A functional chimaeric S-layer–enhanced green fluorescent protein to follow the uptake of S-layer-coated liposomes into eukaryotic cells

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The chimaeric gene encoding a C-terminally truncated form of the S-layer protein SbpA of *Bacillus sphaericus* CCM 2177 and the EGFP (enhanced green fluorescent protein) was ligated into plasmid pET28a and cloned and expressed in *Escherichia coli*. Just 1 h after induction of expression an intense EGFP fluorescence was detected in the cytoplasm of the host cells. Expression at 28 °C instead of 37 °C resulted in clearly increased fluorescence intensity, indicating that the folding process of the EGFP moiety was temperature sensitive. To maintain the EGFP fluorescence, isolation of the fusion protein from the host cells had to be performed in the presence of reducing agents. SDS/PAGE analysis, immunoblotting and N-terminal sequencing of the isolated and purified fusion protein confirmed the presence of both the S-layer protein and the EGFP moiety. The fusion protein had maintained the ability to self-assemble in suspension and to recrystallize on peptidoglycan-containing sacculi or on positively charged liposomes, as well as to fluoresce. Comparison of fluorescence excitation and emission spectra of recombinant EGFP and $rSbpA_{31\text{-}1068}$/EGFP revealed identical maxima at 488 and 507 nm respectively. The uptake of liposomes coated with a fluorescent monomolecular protein lattice of $rSbpA_{31\text{-}1068}$/EGFP into HeLa cells was studied by confocal laser-scanning microscopy. The major part of the liposomes was internalized within 2 h of incubation and entered the HeLa cells by endocytosis.

Key words: confocal laser-scanning microscopy, fluorescence microscopy, enhanced green fluorescent protein (EGFP), self-assembly, S-layer fusion protein, S-liposome.

INTRODUCTION

Many bacteria and archaea possess crystalline cell-surface layers (S-layers) as their outermost cell envelope component [1,2]. S-layers consist of a uniform species of protein or glycoprotein subunit and within an S-layer there is only one type of subunit. They are capable of self-assembling *in vitro* into highly porous, regularly structured lattices exhibiting oblique, square or hexagonal symmetry. The high density of functional groups in the S-layer lattice as well as the ability to recrystallize into regular arrays on solid supports (e.g. gold chips, silicon wafers, silanized glass) or on interfaces (e.g. lipid films or liposomes) [3–6] opened a promising application potential in nanobiotechnology and biomimetics.

The gene encoding the S-layer protein SbpA of *Bacillus sphaericus* CCM 2177 has been sequenced previously (GenBank accession no. AF211170), ligated into plasmid pET28a and cloned and expressed in *Escherichia coli* [7]. The entire *sbpA* sequence of 3804 bp encodes a 1268 amino-acid-long protein with a theoretical molecular mass of 132 062 Da. The N-terminus of the S-layer protein SbpA carries an SLH (S-layer-like homologous) domain, which is involved in anchoring the subunits to the peptidoglycan backbone via a pyruvylated SCWP (secondary cell-wall polymer) [8]. Investigation of the structure–function relationship of SbpA revealed that the deletion of 200 C-terminal amino acids significantly increased the accessibility of the C-terminus, but did not interfere with the self-assembly properties of this S-layer protein. Based on the C-terminally truncated form $rSpbA_{31\text{-}1068}$, S-layer fusion proteins were constructed which comprised the sequence of the major birch pollen allergen Bet v1 [7], a single hypervariable region of a heavy-chain camel antibody specifically recognizing either lysozyme [9] or prostate-specific antigen [10] and the F_c-binding Z-domain of protein A [11]. Due to their ability to recrystallize into monomolecular protein lattices on solid supports precoated with SCWP while presenting the functional domains on the outermost surface, S-layer fusion proteins are currently exploited as sensing layers for biochip development or IgG-binding layers in novel immunoadsorbents [11].

Artificial lipid vesicles termed liposomes are widely used as delivery systems for enhancing the efficiency of various biologically active molecules [12]. These vesicles can encapsulate water-soluble drugs in their aqueous compartment and lipid-soluble substances within the lipid bilayer itself [13]. Currently, liposomes are used as drug delivery vehicles to transport therapeutic agents to disease sites *in vivo*. These agents include small molecule drugs used in cancer chemotherapy and genetic drugs as plasmids encoding therapeutic genes [14]. Generally, liposomes release their contents by interaction with target cells, either by adsorption, endocytosis, lipid exchange or fusion [15].

In previous studies, the S-layer proteins of *B. coagulans* E38-66 and *Geobacillus stearothermophilus* PV72/p2 were recrystallized on positively charged liposomes composed of DPPC (dipalmitylphosphatidylcholine), cholesterol and hexadecylamine in a molar ratio of 10:5:1 [3,16]. Such S-layer-coated liposomes (S-liposomes) with a diameter of 50–200 nm represent simple model systems resembling the features of virus envelopes. Therefore S-liposomes could reveal a broad application potential,

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; DPPC, dipalmitylphosphatidylcholine; DTT, dithiothreitol; EGFP, enhanced green fluorescent protein; rEGFP, recombinant EGFP; GPC, gel-permeation chromatography; SCWP, secondary cell-wall polymer; GdnHCl, guanidine hydrochloride; TBS, Tris-buffered saline; 3D, three-dimensional.

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particularly as drug-delivery systems or in gene therapy [17]. Liposomes coated with S-layer protein possessed significantly enhanced stability towards thermal and mechanical stress factors [4]. For chemical functionalization, the S-layer lattice was cross-linked with BS^3 [bis(sulphosuccinimidyl)substrate], biotinylated with *p*-diazobenzoyl biocytin and exploited for covalent binding of functional macromolecules via the avidin- or streptavidin-biotin bridge [5]. To avoid chemical modification reactions and to prevent the diffusion of potentially toxic agents through the lipid bilayer into the interior of the vesicles, a genetically engineered S-layer fusion protein incorporating the sequence of core–streptavidin was constructed and used for recrystallization on the liposomes. Since the S-layer subunits attached with the outer surface, N-terminal S-layer fusion proteins were chosen that left the fused core–streptavidin moiety exposed to the ambient environment, and therefore accessible for binding of biotinylated molecules [18].

In the present study, the nucleotide sequence encoding the EGFP (enhanced green fluorescent protein) was fused to the 3′ end of the sequence encoding $rSbpA_{31\text{-}1068}$. The chimaeric gene coding for the S-layer fusion protein $rSbpA_{31\text{-}1068}$/EGFP was cloned in plasmid pET28a and expressed in *E. coli*. To maintain the *in vitro* EGFP-fluorescence capability of the fusion protein, an appropriate isolation and purification protocol had to be established. The self-assembly and recrystallization properties of the purified fusion protein were tested and the fluorescence of the EGFP portion was investigated. Finally, $rSbpA_{31\text{-}1068}$/EGFP was recrystallized on liposomes and the uptake by eukaryotic cells and their localization in the cytosol were investigated.

MATERIAL AND METHODS

Bacterial strains, plasmids, DNA manipulation and culture conditions

For PCR amplification of the EGFP gene the plasmid pEGFP-N1 (Clontech, Palo Alto, CA, U.S.A.) was used. For cloning, *E. coli* TG1 cells were transformed with the recombinant plasmids pET28a/EGFP or pET28a/$rSbpA_{31\text{-}1068}$/EGFP. For expression, *E. coli* BL21(DE3) was chosen as a host strain for the constructs pET28a/EGFP and pET28a/$rSbpA_{31\text{-}1068}$/EGFP. *E. coli* was grown on Luria–Bertani medium (Invitrogen, Carlsbad, CA, U.S.A.) or on modified M9ZB medium [18a] for cloning experiments at 37 °C and for expression experiments at 28 °C. For selection of transformants harbouring the pET28a derivative, kanamycin was added to the medium to a final concentration of 30 μg/ml. Chromosomal DNA of *B. sphaericus* CCM 2177 was prepared by using Genomic Tips 100 (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Digestion of DNA with restriction endonucleases, separation of DNA fragments and transformation procedures were performed as described previously [19]. DNA fragments were recovered from agarose gels by using Qiaex II Gel Extraction Kit (Qiagen).

Cloning of the recombinant plasmid pET28a/EGFP

For PCR amplification of the EGFP gene from plasmid pEGFP-N1, the oligonucleotide primers *Bam*HI/EGFP-forward (5′-CGC *GGA TCC* ATG GTG AGC AAG GGC GAG-3′) and *Xho*I/EGFP-reverse (5′-GAC CG*C TCG AG*T TAC TTG TAC AGC TCG TCC ATG-3′), which introduced the restriction sites *Bam*HI (italic) at the 5′ end and *Xho*I (italic) at the 3′ end of the coding sequence respectively, were used. For cloning, the gel-purified PCR fragment was inserted into the corresponding restriction sites of plasmid pET28a, and the derivative pET28a/EGFP was established in *E. coli* TG1.

Cloning of the recombinant plasmid pET28a/$rSbpA_{31\text{-}1068}$/EGFP

For PCR amplification of the *sbpA* derivative encoding $rSbpA_{31\text{-}1068}$, DNA isolated from whole cells of *B. sphaericus* CCM 2177 was used as a template and the oligonucleotide primers *sbpA*37-forward (5′-CGG AAT T*CC ATG G*CG CAA GTA AAC GAC TAT AAC AAA ATC-3′) and *sbpA*41-reverse (5′-CGC *GGA TCC* TTC TGA ATA TGC AGT AGT TGC TGC-3′) were chosen. The oligonucleotide primers *sbpA*37-forward and *sbpA*41-reverse introduced an *Nco*I restriction site (italic) at the 5′ end and a *Bam*HI restriction site (italic) at the 3′ end of the coding sequence, respectively. For cloning, the gel-purified PCR fragment was inserted into the corresponding restriction sites of the recombinant plasmid pET28a/EGFP, and the obtained derivative pET28a/$rSbpA_{31\text{-}1068}$/EGFP was established in *E. coli* TG1.

Heterologous expression of the fusion protein $rSbpA_{31\text{-}1068}$/EGFP

For expression, the recombinant plasmid pET28a/$rSbpA_{31\text{-}1068}$/EGFP was transformed into *E. coli* BL21(DE3). The plasmid stability test was performed as described previously [20]. Expression of the gene encoding $rSbpA_{31\text{-}1068}$/EGFP was induced by the addition of isopropyl β-D-thiogalactoside (Gerbu, Gaiberg, Germany) to a final concentration of 1 mM at an attenuance (D_{600}) of 1. Expression was carried out at 28 °C for 5 or 18 h, respectively. Samples (2 ml) were taken before and at 1, 5 and 18 h after induction of gene expression. Preparation of biomass samples and SDS/PAGE were performed as described previously [20]. For electron-microscopic investigation, whole cells of *E. coli* BL21(DE3) were fixed and embedded in Spurr resin and subjected to ultrathin sectioning according to procedures described previously [21].

Isolation, purification and immunoblotting of the fusion protein $rSbpA_{31\text{-}1068}$/EGFP

After 18 h of expression, isolation of $rSbpA_{31\text{-}1068}$/EGFP from *E. coli* BL21(DE3) cells was performed using B-PER Bacterial Protein Extraction Reagent (Pierce, Rockford, IL, U.S.A.) following a modified protocol. At first, biomass pellets (2 g) were resuspended in 20 ml of B-PER containing 1 mM DTT (dithiothreitol). After incubation for 10 min at room temperature the solution was centrifuged (20 000 ***g***, 15 min, 4 °C). Subsequently, the pellet was resuspended in 20 ml of B-PER containing 1 mM DTT and 4 mg of lysozyme (Sigma, Munich, Germany) and incubated for 5 min at room temperature. After sonication (Branson Sonifier; 4×10 s), 20 μl of a 1 mg/ml DNAseI solution (Roche, Basel, Switzerland) and 1 ml of a 0.1 M $MgSO_4 \cdot 7H_2O$ solution were added and the suspension was incubated for 30 min at room temperature. Subsequently, 100 ml of B-PER (diluted 1:10 in distilled water) containing 1 mM DTT were added and the solution was centrifuged (20 000 ***g***, 20 min, 4 °C). After two washing steps with 30 ml of diluted B-PER containing 1 mM DTT and one washing step with 30 ml of 50 mM Tris/HCl buffer (pH 7.2) with 1 mM DTT, the suspension was centrifuged (20 000 ***g***, 20 min, 4 °C) and the obtained pellet was resuspended in 3.5 ml of 4 M GdnHCl (guanidine hydrochloride) in 50 mM Tris/HCl buffer (pH 7.2) and stirred for 10 min at room temperature. The suspension was diluted to a final GdnHCl concentration of 2 M. To remove peptidoglycan

fragments, the suspension was centrifuged (36 000 ***g***, 30 min, 4 °C), the supernatant was filtered through an RC membrane of pore size 0.45 μm (Minisart RC 25) and the filtrate was subjected to GPC (gel-permeation chromatography) using a Superdex 200 column (Amersham Biosciences, Little Chalfont, Bucks., U.K.) equilibrated in degassed 2 M GdnHCl in 50 mM Tris/HCl buffer (pH 7.2) with 150 mM NaCl and 1 mM DTT, for separation. Fractions containing the fusion protein rSbpA$_{31\text{-}1068}$/EGFP were pooled, dialysed against degassed 10 mM Tris/HCl buffer (pH 7.2) containing 1 mM DTT for 18 h at 4 °C, lyophilized and stored at − 20 °C. N-terminal sequencing of the fusion protein was performed as described previously [22]. Immunoblotting with a polyclonal rabbit antiserum raised against the S-layer protein of *B. sphaericus* CCM 2177 was carried out as described previously [22]. For detection of the EGFP portion, the fusion protein rSbpA$_{31\text{-}1068}$/EGFP was subjected to SDS/PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell Protean) by semi-dry blotting as described previously [22]. After blocking with 2 % Top Block (Fluka, Buchs, Switzerland) in 0.1 M TBS (Tris-buffered saline) for 2 h at room temperature, the membrane was incubated with primary rabbit anti-GFP antibody (Molecular Probes) diluted 1:5000 in 2 % Top Block in 0.1 M TBS for 1 h at room temperature and washed three times with washing buffer (0.5 % Tween 20 in 0.1 M TBS). After incubation with secondary goat anti-rabbit IgG–alkaline phosphatase-conjugated antibody (Sigma) diluted 1:20 000 in 2 % Top Block in 0.1 M TBS for 1 h at room temperature and three washing steps, detection was accomplished by treatment with 5-bromo-4-chloro-3-indolylphosphate and Nitro Blue Tetrazolium chloride (Roche).

Heterologous expression, isolation, purification and immunoblotting of rEGFP (recombinant EGFP)

Heterologous expression of rEGFP in *E. coli* BL21(DE3), plasmid stability test, preparation of biomass samples and SDS/PAGE were performed as described for the fusion protein rSbpA$_{31\text{-}1068}$/EGFP. Isolation and purification of rEGFP from the host cells was performed as described previously for soluble recombinant protein [23]. Immunoblotting of purified rEGFP using the rabbit anti-GFP antibody was preformed as described for rSbpA$_{31\text{-}1068}$/EGFP.

Comparison of excitation and emission spectra of rEGFP and rSbpA$_{31\text{-}1068}$/EGFP

For measurement of excitation and emission spectra of rEGFP and rSbpA$_{31\text{-}1068}$/EGFP with the luminescence spectrometer LS 55 (PerkinElmer, Shelton, CT, U.S.A.), 0.5 mg of GPC-purified rEGFP or rSbpA$_{31\text{-}1068}$/EGFP, were dissolved in 4 M GdnHCl in 50 mM Tris/HCl buffer (pH 7.2) containing 1 mM DTT. The solutions were dialysed against distilled and degassed water containing 1 mM DTT for 18 h at 4 °C. After centrifugation (16 000 ***g***, 5 min, 4 °C), the protein concentration of the clear supernatant containing either rEGFP or non-assembled rSbpA$_{31\text{-}1068}$/EGFP was determined by the bicinchoninic acid method [24]. Both protein solutions were dissolved to a final concentration of 0.12 pM and each placed in a quartz cuvette (1 cm). Fluorescence spectroscopy of the protein samples was carried out by wavelength scan with a scan speed of 500 nm/min. The band pass for both the excitation and the emission monochromators was 5 nm. Emission spectra were recorded at a fixed wavelength of the excitation maximum, and excitation spectra at a fixed wavelength of the emission maximum.

Investigation of the fluorescence properties of rSbpA$_{31\text{-}1068}$/EGFP self-assembly products and of the fusion protein recrystallized on peptidoglycan-containing sacculi and on liposomes

For production of self-assembly products, 3 mg of the GPC-purified rSbpA$_{31\text{-}1068}$/EGFP fusion protein was dissolved in 1 ml of 4 M GdnHCl in 50 mM Tris/HCl buffer (pH 7.2) containing 1 mM DTT. The solution was dialysed against 10 mM $CaCl_2$ in distilled and degassed water containing 1 mM DTT for 18 h at 4 °C. Negative staining of the suspension was performed as described previously [25]. The fluorescence of self-assembly products formed by rSbpA$_{31\text{-}1068}$/EGFP was investigated using a fluorescence microscope Nikon Eclipse ME600 with the filter combination B-2A. Recrystallization of rSbpA$_{31\text{-}1068}$/EGFP on peptidoglycan-containing sacculi of *B. sphaericus* CCM 2177 was as described previously [7], except that the sample was dialysed against 10 mM $CaCl_2$ in degassed distilled water containing 1 mM DTT. Fluorescence properties of the recrystallization products were examined as described above. Liposomes exhibiting a positive net charge were prepared from a lipid mixture composed of 20 μmol of DPPC, 10 μmol of cholesterol and 8 μmol of hexadecylamine according to the procedure described previously [5]. For disintegration, 1 mg of lyophilized rSbpA$_{31\text{-}1068}$/EGFP was dissolved in 400 μl of 2 M GdnHCl in 50 mM Tris/HCl buffer (pH 7.2) containing 150 mM NaCl. The solution was dialysed against 10 mM NaCl in degassed distilled water containing 0.5 mM DTT for 1 h at 22 °C. After centrifugation (13 000 ***g***, 15 min, 4 °C), the supernatant, containing monomers of rSbpA$_{31\text{-}1068}$/EGFP with a concentration of 19 nmol/ml, was mixed with 100 μl of liposome suspension containing 6 μmol of DPPC/ml. Recrystallization of the fusion protein rSbpA$_{31\text{-}1068}$/EGFP on liposomes was carried out in a Test Tube Rotator Type 3025 [GFL (Gesellschaft für Labortechnik mbH, Burgwedel, Germany] at 22 °C for 18 h. Excess protein was removed by centrifugation (13 000 ***g***, 5 min, 22 °C). The formation of the square lattice structure on liposomes was determined by electron microscopy of negatively stained preparations according to Küpcü et al. [3]. Fluorescence properties of rSbpA$_{31\text{-}1068}$/EGFP-coated liposomes were investigated using a Nikon Eclipse ME600 fluorescence microscope with the filter combination B-2A.

Uptake of positively charged liposomes coated with rSbpA$_{31\text{-}1068}$/EGFP by eukaryotic cells investigated by 3D (three-dimensional) confocal laser-scanning microscopy

HeLa cells (a human cervical carcinoma cell line) were grown at 37 °C on fibronectin (10 μg/ml in PBS)-coated glass chamber slides in DMEM (Dulbecco's modified Eagle's medium; Invitrogen) containing 10 % fetal bovine serum, antibiotics and glutamine in a 5 % CO_2 atmosphere until $\approx$80 % confluence (4×10^4 cells). After a washing step with 200 μl of fresh DMEM medium without fetal bovine serum, the HeLa cells were incubated with 11 nmol of rSbpA$_{31\text{-}1068}$/EGFP recrystallized on positively charged liposomes containing 1 nmol of DPPC in a final volume of 100 μl of DMEM in a 5 % CO_2 atmosphere for 2 h at 37 °C. For confocal laser-scanning microscopy analysis, the HeLa cells which had been incubated with S-liposomes were washed three times with 200 μl of PBS before fixation with 4 % paraformaldehyde in PBS for 15 min at room temperature. After washing the cells with PBS, the nuclei of the HeLa cells were stained with DAPI (4′,6-diamidino-2-phenylindole; diluted 1:1000 in PBS) for 10 min at room temperature. The plasma membrane of the HeLa cells was labelled by transferrin–tetramethylrhodamine conjugate (0.5 mg/ml in PBS; Molecular Probes) for 1 h at room temperature. After staining procedures,

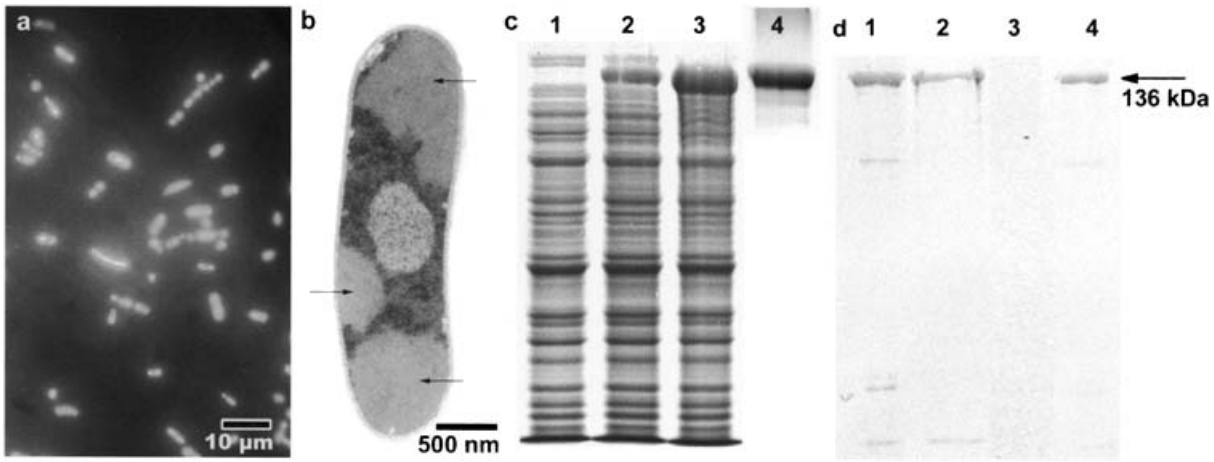

Figure 1 ***E. coli*** **BL21(DE3) cells carrying plasmid pET28a/rSbpA$_{31\text{-}1068}$/EGFP after induction of expression of the chimaeric gene**

(**a**) Fluorescence micrograph of biomass samples 18 h after induction of expression. The fluorescent fusion protein rSbpA$_{31\text{-}1068}$/EGFP was accumulated in inclusion bodies within the cytoplasm of the *E. coli* host cells. (**b**) Electron micrograph of ultrathin-section preparations 5 h after induction of expression. Inclusion bodies are indicated by arrows. (**c**) SDS/PAGE pattern of whole-cell extracts before (lane 1) and 1 h (lane 2) and 5 h (lane 3) after induction of expression. Lane 4, fusion protein rSbpA$_{31\text{-}1068}$/EGFP purified by GPC. (**d**) Immunoblot analysis using a polyclonal rabbit antiserum raised against the S-layer protein SbpA from *B. sphaericus* CCM 2177. SDS extracts of the S-layer protein SbpA from *B. sphaericus* CCM 2177 (lane 1) and rSbpA$_{31\text{-}1068}$/EGFP (lane 2). Immunoblot analysis using antibody rabbit-anti-GFP. SDS extracts of SbpA (lane 3) and rSbpA$_{31\text{-}1068}$/EGFP (lane 4).

HeLa cells adhering to chamber slides were washed twice with PBS, embedded in Decal mounting fluid and overlaid with cover slides. 3D fluorescence images were acquired using a confocal laser scanning microscope (model 510; Carl Zeiss, Jena, Germany) running on Advanced Imaging Microscopy software 3.0. The EGFP part of the fusion protein was excited using an Ar 488 nm laser at 0.062 mW, and rhodamine was excited using a He/Ne 543 nm laser at a power of 0.515 mW. A Plan-Neofluar 40×/1.3 oil objective lens was used with the scanning zoom set to 3 or 4, depending on the respective field of view. To increase the specificity of fluorescence detection a MultiTrack configuration was used, where both colours where excited and detected one by one rather than simultaneously. z-Stacks of cells loaded with rSbpA$_{31\text{-}1068}$/EGFP-coated liposomes were recorded under strictly confocal conditions, i.e. pinhole size close to 1 Airy unit. The first and last slice was set manually so that the first focal plain was slightly above and the last focal plain was slightly below the cells under investigation. Pinhole size and *z*-interval were optimized for an overlay of succeeding images in the *z*-stack of 1/3, thereby allowing the reconstruction algorithm a solid shape interpolation. 3D reconstruction was done for panorama views (360°) with angles of 6° between succeeding projections. Background noise in the images was reduced by a low-pass filter (with 7 × 7 matrix) for GFP and a median filter for rhodamine, in order to improve the quality of 3D reconstructions.

RESULTS

Cloning and expression of the chimaeric gene encoding rSbpA$_{31\text{-}1068}$/EGFP

The PCR product, which was obtained by amplification of the EGFP gene using the primers *Bam*HI/EGFP-forward and *Xho*I/EGFP-reverse, was ligated into plasmid pET28a. After cloning in *E. coli* TG1 and isolation of the amplified recombinant plasmid pET28a/EGFP, the gene encoding the C-terminally truncated S-layer protein rSbpA$_{31\text{-}1068}$ was ligated via the corresponding restriction sites into plasmid pET28a/EGFP. For heterologous expression of the chimaeric insert, the resulting plasmid pET28a/rSbpA$_{31\text{-}1068}$/EGFP was established in *E. coli* BL21(DE3). To achieve correct folding of the EGFP portion in the fusion protein, expression was carried out at a constant temperature of 28 °C instead of the usual 37 °C, which is optimal for the growth of the host cells. Just 1 h after induction of expression an intense EGFP fluorescence of the *E. coli* BL21(DE3) cells was detected by fluorescence microscopy. Extension of the expression time up to 18 h at 28 °C led to a clearly increased fluorescence of the host cells (Figure 1a), whereas expression at 37 °C revealed an obvious decrease in the fluorescence intensity (results not shown). Electron microscopy of ultrathin sectioned *E. coli* BL21(DE3) cells embedded in Spurr resin 5 h after induction of expression revealed the accumulation of inclusion bodies within the cytoplasm of the host cells (Figure 1b). Analysis of the expression of the chimaeric gene encoding the fusion protein rSbpA$_{31\text{-}1068}$/EGFP by SDS/ PAGE 1 h after induction of expression revealed an additional protein band with an apparent molecular mass of 136000 Da (Figure 1c, lane 2) The intensity of this protein band increased with time of expression (Figure 1c, lane 3). *E. coli* BL21(DE3) cells carrying plasmid pET28a/rSbpA$_{31\text{-}1068}$/EGFP, harvested before the addition of isopropyl β-D-thiogalactoside, showed no additional protein band on SDS gels (Figure 1c, lane 1).

Isolation, purification and immunoblotting of the rSbpA$_{31\text{-}1068}$/EGFP fusion protein

For isolation of rSbpA$_{31\text{-}1068}$/EGFP from the *E. coli* BL21(DE3) host cells, the B-PER Protein Extraction Reagent was used and an appropriate isolation protocol was established. To protect the EGFP portion against oxidation, all buffers and solutions contained 1 mM DTT. Investigation of samples taken during the isolation procedure (after lysozyme treatment, after sonication, before and after extraction of the water-insoluble pellet with 4 M GdnHCl) revealed that EGFP fluorescence was maintained under reducing experimental conditions. In contrast, purification of rSbpA$_{31\text{-}1068}$/EGFP without addition of DTT resulted immediately in a decrease in EGFP fluorescence (results not shown). SDS/PAGE analysis showed that the fusion protein had accumulated in the insoluble fraction of the lysed *E. coli* BL21(DE3) cells (results not shown), which was in agreement with the data from ultrathin sections, clearly indicating the formation of inclusion bodies. After purification of

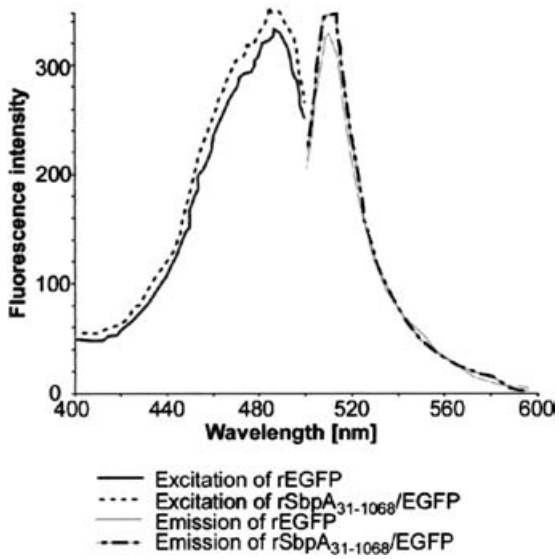

Figure 2 Fluorescence excitation and emission spectra of rEGFP and of the fusion protein rSbpA$_{31\text{-}1068}$/EGFP

Both proteins were applied in the same molar ratio and exhibited identical excitation peaks at 488 nm and emission peaks at 507 nm. In comparison with rEGFP, the fusion protein rSbpA$_{31\text{-}1068}$/EGFP showed no decrease in fluorescence intensity.

rSbpA$_{31\text{-}1068}$/EGFP by GPC, a single protein band with an apparent molecular mass of 136000 Da was detected on SDS gels (Figure 1c, lane 4). On immunoblots, a strong cross-reaction was observed between this protein band and the polyclonal rabbit antiserum raised against the S-layer protein SbpA of *B. sphaericus* CCM 2177 (Figure 1d, lane 2), as well as with the rabbit anti-GFP antibody (Figure 1d, lane 4). The S-layer protein SbpA from *B. sphaericus* CCM 2177, which was used as a control, showed a strong cross-reaction with the polyclonal rabbit antiserum (Figure 1d, lane 1) and no reaction with rabbit anti-GFP (Figure 1d, lane 3). N-terminal sequencing of purified rSbpA$_{31\text{-}1068}$/EGFP revealed that the first five amino acids (AQVND) were identical to those of wild-type SbpA.

Comparison of excitation and emission spectra of rEGFP and rSbpA$_{31\text{-}1068}$/EGFP

Comparative studies of excitation and emission spectra of rEGFP and rSbpA$_{31\text{-}1068}$/EGFP revealed that both proteins showed identical excitation peaks at 488 nm and emission peaks at 507 nm (Figure 2). When both recombinant proteins were applied in the same molar ratio, in comparison with rEGFP, the fusion protein rSbpA$_{31\text{-}1068}$/EGFP showed no decrease in fluorescence intensity.

Investigation of the fluorescence of self-assembly products formed with rSbpA$_{31\text{-}1068}$/EGFP and of the fusion protein recrystallized on peptidoglycan-containing sacculi and on liposomes

Electron microscopical investigations of negatively stained preparations revealed that rSbpA$_{31\text{-}1068}$/EGFP formed self-assembly products, which were flat double- or multi-layer sheets and clearly exhibited the square lattice structure (Figure 3a). Investigation of the rSbpA$_{31\text{-}1068}$/EGFP self-assembly products revealed that the fluorescence activity of the EGFP portion in the fusion protein was not affected by the self-assembly process (Figure 3d). In addition, the fusion protein was capable of recrystallizing into the square lattice structure on peptidoglycan-containing sacculi (Figure 3b) and on positively charged liposomes (Figure 3c). In both cases, the fusion protein retained the ability to fluorescence (Figures 3e and 3f).

Uptake of positively charged liposomes coated with rSbpA$_{31\text{-}1068}$/EGFP by eukaryotic cells investigated by 3D confocal laser-scanning microscopy

Liposomes coated with the S-layer fusion protein rSbpA$_{31\text{-}1068}$/EGFP were applied to HeLa cells. After incubation in DMEM for 2 h, HeLa cells were fixed with paraformaldehyde and the cell membrane was stained with transferrin–tetramethylrhodamine conjugate. For evaluation of the cellular localization and the intracellular fate of the S-liposomes, confocal laser-scanning microscopy was performed. In Figures 4(a) and 4(c), cell compartments indicated by a single arrow show the ongoing interaction between the cell membrane and the green fluorescent S-liposomes. As indicated by double arrows (Figures 4a and 4c), parts of the cell membrane could be stained with rhodamine, which indicated that internalization of the S-liposomes was completed. Furthermore, dark arrowheads in Figures 4(a)–4(c) indicate S-liposomes located in the endosomes or the cytosol. Taken together, confocal images revealed that most of the S-liposomes were internalized within 2 h of incubation and that the major part entered the HeLa cells by endocytosis.

DISCUSSION

By exploiting the specific interaction between the S-layer moiety of various S-layer fusion proteins and the corresponding SCWP, regularly structured protein lattices with the ability to bind biologically active macromolecules could be generated on solid supports such as gold chips, silicon wafers or silanized glas [9]. Currently, such supramolecular structures are used as sensing layers in biochip development, or for the development of novel IgG adsorption systems [11,26].

A further promising application potential can be seen in the development of drug targeting and delivery systems based on liposome–DNA complexes coated with functional S-layer fusion proteins for transfection of eukaryotic cell lines. For that purpose, an S-layer fusion protein generated by fusion of minimum-sized core–streptavidin was recrystallized on positively charged liposomes [18]. By exploiting the streptavidin–biotin interaction as a biomolecular coupling system, the liposome surface was functionalized by specific binding of biotinylated molecules, such as biotinylated peroxidase and biotinylated ferritin [18]. Binding of biotinylated ligands to liposomes coated with the S-layer core–streptavidin fusion protein can be used for enabling receptor-mediated uptake into eukaryotic cells.

GFP, originally isolated from the jellyfish *Aequorea victoria*, has become an invaluable tool in cell biology, since its intrinsic fluorescence can be visualized in living cells [27,28]. In recent years, biotechnology has taken advantage of this feature of GFP by exploiting it as an *in vivo* marker for gene expression and protein localization [29–32]. In previous studies, it was demonstrated that codon alterations within the gene encoding GFP improved expression levels in mammalian systems [33,34] as well as in bacteria [35]. The EGFP is a red-shifted GFP-derivative possessing a 30 times brighter fluorescence intensity at 488 nm than wild-type GFP, caused by a double-amino-acid substitution of Phe-64 to Leu and Ser-65 to Thr [36]. In addition, when produced in *E. coli*, folding of the mutant protein was more efficient than folding of wild-type GFP, which leads to a further increase in the brightness of EGFP [36].

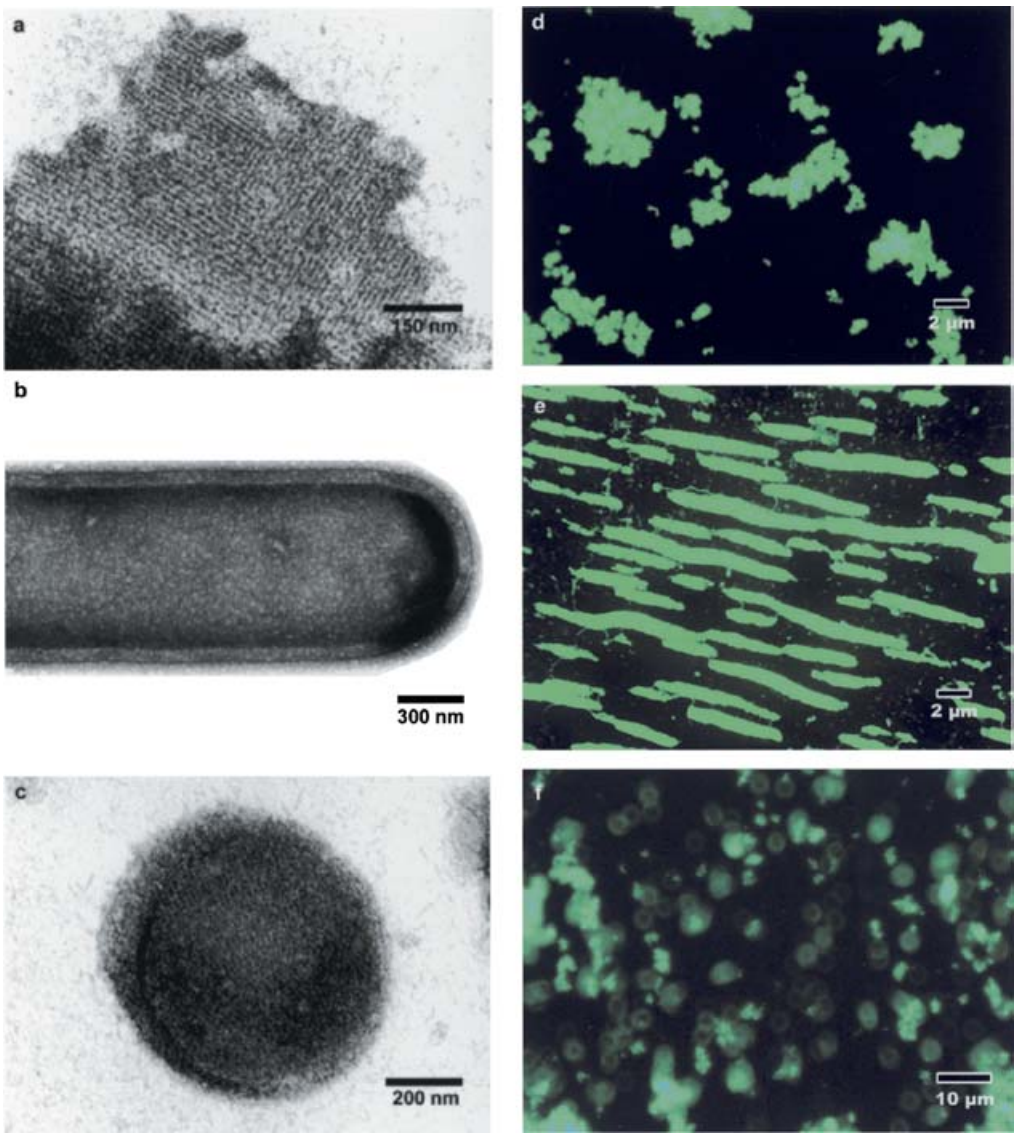

Figure 3 Electron micrographs of negatively stained preparations (a–c) and fluorescence micrographs (d–f)

(**a**, **d**) Self-assembly products formed by $rSbpA_{31-1068}$/EGFP exhibiting the square S-layer lattice structure. (**b**, **e**) $rSbpA_{31-1068}$/EGFP recrystallized on peptidoglycan-containing sacculi of *B. sphaericus* CCM 2177. (**c**, **f**) Positively charged liposomes coated with the fusion protein $rSbpA_{31-1068}$/EGFP.

In the present study, genetic approaches were used to fuse EGFP to the C-terminally truncated S-layer protein $rSbpA_{31-1068}$. After cloning and heterologous expression of the chimaeric gene encoding $rSbpA_{31-1068}$/EGFP, the fusion protein was located in inclusion bodies within the cytoplasm of the *E. coli* cells. These findings are in accordance with data previously obtained with the recombinant fusion proteins $rSbpA_{31-1068}$/Betv1 and $rSbpA_{31-1068}$/cAb-Lys3 expressed in *E. coli* [7,9]. As confirmed by fluorescence microscopy, 1 h after induction of gene expression, *E. coli* cells accumulating $rSbpA_{31-1068}$/EGFP showed a bright EGFP fluorescence within the area of the inclusion bodies. Performing gene expression at 28 °C instead of 37 °C, as usual for *E. coli* expression strains, revealed that the EGFP fluorescence intensity of the fusion protein was very temperature sensitive. Interestingly, fusion protein synthesized at 28 °C was stable and fluorescent for weeks at 4 °C, indicating that the temperature sensitivity was restricted to the folding process within the *E. coli* host cells. These findings were in accordance with the previously reported temperature sensitivity of a GFP–nucleoplasmin fusion protein [37], as well as of a chimaeric protein consisting of the secretory protein chromogranin B and GFP produced to observe secretory pathways in HeLa cells [38]. Comparison of excitation and emission spectra of rEGFP and $rSbpA_{31-1068}$/EGFP indicated identical maxima at 488 and 507 nm, respectively. In comparison with rEGFP, the fusion protein $rSbpA_{31-1068}$/EGFP showed no decrease in fluorescent intensity. Electron-microscopic investigation of self-assembly products and $rSbpA_{31-1068}$/EGFP recrystallized on peptidoglycan-containing sacculi revealed that the self-assembly process did not interfere with the fluorescence properties of the EGFP portion in the fusion protein. In a previous study, a fusion protein comprising GFP and SAC, the C-terminal cell-wall-targeting domain of *Lactobacillus acidophilus* ATCC 4236, was expressed in *E. coli* [39]. Because of the absence of the self-assembly domain, the fusion protein was not capable of forming a regularly structured protein lattice. To our knowledge, $rSbpA_{31-1068}$/EGFP is the first

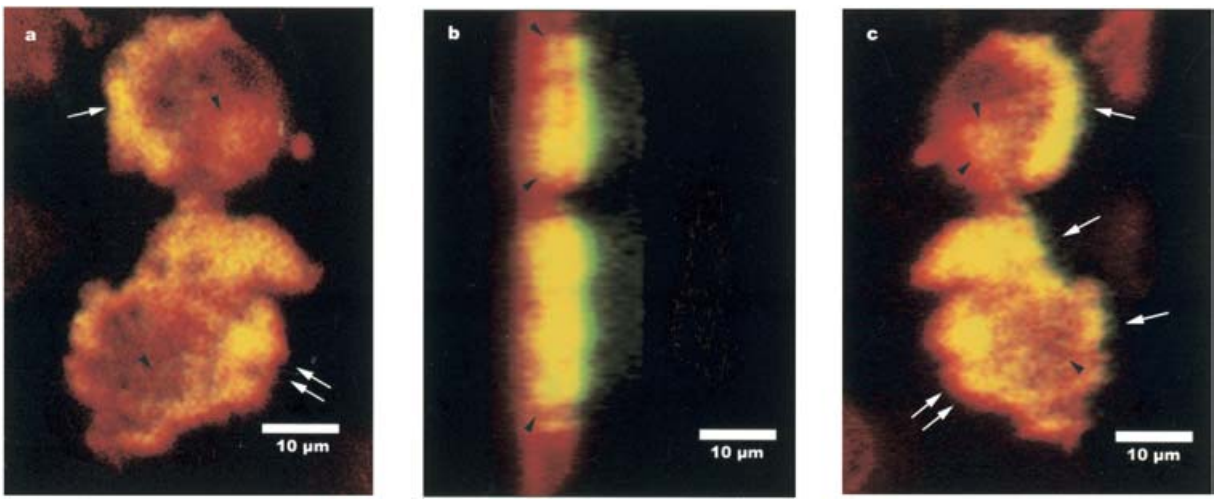

Figure 4 Confocal image and 3D reconstruction of membrane-labelled HeLa cells incubated with S-liposomes in DMEM for 2 h, acquired by confocal laser-scanning microscopy

(**a**) Two-dimensional image captured when focused on the plane of highest intensity. (**b**, **c**) 3D reconstruction based on a confocal *z*-stack. The 3D projections represent views at an anticlockwise rotation around the *y*-axis by 96° (**b**) and by 162° relative to the focal plane (**c**). Green fluorescence indicates $rSbpA_{31\text{-}1068}$/EGFP recrystallized on liposomes; red shows rhodamine fluorescence of the plasma membrane of HeLa cells.

S-layer fusion protein to maintain both EGFP fluorescence and the ability to recrystallize into regularly structured arrays. Because of their fluorescence ability, positively charged liposomes coated with $rSbpA_{31\text{-}1068}$/EGFP represent a useful tool to visualize the uptake of S-liposomes into eukaryotic cells.

With regard to further experiments, the most interesting advantage of the new fusion protein can be seen in recrystallization of $rSbpA_{31\text{-}1068}$/EGFP in combination with other S-layer fusion proteins (e.g. rSbpA–streptavidin) on the same liposome surface. The uptake of these specially coated liposomes by target cells and the functionality of transported drugs could be investigated simultaneously without using any additional labelling.

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REFERENCES

1 Sára, M. and Sleytr, U. B. (2000) S-layer proteins. J. Bacteriol. **182**, 859–868
2 Sleytr, U. B., Sára, M., Pum, D., Schuster, B., Messner, P. and Schäffer, C. (2003) Self assembly protein systems. Microbial S-layers. In Biopolymers, vol. 7 (Steinbüchel, A. and Fahnestock, S., eds.), pp. 285–338, Wiley-VCH, Weinheim
3 Küpcü, S., Sára, M. and Sleytr, U. B. (1995) Liposomes coated with crystalline bacterial cell surface protein (S-layer) as immobilization structures for macromolecules. Biochim. Biophys. Acta **1235**, 263–269
4 Mader, C., Küpcü, S., Sára, M. and Sleytr, U. B. (1999) Stabilizing effect of an S-layer on liposomes towards thermal or mechanical stress. Biochim. Biophys. Acta **1418**, 106–116
5 Mader, C., Küpcü, S., Sleytr, U. B. and Sára, M. (2000) S-layer-coated liposomes as a versatile system for entrapping and binding target molecules. Biochim. Biophys. Acta **1463**, 142–150
6 Schuster, B. and Sleytr, U. B. (2000) S-layer-supported lipid membranes. J. Biotechnol. **74**, 233–254
7 Ilk, N., Völlenkle, C., Egelseer, E. M., Breitwieser, A., Sleytr, U. B. and Sára, M. (2002) Molecular characterization of the S-layer gene, *sbpA*, of *Bacillus sphaericus* CCM 2177 and production of a functional S-layer fusion protein with the ability to recrystallize in a defined orientation while presenting the fused allergen. Appl. Environ. Microbiol. **68**, 3251–3260
8 Ilk, N., Kosma, P., Puchberger, M., Egelseer, E. M., Mayer, H. F., Sleytr, U. B. and Sára, M. (1999) Structural and functional analyses of the secondary cell wall polymer of *Bacillus sphaericus* CCM 2177 that serves as an S-layer-specific anchor. J. Bacteriol. **181**, 7643–7646
9 Pleschberger, M., Neubauer, A., Egelseer, E. M., Weigert, S., Lindner, B., Sleytr, U. B., Muyldermans, S. and Sára, M. (2003) Generation of a functional monomolecular protein lattice consisting of an S-layer fusion protein comprising the variable domain of a camel heavy chain antibody. Bioconjug. Chem. **14**, 440–448
10 Pleschberger, M., Saerens, D., Weigert, S., Sleytr, U. B., Muyldermans, S., Sára, M. and Egelseer, E. M. (2004) An S-layer heavy chain camel antibody fusion protein for generation of a nanopatterned sensing layer to detect the prostate-specific antigen by surface plasmon resonance technology. Bioconjug. Chem., in the press
11 Völlenkle, C., Weigert, S., Ilk, N., Egelseer, E. M., Weber, C., Loth, F., Falkenhagen, D., Sleytr, U. B. and Sára, M. (2004) Construction of a functional S-layer fusion protein comprising an IgG-binding domain for the development of specific adsorbents for extracorporeal blood purification. Appl. Environ. Microbiol., in the press
12 Lasic, D. D. and Papahadjopoulos, D. (1995) Liposomes revisited. Science **267**, 1275–1276
13 Lasic, D.D. (1998) Novel applications of liposomes. Trends Biotechnol. **16**, 307–321
14 Templeton, N. S. and Lasic, D. D. (1999) New directions in liposome gene delivery. Mol. Biotechnol. **11**, 175–180
15 Ostro, M. J. and Cullis, P. R. (1989) Use of liposomes as injectable-drug delivery systems. Am. J. Hosp. Pharm. **46**, 1576–1587
16 Küpcü, S., Lohner, K., Mader, C. and Sleytr, U. B. (1998) Microcalorimetric study on the phase behaviour of S-layer coated liposomes. Mol. Membr. Biol. **15**, 69–74
17 Sleytr, U. B., Messner, P., Pum, D. and Sára, M. (1999) Crystalline bacterial cell surface layers (S-layers): from supramolecular cell structure to biomimetics and nanotechnology. Angew. Chem. Int. Ed. **38**, 1034–1054
18 Moll, D., Huber, C., Schlegel, B., Pum, D., Sleytr, U. B. and Sára, M. (2002) S-layer-streptavidin fusion proteins as template for nanopatterned molecular arrays. Proc. Natl. Acad. Sci. U.S.A. **99**, 14646–14651
18a Studier, F. W., Rosenberg, A. H., Dunn, J. J. and Dubendorff, J. W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185**, 60–89
19 Sambrook, J., Fritsch, F. E. and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, Cold Spring Harbour Press, Cold Spring Harbour, NY
20 Jarosch, M., Egelseer, E. M., Mattanovich, D., Sleytr, U. B. and Sára, M. (2000) S-layer gene sbsC of *Bacillus stearothermophilus* ATCC 12980: molecular characterization and heterologous expression in *Escherichia coli*. Microbiology **146**, 273–281
21 Messner, P., Hollaus, F. and Sleytr, U. B. (1984) Paracrystalline cell wall surface layers of different *Bacillus stearothermophilus* strains. Int. J. Syst. Bacteriol. **34**, 202–210
22 Egelseer, E. M., Schocher, I., Sleytr, U. B. and Sára, M. (1996) Evidence that an N-terminal S-layer protein fragment triggers the release of a cell-associated high-molecular-weight amylase in *Bacillus stearothermophilus* ATCC 12980. J. Bacteriol. **178**, 5602–5609
23 Jarosch, M., Egelseer, E. M., Huber, C., Moll, D., Mattanovich, D., Sleytr, U. B. and Sára, M. (2001) Analysis of the structure-function relationship of the S-layer protein SbsC of *Bacillus stearothermophilus* ATCC 12980 by producing truncated forms. Microbiology **147**, 1353–1363

24 Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985) Measurement of protein using bichinonic acid. Anal. Biochem. **150**, 76–85
25 Pum, D., Sára, M. and Sleytr, U. B. (1989) Structure, surface charge, and self-assembly of the S-layer lattice from Bacillus coagulans E38-E66. J. Bacteriol. **171**, 5296–5303
26 Breitwieser, A., Egelseer, E. M., Moll, D., Ilk, N., Hotzy, C., Bohle, B., Ebner, C., Sleytr, U. B. and Sára, M. (2002) A recombinant bacterial cell surface (S-layer)-major birch pollen allergen-fusion protein (rSbsC/Bet v1) maintains the ability to self-assemble into regularly structured monomolecular lattices and the functionality of the allergen. Protein Eng. **15**, 243–249
27 Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. and Prasher, D. C. (1994) Green fluorescent protein as a marker for gene expression. Science **263**, 802–805
28 Dickson, R. M., Cubitt, A. B., Tsien, R. Y. and Moerner, W. E. (1997) On/off blinking and switching behaviour of single molecules of green fluorescent protein. Nature (London) **388**, 355–358
29 Bellucci, M., De Marchis, F., Mannucci, R. and Arcioni, S. (2003) Jellyfish green fluorescent protein as a useful reporter for transient expression and stable transformation in *Medicago sativa* L. Plant Cell Rep. **22**, 328–337
30 Dabiri, G. A., Ayoob, J. C., Turnacioglu, K. K., Sanger, J. M. and Sanger, J. W. (1999) Use of green fluorescent proteins linked to cytoskeletal proteins to analyze myofibrillogenesis in living cells. Methods Enzymol. **302**, 171–186
31 Ko, J. K., Choi, K. H., Kim, H. J., Choi, H. Y., Yeo, D. J., Park, S. O., Yang, W. S., Kim, Y. N. and Kim, C. W. (2003) Conversion of Bfl-1, an anti-apoptotic Bcl-2 family protein, to a potent pro-apoptotic protein by fusion with green fluorescent protein (GFP). FEBS Lett. **551**, 29–36
32 Olson, K. R., McIntosh, J. R. and Olmsted, J. B. (1995) Analysis of MAP 4 function in living cells using green fluorescent protein (GFP) chimeras. J. Cell Biol. **130**, 639–650
33 Yang, T. T., Cheng, L. and Kain, S. R. (1996) Optimized codon usage and chromophore mutations provide enhanced sensitivity with the green fluorescent protein. Nucleic Acids Res. **24**, 4592–4593
34 Zolotukhin, S., Potter, M., Hauswirth, W. W., Guy, J. and Muzyczka, N. (1996) A "humanized" green fluorescent protein cDNA adapted for high-level expression in mammalian cells. J. Virol. **70**, 4646–4654
35 Tsien, R. Y. (1998) The green fluorescent protein. Annu. Rev. Biochem. **67**, 509–544
36 Cormack, B. P., Valdivia, R. H. and Falkow, S. (1996) FACS-optimized mutants of the green fluorescent protein (GFP). Gene **173**, 33–38
37 Lim, C. R., Kimata, Y., Oka, M., Nomaguchi, K. and Kohno, K. (1995) Thermosensitivity of green fluorescent protein fluorescence utilized to reveal novel nuclear-like compartments in a mutant nucleoporin NSP1. J. Biochem. (Tokyo) **118**, 13–17
38 Kaether, C. and Gerdes, H. H. (1995) Visualization of protein transport along the secretory pathway using green fluorescent protein. FEBS Lett. **369**, 267–271
39 Smit, E., Oling, F., Demel, R., Martinez, B. and Pouwels, P. H. (2001) The S-layer protein of *Lactobacillus acidophilus* ATCC 4356: identification and characterisation of domains responsible for S-protein assembly and cell wall binding. J. Mol. Biol. **305**, 245–257

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