# Surface Location of Individual Residues of SlpA Provides Insight into the Lactobacillus brevis S-Layer<sup>∇</sup>

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Bacterial surface layer (S-layer) proteins are excellent candidates for in vivo and in vitro nanobiotechnological applications because of their ability to self-assemble into two-dimensional lattices that form the outermost layer of many Eubacteria and most Archaea species. Despite this potential, knowledge about their molecular architecture is limited. In this study, we investigated SlpA, the S-layer protein of the potentially probiotic bacterium Lactobacillus brevis ATCC 8287 by cysteine-scanning mutagenesis and chemical modification. We generated a series of 46 mutant proteins by replacing single amino acids with cysteine, which is not present in the wild-type protein. Most of the replaced amino acids were located in the self-assembly domain (residues 179 to 435) that likely faces the outer surface of the lattice. As revealed by electron microscopy, all the mutant proteins were able to form self-assembly products identical to that of the wild type, proving that this replacement does not dramatically alter the protein conformation. The surface accessibility of the sulfhydryl groups introduced was studied with two maleimide-containing marker molecules, TMM(PEG)<sub>12</sub> (molecular weight [MW], 2,360) and AlexaFluor488-maleimide (MW = 720), using both monomeric proteins in solution and proteins allowed to self-assemble on cell wall fragments. Using the acquired data and available domain information, we assigned the mutated residues into four groups according to their location in the protein monomer and lattice structure: outer surface of the lattice (9 residues), inner surface of the lattice (9), protein interior (12), and protein-protein interface/pore regions (16). This information is essential, e.g., in the development of therapeutic and other health-related applications of *Lactobacillus* S-layers.

Bacterial surface layers (S-layers) are cell envelope structures ubiquitously found in gram-positive and gram-negative bacteria as well as in Archaea. S-layers are composed of identical (glyco)protein subunits with a molecular mass in the range of 40 to 200 kDa. The proteins self-assemble into two-dimensional crystalline structures with oblique (p1, p2), square (p4), or hexagonal (p3, p6) symmetry, covering the entire cell surface. The subunits are held together and attached to the underlying cell wall by noncovalent interactions and they have an intrinsic ability to spontaneously form regular layers in solution and on solid supports (24). S-layers have been shown to have roles in the determination and maintenance of cell shape as virulence factors, as mediators of cell adhesion, and as regulators of immature dendritic and T cells. Moreover, they can also function as a protective coat, molecular sieve, murein hydrolase, and ion trap (4, 8, 13, 17, 19, 25, 29).

S-layer proteins have several properties that make them an attractive target for the development of nanobiotechnological applications both in vivo and in vitro. In particular, a high

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number of protein subunits are displayed at the bacterial cell surface. Moreover, the protein subunits are able to spontaneously self-assemble into a regularly arranged lattice structure both in solution and on solid supports (1, 27, 30, 31). However, despite the high prevalence of S-layers in nature, their molecular structure remains poorly elucidated. In particular, knowledge about the spatial organization of amino acid residues in S-layer proteins or the interactions between these residues and other subunits is limited. The poor solubility of protein assemblies and the absence of stoichiometrically defined oligomers have hindered attempts to apply nuclear magnetic resonance or hydrogen/deuterium exchange mass spectroscopy. In addition, the intrinsic property of S-layer proteins to form twodimensional lattices has hampered efforts to obtain three-dimensional crystals required for X-ray crystallography (12, 31). To our knowledge, only part of the structure of one S-layer protein, SbsC of Geobacillus stearothermophilus, has been determined by X-ray crystallography (18). Since high-resolution, three-dimensional structural data are mostly lacking, traditional mutation-based techniques are presently the methods of choice. In cysteine-scanning mutagenesis (CSM), a series of mutant proteins is generated by replacing single residues with cysteine, which contains a sulfhydryl group amenable to further chemical modification. The spatial locations of amino acid residues within the S-layer protein SbsB of gram-positive thermophile *G. stearothermophilus* PV72/p2 have been analyzed by CSM. A total of 75 residues out of 920 were studied, identifying 23 residues located at the surface of protein monomers, five of those located on the outer surface of the protein lattice (10). These mutant proteins were subsequently analyzed by a cross-linking screen to assess residues accessible in monomeric form to the protein/protein interface and the inner surface of the lattice (12).

In the genus Lactobacillus, S-layers have been found in several species. S-layer protein genes have been sequenced from L. brevis, L. helveticus, and L. acidophilus group organisms. Sequence similarity between Lactobacillus S-layer protein genes can be found only between closely related Lactobacillus species. Therefore, the primary sequences of Lactobacillus Slayer proteins show extensive variability, with the number of identical amino acids varying from 7 to 100% between different proteins. As a group, Lactobacillus S-layer proteins differ from those of most other bacteria in their smaller sizes (25 to 71 kDa) and higher calculated isoelectric point (pI) values (9.4 to 10.4) (1). The presence of two or more S-layer protein genes in the same strain is common in lactobacilli (5, 6, 11, 28, 35); however, only one S-layer protein gene, *slpA*, has so far been described to be present in the genome of L. brevis ATCC 8287. SlpA is a 435-amino-acid, 46-kDa S-layer protein that assembles into a lattice of oblique symmetry on the bacterial surface (2, 36). L. brevis ATCC 8287 has GRAS (generally recognized as safe) status and has been shown to possess probiotic properties (21), which make SlpA a very attractive subject, e.g., in the development of live oral vaccines. Moreover, a recent report using differential scanning calorimetry suggests that in comparison with other S-layer proteins, SlpA is resistant to high temperatures (21). This thermal stability could prove potentially useful in a variety of in vitro S-layer applications currently being planned or under development (27, 30, 31). Recently, SlpA was characterized to consist of an N-terminal cell wall binding domain (residues 1 to 178) and a C-terminal self-assembly domain (179 to 435) (3). For the development of applications that take advantage of these characteristics, further investigation of SlpA at the molecular level is essential.

Herein, we use CSM and targeted chemical modification to assign 46 amino acid residues of SlpA to spatial locations in the protein monomer and in the lattice according to their surface accessibility. We focused mainly on the self-assembly domain, the region facing the outer surface of the protein lattice and thus most amenable to insertions and chemical modification. Two different marker molecules were used to modify cysteinecontaining mutant proteins that were either in solution or attached to the cell wall. The results were subsequently evaluated taking advantage of the recent new information on SlpA domain boundaries (3). We were able to distinguish residues located in the outer and inner surfaces of the lattice, protein interior, and interface/pore regions. The information gathered here can be used in the development of further biotechnological and nanobiological applications, both in vitro and in vivo, that benefit from a thermostable S-layer protein from a GRAS bacterium with health-beneficial properties.

## MATERIALS AND METHODS

Bacterial strains and culture conditions. Escherichia coli XL1-Blue supercompetent cells (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB *lacl*<sup>q</sup>ZΔM15 Tn10 (Tet<sup>r</sup>)]) were used in the generation of mutations, and *E. coli* strain BL-21(DE3) [B F<sup>-</sup> *dcm ompT hsdS*( $r_B^- m_B^-$ ) gal  $\lambda$ (DE3)] was used to express the proteins altered. Both strains were from Stratagene. *E. coli* XL-1 Blue was grown in Luria-Bertani medium, BL-21(DE3) in M9ZB medium (32), and *L. brevis* ATCC 8287 in MRS broth (Difco) at 37°C. When appropriate, kanamycin was used at a concentration of 30 µg/ml.

**Routine DNA manipulations and transformation.** Routine molecular biology techniques were used essentially as described previously (23). Plasmid DNA was isolated using the Wizard Minipreps kit (Promega). The PCR products were purified with the QIAquick PCR purification kit (Qiagen). DNA restriction and modification enzymes were used as recommended by the manufacturers (Stratagene, New England Biolabs). The PCR was carried out with DyNAzyme II DNA polymerase as recommended by the manufacturer (Finnzymes). *E. coli* XL1-Blue supercompetent cells were transformed as recommended by the supplier (Stratagene). BL21(DE3) cells were transformed as described previously (23).

Oligonucleotides and DNA sequencing. Oligonucleotides (Oligomer, Helsinki, Finland, and Sigma-Aldrich) used in this work are listed in Table 1. Sequencing of the mutation sites was performed using an ABI Prism 310 genetic analyzer in combination with the DNA sequencing kit for BigDye Terminator cycle sequencing (Applied Biosystems). The sequences of the entire gene constructs were verified by the Sequencing Laboratory of the Institute of Biotechnology, University of Helsinki, Helsinki, Finland.

**Protein analysis.** Protein concentrations were determined by Bio-Rad protein assays (Bio-Rad) using bovine serum albumin as a standard. The protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (14) and stained with Coomassie brilliant blue.

**Construction of SlpA mutant proteins.** Single amino acid mutations were made for 46 amino acid residues (23 threonine, 17 serine, 3 alanine, 2 valine, and 1 leucine) that were dispersed throughout the SlpA amino acid sequence, concentrating on the self-assembly domain defined (3). Site-directed mutagenesis was carried out based on the QuikChange II kit (Stratagene) with the following modifications: as a polymerase, Phusion DNA polymerase (Finnzymes) was used according to the instructions supplied by the manufacturer, and in addition, DpnI digestion was extended to 3 h at 37°C. The PAGE-purified oligonucleotides required were from Sigma-Aldrich. Plasmid pKTH5199 (3), encoding the mature SlpA protein with an N-terminal His tag under a T7 promoter in a pET28a(+) backbone (Novagen) was used as a template in all mutagenesis reactions. The mutations were verified by performing colony PCRs on colonies observed on transformation plates using primers 1568 and 1569, followed by sequencing the resulting PCR products containing the recombinant SlpA gene with primers 622, 1568, and 1569.

Heterologous expression of sequences encoding SlpA mutant proteins. Gene expression was carried out using the BL21(DE3) strain as described in the pET system manual (Novagen). Briefly, the expression of SlpA mutant proteins was induced by adding isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) at a concentration of 1.0 mM to the medium of exponentially growing *E. coli* strains that harbored an expression plasmid containing the mutation desired. After IPTG was added, the incubation was continued for 5 h. The cells were harvested by centrifugation and the pellets (typically ~1.5 g [wet weight]/200 ml of culture) stored at  $-80^\circ$ C until used.

For the purification of mutant proteins, the cells were resuspended in doubledistilled water and disrupted with a Branson Sonic Power sonicator (Branson Ultrasonic Corp.), followed by centrifugations for 5 min at  $3,000 \times g$  and 20 min at  $15,000 \times g$ . The SlpA mutant proteins were purified from the latter supernatant in the presence of 4 M guanidine hydrochloride (GHCl) with a His Trap HP column according to the instructions given by Amersham Biosciences (binding and washing buffer, 20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole [pH 7.4]; elution buffer, same but 0.5 M imidazole). After purification, the fractions containing the SlpA mutant protein were dialyzed overnight at +4°C against a buffer containing 10 mM Tris-HCl (pH 7.5) and 0.1 mM dithiothreitol (DTT). The purity of the proteins isolated was checked by SDS-PAGE.

Analysis of the lattice formed by wild-type and mutant proteins by EM and small-angle X-ray scattering (SAXS). Suspensions of all 46 heterologously expressed, isolated, and dialyzed SlpA mutant proteins were examined by negative staining to visualize the self-assembly products by electron microscopy (EM) as described previously (3, 20).

The SAXS measurements were performed using a conventional X-ray tube with CuK<sub> $\alpha$ </sub> radiation (wavelength, 1.54 Å). The intensity curves were measured with a HI-STAR area detector at a sample-to-detector distance of 50 cm. The magnitudes of the scattering vector  $k [k = (4\pi \sin \theta)/\lambda$ , where 20 is the scattering angle and  $\lambda$  is the incident wavelength] were 0.02 to 0.42 Å<sup>-1</sup> (34). The samples were centrifuged into pellets. The SAXS intensities of the pellets were measured TABLE 1. Oligonucleotides used in this study<sup>a</sup>

62*       AACTGATGGTACAAAGGCAGG         156*       GCTAGTATTGCTCACGAGG         156*       GCTAGTATTGCTCACGAGG         197C       GGTTGTTATGCTCACGAGGCAGGTGTGTAAGGGTGCTAAG         197C       GGTTGTCGCTCTAAGGCTGTGTATGGCTAGGATGCCAGT         197C       GGTTGTCGCTCTAAGGCTGTGTATGGCTAGGGTGTAGGGTGTAGGGTGCTAGGAGC         197C       GGTTGCGTCTAAGGCTGTGTTGTGTGTGTGTGTGTGTGTG	Oligonucleotide	Sequence $(5'-3')$
1568*	622*	AACTGATGGTACAAAGGCAGG
1569*       TAATACGACTCACTATAGGG         T035C       GCTTTATACACGAAGCCAGGTTGTGTAAGGGTGCTAAG         T047C       GGTTGTCGCTTCTAAGGCCTGGTTAGGCTAAGTAGCTAC         S073C       CACTAACCCTGGTTGGTATGGCTTAAGGCTGATTAGGCT         S073C       CACTAACCCTGGTTGGAAGGGTTATGGTGTTGTGAGAGGAGT         T108C       GGTTACAAGTCTGCTGAAAGGCTGATTAAGGCTGATTAGGGTGTGGTATTGCC         S145C       CACGAAGCAAGTAAAGTTGCGTTACTAACGGTGGTGGTATTAGGGGTGG         S179C       CTATCACGTATGGGTACTAACGGTGGTGGTATTAGTGGG         S179C       CTATCACGTAACTGGTACTAACGGTGGGTGGTATTAGTGGG         S182C       GGTTCTCACTGGATCTACTGCTCGTGCCACGAGTCGC         S192C       CTGGTAAGGCTTCGTGTGGCACGAGCAAGGTACGGTGC         S192C       GGTTCTCAACTGGATCTGCTGGCGCACGAAGGATAACGG         S299C       GGTTCTCAACTGGATACTGCTGGCGCCAAGGATAACGGG         S299C       GGTTCTCAACTGGATACTGGTGGCACGCAAGGATAACGGG         S299C       GGTTCCAACTGGATGAAGGGATGGAAGGAACACTGGG         S299C       GGTTCCAACTGGATGAAGGGATAGCAACCAAGGG         S299C       GGTTCCAACTGGATGAAGGGATGGAAGCAACGATGGGGG         S299C       CGGTACAAGGCAGCGGTGAAGGGAAGCAACGTGGGG         S299C       GGTTCCAACTGGATGGATAGGTAGCAACGAAGGATAACGGG         S299C       CGGTACAAGGCACGATGGTTGAGGAACCATTGGG         S299C       CGGTACAAGGCACGATGATTGATTGGATGGAGCACACGTGGGGGATAAGGGGGCCCAAGGCTCAACTGGATGGCCCAAGGGCCCACGGCGCCAAGGGTAA	1568*	GCTAGTTATTGCTCAGCGG
T035C       GCTTTATACACGAAGCCAAGCTAGGTTGTATAAGGGTGCTAAAG         T047C       GGTTGTGCTTCTAAGGCTTGTGCTTATAGCTAGTTC         S073C       CACTAACCGTGGTGCGTTGTGCTAAGGCTTGACGATG         S145C       CAAGGCAAGTAAAGCTGTACTAAGGCTGTGATAGGCG         S145C       CTATCACGTATGCTGCTAAAGGTGTGTGTTGTAACGATG         S174C       CTATCACGTAACGGTAGTGGTGTGGTGTTGGTTAGTGGGTG         S179C       CTATCACGTAACGGTGGTGTGGTGGTGTGGTTAGTGGGG         S182C       GGTTGATTACTGGGTGAGTGTTGTGGTCAGTACTGCG         S182C       GGTTGGATTACTGGGTGGTGTGGTGTGGTGTGGTGCAGTACTGCG         S192C       CTGGTAAGGGCTTCTGGTACTGGTGGTCGTGTCAGTACTGCG         S192C       GGTTGGAATGGCGTTGGTGCACTGATACTGGGGGGTGGGGAAGCGAACCGG         S209C       GGTCGTGCAACGGATAAGTGGCCTGCAGAAGCAGCG         S219C       CGGTCGATGAGTAAGTGGATACGGCAACAGCG         S219C       CGGTCGATGAGTAAGGGCAGCAGCAAGCGGCAGCAAGGG         S219C       CGGTCGAGTGATAAGGCGCGCGATCAAGTGGCAACCGAGCAACGG         S219C       CGGTCGAGTAGAGGCAGCTGCTGAAGGCAGCAGCAGCG         S246C       CGGTCCGATGAAGGCAGCGGTTGCAAGGCATAAGGCC         S246C       CTGCTCTGGAGCGGATCAAGTGTAAGCGGATAAGGCAGCGGGGGGGG	1569*	TAATACGACTCACTATAGGG
TH4C	T035C	GCTTTATACACGAAGCCAGGTTGTGTTAAGGGTGCTAAG
Si73C.       CACTAACCGTGGTTGCGTTACTACGTGTGTGTACCAGTG         T198C.       GGTATCAAGCTGCTGCAAAGGTACTAAGGCTGGATATGCC         S145C.       CAAGGCAAGTAAAGTTGCCTGTAAACGTGTGGTAACGATG         S179C.       CTATCACGTAATGCTGCAACAGGTACTAGGGGTGGTATAGTGGTG         S179C.       CTATCACGTAATGCGTACTAACGGTAGTGGTATTAGTGG         S179C.       CTATCACGTAATGCGTACTAACGGTAGTGGTATAGTGC         S179C.       CTGGTAAGGGCTTCGTGCTACTACGGCTACTGGCAGTACTGG         S179C.       CTGGTAAGGGCTTCGTGACTGCGCAGTACTGGGTACG         S179C.       CGGTTCGATCGTGTGTGGCCAGTACTGGGCAGTACTGGGG         S179C.       CGGTTCGATGTGCACTGCCAGTACTGGGCAGTACTGGGG         S198C.       GGTACACAAGTACTGGTGTACGCAGTACTGGGAACCAGG         S209C.       GGTCTGCAACTGATAAGTGCCAACACG         S209C.       CGACTGATGAGCACCCAAGTGGTGAGCAGCAACGG         S209C.       CGGTCGATGAGGCACCAAGTGGCAACAGGG         S209C.       CGGTCGATGAAGGCGCGCGATCAAGTGGCAACACGG         S209C.       CGGTCGATGAAGGCGCGCATCAAGTGGCAACACGG         S209C.       CGGTACAACGCCGACTCAAGTGGGATAAGGCGCACACGG         S209C.       CGGTCGATGAAGGCAGCCTACAGGCGATAAGGCG         S209C.       CGGTCCAACTGATGGAGCAACAGGGC         S246C.       CGGTCGCGATCAAGGTGGAACAGCTGGGGCATAAGGCAACGCC         S246C.       CTACCACACGCGATCACCTGCCTGGGGGCCCTCTGGTACAGCGC         S296C.       CGTAAGCGG	T047C	GGTTGTCGCTTCTAAGGCTTGTATGGCTAAGTTAGCTTC
Ti98C	S073C	CACTAACCGTGGTTGCGTTTACTACCGTGTTGTAACGATG
Si4sc.       CAAGGCAAGTAAAGTTICCCTITATGGTGCTACTAAGGAC         T174C.       CTATCACGTATGTGCTACTAACGGTAGTGGTATTAGTGG         S179C.       CTATCACGTATGTGCTACTAACGGTAGTGGTATTAGTGG         S179C.       GCTACTAACGGTAGTGGTATTGTGGTATTAGTGG         S182C.       GGTACTAACGGTAGTGGTATTGTGGTGTGGATTACCC         S197C.       CTGGTAAGGGCTTCGTACTACTGCTACTGGTGCACTGGTGG         S198C.       GGCTTCATACTGCTGACTACTGCTGCACAGTACTGGTGG         S209C.       GGTCTGTCAACTGGTACGGTCTGTGCACAGTACCAACGA         S209C.       GGTCTGTCAACTGGTAAGGTCACGACAACGAACCAACG         S213C.       CTGTCAACTGATAAGTCAGTTACAGCAACCAACGAACAGAAACGGC         S246C.       CGGTACAAAGGCCGCCCCATCAAGTTGGCACTGACACGAAACGGC         S246C.       CGGTACAAGGCCGCCCCCCAATCAATGGTGCACGCAACGG         S246C.       CGTCCTGTACACTCAAGTCAATGCTGCGGTAACGCC         S268C.       CTCACTGTGCAACGCATCAATGGTGCAGGCGCCCC         S268C.       CCTCACTGCTGACACTCAATGGTAACCGGTAACGGCGCC         S288C.       CCTACAGGGTACACTGGTTGCAAGCCTGCAGGTAACGGGTTAC         S291C.       GGTCGCTTGAAGCCTGCTTGTATGGGTGTAACGGGTGTAC         S292C.       GGTCGCTTGAAGCCTGGTTGCAAGGCTAACGGTGATGC         S286C.       CTACCTAGTGCTGAAGCCTGCCTGGTAACCATGGGTTACGCAGG         S291C.       GGCTAGCTGTTGAACGCTGCTGGGTGGACCCTGGTACGC         S291C.       GGTCGCTTGAAGCTGCTGGTTGCAAGGCGAGTTGGCTGACGCAGTGC	T108C	GGTATCAAGTCTGCTGAAACGTGTACTAAGGCTGATATGCC
T174C CTATCACGTACTGGCTACTAACGGTAGTGGTATTAGTGGTTG S179C CTATCACGTAACTGGCTACTAACGGTTGTGGTATTAGTGGTG S182C CGCACTACACGGTAGTGGTATTGGTGGGTATTAGTGG S182C GGTCGTGAAGGGCTTCTGTAACGGCTTCGGTAGCGCTACTGGT S197C CGGTGGAGGGCTCTGTAAGGGCTTCGGTAGCGGTAC S197C CGGTGGTGGCGTGGTAAGGGCTTCGGTAGCGACGTACTGGTG S197C CGGTCGTCACTGGTGCTGCTGGTGCCAGTACTGGTGG S205C GGTCACCAGTACTGGTGGCGTGGCACGTAAAGTCG S209C GGTCTGTCAACTGGTGGTGCTGGCACGTAAAGTCAG S209C CGGTCGTCAACTGGTGGCGTGTGCAGCGACACACGA S209C CGGTCGTCAACGGAGTGGTGTGAGGCAACGACGATAACAGTG S232C CGGCTGACGAGTGGTGTGAGGCAACGCAGCAACAGG S232C CGGCTGACGAGTGGTGTGAAGGTAAGGCAACACGGG S246C GGTCACAGGGTGTGAAGGTGGTGCAACGCTAAGGC S246C CGGATAAAGGCGGCGCACTCAAGTGGTGGCGGCGACGAAGGC S246C CGGATAAGGCGGCGCAATCAATGGCGCTCAGGG S246C CGGATAAGGCGCGCGAATCAATGGCGCTCAGGG S246C CGGTACACGGTACGCAGCAACAAGCG S288C CTACACGGTACCAGCATCAATGGCGCTAAGGCAACGCG S288C CGCCACCGGGTTACACTGATGTAGCCCAACGG S289C CGCCAGCGGGTCACGTATGTAGCCCAACGG S289C CGCCAGCGGGTCACGTGTGGCAGCACCAGGT S291C GGTTAGGGCTACACTGATGTGGCAGCACGGG S393C GGGTCGCGGTGCGGGTACACTGAGGCGCGCGGTTAC S291C GGACCCCGGTTACGAGGCTGCGGGTACCGGGTTAC S390C GGGCCGGCGCGCGGTACGCGGTTACC S390C CGGACCCCGGTAAGGCTGGCGGACGCGGCGCGGTTAC S390C CGGACCCCGGTAAGGCTGGCGGCCAGCTCAGCCAGCGCACGCC S330C CGGTCACGGTGCGGGGCCAGCGCGCGGTTACC S330C CGGGTCACGGTAACGGCTGGGGGCCAGGTACCCCGGGC S333C CGGGCCAGGCCACGGCTAATGCAACGGCTACGCCACGC S330C CGGGCAAGGCCAAGGCTAAGGTAACGGCCAACGCAACCAC S390C CGGGCCAAGTTGGCGCGCACGCTCATTAACGGCC S330C CGGGCAAGGCTAATGGAAGGCCAAGGCTAAGGATACCAC S330C CGGGCAAGGTCAGGTGAAGGCCAAGGCTAAGGATACCAC S330C CGGGCAAGGTCGGGGTGGACGGCGCAGGCCAAGGCCAACGC CGGGCAAGGTCAGGGGCAAGGGCTAAGGAAAGGGCCAACGGCAACGCACCC CGGGCAAGGTCAGGGCCAAGGCCAGGCC	S145C	CAAGGCAAGTAAAGTTTGCCTTTATGGTGTTGCTAAGGAC
S179C       CTATCACGTAACTGCTACTAACGGTTGTGGTATTAGTGG         S182c	T174C	CTATCACGTATGTGCTACTAACGGTAGTGGTATTAGTGGTTG
Si82C	\$179C	CTATCACGTACTGCTACTACGGTTGTGGTATTAGTGG
A187C.       GGTTGGATTTACTGTGGTAAGGGCTTCAGTACTACTGC         S192C.       CTGGTAAGGGCTTCAGTACTGGTACTACTGGTAC         S198C.       GGCTTCAGTACTGGTGGTTGGTGCCAGTACAGTGAGG         S209C.       GTACACAGTGATACTGGTGGTGGTGCAGCTACAGGAACCAACG         T131C.       CTGTCAACTGATAAGTGCGTTACAGCAACCAACGG         S232C.       CGACGACGACCACGGCATCCAGGTACAGCAACCAGGG         S246C.       GGTACAAAGGCAGCAGTAGTGAGGTACAGCGAACCAACG         S246C.       CGACGACAGGCACCAACTGGTGGTGCAGCTAAAGGCC         A264C.       CGCTCTCCAAGTGGTACAACTGGTGGTACAACTGGCG         S288C.       CCTAACTCAATGCTACAGCTACAGTCAAGTCAAGCCTAGCG         S288C.       CCTAACTCAATGCTGCAGCACAAGCCTTGCGGGTACACTGG         S291C.       GCCTAAGCGGTTACACTGGTTGGCAGCACCAAGGTTACA         S291C.       CCCTAAGCGGTTACACTGGTTGCAGGACGCACTTCTAAGG         S303C.       GGTGGCTTTGACACTGGTTGCGGGGACTCCTGTTAC         S308C.       GGTGGCTTACCTGTTGACGGGACCCCGTTCAAGGATACAGCGGATGC         S308C.       GGACTCCTGTTTGACAGCGGACAGTCCTGTACGGATGCCAGGC         S330C.       CGACCCTGCTAATGGATGGTGCTGGCGACAGCGACGCGGACGGA	\$182C	GCTACTAACGGTAGTGGTATTTGTGGGTTGGATTTACGC
Si92C.       CTGGTAAGGGCTTCTGTACTACTGCTACTGGTAC         T198C       GGCTTCAGTACTACTGCTGCTACTGGTACTAGTAGTGG         S205C       GTAACAAGTACTTGGTGGTCTGTGCACTGATAAGTCAG         S209C       GGTCTGTCAACTGATAAGTGCGTTACAGCAACCAACG         S212C       CTGTCAACTGATAAGTCAGTAAGCAACGAACGAACGA         S232C       CGACTGATGGCACTCAAGTGGGCGTTGAAGGCAACGATGACAGTG         S232C       CGACTGATGGCACTCAAGTGGGCATCAAGGCAACGACGG         S246C       CGTACAAAGGCAGCCGCCGATCAATGTGTACACGATAAGGC         S256C       CTGACTAAGGCAACGCTGCCGGTACACTGGAGC         S268C       CTGACTCAAGGGGTTGCAACGCTTGCGGTACACTG         T273C       GCCTAAGCGGTTACACGCTTGCTGGTAAGCTGCAGG         S291C       GTTTACGCTACGGGTGCACAGCTTGCTAGGTAACCACAGTTTAC         S291C       GTTTACGCTACGGTTGCATGGTAACGCACAGTTTAC         S291C       GGTTCGGTGGCAGGGTCGCGGGACCCCTGTTAC         S291C       GGTTCAGTTGATCGCCAGGCGCAGCTACTCTAAGG         S303C       GGGCCCCTGTTTGATAGGTCACGCGGATGCCAGGTTAC         T308C       GGACTCCTGTTGATACGCATTGGTAACGCAGAGTGCC         S303C       GGACTCCTGTTGATGCTTGGCAGGTTAAGGTAACGAC         S330C       CGACTGCTGTTGTTGCAAGGGCTAATGGAGCAACCAC         S330C       GGACTCCTGTTGTTGCAAGGGTAAGGTAACCAAC         S330C       GTAACCACTGTTGATGCTGCAGGTTGTAAGTAACGAGCCAACC         S3335C       GTAAGTCACTGTTGAAGGGTCAACTGACG	A187C	GGTTGGATTACTGTGGTAAGGGCTTCAGTACTGC
T198C       GGCTTCAGTACTACTGCTACTGGTTGCCAAGTACTTGGTGG         S209C       GTACACAAGTACTTGGTGGTTGTGCCAAGTAATAGTCAG         S209C       GGTCTGTCAACTGATAAGTCAGTAGCAACGATAACAGT         T213C       CTGTCAACTGATAAGTCAGTTACAGCAACGAACGATACAGT         S232C       CGACTGATGGCACTCAAGTTGGTTGCAACCAACGTGGGG         S246C       GGTACAAGGCCAGCCAACTGAAAGGCAGCAACGTGGGG         S246C       CGATAAGGCCGCCGATTCAATGGTACTGGGGC         A264C       CTGCTCTTGAAGCCGCAATCAATTGTAACCACGC         A264C       CTGCTCTTGAAGCCGCAATCAATTGTAACCACGG         S288C       CTACATCAATGCTAACAAGTGTGCAACACGGG         S281C       CCCCAAGCGGTTACACTGGTTGCAACGCCAACCAGCTGCTACGGG         S291C       GTTTACGCTACTGTTTGCAAGCCGCAACCAAGTTTAC         S291C       GTTTACGCTACTGTTGCAGGGCACCCTGATTACGGG         S303C       GGTCGCCTACTGTTGGAGGCACCCTGATTACAGGG         S303C       GGACTCCTGTTTGAAGGTCGCGGGACTCCTGTTAC         T308C       GGACTCCTGTTTGAAGGTGCAGGCTAACGGTATACCAC         S330C       CCACCTAGCTAGCATGCAAGTGTGTACAGCGCAAGCACATACCAC         S330C       CGATACCACTGCTAATGGTGCACGCAGCCAAGCTAACGAC         S330C       CGATACCACTGTTAGCGTGCACGCGCACGCCAGGCAACCACC         S330C       CGATACCACTGTGTCACGGTTGATAGTGGCGCAAACCAC         S330C       CGACTACCACTGTTACACGTGCACAGTTGGCTGAAACAC         S333C       GGCTCAACTTACACTGCCA	S192C	CTGGTAAGGGCTTCTGTACTGCTACTGGTAC
S205c       GTACACAAGTACTIGGTGGTGTGTGCACTGATAAGTCAG         S209c       GGTCGTCAACTGATAAGTGCGTTACAGCACGACAGAG         S212c       CTGTCAACTGATAAGTCAGTTACAGCATCCAACGGT         S232c       CGACTGATGGCACTCAAGTTGGTGCACAGCAACACGGG         S246C       GGTACAAAGCAGCACTCAAGTTGAGCACACACTGGGG         S246C       CGATAAAGCCGCGCGATCAATGTGCTCTGAAGGCGACAGGG         S26c       CTACATCAATGCTAAGCCGGTTAACGGTAAGGGC         A264C       CTGCTCTTGAAGCCTAACAGCCTGCGGTTACACTG         T273c       GCCTAATGCTACATGCTACAGGCTTGCAGGGTACACTG         T273c       GCCTAAGCGCACACGGTTGCAAGGGACTCCTGTAAGGTACAGG         S291C       GTTACGCCACAGTGTTGCAAGCAGCAGCAGCTTAC         S291C       GGTCGCTTTGAAGGTCTGCGGGGACTCCTGTTAC         S303C       GGTCGCTTTGAAGGTCTAGCGGGACTCCTGTTAC         S304C       GGACTCCTGTTTGTACTGCATGGACAGCGGAGCGCGGAGC         S305C       GGACTCCTGTTTGTACGCATGGACGCGGAGCCGGAGC         S306C       CCACTGATGCATAGATAGTTGCGCGGACTCACTGATGC         S305C       GGTACACTGGTTGCAGCGGACTCACGGAGCGAGC         S306C       CCACTGATGCGATAAGGAGCTAACGAGCTAACGACCC         S306C       CCACTGATGCGATAAGGAGCTAACGAGCTAACGACCC         S306C       CGATCCCGATTGCGTGCTGCTGTTGCAGCTAACGACACCC         S306C       CGATCCCGTTGTTGCAGCTGGTGCTAAGCACCCCCACACACCACCCC         S3305C       GGTAACCACTGCTGCTGCTGCTATACC	T198C	GGCTTCAGTACTACTGCTACTGGTTGCCAAGTACTTGGTGG
S209C       GGTCTGTCAACTGATAAGTGGGTTACAGCAACCAACG         S219C       CTGTCAACTGATAAGTGGTGTGTGCGCAACCAACGATAACAACG         S222C       CGACTGATGGCACTCAAGTTGGTTGCAACCATTGGG         S232C       CGACTGATGGCACTCAAGGTGGTTGCAACCATTGGG         S246C       GGTACAAAGGCAGGTTGTAAGGTAAGCGATAAGGC         T256C       CCGATAAAGGCCGCCGATCAATGTGCTCTTGAAAGCC         A264C       CTGCTCTTGAAGCCTACATACATGCTGCACCTAGCG         S288C       CTACATCAATGCTTACAAGCCTACCAGGTTACACTG         T273C       GCCTAGCGGTTACACTGGTTGTACACTGGTGCACCTGAGG         S291C       GGTTACCGTATGCTACCAGGTCCTGTTACACTGGTGCAGG         S203C       GGTCGCTTGGAAGCTTGCCAGGCTACCTGTTAC         S303C       GGTCGCTTGTAACATGCTACAGCTGACTGCTGAGC         S303C       GGACTCCTGTTTGAAGCTACAGCTGATGCAACAGCTGATGC         T312C       GGACTCCTGTTTACTACTGCATTGGACAGCTGATGC         T308C       GGACTCCTGTTTACTACTGCATTGACAGCTGATGC         S300C       CCAACGCTAATGATACAGCTGATTGACAACGGTGAACCAC         S300C       CCAACCGCTGATGCAATGAAGGTGCAACAGCTCAATGAACACC         S300C       CCAACGCTCATTGCAGCTGATGCAAGCTAACAGCTCAATAACAGC         T342C       CAACAGTCTTATGCTGCTGGTGTAAGAAGTGCCAATACAACCAC         S330C       GGGTCAACTCATTAGCTGCACGAGTGCAATACAACCAC         T349C       CTAAGTGTGTACAGCTGGTGTGCTCAATTAACTGCATTGGGTGAAAGAGCTAACCAC         T349C	\$205C	GTACACAAGTACTTGGTGGTGGTGCACTGATAAGTCAG
337.2.       CIGTCAACTGATAAGTCAGTTACAGCATGCAACGATAACAGTG         5232C.       CGACTGATGGCACTCAAGTTGGTTGCAACGATAACGG         5246C.       GGTACAAAGGCAGCATCAAATGTGCTTGCAACGATAAGGC         7256C.       CGATAAGGCCGCCGATCAATGTGCTAGCAACACTTGGG         5268C.       CTACATGCTAAGCCTACATGAGTTAACACTG         7273C.       GCCTAGCGGTACAATGTGCTAACACGGGTAACACGGT         7273C.       GCCTAGCGGTTGCAAGGCTTGCGGGTAACACGGTACACGGG         7281C.       CCCTAATGCTGCAGGATGCTTGCGAGGACCCTGTTAC         7291C.       GTTTACGCTACGGTTTGCAAGGCTGCAGGCGCACTCCTGTTAC         7308C.       GGTCGCTTTGAAGGTCTGCCGGGACTCCTGTTAC         7308C.       GGACTCCTGTTTGTACTACTGCATGGCTAACGGCGATGC         7312C.       GGACTCCTGTTTGTACTACTGCATTGGTAACGCTGATGC         7308C.       GGACTCCTGTTTGACAGCTGATGGTAACGGCGATGC         7312C.       GGACTCCTGTTGCAAGCTGATGGTAATGATAGGTGCAGCAACGC         7308C.       CGACTCGTATGCATGCAGCTGATGAAGGTGGCAGTAACGCTGCC         7312C.       CCACCGATGCATTACACTGGTTGTGTCTGTGCAGGCGC         7330C.       CCACGTGATGCATTACACGCTGATTGAAGGTGCAGCTAACGCC         7320C.       CAACAGTCTATGCTGCTGCTGGTTGTAAGGTCGGCGCCAATTAACCAC         7342C.       CTAACGTTAGCTGCTGCTGGTTGTAACGTCTGAGTGAAGGCCCAATCACG         7330C.       CCACAGTCGTGCTCAATTAACATGATTGACGTGAACGGGGGCAATCGTGGTGGCAATTAACGCACT         7342C.       CAAACGGTCATGGTGCCAATTAACT	\$205C	GGTCTGTCA ACTGATA AGTGCGTTA CAGCA ACCA AC
1110.       CGACTGATGGCACTCAAGTTGGTTGCAACACTGGG         5232C.       CGACTGATGGCAGTGTAAGGTTGCAACACTGGGG         5244C.       CGGTACAAAGGCCACGATCAATGTGCAAGCCAAGGC         7256C.       CGATAAAGCCTACAATGGTAACGCTAAGGCCAAGGC         726C.       CTACATCAATGCTAAGCCTACATGGTAACAAGCCTACGG         5282C.       CTACATCAATGCTGCAGATGCTTGTAACAAGCCTACGG         5286C.       CTACATCAATGCTGCAGATGCTTGCAAGCGCTACCTG         7273C.       GCCTAGCGGTTACACTGTTGCAAGCAGCTTACTGCAG         7281C.       CCCCTAATGCTGCCAGGTACCTGTATGGTAACCACAGTTAC         5291C.       GTTTACGCTACTGGTTGCAAGCAGCTCATTGTACGCAG         5303C.       GGTCGCTTTGAAGGTCGCGGGGACTCCTGTTAC         7308C.       GGACTCCTGTTTGTACTGCAGGTGACGCGCGCGCGCGCGC	T213C	CTGTCA ACTGATA AGTCA GTTA CAGCATGCA ACGATA ACAGTG
S22C       CGACTGATAGGCAGGTTGTAAGGTAAGGCATAAGGC         S246C       CGATAAGGCAGGTTGTAAGGTAAGGCATAAGGC         T256C       CGATAAGGCCGCCGATCAATGGTACAGCGATAGGC         A264C       CTGCTCTTGAAGCCTACATCGATTGTAACAGCCTAGCG         S268C       CTACATCAATGCTAACAAGCCTTGCGGTTACACTGG         T231C       GCCTAAGCGTTACACTGATTGTAACCCTAATGCTACGGTACACAAGGTTAC         S291C       GTTACGCTAATGCTGCAGGAGCTCCTGTTAGC         T308C       GGACCCTGTTTGAAGGTCGCGGGACTCCTGTTAC         T308C       GGACTCCTGTTTGTACTACGATTGACAACGCTGATGC         T312C       GGACTCCTGTTACTACGCATGGATGTACGAGGTGCAGC         X300C       CGACTCCTGTTACTACGCATGGAGTGTAAGGTAGCAGC         X300C       CGACTCCTGTTACTACGGCTGCAGGTGAAGGTGCCACC         S333C       GTAGTTCTGCGCAGGCTGCAAGGTTAACGATACCAC         S330C       CCGATACCACTGCTAATGGTTGTCTGTGCGGGC         S330C       CCGATACCACTGCTAATGGTGTGCAGCTAACGATACCAC         S330C       CCGATACCACTGCTAATGGTGCTAACGATACCAC         S330C       CCGATACCACTGCTAATGGTGCTACAGTTGCCAGGCTGACACC         S330C       CCGATACCACTGCTAATGGTGCACAGTTAACGGTGGTGCAGGC         S330C       CCTAGCGGTGATGTGCAGCTGCTGTTGTAAGGTGGCGGC         S330C       CGAATCCGGCTGCTGCTGCTGGTGGTGCTAGCTGGCTGGTGGTGGG         S330C       CGAATCGGACTAACGGCCGCACGAGCTGCCTGGTGCTGGTGGTGGCGGGGTGGAAGGGCCAACGGGGTGAAGGGCTAACGGCTGGCT	1215C \$232C	CGACTGATGCACCAAGTTGCTTGCAACACTGGG
32-90.       COTACAAAGCCGCGATCAATGGTCTTGAAGCC         7256C       .CGATAAGGCCGCCGATCAATGGTCAAGCC         A264C       .CTGCTCTGAAGCCTACAATGGTCAAGCCTAGCG         S268C       .CTACATCAATGCTAACAAGCCTTGGGGTACACTGG         T275C       .GCCTATGCGGGTACACTGATGGTAACACAGGTTACACTG         T273C       .GCCTATGCGGGTACACTGGTAGGTAACACAGTTAC         S291C       .GCCTAATGCTGCAGAGGCTGCCTAGTGGTAACACAGTTAC         S291C       .GGTTACGCGCTTTGAAGGCTGCGGGGACTCCTGTTAC         S303C       .GGTCGCTTTGAAGGTCTGCCGGGACTCCTGTTAC         T308C       .GGACTCCTGTTACTGCCATGGCAGCACAGCTGATGC         A316C       .CTACTGCATTGACTACGCGGATGCTGCAGGTGATACCAC         S300C       .CGATACACACTGCTAATGGTGTGTCGTGCAGGC         S330C       .GGACTCTGTTGCAGGCTGCACAGCTAATGGAACCGATACCAC         S330C       .CGATACCACTGCTAATGGTGTGTGTGAGGCC         S330C       .CGATACCACTGCTAATGGTGGTGGAAGTGACCAC         S330C       .CGATACCACTGCTAATGGTGCCGACAGCTAATCAACG         S330C       .CGAGTACCACTGCTAATGGTGCCACAGCCTAACCAC         S330C       .CGATACCACTGCTAATGGTGCACAGTCAAGGTGGCCGC         S330C       .CGAAGTTCGCTTGGCGGTGGACGCCAAGCGCTAACGAC         S330C       .CGAAGTCCGGTGCTAATGGCGCCAAGGCCGCAACGCCC         S330C       .CGAAGTGCGCTGGCTGGCTGGCTGAAGCGCCAACGCGCGGC         S335C       .GTAGTTCGTGCAAGTGTGCCACTGGCTGCCAATTAACGC	S252C S246C	GGTACA AAGCCAGGTTGTAAGCTAAGCGATAAGCC
A264C       CGGCTTGAAGCCTACATTGATACAAGCCTAGCG         S268C       CTACATCAATGCTAACAAGCCTTACAATTGTAACAAGCCTAGGG         T273C       GCCTAAGCGGTTACACTGGATGTAACACGGATTACACTG         T281C       .CCCTAATGCTACAGTGTAGGAACACACGTTTAC         S303C       GTTTACGCTACTGTTTGCAAGGGCTACCTCTTTAC         T308C       .GGACTCCTGTTTGAAGGCTGACGGGACTCCTGTTAC         T308C       .GGACTCCTGTTACTACTGCATTGGACGGGACTCCTGTTAC         T308C       .GGACTCCTGTTACTACGCATTGGACGGGACTCCAGTGGC         A316C       .CTACTGCATTGCATAGGTATGGTAATGATAAGGTTGCAGC         A316C       .CTACTGCATGCTAATGGTTGTACGGCTGATGGTAAGGAACCAC         S300C       .CGAATACCACTGCTAATGGTTGTACGGCGGGG         S335C       .CGATACCACTGCTAATGGTTGTAAGTTAGCTGCGTGG         S336C       .CTAAGTTGCGGGGGAACTCAGGTGGAAAAGGGTCAATGGACGAACCAC         S330C       .CCAACAGTCTATGCTGGCGGGTGTAAAGTTGGCCAATAACAAC         S336C       .CTAAGTGGTGAAAAGGGTCAATGGTGCACTTAACCACGGG         S336C       .CTAAGTGGTGAAAAGGGTCAATGGTGCACATTAACCACCAC         S330C       .CTAAGTGGTGAAAGGGTCAATGGTTGCACTTAACTGCCATCG         S336C	T256C	CGATA AGGCCGCCGATA AGGCCCCCC
AD9C       CTOCTECTOAAGCCTTGCAGCATCAGCTTGCAGCTAGCCTG         S268C       CTACATCAATGCTGAACAGCCTTGCGGGTTACACTG         T273C       GCCTAGCGGTTACACTGTATGTAACCCTATGCTAAGCCTGG         T281C       CCCTAATGCTGCAGAGTGCTTGCTATGGTAACCACAGTTAC         S291C       GTTTACCGTACTGTTTGCAGCAGCAGCTACTTCTAAGG         S303C       GGTCGCTTTGAAGGTCTGCGGGACTCCTGTTAC         T308C       GGACTCCTGTTACTACTGCATTGCATGAGCTGATGC         T312C       GGACTCCTGTTACTACTGCATTGTAATGATAAGGTTGCAGC         S300C       CTACTGCATGATGACTACAGCTGATGCAACGATACCAC         S302C       CCAATGCTGATGCTAATGATAAGTGTGCAGCTAACGATACCAC         S308C       CTACTGCATGATGATGATAGAGTGTGCAGCTAACGATACGAC         S309C       CCAACTGGTGATGCTAATGATGCAGCAGCACAC         S300C       CCAACAGTCTATGCTACTGCTAGCGAGCCAACGACTACGGCGCCACAGCTATGCAGCAC         S300C       CCAACAGTCTATGCTGCTGGGTGTAAGTTGGCACATTAACGCGCGCG	1250C	
2000C.       GCTACATGCTAATGCTAATGCTACACTGTACGTGCAG         T273C.       GCCTAGCGGTTACACTGTATGTAACCCTAATGCTGCAG         7281C.       CCCTAATGCTGCAGAGTGCTTGCTAGGTAACACCAGTTTAC         S201C.       GTTTACGCTACTGTTTGCAAGGCAGCTCCTGTTAC         T308C.       GGTCGCTTTGAAGGTCTGCGGGACTCCTGTTAC         T308C.       GGACTCCTGTTACTACTGCATTGACAGCTGATGGC         A316C.       CTACGCTGATGCTAATGATAAGGTTGAAGGATGCAGC         V300C.       CGACTCCTGTTACTACTGCAGCTGATGGCAGCTAACGATACCAC         S303C.       CGATACCACTGCTAATGATAAGGTGTGCAGGC         S303C.       CGATACCACTGCTAATGATAAGGTGTGCAGGC         S303C.       CGATACCACTGCTAATGATAAGGTGTGCAGGTGAGTACCAC         S303C.       CGATACCACCTGCTAATGGTGCTGAGCTAACGATACCAC         S303C.       GTAGTTCTGTTGCAGCTGGTGCACAGTCTATGCGCGCGG         S330C.       CGATACCACCACGCTAATGGTGCTCAGTATAGCTGCGGG         S332C.       GTAGTTCTGTTGCAGCTGGTGATAGGTGCTCAATTAACAAC         S42C.       CAACAGTCTATGCTGCTCAATTAACTGCCAACTAACGG         S438C.       GGGTCAAGTTGCCAATTAACGTGACTGACTGATACTGGATACGACTTTGG         S42C.       GACTGGTCAATTACTGCCAATTAACTGCAACTAACGGATACCGCATCG         S439C.       GGGTCAAGTTGCACATTAACTGGCACATACCGATTTGGCTGCTCTTGCAATACGGCATACGGC         S438C.       GGGTCAAGTTGCCCAATTAGTGGCACAGTACCGGATACGGACTACGG         S439C.       GGAACTAACGGCATACGGACTTGCGCTTCACTGGAAAGGACGCACC	A204C	CTACATCA ATCCTA A CCTTCCCCTA CACTG
123C       CCCTAATGCTGCAGATGCTTGCTATGGTAACACAGTTTAC         5291C       GTTTACGCTAATGCTGCTGCTGGCTGCTACCACACACAGTTTAC         5303C       GGTCGCTTTGAAGGTCTGCCGGGACTCCTGTTAC         1308C       GGACTCCTGTTGTTGCAAGGTGACAGCTGACGC         7312C       GGACTCCTGTTGTACTGCAATGGTAACGACTGATGC         7312C       GGACTCCTGTTACTACCGCATTGTGACACGCTGATGC         7312C       CGACTCCTGTTACTACAGCTGATAGGATACAGCTGATGC         7312C       CGACTCCTGTTAGCAAAGCTGTGACAGCTGAATGGATACGACACGATACCAC         7320C       CCACTGCTAATGATAAGTGTGCACGCTAACGATACCACTACCAC         7330C       CCGATACCACTGCTAATGATAAGTGTGCACGCTGATGCAGCGC         7342C       CAACAGTCTATGCTGCTGGTTGTAAGTTGGCTCAATTAACAAC         7349C       CCTAAGTTGGCTCAATTAACATGGACTTGACTGGGTGAAAAGG         V358C       GGGTCAAGTTGGCCACATTAACACTGCCATCGATTTGGG         7362C       GGGGTCAAGTTGGCCACATTATGGCCACCGATACTGATTTGG         7366C       GGCACACATTAACTGCCATCGATTGTGGAACAGCGCTACG         7376C       GGAACTACGACTTACTGCTTCACTGGAACAAGGAACGCTACC         7390C       CAATAAGGACTGTCCTGCTTCATTGGTAAGCAATAAGGAACCC         7390C       CAATAAGGACTGCTGCTTCATTGGTTCAAATTAACAGCAAGAAGCAACCC         7390C       CAATAAGGACTACCACTACTGCTTTCAATTGCAAGTAAGGAAGCACTACCC         7390C       CAATAAGGACTGCCTGCTTGTTCAAATGCAAGTAACCC         7400C       CAATAAGGACTACCACTACCGTTGTTCAATGGCAAGTACCAC	5200C	
1201C	T2/JC	
S291C.       GGTCGCTTTGAAGGTCTGCAGGGACTCCTGTTAC         T308C.	1201C	
3505c	5291C	
1308C	5303C	
1312C       CTACTGCATTGACTACAGCTGATTGTAATGATAAGGTGCAGC         A316C       CAGCTGATGCTAATGATAAGTGTGCAGCTAACGATACCAC         S330C       CGATACCACTGCTAATGGTTGTTCTGTTGCAGGC         S335C       GTAGTTCTGTTGCAGGCTGCACAGTCTATGCTGGTGG         T342C       CAACAGTCTATGCTGCTGGGTTGTAAGTTGGCTCAATTAACAAC         T349C       CTAAGTTGGCTCAATTAACATGTGACTGGAAAAAGG         V358C       GACTGGTGAAAAGGGTCAATGTGCACATTAACTGCCATC         T362C       GGGTCAAGTTGTCACATTATGTGCCATCGATACTGATTGG         T362C       GGGGTCAAGTTGACTGCACATTAACTGCCATCGATACGATTTGG         T372C       CACCGATACTGATTGCACGCTTGCTCACTGGAACTACGGACTACG         T372C       CACCGATACGATTCACTGGATGTGGCATCGGAACTACCACTAC         T390C       GGAACTACGACTTACCACTACTGTACAGTACGACTTACCATACAGAGCACTACC         S397C       CAATAAGGACTGCCGCTGCTTCTCAAATGCAAGTACCAC         S400C       CAATAAGGACTGCTGCTTCTTCAAATGCAAGTACCAC         S409C       GTACCCAATTAGGTGCACGTGCTGCTTGTTCAAATGCAAGTACCC         S409C       GTACCCAATTGGTTGCACGTGCTACTGGTACTTACGTACCTAC         T418C       GTACCCAATTGGTACTGCTACCTGCTACTGTTAACGGAACTACC         S409C       GTACCCAATTGGTACTGCTACCTGCTAACC         S409C       GTACCCAATTGGTACTTGACTGCTACCTGGTACCTGCTACC         S409C       GTACCCAATTGGTACTTGGTACCTGCTACTGCTACCTGTAACGGTAAGTC         S4092       GTACCCCATTGGTACTTGCACGGTACTTAACTGCTACC	T 308C	
A316C       CTACTGCATTGCATGCATGCATGCAGGCTAATGCTAACGCTGATGCTAACGCT         V320C       CGATACCACTGCTAATGGTTGTTCTGTTGCAGGC         S330C       GTAGTTCTGTTGCAGGCTGCACAGTCTATGCTGCTGG         S342C       CAACAGTCTATGCTGCTGGTTGTAAGTTGGCCACTGCTGG         T342C       CCAACAGTCTATGCTGCTGGTTGTAAGTTGGCCACTGATAACAGC         T349C       CTAAGTTGGCTCAATTAACATGTGACTGACTGACAAAAGG         V358C       GACTGGTGAAAAGGGTCAATGTGTCACATTAACTGCCATC         T362C       GGGTCAAGTTGTCACATTAACTGCCATCGATACTGATTTGG         T366C       GTCACATTAACTGCCATCGATTGTGACTGGAAGAGCGCTACG         T372C       CATCGATACTGATTGGAGAGACGCTTGCTCACTGGAACTACG         T376C       GACCGTACGTTCACTGGATGTGCACTTACTATTCAGATC         L383C       GGAACTACGACTTACTATTCAGATTGTGGTAAAGGACAGTGC         S397C       CAATAAGGACATACCACTACCACTACTGTTACAATAAGGACAGTGC         S400C       CAATAAGGACAGTGCTGCTTCTTCAAATGCAAGTACCACTACC         S400C       CAATAAGGACAGTGCTGCTTGCTTCTAAATGCAAGTACCC         S400C       GTACCCAATTTGGTTGCAACGTCACTGGTACTTTAACTGCTACC         T405C       GTACCCAATTGGTTGCAACGTCACCTGGTACTTTAACTGCTACCT         S400C       CAATAAGGACAGTGCTGCTTCTTCAAATGCAAGTACCC         S400C       CAATAAGGACAGTGCTGCTTGTTAACTGCACTTGGTTCAAACG         S409C       GTACCCAATTTGGTTGCAACGTCACTGGTACTTTACCTGTACCGTGCTACCTGTTATACTGCTACC         T414C       CAAACGTCACTGGT	1312C	
V30CCAGC IGAIGCIAAIGATAAGIGIGCAGCIAACGATACCACS330CCGATACCACTGCTAATGGTTGTGCAGGCS332CGTAGTTCTGTTGCAGGCTGCACAGTCTATGCTGCTGGS332CCTAAGTTCGTTGCAGGCTGCACAGTCATGGCTGGTGAAAAGGT342CCAACAGTCTATGCTGCTGGTGAAAGTGGACTGACTGGACTGGTGAAAAGGV358CGACTGGGTGAAAAGGGTCAATGTGCACATTAACTGCCATCT362CGGGTCAAGTTGTCACATTATGTGCCATCGATACTGATTTGGT366CGTCACATTAACTGCCATCGATTGTGACTGGAAGACGCTACGT372CCATCGATACTGATTTGGAAGACGCTTACTGTACAGTACCGT376CGGACGCTACGTTCACTGGATGTACGACTTACTATTCAGATCL383CGGAACTACGACTTACTATTCAGATTGTGGTAAAGCATACCACTACT390CGGAACTACGACTACCACTACTGTTACACTTACAATACGAAGTACCACTACS307CCAATAAGGACATACCACTACTGTTCTTCAAATGCAAGTACCCS400CCAATAAGGACAGTGCTGCTTGTTCAAATGCAAGTACCCT405CGTACCCAATTTGGTTGCAACTGGTACTTACAATTGGTTCAAACGS409CGTACCCAATTTGGTTGCAACTGGTACTTTAACTGCTACT414CCAAACGTCACTGGTTGTTAACTGCTACTGGTACTTAACGTACT4142GTCACTGGTACTTTAACTGCTACCTTGTTAAGGTAAGTCS424CCCCTTGTTATGGGTAAGTGTACTGGTTCAACT431CGTAAGTCTACTGCTACTGCTACTGGTTACTTGGTTCAACC	A310C	
S330C	V 320C	
S3352	\$330C	
1342CCAACAGTCTATGCTGCTGGTTGTAAGTTGGCTCAATTAACAAGTT349CCTAAGTTGGCTCAATTAACAGTGTGACTTGACTGATAACAGGGT349CGACTGGTGGAAAAGGGTCAATTAACTGACTGACTGATAGTGCAATGV358CGACTGGTGAAAAGGGTCAATGTGTCACATTAACTGCCATCT362CGGGTCAAGTTGTCACATTATGTGCATCTGATTTGGT366CGTCACATTAACTGCCATCGATACTGATTTGGAAGACGCTACGT372CCATCGATACTGATTTGGAAGACGCTTACTGGAACTACGT376CGACGCTACGTTCACTGGATGTACGACTTACTATTCAGATCL383CGGAACTACGACTTACTATTCAGATTGTGGTAAAGCATACCACTACT390CGGTAAAGCATACCACTACTGTTACACTTACAATAAGGACAGTGCS397CCAATAAGGACTGTGGTGCTGCTTCTTCAAATGCAAGTACCCS400CGTGCTGCTTCTTCAAATGCAAGTACCACT405CGTGCTGCTTCTTCAAATGCAAGTACCACT414CCAAACGTCACTGGTTGTTGCAACGTCACTGGTACTTTAACTGCTACT418CGTCACTGGTACTTTAACTGCTTACCTTGTTATGGGTAAAGTCS424CCCCTTGTTATGGGTAAGTGCTACTGCTACTGCTAACT431CGTAAGTCTACTGCTACTGCTACTGGTTGACTTGGTTCAAC	5335C	
1349CCTAAGTIGGCTCAATTAACATGTGACTIGACTIGACAAGGV358CGACTGGTGAAAAGGGTCAAATGTGTCACATTAACTGCCATCT362CGGGTCAAGTTGTCACATTATGTGCCATCGATACTGACTTGGGT366CGTCACATTAACTGCCATCGATTGTGATTTGGAAGACGCTACGT372CCATCGATACTGATTTGGAAGACGCTTGCTTCACTGGAACTACGT376CGACGCTACGTTCACTGGATGTACGACTTACTATTCAGATCL383CGGAACTACGACTTACTATTCAGATTGTGGTAAAGGACAGTGCT390CGGTAAAGCATACCACTACTGTTACACTTACAATAAGGACAGTGCS397CCAATAAGGACTGTGCTGCTTCTTCAAATGCAAGTACCCS4000GTGCTGCTTCTTCAAATGCAAGTACCCT405CGTGCTGCTTCTTCAAATGCAAGTACCACTACT414CCAACGTCACTGGTTGCTGCTTGTTCAAATGCAAGTACCGS409CGTACCCAATTTGGTTGCAACGTCACCTTGTTAGGGTACTTTAACTGCTACT414CCAAACGTCACTGGTTGTTTAACTGCTACCTTGTTATGGGTAAGTCS424CCCCTTGTTATGGGTAAGTGCAACTGCTAACT431CGTAAGTCTACTGCTACTGCTAACTGGTTGACTTGGTTCAAACG	1342C	
V38CGACIGGIGAAAAGGGICAAIGIGICACATTAACIGCCATCT362CGGGTCAAGTTGTCACATTATGTGCCATCGATTACTGATTTGGT366CGTCACATTAACTGCATCGATTGTGAAGACGCTACGT372CCATCGATACTGATTTGGAAGACGCTTACTATTCAGGACTACGT376CGACGCTACGTTCACTGGATGTACGACTTACTATTCAGATCL383CGGAACTACGACTTACTATTCAGATTGTGGTAAAGCATACCACTACT390CGGTAAAGCATACCACTACTGTTACACTTACAATAAGGACAGTGCS397CCAATAAGGACTGTGCTGCTTCTTCAAATGCAAGTACCCS400CGTGCTGCTTCTTCAAATGCAAGTACCCT405CGTACCCAATTAGGACGTGCTGCTGCTGCTACAATTGGTTCAAACGS409CGTACCCAATTGGTTGCAACGTGACTTTAACTGCTACGTACT414CCAAACGTCACTGGTTGTTTAACTGCTACCTTGTTATGGGT418CGTCACTGGTACTTTAACTGCTTGCTACTGGTAAGGCS424CCCCTTGTTATGGGTAAGTGCAACTGCTAACT431CGTAAGTCTACTGCTACTGCTAACTGGTTCAAC	1349C	
T362C       GGGTCAAGTIGICACATTAIGIGCCATCGATTGGATACIGATITGGG         T366C       GTCACATTAACTGCCATCGATTGTGATTTGGAAGACGCTACG         T372C       CATCGATACTGATTTGGAAGACGCTTACTACTGGAACTACG         T376C       GACGCTACGTTCACTGGATGTACGACTTACTATTCAGATC         L383C       GGAACTACGACTTACTATTCAGATTGTGGTAAAGCATACCACTAC         T390C       GGTAAAGCATACCACTACTGTTACAATGCAAGTACCC         S400C       CAATAAGGACAGTGCTGCTTCTTCAAATGCAAGTACCC         S400C       GTGCTGCTTCTTCAAATGCAAGTACCC         S400C       GTGCTGCTTCTTCAAATGCAAGTACCC         S400C       GTACCAATTAGGACAGTGCTGCTTGTTCAAATGCAAGTACCC         T414C       GTACCAATTGGTTGCAACGTCACTTGGTACTTTAACTGCTACGGA         T414C       CAAACGTCACTGGTTGTTTAACTGCTACCTTGTTATGGGTAAGTC         S424C       GTCACTGGTACTTTAACTGCTTGCCTGCTACTGCTAAC         T431C       GTAAGTCTACTGCTACTGCTACTGGTTCAACG	V358C	
T366CGTCACATTAACTGCCATCGATTGGATTTGGAAGACGCTACGT372CCATCGATACTGATTTGGAAGACGCTTGCTTCACTGGAACTACGT376CGACGCTACGTTCACTGGATGTACGACTTACTATTCAGATCL383CGGAACTACGACTTACTATTCAGATGTGGTAAAGCATACCACTACT390CGGTAAAGCATACCACTACTGTTACACTTACAATAGGACAGGCGS397CCAATAAGGACTGTGCTGCTTCTTCAAATGCAAGTACCCS400CCAATAAGGACAGTGCTGCTTGTTCAAATGCAAGTACCCT405CGTGCTGCTTCTTCAAATGCAAGTACCCT414CCAACCCAATTTGGTTGCAACGTCACTTGGTACACTTAACTGCTACT414CCAAACGTCACTGGTTGTTTAACTGCTACCCTTGTTATGGGT418CGTCACTGGTACTTTAACTGCTTGCTTATGGGTAAGTCS424CCCCTTGTTATGGGTAAGTGTACTGCTACTGCTAACT431CGTAAGTCTACTGCTACTGCTAACTGGTTCAAC	T362C	GGGICAAGIIGICACAIIAGIGCCAICGAIACIGAIIIGG
T37/2C       CATCGATACTGATTTGGAAGACGCTTGCTTCACTGGAACTACG         T376C       GACGCTACGTTCACTGGATGTACGACTTACTATTCAGATC         L383C       GGAACTACGACTTACTATTCAGATTGTGGGAACACCACTAC         T390C       GGTAAAGCATACCACTACTGTTACACTTACAATAAGGACAGTGC         S397C       CAATAAGGACTGTGCTGCTTCTTCAAATGCAAGTACCC         S400C       CAATAAGGACAGTGCTGCTTGTTCAAATGCAAGTACCC         T405C       GTGCTGCTTCTTCAAATGCAAGTACCC         T414C       GTACCCAATTTGGTTGCAACGTCACTGGTACTTTAACTGCTAC         T418C       GTCACTGGTACTGGTTGTTAACTGCTACCCTTGTTATGGG         T418C       GTCACTGGTACTTTAACTGCTTGCCTTGTTATGGGTAAGTC         S424C       CCCTTGTTATGGGTAAGTGTACTGCTACTGCTAAC         T431C       GTAAGTCTACTGCTACTGCTAACGGTTGACTTGGTTCAAC	Т366С	
T37/6C       GACGCTACGTTCACIGGATGTACGACTTACTATTCAGATC         L383C       GGAACTACGACTTACTATTCAGATTGTGGGAAAGCATACCACTAC         T390C       GGTAAAGCATACCACTACTGTTACACTTACAATAAGGACAGTGC         S397C       CAATAAGGACTGTGCTGCTTCTTCAAATGCAAGTACCC         S400C       CAATAAGGACAGTGCTGCTTGTTCAAATGCAAGTACCC         T405C       GTGCTGCTTCTTCAAATGCAAGTACCC         T405C       GTGCTGCTTCTTCAAATGCAAGTTGCCAATTTGGTTCAAACG         S400PC       GTACCCAATTTGGTTGCAACGTCACTGGTACTTTAACTGCTAC         T414C       CAAACGTCACTGGTTGTTTAACTGCTACCCTTGTTATGGG         T418C       GTCACTGGTACTTTAACTGCTTGCCTTGTTATGGGTAAGTC         S424C       CCCTTGTTATGGGTAAGTGTACTGCTACTGCTAAC         T431C       GTAAGTCTACTGCTACTGCTAACGGTTGACTTGGTTCAAC	T372C	
L383C       GGAACTACGACTTACTATTCAGATTGTGGTAAAGCATACCACTAC         T390C       GGTAAAGCATACCACTACTGTTACACTTACAATAAGGACAGTGC         S397C       CAATAAGGACTGTGCTGCTTCTTCAAATGCAAGTACCC         S400C       CAATAAGGACAGTGCTGCTTGTTCAAATGCAAGTACCC         T405C       GTGCTGCTTCTTCAAATGCAAGTAGCCAATTGGTTCAAACG         S409C       GTGCTGCTTCTTCAAATGCAAGTTGCCAATTGGTTCAAACG         T414C       GTACCCAATTTGGTTGCAACGTCACCTTGTTAACTGCTAC         T418C       GTCACTGGTACTTTAACTGCTTGCCTTGTTATGGGTAAGTC         S424C       CCCTTGTTATGGGTAAGTGTACTGCTACTGCTAAC         T431C       GTAAGTCTACTGCTACTGCTAACGGTTGACTTGAAC	Т376С	
T390C	L383C	GGAACTACGACTTACTATTCAGATTGTGGTAAAGCATACCACTAC
S397CCAATAAGGACIGIGCIGCITCITCAAATGCAAGTACCC S400CCAATAAGGACAGTGCTGCTTCITCAAATGCAAGTACCC T405CGTGCTGCTTCTTCAAATGCAAGTTGCCAATTGGTTCAAACG S409CGTACCCAATTTGGTTGCAACGTCACTGGTACTTTAACTGCTAC T414CCAAACGTCACTGGTTGTTTAACTGCTACCCTTGTTATGGG T418CGTCACTGGTACTTTAACTGCTTGCCTTGTTATGGGTAAGTC S424CCCCTTGTTATGGGTAAGTGTACTGCTACTGCTAAC T431CGTAAGTCTACTGCTACTGCTAACGGTTGACTTGGTTCAAC	Т390С	
S400C       CAATAAGGACAGTGCTGCTTGTTCAAATGCAAGTACCC         T405C       GTGCTGCTTCTTCAAATGCAAGTTGCCAATTTGGTTCAAACG         S409C       GTACCCAATTTGGTTGCAACGTCACTGGTACTTTAACTGCTAC         T414C       CAAACGTCACTGGTTGTTTAACTGCTACCCTTGTTATGGG         T418C       GTCACTGGTACTTTAACTGCTTGCCATGGTAGTTATGGGTAAGTC         S424C       CCCTTGTTATGGGTAAGTGTACTGCTACTGCTAAC         T431C       GTAAGTCTACTGCTACTGCTAACGGTTGTTCAAC	S397C	CAATAAGGACTGTGCTGCTTCTTCAAATGCAAGTACCC
T405C       GTGCTGCTTCTTCAAATGCAAGTTGCCAATTTGGTTCAAACG         S409C       GTACCCAATTTGGTTGCAACGTCACTGGTACTTTAACTGCTAC         T414C       CAAACGTCACTGGTTGTTTAACTGCTACCCTTGTTATGGG         T418C       GTCACTGGTACTTTAACTGCTTGCCTTGTTATGGGTAAGTC         S424C       CCCTTGTTATGGGTAAGTGTACTGCTACCGCTACC         T431C       GTAAGTCTACTGCTACCGCTACTGGTTCAAC	S400C	CAATAAGGACAGTGCTGCTTGTTCAAATGCAAGTACCC
S409CGTACCCAATTTGGTTGCAACGTCACTGGTACTTTAACTGCTAC T414CCAAACGTCACTGGTTGTTTAACTGCTACCCTTGTTATGGG T418CGTCACTGGTACTTTAACTGCTTGCCTTGTTATGGGTAAGTC S424CCCCTTGTTATGGGTAAGTGTACTGCTACTGCTAAC T431CGTAAGTCTACTGCTAACGGTTGTACTTGGTTCAAC	T405C	GTGCTGCTTCTTCAAATGCAAGTTGCCAATTTGGTTCAAACG
T414CCAAACGTCACTGGTTGTTTAACTGCTACCCTTGTTATGGG T418CGTCACTGGTACTTTAACTGCTTGCCTTGTTATGGGTAAGTC S424CCCCTTGTTATGGGTAAGTGTACTGCTACTGCTAAC T431CGTAAGTCTACTGCTAACGGTTGTACTTGGTTCAAC	S409C	GTACCCAATTTGGTTGCAACGTCACTGGTACTTTAACTGCTAC
T418CGTCACTGGTACTTTAACTGCTTGCCTTGTTATGGGTAAGTC S424CCCCTTGTTATGGGTAAGTGTACTGCTACTGCTAAC T431CGTAAGTCTACTGCTAACGGTTGTACTTGGTTCAAC	T414C	CAAACGTCACTGGTTGTTTAACTGCTACCCTTGTTATGGG
S424CCCCTTGTTATGGGTAAGTGTACTGCTACTGCTAAC T431CGTAAGTCTACTGCTAACGGTTGTACTTGGTTCAAC	T418C	GTCACTGGTACTTTAACTGCTTGCCTTGTTATGGGTAAGTC
T431CGTAAGTCTACTGCTAACGGTTGTACTTGGTTCAAC	S424C	CCCTTGTTATGGGTAAGTGTACTGCTACTGCTAAC
	T431C	GTAAGTCTACTGCTACTGCTAACGGTTGTACTTGGTTCAAC

<sup>*a*</sup> An asterisk denotes a sequencing oligonucleotide. For mutagenesis oligonucleotides, only the forward oligonucleotide is shown; reverse oligonucleotides are reverse complements of the forward sequences shown.

using cell wall fragments without protein as the background pellet. Each sample was measured in 3-h sequences, and one to seven intensity curves were summed together, depending on the sample.

Solvent accessibility of monomeric mutant proteins. Monomeric, DTT-free protein solutions were obtained by centrifuging the protein suspensions for 15 min at  $16,000 \times g$ , washing the protein pellet twice with 10 mM Tris-HCl (pH 7.5), and collecting the last supernatant. These preparations were immediately labeled with maleimide-containing reagents.

The cysteine-containing proteins in monomeric form were modified with branched methyl-capped polyethylene glycol (mPEG)-maleimide TMM(PEG)<sub>12</sub> (molecular weight [MW] = 2,360.75; Pierce) by using the molecular ratio of 1:100. In a 24-µl reaction mixture, protein was used at the molarity of 1.75 µM, mixed with 175 µM of TMM(PEG)<sub>12</sub>, and incubated at room temperature for 5 min or 1 h. The reaction was stopped by adding *N*-ethyl maleimide (NEM; Sigma-Aldrich) in 60-fold molar excess. The proteins modified were visualized with

Coomassie blue R-250 stain (Santa Cruz Biotechnology) and analyzed using Alpha Ease FC (Alpha Innotech) or AIDA image analyzer (Raytest) software.

The cysteine-containing proteins in monomeric form were modified with AlexaFluor488 C<sub>5</sub>-maleimide (MW = 720.66; Molecular Probes) using the molecular ratio of 1:1. In a 48-µl reaction mixture, each protein was used at a molarity of 1.75 µM and the reaction mixture was incubated on ice for 10 s, 30 s, or 120 s. The reaction was stopped by adding NEM in 600-fold molar excess. The proteins modified were run on SDS-PAGE gels without  $\beta$ -mercaptoethanol. The bands were visualized using the FLA-5100 imaging system with FLA Fluor Stage 4046 tray and 473-nm laser (Fujifilm) and analyzed with AIDA Image Analyzer software.

Solvent accessibility of cell wall-bound mutant proteins. Isolated cell wall fragments of *L. brevis* ATCC 8287 and the cell wall-bound protein preparations were obtained as described previously (3). The cell wall-bound cysteine-containing proteins were modified with  $TMM(PEG)_{12}$  using the calculated amount of 2  $\mu$ g of each mutant protein on the cell wall fragments and the molecular ratio of



FIG. 1. Electron micrographs of negative-stained self-assembly products of heterologously expressed, His-tagged nonmutated SlpA (A) and SlpA mutant proteins S182C (B), S232C (C), and A264C (D) show the same type of oblique lattice.

1:100 [protein/TMM(PEG)<sub>12</sub>] in the total reaction mixture volume of 16  $\mu$ l. The reaction mixtures were incubated for 1, 3, or 5 min at room temperature and stopped by NEM as described above, and the modified proteins were analyzed by SDS-PAGE as described for the monomeric proteins.

Analysis of primary amino acid sequences. The predictions of the cleavage sites of trypsin were obtained using the PeptideCutter program of the ExPASy server (http://au.expasy.org/tools/peptidecutter) (9). The predictions of second-ary structure and surface accessibility, as well as the pI value, were obtained using the PredictProtein program (www.predictprotein.org) (22). In addition, SA BLE (http://sable.cchmc.org) and JNET (www.compbio.dundee.ac.uk/~www-jpred) predictions of surface accessibility were compared with the results obtained in this work.

### RESULTS

**Construction of mutant proteins.** We generated a series of 46 mutant proteins of the *L. brevis* ATCC 8287 S-layer protein SlpA in which a single amino acid residue was changed into a cysteine. All the mutant proteins were derived from a mature wild-type 435-amino-acid SlpA lacking the signal peptide but containing an N-terminal His-tag sequence.

All the mutant proteins were generated by site-directed, PCR-based mutagenesis and were verified by DNA sequencing. We targeted mostly serine and threonine, polar residues often concentrated on the surface of proteins (15, 33) and abundant in the SlpA protein sequence. In addition, the conservative replacement of serine or threonine with cysteine is likely to cause minimal changes to the protein self-assembly process (10). Of the 46 mutant proteins generated, all contained only the single residue substitution desired in their coding region, except for one mutant (S246C) protein, which contained an additional spontaneous, but benign, point mutation (T348A).

The proteins were produced using IPTG induction in the *E. coli* BL21(DE3) expression strain and purified by affinity chromatography using His-tag columns. The average protein yield from a 200-ml culture was  $\sim$ 5 mg, and the purity of the mutant proteins was verified by SDS-PAGE. Under reducing conditions (β-mercaptoethanol or DTT), the migration pattern of all



FIG. 2. Small-angle X-ray scattering intensities of SlpA cysteine mutant proteins on the *Lactobacillus brevis* cell wall fragments. The curves from top to bottom: nonmutated SlpA and S145C, A187C, T256C, T281C, S291C, T312C, T362C, L383C, and T405C mutant proteins.

the mutant proteins was identical to that of wild-type SlpA, corresponding to a 46-kDa protein. In the absence of reducing agents, an additional minor band of  $\sim 100$  kDa was observed in all the mutant protein samples and likely represented a dimeric protein formed through the cysteine sulfhydryl groups.

Analysis of the lattice formed by the wild-type and mutant proteins by EM and SAXS. To test whether the SlpA mutant proteins had the capacity to form self-assembly products, denatured proteins were dialyzed and the resulting precipitates were analyzed by negative staining and EM. A regular pattern of oblique lattice symmetry essentially identical to that formed by nonmutated SlpA (Fig. 1A) was detected for all the SlpA mutant proteins. Examples of such lattice formation are shown in Fig. 1B, C, and D.

The ability of nonmutated SlpA to form lattice on cell wall fragments has been examined by cryo-EM and SAXS analysis (P. Jääskeläinen et al., unpublished data). To validate the ability of SlpA mutant proteins to form lattice in a similar manner, selected SlpA mutants were analyzed with SAXS, the results of which are summarized in Fig. 2. All SAXS intensity curves showed indistinguishable diffraction maxima, indicating that all of the mutant proteins examined displayed crystal properties essentially identical to those of the nonmutated SlpA.

Solvent accessibility of monomeric mutant proteins. To assess the surface accessibility of the residues altered by cysteine substitution, the SlpA mutant proteins were subjected to sulfhy-dryl-specific modification as monomeric proteins in an aqueous solution. Two differing modification systems were used: a branched mPEG-maleimide reagent, TMM(PEG)<sub>12</sub>, with a molecular mass of ~2.4 kDa and a smaller labeling reagent, Alexa-Fluor488-maleimide, with a molecular mass of 0.7 kDa. Both markers are hydrophilic, making them more likely to react with residues localized on the outer surface of the protein.

The extent to which the monomeric proteins were modified by labeling with TMM(PEG)<sub>12</sub> was monitored at the 5-min and 1-h time points by SDS-PAGE (Fig. 3, PEG-mono), and the TMM(PEG)<sub>12</sub>-modified SlpA proteins (~48.5 kDa) were distinguished from nonmodified proteins (46 kDa) by differential



FIG. 3. Examination of the surface accessibility of cysteine sulfhydryl groups of SlpA monomers in solution and proteins assembled on cell wall fragments using a gel shift assay. PEG-maleimide modification of proteins as monomers (PEG-mono) and assembled on cell wall fragments (PEG-CW) yield a band migrating slower (B) than nonmodified SlpA (A) on an SDS-PAGE gel. Modification with Alexa-Fluor488-maleimide yields a band that is not distinguishable from that of a nonmodified protein by migration. However, modified protein can be visualized with a fluorescent filter (Alexa-1) and the band can be compared with the protein band visualized with Coomassie stain (Alexa-2). A minor band corresponding to the protein dimer (C) is visible especially in nonmodified samples. The bands of mutant T366C are shown here as an example.

migration. The results of the 5-min reaction are summarized in Fig. 4A and generally indicated that the modification reaction was rapid. In 11 of the 46 mutant proteins studied, more than 75% of the protein molecules were already modified after 5 min of incubation. However, the differences in the speed and extent of modification between individual mutant proteins were noticeable, e.g., eight mutant proteins were less than 25% modified after 5 min of incubation. The amount of modification also varied notably between mutant proteins with cysteine residues located close to each other in the primary amino acid sequence (e.g., mutant proteins T273C/T281C, A316C/V320C, and T372C/T376C). After 1 h of incubation, the differences between mutant proteins were less pronounced, as 42 of 46 mutant proteins were at least 50% modified. However, two mutant proteins, T281C and V320C, remained less than 25% modified even after 1 h.

Monomeric proteins labeled with AlexaFluor488-maleimide for 10, 30, or 120 s were analyzed by SDS-PAGE and visualized under 473-nm light with a fluorescent filter. Fluorescence signal levels were compared with protein concentrations determined by Coomassie staining of the same gels (Fig. 3, Alexa-1 and Alexa-2, respectively). Due to a much higher reaction rate with AlexaFluor488-maleimide than with the larger PEG-maleimide, several proteins reached their maximal modification state in 10 s, and after 30 s, further modification was negligible. The results of the 30-s reaction are shown in Fig. 4B. Compared with PEG-maleimide, the modification of cysteine residues with AlexaFluor488-maleimide was generally either very strong or almost minimal. The difference between the residues located close to each other (e.g., T308C/T312C and V358C/ T362C) was even more pronounced when modified with AlexaFluor488. Still, the most extensively modified residues were the same using both modifying reagents, as were the least modified residues. Taking the two experiments together, 24 of the 46 (52%) mutant proteins tested had cysteine residues that were highly accessible (on average, modified >67% of the most modified residue), while 10 (22%) were moderately accessible (modified 33 to 67% of the most-modified residue) and 11 (24%) were poorly accessible (modified <33% of the most-modified residue). One mutant (T281C) had a cysteine residue that was classified as totally inaccessible in the conditions used.

**Solvent accessibility of cell wall-bound mutant proteins.** To determine the surface accessibility of the proteins attached to the cell wall, mutant SlpA proteins assembled on the surfaces of isolated cell wall fragments of *L. brevis* were modified with PEG-maleimide. The labeling reactions (1, 3, or 5 min) were carried out with molecular ratios similar to that for the monomeric protein experiment and analyzed by SDS-PAGE (Fig. 3, PEG-CW). All of the mutant proteins were still in the linear range of modification between the 3-and 5-min time points.

A summary of the results of the 5-min reaction is shown in Fig. 4C. Overall, 9 mutant proteins (20%), all with residues classified as highly accessible in the monomer experiment, had residues regarded as very accessible (modified at least 50% of the most-modified residue), while 7 (15%) were moderately accessible (modified 20 to 50% of the most-modified residue), 7 (15%) poorly accessible (modified 0.1 to 20% of the most-modified residue), and 23 mutant proteins (50%) had residues that were entirely inaccessible in the conditions used. Repeated experiments on mutant proteins with very accessible residues yielded practically identical results, indicating very good reproducibility.

Figure 5 depicts the comparison of the mutant proteins modified with the three different labeling reactions. Overall, the results from the three labeling reactions correlate with one another. The self-assembly domain of SlpA can be divided into different segments of approximately 20 to 30 amino acids based on their accessibility. These segments have fairly well-defined boundaries, as indicated by the fact that in several cases residues located close to each other can have very different values of accessibility (e.g., A316C/V320C and T372C/T376C).

All 12 residues that were poorly accessible in protein monomers were also poorly accessible in cell wall-bound proteins, indicating a location in the protein interior. Contrastingly, the residues most accessible to modification in the cell wall-bound SlpA clustered into four segments located in the middle of the self-assembly domain: residues 256 to 273, 303 to 316, 335 to 349, and 362 to 372. Notably, a cluster between residues 400 to 414, while accessible in the monomer, was poorly accessible in the cell wall-bound SlpA.

Nine mutated residues that are located in the cell wallbinding domain (1 to 178) were highly or moderately accessible in the monomeric SlpA but almost completely inaccessible in the cell wall-bound protein. In addition, residues located at the N-terminal end (S179C, S182C, and A187C) of the self-assembly domain (179 to 435) shared the same pattern of accessibility, suggesting that in the protein lattice, these residues are located in the inner surface of the S-layer lattice facing the cell wall. Located deeper within the self-assembly domain, residues S192C, T198C, S205, S209, and T213C also showed remarkably lower accessibility as cell wall-bound protein than as monomeric proteins. Moreover, several other residues (S232, T273C, S291C, S400C, T405C, and S409C) displayed a prominent decrease in





FIG. 4. Accessibility of the sulfhydryl group of cysteine in the SlpA mutant proteins modified. Monomeric proteins in solution modified with PEG-maleimide for the 5-min reaction (A) and AlexaFluor488-maleimide for the 30-s reaction (B). (C) Proteins assembled on cell wall fragments modified with PEG-maleimide (5-min reaction). Bars indicate the proportion of modified protein from total protein (A and C) or the intensity of the fluorescence marker signal compared with the highest signal observed (B).

accessibility from monomeric to cell wall-bound protein, suggesting their localization at the protein-protein interface.

## DISCUSSION

To better understand the molecular structure of the L. brevis S-layer protein SlpA and the roles of individual amino acid residues within the structure, we generated a series of proteins in which a single amino acid residue was replaced with cysteine. All 46 mutant proteins generated were able to form self-assembly products in an aqueous solution, indicating that mutations introduced do not markedly affect the conformation of SlpA. In contrast, a similar study of *G. stearothermophilus* S-layer protein SbsB reported that three of 75 mutant proteins showed reduced ability to form regular lattices. However, due to random PCR errors and a restriction enzyme-dependent screening method, all SbsB mutant proteins contained another amino acid change adjacent to the cysteine, possibly affecting their lattice-forming properties (10).

The high number of surface-accessible residues in monomeric



FIG. 5. Comparison of the surface accessibility of mutated amino acid residues modified. Relative accessibility is given as a value between 0 and 1 and denotes the proportion of protein that is modified after 5 min (PEG-maleimide) or 30 s (AlexaFluor488-maleimide) of reaction from the total protein. The solvent accessibility of cysteine residues in protein monomers is shown as dotted black (PEG-maleimide) and solid gray (AlexaFluor488-maleimide) lines. The accessibility of residues in cell wall-bound proteins modified with PEG-maleimide is shown as a white line.

protein is in accordance with results derived from *G. stearothermophilus* SbsB (10). All of the residues that were almost or totally inaccessible in the monomeric form were found within the C-terminal half of the protein, suggesting that these residues are located inside a structurally compact region of the self-assembly domain. This finding is consistent with a previous trypsin cleavage experiment (3), which identified a trypsin-resistant region encompassing residues (190) 209 to 423 (see below).

The ability of the recombinant SlpA protein to self-assemble in dialysis as a lattice on isolated cell wall fragments has been shown using cryo-EM and SAXS (Jääskeläinen et al., unpublished). As was confirmed by SAXS analysis, crystals of mutated proteins that are attached to cell wall fragments are similar to those formed by nonmutated SlpA. Thus, the surface accessibility of mutated residues within a lattice structure could be analyzed. In general, the observed accessibility of all the mutated residues was lower when attached to the cell wall than as monomers in solution. The differences in reaction kinetics and the more constrained structure of the lattice compared with the protein monomer in solution obviously contribute to the reduced level of accessibility observed. Additional reasons for this phenomenon include obstruction of the inner surface of the protein lattice by the cell wall, as well as part of the monomer surface being shielded at the subunit-subunit interface of the lattice or obstructed in lattice pores (10, 12).

As evidenced by the inaccessibility of the residues located in the cell wall-binding domain (residues 1 to 178) while SlpA is attached to a cell wall fragment, TMM(PEG)<sub>12</sub> is not able to permeate the cell wall and gain access to the cysteine residues in the conditions used. This is feasible since, assuming that it has a spherical shape, a 2.5-kDa PEG polymer has a hydrodynamic diameter of approximately 3.0 nm (26), which is significantly smaller than the 5.2 nm of a 5-kDa PEG used with G. stearothermophilus (10) but between the exclusion limits of isolated cells walls from Bacillus megaterium (2.2 nm) (26) and Bacillus subtilis (4.2 nm) (7). Based on the hydrodynamic diameter, it can be assumed that a 2.5-kDa PEG occupies an area of  $\sim 9 \text{ nm}^2$ . Compared to the lattice constants of SlpA  $(a = 10.38 \text{ nm}, b = 6.36 \text{ nm}, 72.7^{\circ} \text{ for nonmutated protein}) (3),$ it is likely that only the residues facing the largest pores of the lattice are accessible to  $TMM(PEG)_{12}$ .

Based on the accessibility data, the mutated residues can be

divided into four groups (Table 2) according to different locations within the protein lattice. (i) Residues inaccessible to modification even in the monomeric form of the protein (modified less than 30% of the most-modified residue) are likely located within the interior of the protein. (ii) Residues that are highly accessible in both monomeric and cell wall-bound forms of the protein (modified more than 50% of the most-modified residue in the cell wall-bound form) are likely located on the outer surface of the protein lattice. (iii) Residues located in the cell wall-binding domain (1 to 178) that showed medium to high accessibility in the monomer experiment yet had only poor accessibility while the protein was bound to the cell wall are likely located on the inner surface of the protein lattice. In addition, this assignment likely applies to residues located in the immediate N-terminal end of the self-assembly domain (S179C, S182C, and T187C). (iv) Residues located further into the self-assembly domain showed markedly better accessibility in the monomer experiment than when the protein was bound to the cell wall. In this fourth group, the residues could be located in the protein-protein interface between subunits, flanking the pores of the S-layer lattice, or possibly in the inner surface of the lattice facing the cell wall.

We also analyzed the sequences surrounding the mutated residues in a seven-amino-acid window (Table 2). Each group, formed on the basis of experimental results, has a distinct profile of amino acid composition, thus supporting the assignments made. Notably, the amount of hydrophobic (nonpolar) amino acids is fairly constant (42 to 46% of the residues) in all the groups. While contradictory to the results derived from *G. stearothermophilus* SbsB (12), this is not surprising given that in general hydrophobic residues are evenly spread throughout the SlpA amino acid sequence, as evidenced by a hydropathy plot which reveals no extensive hydrophobic or hydrophilic regions (data not shown). However, there are remarkable differences between the groups in the amounts of polar and charged amino acids, as well as in the net charge of the seven-amino-acid sequences.

On average, the sequences around residues mapped to the protein interior have 14% of charged residues and similar amounts of polar and hydrophobic residues (43% each). The net charge is slightly positive (+0.33). As can be expected, the residues mapped to the outer surface of the lattice are in a

Location and mutant no.	Sequence <sup>a</sup>	No. of charged amino acids	No. of polar amino acids	No. of nonpolar amino acids	Net charge	% Charged residues	% Polar residues	% Nonpolar residues	
Interior $(n = 12)$									
246	KAGSKVS	2	2	3	+2	28.6	28.6	42.9	
281	ADATYGN	1	3	3	-1	14.3	42.9	42.9	
308	TPVTTAL	0	3	4	0	0.0	42.9	57.1	
320	NDKVAAN	2	2	3	0	28.6	28.6	42.9	
330	ANGSSVA	0	3	4	0	0.0	42.9	57.1	
358	KGOVVTL	1	2	4	+1	14.3	28.6	57.1	
376	FTGTTTY	0	5	2	0	0.0	71.4	28.6	
383	VSDI GKA	2	2	2	Ő	28.6	28.6	42.0	
300	VHVTVTV	1	6	0	±1	14.3	20.0 85 7	-2.5	
207	NKDSAAS	1	2	2	0	28.6	42.0	28.6	
397 410	ITATIVM	2	5	2	0	28.0	42.9	20.0	
418	LIAILVM	0	2	2	0	0.0	28.0	/1.4	
424	MGKSIAI	1	3	3	$\pm 1$	14.3	42.9	42.9	
Avg					+0.33	14.3	42.9	42.9	
Interface/pore $(n = 16)$			_						
192 <sup>b</sup>	KGFSTTA	1	3	3	+1	14.3	42.9	42.9	
198 <sup>b</sup>	ATGTQVL	0	3	4	0	0.0	42.9	57.1	
$205^{b}$	GGLSTDK	2	2	3	0	28.6	28.6	42.9	
209 <sup>b</sup>	TDKSVTA	2	3	2	0	28.6	42.9	28.6	
213 <sup>b</sup>	VTATNDN	1	4	2	-1	14.3	57.1	28.6	
$232^{b}$	OVGSNTW	0	5	2	0	0.0	71.4	28.6	
264 <sup>c</sup>	YINANKP	1	3	3	+1	14.3	42.9	42.9	
$273^{b}$	YTVTNPN	0	5	2	Ō	0.0	71.4	28.6	
201 <sup>b</sup>	ATVSOAA	0	3	1	0	0.0	/1.4	57.1	
2020	LEVECTE	0	2	4	1	14.2	78.6	57.1	
2406	OUTTOUT	1	<u>∠</u> 4	4	⊤1 1	14.3	20.0	28.6	
349 <sup>2</sup>	QLIIDLI	1	4	2	-1	14.5	57.1	28.0	
400	SAASSNA	0	4	3	0	0.0	57.1	42.9	
405	NASTQFG	0	4	3	0	0.0	57.1	42.9	
409 <sup>o</sup>	QFGSNVT	0	4	3	0	0.0	57.1	42.9	
$414^{c}$	VTGTLTA	0	3	4	0	0.0	42.9	57.1	
431 <sup>c</sup>	ANGTTWF	0	4	3	0	0.0	57.1	42.9	
Avg					+0.06	8.0	50.0	42.0	
Outer surface $(n = 9)$									
256	ADOTALE	2	2	3	-2	28.6	28.6	42.9	
268	NKPSGYT	1	4	2	+1	14.3	57.1	28.6	
312	TALTTAD	1	3	2	_1	14.3	12.0	42.0	
216	TADANDK	1	2	2	_1	14.5	78.6	42.9	
225	VACSTVV	5	2	4	-1	42.9	28.0	20.0	
242	VAUSIVI	0	5	4	0	0.0	42.9	37.1 71.4	
342	AAGIKLA	1	1	5	+1	14.5	14.3	/1.4	
362	VILIAID	1	2	4	-1	14.3	28.6	57.1	
366	AIDTDLE	3	1	3	-3	42.9	14.3	42.9	
372	EDATFTG	2	2	3	-2	28.6	28.6	42.9	
Avg					-0.89	22.2	31.7	46.0	
Inner surface $(n = 9)$						<b>8</b> 0 (			
35	KPGTVKG	2	1	4	+2	28.6	14.3	57.1	
47	SKATMAK	2	2	3	+2	28.6	28.6	42.9	
73	NRGSVYY	1	4	2	+1	14.3	57.1	28.6	
108	AETTTKA	2	3	2	0	28.6	42.9	28.6	
145	SKVSLYG	1	3	3	+1	14.3	42.9	42.9	
174	YHVTATN	1	4	2	+1	14.3	57.1	28.6	
179	TNGSGIS	0	4	3	0	0.0	57.1	42.9	
182	SGISGWI	n n	2	4	õ	0.0	42.0	57.1	
102	WIVACKC	1	2	т Л	1	1/ 2	78.5	57.1	
107	WITAUNU	1	2	4	1.1	14.3	20.0	57.1	
Avg					+0.89	15.9	41.3	42.9	
Full-length SlpA						17.5	41.6	40.9	
Self-assembly domain						14.0	44.7	41.3	

TABLE 2.	Mutant	proteins	generated	grouped	according to	their	accessibility	and	the amino	acid	compositions	of insertion	sites	using s	even-
						amin	o-acid windo	ws							

<sup>*a*</sup> Mutated residues are in bold. <sup>*b*</sup> Putative interface. <sup>*c*</sup> Putative pore.



FIG. 6. Solvent accessibility of mutated residues in the self-assembly domain of SlpA compared with PredictProtein predictions of secondary structure (horizontal block arrows indicate  $\beta$ -strands). Mutated residues are shaded according to their assignment: black, outer surface; medium gray, pore/interface; light gray, protein interior; and white, inner surface. Observed and additional predicted (PeptideCutter) cleavage sites of trypsin are marked with small vertical arrows in black and white, respectively.

more hydrophilic and charged (22% of the residues) environment with a negative net charge (-0.89 on average). These results are in accordance with those for SbsB, in which the residues assigned to the outer surface have an average net charge of -1.75 and 39% of the residues within the window are charged (12). Interestingly, the self-assembly domain (residues 179 to 435) of SlpA has a low calculated pI of 5.04.

Also in agreement with the results from the SbsB study (12), residues mapped to the inner surface of the lattice (from the N terminus up to T187C) share a similar, positively charged (SlpA, +0.89, and SbsB, +0.3, on average) amino acid composition. This suggests that SlpA S-layers could be assembled on a negatively charged support. Contrastingly, the surrounding environment of the residues in the interface/pore group appears to be almost totally devoid of charge with an average net charge of +0.06 and 7.6% charged residues. These numbers are notably different from the interface residues of SbsB (+0.5 net charge, 29% charged residues). However, with SbsB only four such residues were detected, limiting the statistical value of average composition (12).

Finally, the interface/pore group could be divided further into distinct subgroups. The difference in accessibility between monomeric and cell wall-bound protein is likely to be more substantial with the residues located at the interface than with residues flanking the pores of the lattice or located within the depressed areas on the protein surface. In addition, residues situated in the depressions of the protein surface are likely to be relatively more reactive toward the smaller AlexaFluor488maleimide marker than the larger-sized TMM(PEG)<sub>12</sub>. According to these two criteria, the residues could be divided further into the putative interface and pore subgroups shown in Table 2. For further characterization of these residues, a cross-link assay detecting residues located in the protein-protein interface (12) could be conducted in a future study.

A previous study on SlpA used a hydrophilicity profile to predict suitable, exposed insertion sites from the primary protein sequence, and introduced an 11-amino-acid VP1 epitope at positions A49/K50, K251/A252, A316/N317, and D365/T366. Of these sites, only K251/A252 and D365/T366 yielded an antibody response (2). In contrast, our results suggest that A316C and T366C are among the most accessible residues

studied. This discrepancy is probably due to either aberration of the protein structure caused by the peptide insert or the difference in the interactions involved in an epitope-antibody binding compared with a sulfhydryl group-maleimide reaction.

The SlpA protein is known to contain two trypsin-resistant fragments, encompassing amino acids 190 to 423 and 209 to 423, suggesting the existence of a compact domain structure between these residues (3). In addition, there are 13 other potential cleavage sites located within the self-assembly domain of SlpA (Fig. 6). One of these sites is situated next to a residue mutated in our study (V320C) that remains almost completely nonmodified in monomeric protein, indicating a buried location deep inside a compact protein structure. Nine other cleavage sites are located close to or in between residues assigned to either the interior or interface/pore, presumably inaccessible to trypsin. However, two putative cleavage sites (residues 266 and 343) are located within the window of residues assigned to the outer surface of the protein and another (residue 251) at an evidently accessible VP1 insertion site, K251/A252 (2). These results reinforce the fact that surface accessibility can be measured with several different methods and that the accessibility of a sulfhydryl group of a given residue to a small maleimide derivative does not always correlate with the accessibility of a nearby peptide bond to a much larger trypsin molecule (MW = 24,000). Interestingly, mutated residue S424C is only poorly accessible in a protein monomer despite being situated next to the functional trypsin cleavage site at 423. The reason for this discrepancy might be the difference in molecular concentrations used, the reaction time, or the higher reaction temperature used with trypsin. A cleavage site close to the unanchored C terminus of the protein could be made accessible simply by increased thermal motion at 37°C.

The prediction of the secondary and tertiary structures of S-layer proteins with algorithms developed with other types of proteins has been difficult. With SlpA, different programs and settings typically yield vastly different types of secondary structure predictions. However, a recent Fourier transform infrared spectroscopy study of SlpA estimated that the protein consists of  $0\% \alpha$ -helix,  $50.2\% \beta$ -strand, and  $49.8\% \beta$ -turn and nonordered structures (16). Reasonably well in accordance with these results, PredictProtein (22) estimates for the whole pro-

tein were 0%  $\alpha$ -helix, 59.1%  $\beta$ -strand, and 40.9% loop, i.e., other structures. Figure 6 depicts a projection of the mutant assignments made with the secondary structure of the selfassembly domain of SlpA predicted from the amino acid sequence by PredictProtein. Overall, the assignments made in our study are in agreement with the secondary structure predictions. Excluding S335C, T362C, and T366C, residues assigned to the outer surface of the lattice are located in the predicted loops. In contrast, with the exception of T376C and S424C, residues assigned to the protein interior are located within the predicted  $\beta$ -strands. Residues assigned to interface/ pore are located within both loops and  $\beta$ -strands predicted. Furthermore, as the prevalence of the  $\beta$ -strand with Predict-Protein is higher than the actual observed prevalence of  $\beta$ -strands, it is possible that the  $\beta$ -strands predicted to cover residues 330 to 340 and 357 to 367, spanning residues assigned to the outer surface, are shorter than predicted or do not exist in the actual protein.

In contrast to secondary structure, the prediction of solvent accessibility from the amino acid sequence by PredictProtein poorly matches the results obtained in our cysteine-labeling mutant study. The solvent accessibility predictions of other programs tested (SABLE and JNET) were even worse at reflecting the experimental results obtained (data not shown), further confirming that prediction programs are presently unable to accurately predict the solvent accessibility profile for SlpA or, presumably, other S-layer proteins. In the absence of three-dimensional crystals required for structural determination by X-ray crystallography, CSM remains one of the most reliable methods for assessing the surface accessibility of individual amino acid residues. Furthermore, preliminary studies on live bacteria indicate that epitopes inserted into residues of similar surface accessibility can yield markedly different antibody responses (S. Åvall-Jääskeläinen, personal communication). This phenomenon may be caused by the spatial constraints of the inserted epitope or the effect of the epitope on the refolding of the protein on the bacterial surface. Thus, the identification of several surface-accessible residues-and putative insertion sites-is crucial for potential future applications that utilize live bacteria.

Our results, gained from 46 single-cysteine mutant proteins of SlpA, further confirm the two-domain model of the protein. In addition, we were able to classify the mutated residues according to their solvent accessibility into four different groups: outer surface, pores/protein interface, inner surface of the lattice, and protein interior. The amino acid compositions around mutated residues as well as the predicted secondary structure support the assignments made based on observed accessibility. These results can be used to develop an accurate model for S-protein structure prediction. The residues placed on the outer surface have practical value in potential further applications, e.g., the surface display of effector molecules. In addition, the residues located at the outer surface could be further modified, e.g., to immobilize bacterial cells to solid support. Moreover, the residues located at the protein-protein interface could be further examined to elucidate the interactions involved in the formation of a two-dimensional lattice.

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