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In Vivo Expression of the *Lactobacillus brevis* S-Layer Gene

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Lactobacillus brevis **possesses a surface layer protein (SlpA) with tightly regulated synthesis. The *slpA* gene is expressed by two adjacent promoters, P1 and P2. The level of P2-derived transcripts was approximately 10 times higher than that of P1-derived transcripts throughout the entire growth of *L. brevis*. The half-lives of *slpA* transcripts were shown to be exceptionally long (14 min).**

Surface layer proteins, forming regular crystalline arrays on cell surfaces, have been identified from over 300 different species, including both gram-positive and gram-negative eubacteria and archaebacteria (9). S-layers are composed of subunits of a single protein or glycoprotein with a size range of 40 to 200 kDa, and they commonly contain a high proportion of acidic, hydrophobic, and hydroxy amino acids. S-layers may function as protective barriers and molecular sieves, promote cell adhesion and surface recognition, and maintain cell shape and envelope rigidity. Although the morphological properties of S-layers have been extensively studied for a wide range of microorganisms, relatively little is known about the regulatory mechanisms involved in their biosynthesis and transport. The requirement of a very large number (5×10^5) of S-layer subunits per cell implies highly efficient expression and secretion signals, which may be utilized in various biotechnological applications.

Among lactic acid bacteria, the S-layer seems to be a typical surface structure in several *Lactobacillus* species, e.g., in *L. acidophilus*, *L. helveticus*, *L. casei*, *L. brevis*, *L. buchneri*, *L. fermentum*, *L. bulgaricus* (9), and *L. plantarum* (10). Since several *Lactobacillus* species with an S-layer structure play essential roles in many fermentation processes of the food industry, silage fermentations, and human and animal probiotics, elucidation of the characteristics of lactobacillar S-layers has become increasingly important. We have earlier isolated and purified an S-layer protein from *L. brevis* ATCC 8727 and characterized its gene and mRNA (12). Two genes, *slpA* and *slpB*, encoding a functional S-layer protein and representing a silent gene, respectively, from *L. acidophilus* ATCC 4356 have also been characterized (2, 3). Furthermore, the DNA sequences of the S-layer genes from two *L. helveticus* strains have been deposited in the EMBL Nucleotide Sequence Data Library (accession numbers X91199 and X92752). Comparison of these four lactobacillar S-layers reveals that even though substantial sequence similarity is not shown, they all have a high content of basic amino acid residues. This seems to be a very distinct characteristic of the *Lactobacillus* S-layers, since the S-layer proteins analyzed from other bacteria commonly contain a high proportion of acidic amino acids.

Genetic characterization of the *slpA* gene from *L. brevis* ATCC 8727 revealed two adjacent promoters (P1 and P2) with the conserved hexanucleotide −10 and −35 regions typical to prokaryotic promoters, the consensus ribosome binding site, the ATG start codon, and a signal sequence with the capacity to encode a leader peptide of 30 amino acids. The structural *slpA* gene has the capacity to encode a 45-kDa mature S-layer protein followed by a strong transcription terminator sequence downstream from two translation stop codons (12).

In this study we have further characterized in vivo expression of the *L. brevis* S-layer protein, measured the half-life of *slpA* transcripts, and determined the usage of the two *slpA* promoters as a function of growth.

Expression of the *slpA* gene. *L. brevis* cells were grown in MRS broth (Difco) at 37°C without shaking. The accumulation of the S-layer protein, level of *slpA* mRNA, and pH as a function of growth were determined by collecting cell samples at 2-h intervals for 24 h following the inoculation (Fig. 1). Proteins released from the intact cells with the Laemmli sample buffer were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8) and stained with Coomassie blue, and then the gels were scanned with a densitometer (Fig. 1A). The major protein band in these gels was the S-layer protein with a molecular mass of 45 kDa. The amount of SlpA increased up to the stationary phase of growth and remained then at a relatively constant level (Fig. 1A). Total RNA was isolated from the *L. brevis* cells (11); this was followed by agarose gel electrophoresis (7) and blotting to a nylon filter (Zeta-Probe; Bio-Rad) for Northern hybridization. A 1.2-kb PCR fragment, amplified from the *slpA* gene region with the primer pair 5′-TT(A/G)TT(A/G)TAIGT(A/G)TAIG T(A/G)TA(A/G)TG(A/G)TAIGC-3′ and 5′-TA(T/C)GCIAC IGCIGGIGCITA(T/C)(T/A)(C/G)IACI(T/C)TI-3′, was used as the hybridization probe after labeling with [α-^{32}P]dCTP (>3,000 Ci/mmol). Quantitation of the *slpA* transcripts (Fig. 1) was performed with a liquid scintillation counter (Wallac 1410) from the *slpA* mRNA bands cut from the hybridization filter. Comparison of the relative amounts of the S-layer protein and *slpA* mRNA showed that the kinetics of the accumulations of the *slpA* mRNA and protein correlated well up to the onset of the stationary phase (Fig. 1A). The level of *slpA* mRNA increased for up to 12 h and then sharply decreased. The mRNA decay was, however, slower than expected from the half-life of *slpA* transcripts (see Fig. 3). This suggests that residual transcription continues even though the total amount of the S-layer protein does not further increase at the stationary phase. In addition to our studies on the in vivo transcription of S-layer genes, only the S-layer mRNA of *Aeromonas salmonidica* has been described in the literature. The level of transcripts derived from the *A. salmonidica* S-layer protein gene, *vapA*, was found to be highest at the mid-log phase of growth, whereas a relatively sharp decline of *vapA* transcripts occurred already at the late exponential phase (5).

No release of the S-layer protein into the supernatant fractions was found at any of the growth phases studied, confirming

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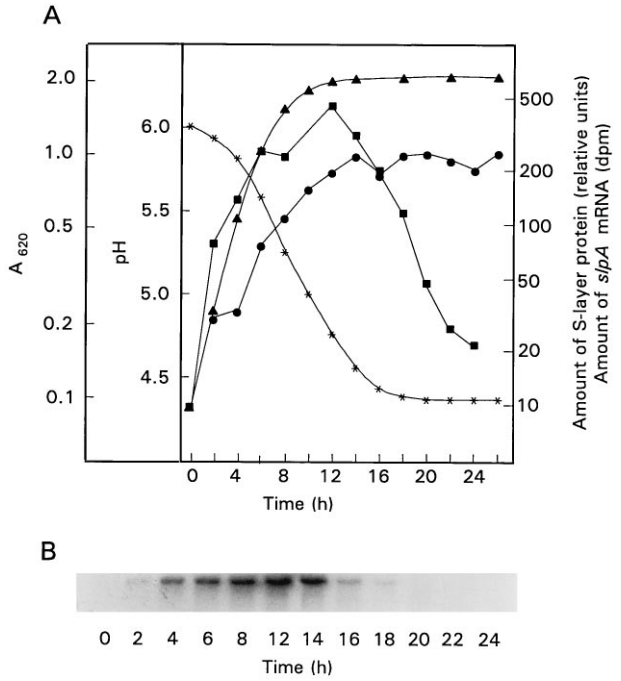

FIG. 1. Expression of the *L. brevis slpA* gene as a function of growth. *L. brevis* cells were propagated in MRS broth at 37°C. (A) Triangles, cell growth measured as change of absorbance at 620 nm; stars pH; circles, relative amounts of S-layer protein per volume determined by densitometric scanning of Coomassie-blue stained gels from SDS-PAGE; squares, relative amounts of *slpA* mRNA quantified by liquid scintillation counting of the Northern blots shown in panel B. (B) Northern blot analysis of *slpA* mRNA from cell samples of equal volumes taken at the indicated times of growth. Hybridization was with a ^{32}P-labeled 1.2-kb PCR probe from the *slpA* gene region.

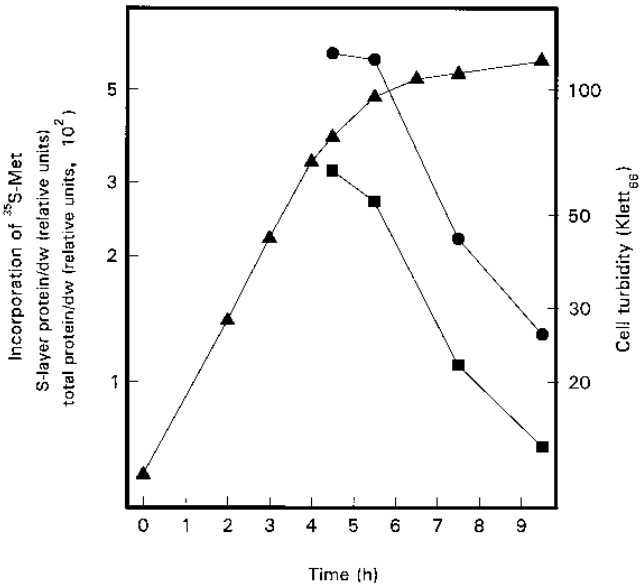

FIG. 2. Incorporation of [^{35}S]Met in *L. brevis* total cellular and S-layer proteins. Samples were withdrawn for labeling at the cell density of 77 Klett (filter 66) units (0 h) and 1, 3, and 5 h thereafter. De novo synthesis of proteins was determined by pulse-labeling with [^{35}S]Met for 10 min. The incorporation of [^{35}S]Met in the cell fractions (circles) and S-layer protein (squares) was determined by liquid scintillation counting from trichloroacetic acid precipitates and SDS-PAGE gel slices carrying the S-layer protein band, respectively. Triangles, cell densities. Values represent averages of two independent determinations. dw, dry weight.

the earlier observations (12) and suggesting a tight regulation of the S-layer synthesis and assembly. The regulation of SlpA synthesis was further studied by biosynthetic labeling of the *L. brevis* proteins. For examining the the de novo protein synthesis in different stages of growth, pulse-labeling with L-[^{35}S]methionine (>1,000 Ci/mmol) was used. The *L. brevis* cells were grown to the exponential phase (77 Klett [filter 66] units) in MRS broth. Cell samples were withdrawn at four time points, resuspended in methionine assay medium (Difco), incubated for 15 min, and labeled with [^{35}S]Met (50 μCi) at 37°C for 10 min. Incorporation of the [^{35}S]Met into the total cellular protein fraction and into the S-layer protein was measured by trichloroacetic acid precipitation and SDS-PAGE (8), respectively, followed by liquid scintillation counting of the precipitates on glass filters (Whatman) and cut gel slices. The amount of [^{35}S]Met incorporation per cell dry weight (Fig. 2) showed only a slightly faster decrease in the SlpA synthesis than in the total cellular protein synthesis at the late exponential phase, although this ratio may have been affected by the differences in the turnover numbers. Furthermore, Breitwieser et al. (4) have demonstrated the presence of substantial amounts of S-layer subunits on the inner surface or within the peptidoglycan layer in *Bacillus stearothermophilus*, suggesting an intermediate phase between the synthesis and final location of the S-layer protein. This has also been quite commonly observed in S-layers of other gram-positive eubacteria (4). However, in *L. brevis* over 95% of the S-layer subunits could be released with the SDS-PAGE sample buffer from intact cells (12), as Western blot analysis of intact and disrupted cells indicated (data not shown). Thus, it appears that essentially no accumulation of the *L. brevis* S-layer subunits took place inside the peptidoglycan layer prior to translocation to the outer surface.

Half-life of *slpA* mRNA. The stability of *slpA* transcripts was studied by inhibiting the transcription with rifampin (200 μg/ml; Sigma) in the *L. brevis* cells grown to the logarithmic phase (A_{620} = 0.642), followed by sample withdrawals up to 32 min and determination of the time-dependent decay of the *slpA* mRNA band with Northern blot analysis (Fig. 3A). The 1.2-kb *slpA* probe was labeled with [α-^{32}P]dCTP (>3,000 Ci/mmol). The amount of radioactivity bound to *slpA* mRNA was determined with a liquid scintillation counter. The curve of mRNA decay (Fig. 3B) showed that the half-life of the *slpA* transcripts was 14 min. Compared to typical prokaryotic mRNAs on the basis of half-life, the *slpA* transcripts are exceptionally stable. The lack of stability studies of other mRNAs from lactic acid bacteria prevents further comparisons in this group of bacteria. Furthermore, the transcripts of *A. salmonidica vapA* have also been shown to be very stable. The half-lives of *vapA* mRNA were 22 and 11 min when *A. salmonidica* was grown at 15 and 20°C, respectively (5). The long half-lives of these two S-layer mRNAs from two very different organisms may indicate that high mRNA stability is a general feature for S-layer mRNAs. Because they mediate the synthesis of major structural component of the cell, the high stability of S-layer mRNAs is not entirely unexpected.

Usage of P1 and P2 promoters. The −35 regions of the two promoters of *L. brevis slpA* are located 163 (P1) and 77 (P2) nucleotides upstream of the start codon (12). A primer extension analysis performed previously with a total RNA extract of exponentially growing *L. brevis* cells revealed that both promoters are used at the logarithmic phase (12). To determine how these promoters are used in the growth phase-specific expression of the *slpA* gene, Northern and dot blot analyses were performed. Several dilution series of total RNA samples isolated from *L. brevis* cells as a function of growth were hy-

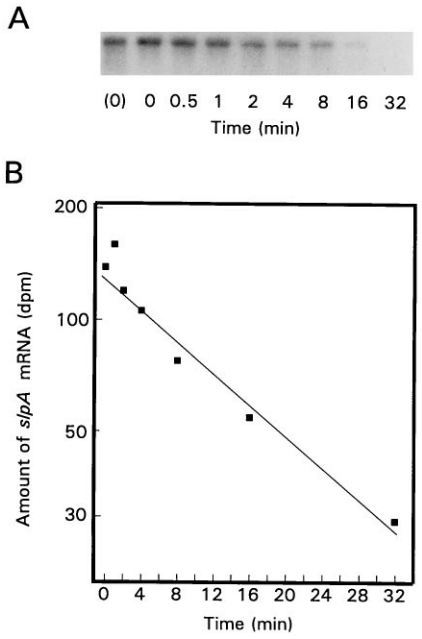

FIG. 3. Determination of the half-life of *slpA* mRNA. (A) Northern analysis of *L. brevis* RNA was done with the ^{32}P-labeled 1.2-kb PCR fragment probe amplified from the *slpA* gene region. Lanes refer to total RNA analyzed from the cell samples collected immediately before [(0) min] and at the indicated times after addition of rifampin. (B) Quantitation of the full-size *slpA* mRNA from the Northern blots of panel A by liquid scintillation counting.

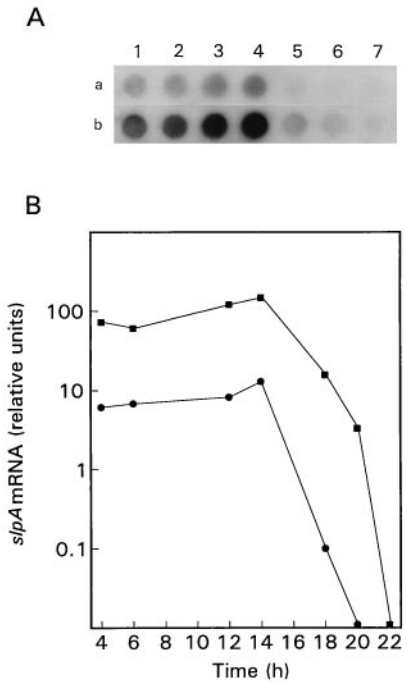

FIG. 4. Use of *slpA* promoters P1 and P2 as a function of growth. (A) Dot blot hybridization of RNA samples isolated from equal volumes of the *L. brevis* cells after 4, 6, 12, 14, 18, 20, and 22 h of growth (lanes 1 to 7, respectively). Two oligonucleotide probes, p1 and p2, detecting transcripts from P1 (a) and P1 and P2 (b), respectively, were labeled with DIG. (B) Relative amounts of transcripts derived from P1 (circles) and P2 (squares). The RNA dot blots (A) were scanned with a densitometer; this was followed by subtraction of the respective values of P1-derived transcripts (a) from those of P1 plus P2 (b).

bridized with two 20-mer oligonucleotide probes labeled with digoxigenin-ddUTP (DIG; Boehringer Mannheim). Hybrid detection was with a DIG luminescence detection kit (Boehringer Mannheim). The target region of probe p1 (5′-AGCGG ACCCGATCAGCTATC-3′) is located between the two promoters, allowing hybrid formation only with the *slpA* mRNA derived from P1. The other probe, p2 (5′-CAAAGCTCAAT GCGGCAAGG-3′), hybridized with nucleotides 79 to 98 downstream of the transcription start site of promoter P2, thus leading to the detection of both *slpA* mRNA types. Both probes had an identical melting temperature, and they were tested to hybridize with an equal efficiency to *slpA* DNA samples. Northern analysis indicated that the P2 promoter is efficiently used during both the logarithmic and early stationary phases whereas *slpA* mRNA derived from P1 was only weakly detected with the p1 probe (data not shown). Dot blot hybridization followed by densitometric scanning of the films showed that transcripts derived from both promoters could be detected throughout the entire growth phase (Fig. 4). However, transcripts derived from promoter P2 were predominant and indicated about 10 times more frequent promoter usage than found with P1 at both the logarithmic and stationary phases of growth. Furthermore, the results suggested that residual mRNA synthesis from P2 continued for a longer period than that from P1 at the stationary phase. S-layer genes carrying multiple promoters have also been described in *Bacillus brevis* (1, 6). The usage of three promoters in the expression of the *cwp* operon of *B. brevis*, two of which are frequently used has been described. Promoter P2 of *B. brevis cwp* is constitutively used whereas P3 is preferentially used at the exponential phase of growth (1).

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