Are the Surface Layer Homology Domains Essential for Cell Surface Display and Glycosylation of the S-Layer Protein from *Paenibacillus alvei* CCM 2051^T?

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Paenibacillus alvei CCM 2051^T cells are decorated with a two-dimensional (2D) crystalline array comprised of the glycosylated S-layer protein SpaA. At its N terminus, SpaA possesses three consecutive surface layer (S-layer) homology (SLH) domains containing the amino acid motif TRAE, known to play a key role in cell wall binding, as well as the TVEE and TRAQ variations thereof. SpaA is predicted to be anchored to the cell wall by interaction of the SLH domains with a peptidoglycan (PG)-associated, nonclassical, pyruvylated secondary cell wall polymer (SCWP). In this study, we have analyzed the role of the three predicted binding motifs within the SLH domains by mutating them into TAAA motifs, either individually, pairwise, or all of them. Effects were visualized *in vivo* by homologous expression of chimeras made of the mutated S-layer proteins and enhanced green fluorescent protein and in an *in vitro* binding assay using His-tagged SpaA variants and native PG-containing cell wall sacculi that either contained SCWP or were deprived of it. Experimental data indicated that (i) the TRAE, TVEE, and TRAQ motifs are critical for the binding function of SLH domains, (ii) two functional motifs are sufficient for cell wall binding, regardless of the domain location, (iii) SLH domains have a dual-recognition function for the SCWP and the PG, and (iv) cell wall anchoring is not necessary for SpaA glycosylation. Additionally, we showed that the SLH domains of SpaA are sufficient for *in vivo* cell surface display of foreign proteins at the cell surface of *P. alvei*.

Bacterial cell surface layers (S-layers) are a distinct type of cell surface decoration (1) enabled by monomolecular self-assembly of individual (glyco)proteins on the supporting cell envelope layer into a two-dimensional (2D) crystalline array exhibiting periodicity on the nanometer scale. These structures hold a great promise for *in vivo* cell surface display of biofunctional epitopes by protein and/or glycosylation engineering, with possible relevance for basic as well as applied research, encompassing biotechnology and therapy. As a basis, a detailed understanding of the mechanisms underlying S-layer protein display on the bacterial cell is required. Above that, the involvement of S-layers in cell adhesion and surface recognition and their function as virulence factors (2–5) have prompted in-depth research on that subject.

Bacterial cell surface display is an evolutionary optimized strategy to express molecules of interest on the exterior of cells by using natural microbial functional components. Many of these cell surface anchors are proteins exhibiting functions in pathogenesis or cell wall maintenance; they are either membrane associated or integrated or cell wall associated, with either covalent or noncovalent linkage (6, 7). Also other components, such as choline residues of (lipo)teichoic acids, can be involved in the binding mechanism (8). For S-layers of Gram-positive bacteria, still another binding mechanism exists: S-layers are noncovalently attached to the bacterial cell wall via a lectin-type-like binding to a peptidoglycan (PG)-associated, nonclassical secondary cell wall polymer (SCWP) (9). A specific mode for that interaction to be exerted is the utilization of S-layer homology (SLH) domains as cell wall targeting modules that recognize a distinct class of SCWP that needs to be pyruvylated by the activity of the polysaccharide pyruvyltransferase CsaB (cell surface attachment) (10, 11). Apart from S-layers, SLH domains have been found in a number of extracellular proteins, such as enzymes and outer membrane proteins from both Gram-positive and Gram-negative bacteria (12),

which pinpoints the wider relevance of this cell surface display strategy. SLH domains are often present in three copies and are located at either the C or the N terminus of the respective proteins. While the overall sequence similarity of SLH domains is rather low, the highly conserved four-amino-acid motif TRAE has been found to play a key role in the binding function to SCWP (13, 14). The crystal structure of one S-layer protein from *B. anthracis* has recently been elucidated, showing the SLH domains arranged in 3-fold pseudosymmetry with the TRAE (or similar) motifs arranged so they would be accessible to the SCWP (13).

The S-layer protein SpaA of the Gram-positive bacterium *Paenibacillus alvei* CCM 2051^T is an ideal model to study SLH domain-mediated S-layer surface display, because it offers the opportunity to perform *in vivo* and *in vitro* studies and, since SpaA is a naturally O-glycosylated protein, to assess the relevance of cell surface anchoring for S-layer protein glycosylation. SpaA is a 983-amino-acid protein with a typical Gram-positive N-terminal signal peptide (residues 1 to 24) followed by three SLH domains containing the predicted binding motifs TRAE in SLH domain 1 (residues 25 to 65), TVEE in SLH domain 2 (residues 82 to 129), and TRAQ in SLH domain 3 (residues 140 to 181) (15). It is important to note that variations in the TRAE motif have also been reported to be functional in *Thermoanaerobacterium thermosulfurigenes* EM1 (14), whereas mutation of the TRAE motifs to

Received 16 August 2012 Accepted 19 November 2012

Published ahead of print 30 November 2012

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doi:10.1128/JB.01487-12

TAAA showed drastically reduced binding to SCWP (14). The SCWP of *P. alvei* CCM 2051^T is a polysaccharide with the structure [(Pyr4,6)-β-D-ManpNAc-(1→4)-β-D-GlcpNAc-(1→3)]_{n-11}-(Pyr4,6)-β-D-ManpNAc-(1→4)-α-D-GlcpNAc-(1→ (16), which is linked via a phosphate-containing bridge to muramic acid residues of the PG backbone (9, 16). We proposed that several genes that are clustered around the *spaA* gene constitute the SCWP biosynthesis locus of *P. alvei* CCM 2051^T, with a *csaB* homolog being one of these genes (15).

Cell-envelope-anchored SpaA is O-glycosylated with a polysaccharide consisting of, on average, 23 [→3)-β-D-Galp-(1→)-[α-D-Glcp-(1→6)]→4)-β-D-ManpNAc-(1→] repeating units that is linked by the adaptor saccharide -[GroA-2→OPO₂→4-β-D-ManpNAc-(1→4)]→3)-α-L-Rhap-(1→3)-α-L-Rhap-(1→3)-α-L-Rhap-(1→3)-β-D-Galp-(1→ to at least two distinct tyrosine residues at positions 47 and 155 of the S-layer preprotein (15, 17). While we already have a clear picture of how the S-layer protein glycosylation pathway proceeds based on the genetic machinery encoded in an S-layer glycosylation gene locus (18), we propose that transfer of the completed S-layer glycan onto the protein occurs cosecretionally in an ATP-dependent manner, with details about the cellular prerequisites of this step being currently unknown. Thus, the important question arises—is cell surface display of SpaA a prerequisite for O-glycosylation? Shedding light onto this aspect would advance the knowledge of protein O-glycosylation in bacteria in general (for reviews, see references 19 and 20).

In this study, we have analyzed the role of the three predicted binding motifs within the SLH domains of SpaA by mutating them into TAAA motifs, either individually, pairwise, or all of them. The effects were visualized *in vivo* by homologous expression of chimeras made of the mutated S-layer proteins and enhanced green fluorescent protein (EGFP) and in an *in vitro* binding assay using His-tagged SpaA variants and native PG-containing cell wall sacculi that either contained SCWP or were deprived of it. To mimic a more natural situation, the binding capacity of *P. alvei* cells that have been stripped off of the S-layer for recombinant SpaA was investigated. Additionally, we showed that the SLH domains of SpaA are sufficient for *in vivo* cell surface display of foreign proteins at the cell surface of *P. alvei* CCM 2051^T.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. *P. alvei* CCM 2051^T was obtained from the Czech Collection of Microorganisms (CCM) and grown at 37°C and 200 rpm in Luria-Bertani (LB) broth or on LB agar plates supplemented with 10 μg/ml chloramphenicol (Cm), when appropriate. *Escherichia coli* DH5α cells (Invitrogen) and *E. coli* BL21(DE3) cells (Invitrogen) were cultivated at 37°C and 200 rpm in LB medium supplemented with 30 μg/ml chloramphenicol (Cm) and 50 μg/ml kanamycin (Km), respectively. *Geobacillus stearothermophilus* NRS 2004/3a (21) was grown on modified S-VIII medium at 55°C (22).

All strains and plasmids used in this study are listed in Table 1.

General molecular methods. Genomic DNA of *G. stearothermophilus* NRS 2004/3a and of *P. alvei* CCM 2051^T was isolated by using the Genomic Tip 100 kit (Qiagen) according to the manufacturer's instructions, except that for the latter organism, cells were broken by repeated freezing and thawing cycles as described previously (23). All enzymes were purchased from Fermentas. The GeneJET gel extraction kit (Fermentas) was used to purify DNA fragments from agarose gels and to purify digested plasmids and oligonucleotides. Plasmid DNA from transformed

cells was isolated with the GeneJET plasmid miniprep Kit (Fermentas). Agarose gel electrophoresis was performed as described elsewhere (24). Primers for PCR and DNA sequencing were purchased from Invitrogen (Table 2), and PCR conditions were optimized for each primer pair. PCR was performed using the Phusion high-fidelity DNA polymerase (Fermentas) and the thermal cycler MyCycler (Bio-Rad). Amplification products were purified using the GeneJET gel extraction kit (Fermentas). Transformation of chemically competent *E. coli* DH5α cells was done according to the manufacturer's protocol (Invitrogen). Transformation of *P. alvei* CCM 2051^T wild-type cells and *P. alvei* CCM 2051^T Δ*slhA* cells (possessing higher transformation efficiency than the wild-type cells) was performed as described by Zarschler et al. (23). Transformants were screened by PCR using the RedTaq Ready Mix PCR mixture (Sigma-Aldrich), and recombinant clones were analyzed by restriction mapping and confirmed by sequencing (LGC).

Plasmid construction for *in vivo* studies. Site-directed mutagenesis of the predicted binding motifs TRAE, TVEE, and TRAQ in the first, second, and third SLH domains (shown as subscript 1, 2, or 3) of the *spaA* gene, respectively, into TAAA motifs was done individually (TAAA₁, TAAA₂, TAAA₃), pairwise (TAAA₁₂, TAAA₁₃, TAAA₂₃), or completely (TAAA₁₂₃) by overlap extension PCR. Two parts of the *spaA* sequence were amplified separately—one comprising the part upstream of the mutation site and the other being the respective downstream part. The forward primer of the downstream part and the reverse primer of the upstream part were overlapping and included the mutations that were consequently introduced in both stretches.

For homologous expression of the mutated and truncated *spaA* variants as chimeras with enhanced EGFP, including a GAGGAGGT linker, PCRs were performed using the primer pairs SP_SpaA_for_SphI/SpaA_MutTRA_E_rev (fragment 1) and SpaA_MutTRA_E_for/SpaA_Link_E_KpnI_rev (fragment 2), SP_SpaA_for_SphI/SpaA_SM_TVEE_rev (fragment 1) and SpaA_SM_TVEE_for/SpaA_Link_E_KpnI_rev (fragment 2), and SP_SpaA_for_SphI/SpaA_MutTRAQ_rev (fragment 1) and SpaA_MutTRAQ_for/SpaA_Link_E_KpnI_rev (fragment 2).

In a second round of PCR, the two fragments (numbered 1 and 2, as described above) were mixed and the whole mutated *spaA* gene was amplified using the primer pair SP_SpaA_for_SphI/SpaA_Link_E_KpnI_rev. As a control, native *spaA* was amplified from *P. alvei* genomic DNA using the primer pair SP_SpaA_for_SphI/SpaA_Link_E_KpnI_rev. For amplification of a truncated *spaA* variant lacking the sequence encoding the signal peptide and the SLH domains, the primer pair SpaA_w/o-SP_w/o-SLH_for/SpaA_Link_E_KpnI_rev was used.

The different *spaA* amplification products were digested with SphI/KpnI and inserted into the linearized vector pEXALV_EGFP. For vector construction, EGFP was amplified from plasmid pEGFP-N1 using the primers KpnI_EGFP_for and EGFP_SacI_rev, digested with KpnI and SacI, and cloned into the KpnI/SacI-linearized *P. alvei* vector pEXALV (23). The resulting plasmids encoding the chimeras (Table 1) were named pEXSMut1E (TAAA₁), pEXSMut2E (TAAA₂), pEXSMut3E (TAAA₃), pEXSMut4E (TAAA₁₂), pEXSMut5E (TAAA₁₃), pEXSMut6E (TAAA₂₃), pEXSMut7E (TAAA₁₂₃), pEXCytE (truncated *spaA*), and pEXALV_SP_SpaA_EGFP (native *spaA*) (Table 1) and transformed into *P. alvei* CCM 2051^T Δ*slhA* cells (23).

For the construction of the TAAA₁₂ mutant, fragment 1 was amplified using the primer pairs SP_SpaA_for_SphI/SpaA_SM_TVEE_rev with the pEXSMut1E (TAAA₁) plasmid as the template, and fragment 2 was amplified using the primer pairs SpaA_SM_TVEE_for/SpaA_Link_E_KpnI_rev with *P. alvei* genomic DNA as the template. For the construction of the TAAA₁₃ mutant, fragment 1 was amplified using the primer pair SP_SpaA_for_SphI/SpaA_MutTRAQ_rev with plasmid pEXSMut1E (TAAA₁) as the template, and fragment 2 was amplified using the primer pair SpaA_MutTRAQ_for/SpaA_Link_E_KpnI_rev with *P. alvei* genomic DNA as the template. For the construction of the TAAA₂₃ mutant, fragment 1 was amplified using the primer pair SP_SpaA_for_SphI/SpaA_MutTRAQ_rev with plasmid pEXSMut2E (TAAA₂) as the tem-

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or relevant characteristic(s)	Source or reference
Strains		
<i>P. alvei</i> CCM 2051 ^T	Wild-type isolate; Km ^r	CCM
<i>G. stearothermophilus</i> NRS 2004/3a	Wild-type isolate	NRS
<i>E. coli</i> DH5α	F [−] φ80Δ <i>lacZ</i> M15 (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (r _K [−] m _K [−]) <i>phoA supE44 thi-1 gyrA96 relA1</i> mutant	Invitrogen
<i>E. coli</i> BL21(DE)	F [−] <i>ompT hsdS</i> (r _B [−] m _B [−]) <i>gal dcm</i> (DE3)	Invitrogen
<i>P. alvei</i> CCM 2051 ^T <i>slhA</i> ::LL.LtrB	<i>P. alvei</i> CCM 2051 ^T carrying targetron insertion at <i>slhA</i> locus; Km ^r	B. Janesch et al., unpublished data
Plasmids		
pEXALV	pNW33N carrying <i>sgsE</i> S-layer gene promoter of <i>G. stearothermophilus</i> NRS 2004/3a; Cm ^r	23
pET28a	Expression vector with His ₆ tag; Kan ^r	Novagen
pEGFP-N1	Expression vector for mammalian cells encoding red-shifted variant of wild-type GFP	BD Biosciences
pEXALV_EGFP	<i>P. alvei</i> CCM 2051 ^T carrying EGFP gene	This study
pEXSMut1E	pEXALV carrying fusion construct of <i>spaA</i> mutated in TRAE motif and EGFP gene; Cm ^r	This study
pEXSMut2E	pEXALV carrying fusion construct of <i>spaA</i> mutated in TVEE motif and EGFP gene; Cm ^r	This study
pEXSMut3E	pEXALV carrying fusion construct of <i>spaA</i> mutated in TRAQ motif and EGFP gene; Cm ^r	This study
pEXSMut4E	pEXALV carrying fusion construct of <i>spaA</i> mutated in TRAE and TVEE motifs and EGFP gene; Cm ^r ; parental strain with pEXSMut1E	This study
pEXSMut5E	pEXALV carrying fusion construct of <i>spaA</i> mutated in TRAE and TRAQ motifs and EGFP gene; Cm ^r ; parental strain with pEXSMut1E	This study
pEXSMut6E	pEXALV carrying fusion construct of <i>spaA</i> mutated in TVEE and TRAQ motifs and EGFP gene; Cm ^r ; parental strain with pEXSMut2E	This study
pEXSMut7E	pEXALV carrying fusion construct of <i>spaA</i> mutated in TRAE, TVEE, and TRAQ motifs and EGFP gene; Cm ^r ; parental strain with EXSMut6E	This study
pEXCytE	pEXALV carrying fusion construct of truncated <i>spaA</i> and EGFP gene; Cm ^r	This study
pEXALV_SP_SpaA_EGFP	pEXALV carrying fusion construct of mutated <i>spaA</i> and EGFP gene; Cm ^r	This study
pETSMut1H	pET28a carrying His-tagged <i>spaA</i> mutated in TRAE motif; Kan ^r ; parental strain with pEXSMut1E	This study
pETSMut2H	pET28a carrying His-tagged <i>spaA</i> mutated in TVEE motif; Kan ^r ; parental strain with pEXSMut2E	This study
pETSMut3H	pET28a carrying His-tagged <i>spaA</i> mutated in TRAQ motif; Kan ^r ; parental strain with pEXSMut3E	This study
pETSMut4H	pET28a carrying His-tagged <i>spaA</i> mutated in TRAE and TVEE motifs; Kan ^r ; parental strain with pEXSMut4E	This study
pETSMut5H	pET28a carrying His-tagged <i>spaA</i> mutated in TRAE and TRAQ motifs; Kan ^r ; parental strain with pEXSMut5E	This study
pETSMut6H	pET28a carrying His-tagged <i>spaA</i> mutated in TVEE and TRAQ motifs; Kan ^r ; parental strain with pEXSMut6E	This study
pETSMut7H	pET28a carrying His-tagged <i>spaA</i> mutated in TRAE, TVEE, and TRAQ motifs; Kan ^r ; parental strain with pEXSMut7E	This study
pET28a_SpaAdSLH_6His	pET28a carrying truncated His-tagged <i>spaA</i> gene of <i>P. alvei</i> CCM 2051 ^T ; Kan ^r	This study
pET28a_SpaA_6His	pET28a carrying His-tagged <i>spaA</i> gene of <i>P. alvei</i> CCM 2051 ^T ; Kan ^r	This study
pEXALV_SpaA_SgsE3Z+	pEXALV carrying fusion construct of truncated <i>spaA</i> (aa 1–350) and <i>sgsE</i> 3Z+ (aa 542–903) ^a	This study
pEXALV_SP_SgsE3Z	pEXALV carrying fusion construct of the signal peptide of <i>spaA</i> (aa 1–24) and <i>sgsE</i> 3Z+ (aa 542–903)	This study

^a aa, amino acids.

plate, and fragment 2 was amplified using the primer pair SpaA_MutTRAQ_for/SpaA_Linker_KpnI_rev with *P. alvei* genomic DNA as the template. Digestion, ligation, and transformation were done as described above. The resulting plasmids (Table 1) were named pEXSMut4E (TAAA₁₂), pEXSMut5E (TAAA₁₃), and pEXSMut6E (TAAA₂₃).

For the construction of the TAAA₁₂₃ mutant, fragment 1 was amplified using the primer pair SP_SpaA_for_SphI/SpaA_MutTRAE_rev with *P. alvei* genomic DNA as the template, and fragment 2 was amplified using the primer pair SpaA_MutTRAE_for/SpaA_Linker_KpnI_rev with plasmid pEXSMut6E (TAAA₂₃) as the template. Digestion, ligation, and transformation were done as described above. The resulting plasmid (Table 1) was named pEXSMut7E (TAAA₁₂₃).

Recombinant production of His-tagged SpaA variants for *in vitro* assays. All SpaA variants used for *in vitro* assays were produced as His₆-tagged constructs for detection purposes. The C-terminal His₆ tag was fused to the sequence of mutated *spaA* by PCR using the primers SpaA_w/oSP_BsaI/NcoI_for/SpaA_His_XhoI_rev for whole *spaA* and SpaA_w/oSP_w/oSLH_BsaI/NcoI_for/SpaA_His_XhoI_rev for an *spaA* variant lacking the sequence coding for the signal peptide and the SLH domains, using the respective pEXALV-based constructs as the templates (described above).

The His₆-tagged *spaA* amplification products were digested with BsaI/XhoI and cloned into the NcoI/XhoI-linearized vector pET28a(+) (Novagen). The corresponding plasmids were named pETSMut1H through

TABLE 2 Oligonucleotide primers used for PCR amplification

Primer	Sequence (5'→3') ^a	Comment
SP_SpaA_for_SphI	aatcaGCATGCAGAAAAGATTGGCCCTTCTGC	
SpaA_w/oSP_w/oSLH_for	aatcaGCATGCAAGCAGCATTTCAGC	
SpaA_MutTRAE_for	GGTCAAGATATGACTGCAGCTGCATTTCGCTAAAGTTC	Used for overlap extension PCR to mutate TRAE to TAAA
SpaA_MutTRAE_rev	CAAGAACTTTAGCGAATGCAGCTGCAGTCATATC	Used for overlap extension PCR to mutate TRAE to TAAA
SpaA_SM_TVEE_for	CAACGGTAAAATCACAGCAGCCGAGCATCCAAACTTTGGTTACTG	Used for overlap extension PCR to mutate TVEE to TAAA
SpaA_SM_TVEE_rev	CCAAAGTTTGGATGCTGCGGCTGCTGTGATTTTACCGTTGAAGTC	Used for overlap extension PCR to mutate TVEE to TAAA
SpaA_MutTRAQ_for	GCAAATGCAACTGCAGCTGCATTGGTAGAAGCAGC	Used for overlap extension PCR to mutate TRAQ to TAAA
SpaA_MutTRAQ_rev	GCTTCTACCAATGCAGCTGCAGTTGCATTGCTTTAG	Used for overlap extension PCR to mutate TRAQ to TAAA
SpaA_Linker_KpnI_rev	aatcaGGTACCTCCAGCACCGCCGGCACCTTACCGGAGTATGTTCCAGG	
KpnI_EGFP_for	aatcaGGTACCATGGTGAGCAAGGGCGAGG	
EGFP_SacI_rev	aatcGAGCTCTCACTTGTACAGCTCGTCCATGCC	
SpaA_w/oSP_BsaI_NcoI_for	aatcaGGTCTCCCATGGCTGACGCAGCAAAAACAACCTCAAG	
SpaA_w/oSP_w/oSLH_BsaI_NcoI_for	aatcaGGTCTCCCATGGCAGCATTTGCAGCTGACGAAATG	
SpaA_His_XhoI_rev	aatcaCTCGAGTTAATGGTGATGGTGATGGTGCTTACCGGAGTATGTTCCAGG	
SpaA_SgsE_OE_for	GTGAAGTTCACGGACTTGGCGGGCAATAGCGGGGATGC	
SpaA_SgsE_OE_rev	GCTATTGCCCGCCAAAGTCCGTGAACCTTCACTTTTTCATC	
SgsE_rev_KpnI	aaGGTACCTTATTTTGTACGTTTACAACAGTAGC	
SP(SpaA)_SgsE3Z+_SphI_for	aatcGCATGCAGAAAAGATTGGCCCTTCTGCTTCCGTTGCTATGGCGTTCTC TATGTTTCGAAACGTAGCTTTCGGTGACTTGGCGGGCAATAGCGGG	

^a Artificial restriction sites are underlined. Lowercase letters indicate artificially introduced bases to improve restriction enzyme cutting.

pETSMut7H and pET28a_SpaAdSLH_6His, respectively (Table 1), and transformed into *E. coli* BL21(DE3) cells. Subsequently, freshly transformed cells were grown in LB medium (24) to the midexponential growth phase (optical density at 600 nm [OD₆₀₀] of ~0.6), protein expression was induced with a final concentration of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and cultures were grown for additional 4 h at 37°C and 200 rpm. Cells were harvested by centrifugation (4,500 × g, 30 min, 4°C), and pellets were stored at –20°C until use.

Preparation of crude extracts of recombinant S-layer protein for *in vitro* studies. *E. coli* BL21(DE3) cells carrying pET28a-based constructs encoding full-length, His₆-tagged SpaA, variants thereof, and His₆-tagged SpaA lacking the SLH domains, respectively, were grown in 400 ml of LB medium, and protein expression was induced as described above. Crude cell extracts expressing the recombinant proteins were prepared by resuspending the cell pellets in either 1 ml of phosphate-buffered saline (PBS) buffer or, for *in vitro* binding assays, in 25 mM Tris-HCl (pH 8.0), containing 1 mg lysozyme and 50 μl of bacterial protease inhibitor each. Extracts were incubated on ice for 30 min followed by sonication on ice (six times for 10 s with a 5-s pause each). Subsequently, the suspension was centrifuged (16,100 × g, 30 min, 4°C), and the supernatant containing the recombinant protein was used for further studies.

Preparation of S-layer protein and native peptidoglycan-containing cell wall sacculi. S-layer protein and protein-free PG-containing cell wall sacculi, including native SCWP [PG(+)] of *P. alvei* CCM 2051^T, were prepared essentially as published previously (25). Briefly, cells were grown until the late exponential phase and broken by 10 freezing and thawing cycles because of their lysozyme resistance. After centrifugation (35,000 × g, 30 min, 4°C), the pellet was resuspended in 50 mM Tris-HCl (pH 7.2) (buffer A), containing 0.5% Triton X-100, and incubated for 10 min at 20°C. The pellet was washed four times with buffer A and once with Milli-Q water and finally resuspended in 10 pellet volumes (wt/vol) of 5 M guanidine hydrochloride (GdHCl) in buffer A followed by stirring for 20 min at 20°C. After centrifugation, the supernatant containing the S-layer protein was dialyzed (dialysis tubing cutoff of 12 to 16 kDa; Biomol) against Milli-Q water. The pellet was washed once with 5 M GdHCl in buffer A and twice with buffer A. One hundred milligrams (wet weight) of that preparation was poured into a boiling 1% (wt/vol) SDS solution and incubated for 30 min with stirring at 100°C. The resulting pellet [PG(+)] was washed repeatedly with Milli-Q water to remove traces of SDS.

The extraction of SCWP from native PG-containing cell wall sacculi [PG(–)] was done by treatment with 48% hydrofluoric acid (HF) for 96 h at 4°C as described previously (26).

Lyophilized PG-containing cell wall sacculi with [PG(+)] and without [PG(–)] SCWP were stored at –20°C.

Preparation of peptidoglycan of *E. coli* DH5α. *E. coli* DH5α cells were grown until the late exponential phase and lysed by sonication (twice for 60 s with a 60-s pause). After centrifugation (35,000 × g, 30 min, 4°C), the pellet was resuspended in buffer A, containing 0.5% Triton X-100, and incubated for 10 min at 20°C. The pellet was washed four times with buffer A and once with Milli-Q water. One hundred milligrams (wet weight) of that preparation was treated with SDS followed by treatment as described above. Lyophilized *E. coli* DH5α PG was stored at –20°C.

Interaction studies of SLH-containing SpaA variants and peptidoglycan-containing cell wall sacculi. The different His₆-SpaA variants were tested for their ability to interact with isolated PG-containing cell wall sacculi from *P. alvei* CCM 2051^T, PG(+) and PG(–), as well as with *E. coli* DH5α PG, according to a published protocol (14). A solution of the different His₆-tagged SpaA variants (final concentration, ~0.4 mg/ml) in 25 mM Tris-HCl (pH 8.0), containing 0.2% (vol/vol) Tween 20 was incubated at 37°C for 1 h with lyophilized PG preparations (0.2 mg) in a total volume of 125 μl. The mixture was centrifuged (16,100 × g, 20 min, 4°C), yielding a supernatant containing unbound protein and a pellet of the insoluble cell wall components, including attached protein. The pellet was washed twice and suspended in incubation buffer, and fractions were investigated by SDS-PAGE followed by Western immunoblotting using anti-His₆ mouse antibody (Roche). Mixtures without PG were used as a control. Detection was done with the Li-Cor Odyssey infrared imaging system using goat anti-mouse IgG-IRDye 800CW conjugate (Li-Cor). The integrated intensity of detected bands was determined using the Li-Cor Odyssey Application software 3.0.21. The pellet fraction and the supernatant fraction of the reaction as well as the control (lacking the PG) were quantified, and the value of precipitated protein in the control was subtracted from that in the reaction pellet. For comparability of data sets, the sum of the pellet fraction and the supernatant fraction of each reaction was set to 100%. Experiments were performed in duplicate, and standard deviations were calculated.

Interaction studies of recombinant protein with GdHCl-extracted *P. alvei* cells. Cells from a 4-ml overnight culture of *P. alvei* CCM 2051^T were harvested (OD₆₀₀ ~3.0) and washed twice with PBS. The pellet was resuspended in 1 ml of 5 M GdHCl and incubated at 25°C for 30 min with gentle shaking to extract the S-layer protein. The suspension was centrifuged (16,100 × g, 10 min, 25°C), the supernatant was discarded, and the pellet was washed with PBS followed by resuspension in 500 µl of LB medium. Subsequently, 250 µl of cell suspension was added to 4.75 ml of fresh LB medium and 50 µl of crude protein extracts (final concentration, ~0.4 mg/ml) of *E. coli* BL21(DE3) cells expressing either full-length His₆-tagged SpaA or His₆-tagged SpaA lacking the SLH domains. As a control, 5 ml of LB medium was incubated with crude extracts of the recombinant proteins without addition of *P. alvei* CCM 2051^T cells. Incubation was continued for 8 h at 37°C, which covers the logarithmic growth phase (final OD₆₀₀ ~0.8). The mixture was centrifuged (16,100 × g, 10 min, 25°C), yielding a supernatant with unbound proteins and a pellet with cells, including attached protein. The pellet was washed twice with PBS buffer and suspended in Laemmli buffer (27). Proteins contained in the supernatant were extracted with trichloroacetic acid (TCA), and fractions were investigated by SDS-PAGE followed Western blotting using anti-His₆ mouse antibody.

Construction of a chimeric gene for heterologous cell surface display. A chimeric gene encoding the 350 N-terminal amino acids of SpaA (comprising the signal peptide and all three SLH domains) and the C-terminal portion (amino acids 542 to 903) of the S-layer protein SgsE from *G. stearothermophilus* NRS 2004/3a was constructed by overlap extension PCR using the primer pair SP_SpaA_for_SphI/SpaA_SgsE_OE-rev for amplification of the *spaA* fragment from genomic DNA of *P. alvei* CCM 2051^T and the primer pair SpaA_SgsE_OE-for/SgsE_rev_KpnI for amplification of the *sgsE* fragment from genomic DNA of *G. stearothermophilus* NRS 2004/3a. The two fragments were mixed, and the chimeric *spaA-sgsE* 3Z+ gene was amplified using the SP_SpaA_for_SphI forward primer and SgsE_rev-KpnI reverse primer. As a control, a chimera was constructed without SLH domains, using the primers SP(SpaA)_SgsE3Z+_SphI_for/SgsE_rev_KpnI.

The purified amplification products were digested with SphI/KpnI and inserted into the linearized and dephosphorylated vector pEXALV, resulting in plasmids pEXALV_SpaA_SgsE3Z+ and pEXALV_SP_SgsE3Z+, which were subsequently transformed into *P. alvei* CCM 2051^T wild-type cells (23).

Immunofluorescence microscopy. The *in vivo* cell surface display of the SpaA-SgsE 3Z+ chimera was analyzed by fluorescence microscopy using polyclonal anti-SgsE rabbit antiserum and a fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit secondary antibody (Sigma). *P. alvei* CCM 2051^T cells harboring pEXALV_SP_SgsE3Z+ were harvested at an OD₆₀₀ of ~0.6 and washed three times with PBS. After resuspension in 100 µl of PBS, 10 µl of anti-SgsE antibody was added and the mixture was incubated for 1.5 h at 25°C on a horizontal shaker. After being washed three times, the cells were resuspended in 100 µl of PBS containing 5 µl of an FITC-labeled secondary antibody and incubated for an additional 1.5 h. After being washed, the cells were resuspended in 100 µl of PBS and analyzed by fluorescence microscopy.

P. alvei CCM 2051^T cell suspensions (5 µl each) expressing the pEXALV_SpaA_SgsE3Z+ and pEXALV_SP_SgsE3Z+ plasmids, respectively (Table 1), were mixed with 5 µl of a solution containing 0.9% molten agarose, 40 mM Tris-HCl (pH 8.0), 20 mM acetic acid, and 1 mM EDTA (pH 8.0) and preheated to 55°C. The samples were covered immediately with glass to immobilize the cells in the solidified agarose. The samples were analyzed using a Nikon Eclipse TE2000-S fluorescence microscope at a magnification of ×100 using an oil immersion objective and, in the case of the FITC-labeled SpaA-SgsE 3Z+ chimera, the FITC filter block. Pictures were taken with a Nikon Digital Sight DS-Qi1Mc camera and the NIS-Elements 3.22 imaging software.

General and analytical methods. SDS-PAGE was carried out according to a standard protocol (27) using a Protean II electrophoresis appara-

tus (Bio-Rad). Protein bands were visualized with Coomassie brilliant blue G250 staining reagent. TCA precipitation of proteins was performed according to Sanchez (28). For Western blotting of proteins onto a polyvinylidene difluoride membrane (Bio-Rad), a Mini Trans-Blot cell (Bio-Rad) was used. Detection of EGFP in SpaA chimeras was done with the Li-Cor Odyssey infrared imaging system using anti-GFP mouse antibody (Roche) in combination with goat anti-mouse IgG-IRDye 800CW conjugate (Li-Cor).

PG-containing cell wall sacculi with [PG(+)] or without [PG(−)] SCWP were analyzed for their content of amino sugars and diaminopimelic acid (DAP) after hydrolysis of 1 mg of sample each with 200 µl of 4 N HCl for 4 h at 110°C (26, 29). Dried samples were resuspended in 200 µl of 0.2 M sodium citrate buffer (pH 2.2) (Biochrom) and analyzed on a Biochrom 30 amino acid analyzer.

RESULTS

Two functional SLH domains are sufficient for binding of SpaA to peptidoglycan-containing cell wall sacculi *in vitro*. To investigate the individual and concerted roles of the TRAE, TVEE, and TRAQ motifs that are located in the first, second, and third SLH domains of SpaA, respectively, for the binding of the S-layer protein to the cell envelope, the motifs were mutated into TAAA sequences. In analogy to what has been described for *T. thermosulfurigenes* EM1 (14), it was assumed that the TAAA motif would render the respective SLH domain nonfunctional for binding to PG-associated SCWP.

The complete set of mutated recombinant SpaA variants (TAAA₁, TAAA₂, TAAA₃, TAAA₁₂, TAAA₁₃, TAAA₂₃, and TAAA₁₂₃) was tested for the ability to bind to native PG-containing cell wall sacculi [PG(+)] of *P. alvei* CCM 2051^T, which were used to mimic the native situation. The results from the *in vitro* binding assay, as evidenced by the densitometric quantification of Western immunoblots, indicated that all single mutants (TAAA₁, TAAA₂, and TAAA₃) retained their ability to bind to PG(+) material of *P. alvei* CCM 2051^T, with the best binding observed for TAAA₂ (~88%) and decreased binding for TAAA₃ and TAAA₁ (~51% and ~38%, respectively). In contrast, the double mutants TAAA₁₂ and TAAA₂₃ exhibited drastically impaired binding of ~6%, each, while TAAA₁₃ had completely lost binding capability (Fig. 1A). The same applied for the triple mutant (compare with Table 3). The controls, recombinant full-length SpaA and N-terminally truncated SpaA lacking the SLH domains, confirmed the binding hypothesis, with the recombinant S-layer protein having affinity to PG(+) cell envelope material (99% binding) and the truncated S-layer protein being nonreactive with that material (Fig. 1A).

To test if the binding of the S-layer protein was specific for the SCWP associated with the native PG of *P. alvei* CCM 2051^T, the cell walls were treated with HF to liberate the covalently bound SCWP, yielding PG(−) cell envelope material (26). Analysis of that material for the molar content of GlcNH₂, DAP, and ManNH₂ revealed a ratio of 1.3:1:0 in comparison to a ratio of 1.9:1:0.7 for PG(+) cell envelope material, with DAP being arbitrarily set to 1.0, GlcNH₂ being indicative of the PG backbone, and ManNH₂ serving as a lead component for SCWP. This confirmed that after HF treatment, the cell wall sacculi were completely SCWP free. The use of that PG(−) cell envelope material in the *in vitro* binding assay clearly showed that neither the different TAAA mutants nor truncated SpaA could bind to the support (Fig. 1B and Table 3). In contrast, recombinant full-length SpaA still showed ~37% binding to PG(−) (Fig. 1B).

FIG 1 Functional SLH domains are required for *in vitro* binding of SpaA to cell wall sacculi. Binding of native SpaA and SpaA variants to PG(+) and PG(−) cell wall sacculi of *P. alvei* as well as to PG of *E. coli* DH5α was tested. S-layer protein was incubated with (a) and without (b) cell wall sacculi. After incubation, the reaction mixtures were centrifuged to separate cell walls (with bound protein) from unbound protein. Analysis was done by SDS-PAGE (8 to 10%) followed by Western blotting using anti-His₆ antibody. Ten microliters of each sample was loaded onto the gel. L, PageRuler Plus prestained protein ladder (Fermentas); S, supernatant; P, pellet; w/o, without. The results of the Western blots used for quantification are summarized in Table 3. The figure represents one of at least two independent repeats of the experiment. If more than one band is visible, the band in accordance is marked with an asterisk.

Based on that finding, we were interested to see whether recombinant SpaA would bind to the PG of a heterologous, non-SCWP-expressing organism, since with a PG-binding domain, the protein should be able to be pulled down by a heterologous organism's PG. Quantification of the *in vitro* binding of the recombinant S-layer protein and of the triple mutant TAAA₁₂₃ to the PG of *E. coli* DH5α showed binding capabilities of ~9% and 4%, respectively. In contrast, truncated SpaA lacking the SLH domains did not bind at all (Table 3).

The combined results from the *in vitro* experiments suggest

TABLE 3 Quantification of the *in vitro* binding assay of the S-layer protein SpaA and variants with mutations in the three predicted binding motifs TRAE, TVEE, and TRAQ

Variant ^a	% binding to ^b :		
	PG(+)	PG(−)	<i>E. coli</i> DH5α PG
TAAA ₁	37.4 ± 9.8	3.5 ± 5.0	
TAAA ₂	87.9 ± 1.6	0.6 ± 0.6	
TAAA ₃	50.5 ± 16.4	0.0 ± 0.0	
TAAA ₁₂	6.3 ± 8.8	5.0 ± 0.1	
TAAA ₁₃	0.6 ± 0.9	0.0 ± 0.0	
TAAA ₂₃	6.4 ± 7.7	0.9 ± 1.3	
TAAA ₁₂₃	2.3 ± 0.6	0.0 ± 0.0	3.7 ± 0.2
SpaA_w/oSP and SpaA_w/oSLH	0.0 ± 0.0	0.6 ± 0.8	0.4 ± 0.7
SpaA recombinant	99.1 ± 1.3	36.6 ± 3.5	9.1 ± 1.6

^a All amino acid motifs were mutated into TAAA, in either one (TAAA₁, 2, or 3), two (TAAA₁₂, 13, or 23), or all three (TAAA₁₂₃) SLH domains. w/o, without; SP, signal peptide.

^b Data are shown as means ± standard deviations from at least three independent experiments.

that for anchoring of the SpaA S-layer protein to the native PG layer [PG(+)], at least two functional SLH domains are necessary and that the *P. alvei* CCM 2051^T SLH domains obviously have a dual-recognition function, both for the SCWP and for the PG. This corroborates the initial concept of SLH domain-mediated cell envelope binding, which implicated binding to PG (30–33).

Inactivation of two SLH domains results in loss of binding of SpaA to the cell envelope and secretion of glycosylated SpaA *in vivo*. To investigate whether the *in vitro* data are representative of the *in vivo* situation and whether SpaA glycosylation is coupled to anchoring of the S-layer protein to the cell envelope, SpaA-EGFP chimeras were constructed, including mutated (TAAA₁, TAAA₂, TAAA₃, TAAA₁₂, TAAA₂₃, TAAA₁₃, and TAAA₁₂₃), full-length, and N-terminally truncated SpaA. Expression of chimeras was done in *P. alvei* CCM 2051^T, and cells were harvested at an OD₆₀₀ of ~1.0. The chimeras were analyzed for cell surface display and their glycosylation status after centrifugation, assuming that in a case in which cell envelope anchoring occurred, the respective chimera should be detectable in the pellet, whereas it should be detectable in the TCA-precipitated supernatant if no binding had occurred. It has to be considered that the pellet fraction includes cytoplasmic material, but as was shown for SpaA lacking the signal peptide and lacking the SLH domains, cytoplasmic SpaA is always unglycosylated. Consequently, glycosylated SpaA contained in the pellet fraction is surface located.

Western immunoblotting using anti-EGFP antibody, in which the glycosylation status was inferred from the molecular mass of the detected SpaA band in comparison to the wild-type situation, supported the *in vitro* experiments, except for the TAAA₁ single mutant, for which only ~38% binding was detected in the *in vitro*

FIG 2 Inactivation of two SLH domains results in loss of binding and secretion of glycosylated SpaA. Presented are Western immunoblots (anti-EGFP antibody) showing the binding affinity of wild-type and mutant SpaA-EGFP chimeras to the cell surface of *P. alvei* CCM 2051^T Δ slhA cells. Ten microliters (0.5 OD unit) of the pellet and 20 μ l of 2-fold-concentrated TCA extracts of the supernatant were applied to the SDS-PAGE (8 to 10%). (A) TAAA₁; (B) TAAA₂; (C) TAAA₃; (D) TAAA₁₂; (E) TAAA₁₃; (F) TAAA₂₃; (G) TAAA₁₂₃; (H) wild-type S-layer glycoprotein (control); (I) S-layer protein without signal peptide and SLH domains. The additional bands present in the pellet fraction of TAAA₁₂₃ (H) might be caused by cross-reactivity of the GFP-antibody with the wild-type S-layer bands of *P. alvei*, since these bands migrate at a lower molecular mass than the labeled bands present in the supernatant fraction. S, supernatant; P, pellet; WT, wild type; w/o, without.

experiment (Fig. 1A), while in the *in vivo* experiment, most of the protein could be recovered from the pellet (Fig. 2A). For the TAAA₂ single mutant (Fig. 2B) and for the positive control (native SpaA-EGFP without mutation) (Fig. 2D), the protein was found in the pellet, indicative of binding of the protein to the cell surface. For the TAAA₃ single mutant, protein could also be detected in the supernatant (Fig. 2C), as could be expected from 51% binding efficiency in the *in vitro* experiment (compare with Table 3).

For the double (Fig. 2E to G) and triple (Fig. 2H) mutants, the protein was detected in the supernatant, showing the loss of ability to bind to the cell surface.

Interestingly, the EGFP-fusion proteins from the variants that were not anchored to the cell surface were glycosylated (as inferred from an aberrant molecular mass upshifted to ~190 and ~270 kDa on SDS-PAGE, while the fusion protein of the variant without a signal peptide and without SLH domains was retained in the cytoplasm and thus was nonglycosylated, as evidenced by migration at ~135 kDa (Fig. 2I), which corresponds well to the calculated molecular mass of the chimera. Thus, the S-layer protein glycosylation event seems to be independent of the anchoring of the target protein to the cell envelope, but requires export of the protein across the cytoplasmic membrane.

The *in vivo* binding data support the results from the *in vitro* experiments. (For a summary of the *in vitro* experiments, see Table 3.) This also shows that neither the EGFP portion nor the His₆ tag included in the different SpaA variants used in the *in vivo* and *in vitro* studies, respectively, compromises the conformation of the SLH domains to such an extent that the native binding behavior would be affected.

Anchoring of recombinant SpaA to GdHCl-extracted *P. alvei* CCM 2051^T wild-type cells. In a setting that mimicked a “semi-native” situation, *P. alvei* CCM 2051^T wild-type cells that had been previously treated with 5 M GdHCl to extract the S-layer glycoprotein were used as a support for binding of recombinant, His₆-

tagged full-length SpaA and the N-terminally truncated SpaA counterpart. Assuming that it takes a certain period of time until the stressed *P. alvei* CCM 2051^T cells (which under favorable conditions have a generation time of ~75 min) are covered with S-layer glycoprotein again, solutions of the recombinant SpaA protein were added to LB medium containing “naked” *P. alvei* CCM 2051^T cells.

Since the read-out principle of the experiment was based on centrifugation followed by Western immunoblotting analysis of the pellet and the supernatant, the inherent self-assembly tendency of the His₆-tagged full-length SpaA and His₆-tagged SpaA devoid of SLH domains was tested under identical reaction conditions to rule out that pellet originated from self-assembled rather than bound SpaA protein (Fig. 3).

Full-length, recombinant SpaA protein was clearly able to bind to “naked” *P. alvei* CCM 2051^T cells, albeit it has to be considered that due to the 8 h of cultivation of the reaction mixtures after addition of SpaA, a reasonable production of S-layer glycoprotein has already taken place (Fig. 3, lane 2). Thus, there was also a lot of unbound protein found in the supernatant of the reaction (Fig. 3, lane 3), which was probably due the fact that the recombinant protein was displaced by the newly synthesized glycoprotein; on the other hand, it could also be caused by the surplus of added protein. In contrast, for the truncated S-layer variant lacking the SLH domains no binding could be observed (Fig. 3, lane 6); the added protein was exclusively found in the supernatant (Fig. 3, lane 7). Aggregation of protein due to the self-assembly capability of the S-layer proteins did not take place, either for the full-length protein or for the truncated variant, as evidenced by the analysis of respective pellets from assay mixtures that did not contain bacterial cells (Fig. 3, lanes 4 and 8). Thus, the observed effect was due to binding of the recombinant, full-length S-layer protein to the viable *P. alvei* CCM 2051^T cells, corroborating the requirement of the SLH domains for cell envelope anchoring. However, this pro-

FIG 3 Anchoring of recombinant SpaA to GdHCl-extracted *P. alvei* CCM 2051^T cells. Presented are the results from SDS-PAGE and Western immunoblotting (anti-His₆ antibody) showing the affinity of recombinant full-length His₆-tagged SpaA or recombinant His₆-tagged SpaA lacking the SLH domains to *P. alvei* CCM 2051^T cells extracted with GdHCl. Incubation was done for 8 h at 37°C (final OD₆₀₀, ~0.8). Ten microliters (0.5 OD unit) of the pellet and 10 μ l of TCA extracts of the supernatant were applied to the SDS-PAGE (10%). Lane 1, PageRuler Plus prestained protein ladder; lane 2, pellet of cells incubated with recombinant full-length His₆-tagged SpaA; lane 3, TCA-precipitated supernatant of cells incubated with recombinant full-length His₆-tagged SpaA; lane 4, pellet of control for lane 2 without cells; lane 5, TCA-precipitated supernatant of control for lane 3 without cells; lane 6, pellet of cells incubated with recombinant His₆-tagged SpaA lacking the SLH domains; lane 7, TCA-precipitated supernatant of cells incubated with recombinant His₆-tagged SpaA lacking the SLH domains; lane 8, pellet of control for lane 6 without cells; lane 9, TCA-precipitated supernatant control for lane 7 without cells. The figure represents one of two independent repeats of the experiment.

tein was not glycosylated, as indicated by a molecular mass of ~107 kDa corresponding to the nonglycosylated His₆-tagged SpaA protein (calculated molecular mass based on amino acid sequence). This observation supports our hypothesis of cosecretory glycosylation of SpaA in a pre- or even unfolded state prior to the formation of the 2D crystalline array on the bacterial cell surface.

Display of a heterologous protein on the cell surface of *P. alvei* CCM 2051^T via SLH domains. Based on previous reports on the use of SLH domains for anchoring of heterologous proteins on the cell surface of *Bacillus anthracis* (34, 35), it was investigated whether proteins fused to the SLH domains of SpaA could be synthesized and efficiently exposed on the cell surface of *P. alvei* CCM 2051^T. For proof of concept, a chimera was constructed comprising the signal peptide and SLH domains of SpaA and the C-terminal portion SgsE 3Z+ of the S-layer protein of *G. stearothermophilus* NRS 2004/3a (Fig. 4A). Analysis of *P. alvei* CCM 2051^T cells was done by fluorescence microscopy using an antibody raised against SgsE and an FITC-labeled secondary antibody for detection.

The SpaA-SgsE chimera was exclusively detectable on the outermost surface of *P. alvei* CCM 2051^T cells (Fig. 4B). We also checked proteins carrying the SpaA signal peptide but lacking the SLH domains for binding, and in this case, no cells with bound protein could be detected (data not shown). This confirmed that the chimera indeed bound in an almost even distribution to the cell surface via the SLH domains, as inferred from the appearance of a continuous fluorescent halo around the cells, and neither formed aggregates nor self-assembled protein at the cell surface. Western immunoblotting of the same samples confirmed that the SpaA-SgsE hybrid was found in the pellet (Fig. 4C, lane 2), with

the molecular mass of the hybrid corresponding well to the calculated molecular mass of 77 kDa. It was also evident that the SpaA-SgsE hybrid was not very stable, because several degradation bands could be detected. In contrast, the truncated hybrid, which only contained the signal peptide of SpaA but not the SLH domains, was found exclusively in the TCA-precipitated supernatant (41-kDa calculated molecular mass) (Fig. 4C, lane 5). Interestingly, also in the unbound fraction of the full-length hybrid, a protein band appeared at almost the same height, which might be indicative of the fusion site being a preferred cleavage site of the hybrid. (The calculated molecular masses of the SpaA protein and the SpaA part of the chimera are ~39 kDa each.) This underlines the fact that only a protein that is targeted to the cell surface by a signal peptide and carries functional SLH domains is able to bind to the cell surface; a protein without SLH domains is released to the supernatant.

DISCUSSION

SLH domains, and especially the conserved four-amino-acid motif TRAE, which is located at the beginning of the α -helix within these domains (12), are a strategy for anchoring of proteins to the bacterial cell envelope (11, 13, 14, 34–39). This strategy applies for many S-layer proteins of Gram-positive bacteria, which are the focus of this study, exemplified by the SpaA glycoprotein of *P. alvei* CCM 2051^T. While it was initially assumed that SLH domains bind directly to the PG layer of the cell wall, it is now established knowledge that binding is mediated via a PG-associated, nonclassical pyruvylated SCWP (10, 11, 13, 25, 29, 36, 38–41). It was proposed that the apparent functional unit of SLH domains is an oligomeric structure (12), which is in concordance with the usual presence of three SLH domains. For the SCWP, a particular spatial role was suggested that promotes the ordered deposition of SLH domain proteins, thereby influencing proper cell wall assembly (42). While some structural commonalities can be found within SCWPs of different bacteria, especially regarding the composition of the backbone (9), it was only recently found in *Bacillus cereus* that the synthesis of SCWP can be dependent on the growth state and the lifestyle of the bacterium, with the possibility of the synthesis of a second SCWP under biofilm conditions in addition to the constitutive SCWP that is expressed at the cell surface (43).

In this study, we investigated the functional role of the conserved TRAE motif and the TVEE and TRAQ variations thereof within the three N-terminal SLH domains of S-layer protein SpaA of *P. alvei* CCM 2051^T for binding to its pyruvylated SCWP. In contrast to the previously reported requirement of three functional SLH domains as SCWP targeting modules in the cell envelope of *T. thermosulfurigenes* EM1 (14) or *Bacillus anthracis* (13), we demonstrate here by *in vitro* and *in vivo* experiments that for anchoring of SpaA, two functional SLH domains are sufficient, albeit the single mutants TAAA₁ and TAAA₃ showed only 42% and 58%, respectively, of the binding of TAAA₂ (Table 3). Since in the TVEE motif (domain 2) the arginine residue, to which high importance for the promotion of strong binding was attributed in a study with *Thermoanaerobacterium sulfurigenes* EM1 (14), is missing, the influence of mutation on its binding efficiency was only minor, underpinning the previous findings of May et al. (14). The double mutants resulted in a drastic loss of binding capacity in all three combinations, with TAAA₁₂ and TAAA₂₃ possessing residual binding of ~6.5% each. The finding that there is an additive defect with mutated SLH domain variants is different from

FIG 4 Cell surface anchoring of the SpaA-SgsE chimera. (A) Schematic drawing of the SpaA-SgsE fusion construct. (B, upper panel) Immunofluorescence microscopy of *P. alvei* CCM 2051^T wild-type cells displaying the SpaA-SgsE chimera. (B, middle panel) SgsE 3Z+ carrying the SpaA signal peptide but lacking the SLH domains, (B, lower panel) *P. alvei* CCM 2051^T wild-type cells harboring pEXALV (control). For immunofluorescence staining of surface-located SpaA-SgsE, cells were probed with the FITC conjugate. The FITC long pass (LP) filter block was used for detection of FITC-labeled chimeric protein. Corresponding bright-field images of the same cells are shown on the left, the image taken in the FITC channel is shown in the middle, and an overlay is shown on the right side. (C) Western immunoblot showing the binding affinity of the SpaA-SgsE and SP-SgsE chimeras expressed in *P. alvei* CCM 2051^T cells. Ten microliters (0.5 OD unit) of the pellet and 5 μ l of TCA extracts of the supernatant were applied to the SDS-PAGE (10%). 1, PageRuler Plus prestained protein ladder; 2, pellet of cells expressing chimeric SpaA-SgsE; 3, TCA-precipitated supernatant of cells expressing chimeric SpaA-SgsE; 4, pellet of cells expressing SP-SgsE; 5, TCA-precipitated supernatant of cells expressing SP-SgsE. The figure represents one of two independent repeats of the experiment.

the results seen in *T. thermosulfurigenes* (14), in which a functional TRAE motif was found to be necessary in all three SLH domains.

The situation in *P. alvei* CCM 2051^T might put our current understanding of SLH domain-mediated protein display in *Bacillus* species in another perspective. This is also emphasized by the proposal of a dual-recognition function of the *P. alvei* CCM 2051^T SLH domains for both the SCWP and the PG. Our data clearly showed that full-length SpaA retained ~37% of its binding function to PG-containing cell wall sacculi, even after complete removal of SCWP, and also showed binding to the PG of *E. coli* DH5 α , although this was reduced by a factor of 4. Still, binding to PG(–) sacculi was only observed for full-length SpaA protein; mutations in the SLH domains or truncation of the protein rendered it incapable of binding. Thus, we suggest that the mechanism underlying the interaction of the SLH domains with the PG is similar to that with the SCWP. There is a report in the literature supporting this assumption (33). It was originally proposed that

the N-terminal part of the S-layer protein SbsB from *G. stearothermophilus* PV72/p2 carries two binding domains, one for the SCWP and another for the PG (33); however, this could not be verified by real-time surface plasmon resonance measurements, which showed that the SLH domain of SbsB was exclusively responsible for binding to the SCWP (39). On the other hand, there are a few reports on cell surface proteins binding to the PG without involvement of an SCWP, including SdbA, a scaffoldin dockerin binding protein, and the two S-layer proteins Slp1 and Slp2 from *Clostridium thermocellum* (44, 45).

Pyruvylation of ManNAc residues present in the backbone structure seems to be a feature of many *Bacillus* SCWPs (9, 16, 46, 47), possibly pinpointing an ancestral origin of this epitope that is necessary for the interaction between the SCWP and SLH domains (10, 38). There is a strong correlation between the presence of the *csaB* gene encoding a putative pyruvyltransferase catalyzing the addition of pyruvate residues onto ManNAc and the nonco-

valent anchoring mechanism via SLH domains (11). To directly analyze the contribution of the pyruvate residues for the interaction with the SLH domains, we attempted to inactivate *csaB*; this, however, generated a lethal phenotype of *P. alvei* CCM 2051^T (B. Janesch, unpublished data). This is in line with the impossibility of inactivating *spaA* (B. Janesch, unpublished data), which indicates that cell envelope integrity is essential for the viability of *P. alvei* CCM 2051^T. In this context, it is interesting to note that for *B. anthracis*, no null mutations of *tagO* and *tagA*, which mediate assembly of linkage units and tether pyruvylated SCWP to the *B. anthracis* envelope, could be obtained (10). Sequence analogues of these genes are encoded in the predicted SCWP biosynthesis locus of *P. alvei* CCM 2051^T flanked by *csaB* and *spaA* (15).

The experimental design of this study also allowed assessment of the importance of anchoring of the S-layer protein SpaA to the cell envelope for O-glycosylation. This was especially interesting because, while *P. alvei* CCM 2051^T and *G. stearothermophilus* NRS 2004/3a are among the best-studied model organisms for bacterial O-glycosylation, no detailed information is available on where and how the final transfer of the elongated S-layer glycan chain onto the protein occurs (18, 23, 48). *In vivo* experiments with the different variants of SpaA-EGFP chimeras conducted in the course of this study not only confirmed our *in vitro* data but also clearly showed that the S-layer protein was glycosylated independent of anchoring of SpaA to the cell surface. This is derived from the fact that glycosylated SpaA could be detected in the supernatant of those SpaA mutants that have lost their ability to bind to the cell surface. This suggests that the transfer of the complete S-layer glycan chain could occur cosecretionally.

Summarizing, we have shown that (i) the conserved TRAE motif as well as the TVEE and TRAQ motifs are critical for the binding function of SLH domains, (ii) the presence of functional binding motifs in two SLH domains is sufficient to anchor the S-layer protein SpaA to the cell envelope, (iii) the SLH domains have a dual-recognition function for the SCWP and the PG, (iv) the N-terminal portion of SpaA comprising the signal peptide and the three SLH domains are sufficient for *in vivo* cell surface display of foreign proteins at the cell surface of *P. alvei*, and (v) anchoring of SpaA to the cell envelope is not a prerequisite for protein glycosylation.

From the understanding of the mechanism underlying SLH domain-mediated cell surface display in combination with target-oriented modification of the displayed glycans, novel concepts in (nano)biotechnology may emerge in the future.

ACKNOWLEDGMENTS

We gratefully acknowledge the expert technical assistance of Sonja Zayni.

Financial support came from the Austrian Science Fund, projects P20745-B11 (to P.M.) and P21954-B20 (to C.S.), and the Hochschuljubiläumsstiftung der Stadt Wien, project H-2136/2010 (to B.J.).

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