Phage display of an intracellular carboxylesterase of Bacillus subtilis: a comparison of the Sec and Tat pathway export capabilities

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Phage display of an intracellular carboxylesterase of *Bacillus subtilis*: a comparison of the Sec and Tat pathway export capabilities

Using the phage display technology proteins can be displayed at the surface of bacteriophages as a fusion to one of the phage coat proteins. Here, we describe the development of this method for the fusion of an intracellular carboxylesterase of *Bacillus subtilis* to the phage minor coat protein g3p. Carboxylesterase A was cloned in the g3p-based phagemid pCANTAB 5E upstream of the sequence encoding the phage g3p and downstream of a signal peptide-encoding sequence. The phage-bound carboxylesterase was correctly folded and fully enzymatically active as determined from the hydrolysis of the naproxen methyl ester with $K_m$ values of 0.15 mM and 0.22 mM for the soluble and phage displayed carboxylesterase, respectively. The signal peptide directs the encoded fusion protein to the cell membrane of *Escherichia coli*, where phage particles are assembled. In this study, we assessed the effects of several signal peptides, both Sec- and Tat- dependent, on the translocation of the carboxylesterase in order to optimise the phage display of this enzyme normally restricted to the cytoplasm. Functional display of *Bacillus* carboxylesterase A could be achieved when Sec-dependent signal peptides were used. Although a Tat-dependent signal peptide can direct carboxylesterase translocation across the inner membrane of *E. coli*, proper assembly into phage particles does not seem to occur.

Introduction

In the past decade, the most remarkable successes from protein engineering have been the result of combining random mutagenesis and screening by means of a high-throughput assay. Unfortunately, for many enzymes, such as esterases and lipases, no high-throughput methods are available and, consequently, the evaluation of the enantioselectivity of lipases and esterases is dependent on time-consuming assays. Thus, it would be highly advantageous if the screening process could be combined with a rapid selection method that limits the amount of mutants to be assayed.

Phage display is a well-defined technique that has lead to a breakthrough in selection methodology for enzymes with desirable properties from a pool of mutants. Derivatives of M13 filamentous phages, a phage particle with a single stranded genome encapsulated by the phage coat proteins, are most commonly used for display in *Escherichia coli*. Enzymes can be expressed as a fusion to one of the M13 phage coat proteins, such as the g3p protein. As phage particles are assembled in the cell envelope of *E. coli*, translocation of the g3p fusion protein across the inner membrane of *E. coli* is a prerequisite for proper phage display. The g3p protein is synthesised with an 18-residue amino-terminal signal peptide that targets this protein to the *E. coli* Sec machinery for membrane insertion. Theoretically, any protein fused to the amino-terminal region of the g3p protein that is efficiently translocated across the inner membrane and that is able to enter the phage assembly site can be presented as a fusion protein on M13 phages.
Today, phage display is applicable for the selection of small peptides, antibody fragments and enzymes. For instance, many enzymes, such as amylases, β-lactamases, lipases, and transferases, have successfully been displayed on bacteriophages. Most of these are extracellular enzymes exported from the cytoplasm of the homologous host in a signal peptide-dependent manner. An important exception is the glutathion-S-transferase from *Schistosoma japonicum*.

In the general procedure for phage display, a gene of interest is cloned in a phagemid vector downstream of the signal sequence of *g3p* or *pelB* in order to direct the corresponding protein through the cell membrane via the Sec-dependent translocation pathway. Apart from the Sec pathway, which transports unfolded proteins over the inner membrane of *E. coli*, the twin-arginine translocation (Tat) pathway can be distinguished. In sharp contrast to the Sec routing, the Tat pathway of *E. coli* seems to accept only folded proteins for membrane translocation. In this context, we were curious to know whether translocation of a *g3p* fusion protein via the Tat pathway of *E. coli* would result in productive phage display of a cytoplasmic protein. To verify this idea, we compared the capability and effectiveness of the Sec-specific signal peptides of *E. coli* TEM-β-lactamase (SpBla) and *g3p* (SpG3p) and the Tat-specific signal peptide of the *E. coli* trimethylamine N-oxide (TMAO) reductase (TorA; the signal peptide is referred to as SpTor) in the export and phage display of *B. subtilis* carboxylesterase NA (CesA) and lipase A (LipA). SpBla was used, as its Sec-specificity is very well documented, whereas SpTor was selected for this purpose, because it was previously shown to direct the export of heterologous or truncated proteins. Furthermore, LipA of *B. subtilis*, a lipase with a twin arginine signal peptide yielding partial dependence on TatC, was used as a control protein for display, because functional phage display of this enzyme with the help of the *g3p* signal peptide was recently demonstrated. Importantly, the functional phage display of both CesA and LipA is of particular biotechnological interest as the corresponding displayed fusion proteins can be used for the selection of improved variants for the enantioselective conversion of several interesting pharmaceutical compounds as was recently shown for the selection of an *S*-(+)-1,2-O-isopropylidene-*sn*-glycerol specific LipA mutant.

**Experimental procedures**

**Plasmids, bacterial strains and media**

The plasmids and bacterial strains that were used in the present study are listed in table I. *E. coli* HB2151ΔtatC was constructed by P1 transduction of the *tatC::Spec* allele. Helper phage M13K07 and pCANTAB 5E were purchased from Pharmacia (Amersham Pharmacia Biotech, Uppsala, Sweden). Genencor International (Leiden, The Netherlands) provided a fermentor broth of the strain *B. subtilis* 1051, producing LipA. LB medium contained: Bactotrypton (1% w/v), Bacto yeast extract (0.5% w/v) and sodium chloride (0.5% w/v); 2xTY medium contained: Bactotrypton (1.6% w/v), Bacto yeast extract (1% w/v) and sodium chloride (0.5% w/v). Antibiotic agents (Duchefa Biochemie, Haarlem, The Netherlands) were used in the following concentrations: ampicillin 100 μg.mL⁻¹, kanamycin 50 μg.mL⁻¹.
Table I: Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
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<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG-1</td>
<td>supE K-12, Δ(lac-pro), thi, hdsD5/F’, traD36, proAB, laqP, lacZΔM15</td>
<td>Amersham Pharmacia</td>
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<td>Biotech, Uppsala, Sweden</td>
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<td>HB2151ΔtatC</td>
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<td>This work</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pCANTABSpBla CesA</td>
<td>pCANTAB 5E derivative containing the B. subtilis 168 cesA gene downstream of the bla signal sequence</td>
<td>This work</td>
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<tr>
<td>pCANTABSpG3p CesA</td>
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<td>This work</td>
</tr>
<tr>
<td>pCANTABSpTorA CesA</td>
<td>pCANTAB 5E derivative containing the B. subtilis 168 cesA gene downstream of the torA signal sequence</td>
<td>This work</td>
</tr>
<tr>
<td>pCANTABSpBla LipA</td>
<td>pCANTAB 5E derivative containing the B. subtilis 168 lipA gene downstream of the bla signal sequence</td>
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<td>pCANTAB 5E derivative containing the B. subtilis 168 lipA gene downstream of the torA signal sequence</td>
<td>This work</td>
</tr>
</tbody>
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**Chemicals**
The methyl ester of S-naproxen was provided by Prof. H.V. Wikström (Department of Medicinal Chemistry, University of Groningen, Groningen, The Netherlands). Butyrate esters of both enantiomers of 1,2-O-ispropyldiene-sn-glycerol (IPG) were provided by Prof. M.T. Reetz (Max-Planck Institut für Kohlenforschung, Mülheim, Germany). p-Nitrophenyl caprylate was purchased from Sigma Chem. Co. (Axel, The Netherlands).

**DNA techniques**
Recombinant DNA techniques were performed as described by Sambrook et al 176. Plasmid DNA was prepared as described by Birnboim & Doly 177. DNA purification was performed using the Qiaquick Gel Extraction kit (Qiagen, Hilden, Germany).

**Construction of the phagemids**
The LipA encoding gene (lipA) was cloned in the phagemid pCANTAB 5E, downstream of a modified g3p signal sequence, as described previously 103. The cesA gene (cesA) sequence
was amplified from the chromosomal DNA of *B. subtilis* 168 using *nap*for1 (5’-GCATGAATCAGGCCAGCCATGCGCAAAACCATTCATCTAGTATTCC-3’) and *nap*rev1 (5’-GATCGTTAGAATGCAGCCGCCATGGCACAAAACCATTCATCTAGTATTCC-3’) primers. PCR was performed using *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA). The amplified gene fragment was cloned into *E. coli* TG-1 into the *Sfi*I and *Eco*52I sites of a modified pCANTAB vector \(^{202}\). To exchange the g3p signal sequence (VKKLLLFAITLLFAIPLVVPFYAAQPAMA) for the signal sequences of *E. coli* SpBla (MSIQHFRVALIPFFAFCLPAMA) or *E. coli* SpTorA (MNNNDLFQASRRRLFAQLGGLTVAGMLGPSLLLTPRRATAAMA), a PCR was performed using primers 5’-CCCAAGCTTGGTACCGTTGGAGCCTTTTTTTTGGAGATTTTTTCAACATGAAATTTG TAATAAAGAAGG-3’ in combination with 5’-GCCGCCATGCGAGGCAAATGC-3’, and primers 5’-CCCAAGCTTGGTACCGTTGGAGCCTTTTTTTTGGAGATTTTTTCAACATGAAATTTG TAATAAAGAAGG-3’ in combination with 5’-GCCGCCATGCGAGGCAAATGC-3’, and primers 5’-GCCGCCATGCGAGGCAAATGC-3’ for the amplification of SpBla from template pUC18 \(^{203}\) and SpTorA from pJDT1 \(^{204}\), respectively. The PCR programme consisted of two steps. In the first step 4 min at 94°C, followed by 8 cycles of 1 min at 94°C, 2 min 50°C and 1 min at 72°C were adopted. Then, 5 pmol of the primer commonsmall (5’-CCCAAGCTTGGTACCGTTGGAGCCTTTTTTTTGGAGATTTTTTCAACATGAAATTTG TAATAAAGAAGG-3’), was added and 22 cycles of 1 min at 94°C, 2 min 50°C and 1 min at 72°C were applied. At the end, DNA synthesis was finished for 10 min at 72°C. The amplified gene fragments cloned in *E. coli* TG-1 into the *Hind*III and *Nco*I sites of pCANTABSpG3pCesA and pCANTABSpG3pLipA, respectively.

**Isolation of the periplasmic fraction, spheroplasts and whole cell extracts**

*E. coli* HB2151 was grown in 50 mL tubes containing 10 mL 2xTY medium, ampicillin and 1 mM of IPTG. The cultures were incubated at 37°C at 250 rpm for 16 h. The OD\(_{600}\) was recorded and the cells were harvested and resuspended in 10 mM Tris HCl, pH 7.4. After centrifugation, the cells were resuspended in 200 μL buffer containing 10 mM Tris HCl, pH 8.0, 25% sucrose, 2 mM ethylene diamine tetra acetic acid (EDTA) and 0.5 mg.mL\(^{-1}\) lysozyme. After incubation on ice for 20 min, 50 μL buffer containing 10 mM Tris HCl, pH 8.0, 20% sucrose and 125 mM MgCl\(_2\) was added. The suspension was centrifuged at 12,000 x g for 10 min and the supernatant, representing the periplasmic fraction, was isolated and used as enzyme solution in the activity assay. The pellet was resuspended in 200 μL buffer containing 50 mM Tris HCl, pH 8.4, and 2 mM EDTA. The suspension, representing the spheroplasts, was used as enzyme solution in the activity assay. The protein content of each fraction was determined by a Bradford assay in duplicate using bovine serum albumin (BSA) as a standard (Pierce, Rockford, Illinois, USA). To obtain whole cell extracts, cells were centrifuged at 12,000 x g and taken up in 200 μL buffer containing 50 mM Tris HCl, pH 8.4, and 2 mM EDTA.

**Phage rescue**

10\(^{10}\) helper phages were added to exponential phase growing *E. coli* TG-1 cells transformed with the plasmids mentioned in table I (phage-to-bacterium ratio 30:1), followed by 16 h of
growth at 28°C in a glucose depleted 2xTY medium containing ampicillin and kanamycin. Phages were precipitated by the addition of 5% w/v polyethylene glycol (PEG4000) in 2.5 M NaCl. After centrifugation, the phages were resuspended in 2 mL 10 mM Tris HCl buffer, pH 7.4, containing 1 mM EDTA, and filtered through a 0.45 µm filter (Millipore, Bedford, MA, USA). The number of phage particles in the suspension was determined by absorption spectroscopy according to using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, USA). The protein content was also determined by performing a Bradford assay using BSA as a standard.

Electrophoresis
Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) was performed on 11% separating and 4% stacking gels for LipA, and 12.5% separating and 3% stacking gels for CesA. Molecular mass markers were purchased from Bio-Rad. After electrophoresis, proteins were blotted to nitrocellulose and immunostained with a rabbit antiserum against LipA or CesA or with mouse monoclonal antibodies against g3p (PSKAN3; MoBiTec, Göttingen, Germany). Detection of the antibody was performed with alkaline phosphatase-conjugated antibodies against rabbits (LipA and CesA antiserum) or mice (g3p antibody).

Enzyme kinetics
Enzymatic activity of LipA was determined spectrophotometrically by the p-nitrophenyl caprylate assay as described previously. Esterase activity was determined using a naproxen methyl ester assay. The Michaelis-Menten constant (Km), specific activities and turnover numbers of the soluble and phage-bound CesA (phages were produced using E. coli TG-1 transformed with pCANTABSpBlaCesA) were determined with S-naproxen methyl ester substrate concentrations between 0.25 mM and 0.75 mM. All data were expressed as mean ± SEM (n = 3). The statistical significance of differences was tested at a significance level of p < 0.05 using a two-tailed Student's t-test.

Results

Construction of the phagemids
With the ultimate aim to display LipA and CesA of B. subtilis on M13 phages, the corresponding genes were cloned in the phagemid pCANTAB 5E, downstream of a modified g3p signal sequence and upstream of a collagenase cleavage site, six consecutive histidine residues (His-tag), an amber stop codon, and the sequence encoding residues 3-406 of the g3p coat protein. The original g3p maturation site in this phagemid, SHS, was modified to AAQPAMA in order to better resemble the consensus for signal peptidase I cleavage. The His-tag can be used for the purification of wild-type and mutant enzymes. The collagenase cleavage site can be used for phage rescue during phage display selection.
In order to compare the effect of different Sec- and Tat-specific signal peptides in phage display, the sequences encoding the signal peptides SpBla and SpTor were used to replace the g3p signal sequence in pCANTABSpG3pLipA. The resulting constructs were stably maintained in \textit{E. coli} HB2151 and \textit{E. coli} HB2151ΔtatC.

**Export and phage display of \textit{Bacillus} LipA**

**Processing of LipA**

The periplasmic fraction, spheroplasts and whole cell extracts of \textit{E. coli} HB2151 and \textit{E. coli} HB2151ΔtatC transformed with the plasmids pCANTABSpBlaLipA, pCANTABSpG3pLipA, or pCANTABSpTorALipA were isolated to determine the processing of LipA and the activity towards \(p\)-nitrophenyl caprylate. As the TAG stop codon is not suppressed in these strains, the different forms of LipA encoded by the three plasmids will not be fused to g3p. SDS PAGE under reducing conditions and Western blot analysis with a rabbit antiserum against LipA detected mature LipA at approximately 21 kDa, corresponding to the molecular mass of the His-tagged enzyme (figure 1A, 1C and 1E).

![Figure 1: Detection of LipA in the periplasmic fraction, spheroplasts, and whole-cell extract (10 µg of protein per lane). SDS PAGE (11% gel), Western blotting, and immunostaining with a rabbit antiserum against LipA were performed with the periplasmic fractions (A), spheroplasts (C) and whole-cell extracts (E) of \textit{E. coli} HB2151 and \textit{E. coli} HB2151ΔtatC transformed with pCANTABSpBlaLipA (lanes 1 and 2), pCANTABSpG3pLipA (lanes 3 and 4), or pCANTABSpTorALipA (lanes 5 and 6). Lanes 1, 3, and 5 contained samples from \textit{E. coli} HB2151, and lanes 2, 4, and 6 contained samples from \textit{E. coli} HB2151ΔtatC. Activities of LipA in the periplasmic fraction (B) and in the lysed spheroplasts (D) were determined using \(p\)-nitrophenyl caprylate as the substrate. +, enzyme activity; -, no enzyme activity.](image-url)
The different precursor proteins were detectable at apparent molecular masses of 23 to 27 kDa, depending on the signal peptide used (SpBlaLipA 24 kDa; SpG3pLipA 23 kDa; SpTorALipA 27 kDa, respectively). Notably, for some signal peptide-LipA fusions, the SpTorALipA fusion in particular, several distinct precursor forms were observed. During isolation of the periplasmic fractions, some cell lysis may have occurred as precursor proteins were detectable in these fractions. This effect was most prominent in strains containing pCANTABSpTorALipA, suggesting that the production of the SpTorALipA fusion protein makes the cells more prone to cell lysis, or that the corresponding precursor forms are not efficiently retained in the inner membrane. Conversely, mature LipA was observed in the spheroplasts, which is obviously explained by the presence of membranes in these fractions and the lipophylic behaviour of lipases in general. However, comparison of the periplasmic and spheroplast fractions revealed that, with the exception of the SpTorALipA-producing E. coli strains, the relative amounts of the different precursor proteins were lower in the periplasmic fractions than in the spheroplasts. Most interestingly, LipA was present exclusively in precursor forms in the periplasmic and spheroplast fractions of the ΔtatC mutant transformed with plasmid pCANTABSpTorALipA. As the precise fusion between SpTorA and LipA, encoded by pCANTABSpTorALipA, resulted in the accumulation of relatively high levels of SpTorALipA precursor forms in the wild type E. coli strain, an alternative SpTorALipA fusion was made containing the original signal peptidase recognition site of SpTorA and the first three residues of the mature TorA protein (pCANTABSpTorA-AQAATD-LipA) (figure 2). This, however, did not result in reduced levels of SpTorALipA precursor accumulation. Importantly, none of the two SpTorALipA constructs was processed to mature LipA in the ΔtatC strain, indicating that they require a functional Tat machinery for membrane translocation and subsequent processing by signal peptidase.

Figure 2: TatC-dependent processing of SpTorALipA fusions. SDS-PAGE (11% gel), Western blotting, and immunostaining with a rabbit antiserum against LipA were performed with cell lysates of E. coli HB2151 and E. coli HB2151ΔtatC transformed with pCANTABSpTorA-AQAATD-LipA (lanes 1 and 2, respectively) and cell lysates of E. coli HB2151 and E. coli HB2151ΔtatC transformed with pCANTABSpTorALipA (lanes 3 and 4, respectively).

To investigate the enzymatic activity of LipA in the periplasmic fraction and lysed spheroplasts of E. coli HB2151 and the ΔtatC mutant, the specific activity towards p-nitrophenyl caprylate was determined (figure 1B and 1D). All fractions containing mature LipA were able to hydrolyse the caprylate ester of p-nitrophenol (indicated by a +). Note
that no LipA activity was detectable in fractions derived from the \( \Delta tatC \) mutant producing SpTorALipA (indicated by a -), and that the same was true for fractions of \( E. coli \) strains not producing LipA (data not shown). Together, these results show that LipA precursors are translocated across the membrane and processed to the mature form in \( E. coli \) HB2151 both if using a Sec-dependent or a Tat-dependent signal peptide.

**Phage display of LipA**

\( E. coli \) TG-1 cells, transformed with pCANTABSpBlaLipA, pCANTABSpG3pLipA, or pCANTABSpTorALipA, respectively, were infected with M13K07 helper phages to produce phage particles containing the phagemid genome and a mixture of wild-type g3p and LipA-g3p fusion proteins. The LipA fusion to g3p results from a partial suppression of the TAG stop codon in the \( E. coli \) TG-1 host cells. To visualise the presence of phage-bound LipA, phage particles were analysed by SDS PAGE under reducing conditions and immunoblotting with a rabbit antiserum against LipA (figure 3A).

![Figure 3: Detection of phage-bound LipA. A) LipA-g3p fusion proteins in 0.25-µg phage suspensions were visualized by SDS PAGE, Western blotting, and immunostaining with rabbit antiserum against LipA (αLipA) (left panel) or mouse monoclonal antibodies against g3p (αg3p) (right panel). Phages were isolated from the growth media of \( E. coli \) TG-1 cells transformed with pCANTABSpBlaLipA (SpBla), pCANTABSpG3pLipA (SpG3p), or pCANTABSpTorALipA (SpTorA). B) Hydrolysis of p-nitrophenyl caprylate by phage-bound LipA. +, hydrolysis; –, no hydrolysis.](image)

A LipA-g3p fusion protein was detectable in phages isolated from the growth media of \( E. coli \) TG-1 cells transformed with pCANTABSpBlaLipA or pCANTABSpG3pLipA. This fusion protein had an apparent molecular mass of approximately 85 kDa, which corresponds to the apparent molecular mass of LipA plus a g3p protein on SDS PAGE gels. Notably, the LipA-g3p fusion protein was absent from the phage suspension derived from \( E. coli \) TG-1 cells transformed with pCANTABSpTorALipA. Previously, it has been demonstrated by the determination of the Michaelis-Menten constants, and the turnover numbers of the soluble and phage-bound LipA (phages were produced using \( E. coli \) TG-1 transformed with pCANTABSpG3pLipA) that phage-bound LipA was correctly folded and fully enzymatically active. To assess whether the specific lipase activity in the different phage suspensions corresponded with the amount of fusion protein, the activity towards p-nitrophenyl caprylate was determined. Importantly, figure...
3B demonstrates indeed that the presence of fusion protein correlates with LipA activity. Overall, the highest lipase activity and highest amount of fusion protein were obtained when Sec-specific signal peptides were used. Although the Tat-specific signal peptide of TorA can be used to direct export of LipA of *B. subtilis* to the periplasmic space of *E. coli*, proper phage display of the TatC-dependently exported enzyme seems to be impaired.

**Export and phage display of Bacillus CesA**

**Processing of CesA**

To investigate the export and processing of CesA, the periplasmic fraction, spheroplasts and whole cell extracts of *E. coli* HB2151 and *E. coli* HB2151Δ*tatC* transformed with plasmids pCANTABSpBlaCesA, pCANTABSpG3pCesA, or pCANTABSpTorACesA were isolated, and SDS PAGE under reducing conditions and Western blot analyses were performed with a rabbit antiserum against CesA (figure 4A, 4C and 4E). Mature CesA with the predicted molecular mass of 36 kDa was detectable in both strains. Furthermore, CesA precursor forms were detectable at apparent molecular masses ranging from 38 to 40 kDa (SpBlaCesA, 38 kDa; SpG3pCesA, 38 kDa; SpTorACesA, 40 kDa, respectively) in most periplasmic fractions and spheroplasts. Again, precursor forms of CesA were detectable in the periplasmic fractions, indicating that the *E. coli* strains were either subject to lysis upon spheroplasting, or that the precursors are not effectively retained in the inner membrane. Importantly, figure 4 shows that the cytoplasmic protein CesA of *B. subtilis* can be exported to the periplasm of *E. coli* using Sec-specific signal peptides. Mature CesA could be detected in the periplasmic fraction of *E. coli* HB2151 cells producing SpTorACesA as well, though most of the mature CesA was present in the cytoplasmic fraction of these cells. In contrast, the SpTorACesA produced in the Δ*tatC* mutant remained mainly in the precursor form, which is consistent with the fact that SpTorA is a Tat-specific signal peptide. These observations suggest that CesA can be translocated across the membrane via the Tat machinery.

Determination of the IPG hydrolysing activity of CesA in the periplasmic fraction and lysed spheroplasts of *E. coli* HB2151 and the Δ*tatC* mutant revealed that an endogenous IPG hydrolase is present in the cytoplasm of the *E. coli*. This complicated the determination of the specific CesA activity in the lysed spheroplast fractions. In contrast, no endogenous IPG hydrolase activity was detectable in the periplasmic fractions of *E. coli* HB2151 and the Δ*tatC* mutant (figure 4B). Although the level of IPG conversion was very low, enzymatic activities could be demonstrated in most of the periplasmic fractions of *E. coli* HB2151 and the Δ*tatC* mutant (enzymatic activity indicated by a +, higher activity indicated by ++). No detectable CesA activity was, however, observed in the periplasmic fraction of *E. coli* HB2151 Δ*tatC* transformed with plasmid pCANTABSpTorACesA (indicated by a -). In a further attempt to determine the activity of CesA in lysed spheroplasts, the specific activity towards methyl ester of S-naproxen was explored (figure 4D). Although the rate of conversion to S-naproxen was low, CesA activity could be demonstrated in most of the lysed spheroplast fractions. However, no S-naproxen methyl ester hydrolysis was detectable in the lysed spheroplast fraction of the Δ*tatC* mutant transformed with pCANTABSpTorACesA. These findings imply that the CesA precursor forms detectable in these spheroplasts and periplasmic cell fractions are not enzymatically active.
Figure 4: Detection of CesA in the periplasmic fraction, spheroplasts, and whole-cell extract (20 µg of protein per lane): SDS-PAGE (12.5% gel), Western blotting, and immunostaining with a rabbit antiserum against CesA of the periplasmic fractions (A), spheroplasts (C), and whole-cell extracts (E) of E. coli HB2151 and E. coli HB2151ΔtatC transformed with pCANTABSpBlaCesA (lanes 1 and 2), pCANTABSpG3pCesA (lanes 3 and 4), or pCANTABSpTorACesA (lanes 5 and 6). Lanes 1, 3, and 5 contained samples from E. coli HB2151, and lanes 2, 4, and 6 contained samples from E. coli HB2151ΔtatC. (B) Hydrolysis of racemic esters of 1,2-O-ispropyldiene-sn-glycerol butyrate. (D) Hydrolysis of the methyl ester of S-naproxen. +, enzymatic activity; ++, higher enzymatic activity; –, no enzymatic activity.

**Phage display of CesA**

Phage particles were produced using E. coli TG-1 cells transformed with pCANTABSpBlaCesA, pCANTABSpG3pCesA, or pCANTABSpTorACesA. SDS-PAGE under reducing conditions and Western blot analysis with mouse monoclonal antibodies with a rabbit antiserum against CesA and against g3p were performed. The apparent molecular mass of the CesA-g3p fusion protein was approximately 95 kDa, which corresponds to the masses of CesA plus a g3p protein. This fusion protein was detected with the CesA antibody at 95 kDa (figure 5A). Two specific protein bands reacting with the antibodies against the g3p coat protein were detectable (figure 5B). These bands corresponded to g3p (apparent molecular mass of 65 kDa) and to a CesA-g3p fusion protein. Notably, the CesA-g3p fusion protein was not detectable in the phage suspension derived from cells producing SpTorACesA. The reaction of αCesA with bands around 65 kDa and 35 kDa reflects, most likely, the presence of degradation products of the CesA-g3p fusion protein. Despite the fact that only very low levels of (S)-naproxen methyl ester hydrolysis were detectable, CesA activity could be demonstrated for both phage suspensions derived from cells producing CesA-g3p with Sec-specific signal peptides.
Figure 5: Detection of phage-bound CesA. A) CesA-g3p fusion proteins in 0.25-μg phage suspensions were visualized by SDS PAGE, Western blotting, and immunostaining with rabbit antisera against CesA (αCesA) (left panel) or mouse monoclonal antibodies against g3p (αg3P) (right panel). Phages were isolated from the growth media of E. coli TG-1 cells transformed with pCANTABSpBlaCesA, pCANTABSpG3pCesA, or pCANTABSpTorACesA. (B) Hydrolysis of the methyl ester of (S)-naproxen by phage-bound CesA. +, enzymatic activity; –, no enzymatic activity.

**Enzymatic activity of soluble and phage-bound Bacillus CesA**

To investigate whether the kinetic properties of the phage-bound CesA were unaltered, the $K_m$ and the specific activities were measured of both soluble and phage-bound CesA (table 2). The steady state hydrolysis of S-naproxen methyl ester showed that the $K_m$ of the enzyme remained unchanged (being not significantly different ($p > 0.05$)), suggesting that the protein is correctly folded and fully enzymatically active. In contrast, the specific activity of the phage-bound CesA was reduced. This observed difference is likely to result from the different individual weights of the soluble CesA and a phage particle (5.98 x $10^{-17}$ and 2.36 x $10^{-14}$ mg, respectively). Taken together, our present findings show that both LipA and CesA can be effectively exported from the cytoplasm of E. coli, and displayed on M13 phages with the help of Sec-specific signal peptides, but not with the Tat-specific signal peptide of TorA.

**Table II: Enzyme kinetics of CesA from Bacillus subtilis.** (n=3)*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity (U.mg$^{-1}$)</th>
<th>$K_m$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble CesA</td>
<td>12.8 ± 11.3</td>
<td>3.71 ± 4.38</td>
</tr>
<tr>
<td>Phage-bound CesA</td>
<td>0.22 ± 0.07</td>
<td>0.22 ± 0.13</td>
</tr>
</tbody>
</table>

* Statistical significance of differences, $p < 0.05$. 
Discussion

The present studies report for the first time the functional phage display of the cytoplasmic protein CesA of *B. subtilis* as a fusion to the phage M13 minor coat protein g3p. It was tempting to speculate that phage display of heterologous cytoplasmic proteins, such as *Bacillus* CesA, in *E. coli* might be enhanced if the respective fusion proteins were exported from the cytoplasm via the Tat pathway, as the Tat pathway exists for the export of intracellularly folded proteins\textsuperscript{207,208}. Remarkably, our results show that functional phage display of CesA-g3p and LipA-g3p fusion proteins could only be achieved if Sec-specific signal peptides (SpBla and SpG3p) were used for translocation of the fusion protein across the inner membrane of *E. coli*. In marked contrast, the use of the Tat-specific signal peptide SpTorA did not result in functional phage display of these g3p fusion proteins. These results are in accordance with the recently published results of Paschke & Höhne. These authors demonstrated that fusion proteins of mutated GFP with the C-terminal domain of g3p, using a TorA or PelB signal sequence, could not sufficiently be displayed on phages. However, phage display was ultimately achieved by transporting g3p and GFP to the periplasm independently, followed by combination using a coiled coil/disulfide strategy. The authors suggest that the unfolded g3p domain is not suitable for Tat-dependent export\textsuperscript{109}.

At present, the exact reason why Tat-specific export of the tested g3p fusion proteins did not result in their incorporation into phages remains unclear. The assembly of M13 phages occurs at sites in the cell envelope where the inner and outer membranes are in close contact\textsuperscript{209}. Prior to incorporation into the phage particle, all phage proteins are assembled in the inner membrane\textsuperscript{210,211}. Specifically, the g3p protein requires the Sec pathway for inner membrane assembly\textsuperscript{191}. Thus, at least two possible explanations for the ineffectiveness of SpTorA in phage display are conceivable. Firstly, the bacterial Tat machinery seems to accept only folded proteins for translocation\textsuperscript{195}, which may have a negative impact on the assembly of g3p fusion proteins into phages. Possibly, the CesA-g3p and LipA-g3p fusion proteins are only competent for assembly into phages if they are translocated via the Sec machinery in an unfolded state. Translocation in a folded state via the Tat machinery might render them incompetent for phage assembly. Secondly, the Tat system may not be able to sort proteins to the specific sites where phage assembly takes place. For example, the Tat pathway may export the g3p fusion proteins to the periplasm. This would hamper the assembly of these fusion proteins into phages, because they need to remain attached to the inner membrane for this purpose. However, mis-sorting of g3p fusion proteins to the periplasm seems somewhat unlikely as it has been demonstrated recently that integral membrane proteins with a carboxyl-terminal membrane anchor (like g3p) can be inserted into the membrane by a Tat-dependent mechanism\textsuperscript{212}. At least in the case of the CesA-g3p fusion, a mis-sorting event seems nevertheless a plausible explanation for the lack of phage incorporation upon Tat-dependent membrane translocation, because the mature CesA (not fused to g3p) that resulted from SpTorACesA processing was in part released into the periplasm. However, most of the mature protein was detected in the spheroplasts. In contrast, the mature forms of CesA that resulted from SpBlaCesA or SpG3pCesA processing were detected abundantly in the periplasmic cell fraction.

Remarkably, cell fractionation experiments revealed that a significant proportion of all hybrid CesA and LipA precursor proteins analysed in these studies was readily released
from spheroplasts into the spheroplast supernatant (i.e. the periplasmic fraction). This suggests that spheroplasts of cells producing these precursor proteins are either sensitive to cell lysis or that the precursor proteins are not effectively retained in the inner membrane of *E. coli* HB2151 and its ΔtatC mutant derivative. Although it is presently difficult to distinguish between these two possibilities, we favour the idea that the precursors are poorly retained. This preference relates to the observation that the endogenous IPG hydrolase activity of *E. coli* HB2151 is detectable only in cytoplasmic cell fractions, but not in periplasmic cell fractions. However, we cannot rule out the possibility that this hydrolase activity is absent from the periplasmic fractions due to the presence of an, as yet, unidentified periplasmic inhibitor. Conversely, the presence of mature LipA and CesA in the cytoplasmic fractions can be explained by the presence of membranes in these fractions and the lipophylic behaviour of these lipases and esterases. Finally, the CesA and LipA activity assays on periplasmic and cytoplasmic fractions of the *E. coli* HB2151 ΔtatC mutant revealed that the SpTor precursor forms of these proteins are enzymatically inactive. Thus, it seems that the fusion of CesA and LipA to the SpTor signal peptide does not only preclude the display of these proteins on M13 phages, but also affects their ability to fold into an enzymatic active form. This could imply that these proteins do not reach the relevant folding catalysts when they are targeted into the Tat pathway. In fact, this may not only be true for mature CesA and LipA, but also for the CesA-g3p and LipA-g3p fusion proteins. If so, the inability of SpTorA to direct functional phage display may relate both to targeting and folding problems.

In conclusion, functional display of the cytoplasmic protein CesA of *B. subtilis* can be achieved when Sec-dependent signal peptides are used for this purpose. Although the use of a Tat-dependent signal peptide, SpTorA, can result in CesA precursor processing, the mature form of this protein remains membrane bound. Proper phage display using SpTorA seems to be impossible for this substrate. It will be a major challenge for future phage display research to elucidate the molecular mechanisms underlying these observations. It is anticipated that such studies will provide novel insights concerning the mechanism of g3p assembly into M13 phages.

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