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Cell surface display of chimeric glycoproteins via the S-layer of *Paenibacillus alvei*

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Abstract

The Gram-positive, mesophilic bacterium *Paenibacillus alvei* CCM 2051^T possesses a two-dimensional crystalline protein surface layer (S-layer) with oblique lattice symmetry composed of a single type of O-glycoprotein species. Herein, we describe a strategy for nanopatterned in vivo cell surface co-display of peptide and glycan epitopes based on this S-layer glycoprotein self-assembly system. The open reading frame of the corresponding structural gene *spaA* codes for a protein of 983 amino acids, including a signal peptide of 24 amino acids. The mature S-layer protein has a theoretical molecular mass of 105.95 kDa and a calculated pI of 5.83. It contains three S-layer homology domains at the N-terminus that are involved in anchoring of the glycoprotein via a non-classical, pyruvylated secondary cell wall polymer to the peptidoglycan layer of the cell wall. For this polymer, several putative biosynthesis enzymes were identified upstream of the *spaA* gene. For in vivo cell surface display, the hexahistidine tag and the enhanced green fluorescent protein, respectively, were translationally fused to the C-terminus of SpaA. Immunoblot analysis, immunofluorescence staining, and fluorescence microscopy revealed that the fused epitopes were efficiently expressed and successfully displayed via the S-layer glycoprotein matrix on the surface of *P. alvei* CCM 2051^T cells. In contrast, exclusively non-glycosylated chimeric SpaA proteins were displayed, when the S-layer of the glycosylation-deficient *wspP* mutant was used as a display matrix.

Keywords

Cell surface display; S-layer; Nanopatterning; Secondary cell wall polymer biosynthesis; *Paenibacillus alvei*

1. Introduction

The presentation of heterologous proteins or peptide epitopes on various cell surfaces by genetic engineering has become an intensely used strategy for a wide range of

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biotechnological applications, including live vaccine development and delivery,¹ antibody production,² peptide library screening,³ whole-cell biocatalysis,⁴ and bioremediation.^{5,6} So far, numerous display systems have been studied for both bacteria and yeast.^{7–12}

Outer membrane proteins, lipoproteins, autotransporters, ice nucleation proteins, flagellae, and fimbriae are the most common anchoring motifs of Gram-negative surface display systems, whereas for Gram-positive bacteria, staphylococcal protein A and different S-layer proteins, such as RsaA of *Caulobacter crescentus* and EA1/Sap of *Bacillus anthracis* have been utilized.^{9,13–19} S-Layers, in general, are among the most frequently observed outermost cell surface structures of bacteria. They are composed of individual protein or glycoprotein species, which have the unique feature of self-assembling into a closed two-dimensional crystalline array with nanometer-scaled periodicity. Thus, this matrix is ideally suited for cell surface display approaches, where strict control over position and orientation of functional epitopes or molecules is desired.²⁰ For *C. crescentus*, the insertion of a protein G IgG-binding domain into certain sites of full length S-layer protein RsaA resulted in functional, immunoreactive surface display at very high density.²¹ For *B. anthracis*, targeting of active levansucrase of *Bacillus subtilis* and immunogenic tetanus toxin fragment C of *Clostridium tetani* to the cell surface was achieved by translational fusion of the target proteins to the three S-layer homology (SLH) domains of EA1 and Sap, respectively.^{15,17}

Amino- and carboxy-terminal SLH domains of an approximately 55 amino acid-long sequence motif, each, have been identified in several S-layer proteins, in many cell wall-bound exoenzymes, and in outer membrane proteins.²² Although the overall sequence similarity of SLH domains is rather low, a highly conserved TRAE motif has been identified to play a key role for the binding function of SLH domains.²³ In the case of S-layer (glyco)proteins, it has been shown that these anchoring modules do not directly bind to peptidoglycan, but to a non-classical secondary cell wall polymer (SCWP)^{24–28} carrying either pyruvate or carboxyl group-containing modifications. In *B. anthracis* and *Thermus thermophilus*, pyruvylation of SCWPs was shown to be dependent on the presence and activity of the polysaccharide pyruvyltransferase CsaB.^{24,26} Further, in different *Bacillus* strains, a perfect correlation between the occurrence of CsaB homologues and the presence of SLH domains exists, leading to the conclusion that the interaction between pyruvylated SCWP and SLH domains is widespread in bacteria and has been conserved during evolution.^{24,26}

In the present study, we lay the groundwork for the development of a peptide/protein and glycan cell surface co-display system based on the glycosylated S-layer protein SpaA of *Paenibacillus alvei* CCM 2051^T. This is a very timely approach, since, due to the complexity of their biosynthesis, glycans have escaped cell surface display approaches, so far. *P. alvei* CCM 2051^T is a mesophilic, Gram-positive bacterium that is covered with an oblique S-layer nanolattice. The S-layer proteins are naturally O-glycosylated, exposing long-chain saccharides with the structure $[-\rightarrow 3)-\beta\text{-D-Galp-(1}[\alpha\text{-D-Glcp-(1}\rightarrow 6)]\rightarrow 4)-\beta\text{-D-ManpNAc-(1}\rightarrow]_{n\sim 23}-[\text{GroA-2}\rightarrow \text{OPO}_2\rightarrow 4-\beta\text{-D-ManpNAc-(1}\rightarrow 4)]-[3)-\alpha\text{-L-Rhap-(1}\rightarrow]_{n=3} 3)-\beta\text{-D-Galp-(1}\rightarrow \text{O})\text{-Tyr}$ to the environment.²⁹ This glyco-nanolattice is anchored to the bacterial cell wall via a pyruvate-containing SCWP with the structure $[(\text{Pyr4,6})-\beta\text{-D-ManpNAc-(1}\rightarrow 4)-\beta\text{-D-GlcpNAc-(1}\rightarrow 3)]_{n\sim 11}-(\text{Pyr4,6})-\beta\text{-D-ManpNAc-(1}\rightarrow 4)-\alpha\text{-D-GlcpNAc-}$

(1→. These pyruvate-containing and, hence, overall anionic glycan chains are linked via phosphate-containing groups to muramic acid residues of the peptidoglycan layer.³⁰

We describe herein (i) the identification of the *spaA* gene coding for the S-layer protein of *P. alvei* CCM 2051^T, (ii) its utilization as an in vivo surface co-display system, and (iii) the identification of several open reading frames in the upstream region of *spaA* coding for enzymes putatively involved in the biosynthesis of the native cell wall anchor of the S-layer nanolattice, which is the pyruvylated SCWP.

For proof of concept, full-length SpaA was expressed with a carboxy-terminal hexahistidine tag and with enhanced green fluorescent protein as a fusion partner, respectively. Either chimeric protein was shown to be displayed on the cell surface and glycosylated by the native glycosylation machinery of *P. alvei* CCM 2051^T. To our knowledge, this is the first report on the co-display of a fused functional epitope and an O-glycosidically linked glycan on a bacterial cell surface in a nanolattice-like fashion. This strategy is opening up new avenues for controlled high-density co-display of protein- and carbohydrate-mediated biological functions with defined orientation and with nanometer-scaled precision. This approach ideally mimics, especially for the glycan-mediated functions, the naturally occurring clustering effect. When the S-layer glycosylation-deficient *wspP* mutant was used for cell surface display, exclusively non-glycosylated chimeric SpaA proteins were obtained.³¹

2. Results

2.1. General description of the organism

The cell surface of the type culture strain *P. alvei* CCM 2051^T (ATCC 6344; DSM 29) is completely covered with an oblique S-layer lattice composed of identical O-glycoprotein species. Various aspects of its crystalline S-layer including ultrastructural characterization,^{32,33} glycosylation analysis,^{29,33} and glycan biosynthesis³⁴ have been investigated in the past. In SDS-PAGE, the S-layer glycoprotein is separated into three bands with apparent molecular masses of approximately 105, 160, and 240 kDa, respectively. The two high molecular mass bands give a positive PAS staining reaction, corresponding to mono- and di-glycosylated S-layer proteins.³¹

2.2. Isolation and molecular characterization of *spaA*

After chemical deglycosylation of the S-layer glycoprotein, Edman degradation revealed the N-terminus of the mature protein to have the amino acid sequence ADAAKTTQEK. Based on this information, the degenerate oligonucleotide primer proof_wSpa_-for was designed by in silico reverse translation. Identification of the entire *spaA* gene was accomplished using a gene walking approach, starting with the primer specific for the N-terminus of *spaA*. The sequence was deposited at GenBank under the accession number FJ751775. The *spaA* gene revealed one ORF extending 2952 nt, encoding a putative protein of 983 amino acids with a calculated molecular mass of 108.55 kDa. The ORF starts with an ATG at nucleotide position 1, preceded by a typical ribosomal binding site (Shine–Delgarno sequence) 19 nt upstream of the start codon and a putative promoter region comprising a –10 sequence

(TTGTATAAT) located 232 nt upstream of the translation start and a putative –35 sequence (TTTACG) starting 252 nt in front of the start codon. 7 nt downstream of the TAA stop codon, a putative ρ -independent transcriptional termination signal was identified. The terminator consists of a palindromic stem loop sequence of 39 nt with a perfect stem of 13 nt. The average G+C content of the whole *spaA* gene was calculated to be 38.6%.

2.3. Description of the S-layer protein SpaA

The amino acid sequence ADAAKTTQEK obtained by amino-terminal sequencing of chemically deglycosylated, mature SpaA was identified at positions 25–34 of the *spaA* gene product, indicating that the first 24 amino acids of SpaA constitute a signal sequence, which is cleaved at the Gly-Ala motif during biosynthetic protein processing. The overall amino acid composition of mature SpaA is within the typical data reported for S-layer proteins of Gram-positive bacteria,³⁵ exhibiting a high content of hydrophobic and acidic amino acids and lacking cysteine. Mature SpaA has a calculated theoretical molecular mass of 105.95 kDa and a pI of 5.83. A conserved motif search revealed the presence of three SLH domains (PF00395) at the amino-terminal region of the S-layer protein; these comprise the amino acid stretches aa 25–65, aa 82–129, and aa 140–181, respectively (Table 1). The comparison of the three SLH domains with the SLH domain profile is depicted in Figure 1.

A homology search using the BLASTP program showed that the amino acid sequence of SpaA shows moderate identities with other surface exposed proteins of different *Bacillaceae*. A phylogenetic tree was constructed by pairwise alignment of SpaA with the first 100 proteins of this BLAST search (Fig. 2). The rooted tree shows that SpaA is closely related to S-layer domain proteins of *Paenibacillus* sp. JDR-2 and *Geobacillus* sp. Y412MC10 as well as to an S-layer domain-containing protein of *Paenibacillus* sp. oral taxon 786 strain D14. A rather distant relationship could be observed between SpaA and several cell surface proteins of different clostridia. The global identity and similarity values of SpaA compared with other well-characterized S-layer proteins are summarized in Table 2. Considering these data, it is obvious that regions of high homology are neither present in the N-termini, responsible for binding of the S-layer protein to the cell wall, nor in the residual amino acid sequence. This corroborates the general concept of low primary sequence similarity of S-layer proteins, even of those of phylogenetically closely related bacteria.³⁶

2.4. Description of the SCWP biosynthesis locus

Upstream and downstream of the *spaA* gene, several genes were identified, which are most likely involved in the SCWP biosynthesis of *P. alvei* CCM 2051^T (Fig. 3). By in silico analysis using the *Escherichia coli* σ^{70} promoter consensus sequence, putative promoter sites located upstream of *orf1*, *tagO*, *slhA*, *spaA*, and *orf7* were recognized. In addition to the ρ -independent bacterial terminator downstream of *spaA*, a single terminator was identified downstream of the *slhA* gene. The prediction of transcription units for the complete ~14-kb DNA fragment resulted in a polycistronic RNA containing *orf1*, *csaB*, and *tagA* in addition to four separate monocistronic RNAs for *tagO*, *slhA*, *spaA*, and *orf7*. The putative gene products of the SCWP biosynthesis locus have been analyzed by extensive database comparison and are discussed in the order of their appearance within the analyzed region.

2.4.1. *orf1*—The gene product of *orf1* contains eight potential transmembrane domains at its carboxy-terminal part and several domains of unknown functions (DUFs) also found in putative bacterial cell surface and hypothetical transmembrane proteins. The signal peptide of 23 amino acids is followed by a 340 amino acid-containing loop potentially facing the cell exterior. The Conserved Domain Finder (National Center for Biotechnology Information) found a weak similarity to the sublancin ABC transporter SunT of *B. subtilis* 168, suggesting that this protein might act as an exporter³⁷ like CsaA in the biosynthesis of other pyruvylated SCWPs.^{24,26}

2.4.2. *csaB*—Throughout the whole gene *csaB* is highly homologous to genes coding for pyruvyltransferases (CsaB) in various *Bacillus* strains, with the translation product containing a polysaccharide pyruvyltransferase domain. In *B. anthracis* and *T. thermophilus*, CsaB is involved in the addition of pyruvyl groups to the SCWP, a frequently necessary reaction for anchoring of cell wall associated proteins containing SLH domains.^{24–27} The presence of a pyruvate-containing SCWP in *P. alvei* CCM 2051^T suggests that CsaB is responsible for SCWP-pyruvylation in this organism.³⁰

2.4.3. *tagA* and *tagO*—The deduced 252- and 377-amino acid proteins encoded by *tagA* and *tagO* reveal high similarity to the glycosyltransferases TagA and TagO of different *Bacillaceae*, respectively. In *B. subtilis*, both enzymes are involved in the biosynthesis of teichoic acids, where TagO couples *N*-acetylglucosamine (GlcNAc) to the membrane-embedded lipid undecaprenylpyrophosphate and TagA catalyzes the addition of *N*-acetylmannosamine (ManNAc) to produce the lipid-linked GlcNAc-ManNAc disaccharide.^{38,39} Since a ManNAc-Glc-NAc backbone disaccharide motif is found in the SCWP of *P. alvei* CCM 2051^T, it is conceivable that both enzymes are involved in the biosynthesis of this cell wall polysaccharide.^{29,38}

2.4.4. *slhA*—The *slhA* gene product contains three carboxy-terminal SLH domains and is similar to SLH domain-containing proteins of various Gram-positive bacteria. In the central part of the putative protein, a bacterial Ig-like domain found in a variety of bacterial surface proteins is identified.⁴⁰ A galactose-binding domain (CBM6) typical for proteins binding to specific ligands, such as cell-surface-attached carbohydrate substrates, was detected in the amino-terminal part. This observation leads to the suggestion that SlhA might be a cell surface-anchored exoenzyme or a receptor.

2.4.5. *orf7*—No homology was found for this incomplete ORF or its putative protein product.

2.5. Expression and display of chimeric SpaA constructs on the cell surface

The expression of the hexahistidine tagged S-layer protein SpaA_6HIS and the chimeric fusion protein SpaA_EGFP in *P. alvei* CCM 2051^T wild-type and in *wsfP*::Ll.LtrB mutant cells, respectively, was performed constitutively from the vector pEXALV and confirmed by immunoblot analysis of whole-cell lysates using anti-His-tag and anti-GFP antibody, respectively (Fig. 4A and B). In either case, three bands, most possibly corresponding to the non-glycosylated, mono-glycosylated, and di-glycosylated forms³¹ of SpaA_6HIS and

SpaA_EGFP were detected when *P. alvei* CCM 2051^T carrying either pEXALV_SP_SpaA_6HIS or pEXALV_SP_SpaA_EGFP, respectively, was used as an expression host, while no such proteins were detected with *P. alvei* CCM 2051^T carrying the parental plasmid pEXALV (data not shown). Since the transfer of the completed glycan chain to the S-layer protein is predicted to occur either co-secretionally or on the external face of the cytoplasmic membrane,⁴¹ these results indicate that SpaA_6HIS and SpaA_EGFP are correctly expressed and targeted to the cell surface of *P. alvei* CCM 2051^T. For *P. alvei* CCM 2051^T *wsfP::Li.LtrB* mutant cells, which are, due to the deletion in the initiation enzyme of S-layer glycan biosynthesis, of a glycan-deficient phenotype, only a single band corresponding to non-glycosylated SpaA_6HIS and SpaA_EGFP was detected (Fig. 4). Semi-quantitation of the amount of produced recombinant S-layer fusion proteins was performed from Western blots developed with fluorescence-labeled antibodies using the Li-Cor Odyssey Application software. Comparing the expression levels of the recombinant non-glycosylated chimera from *P. alvei* CCM 2051^T *wsfP::Li.LtrB* mutant cells with that of the corresponding chimera produced in glycosylation-competent *P. alvei* CCM 2051^T cells revealed equal amounts of S-layer fusion proteins, regardless of the glycosylation status. This was inferred from the combined intensities of the non-, mono-, and di-glycosylated bands (Fig. 4A and B, lane 1) being identical to that of the single band originating from the glycosylation-deficient mutant (Fig. 4A and B, lane 2). This indicates that the developed system is well suited for peptide/protein and glycan co-display.

To confirm the surface localization of the chimeric S-layer proteins, immunofluorescence microscopy was used (Fig. 5). For SpaA_6HIS, *P. alvei* CCM 2051^T wild-type cells were probed and fluorescently stained with the penta-His Alexa Fluor 555 conjugate. Cells harboring pEXALV_SP_SpaA_6HIS were brightly fluorescent, indicating that SpaA_6HIS was successfully displayed on the surface (Fig. 5C). Surface display of SpaA_EGFP was confirmed by direct fluorescence microscopy (Fig. 5D). *P. alvei* CCM 2051^T wild-type and *wsfP::Li.LtrB* mutant cells (not shown) showed identical results in immunofluorescence microscopy, whereas cells carrying the parental plasmid pEXALV were not stained at all (Fig. 5A and B).

These results show the proper and efficient display of SpaA_6-HIS and SpaA_EGFP on the cell surface of *P. alvei* CCM 2051^T simultaneously with the native S-layer glycans while maintaining the S-layer inherent feature of a dynamically closed two-dimensional nanolattice covering the entire bacterium.

3. Discussion

Monomolecular isoporous S-layers cover many bacterial cells in the form of nanolattices, that is, two-dimensional crystalline arrays with nanometer-scale periodicity. An intact closed S-layer nanolattice on an average-sized, rod-shaped bacterium consists of ~500,000 monomers making it ideally suited for highly efficient display of a significant number of functional epitopes in defined and precise orientation.⁴²

In this report, we describe the identification of the structural gene encoding the S-layer protein SpaA of *P. alvei* CCM 2051^T and the development of an *in vivo* surface co-display

system using this protein as a cell wall anchor. This was exemplified by the presentation of a heterologous peptide epitope and a functional protein, respectively, in addition to the native S-layer glycan chain (Fig. 6). The strategy is based on the continuous expression of plasmid-encoded S-layer chimera in *P. alvei* CCM 2051^T using a constitutive promoter and its export, glycosylation as well as surface anchoring, in competition with the wild-type S-layer glycoprotein.

For proof of concept, the hexahistidine tagged S-layer protein SpaA_6HIS as well as the chimeric fusion protein SpaA_EGFP was constructed and their cell surface localization was demonstrated by immunofluorescence staining and fluorescence microscopy. The used *P. alvei* CCM 2051^T surface display vector pEXALV_SP_SpaA can be generally utilized for translational fusion of various functional epitopes to the C-terminus of SpaA, their subsequent export and surface presentation. By immunoblot analysis, for both surface-displayed constructs—SpaA_6HIS and SpaA_EGFP—a non-glycosylated, mono-glycosylated, and di-glycosylated form resulting from the native S-layer protein O-glycosylation system of wild-type *P. alvei* CCM 2051^T was detected, which reflects the native SpaA O-glycosylation pattern. This indicates the suitability of this system for the future in vivo cell surface co-display of engineered, bioactive glycan structures based on the native S-layer glycans, in addition to peptide/protein epitopes. Since the ongoing investigation of the S-layer glycosylation machinery of *P. alvei* CCM 2051^T revealed some promising insights,⁴¹ this bacterium is a prime candidate for the design and presentation of S-layer neoglycoproteins by means of genetic carbohydrate engineering.⁴³ Thereby, the S-layer glycosylation deficient mutant strain *wsfP*::Ll.LtrB, carrying an insertion in the *wsfP* gene coding for the initiation enzyme of S-layer glycan biosynthesis, provides the possibility of turning on the functional glycosylation of any SpaA chimera, when the activity of WsfP is reconstituted by plasmid-based expression of *wsfP*.³¹

Besides the attachment of a tailor-made, functionalized S-layer protein to the cell envelope, secretion into the medium can also be a desired option for specific purposes, avoiding time- and cost-consuming purification steps. In this context, the anchoring mechanism of the S-layer protein was investigated, starting with the observation of three SLH domains located at the N-terminus of SpaA. The typical, highly conserved TRAE motif could be identified in the first SLH domain (aa 25–65) of SpaA. In the second (aa 82–129) and third (aa 140–181) SLH domain, two different variants of this motif, TVEE and TRAQ, are present. However, these motif variants have also been reported to be functional.²³ Since these SLH domains are known to play a key role in mediating the cell wall binding of various proteins by interacting with SCWPs carrying pyruvate modifications, it was not surprising to identify a pyruvyltransferase encoding gene in the upstream region of *spaA*. Furthermore, with *tagA* and *tagO*, both coding for glycosyltransferases, two additional enzymes obviously involved in SCWP biosynthesis of *P. alvei* CCM 2051^T were identified. Due to the similarity of TagO to an undecaprenylphosphate *N*-acetylglucosaminyl-1-phosphate transferase, it can be assumed that TagO attaches GlcNAc to the membrane-embedded lipid carrier undecaprenylpyrophosphate at the cytoplasmic side of the membrane. The next step in the biosynthesis pathway would be the addition of ManNAc catalyzed by the *N*-acetylglucosaminyl diphosphoundecaprenol *N*-acetyl-β-D-mannosaminyltransferase TagA, to

produce the lipid-linked GlcNAc-ManNAc disaccharide. The transfer of the pyruvate modification to ManNAc residue is accomplished by the pyruvyltransferase CsaB. Additional enzymes required for the export and for the formation of the covalent linkage between SCWP and peptidoglycan could not be identified, so far. The prediction of transcription units for the SCWP biosynthesis locus showed that *orf1*, *csaB*, and *tagA* are located on a polycistronic mRNA. This finding is supported by the closed spacing and the absence of a promoter consensus sequence between these three genes. The role of the gene product of *orf1* being part of this polycistronic mRNA remains, due to the lack of reliable sequence similarities, speculative and needs to be further investigated. The encoded protein could be possibly involved in the export of the pyruvylated polymer. *TagO*, *slhA*, and *spaA* are predicted to be transcribed monocistronically. The presence of three SLH domains located at the C-terminus of SlhA argues for its surface exposure and turns this protein into an eligible candidate for co-display of chimeric SpaA- and SlhA proteins interacting with each other or binding a target molecule in two different manners.

In conclusion, we have demonstrated the in vivo cell surface co-display of a heterologous peptide epitope and a functional protein fused to the N-terminus of the S-layer glycoprotein SpaA of *P. alvei* CCM 2051^T in addition to the S-layer glycans. The developed strategy is the starting point for the future in vivo presentation of different peptides and proteins combined with bioactive glycans, which may have great value in the fields of receptor mimics, vaccine development, or drug delivery.

4. Experimental

4.1. Bacterial strains and growth conditions

P. alvei CCM 2051^T (Table 3) was obtained from the Czech Collection of Microorganisms (CCM, Brno, Czech Republic) and grown at 37 °C and 200 rpm in Luria–Bertani (LB) broth or on LB agar plates supplemented with 10 µg mL⁻¹ chloramphenicol (Cm), when appropriate. *E. coli* DH5α (Invitrogen, Lofer, Austria) was grown in LB broth at 37 °C supplemented with 30 µg mL⁻¹ Cm, when appropriate.

4.2. Analytical and general methods

Genomic DNA of *P. alvei* CCM 2051^T was isolated as described recently.³¹ Restriction and cloning enzymes were purchased from Invitrogen. The MinElute gel extraction kit (Qiagen, Vienna, Austria) was used to purify DNA fragments from agarose gels, and the MinElute reaction cleanup kit (Qiagen) was used to purify digested oligonucleotides and plasmids. Plasmid DNA from transformed cells was isolated with the Plasmid Miniprep kit (Qiagen). Agarose gel electrophoresis was performed as described elsewhere.⁴⁴ Transformation of *E. coli* DH5α was done according to the manufacturer's protocol (Invitrogen). Transformants were screened by in situ PCR using RedTaq ReadyMix PCR mix (Sigma–Aldrich, Vienna, Austria); recombinant clones were analyzed by restriction mapping and confirmed by sequencing (Agowa, Berlin, Germany). Transformation of *P. alvei* CCM 2051^T was performed as described recently.³¹ SDS–PAGE was carried out according to a standard protocol⁴⁵ using a Protean II electrophoresis apparatus (Bio-Rad, Vienna, Austria). Protein bands were visualized with Coomassie Brilliant Blue G250 staining reagent. Western

blotting using a Mini Trans-Blot Cell (Bio-Rad) was performed to transfer the proteins to a polyvinylidene difluoride membrane (Bio-Rad). Anti-GFP mouse antibody (Roche, Vienna, Austria) and anti-His-tag mouse antibody (Novagen, Darmstadt, Germany) were used in combination with goat anti-mouse IgG IR Dye 800CW conjugate (Li-Cor, Lincoln, NB, USA) to detect EGFP and hexahistidine epitopes using the Li-Cor Odyssey Infrared Imaging System (Li-Cor). The integrated intensity of detected bands was determined using the Li-Cor Odyssey Application Software 3.0.21. Isolation and purification of S-layer glycoprotein essentially followed published methods.⁴⁶ Qiagen's Ni-NTA Spin Kit was used to purify hexahistidine-tagged S-layer glycoprotein under denaturing conditions. Deglycosylation of S-layer glycoprotein and amino-terminal sequencing (Edman degradation) were performed as published previously.⁴⁷

4.3. PCR and DNA sequencing

PCR (My Cycler™, Bio-Rad) was performed using the Herculase® II Fusion DNA Polymerase (Stratagene, La Jolla, CA, USA). For each primer pair (Table 4), PCR conditions were optimized, and amplification products were purified using the MinElute PCR purification kit (Qiagen). Primers for PCR and DNA sequencing were purchased from Invitrogen. For sequence determination of the *spaA* gene including upstream and downstream regions, chromosome walking was applied as previously described.^{48,49}

4.4. Sequence analysis

Nucleotide and protein sequences were analyzed using the BLASTN and BLASTP sequence homology analysis tools (National Center for Biotechnology Information, Bethesda, MD, USA). Open reading frames in the DNA sequence were identified by using the Clone Manager Professional Suite (SECentral, Cary, NC, USA) and the ORF Finder analysis tool (National Center for Biotechnology Information). For the identification of putative protein transmembrane-spanning domains and the presence and location of signal peptide cleavage sites, the TMHMM Server v. 2.0 transmembrane prediction program and the SignalP 3.0 Server (Center for Biological Sequence Analysis, Technical University of Denmark, Lyngby, Denmark) were used, respectively. The G+C content of a certain DNA sequence was determined using the GC Content and GC Skew program (Nano+Bio-Center, University of Kaiserslautern, Germany). For in silico reverse translation the Sequence Manipulation Suite was used.⁵⁰ Bacterial promoters, transcriptional terminators, operons, and genes were predicted by the BProm and FindTerm modules of the FGenesB gene prediction program in Molquest software (SoftBerry, Mount Kisco, NY, USA). The presence of conserved motifs in a given protein sequence was analyzed by the Pfam protein families database,⁵¹ the SUPERFAMILY database,⁵² and the Conserved Domain Finder of the National Center for Biotechnology Information.^{53–55} Physical and chemical parameters for a given protein were calculated using the ProtParam tool.⁵⁶ For pairwise alignments of certain protein sequences, the EMBOSS Pairwise Alignment Algorithms were used.⁵⁷

The phylogenetic tree was calculated with the Blast Tree View Widget using the Neighbor Joining method⁵⁸ and visualized using iTOL.⁵⁹ Sequences with more than 0.85 difference were removed from the treeview.

4.5. Construction of the SpaA surface display constructs

The *P. alvei* expression vector pEXALV was used for construction of all SpaA surface display constructs. The carboxy-terminal hexahistidine tag was fused to the coding sequence of *spaA* by PCR using primers SP_SpaA_*SphI*_for and SpaA_6HIS_STOP_*KpnI*_rev, with genomic DNA of *P. alvei* CCM 2051^T as template. The ~3000-bp PCR product was digested with *SphI* and *KpnI* and ligated into *SphI/KpnI*-linearized and dephosphorylated plasmid pEXALV. This construct was named pEXALV_SP_SpaA_6HIS. For the construction of an S-layer-EGFP fusion protein, the DNA fragment encoding the *spaA* gene lacking the TAA stop codon was amplified by PCR using primers SP_SpaA_*SphI*_for and SpaA_noSTOP_*PstI*_rev with genomic DNA of *P. alvei* CCM 2051^T as template. The ~3000-bp PCR product was digested with *SphI* and *PstI* and ligated into *SphI/PstI*-linearized and dephosphorylated plasmid pEXALV. This construct was named pEXALV_SP_SpaA. The 742-bp *egfp* fragment was amplified by PCR from plasmid pEGFP-N1 using the primers EGFP_for_*PstI* and EGFP_rev_*KpnI*, digested with *PstI* and *KpnI*, and cloned *in frame* into *PstI/KpnI*-linearized and dephosphorylated plasmid pEXALV_SP_SpaA. This construct was named pEXALV_SP_SpaA_EGFP.

4.6. Analysis of cell surface display and immunofluorescence staining of SpaA-constructs

The surface accessibility of the displayed hexahistidine-tagged S-layer protein SpaA_6HIS on intact *P. alvei* cells was analyzed by direct immunofluorescence staining followed by fluorescence microscopy. Briefly, cells transformed with pEXALV_SP_SpaA_6HIS were harvested after expression at an OD₆₀₀ ~0.6, resuspended, and washed three times in phosphate-buffered saline (PBS). After resuspension in 200 µL of PBS, 10 µL of penta-His Alexa Fluor 555 conjugate (Qiagen) was added and incubated for 2 h at room temperature on a horizontal shaker. After washing for three times, the cells were resuspended in 500 µL of PBS and analyzed by fluorescence microscopy. To directly assess the functional surface expression of the chimeric S-layer fusion protein SpaA_EGFP, *P. alvei* cells transformed with pEXALV_SP_SpaA_EGFP were analyzed by fluorescence microscopy. Fluorescence microscope imaging of intact *P. alvei* cells was carried out with a Nikon Eclipse TE2000-S inverted fluorescence microscope with a Hg vapor lamp, a Nikon digital sight DS-Qi1Mc camera, and the NIS-Elements imaging software using the TRITC (540/25 nm for excitation light, 605/55 nm for emission light) filter block for Alexa Fluor 555 and the GFP LP (480/40 nm for excitation light, long pass at 510 nm for emission light) filter block for EGFP.

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Figure 1.

Comparison of the three SLH domains of SpaA with the S-layer homology (SLH) domain profile (PROSITE No.: PS51272). The amino-terminal region starting with the first amino acid of the mature protein (aa 25) is aligned with the PROSITE sequence logo. The total height of an amino acid position depends on the degree of conservation in the corresponding multiple sequence alignment and the height of each letter is proportional to the observed frequency of the particular amino acid.

Figure 2.
Phylogenetic tree of the S-layer protein SpaA of *P. alvei* CCM 2051^T and its closest relatives. The calculation was done with the Blast Tree View Widget using the Neighbor Joining method.

Figure 3.

Genetic organization of the SCWP biosynthesis locus of *P. alvei* CCM 2051^T. Predicted open reading frames are indicated by horizontal arrows with the respective gene designations indicated above the arrow. Genes encoding similar functions in SCWP biosynthesis have a similar gray scaling code. Genes coding for proteins with unknown function are indicated in black. Genes highlighted in gray encode putative glycosyltransferases. White indicates the *csaB* gene encoding the pyruvyltransferase. The structural gene *spaA* is highlighted in lightest gray. Putative promoters and terminators are represented as flags and hairpins, respectively. Monocistronic and polycistronic mRNAs are depicted as vertical black arrows. The graphic representation of the G+C percentages is given below the locus map.

Figure 4.

Western blot with fluorescence detection of chimeric S-layer proteins. (A) SpA₆HIS detected using an anti-His-tag antibody in combination with goat anti-mouse IgG IR Dye 800CW conjugate. (B) Detection of SpA_{EGFP} using an antibody raised against GFP in combination with goat anti-mouse IgG IR Dye 800CW conjugate. The constructs were analyzed by SDS-PAGE (8% gel) and transferred to a PVDF membrane followed by immunoblot detection. Tri-banded appearance corresponds to non-glycosylated (N), mono-glycosylated (M), and di-glycosylated (D)³¹ chimeric SpA proteins produced by *P. alvei*

CCM 2051^T wild-type cells (lanes 1). For *P. alvei* CCM 2051^T *wspP::Ll.LtrB* mutant cells, sole bands corresponding to non-glycosylated SpaA chimera were detected (lanes 2). Two milligrams of intact cells were loaded per well.

Figure 5.

Immunofluorescence microscopy of *P. alvei* CCM 2051^T wild-type cells displaying chimeric S-layer glycoproteins. (A and B) Control *P. alvei* CCM 2051^T wild-type cells harboring pEXALV. Recombinant *P. alvei* CCM 2051^T wild-type cells harboring (C) pEXALV_SP_SpaA_6HIS, and (D) pEXALV_SP_SpaA_EGFP. For immunofluorescence staining of surface-located SpaA_6HIS, cells were probed with the penta-His Alexa Fluor 555 Conjugate (Qiagen). The TRITC and the GFP LP filter blocks were used for detection of Alexa Fluor 555 (A and C) and EGFP (B and D), respectively. Corresponding brightfield images of the same cells are shown in the left. The immunofluorescence images of the *wsfP::Ll.LtrB* mutant strain are identical to those observed for wild-type cells and hence not shown.

Figure 6. Strategy for in vivo cell surface co-display of functional epitopes on *P. alvei* CCM 2051^T cells. Schematic picture showing the cell wall profile with the peptide/protein epitope and the glycans being presented in nanopatterned fashion to the exterior of the bacterial cell. Non-glycosylated (N), mono-glycosylated (M), and di-glycosylated (D)³¹ chimeric SpaA proteins are distributed equally over the cell surface.

Table 1

Predicted gene products encoded by the SCWP biosynthesis locus of *P. alvei* CCM 205^T and S-layer protein structural gene *spaA* together with database homologies

ORF	orf1			
Length/mol. mass	657/73.18			
Conserved motifs and regions	Signal peptide 1–23			
	DUF916 (PF06030) 1–30			
	DUF204 (PF02659) 341–395			
	DUF808 (PF05661) 460–641			
	DUF981 (PF06168) 521–558			
Related proteins	DUF2061 (PF09834) 608–650			
	Name/putative function			
	Hypothetical protein GYMC10DRAFT_1551	Organism	Identity/similarity (%)	Accession No.
ORF	Hypothetical protein Pjdr2DRAFT_3486			
	cscB			
Length/mol. mass	396/43.59			
Conserved motifs and regions	PS_pyruv_trans (PF04230) 7–357			
Related proteins	Name/putative function			
	Polysaccharide pyruvyl transferase	Organism	Identity/similarity (%)	Accession No.
	CsaB protein			
	tagA			
Length/mol. mass	252/28.69			
Conserved motifs and regions	Glyco_tran_WecB (PF03808) 60–231			
Related proteins	Name/putative function			
	Glycosyl transferase, WecB/TagA/CpsF family	Organism	Identity/similarity (%)	Accession No.

NP_835080

45/65

Bacillus cereus ATCC 14579

N-Acetylglucosaminyldiphosphoundecaprenol *N*-acetyl-β-D-mannosaminyltransferase

ORF

tagO

Length/mol. mass

377/40.47

Signal peptide 1–33

Glycos_transf_4 (PF00953) 84–247

Conserved motifs and regions

Related proteins

TagO protein

Undecaprenyl-phosphate *N*-acetylglucosaminyl 1-phosphate transferase

ORF

sllA

Length/mol. mass

1335/148.46

Signal peptide 1–31

Galactose-binding domain-like (CBM6) 91–200

Conserved motifs and regions

Big_3 (PF07523) 493–513

SLH (PF00395) 1125–1169

SLH (PF00395) 1198–1242

SLH (PF00395) 1267–1319

Related proteins

Name/putative function

S-layer domain protein

Hypothetical protein BBR47_54190

ORF

spaA

Length/mol. mass

983/108.55

Signal peptide 1–24

SLH (PF00395) 25–65

SLH (PF00395) 82–129

SLH (PF00395) 140–181

Related proteins

Name/putative function

S-layer domain protein

Identity/similarity (%)

Accession No.

Organism

Bacillus megaterium DSM319

53/72

Lysinibacillus sphaericus C3-41

52/72

Identity/similarity (%)

Accession No.

Organism

Paenibacillus sp. IDR-2

49/63

Brevibacillus brevis NBRC 100599

46/61

Identity/similarity (%)

AccessionNo.

Organism

Geobacillus sp. Y412MC10

43/59

YP_003245899

ORF	S-layer domain-containing protein		43/59	ZP_04851624
	<i>orf7</i>			
Length/mol. mass	468/52.58			
Conserved motifs and regions
Related proteins	Signal peptide 1–20		Identity/similarity (%)	Accession No.
	Name/putative function			
	Hypothetical protein GYMC10DRAFT_0729			
	Hypothetical protein Pjdr2DRAFT_3479		24/44	ZP_03036896
	Organism		24/44	ZP_02848309
	<i>Geobacillus</i> sp. Y412MC10			
	Paenibacillus sp. JDR-2			

Table 2

Amino acid sequence comparison between SpaA and different well-characterized S-layer proteins using the EMBOSS Pairwise Alignment Algorithms (Global alignment method, Blosum62 matrix)

Protein	Organism	Accession no.	SLH domains	Analyzed region	Identity%	Similarity%
SbpA	<i>Lysinibacillus sphaericus</i> CCM 2177	AAF22978	33–76 92–135 152–199	1–1268 1–200	18.8 26.6	29.1 38.3
Surface layer protein	<i>Lysinibacillus sphaericus</i> 2362	AAA50256	32–76 92–135 152–199	1–1176 1–200	26.5 26.7	39.3 38.5
SlpC	<i>Lysinibacillus sphaericus</i> C3–41	ABQ00414	32–76 92–135 152–199	1–1176 1–200	26.5 26.7	39.3 38.5
Sap	<i>Bacillus anthracis</i> strain Sterne	YP_027117	33–76 94–135 155–197	1–814 1–200	22.2 22.6	34.8 36.7
EA1	<i>Bacillus anthracis</i> strain Sterne	YP_027118	33–76 94–136 156–197	1–862 1–200	21.8 23.1	36.1 39.1
CTC protein	<i>Bacillus thuringiensis</i> CTC ssp. <i>finitimus</i>	CAA09981	33–76 94–135 155–198	1–816 1–200	20.7 22.6	34.0 37.4
SlpA	<i>Bacillus thuringiensis</i> NRRL 4045 ssp. <i>galleriae</i>	CAB63252	33–76 94–136 156–197	1–821 1–200	22.2 24.4	35.3 36.4
SbsB	<i>Geobacillus stearothermophilus</i> PV72 p2	CAA66724	33–76 92–133 150–191	1–920 1–200	21.8 21.0	36.1 36.1
Middle wall protein precursor	<i>Brevibacillus brevis</i> 47	AAA22760	32–72 93–136 —	1–1053 1–200	20.3 27.1	31.8 44.9
S-Layer protein precursor	<i>Thermoanaerobacter kivui</i>	AAA21930	30–73 95–137 —	1–762 1–200	19.2 27.4	29.5 38.4
P100 protein	<i>Thermus thermophilus</i> HB8	CAA40609	24–67 — —	1–917 1–200	21.1 27.2	34.2 45.6
SlpA	<i>Clostridium thermocellum</i> NCIB 10682	AAC33404	— 94–134 —	1–1036 1–200	23.7 18.6	40.4 29.6

For analysis of full length proteins, SpaA from aa 1 to 983 was used and for comparison of the amino-terminal parts aa 1–200 were included.

Table 3**Bacterial strains and plasmids used in this study**

Strain or plasmid	Genotype and/or relevant characteristics	Source or reference
<i>Strains</i>		
<i>P. alvei</i> CCM 2051 ^T	Wild-type isolate, Km ^r	Czech collection of microorganisms (CCM)
<i>P. alvei</i> CCM 2051 ^T <i>wsfP</i> ::Ll.LtrB	S-Layer glycosylation deficient mutant carrying a targetron insertion at the <i>wsfP</i> gene; Km ^r	Ref. 31
<i>Escherichia coli</i> DH5α	F ⁻ ϕ 80dlacZ <i>M15</i> (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (rK ⁻ mK ⁻) <i>phoA supE44 thi-1 gyrA96 relA1 λ</i> ⁻	Invitrogen
<i>Plasmids</i>		
pEXALV	<i>P. alvei</i> expression vector	Ref. 31
pEGFP-N1	Expression vector for mammalian cells encoding a red-shifted variant of wild-type GFP	BD biosciences
pEXALV_SP_SpaA_6HIS	pEXALV carrying the his-tagged <i>spaA</i> gene of <i>P. alvei</i> CCM 2051 ^T	This study
pEXALV_SP_SpaA	pEXALV carrying the <i>spaA</i> gene of <i>P. alvei</i> CCM 2051 ^T lacking the TAA stop codon	This study
pEXALV_SP_SpaA_EGFP	pEXALV carrying a <i>spaA-egfp</i> fusion construct	This study

Table 4

Oligonucleotide primers used for PCR amplification reactions

Oligonucleotide	Sequence (5'→3') ^a
proof_wSpa_for	GCIGAYGCIGCIAARACIACICARG
SP_SpaA_SphI_for	aatcaGCATGCAGAAAAGATTGGCCCTTCTGCTTTCCG
SpaA_6HIS_STOP_KpnI_rev	aatcaGGTACCttaatggtgatggtgatggtgCTTACCGGAGTATGTTCCAGGAAGG
SpaA_noSTOP_PstI_rev	aatcaCTGCAGCTTACCGGAGTATGTTCCAGGAAGG
EGFP_for_PstI	aatcaCTGCAGATGGTGAGCAAGGGCGAGGAGC
EGFP_rev_KpnI	aatcaGGTACCCTTACTTGTACAGCTCGTCCATGCC

^a Artificial restriction sites are underlined.