Heterologous expression of the surface-layer-like protein SIIB induces the formation of long filaments of *Escherichia coli* consisting of protein-stabilized outer membrane

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*Escherichia coli* is one of the best studied micro-organisms and is the most widely used host in genetic engineering. The Gram-negative single cells are rod-shaped, and filaments are usually not found. Here, we describe the reproducible formation of elongated *E. coli* cells. During heterologous expression of the silent surface (S)-layer protein gene *sllB* from *Lysinibacillus sphaericus* JG-A12 in *E. coli* BL21(DE3), the cells were arranged as long chains which were surrounded by highly stable sheaths. These filaments had a length of >100 µm. In the stationary growth phase, microscopic analyses demonstrated the formation of unusually long transparent tube-like structures which were enclosing separate single cells. The tube-like structures were isolated and analysed by SDS-PAGE, infrared-spectroscopy and different microscopic methods in order to identify their unusual composition and structure. The tube-like structures were found to be like outer membranes, containing high levels of proteins and to which the recombinant S-layer proteins were attached. Despite the entire structure being indicative of a disordered cell division, the bacterial cells were highly viable and stable. To our knowledge, this is the first time that the induction of drastic morphological changes in *E. coli* by the expression of a foreign protein has been reported.

INTRODUCTION

*Escherichia coli* is one of the best studied micro-organisms. These rod-shaped enterobacteria have dimensions of 1.1–1.5 µm × 2.0–6.0 µm (Orskov, 1984) and are peritrich flagellated. Naturally it colonizes the colon of mammals and is pathogenic in some cases. Filamentous forms have only been described in a few studies and occur only under special culture conditions or in genetically modified strains (Koch et al., 1987; Painbeni et al., 1997; Parker et al., 1992; Preusser, 1959). *E. coli* B 834 is a genetically modified strain that is used in basic research as a model organism in the investigation of bacterial genetics, physiology and molecular biology. *E. coli* BL21(DE3), an *E. coli* strain derived from the B 834 strain, is widely used as a host for heterologous expression of proteins of interest. However, misfolding of the expressed recombinant proteins frequently occurs in *E. coli*, causing the formation of inclusion bodies and often complicating their preparation. In particular, expression of bacterial surface layer (S-layer) proteins has failed in many cases (Boot et al., 1993; Bowditch et al., 1989; Kuen et al., 1996). The cost-efficient and large-scale production of recombinant proteins is of great interest because of the high application potential of bacterial S-layers (Raff et al., 2003; Sára et al., 2005).

S-layers are proteinaceous 2D paracrystalline structures which appear in many bacteria and as universal attributes in all archaea (Sleytr & Messner, 1988; Sleytr & Beveridge, 1999). These are probably the basic and oldest forms of biological cell envelopes and are mainly composed of protein or glycoprotein monomers. They also have the ability to self-assemble into 2D arrays (Bahl et al., 1997; Sára & Sleytr, 2000; Sidhu & Olsen, 1997). Several functions are ascribed to S-layers. They can work as a molecular sieve (Sára & Sleytr, 1987) and as virulence factors in several pathogenic bacteria (Ishiguro et al., 1981; Lewis et al., 1987; Mesnage et al., 2001). They are also able to bind toxic heavy metal ions (Merroun et al., 2005; Raff, 2002). They can potentially be used as ultrafiltration membranes (Sára et al., 1996), drug microcontainers (Schuster et al., 2008), filter materials (Raff et al., 2003) or patterning structures in nanotechnology (Fahmy et al.,...
These applications require an efficient, inexpensive and reproducible synthesis of S-layer proteins, ideally by heterologous expression. The S-layer protein SbsA of the Geobacillus stearothermophilus (formally Bacillus stearothermophilus) has successfully been expressed in E. coli. Following expression, sheet-like intracellular structures have been monitored, indicating the self-assembly of recombinant S-layer proteins in the cytosol (Kuen et al., 1996). Expression of the S-layer protein SlfB of the Lysinibacillus sphaericus JG-A12 has also been successful (Pollmann & Matys, 2007), but structural changes in S-layer proteins have been found after subsequent purification.

In the present study, we expressed the S-layer-like protein Slb of the bacterial strain L. sphaericus JG-A12 in E. coli BL21(DE3). L. sphaericus JG-A12 was isolated from the uranium mining waste pile Haberland located near Johanngeorgenstadt in Germany, and used previously for radioecological studies. These Gram-positive bacterial cells express the functional S-layer protein Slb which assembles into a protein array of square symmetry. It is composed of identical protein monomers (Raff, 2002) and binds high amounts of uranium very efficiently (Raff et al., 2004). In addition to the functional gene slb, the silent S-layer-like gene slb, which has all the typical features of S-layer genes, is encoded by the strain (Pollmann et al., 2005). Presumably, slb is expressed under certain conditions but its expression has not been demonstrated yet.

We have expressed slb in order to study the properties of the gene product in comparison with the S-layer protein Slb. Surprisingly the expression of slb induces drastic morphological changes in E. coli cells. Instead of the typical rod-shaped single E. coli cells, filaments longer than 100 µm are formed in the exponential growth phase. Biochemical, microscopic and infrared (IR) spectroscopic analyses demonstrate that these filaments, which develop in the stationary growth phase to tube-like structures, are composed of an outer membrane that encloses single cells. These results indicate severe influences of recombinant protein expression on cell division processes and raise new questions about the regulation of cell division and the formation of outer membranes at the molecular level. Despite the expressed S-layer proteins being present mainly in the cytosol, they are found to be associated with the tubes that morphologically represent an extracellular locus. This could imply a yet uncharacterized pathway for the cellular export of heterologously expressed recombinant proteins as described by Ni & Chen (2009). The S-layer tube-like structures may provide an interesting matrix for technical applications such as the development of microcontainers or hollow microwires.

METHODS

Bacterial strains and culture conditions. Lysinibacillus sphaericus JG-A12 is a natural isolate from the uranium mining waste pile of Haberland located near the town of Johanngeorgenstadt, Germany (Selenska-Pobell et al., 1999). Bacterial cells were routinely grown in nutrient broth medium. Host strains of E. coli NovaBlue GigaSingles (Novagen), an E. coli K-12 derivative strain, and E. coli BL21(DE3) (Novagen), an E. coli B 834 derivative strain, were routinely grown in Luria–Bertani (LB) medium.

Preparation of DNA, cloning, gene expression and membrane purification. As a template for PCR amplification, genomic DNA of L. sphaericus JG-A12 was isolated and purified using the NucleoSpin tissue kit (Macherey-Nagel) according to the manufacturer’s instructions. The S-layer protein gene slb was amplified from the chromosomal DNA of L. sphaericus JG-A12 using the primer pair Lic93f (5'-GAC GAC GAC AAG ATG/GCA GGA TTC TCA GAT GTA GCA-3') and Lic_PIHs (5'-GAG GAG AAG CCC GGT/TTA TGG AGT TGG CTT TAC TGT AAT A-3') for the construction of the N-terminal His-tagged recombinant protein Slb_1, and the primer pair Lic704f (5'-GAG GAC GAC AAG ATG/ATC AAC AAC ACA ACT GTT GAA-3') and Lic_PIHs for the construction of the N-terminal truncated and His-tagged recombinant protein Slb_2.

The gfp gene was amplified from the pGFP-vector DNA (Clontech) using the primer pair LiGFP_BamHI_F (5'-AAA GGA TCC ATG ATC AAC AAC ACA ACT GTT GAA-3') and LiGFP_EagI_R (5'-TTC CGG CTA TTT GTA TAG TTA ATC CA-3') for the construction of the N-terminal His-tagged recombinant fusion protein Slb2-GFP (Fig. 1).

PCR and cloning. PCR amplifications were performed as described previously (Pollmann & Matys, 2007). Positive, purified PCR products were cloned in the vector pET-30 EK/LIC (Novagen) according to the manufacturer’s instructions. The construct was transformed into E. coli NovaBlue (Novagen) as a non-expression host. The plasmids from positive clones were isolated using the

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**Fig. 1.** Comparison of the primary structures of the natural silent S-layer protein and its resultant newly designed fragments. SP, Signal peptide; NTD, N-terminal domain; CD, central domain; CTD, C-terminal domain; GFP, green fluorescent protein; Pos., position of the amino acids in the protein.
Wizard plus SV miniprep DNA-purification system (Promega) and transformed into E. coli BL21(DE3) as an expression host.

SllB_2 was fused with the gfp gene to create the construct SllB2–GFP. The multiple-cloning site of the pET-30 Ek/LIC vector was checked for correct ligation by using PCR and sequencing with T7-primers. Direct sequencing of the PCR products was performed with a BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems), using the PCR amplification primer pair (Sanger et al., 1977).

**Expression.** For each E. coli BL21(DE3) construct, separate flasks containing 100 ml LB medium supplemented with kanamycin (25 μg ml⁻¹) were inoculated with 5 ml of an LB-grown preculture of the construct. The cultures were incubated at room temperature or at 37 °C. After 2 h growth, recombinant protein expression was induced by the addition of 0.1 mM IPTG.

Protein isolation was performed as described previously (Pollmann & Matys, 2007). The protein profile of the supernatant, cell extract and cell debris were investigated by SDS-PAGE as described by Laemmli (1970). Expression of SllB was controlled by immunoblotting using polyclonal antibodies raised against the S-layer protein of L. sphaericus JG-A12 (Pineda Antikoerper Service) and the Bio-Rad immune-blot assay kit according to the manufacturer’s instructions.

Recombinant proteins were quantified by the SYPRO-Ruby protein gel stain (Bio-Rad) and the Bio-Rad protein assay according to the manufacturer’s instructions. The SDS-PAGE gels stained with SYPRO-Ruby were analysed with the versa DOC visualization system (Bio-Rad) with the programs PD Quest and Quantity One.

**Tube purification.** After overnight incubation, the cells were harvested by centrifugation (10 000 g, 10 min, 4 °C) and washed twice with 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na₂HPO₄ and 1.47 mM KH₂PO₄ (pH 7.4). The cells were suspended in 10 ml 40 % sucrose and incubated with stirring at 250 r.p.m. for 1 h at room temperature. The cells were concentrated by centrifugation (10 000 g, 10 min, 4 °C) and the supernatant was transferred to 1 ml an empty tube. After centrifugation for 30 min at 12 000 g and 4 °C, the supernatant was removed and the first and second pellets were suspended in 10 ml 6 M urea. After 1 h incubation at room temperature with stirring, the cells were collected by centrifugation (3000 g, 15 min, 4 °C). Each supernatant was centrifuged again at 12 000 g for 30 min at 4 °C. The tube pellets were washed twice with de-ionized water and analysed by SDS-PAGE, IR-spectroscopy, different microscopic methods and N-terminal sequencing of proteins using an ABI 494A Procise HT sequencer (Applied Biosystems).

**Isolation of E. coli membranes.** Cells were grown overnight and harvested from 100 ml culture by centrifugation (6500 g, 15 min, 4 °C) and suspended in 20 ml resuspension buffer [100 mM NaH₂PO₄-H₂O, 10 mM Tris base and 0.5 M NaCl (pH 8.0)]. The cells were disrupted by sonication (Sonifier W250-D, Branson) 5–6 times at 60 % amplitude for 30 s and the lysate was centrifuged for 15 min at 3000 g. Subsequently the supernatant was centrifuged at 12 000 g for 20 min at 4 °C. After centrifugation, the supernatant included most of the membrane components and the pellet included most of the cell wall components. The supernatant was centrifuged at 48 000 g for 1 h at 4 °C. The supernatant was removed and the pellet was washed twice in 10 ml resuspension buffer. After each washing step, the membranes were again centrifuged at 48 000 g for 1 h at 4 °C. Finally, the membrane pellet was transferred to 1 ml resuspension buffer and used for protein analyses.

**Preparation and characterization of different cell protein fractions.** Cells were grown overnight at room temperature and 40 ml of a well-grown culture was harvested by centrifugation at 10 000 g for 10 min at 4 °C. The proteins released to the medium, the proteins in the periplasmic protein fraction and the cytoplasmic proteins were isolated as described previously in the pET System Manual (Novagen) and were studied by protein assays.

**/β-Galactosidase assay.** All measurements of enzyme activities were carried out in a μQuant Microplate Spectrophotometer with the setting KC4 (Bio-Tek Instruments) and performed as previously described (Miller, 1972).

**Microscopic methods.** Light microscope images of the cells and purified tubes were taken with the Olympus BX61 microscope (Olympus Life Science) in phase-contrast mode. Cells expressing GFP fusion proteins were visualized by transmission through the GFP-Filter U-MNIBa2 (Olympus Life Science).

The L-7007 live/dead BacLight bacterial viability Kit (Molecular Probes) was used in order to distinguish living and dead bacteria. Microscopic analyses were done at extension/emission wavelengths of 480/500 nm for the SYTO 9 stain and at 490/635 nm for propidium iodide.

Atomic force microscopy (AFM) images of filaments and tubes were taken with the MFP-3-D-Bio (Asylum Research) using AC-mode in air and in liquid. The in-air samples were applied on silicon wafers, dried and scanned using OMCL AC240 cantilever (Olympus Life Science). The in-liquid filaments and tube-like structures were incubated overnight at room temperature to be fixed with 2 % glutaraldehyde in PBS, immobilized on silicon wafers and scanned in buffer using OMCL AC40 and OMCL TR400 cantilever in AC-mode (Olympus Life Science).

For transmission electron microscopy (TEM) investigations, the samples were first fixed with 2 % glutaraldehyde in PBS at room temperature, and then fixed with 1 % osmium tetroxide before being dehydrated in an increasing concentration of acetone (including a staining step with 1 % uranylacetate) and embedded in Epoxy resin (Serva) according to the method described by Spurr (1969). Ultrathin sections (about 50–300 nm) of samples were prepared with a Leica EM UC6 ultramicrotome (Leica Microsystems) using a diamond knife (Diatome), and mounted on pialoform-coated copper grids (Plano). The air-dried samples were stored under dry conditions. The investigations were carried out with a Titan 80–300 transmission electron microscope (FEI).

**IR-spectroscopy.** The tubes of SllB_1 and SllB2–GFP were treated with chloroform and methanol for total lipid extraction (Bligh & Dyer, 1959). The resulting lipidic and lipid-free phases, as well as the precipitated tube-associated proteins, were analysed by IR-spectroscopy. Aliquots (10–20 μl) of the respective fractions were dried on a diamond ATR-cell (Retsch) and measured at room temperature. Spectra were calculated from averaging 256 interferograms recorded at 2 cm⁻¹ resolution with a vector22 Fourier-transform infrared spectrometer (Bruker).

**RESULTS**

**Expression of the sllB genes and purification.**

The PCR products encoding the predicted silent S-layer-like protein SllB, a fragment of SllB and an SllB2–GFP fusion protein were cloned and expressed in E. coli BL21(DE3). The successful cloning was confirmed by partial sequencing. The expression of these constructs was induced by IPTG and confirmed by SDS-PAGE (Table 1).
Compared with cell extracts of *E. coli* Bl21(DE3), the sllB-expressing samples showed additional dominant high-molecular-mass protein bands that corresponded approximately to the predicted molecular mass of the constructs (Fig. 2). On immunoblots, a strong cross-reaction was observed between these protein bands and the polyclonal rabbit antiserum raised against SlfB of *L. sphaericus* JG-A12, confirming the expression of the SllB proteins (data not shown).

The concentration of the fusion proteins was determined by the SYPRO-Ruby protein stain and the Bradford assay. From 100 ml of cultures we obtained approximately 14.5 mg (25 % of total protein amount), approximately 18 mg (36 % of total protein amount) and approximately 12 mg (18 % of total protein amount) of the constructs SllB_1, SllB_2 and SllB2–GFP, respectively.

The SllB_2-expressing *E. coli* BL21(DE3) strain had the most efficient gene expression and formed the longest tube-like structures with the highest stability. For these reasons, this construct was chosen for fusion with *gfp* (SllB2–GFP) for further fluorescence microscopy-based localization analyses.

### Microscopic characterization of cell morphologies

Usually, cells of wild-type *E. coli* BL21(DE3) are rod-shaped, 1–2 μm long and 0.5–0.7 μm wide (Fig. 3a–c). These typical cell morphologies drastically changed upon expression of the S-layer-like protein SllB. When growing at room temperature (below 25 °C), recombinant *E. coli* BL21(DE3) cells expressing SllB were elongated and formed filaments of 5–200 μm in length during early exponential phase (Fig. 3d). During late exponential or early stationary phase, filaments appeared as transparent tubes containing single cells, which were, on average, 4–5 μm long and 0.8–1 μm wide (Fig. 3e, f). The single cells were separated by transparent cell-free areas (Fig. 3d–f). Live/dead tests proved the intactness of the cells in these growth phases, indicating that their viability is not disturbed by the unusual cell morphology. In stationary phase, some of the tube-like structures appeared to be empty, containing no cells or only cell fractures. The tube-like structures were found in each growth phase and were very stable against treatment with several chemicals such as SDS, Triton or urea.

To avoid drying artefacts, fixed cells were imaged in liquid after immobilization on silicon surfaces. More detailed AFM analyses in tapping mode determined that the thickness of the fixed tubes was approximately 75 nm (150 nm as double layer) (Fig. 4c, d, f, g). The thickness of the fixed filled filaments next to the empty tubes was approximately 600 nm (Fig. 4c, f). In comparison, wild-type *E. coli* BL21(DE3) cells exhibited a similar average thickness of about 600 nm from AFM height analyses (Fig. 4e, h). Air-dried samples were also imaged by AFM (Fig. 4b). This AFM height image revealed that the filaments were composed of bacterial cells that were separated by cell-free areas and surrounded by membrane-like structures.

In order to compare these structures with the wild-type strain, microscopic analyses of *E. coli* BL21(DE3) growing at room temperature were performed and samples were taken at different growth phases. In the early exponential growth phase, the cells of wild-type *E. coli* BL21(DE3) form filaments of 10–50 μm in length (Fig. 3a). However, when reaching the stationary growth phase, these filaments decompose to single cells of typical *E. coli* size with

### Table 1. Overview of S-layer proteins, cloned S-layer fragments and fusion proteins

<table>
<thead>
<tr>
<th>S-layer protein</th>
<th>Characteristics</th>
<th>Length (aa)</th>
<th>Approx. molecular mass (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SllB</td>
<td>Functional S-layer protein</td>
<td>1238</td>
<td>130</td>
</tr>
<tr>
<td>SllB_1</td>
<td>SllB without signal peptide, with N-terminal His-tag</td>
<td>1101</td>
<td>116</td>
</tr>
<tr>
<td>SllB_2</td>
<td>SllB without N terminus, with N-terminal His-tag</td>
<td>1070</td>
<td>113</td>
</tr>
<tr>
<td>SllB2–GFP</td>
<td>Fusion protein of SllB_2 and GFP</td>
<td>866</td>
<td>92</td>
</tr>
<tr>
<td>SllB2–GFP</td>
<td>Fusion protein of SllB_2 and GFP</td>
<td>1118</td>
<td>120</td>
</tr>
</tbody>
</table>

*Fig. 2.* Expression of S-layer and fusion proteins in *E. coli* BL21(DE3) cells demonstrated by SDS-PAGE analyses of the cell extracts. Lane 1, *E. coli* BL21(DE3) without expression of S-layer proteins; lane 2, SllB_1 (approx. 113 kDa); lane 3, SllB_2 (approx. 92 kDa); lane 4, SllB2–GFP (approx. 120 kDa); M, molecular mass standard.
Dimensions of 1–3 μm long × 0.5–0.7 μm in diameter (Fig. 3b, c). The long filaments and the stable transparent tubes found in sllB-expressing E. coli (Fig. 3e, f) were not observed in the stationary growth phase.

**Purification and characterization of tube-like structures**

Cell-free tubes from each recombinant E. coli strain as well as from the wild-type E. coli B21 BL21(DE3) were prepared by the addition of sucrose and urea to the cells, which were harvested in the stationary growth phase (recombinant E. coli) or in the exponential growth phase (wild-type E. coli). The isolated tubes were analysed by AFM, SDS-PAGE and IR-spectroscopy. SDS-PAGE showed the presence of two major bands (Fig. 5a). The predominant band corresponded to approximately 38 kDa and was assigned to the outer-membrane protein OmpF by N-terminal sequencing. A second dominant band of approximately 35 kDa was assigned to the outer-membrane protein OmpA by N-terminal sequencing. These results indicate that the tubes have a membranous nature. In addition, the recombinant proteins could be detected, suggesting that they are associated with the outer-membrane-like tubular structure. These results were confirmed by SDS-PAGE of isolated membranes (Fig. 5b). Besides the two major bands of OmpF and OmpA, an additional band at approximately 113 kDa in the SllB_1 sample further supported the association of the S-layer protein with the extracted outer membrane.

IR-spectroscopy was performed to derive information about the secondary structure of proteins in the membranous and tubular subfractions and to further assess the chemical composition of the tubes in relation to the normal outer membrane fraction. Fig. 6 (i) presents the IR absorption of the normally expressed S-layer from L. sphaericus. The amid I mode (peptide carbonyl stretching vibration) had contributions from different secondary structural elements which corresponded to ~33 % β-sheet, ~15 % turns and less than 18 % α-helix (Fahmy et al., 2006). The isolated tubes from sllB-expressing E. coli clearly had a complex composition, indicative of a superposition of peptidic amide I and II modes with additional narrow peaks in the 1500 to 1700 cm\(^{-1}\) range [Fig. 6 (ii)]. The latter was observed at 1676, 1625 and 1595 cm\(^{-1}\) free from the superposition with protein absorptions in a lipid-free extract of tubes from the sllB-expressing strain [Fig. 6 (iv)]. These peaks were assigned to residual urea in the tube preparation. Finally, the absorption of the lipidic component was identified after enrichment by organic extraction. It exhibited the typical C=O ester stretching mode and CH\(_3\) deformation vibrations at 1740 and 1465 cm\(^{-1}\), respectively [Fig. 6 (v)]. Quantitatively, this fraction contributes very little to the total absorption of the tubes prepared from either wild-type E. coli BL21(DE3) [Fig. 6 (vi)] or the sllB-expressing strain [Fig. 6 (ii)]. However, the composition of the total absorption of these tube preparations [Fig. 6 (ii)] clearly shows that the presence of an amide I/II-like background in addition to the normal spectral signature of E. coli tubes [Fig. 6 (vi)] is a unique feature of the tubes prepared from the S-layer-expressing strain. Hence, the protein content in the tubes strongly increased when sllB was expressed. We generated a 'pure' spectrum of the enriched protein in the
**Fig. 4.** AFM images of S-layer-expressing *E. coli* BL21(DE3) in stationary phase (a–d) and wild-type *E. coli* BL21(DE3) (e). Height images show an overview of filaments and tubes (a, b) and cells and tubes in detail (c–e). The thickness of native cells and cell-free tubes of SllB-expressing *E. coli* BL21(DE3) (c, d) was determined by generating height profiles (f, g). For comparison, a height profile (h) was also generated from a height image (e) of wild-type *E. coli* BL21(DE3) cells. The black arrows mark filled tubes and the white arrows mark cell-free tube-like structures.

**Fig. 5.** SDS-PAGE of cell-free tubes and isolated membranes from wild-type *E. coli* BL21(DE3) and recombinant *E. coli* BL21(DE3) after expression of SllB, protein fragments or SllB2–GFP fusion protein. (a) SDS-PAGE of cell-free tubes. M, Molecular mass standard; lane 1, *E. coli* BL21(DE3) without expression of an S-layer protein; lane 2, SllB_1; lane 3, SllB_2; lane 4, SllB2–GFP. (b) SDS-PAGE of isolated membranes. Lane 5, *E. coli* BL21(DE3) without expression of an S-layer protein; lane 6, SllB_1. Arrows indicate dominant bands, which were identified as outer-membrane-specific porins by N-terminal sequencing. OmpF and OmpA are typical porins in the outer membrane of *E. coli* cells with a size of 38 kDa and 35 kDa, respectively.
tubes from the sllB-expressing strain by subtracting the absorption of the tubes from a non-expressing E. coli strain. The resulting spectrum [Fig. 6 (iii)] revealed amide I and II bands that are typical of a protein composed predominantly of β-sheets. Intuitively, the spectrum could be assigned to the additionally expressed S-layer. However, comparison with Fig. 6 (i) showed that the spectrum differed from the spectrum of the homologous S-layer from L. sphaericus JG-A12. The shape of the amide I mode with a peak at 1629 cm\(^{-1}\) and a broad shoulder in the 1640–1660 cm\(^{-1}\) range was very similar to published IR spectra of the outer membrane porin OmpF (Terrettaz et al., 2002). Likewise, the pronounced shoulder at 1517 cm\(^{-1}\) in the amide II band is consistent with the relatively high amount of tyrosines in OmpF. The protein absorption in the tubes of the sllB-expressing E. coli BL21(DE3) strain can be explained by the presence of a strongly increased amount of OmpF or an OmpF-like membrane protein, thereby verifying the outer-membrane-like composition of the elongated tubes. This does not exclude the additional presence of the S-layer protein which may contribute to the broad amide I envelope.

**Localization of recombinant proteins**

The fine structure of the tube-like filaments was investigated by TEM. These analyses demonstrated that wild-type E. coli cells formed typical cell walls consisting of cytoplasm and outer membrane enclosing the narrow periplasm. In contrast, recombinant E. coli cells had altered cell membranes and formed an extremely bloated periplasm (Fig. 7). In addition, remarkable layers were visible in the periplasm. These layers resembled membranes, which possibly interacted with recombinant S-layers in the periplasm.

The gfp fusion construct SllB2–GFP was used to visualize the localization of the recombinant proteins in the filaments. The strong fluorescence signal from the single cells in the tubes (Fig. 8b) indicated that most of the recombinant fusion proteins were located in the cytoplasm. The weaker fluorescence signal from the cell-free areas indicated that some of the S-layer proteins were associated with the outer membrane. In addition, a fluorescence signal could clearly be detected from isolated cell-free tubes (Fig. 8c). The recombinant proteins that were released from cells or the periplasm during purification of the cell-free system were probably attached to the tube surface. Overall, the fluorescence images fully agree with the biochemical data and confirm that the recombinant proteins have a high affinity to the outer-membrane-like tubes.

To further localize the recombinant proteins with respect to the cytosol and periplasm, the periplasmic protein fraction was isolated by osmotic shock and separated from the other cell fractions. In addition, the culture medium was concentrated after cultivation and protein expression. The cytoplasmic cell fraction was isolated by treating the cells with BugBuster Protein extraction reagent (Novagen). The isolated protein fractions were analysed by using SDS-PAGE and β-galactosidase assays. The SDS-PAGE gel (Fig. 9) revealed significant differences between the protein profiles. E. coli SllB-2, which has strong expression and tube-forming ability, was selected as a representative example for the S-layer protein-expressing strains. As expected, the recombinant protein was found predominantly in the cytoplasmic protein fraction. The enzyme activity remained almost constant and relatively low over the whole measurement period as a consequence of preparation. However, the periplasm contained a remarkable amount of the expressed protein and the recombinant protein was detected in the medium after 8 h of expression. The different protein profiles indicate that recombinant S-layer proteins pass cell membranes via an unknown mechanism and accumulate in the periplasm.

**Enzyme assays for identification of cell disruption**

The activity of cytoplasmic β-galactosidase was assayed in the different cell fractions in order to investigate whether the release of the expressed S-layer was caused by damage of cell membranes.

Fig. 10 presents the results of the β-galactosidase assays performed with cytoplasmic and periplasmic proteins fractions as well as with the supernatant at different time points during protein expression. While β-galactosidase activity was high in the cytoplasmic fraction, no enzyme activity was detected in the periplasmic fraction or in the supernatant until the end of exponential phase. In combina-
tion with SDS-PAGE analyses (Fig. 9), these data clearly demonstrate that the recombinant S-layers are present in not only the cytoplasm but also the periplasm of intact cells. After 8 h of expression, these proteins were exported to the medium without detectable membrane damage.

**Stability of the tubes**

The tube-forming cells were exposed to both an ultrasonic bath and tip in order to analyse their mechanical stability. The tubes remained intact in the ultrasonic bath, but became disrupted upon exposure of the cells to an ultrasonic tip.

In order to study the chemical stability of the tubes, the cell culture was treated with different reagents in different concentrations and analysed by light microscopy. The tubes resisted treatment with hydrogen bond-disrupting reagents, such as 6 M urea or 6 M guanidine hydrochloride, with complex-forming reagents such as EDTA, or with denaturing agents such as 0.1–1 % SDS over several hours. In contrast, the tubular structure degraded imme-

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**Fig. 7.** TEM images of (a) *E. coli* BL21(DE3) without the SllB-carrying plasmid and (b) *E. coli* BL21(DE3) with the SllB-carrying plasmid after induction of protein expression in the exponential growth phase. OM, Outer membrane; P, periplasm; C, cytoplasm.

**Fig. 8.** Light microscope images of the S-layer–GFP-fusion-protein-expressing *E. coli* BL21(DE3) strain. SllB2–GFP in phase-contrast mode (a), with a GFP filter (b) and of isolated tubes out of SllB2–GFP visualized with a GFP filter (c). The red arrows mark filaments and the white arrows mark empty tube-like structures.
mediately in 30% TCA, 10% barium nitrate or 0.5% Triton X-100.

DISCUSSION

The Gram-negative micro-organism E. coli, with a typical size of 1.1–1.5 × 2.0–6.0 μm (Orskov, 1984), is routinely used for the expression of recombinant proteins and represents one of the best studied organisms with respect to genetics and metabolism. However, many proteins are difficult to express in E. coli. In particular, stable expression of S-layer proteins has failed in many cases, due to the difficulties in cloning or toxicity of gene products (Boot et al., 1993; Bowditch et al., 1989; Chu et al., 1991; Kuen et al., 1996; Peters et al., 1989; Yamagata et al., 1987), misfolding of proteins, or formation of insoluble inclusion bodies (Bingle & Smit, 1994). Like most recombinant proteins, these proteins are found in the cytoplasm. They are not detected in the periplasm or in the culture medium due to the lack of a signal sequence recognition enabling transport across the cell membranes of E. coli (Kuen et al., 1996). Truncated forms of the S-layer protein SbsC of Bacillus stearothermophilus ATCC 12980 are expressed in E. coli HMS174 (DE3) (Jarosch et al., 2001). While these cells demonstrate the presence of self-assembly S-layer products in the cytoplasm, no change was observed in the cell morphology, as demonstrated in this study.

In the present study, we have shown that the expression of the S-layer protein SllB results in the formation of unusually long filaments of E. coli (up to 100 μm). In the late exponential growth phase, these filaments change into tube-like structures, consisting of single cells enclosed by outer membrane-like structures. The elongated E. coli filaments as well as the enclosed single cells are immobile in the late exponential growth phase. Additionally, cell-free tubes were observed in stationary phase. As demonstrated by SDS-PAGE and IR analyses, the monitored tubes are composed of outer membrane and the SllB protein. These filamentous structures can also be found temporarily during the growth of wild-type E. coli BL21(DE3) at temperatures below 25 °C. However, they disaggregate in stationary phase, leaving behind single E. coli cells of normal size. Their persistence beyond the early exponential growth phase in the sllB-expressing strain suggests that the filaments are strengthened by the formation of additional layers, probably outer membranes, and become more stable by the attachment of S-layer proteins. Filamentous forms of E. coli have thus far been described only in a few cases. Preussner (1959) described the formation of E. coli filaments of a length of several tens of micrometre caused by aeration of the cultures with oxygen. The resulting cell shape strongly depended on age and cultivation conditions. Filaments were monitored predominantly in late exponential or stationary growth phase. In the present study, filaments were observed in the early growth phase in normal batch cultures, but their formation depended on temperature. Other E. coli mutants such as the temperature-sensitive E. coli mutant K-12 TOE13, the E. coli strain CS1959 Δrfa (rfaG rfaP) and the E. coli mutants lacking

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**Fig. 9.** Coomassie Brilliant Blue-stained SDS-PAGE gel of proteins of the supernatant (S), the periplasm (P) and the cytoplasm (C) of E. coli SllB_2 at different times throughout growth after induction of expression (T, time in h). M, Molecular mass standard. In the supernatant and periplasmic protein samples, 20 μl was used for preparation. In the preparation of the cytoplasmic protein samples, 2 μl was used. The arrow marks the S-layer protein band.

**Fig. 10.** β-Galactosidase activity in the supernatant (■), the periplasmic (■) and the cytoplasmic (○) protein fractions at different time points. The supernatant was concentrated, periplasmic proteins were isolated by osmotic shock of the cell pellet, and the cytoplasmic proteins were isolated by treatment with BugBuster Protein extraction reagent.

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**F. L. Lederer and others**

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Microbiology 156
the histone-like protein HU can form filaments of a few micrometres in length (Huisman et al., 1989; Koch et al., 1987; Painbeni et al., 1997; Parker et al., 1992). In contrast with the cells in the present study, these mutants were hypersensitive to detergents and exhibited poor growth, and the length of the filaments did not exceed a few micrometres. Morphological changes in the mutants were related to defects in outer membrane synthesis, or to interruption or slowing down of cell division or capsule synthesis during continuous cell growth and DNA replication (Koch et al., 1987; Parker et al., 1992; Preusser, 1959). The expression of S-layer protein may downregulate the cell division process by interacting with membrane proteins involved in synthesis or division of the murein sacculus, or with the membrane itself, or by influencing signal chains in the cytoplasm. Alternatively, recombinant S-layer proteins may influence the replication of bacterial chromosome, i.e. by interacting with nucleoid or nucleoid-associated proteins. However, it is beyond the scope of this paper to elucidate the pathway by which expression of slfB may interfere with cell division.

Here, we have focused on the biochemical composition and secondary structural features of the unusually long, stable and abundant filaments formed in the presence of the SlfB protein.

By using IR analyses, SDS-PAGE and subsequent protein identification of isolated tubes, we demonstrated their membranous origin. SDS-PAGE and GFP labelling have shown that the recombinant S-layer proteins are associated with the tubes and interact with the outer membrane of the cells. In addition, TEM images have revealed an extremely bloated periplasm containing layers, possibly of membranous origin, to which the S-layer proteins were attached. There are several reports describing the interaction of S-layer proteins with lipid membranes (Schuster et al., 2008; Wetzer et al., 1998; Weygand et al., 1999) and lipidic vesicles can be stabilized by coating with S-layer proteins (Schuster et al., 2008). In the context of the high resistance of the tubes against SDS, guanidine hydrochloride, urea and ultrasonic treatment, the above-mentioned work supports the theory that the S-layer protein has a stabilizing effect on the excessively produced outer-membrane-like structures in the filaments of the slfB-expressing E. coli strain formed in the early growth phase.

Interactions of SlfB proteins with the outer membrane require efficient transport across the cytoplasmic membrane. TEM analyses and GFP labelling, as well as analyses of the protein content of the periplasm and the culture medium, have demonstrated that the proteins can be released into the medium without damage to the plasma membrane. This is surprising because E. coli is generally a poor secretor and inadequate secretion is considered to be one of the most significant barriers to using E. coli in applications requiring extracellular production of proteins (Shin & Chen, 2008). Due to the lack of appropriate signal peptides, no specific transport system was involved in the export of the recombinant proteins.

It is possible that SlfB passes the cytoplasmic membrane via passive diffusion or leakage. Secretion of recombinant proteins is often explained by compromising the integrity of the outer membrane as a stress response, leading to high porosity. However, proteins with high molecular mass, such as SlfB, would require a highly porous membrane to enable efficient diffusion. E. coli cells producing SlfB, on the other hand, show high viability, high growth rate and excellent protein expression, making membrane disorders unlikely. This assumption is supported by the enzyme assays. The absence of cytoplasmic enzymes in the periplasm indicates intact cell membranes. There are several reports of non-specific lysis-independent extracellular secretion of both natural and recombinant proteins under shake-flask or bioreactor culture conditions (Nandakumar et al., 2006; Ni & Chen, 2009; Rinas & Hoffmann, 2004; Shin & Chen, 2008). In all these cases, the mechanisms that are responsible for the extracellular secretion remain unknown. However, the studies point to a non-specific transport system (Ni & Chen, 2009). A similar yet unknown non-specific secretion mechanism may occur for recombinant SlfB.

In a previous study, the functional S-layer gene slfB was cloned and expressed in E. coli using the same expression system as in the present work (Pollmann & Matys, 2007). However, production of SlfB was poor and the morphological changes described for SlfB expression were not observed, despite both gene products being closely related and varying only in their C-terminal part.

In conclusion, we have demonstrated that drastic changes in the morphology of E. coli cells are induced by the expression of the S-layer-like protein SlfB. Cells produced stable long tubes exhibiting an outer-membrane-like structure associated with the recombinant protein. To the best of our knowledge, this is the first description of a massive alteration in cell morphology in response to the expression of a recombinant protein. The microtubes are highly interesting for technical applications such as the generation of microcontainers or microwires by metallization. The unexpected extracellular secretion process of recombinant SlfB warrants further investigation.

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