoter (Fig. 6), which itself was threefold higher than that of bacteria transformed with a plasmid containing the LDH-promoter.

**DISCUSSION**

Electron microscopic photographs of bacteria which possess an S-layer show that the complete cell surface is covered with an S-layer during the entire growth cycle. In addition, the presence of free S-protein in the growth medium, besides an intact S-layer, has been reported for several bacteria (1, 4, 31). Information about growth-dependent regulation of S-protein gene expression at transcriptional or translational levels is lacking for most of the known S-proteins. To gain further insight into factors influencing S-protein production, we analyzed the production of the S-protein of *L. acidophilus* ATCC 4356.

Multiple promoter structures have been found before several S-protein genes, including the S-protein of *Lactobacillus brevis* (44). For *Bacillus brevis*, it was shown that at least one of the five identified promoters before the S-protein-encoding operon is active only during the exponential growth phase (1), whereas another promoter is active during all growth phases. The latter promoter is repressed in the presence of divalent cations (2). Sequence analysis of the promoter region of the *L. acidophilus* ATCC 4356 *slp* expression site revealed two potential promoters, P-1 (−228 to −198) and P-2 (−335 to −303) (Fig. 1). These two potential promoters have the same −10 and −35 sequences, showing extensive sequence identity with consensus −10 and −35 promoter sequences of lactobacilli (24, 32). Both the P-1 and P-2 −10 and −35 promoter sequences are preceded by an A+T-rich region (20 bp). This region is known as the UP element and was found to stimulate transcription through interaction with the α subunit of the RNA polymerase in *E. coli* and *Bacillus subtilis* (34). The only difference between the P-1 and the P-2 promoters is the spacing of the −10 and −35 sequences. The −10 and −35 sequences of promoter P-1 have the optimal spacing of 17 nt, whereas the spacing of the P-2 −10 and −35 sequences have the suboptimal spacing of 20 nt (41). Although it is suboptimal, at least one active *L. delbrueckii* promoter is reported to possess a spacing of 20 nt (24). Transcription directed by a multiple promoter structure might contribute to an amount of mRNA higher than that of transcription directed by a single promoter. Another reason for the observed multiple promoter structure might be that the two promoters are active under different conditions. Both primer extension (Fig. 2B) and Northern blot (Fig. 3) analyses showed that only the most downstream promoter (P-1) is active during all growth conditions analyzed. Even in RNA of *L. acidophilus* cells which had been cultivated for 30 min in fresh medium after the S-layer had been extracted by a nonlethal procedure, no transcript from the more distal promoter (P-2) was detected (Fig. 3).

The observation that only one of two promoter sequences of the *L. acidophilus* S-protein gene is active is in contrast to the situation described for *L. brevis*, in which the two identified promoters before the S-protein gene are about equally active during exponential growth (44).

Regulation of promoters is often found to take place by binding of a trans-acting factor (DNA-binding protein) to a hairpin structure which regulates the expression positively (activator) or negatively (repressor). The sequence capable of hairpin formation which is present before promoter P-2 of the

![Diagram](Image)

**FIG. 6.** Reporter protein levels resulting from different promoter constructs in extracts of *L. casei* were analyzed. A broad-host-range plasmid containing a CAT reporter gene was used for expression analysis. The numbers at the bottom of the schematic drawings of the promoter regions represent nucleotides relative to the start of the different ORFs. *L. casei* was transformed with these plasmids. Purified transformants were cultivated in MRS broth, and cell extracts of exponentially growing cultures were analyzed for CAT activity. Values in parentheses are standard deviations of the mean values of CAT activities (n = 6).
slp expression site (Fig. 1) could be part of such a regulation mechanism. Regulation involving a transacting factor (AbcA, a leucine zipper-containing protein) and a potential inverted DNA sequence in the vicinity of one of the promoters of an S-protein gene has recently been described for *Aeromonas salmonicida* (27). It is not yet clear whether the *L. acidophilus* ATCC 4356 promoter P-2 is detectably activated or derepressed under any specific growth condition or whether this potential promoter structure is never used to direct transcription.

Efficient production of a protein encoded by a single gene is often found to be directed by an mRNA which is considerably more stable than average mRNAs of the same species. The half-lives of mRNAs encoding S-proteins from *Caulobacter crescentus* (15) and *A. salmonicida* (10) have been determined and were found to be 10 to 15 min and 22 min, respectively. We leader sequence of mRNAs is known to protect mRNA against DNA sequence in the vicinity of one of the promoters of an leucine zipper-containing protein) and a potential inverted mechanism. Regulation involving a transacting factor (AbcA, a slp

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**A. salmonicida**

half-lives of mRNAs encoding S-proteins from *E. coli* are more stable than average mRNAs of the same species. The pressed under any specific growth condition or whether this secondary structure, the first nucleotides of the 5 end of mRNA of, will fold into an extensive hairpin-like structure. (Fig. 4). According to this computer prediction of the secondary structure, the first nucleotides of the 5' end are involved in stem formation. Base pairing of the 5' end of mRNA has, for *E. coli*, been shown to be a prerequisite for preventing fast degradation of the mRNA by RNase E (8). The ribosome-binding site (RBS) is in the predicted secondary structure exposed in a loop. Exposure of the RBS is expected for a highly translated mRNA, since it has been shown that only an exposed RBS can hybridize with the complementary sequences of the 16S rRNA subunit of the ribosome (37). On the basis of computer analysis, the untranslating leader sequence of mRNA directed by promoter P-2 will also fold into a stable structure. However, in this prediction, the 5' end of the hypothetical mRNA is not part of a stem structure, and the nucleotide sequence which constitutes the RBS forms an internal duplex (data not shown).

The *L. acidophilus* S-promoter can be used in *L. casei* ATCC 393 for heterologous protein production. Bacteria belonging to the *L. casei* species do not possess an S-layer (23) and, in contrast to *L. acidophilus*, can efficiently be transformed with plasmids. Results based on enzymatic detection of a reporter protein (CAT) showed that the S-promoter is highly active in this host, since an activity approximately three times higher than that of the most active homologous *L. casei* promoter known to us was found with this promoter. This efficient production is in part due to the presence of the untranslating leader sequence of the S-protein mRNA, since deletion of part of this sequence diminished the reporter protein activity by nearly twofold (Fig. 6).

It appears that the region encoding the long untranslated leader sequences of the S-protein mRNA has a dual function. In earlier studies, we have shown that the DNA encoding the untranslating leader mRNA is involved in chromosomal recombination (6). A 6.0-kb chromosomal segment (*slp* segment), containing two S-protein-encoding genes in opposite orientations, is inverted in a fraction of the bacterial population. This inversion occurs between two regions with identical sequences (280 nt), which comprise the region encoding the untranslated leader sequence (180 nt) of both S-protein encoding genes. In this study, we have shown that this region is also efficient for reproduction of the S-protein, since it generates a long untranslated leader sequence in S-protein mRNA which can fold into a stable secondary structure.