Force generation in dividing E. coli cells: A handles-on approach using optical tweezers

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Chapter 5: Outer membrane assembly of N- and C-terminal fusions to the OmpA transmembrane domain

Abstract

With the aim of sub-localizing the OmpA transmembrane (TM) domain to the site of constriction (mid-cell) in dividing *Escherichia coli*, we studied the outer membrane (OM) assembly of N- or C-terminal fusions to this domain. Various protein domains were genetically fused to the OmpA TM domain. A rare-sugar (D-allose) binding protein (ALBP), and the fluorescent protein mCherry were used that were expected not to interact specifically with other proteins (non-interacting domains). As mid-cell localization domains, the Pal lipoprotein and the septal targeting domain from AmiC (^{T}amiC), a Tat substrate, were used. As the OmpA TM domain had either a FLAG epitope or a streptavidin binding peptide inserted in a surface-exposed loop, OM assembly could be evaluated using cell surface immunolabeling. Furthermore, the *in vivo* folding state of the OmpA TM domain was determined by a gel-shift assay. In addition, for mCherry fusions, fluorescence microscopy was used to study sub-cellular localization. Our results show that non-interacting fusions to either the N- or the C-terminus of the OmpA TM domain can be assembled in the OM. Interestingly, the localization of the (OmpA-177)-Pal fusion was excluded from mid-cell in the presence of wild type Pal. Finally, we present two specific cases of N-terminal fusions that are not incorporated in the OM, presumably due to specific needs of these fusions.

Introduction

The outer membrane of Gram-negative bacteria contains a variety of proteins (OMPs) that fold as a single protein domain with a compact β-barrel fold. A well-known OMP with an additional C-terminal domain is OmpA, which consists of a N-terminal transmembrane (TM) domain (first 170 residues of the mature protein), connected to a C-terminal periplasmic peptidoglycan (PG)-binding domain. Our goal was to create, in dividing cells, an anchor point in the bacterial outer membrane, such that a micron-sized bead could be attached to a living bacterium and act as both handle and force probe in biophysical experiments. It has been shown that the OmpA TM domain can insert in the OM as efficiently as the full-length protein (Ried et al. 1994), even with an epitope tag inserted into one of its surface-exposed loops (Verhoeven et al. 2008). A possible approach then, is to genetically link the OmpA TM domain to a periplasmic domain that is known to localize to the site of constriction (mid-cell). This requires that the OmpA TM domain (OmpA-177) can insert into the OM with a heterologous domain attached to either its N- or C-terminus.

What would be a suitable mid-cell domain? A high copy number is preferred, preferably $>10^3$, because mid-cell localization and OM insertion at mid-cell of a artificial OMP fusion protein will most likely not be 100% efficient. Assuming only 10% properly inserts and localizes to mid-cell, this would give us $>10^2$ concentrated anchor points, which would allow their detection with conventional fluorescence microscopy. Of course, increasing the number of anchor points at mid-cell also increases the probability of bead attachment.

When this study was initiated, all known periplasmic domains with mid-cell affinity were low copy number ($<10^3$) inner membrane (IM) proteins that extend outwards into the periplasm. Typically, they diffuse around in the IM and dynamically associate with other cell division proteins to form the divisome. One of them, the FtsQ protein, has a copy number of 25-50 (Carson et al. 1991), and consists of an IM α-helix connected to a periplasmic moiety of two sub-domains (van den Ent et al. 2008). For this protein, it has been shown that its C-terminus can be extended with a HSV epitope without interfering with mid-cell localization in the presence of wildtype FtsQ (Vinkenvleugel 2006). Therefore FtsQ was selected as mid-cell localization domain.

As a fusion protein bridging FtsQ and OmpA-177 needs to go through the peptidoglycan layer, we are faced with a potential problem. OM insertion of OmpA-177

when fused to FtsQ should be well timed: it should not occur as long as the FtsQ domain diffuses around, as the fusion might become trapped in the PG mesh. After FtsQ localizes to mid-cell, it should occur as soon as possible, as FtsQ localizes to mid-cell a few minutes before a (phase contrast-) visible constriction appears (Aarsman et al. 2005), at which time the anchor presented by OmpA-177 should be in place for bead tethering.

How to span the periplasm? As the solute-accessible periplasmic width of *Escherichia coli* is estimated at ~15-21 nm (Matias et al. 2003; Collins et al. 2007), and the C-terminus of FtsQ can extend up to 7 nm into the periplasm (van den Ent et al. 2008), a spacer of ~8-14 nm is needed. To prevent proteolysis and/or misfolding it was decided to use native, folded domains instead of unstructured or artificial amino acid sequences. Preferably, the spacer domain should not have any interactions with other cellular components, as these could interfere with mid-cell and/or OM localization. Finally, its N- and C-terminus should be sufficiently separated to act as spacer domain. With these considerations in mind, the periplasmic D-allose binding protein ALBP (Chaudhuri et al. 1999) was chosen. As the D-allose operon is not expressed when its substrate D-allose is absent (Kim et al. 1997), ALBP's main interaction partners will expected to be absent when grown in a defined medium without D-allose. Furthermore, its N- and C-terminus are on opposite sides of the protein roughly 5 nm away (Chaudhuri et al. 1999). To be able to detect the spacer construct, and to facilitate proper folding of the individual domains, a myc epitope is fused as a linker between two ALBP domains.

During the course of this study, two periplasmic mid-cell domains that bind at the OM side of the PG were discovered (Bernhardt and de Boer 2003; Gerding et al. 2007). Such domains could simplify things enormously, as only a single domain has to be fused to OmpA-177, and the risk of entanglement in the PG would be reduced considerably. One of these domains was Pal, a peptidoglycan-associated lipoprotein (Gerding et al. 2007). It appeared an ideal candidate for fusion to OmpA-177, as it is anchored to the OM, its fold was homologous to the periplasmic domain of OmpA and it has a high copy number of 10^4 (Cascales et al. 2002; Gerding et al. 2007). Also, a Pal-mCherry fusion, expressed at levels expected to be $>10^3$ in a ΔPal strain, shows almost exclusive localization to mid-cell (Gerding et al. 2007). This suggests that several thousands of potential binding sites at mid-cell are available, indicating a high mid-cell localization potential for a Pal fusion protein. A second periplasmic domain discovered in recent years was the septal targeting domain

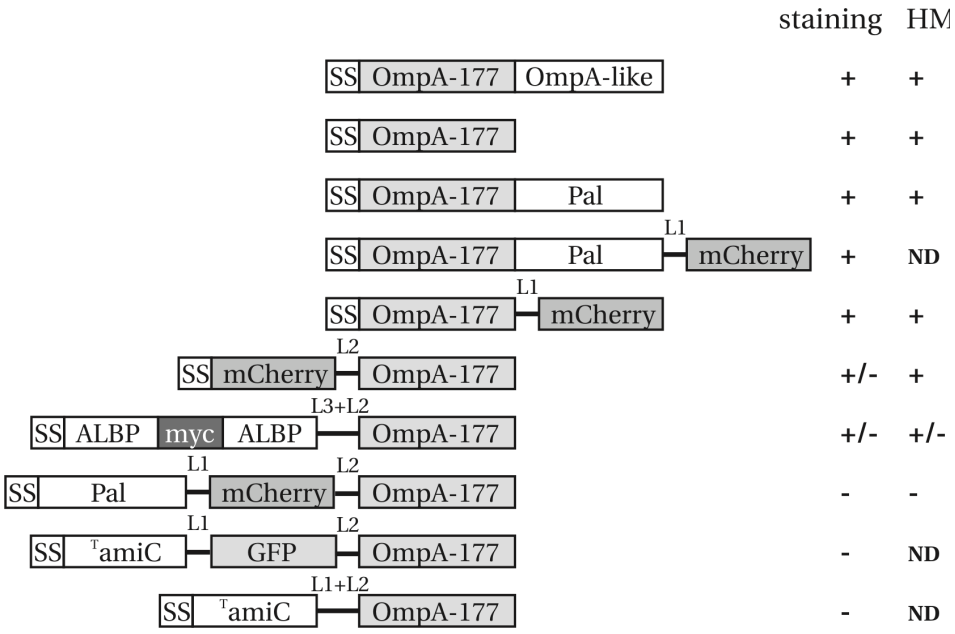


Figure 5.1. All domain fusions to the TM domain of OmpA drawn schematically. SS indicates either the signal sequence of OmpA, DsbA, ALBP, Pal or AmiC, depending on the construct. See **Table II** (Materials and methods) for details. The OmpA-177 TM domain contained either a 3xFLAG or a SA-1 peptide insertion in one of its surface exposed loops. OmpA-like indicates the native periplasmic domain of OmpA. Linker sequences are indicated with L1, L2 and L3 and consist of residues LEDPPAEF, SRAQQ and GSS, respectively. Myc represents the residues (GSST)EQKLISEEDL.

of AmiC (Bernhardt and de Boer 2003). Similar arguments as for Pal can be made for it, with a potential caveat that it is a Tat substrate. However, the Tat system can export fusion proteins, as an AmiC-GFP fusion is fully functional in the periplasm (Bernhardt and de Boer 2003).

We constructed a series of fusion proteins to either N- or C-terminus of the OmpA TM domain (see **Figure 5.1** for a schematic overview of all the constructs). To test the efficiency of OM insertion, OmpA-177 was fused to tandem ALBP domains at its N-terminus (designed to be fused behind the FtsQ protein), and mCherry was fused either to its N- or C-terminus. Next, the mid-cell domains Pal and ^{T}amiC were fused to OmpA-177,

of which Pal to either terminus, and ^{T}amiC only to the N-terminus. Fusions were analyzed using immunoblots, combined with a gel-shift assay (also called heat-modifiability) to test the folding state of the OmpA-177 domain. Furthermore, the epitope in the OmpA-177 was detected on the cell surface by fluorescent labeling. Constructs that carried a mCherry fluorescent protein as part of the domain fusion could be imaged directly, and the localization pattern compared with that of fluorescent staining.

It was found that with either the mCherry or the Pal domain fused to its C-terminus, the OmpA TM domain was able to efficiently insert in the OM. Interestingly, the localization of the (OmpA-177)-Pal fusion was excluded from mid-cell in the presence of wild type Pal, but not in ΔPal background. When a domain was fused to the N-terminus of the OmpA TM domain, non-interacting domains (mCherry, tandem ALBP domains) allowed OM insertion, but with reduced efficiency. However, both for the Pal domain, and the ^{T}amiC domain, no OM incorporation of the OmpA TM domain was observed when fused to the N-terminus of OmpA-177. Instead, these fusions behaved as if the OmpA TM domain was absent. For Pal, the N-terminus, which is lipidated and targeted to the OM by the Lol system, could interfere with OM assembly of the OmpA TM domain. For the targeting domain of AmiC, the Tat system could be incompatible with OM assembly of the OmpA TM domain.

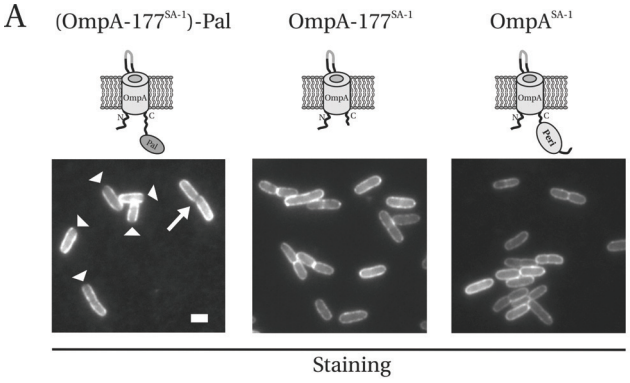
Results

As a starting point we used a construct that consists of the TM domain of OmpA (OmpA-177), either with a streptavidin binding peptide (SA-1) (Bessette et al. 2004) inserted in surface-exposed loop 1 (OmpA-177$^{SA\text{-}1}$) or with a 3xFLAG epitope inserted in surface-exposed loop 3 (OmpA-177^{FLAG}). Both were shown to insert correctly in the OM, although OmpA-177^{FLAG} had reduced protein levels (**Chapter 3**). All constructs were expressed from a IPTG-inducible weakened-pTrc99A vector (Den Blaauwen et al. 2003). It was found that when cells were grown in MOPS glucose defined rich medium (DRu) (Neidhardt et al. 1974), 50-100 μM IPTG was needed to get expression levels similar to the basal expression level when grown in TY medium without IPTG (see Materials & Methods).

In the following, experiments with fusions to OmpA-177^{FLAG} were performed in the wild-type strain LMC500 (MC4100 *lysA*) grown in GB1 minimal medium (defined) or TY medium (undefined). Intact cells were fluorescently labeled with anti-FLAG antibody followed by a fluorescent secondary reporter. Experiments with fusions to OmpA-177$^{SA\text{-}1}$ were performed in wild-type strain MC1061, grown in DRu medium or TY medium. Cells expressing these constructs were labeled directly with fluorescent streptavidin. Both OmpA-177^{FLAG} as well as OmpA-177$^{SA\text{-}1}$, in both genetic backgrounds (LMC500 and MC1061), became homogeneously fluorescent along the perimeter of the cell after fluorescent labeling (**Figure 5.2A**). No essential differences between fusions to either OmpA-177^{FLAG} or OmpA-177$^{SA\text{-}1}$ were observed.

A C-terminal Pal fusion to the OmpA TM domain

An (OmpA-177)-Pal fusion protein was constructed that starts with the signal sequence and TM domain of OmpA, and continues after residue 177 with residues 2-152 of the mature Pal domain (i.e. excluding its signal sequence and the N-terminal lipidated cysteine). At the C-terminus, four residues (LEDP) form part of a linker that allows sub-cloning of the mCherry fluorescent protein (Shaner et al. 2004). Residues 2-39 of Pal are thought to form a unstructured linker region (Cascales and Lloubes 2004). In **Figure 5.1**, all fusions presented in this paper are drawn schematically. Pal Fusions to either OmpA-177^{FLAG} or OmpA-177$^{SA\text{-}1}$ were constructed. Correct expression of the construct was verified on immunoblot for the FLAG fusion in LMC500, and a strong band at 41 kDa was detected (calculated at 39 kDa, but 3xFLAG retards proteins a few kDa) (data not


A
(OmpA-177SA-1)-Pal
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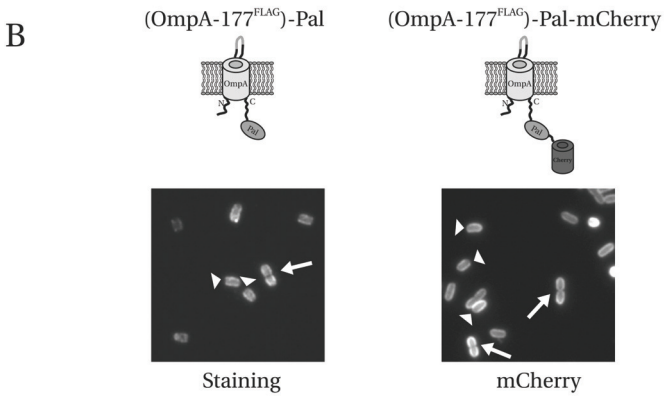
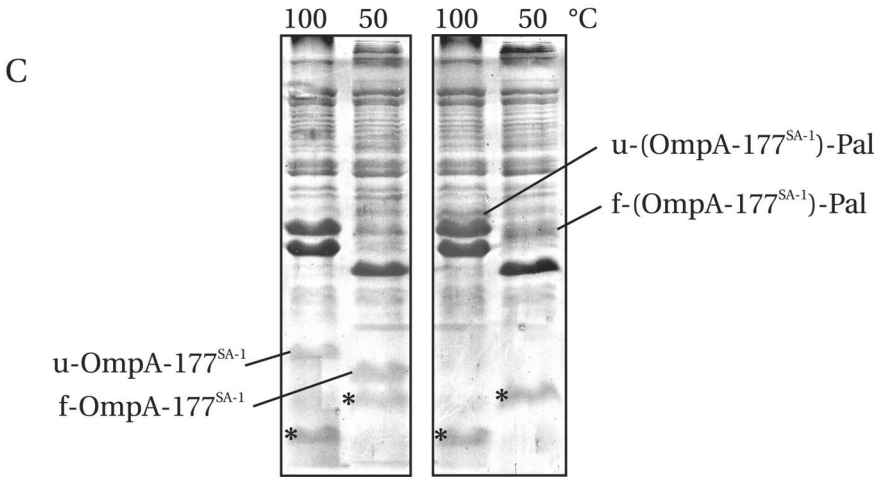

B
(OmpA-177FLAG)-Pal
(OmpA-177FLAG)-Pal-mCherry
OmpA
Pal
mCherry
Staining
mCherry


C
100
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u-(OmpA-177SA-1)-Pal
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Figure 5.2. OmpA-177-Pal fusion inserts in the OM, but does not localize to mid-cell. **(A)** MC1061 cells expressing (OmpA-177^{SA-1})-Pal, OmpA-177^{SA-1} or OmpA^{SA-1} (with linker L1 present at its C-terminus, see Figure 1) were grown to exponential phase in DRu medium at 28°C in the presence of 0.1 mM IPTG to an OD600 of 1.0, and labeled on ice with fluorescent streptavidin for 30 min. The cells were then fixed and imaged. The triangles indicate a reduced fluorescence at one pole of non-dividing cells. The arrow indicates a reduced fluorescence at mid-cell in a dividing cell. **(B)** LMC500 cells expressing (OmpA-177^{FLAG})-Pal were grown to exponential phase in GB1 medium at 28°C in the presence of 0.03 mM IPTG to an OD450 of 0.2 and were labeled with biotinylated anti-FLAG followed by fluorescent streptavidin. The cells were then fixed and imaged. Cells grown under the same conditions expressing (OmpA-177^{FLAG})-Pal-mCherry were fixed and imaged directly. The scale bar is 1 x 2 μm, and exposure time was 470 ms for all images. The triangles indicate a reduced fluorescence at one pole of non-dividing cells. The arrow indicates a reduced fluorescence at mid-cell in a dividing cell.
(C) A Coomassie stained SDS-PAGE gel containing membrane fractions of MC1061 cells expressing either OmpA-177^{SA-1} or (OmpA-177^{SA-1})-Pal grown to exponential phase in DRu medium at 28°C in the presence of 0.1 mM IPTG. Samples were either heated in sample buffer at 99°C for 10 minutes or heated at 50°C for 15 minutes, as indicated. The asterisk indicates an unidentified heat-modifiable band present in both samples. Folded and unfolded β-barrels are indicated with f- and u-, respectively.

shown). Heat modifiability experiments (Verhoeven et al. 2008) of a Coomassie blue stained membrane fraction of cells expressing the OmpA-177^{SA1}-Pal fusion demonstrate that the construct is intact (at 38 kDa, equal to its calculated kDa) and heat-modifiable, and therefore present in the OM, consistent with its detection on the cell surface (**Figure 5.2C**).

(OmpA-177)-Pal in wild-type cells is excluded from mid-cell

The constructs were first studied in a wild-type background (*Pal+*). Surprisingly, the addition of the Pal domain leads to the exclusion of the OmpA TM domain from mid-cell, and from (what is most likely) the newly synthesized poles of newborn cells (**Figure 5.2A**). In contrast, labeling of both a strain expressing the OmpA TM domain without Pal, and a strain expressing the full-length OmpA protein, resulted in homogeneous staining patterns with fluorescence along the cell perimeter. In addition, constricting cells even showed a slightly *increased* fluorescence at mid-cell (approximately a factor of 2, as the intensity increase along two cells lying adjacent is similar, thus most likely caused by the close proximity of two OM's during division).

To exclude a possible artifact of the staining procedure (e.g. further cell growth after

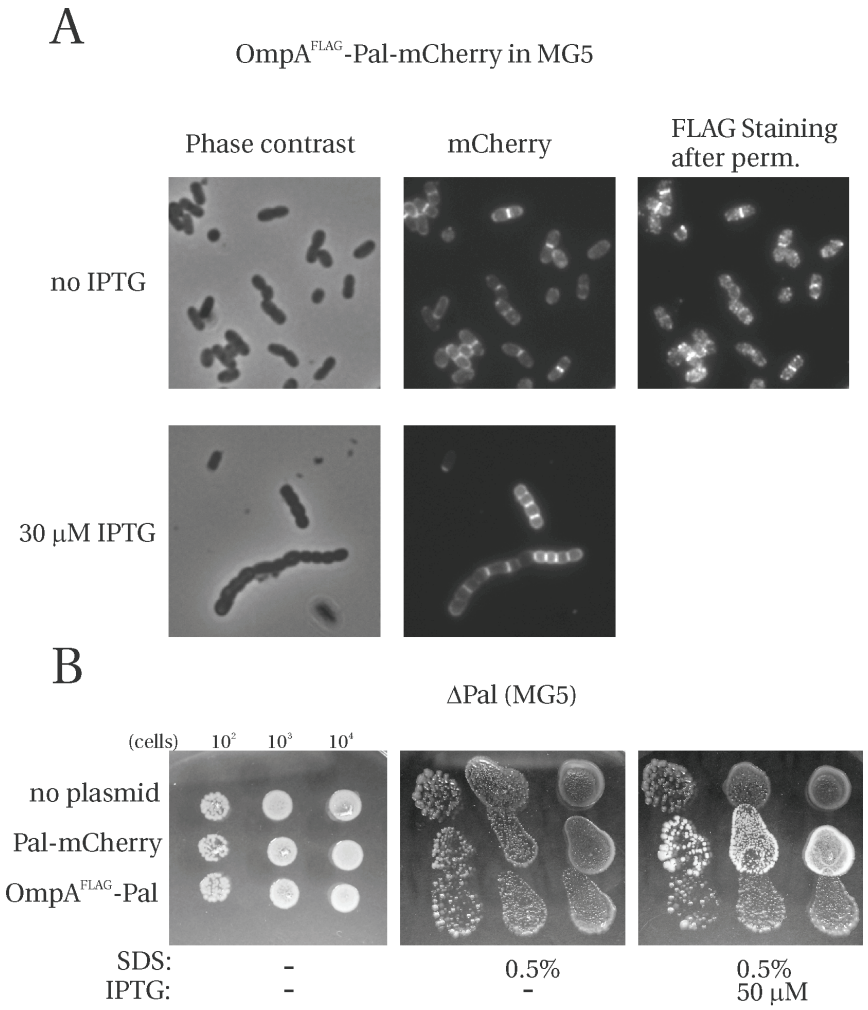

Figure 5.3. OmpA-177^{FLAG}-Pal-mCherry is localized at mid-cell in a ΔPal strain, but not exposed. Cells were grown to exponential phase in GB1 medium at 28°C, fixed, permeabilized, immunolabeled and imaged ("no IPTG") or fixed and imaged directly ("30 μM IPTG"). (A) Exposure times were: mCherry 1 s, FLAG staining (Alexa-488-conjugated antibodies) 330 ms (no induction) and mCherry, 150 ms (30 μM IPTG). (B) SDS hypersensitivity assay. The SDS sensitivity of MG5 (ΔPal) is alleviated by expressing proPal-mCherry, but not OmpA-177^{FLAG}-Pal. The difference became clearer after prolonged incubation at room temperature, therefore the SDS plates were imaged after one week at RT.

washing away the fluorescent probe), we constructed a triple fusion by addition of

mCherry to the C-terminus of Pal, which allows direct visualization of the observed sub-localization pattern. Continuous expression with 50 μM IPTG of the (OmpA-177^{FLAG})-Pal fusion had no effect on growth rate in LMC500. The triple fusion (OmpA-177^{FLAG})-Pal-mCherry was toxic for IPTG concentrations exceeding 30-50 μM. Induction of this triple fusion gave a similar fluorescent mCherry pattern as observed with labeling against the FLAG tag of a fusion without mCherry (**Figure 5.2B**). As expected, staining of the triple fusion against the FLAG tag gave a pattern that co-localized with mCherry fluorescence (data not shown). Therefore, we conclude that the exclusion of (OmpA-177)-Pal from the division site is not an artifact of the staining procedure.

Localization of (OmpA-177)-Pal in ΔPal cells

We reasoned that the OmpA-Pal fusion could not compete with wild-type Pal for septal localization, and therefore also expressed the fusion in a ΔPal strain (MG5). ΔPal cells have an increased sensitivity to detergents such as SDS (Gerding et al. 2007). These authors showed that IPTG-induced expression of a Pal-mCherry fusion from plasmid was able to rescue this hypersensitivity, allowing growth on plates containing 0.5% SDS. As partial induction of (OmpA-177^{FLAG})-Pal (50 μM IPTG) in a wild-type background did not interfere with cell growth, we asked whether it was able to complement the Pal deletion in a similar fashion as Pal-mCherry did. OmpA^{FLAG}-Pal did not complement the ΔPal strain in this assay (**Figure 5.3B**).

Both (OmpA-177^{FLAG})-Pal as well as the triple fusion (OmpA-177^{FLAG})-Pal-mCherry were toxic when grown in the continuous presence of 30 μM IPTG in GB1 medium, and in the case of (OmpA-177^{FLAG})-Pal-mCherry lead to filaments consisting of cells with multiple stalled constrictions at very short intervals, as if length growth was also affected (**Figure 5.3A**). Apparently, in the absence of wild-type Pal, this fusion inhibits completion of constriction, but allows the formation of new constrictions.

When ΔPal cells expressing the (OmpA-177^{FLAG})-Pal-mCherry fusion were grown without induction, mCherry was clearly localized to mid-cell in constricting cells (**Figure 5.3A**). As reported before for ΔPal cells expressing a Tat-exported periplasmic GFP protein (Gerding et al. 2007), fluorescent vesicles accumulated in the medium, and cells had fluorescent blebs (data not shown). Without induction, immunolabeled ΔPal cells expressing OmpA^{FLAG}-Pal (with or without mCherry) presented only a few FLAG spots on the cell surface (data not shown). However, after fixation and permeabilization, a clear

FLAG signal at mid-cell was detected in constricting cells (**Figure 5.3A**, and data not shown). This signal was absent in fixed and permeabilized ΔPal cells without plasmid, where only a homogeneous aspecific background was observed after labeling with anti-FLAG (data not shown). This suggests that OmpA-177^{FLAG} is present at mid-cell, but in the periplasm and not in the OM, as permeabilization of the outer membrane was required for the antibodies to reach the FLAG epitope.

Although as mentioned, continuous growth of the triple fusion in the presence of 30 μM IPTG was toxic, we added 30 μM IPTG to an exponentially growing culture of cells expressing (OmpA-177^{FLAG})-Pal-mCherry, and after 3 hours of induction, labeled living cells with biotinylated anti-FLAG. Again, constricting cells have a strong mCherry band at mid-cell. Instead of a few FLAG spots, at these higher expression levels, staining has become homogeneous along the perimeter of the cell. In this sample, that had not been permeabilized, lysed cells were present at a low frequency (<1%), and only these cells had in addition to a mCherry band also a co-localized FLAG band at mid-cell. This suggests that next to OmpA-177^{FLAG} detected homogeneously on the cell surface, OmpA-177^{FLAG} is also present at mid-cell, but in the periplasm and not in the OM, as only in (partially) lysed cells the antibody could reach the FLAG epitope.

These observations suggest that in these cells, (at least) two populations of (OmpA-177)-Pal were present: (i) (OmpA-177)-Pal that is incorporated randomly in the OM, without any preference for mid-cell, and (ii) (OmpA-177)-Pal that is localized at mid-cell, but is not incorporated in the OM (perhaps due to OM blebbing). We conclude that the Pal domain cannot specifically target OM incorporated OmpA-177 to the site of constriction, presumably because it can neither compete (in wild-type cells) nor replace (in ΔPal cells) wild-type Pal.

A C-terminal mCherry fusion to the OmpA TM domain

We decided to explore the possibilities of using mCherry as a fluorescent protein domain to study OM assembly of OmpA-177 with heterologous domains attached. Furthermore, as mCherry is not expected to interact with endogenous proteins, and becomes fluorescent in the periplasm, it is also an attractive candidate to function as a spacer domain in mid-cell localization fusion constructs. As we already knew that the OmpA TM domain could insert when Pal was fused to its C-terminus, we started by replacing Pal with mCherry. Using dual color fluorescence microscopy imaging, we find that the fusion is exported to the

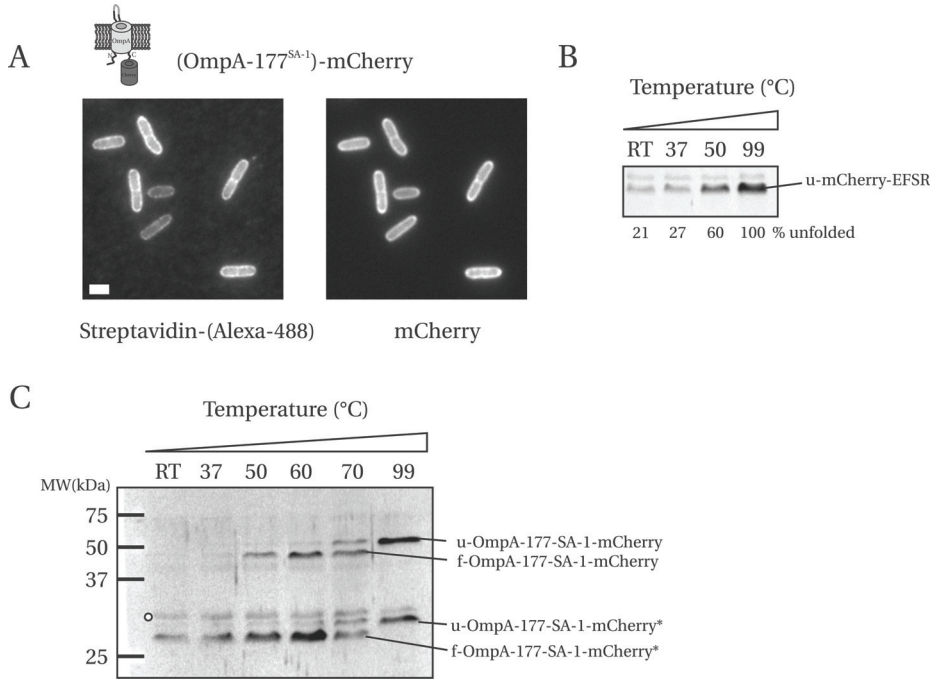

Figure 5.4. Outer membrane assembly of a fusion between the C-terminus of the OmpA TM domain and mCherry. A) Cells grown to exponential phase in DRu medium with 0.1 mM IPTG were labeled with fluorescent streptavidin. Exposure times are as indicated (contrast is enhanced). Scale bar is 1 x 2 μm. B) Heat-modifiability of mCherry-EFSR. Sonicated cell lysate of LMC500 expressing mCherry-EFSR was resuspended in sample buffer and either; not heated (RT), heated at 37°C for 5 min, heated at 50°C for 15 min, or heated at 99°C for 10 min. Shown is an immunoblot probed with anti-DsRed antibody. The faint band present in each lane is aspecific. The unfolded (denatured) mCherry-EFSR band is indicated. Percentage of unfolded mCherry-EFSR are indicated, assuming that after heating at 99°C all protein is unfolded. C) Heat-modifiability of OmpA-177-SA-1-mCherry. Cells from the same culture used for labeling in A) were sonicated and resuspended in sample buffer. Heat treatment as in B), heating at 60°C and 70°C was for 15 min. The folded and unfolded forms of both the intact fusion and the degradation product are indicated by a preceding f- or u-, respectively.

periplasm, demonstrated by the red mCherry fluorescence along the perimeter of the cell (**Figure 5.4A**). Furthermore, we find that the OmpA TM domain is correctly inserted into the outer membrane, demonstrated by the green fluorescent staining of the SA-1 peptide on the cell surface.

To exclude that in the periplasm, the two-domain fusion was proteolytically degraded

into separate domains, heat-modifiability (gel-shift) experiments were performed. First, we checked for a possible heat-modifiability of mCherry, as it also has a β-barrel fold. To this end, we grew cells expressing a cytoplasmic mCherry, lysed them by sonication, and after varying heat treatment, subjected the samples to SDS-PAGE. We find that after heating at 37°C for 5 min in sample buffer (a condition under which GFP retains its fluorescence in-gel (Drew et al. 2006)), in-gel fluorescence of mCherry could be detected (see M&M), but fluorescence was approximately halved after heating at 50°C for 15 min (data not shown). Furthermore, mCherry could be detected on immunoblot using a monoclonal anti-DsRed antibody (**Figure 5.4B**). A band of the expected height (27 kDa) was present that increased in intensity upon heating (the faint band above it was also present in lysate without mCherry). The inverse relation between in-gel fluorescence and detection on immunoblot indicated that the anti-DsRed antibody only recognizes the denatured conformation of mCherry. We conclude that the mCherry β-barrel fold is less stable than that of the OmpA TM domain, because under conditions where the OmpA TM domain is completely stable (50°C for 15 min), the mCherry β-barrel clearly is not (**Figure 5.4B**). Therefore, the anti-DsRed can be used to determine the folding state of the OmpA TM domain, because only denatured mCherry will be visible, and any gel-shifts observed can then be unequivocally attributed to the OmpA TM domain.

To test the heat-modifiability of the OmpA-$177^{SA\text{-}1}$-mCherry fusion, an immunoblot containing cell lysates heated at different temperatures was probed with anti-DsRed (shown in **Figure 5.4C**). In the following, we assume that already at RT, the (folded) OmpA-177 fusion protein, present in the membrane fraction, is solubilized completely in SDS-PAGE sample buffer. At RT and 37°C, only a degradation band at 26 kDa was detected. At 50°C, mCherry starts to unfold, and the fusion becomes visible on blot. From literature and our own experiments, we know that the folded OmpA TM domain does not unfold at all under these conditions. Increasing the temperature further from 50°C to 99°C, the OmpA TM domain unfolds and the fusion shifts to its expected molecular weight of 49 kDa. These results demonstrate that the OmpA TM domain remains heat-modifiable and therefore is correctly assembled into the OM when mCherry is fused to its C-terminus.

With increasing heat-treatment, the degradation band increased in intensity, suggesting that fluorescent mCherry was present in the lysate. Plasmolysis experiments in which the (periplasmic) plasmolysis bays contain fluorescent mCherry (data not shown)

support this. Surprisingly, this degradation band contained some highly stable secondary structure with a half-life of 15 min at 70°C. After boiling this band shifted to 28 kDa. As denatured cytoplasmic mCherry (with 4 extra linker residues at its C-terminus) migrated at 27 kDa, and if we assume that the N-terminus of mCherry remained intact, then at most some 10-15 additional residues are present. This would be comprised of the LEDPPAEF linker, with only the very last C-terminal residues of OmpA-177. As there is no expected tertiary structure present in these residues, the gel-shift is difficult to explain. However, the very C-terminal end of the OmpA TM domain has recently been found to be covalently modified with oligo-(R)-3-hydroxybutyrate (Xian et al. 2007). A possible explanation could be that this modification is somehow responsible for the gel-shift. Alternatively, the degradation product is degraded on both termini, with the antibody epitope still present, and still able to partially fold to shield the antibody epitope at lower temperatures (needed to explain the increase in band intensity with increasing temperature), but with a larger portion C-terminal OmpA residues present, which tertiary structure (β-strands) unfolds upon heating.

We conclude that cells expressing OmpA-177-SA-1-mCherry contain a mixture of intact fusion assembled in the OM, and proteolytically removed, mostly fluorescent mCherry.

An N-terminal mCherry fusion to the OmpA TM domain

Having established that mCherry was suitable for our purpose, we decided to fuse mCherry to the N-terminus of the OmpA TM domain. This requires that mCherry is sandwiched between OmpA and its signal peptide. As a first step, the OmpA signal sequence was cloned upstream of mCherry and its sub-cellular localization was compared to mCherry without signal sequence. Exponentially growing cells in TY medium were fixed and imaged (**Figure 5.5A**). It is clear that introduction of the OmpA signal sequence causes an increase of mCherry at the perimeter of the cells, something one would expect if only a fraction of mCherry has been exported to the periplasm. Line profiles normal to the cell's long axis confirm this visual impression. We verified that observed cell diameters in phase contrast were equal for both strains (~ 1.1 μm).

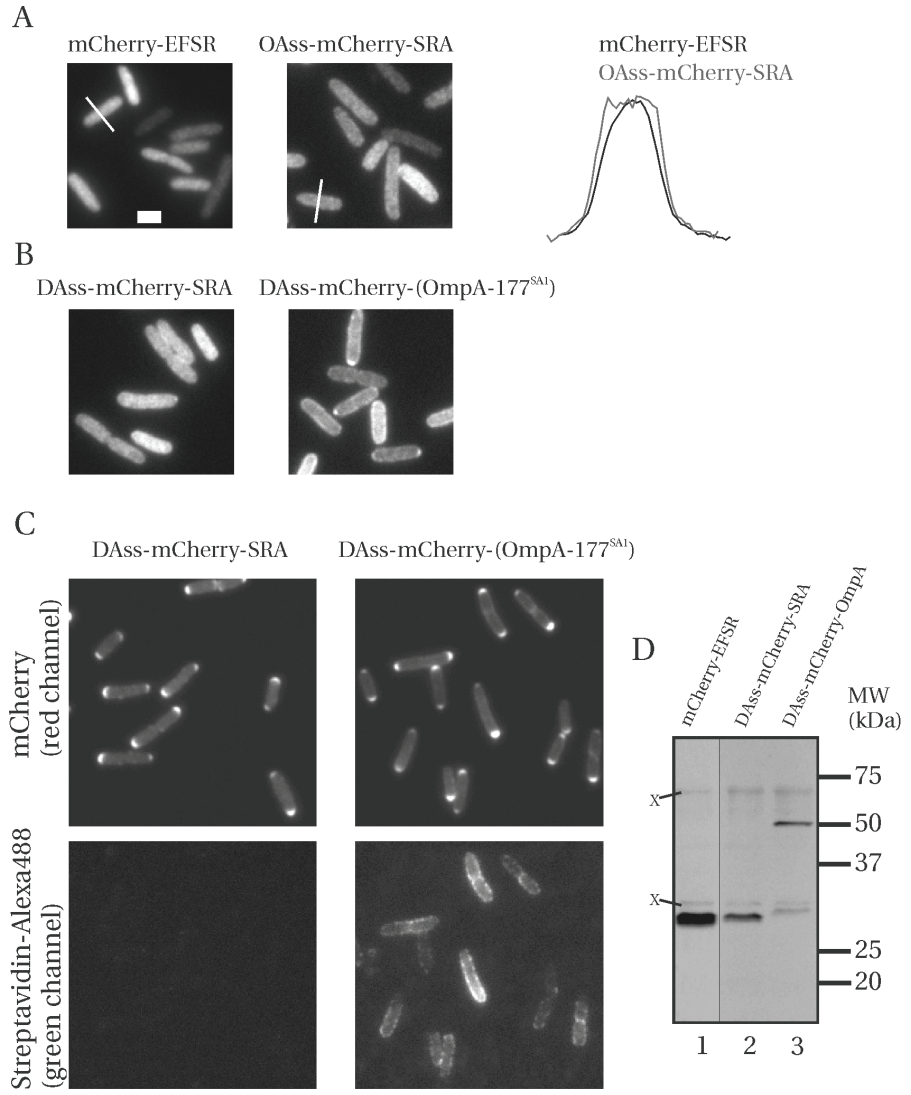

Figure 5.5. Periplasmic export of mCherry and a fusion of mCherry attached to the N-terminus of the OmpA TM domain. **(A)** Cells grown to exponential phase in TY medium at 28°C expressing either cytoplasmic mCherry (with C-terminal linker residues EFSR) or OmpAss-mCherry-SRA. Exposure time was 470 ms. The scale bar is 1 x 2 μm. Representative line profiles through cells are depicted, black line: mCherry-EFSR, gray line: OmpAss-mCherry-SRA. **(B)** Cells expressing either DsbAss-mCherry-SRA or DsbAss-mCherry-(OmpA-177^{SA-1}). Exposure time was 470 ms. **(C)** Cells grown to exponential phase in DRu medium with 0.1 mM IPTG at 28°C expressing either DsbAss-

mCherry-SRA or DsbAss-mCherry-(OmpA-177^{SA-1}). Exposure times: 100 ms (mCherry), 1.5 s (streptavidin-Alexa-488). Both sets of images received the same contrast enhancements allowing comparison of intensities. **(D)** Immunoblot probed with anti-DsRed (1:500). Two aspecific bands are indicated with X. Lane 1: Sonicated lysate of LMC500 cells expressing mCherry-EFSR were grown in TY at 37°C and induced with 0.1 mM IPTG for 2 hours. Lane 2: MC1061 cells expressing DsbAss-mCherry grown in DRu at 28°C with 0.1 mM IPTG. Cells were resuspended in Sonication buffer, frozen, thawed and solubilized in sample buffer. Lane 3: MC1061 cells expressing DsbAss-mCherry-(OmpA-177^{SA-1}).

A possible explanation for the observed inefficient export could be the rapid folding of mCherry in the cytoplasm, combined with the inability of the Sec translocase to unfold it again, possibly due to (covalent) chromophore formation. If this would be the case, a signal sequence that directs mCherry to the *co*-translational SRP pathway could increase export efficiency, as was found for the cytoplasmic protein thioredoxin 1 fused to the DsbA signal sequence (Schierle et al. 2003). Unexpectedly, after replacing the post-translational OmpA signal sequence by the co-translational DsbA signal sequence, export of mCherry to the periplasm did not improve (**Figure 5.5B, left panel**).

Finally, we fused the TM domain of OmpA downstream of DsbAss-mCherry. Surprisingly, this did improve the amount of fluorescent mCherry observed along the perimeter (**Figure 5.5B, right panel**). Maybe the OmpA domain improves translocation, perhaps by reducing the folding rate of mCherry. Less likely, fluorescent mCherry in the cytoplasm becomes associated with the IM via the unfolded SecB-stabilized OmpA peptide moiety.

In TY medium, a mild polar mCherry localization was observed (**Figure 5.5B, right panel**), which increased with increasing expression levels, obtained by growth in DRu medium supplemented with 0.1 mM IPTG (**Figure 5.5C**). To test whether OmpA was able to assemble in the OM, cells were labeled with green fluorescent streptavidin. Dual color fluorescence imaging revealed that the TM domain of OmpA was indeed present in the OM, as a homogeneous staining along the perimeter of the cells was observed. This staining was absent for cells expressing DsbAss-mCherry without the OmpA domain (**Figure 5.5C**). As no increase in staining was observed at the poles, we conclude that apparently, (at least) two distinct populations are present, one that assembles normally in the OM, and one that does not, but accumulates in the cell poles instead.

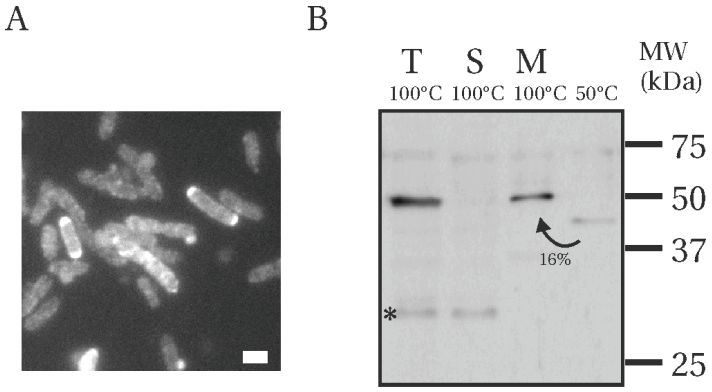

Figure 5.6. DsbAss-mCherry-(OmpA-177^{SA-1}) is heat-modifiable. MC1061 ΔOmpA cells were grown to exponential phase in TY medium and expression was induced with 30 μM IPTG and induced for 1 hour. When the cells had reached OD600~1.0, **(A)** a sample was fixed in 0.25% formaldehyde and imaged. The scale bar is 1 x 2 μm. **(B)** The rest of the cells were sonicated on ice, and total lysate (T) was fractionated in soluble (S) and insoluble (membrane, M) fractions (see M&M). Samples were either heated to 99°C for 10 minutes or to 50°C for 15 minutes, subjected to SDS-PAGE and immunoblotted with anti-DsRed antibody (1:500). The asterisk indicates a degradation product at ~28 kDa.

Immunoblots probed with anti-DsRed (**Figure 5.5D**) revealed that under the growth conditions of **Figure 5.5C**, DsbAss-mCherry migrated as a single band, at the exact same height as mCherry-EFSR (27 kDa). This suggests that DsbAss-mCherry is fully processed, and the accumulation in the poles takes place in the periplasm. The DsbAss-mCherry-OmpA fusion was detected predominantly as intact fusion at 48-50 kDa, with a minor degradation band at 28 kDa.

Finally, it was verified that the fusion detected on the cell surface was intact by heat-modifiability experiments. Cells induced for expression of the DsbAss-mCherry-OmpA fusion were fractionated into soluble and insoluble (membrane) fractions. The membrane fraction was either completely denatured (heating at 99°C for 10 minutes) or heated to 50°C for 15 min. It was found that the intact fusion was heat-modifiable (**Figure 5.6**), suggesting that the OmpA TM domain was properly incorporated in the OM. At first sight, this seems strange, as the fluorescent mCherry in the poles was not detected on the cell surface. However, only denatured mCherry can be detected with the antibody used. Quantifying the relative intensities of detected mCherry on blot at 50°C and at 99°C, we

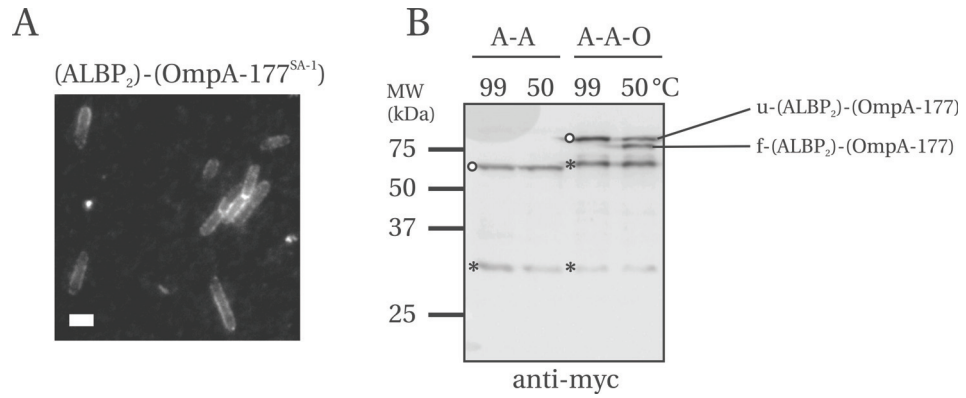

Figure 5.7. A fusion of two ALBP domains to the N-terminus of the OmpA TM domain can insert properly in the OM. **(A)** Cells grown to exponential phase in DRu medium with 0.1 mM IPTG expressing proALBP-myc-ALBP-(OmpA-177^{SA-1}) were labeled with fluorescent streptavidin. Exposure time was 1 s (contrast is enhanced). Scale bar is 1 x 2 μm. **(B)** Immunoblot probed with anti-myc (1 μg/ml). A-A: pro-ALBP-myc-ALBP (expected MW: 63 kDa). A-A-O: A-A fused to the N-terminus of (OmpA-177^{SA-1}) (expected MW: 87 kDa). Samples were resuspended in sample buffer and either heated at 99°C for 10 min or heated at 50°C for 15 min, as indicated. Intact constructs are indicated with an open circle, degradation products with an asterisk. u- indicates unfolded, f- indicates folded.

find that only 16% of all mCherry in the sample is detected at 50°C (whereas cytoplasmic mCherry is 60% denatured under these conditions). The fluorescent aggregates in the poles might not dissociate and denature at this temperature and escape detection.

Thus, we conclude that a Sec-exported, N-terminal mCherry fusion to the OmpA TM domain can be assembled into the OM. However, in contrast with cells expressing a C-terminal OmpA TM domain - mCherry fusion, mCherry fluorescence can accumulate in the poles, depending on growth and induction conditions.

An N-terminal $ALBP_2$ fusion to the OmpA TM domain

Before cloning the spacer construct between FtsQ and the OmpA TM domain, we tested also specifically whether a construct consisting of two ALBP spacer domains in tandem, fused to the N-terminus of OmpA-177^{SA1} is able to insert into the OM. To this end we made a proALBP-myc-ALBP-(OmpA-177^{SA1}) triple domain fusion protein (from here on abbreviated to $(ALBP)_2$-OmpA-177^{SA1}). To determine whether the OmpA TM domain of

this construct was able to insert in the OM, MC1061 cells expressing the $(ALBP)_2$-OmpA-177^{SA1} construct were labeled with fluorescent streptavidin. A staining along the perimeter of the cells showed that the OmpA TM domain was exported successfully and had inserted into the OM (**Figure 5.7A**). However, the fluorescent signal was significantly weaker than obtained for the unfused OmpA TM domain. This could be due to reduced protein levels of the ALBP tandem itself, as addition of the OmpA TM domain does not influence protein levels significantly, as detected with anti-myc (**Figure 5.7B**).

To determine whether the OmpA TM domain detected in the membrane was intact (i.e. with two ALBP domains attached to its N-terminus), heat-modifiability experiments were performed and the constructs were detected on immunoblot via their myc tag between the two ALBP domains (**Figure 5.7B**). Both intact and partially degraded construct were detected, and approximately half of the intact construct shifted position in the gel, indicative of a properly folded and inserted OmpA TM domain. As a control, it was checked that the Sec exported ALBP-myc-ALBP tandem lacking the OmpA TM domain did not show heat-modifiable behavior under the conditions used. Taken together, these data demonstrate that an artificial two-domain N-terminal fusion to the OmpA TM domain can be assembled in the OM.

An N-terminal Pal-mCherry fusion to the OmpA TM domain

It has been shown that a Pal-mCherry fusion protein localized to mid-cell in both wild type (*Pal*+) background as well as in a ΔPal strain (MG5) (Gerding et al. 2007). Furthermore, in the ΔPal strain, induction of Pal-mCherry with 50-100 μM IPTG from a pMLB1113 based vector (Zagursky and Berman 1984; de Boer et al. 1989), resulted in complementation of all phenotypes of a *Pal* deletion tested: Alleviation of SDS hypersensitivity, a "short-and-round" morphology, and a salt-sensitive cell chaining phenotype (Gerding et al. 2007).

To facilitate C-terminal addition of the OmpA TM domain, we cloned the Pal-mCherry into our weakened pTrc vector with a C-terminal *XmaI* site that introduced three extra amino acids (Pal-mCherry-SRA). Growth in TY medium resulted in the expected envelope and mid-cell localization of this construct in both a wild type (LMC500, *Pal*+) background as well as in a ΔPal strain (MG5) (**Figure 5.8A**).

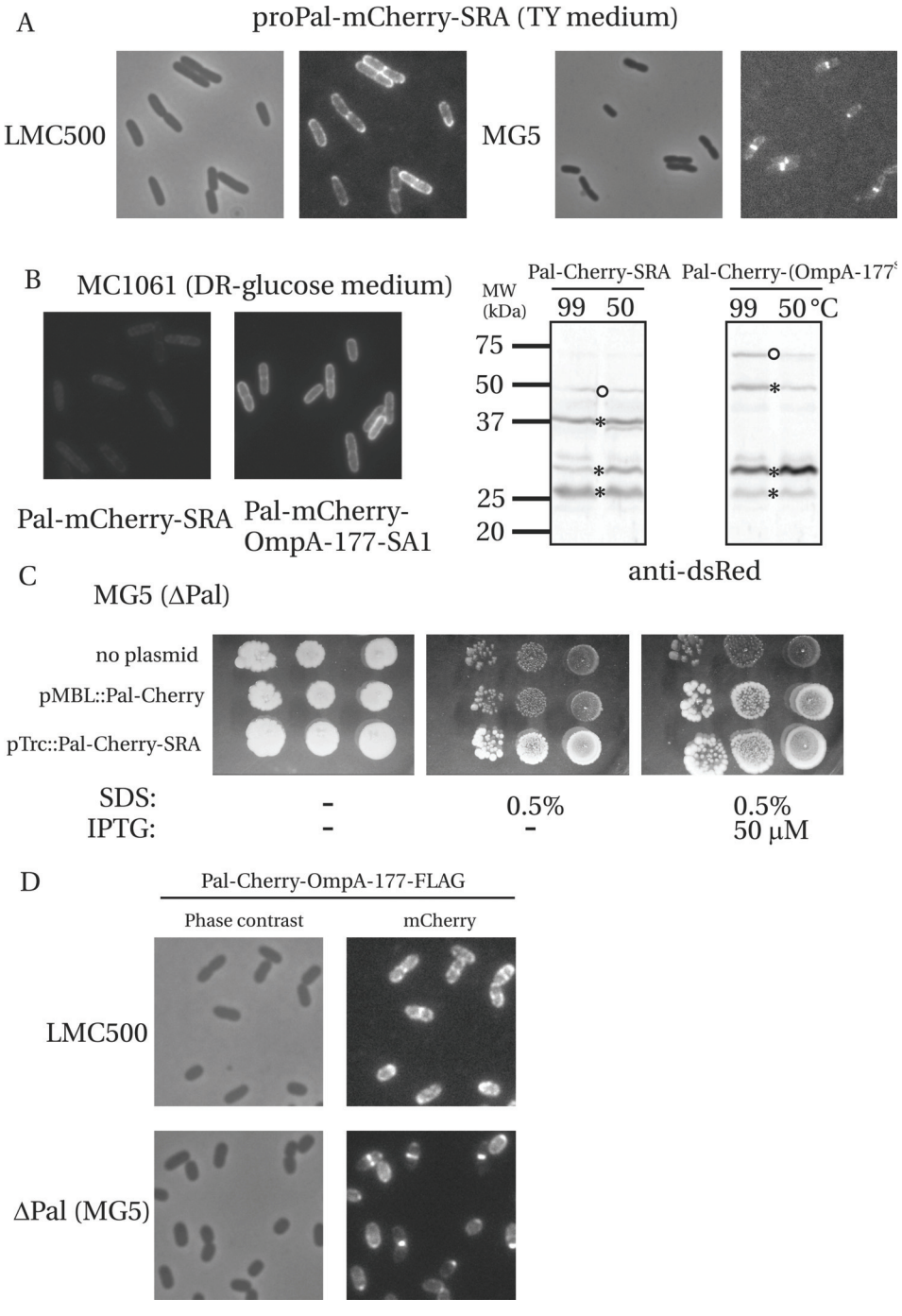
A
proPal-mCherry-SRA (TY medium)
LMC500
MG5
B
MC1061 (DR-glucose medium)
Pal-mCherry-SRA
Pal-mCherry-OmpA-177-SA1
Pal-Cherry-SRA
Pal-Cherry-(OmpA-177
MW (kDa)
99 50
99 50 °C
75
50
37
25
20
anti-dsRed
C
MG5 (ΔPal)
no plasmid
pMBL::Pal-Cherry
pTrc::Pal-Cherry-SRA
SDS: - 0.5% 0.5%
IPTG: - - 50 μM
D
Pal-Cherry-OmpA-177-FLAG
Phase contrast
mCherry
LMC500
ΔPal (MG5)

Figure 5.8. **proPal-mCherry with the OmpA TM domain fused to its C-terminus**. (Pal-) mCherry still localizes to mid-cell, but OmpA is most likely (proteolytically) removed from it. (**A**) LMC500 cells expressing proPal-mCherry-SRA were diluted from o.n. culture and grown to exponential phase in TY medium at 28°C. (Exposure time was 1.5 s, contrast is maximized). right panel: MG5 cells expressing proPal-mCherry-SRA grown in TY medium. (**B**) Immunoblot and fluorescence microscopy MC1061 cells expressing Pal-mCherry-SRA and Pal-mCherry-(OmpA-177^{SA-1}). O.n. cultures grown in DRu medium at 28°C were diluted 500x in fresh DRu medium supplemented with 100 μM IPTG and grown to exponential phase (OD600=1.0). Samples for immunoblotting were taken, before cells were labeled with fluorescent streptavidin (data not shown), fixed and imaged. Exposure time was 2.2 s for both images. Both microscopy images and blots can be compared directly as the same image processing steps were applied to both samples. (**C**) SDS hypersensitivity assay. The SDS hypersensitivity of MG5 (ΔPal) is alleviated by expressing either proPal-mCherry from a pMBL1113 vector (needs IPTG), or proPal-mCherry-SRA from a weakened-pTrc99A vector (no need for IPTG). The complementation effect became clearer after prolonged incubation at room temperature, therefore the SDS plates were imaged after four days at RT. (**D**) Both LMC500 and MG5 cells expressing proPal-mCherry-(OmpA-177^{FLAG}) were grown to pseudo-steady state in GB1 medium at 28°C, fixed and imaged. (Growth details: Cells were diluted 20 000 times from an o.n. GB1 culture grown at 28°C into fresh GB1 supplemented with 10 μM IPTG and harvested when the OD450~0.3). Exposure times were 1 s and 0.47 s for LMC500 and MG5, respectively. Contrast was maximized for each image.

Surprisingly, fluorescence was strongly reduced when Pal-mCherry-SRA expressing cells were grown in both poor and rich defined media: hardly any fluorescence could be detected both after growth to pseudo-steady state in GB1 minimal medium in the presence of 50 μM IPTG, as well as after growth in DRu defined rich medium in the presence of 100 μM IPTG (data not shown). This effect was observed in all three strains used here (MG5, LMC500 and MC1061). An immunoblot probed with anti-DsRed revealed that in MC1061 cells grown in defined rich medium, the Pal-mCherry-SRA protein (expected: 45 kDa, observed 47 kDa) was mostly degraded (main products at 38, 28 and 26 kDa), explaining the low mCherry signal observed with microscopy (**Figure 5.8B**).

In contrast, for the original Pal-mCherry in the pMLB1113 vector (Gerding et al. 2007) in both ΔPal (MG5) and *Pal*+ (LMC500) strains, localization in minimal medium was similar to that observed in TY medium (data not shown). After sub-cloning of the mCherry-SRA fragment into this vector, the “loss of fluorescence” effect was transferred along with it, pinpointing its origin to the three additional C-terminal residues SRA. We are forced to conclude that addition of the these additional residues at the C-terminus makes

the fusion susceptible to proteolytic degradation, depending on the differential expression of proteases and/or chaperones under different growth conditions (see below).

As localization in TY medium was unaffected, we tested functionality of the Pal-mCherry-SRA fusion construct for SDS hypersensitivity complementation. Due to a difference in expression levels of the Pal-mCherry fusions by the promoter on the pMBL1113 and weakened-pTrc99A-based plasmids, complementation occurred already without IPTG, as well as in the presence of 50 μM IPTG (**Figure 5.8C**).

After verifying that our Pal-mCherry-SRA construct was functional, the TM domain of OmpA was fused to the C-terminus of mCherry. mCherry can now be thought of as a linker domain between Pal and the OmpA TM domain. Both fusions with OmpA-177^{FLAG} and OmpA-177^{SA-1} were constructed. Surprisingly, after the addition of the OmpA TM domain, fluorescence in both poor and rich defined media was restored (shown in **Figure 5.8B** for defined rich glucose medium). mCherry localization for the Pal-mCherry-(OmpA-177) fusion was similar to that of Pal-mCherry without the OmpA TM domain (compare **Figure 5.8A** with **5.8D**). Note that in the presence of wild-type Pal (**Figure 5.8B**, also observed for 50 μM IPTG induction in TY medium (data not shown)), (too) high expression levels of fusion protein apparently can obscure the mid-cell localization.

However, on immunoblots of this fusion, either with a SA-1 or FLAG tag, cells contain, next to intact fusion protein, various degradation products (**Figure 5.8B**). Since some intact fusion was detected on immunoblot, and mCherry showed mid-cell localization, we asked whether the OmpA TM domain was sub-localized to mid-cell too. We failed to convincingly detect either the FLAG epitope or the SA-1 epitope on the cell surface after labeling with anti-FLAG or streptavidin, respectively (data not shown). Even permeabilization of cells followed by anti-FLAG staining did not result in a fluorescent staining pattern that co-localized with the mCherry protein (data not shown), as could be observed for the (OmpA-177)-Pal-mCherry fusion (see above). This suggests that the majority of fluorescent mCherry at mid-cell had its OmpA TM domain proteolytically removed. The immunoblot of the triple fusion with the SA-1 epitope in MC1061 provides some support in this direction, as a prominent band is detected at 48 kDa that could correspond to such a degradation product (**Figure 5.8B**).

Finally, as might be expected, for the fusion with the SA-1 epitope in MC1061, none of the mCherry bands, including the intact fusion, showed heat-modifiable behavior,

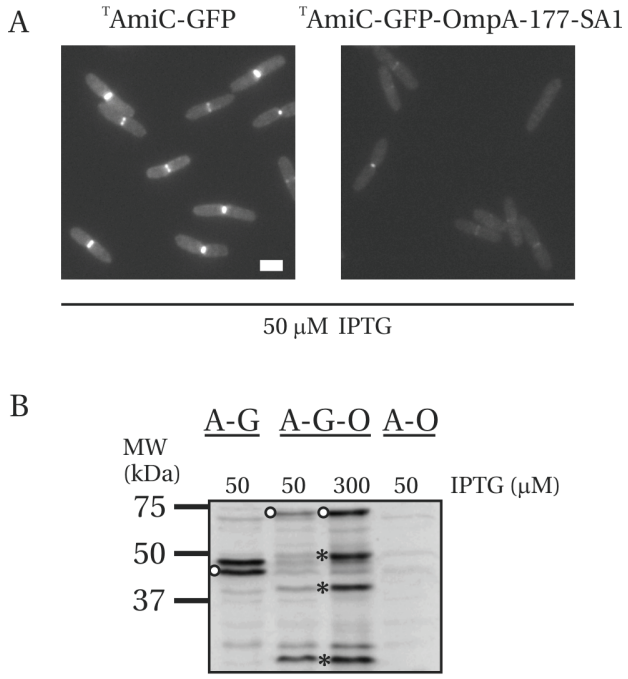

Figure 5.9. Fusions between the septal targeting domain of AmiC and the TM domain of OmpA. **(A)** Cells grown to exponential phase in DRu medium with 50 μM IPTG expressing either ^{T}amiC-GFP or ^{T}amiC-GFP-(OmpA-177^{SA-1}). Exposure time was 1 s. Both images received the same contrast adjustments. Scale bar is 1 x 2 μm. **(B)** Immunoblot probed with anti-GFP (1:1000). A-G: ^{T}amiC-GFP. A-G-O: ^{T}amiC-GFP-(OmpA-177^{SA-1}), A-O: ^{T}amiC-(OmpA-177^{SA-1}). Intact constructs are indicated with an open circle, (main) degradation products with an asterisk.

suggesting that the OmpA TM domain, at least the fraction that had some portion of mCherry attached, was not properly folded (**Figure 5.8B**). We conclude that the OmpA TM domain, when fused to the C-terminus of a Pal-mCherry fusion, is not able to properly insert into the OM and is (therefore?) very susceptible to degradation.

An N-terminal ^{T}amiC fusion to the OmpA TM domain

Finally, we took a gamble and fused a Tat-exported substrate to the N-terminus of OmpA-177. It has been shown that preAmiC consists of three domains: a Tat signal sequence, a septal targeting domain (^{T}amiC) and a catalytic domain (^{C}amiC) (Bernhardt and de Boer 2003). In this paper, it was shown that removal of the catalytic domain leaves the mid-cell

localization in dividing cells unaffected, demonstrated by a ^{T}amiC-GFP (GFPmut2) fusion protein. We have re-created this construct in our own expression vector. Indeed, cells expressing this construct show strong fluorescence at mid-cell, when grown in the presence of 50 μM IPTG in DRu medium (**Figure 5.9A, left panel**). An immunoblot probed with polyclonal anti-GFP (**Figure 5.9B**, first lane) reveals two closely migrating bands also observed by (Bernhardt and de Boer 2003) that were interpreted as processed and unprocessed forms of ^{T}amiC-GFP. We conclude that approximately half of the construct is processed by the Tat system. Then, the TM domain of OmpA was fused to the C-terminus of ^{T}amiC-GFP. Introducing the TM domain of OmpA caused a reduction in GFP fluorescence at mid-cell (**Figure 5.9A, right panel**), and the immunoblot shows that not only intact construct but also a lot of degradation bands are present (**Figure 5.9B**, second lane). The mid-cell localization did not improve upon increasing the induction level to 0.3 mM IPTG (data not shown), and the degradation bands became more pronounced (**Figure 5.9B**, third lane).

Finally, we were unable to detect the OmpA domain in the OM with fluorescent streptavidin labeling (data not shown). We also made a fusion of OmpA directly to the C-terminus of ^{T}amiC, leaving out GFP (used as negative control (fourth lane) in the anti-GFP immunoblot shown in **Figure 5.9B)**. For this construct, we could not detect OmpA in the OM with labeling, either. We conclude that the TM domain of OmpA, when exported via the Tat system as a C-terminal fusion to ^{T}amiC, is not able to properly assemble in the OM.

Discussion

OMP domain organization

Not much is known on to what extent periplasmic domains can be swapped between OMPs, and whether domain order affects OM incorporation. There are several β-barrel outer membrane proteins that contain additional periplasmic domains, attached to either the N- or C-terminus of the TM domain. Examples of TM domains with additional N-terminal domains are the Omp85 protein (5 POTRA domains, involved in OMP biogenesis) (Gentle et al. 2005) and the Imp/LptD protein (contains a single periplasmic domain, involved in LPS biogenesis) (Bos et al. 2004). Also present in the OM are autotransporter proteins, which consist of a N-terminal “passenger domain”, attached to a C-terminal β-barrel TM domain (Bernstein 2007). As the Omp85 protein is required for OM

incorporation of autotransporters (Jain and Goldberg 2007), the OM insertion machinery can insert OMPs that contain an N-terminal domain. A hybrid protein consisting of the signal sequence and two-thirds of the mature portion of periplasmic β-lactamase was fused to the N-terminus of the β-barrel PhoE (Tommassen and Lugtenberg 1984). After proteolytic digestion of membrane fractions, the PhoE moiety was not digested, presumably because it was protected in the outer membrane. Our findings that not only C-terminal, but also N-terminal fusions to the OmpA TM domain are incorporated in the OM further confirm that the Omp85 machinery can incorporate OMPs with N-terminal periplasmic domains. Furthermore, as both fusions to a periplasmic protein (ALBP), and a heterologous protein (mCherry) are assembled in the OM, specific requirements for the N-terminal domain appear to be absent.

Toxicity of fusions

Already in the early 80's, two gene fusions to the OmpA TM domain were constructed (Henning et al. 1983): the first was a fusion between OmpA-228 and 11 of the 12 TM alpha helixes of the *TetA* gene (coding for a inner membrane protein). The second was a fusion between OmpA-228 and a viral capsid protein VP1, followed by 77 residues of VP2A (originating from the FMDV enterovirus, where the proteins are post-translationally cleaved) and 30 codons of vector DNA. Cells expressing these constructs at wild-type OmpA levels were not viable. Experiments with radioactive labeling in mini-cells showed that no intact fusion could be detected, only degradation products. Ignoring for a moment the other potential problems with these particular fusions (partial domains, choosing a hydrophobic membrane protein and a protein that assembles into capsids), it seems important to realize the following. Wild-type OmpA is a very abundant protein, with an estimated copy number of ~10^5 (Henning et al. 1973). As the surface area of the OmpA TM domain is ~ 10 nm^2 (Pautsch and Schulz 1998) this means that the OmpA TM domain occupies roughly 10% of the surface of *Escherichia coli* (area ~10 μm^2). As its C-terminal domain binds PG, it might not be possible to swap its PG-binding domain for an unrelated domain while maintaining wild type expression levels. This could cause structural (growth) problems, with the PG layer closely underneath the OM layer (Matias et al. 2003).

For the C-terminal fusions made, which all were incorporated in the OM to a large extent, expression levels in LMC500 of (OmpA-177)-Pal were tolerated up to 50-100 μM IPTG, whereas continuous expression of (OmpA-177)-Pal-mCherry and (OmpA-177)-

mCherry was tolerated only up to 10 μM IPTG. Maybe Pal can complement the OM tethering of wild-type OmpA, allowing higher expression levels, whereas mCherry obviously cannot. Finally, the increase of toxicity after C-terminal addition of mCherry to (OmpA-177)-Pal might be due to steric hindrance in the crowded space between OM and PG.

Mid-cell exclusion of (OmpA-177)-Pal

Our results show that in a wild type background (both OmpA-177^{FLAG} in LMC500, and OmpA-177^{SA1} in MC1061), (OmpA-177)-Pal is excluded from mid-cell. What could cause this exclusion? If the exclusion is of a physical/space-occupying/steric hindrance type where the presence of wild-type Pal at mid-cell simply does not allow (OmpA-177)-Pal to be present as well, then one would expect the same for the full-length OmpA, as it takes up roughly as much space as (OmpA-177)-Pal. As this is not the case, a difference in properties of the periplasmic OmpA domain and the Pal domain is likely to cause the exclusion. This could, for instance, be a difference in affinity for the peptidoglycan, or an interaction with a (most likely periplasmic) protein, such as TolB. TolB binds Pal with high affinity (Bonsor et al. 2007), and the authors conclude that PG and TolB binding to Pal is mutually exclusive.

How could a Pal interaction cause an exclusion at mid-cell? If wild-type Pal has a higher diffusion rate compared to (OmpA-177)-Pal, then it is expected that when a mid-cell localized Pal binding factor (such as TolB, or a specific high-affinity PG structure) appears during the cell cycle, the faster diffusing wild-type Pal will occupy most of these sites before the slower diffusing (OmpA-177)-Pal has reached them. This reduces the available binding sites for (OmpA-177)-Pal, explaining the reduced presence of this fusion at mid-cell.

This argument requires that Pal binding at mid-cell is not reversible on the timescale of division, i.e. minutes. Furthermore, the diffusion coefficients of (OmpA-177)-Pal and that of lipid-anchored Pal need to differ sufficiently. Reported values for the diffusion coefficient *D* of lipids in both cell membranes as well as artificial membranes fall in the range 1-10 $\mu m^2/s$ (Murase et al. 2004). Therefore, the diffusion coefficient of the lipid-anchored Pal in the periplasm might be similar to that of periplasmic GFP (3 $\mu m^2/s$, (Mullineaux et al. 2006)). Reported *D* values for integral (inner) membrane proteins of *Escherichia coli* are in the range 0.01-0.1 $\mu m^2/s$ ((Deich et al. 2004), (Mullineaux et al.

2006), (Leake et al. 2006)), for an integral OM β-barrel (BtuB) a *D* value of 0.3 $\mu m^2/s$ was measured (Spector & Ritchie, Biophysical Society meeting 2007). As the diffusion of (OmpA-177)-Pal is most likely limited by the diffusion of OmpA-177 in the OM, it is not unreasonable to expect a diffusion constant in the range of 0.1-0.3 $\mu m^2/s$.

The fact that the localization pattern of OmpA-177-Pal is exactly "inverted" compared to wild type Pal (e.g. wild type stays at the new cell poles in daughter cells for some time, OmpA-177-Pal is absent from new cell poles for some time) provides additional evidence that competition with wild type Pal is involved.

In the ΔPal background, the homogeneous FLAG staining of (OmpA-177^{FLAG})-Pal is unexpected, as periplasmic (not properly OM incorporated) (OmpA-177^{FLAG})-Pal localizes to mid-cell, suggesting that the mid-cell localization factors are in place. As (OmpA-177)-Pal does not complement, OM blebbing at mid-cell still is expected to occur, and this might prevent the Pal domain from interacting with its mid-cell interaction factors in the periplasm.

We have excluded the possibility of Pal forming a dimer in these considerations, as for Pal, this is based solely on *in vivo* cross-linking (Clavel et al. 1998), which can lead to loss of specificity when high concentrations (>0.1%) formaldehyde are used (Manting et al. 1997). Both TolB and Pal in the TolB-Pal crystal structure were monomeric (Bonsor et al. 2007), and in a NMR structural study of Pal binding to PG precursor, a single Pal was observed (Parsons et al. 2006).

Why the N-terminal Pal and ^{T}AmiC fusions to the OmpA TM domain are degraded: incompatibility with Lol and Tat systems respectively?

Thus, we find that an (OmpA-177)-Pal fusion is incorporated into the OM, but does not localize to mid-cell. When the domain order is reversed, and OmpA-177 is fused behind a lipoPal-mCherry fusion, lipoPal-mCherry is incorporated in the OM and localizes to mid-cell, but the TM domain of OmpA is not incorporated in the OM and degraded. Perhaps in this case, the TM domain cannot insert autonomously in the OM for some reason, and is subsequently degraded. Alternatively, OM incorporation *in vivo* occurs exclusively via the Omp85 machinery, in which case we could speculate that Omp85 cannot insert the TM domain when it is attached to the lipoPal-mCherry already tethered to the OM.

We found that the OmpA TM domain when exported via the Tat system as a C-

terminal fusion to ^{T}AmiC was not able to assemble in the OM. This could be due to an incompatibility of the (most likely unfolded with SecB bound) OmpA with the Tat system that exports folded domains. However, for the periplasmic EnvC protein, a Sec substrate, it has been shown that Tat export resulted in a functional protein (Bernhardt and de Boer 2004). Perhaps Tat export is not compatible with OMP insertion, for instance, when OMP insertion requires OMPs to arrive in the periplasm in an unfolded state, normally guaranteed by the Sec translocase (Apparently there is some debate whether Tat can export only folded proteins: Gordon Research Conference 2008)

On export to the periplasm of heterologous proteins

Domain fusions that need to be incorporated in the OM, first need to be properly exported to the periplasm. Thus to create a mCherry fusion to the N-terminus of OmpA, first an exported mCherry construct was needed. A few popular signal sequences are most often used for export (OmpA, PelB, PhoA, pIII) of heterologous proteins (Thie et al. 2008). We initially chose the OmpA signal sequence (OmpAss). However, export of OmpAss-mCherry was not very efficient. We reasoned that after translation of the polypeptide, the mature region of the mCherry preprotein could not be kept unfolded by SecB, and might fold into a translocation-incompetent form, perhaps caused by (covalent) chromophore formation in the cytoplasm. As the SRP pathway allows co-translational export, we replaced the OmpAss by the signal sequence of DsbA. This signal sequence is known to direct proteins into the SRP pathway (Schierle et al. 2003), and it was shown that a rapidly folding cytoplasmic protein (thioredoxin) could be successfully exported when fused to the DsbAss but not when fused to MBPss (MBP is exported via the SecB pathway). However, export did not improve. Surprisingly, fusing mRFP1 (from which mCherry is a derivative) to the C-terminus of either full-length MBP or DsbA, or to a Tat signal sequence (TorAss) resulted in a clear fluorescent halo along the perimeter of the cell (Chen et al. 2005). Furthermore, it has recently been shown that the Sec translocase can actively unfold preproteins (Nouwen et al. 2007). Perhaps the presence of an endogenous preprotein (MBP of DsbA), in combination with a slower (fluorescence) maturation rate of mRFP1 compared to mCherry (Shaner et al. 2004), together allow for more time to start translocation before the fluorophore is covalently formed and the preprotein might become translocation-incompetent. As the Tat system exports folded domains, premature folding (suggested to interfere with export) is expected not reduce Tat export efficiency.

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Materials and methods

Strains and growth conditions

E. coli strains (**Table I**) were grown at 37°C in TY medium containing 1% Bacto trypton, 0.5% Bacto yeast extract, 0.5% NaCl and 3 mM NaOH. Alternatively, strains were grown at 28°C in Defined-rich medium with 0.2% glucose as the carbon source (Teknova M2105 kit) and supplemented with 1 mM thiamine-HCl (Sigma). Expression of the constructs was induced by adding up to 1 mM IPTG or 0.02 % L-arabinose, depending on the plasmid vector (plasmids are listed in **Table II**). Antibiotics were ampicillin (100 μg/ml) or Chloramphenicol (25 μg/ml). LMC500 (MC4100 *lysA*) was made chemically competent using the calcium chloride method. MC1061 and its derivative MC1061 ΔOmpA were transformed using electroporation.

MG5 (ΔPal) was made competent by first streaking on a high salt (1% NaCl) TY plate supplemented with 20 ug/ml Kanamycin. From this plate, a TY culture with 0.5% NaCl was inoculated and used to make competent cells using the calcium chloride method. It was found that for plasmid transformation, it was important to use TY plates with 1% NaCl at

Strains	Genotype	Reference
LMC500 (MC4100 *lysA*)	*F⁻, araD139, Δ(argF-lac)U169, deoC1, flbB5301, ptsF25, rbsR, relA1, rpslL150, lysA1*	(Taschner et al. 1988)
MC1061	*F⁻, araD139, Δ(ara-leu)7696, ΔlacX74, galU, galK, hsdR2 (r_{k-} m_{k+}), mcrA0, mcrB1, rpsL, spoT1*	(Casadaban and Cohen 1980)
MC1061 *ΔOmpA*	MC1061 *ΔOmpA*	(Bessette et al. 2004)
MG5	MG1655 *lacIZYA pal*	(Gerding et al. 2007)
DH5α	*F⁻, endA1, hsdR17(r_{k-} m_{k+}), supE44, thi-1, recA1, gyrA, relA1, Δ(lacZYA-argF)U169, deoR, Φ80 lacZΔM15*	Lab collection
DH5α-Z1	DH5α *$LacI_q^+$ $TetR^+$*	(Lutz and Bujard 1997)

Table I. Strains used in this study.

pH 7.0.

In minimal medium (GB1 at 28°C), strain MG5 had a mass doubling rate of ~160-170 min either grown without plasmid in the presence of 20 μg/ml kanamycin or with a plasmid carrying P_{lac}::Pal-mCherry in the presence of 100 μg/ml ampicillin (LMC500 under these conditions grows with a doubling rate of ~85 min).

SDS hypersensitivity complementation assay

This assay was taken from (Gerding et al. 2007). Overnight cultures (grown in TY medium with 1% NaCl) were diluted with TY medium (containing 0.5% NaCl) to an OD600 of $2{\cdot}10^{-4}$, $2{\cdot}10^{-5}$ and $2{\cdot}10^{-6}$ (corresponding to approximately 10^4, 10^3 and 10^2 cells) and 10 μl droplets were pipetted on TY plates with 0.5% NaCl, and optionally SDS (filter sterilized, 0.5%) and/or IPTG (50 μM). Plates were incubated at 28°C (OmpA-177)-Pal) or 30°C (Pal-mCherry-SRA) for 24 hours, followed by a further incubation at RT for one week (OmpA-177)-Pal) or 32 hours (Pal-mCherry-SRA). Plates were imaged with a gel-imager.

Estimation of induction levels

A dense overnight culture grown at 28°C in Defined-rich glucose (DRu) medium of LMC500 cells expressing mCherry was diluted in fresh medium supplemented with increasing amounts of IPTG (between 0 and 1 mM) and grown for 5 hours at 28°C. Cells were fixed and imaged. As a reference, cells grown in TY medium at 28°C were also included. Different regions in the samples (to avoid bleaching) were imaged with increasing exposure times between 22 and 2200 ms, giving a set of images for each induction level. The raw images of DRu-grown cells were then compared to images of TY-grown cells, and for 0.1 mM induction, similar sets of images (fluorescent intensities) were observed.

DNA Constructs

All DNA manipulation, analysis and bacterial transformations were performed according to standard protocols (Sambrook et al., 1989). All PCR fragments were sequenced, either at Baseclear (Leiden) or at the AMC DNA sequencing facility (Amsterdam Medical Centre). Primers were ordered from MWG or Biolegio, and Advantage DNA polymerase (Clontech) or *pfuTurbo* DNA polymerase (Stratagene) was used for the PCR reactions.

Plasmid	Protein	Precursor/ Processed	Reference
pGV10	proALBP	33.1/30.6	This study
pGV11	proALBP-myc-ALBP	65.3/62.8	This study
pGV12	proALBP-myc-ALBP-OmpA-177-FLAG	87.6/85.1	This study
pGV31	proALBP-myc-ALBP-OmpA-177-SA1	86.5/84.0	This study
pGV4	proOmpA-177-3xFLAG	24.1/22.0	(Verhoeven et al. 2008)
pGV28	proOmpA-177-SA1	23.2/21.1	(Verhoeven et al. 2008)
pGV33	proOmpA-SA-1-LEDPPAEF	39.8/37.7	(Verhoeven et al. 2008)
pSAV47	mCherry-EFSR	27.2	(Alexeeva et al.)
pGI1	OmpAss-mCherry-SRA	29.9/27.8	This study
pGI2	DsbAss-mCherry-SRA	29.2/27.2	This study
pGI4	DsbAss-mCherry-OmpA-SA1	50.2/48.2	This study
pGV15	proOmpA-177-3xFLAG-Pal-LEDP	41.1/39.0	This study
pGV16	proOmpA-177-3xFLAG-Pal-mCherry	68.2/66.1	This study
pGV29	proOmpA-177-SA1-Pal-LEDP	40.0 /37.7	This study
pGV30	proOmpA-177-SA1-LEDPPAEF-mCherry	50.7/48.6	This study
pGV19	proPal-LEDP	19.3/17.1	This study
pMG36	proPal-mCherry	46.4/44.2	(Gerding et al. 2007)
pGV21	proPal-mCherry(SM)-SRA	46.8/44.5	This study
pGV23	proPal-mCherry(SM)-OmpA-177-3xFLAG	69.1/66.8	This study
pGV24	proPal-mCherry(SM)-SRA	46.7/44.5	This study
pGV25	proPal-mCherry(SM)-OmpA-177-3xFLAG	69.0/66.8	This study
pGV26	proPal-mCherry-SRA	46.8/44.5	This study
pGV27	proPal-mCherry-OmpA-177-SA1	68.0/65.7	This study
pRD9	pre^{T}AmiC-GFPmut2-SRA	48.6/45.3	This study
pRD10	pre^{T}AmiC-OmpA-177-SA1	43.0/39.7	This study
pRD11	pre^{T}AmiC-GFPmut2-OmpA-177-SA1	69.8/66.5	This study

Table II: Plasmids used in this study. All vectors are based on pTHV037 (Den Blaauwen et al. 2003), except pMG36, pGV24 and pGV25, which are based on pMBL1113 (Zagursky and Berman 1984). Predicted molecular weight before and after signal sequence processing is indicated in kDa.

We used the expression vector pTrc99A (Amann et al. 1988), modified to decrease the basal expression level (i.e. without inducer) to typically a few thousands proteins per cell (pTHV037, (Den Blaauwen et al. 2003)). All plasmids used in this study are listed in **Table I**.

An XhoI site was introduced at the C-terminus of OmpA-177 3xFLAG by PCR on pGV4 (Verhoeven et al. 2008) using primers proOmpANcoIFW and OmpAXhoIPstIRV. This fragment was cloned into pTHV037 using NcoI and PstI sites, resulting in pGV14. Pal excluding its signal sequence and the Cysteine that becomes acylated, was PCR-ed from the chromosome of LMC500 using primers PalXhoIFW and PalBamHIHindIIIRV. The PCR fragment was digested with XhoI and HindIII and ligated into XhoI/HindIII digested pGV14 to form pGV15 (proOmpA-177 L3 3xFLAG-Pal-LEDP). mCherry was PCR-ed from pSAV47 (Alexeeva et al.) using primers mCherryFW and mCherryHindIIIRV. This PCR fragment was digested with BamHI and HindIII and ligated into BamHI/HindIII digested pGV15 to form pGV16 (proOmpA-177 L3 FLAG-Pal-LEDPPAEF-mCherry). The LEDPPAEF linker was copied from (Gerding et al. 2007). OmpA-177-SA1 was PCR-ed from pB33OS1 (Bessette et al. 2004) with primers proOmpANcoIFW and OmpAXhoIPstIRV, digested with NcoI/XhoI and ligated into likewise digested pGV15 to form pGV29. mCherry was PCR-ed from pSAV47 using primers mCherryFW and mCherryXmaIHindIIIRV. OmpA-177 L3 FLAG was PCR-ed from pGV4 with primers proOmpANcoIFW and OmpAEcoRIRV, digested with NcoI/EcoRI and cloned into pTHV37 to form pGV17 (proOmpA-177 Loop 3 FLAG followed by 30 residues from the vector). A mCherry fragment from pGV16 was transferred to pGV17 via EcoRI/HindIII (proOmpA-177 L3 FLAG-mCherry) forming pGV18. OmpA-177-SA1 was PCR-ed from pB33OS1 (Bessette et al. 2004) with primers proOmpANcoIFW and OmpAEcoRIRV, digested with NcoI and EcoRI and ligated into likewise digested pGV18 to form pGV30.

proPal (i.e. including its signal sequence) was PCR-ed from the chromosome of LMC500 using primers proPalNcoIFW and PalBamHIHindIIIRV and ligated to NcoI/HindIII digested pTHV37 to form pGV19 (proPal). OmpA-177 L3 FLAG was PCR-ed from pGV4 with primers OmpAXmaIFW and OmpAHindIIIRV, digested with XmaI/HindIII and ligated into THV37 digested with XmaI/HindIII to form pGV22 (intermediate vector). mCherry was PCR-ed from pSAV47 using primers mCherryFW and mCherryXmaIHindIIIRV. This fragment was digested with BamHI and HindIII and ligated in likewise digested THV037 to form pGV20, containing mCherry-SRA (intermediate

vector). After sequencing, it was found that a silent mutation had been introduced in the mCherry sequence at the last leucine at the C-terminus (...LYKSRA), changing the codon from CTG to CTT (below indicated with (SM)). pGV20 was digested with BamHI and HindIII and the fragment was ligated into likewise digested pGV19 to form pGV21, proPal-mCherry(SM)-SRA. pGV22 was digested with XmaI and HindIII and the fragment was ligated into likewise digested pGV21 to form pGV23. The BamHI/HindIII fragment from pGV20 containing mCherry(SM)-SRA was ligated into likewise digested pMG36 (Gerding et al. 2007) to form pGV24. A BamHI/HindIII fragment from pGV23 containing mCherry(SM)-OmpA177 was ligated into likewise digested pMG36 to form pGV25. To rule out any effect by the silent mutation (SM), a second clone of pGV20 was identified (20.13) that did not contain the SM. A BamHI/HindIII fragment from pGV20.13 was ligated into pGV19 to form pGV26, expressing proPal-mCherry-SRA. OmpA-177-SA1 was PCR-ed from pB33OS1 (Bessette et al. 2004) with primers OmpAXmaIFW and OmpAHindIIIRV, digested with XmaI/HindIII and ligated into likewise digested pGV23 to form pGV27.

proALBP was PCR-ed from the LMC500 chromosome with primers proALBPFW and proALBPRV, ligated into pGEM-T and sequenced. A BspHI/PstI fragment was ligated into NcoI/PstI digested pTHV037 to form pGV10. A SacI/PstI fragment containing myc-ALBP was isolated from pGV9 (See **Chapter 4** for details on construction) and ligated into likewise digested pGV10 to form pGV11. OmpA-177 L3 FLAG was PCR-ed from pGV4 with primers OmpAXmaIFW and OmpAPstIRV, ligated into pGEM-T and sequenced. The XmaI/PstI fragment was isolated and ligated into likewise digested pGV11 to form pGV12. OmpA-177 L1 SA-1 was PCR-ed from pB33OS1 (Bessette et al. 2004) with primers OmpAXmaIFW and OmpAPstIRV, digested with XmaI/PstI, and ligated into likewise digested pGV12 to form pGV31.

Oligos OmpAssFW and OmpAssRV were annealed to form a dsDNA with overhangs as created by NcoI and EcoRI, and ligated into EcoRI/NcoI digested pGV26 to form pGI1. In this construct, the OmpAss is followed by mature OmpA residues APKD, followed by two restriction sites BglII and EcoRI, that result in residues LEF, before the mCherry sequence (including N-terminal methionine) starts. Oligos DsbAssFW and DsbAssRV were annealed to form a dsDNA with overhangs as created by BspHI and NcoI, and ligated into NcoI digested pSAV47 to form pGI2. This vector allows any protein to become DsbAss exported via the NcoI site right after the signal sequence. A portion of mCherry, fused to OmpA177-

SA1 was isolated from pGV27 via PstI/HindIII and ligated into likewise digested pGI2 to form pGI4.

The septal targeting domain of AmiC was PCR-ed from the LMC500 genome with primers AmiC_FW and AmiC_RV, digested with NcoI and EcoRI and ligated into likewise digested pGV26 to form pRD8 (encoding ^{T}amiC-mCherry-SRA). GFPmut2 was PCR-ed from pGV7 (see **Chapter 4** for details on construction) using primers GFP_FW and GFP_RV, digested with EcoRI and HindIII, and ligated into likewise digested pRD8 to form pRD9. OmpA-177-SA1 was PCR-ed from pGI4 with primers prDR_OmpAF_F01 and prDR_OmpAF_R01, digested with EcoRI and HindIII, and ligated into likewise digested pRD9 to form pRD10. The same PCR product of OmpA177-SA1 was also digested with XmaI and HindIII, and ligated into likewise digested pRD9 to form pRD11. A list of primer sequences is available on request.

Preparation of cell lysates

Fresh overnight cultures grown at 37°C were diluted 1000x into 50-100 ml fresh TY medium and cultured at 37°C. Growth was monitored by measurement of the optical density at 600 nm with a spectrophotometer (Perkin-Ellmers). IPTG was added at around an OD600 of 0.1, and when the cells reached an OD600 of 1.0, they were transferred to a 50 ml Falcon tube and put on ice. The cells were then collected by centrifugation for 15 min at 4000 rpm in a tabletop centrifuge at 4°C (Eppendorf). The supernatant was carefully removed, and the cells were resuspended in ice-cold sonication buffer (10 mM Tris-HCl buffer, pH 7.9, supplemented with 1 mM EDTA and 1 tablet of Roche Protease Inhibitor Cocktail), at a concentration corresponding to an OD600 of 250. This cell suspension was transferred to a 2 ml Eppendorf tube, and sonicated on ice with a tip sonicator (Branson) in 4-5 10-second bursts with 10 second cooling in between each burst. Debris and intact cells were pelleted in a 4°C cooled centrifuge at 2700 x g for 2 min. The supernatant was transferred to a 1.5 ml Eppendorf tube and frozen at -20°C as total cell lysate.

Fractionation of cell lysates

After thawing, the cell lysate was diluted to 4 ml (corresponding to an OD600 of 12.5), and 100 µl of this was saved as “total cell lysate”. The samples were pelleted at 45000 rpm (corresponding to 200.000 x g) for 45 min in an ultracentrifuge (Beckman-Coulter). After centrifugation, 500 µl was saved as “supernatant”. The membrane pellet was resuspended

in 100 µl sonication buffer and frozen at -20°C.

SDS-PAGE, in-gel fluorescence and Western blotting

As Protein gel electrophoresis system we used the Bio-Rad mini-gel system protean III. For SDS-PAGE, samples were mixed with sample buffer (end concentration: 62.5 mM Tris pH 6.8, 2% SDS, 10% glycerol, 2% 2-mercaptoethanol) and either heated to 99°C for 5 min or heated to 50°C for 15 min and electrophoresed on 15% polyacrylamide slabs. In-gel fluorescence of GFPmut2 or mCherry was detected using a STORM 860 molecular imager by scanning the complete SDS-PAGE gel still in between glass plates. (For GFPmut2, quantities of 10-20 ng or more could be detected. mCherry was detected only faintly, because a blue excitation source had to be used). The bio-rad semi-dry blotting apparatus was used for immunoblotting. Anti-FLAG and anti-myc monoclonal antibodies were obtained from Sigma and Roche, respectively. The anti-dsRed monoclonal antibody (#632392, Living colors series) was purchased from Clontech. The polyclonal anti-OmpA antibody was a kind gift from A. Driessen (University of Groningen, Netherlands). The bands were detected using the ECL+ chemiluminescence kit (Amersham) and scanning with the STORM 860 fluorescence imager.

Fluorescent labeling of living cells

Cells were put on ice, and an amount of cells equivalent to 1 ml OD600 of 0.2 (around $2{\cdot}10^8$ cells) was taken for labeling. Cells were collected in all cases by centrifugation at 20.000 x g for 3 min at 4°C. The pellet was resuspended in 100 µl PBS with 0.1% BSA. The cells are incubated on ice for 15 min to block aspecific sites on the cell surface. Then, fluorescent Streptavidin (conjugated to either Alexa-488 or Alexa-546, Molecular Probes) was added (45 µg/ml), and incubated for 30 min on ice. PBS (0.85 ml) was added and the cells were pelleted. After a second wash with 0.5 ml PBS, the cells were fixed in 1 ml PBS with 2.8% formaldehyde and 0.042% glutaraldehyde, washed in 1 volume of PBS and resuspended in 0.1 volume PBS. The cells were either imaged directly or stored at 4°C over night before imaging.

Cells expressing fusions with the FLAG tagged OmpA TM domain were labeled in a similar way: Cells were blocked in GB1 with 3% BSA and incubated for 10 min at RT. Then biotinylated anti-FLAG was added (55 µg/ml) and cells were incubated 10 min at RT. After three washes with GB1 with 3% BSA, fluorescent streptavidin was added (5 µg/ml) and

cells were incubated 10 min at RT. After three washes with GB1, cells were fixed in 1 ml GB1 with 2.8% formaldehyde and 0.042% glutaraldehyde, washed in 1 volume of PBS and resuspended in 0.1 volume PBS.

Fluorescence Microscopy

Cells were immobilized on 1% agarose in water slabs-coated object glasses as described by (Koppelman et al. 2004) and photographed with a CoolSnap *fx* (Photometrics) CCD camera mounted on an Olympus BX-60 fluorescence microscope through a UPLANFl 100x/1.3 oil objective (Japan). Images were taken using the public domain program Object-Image2.19 by Norbert Vischer (University of Amsterdam, http://simon.bio.uva.nl/object-image.html), which is based on NIH Image by Wayne Rasband. In all experiments the cells were first photographed in the phase contrast mode. Then a fluorescence image was taken using either a blue excitation/green emission filter cube (green channel, EGFP, ex. 450–490 nm, em. 505-555 nm) or a green excitation/red emission (red channel, U-MNG, ex. 530–550 nm, em. 590 long pass), or both. For dual-color fluorescence imaging, we checked for bleed-through of green fluorophores (GFPmut2, Oregon Green-488 and Alexa-488) on the red channel, and for bleed-through of red fluorophores (Alexa-546 and mCherry) on the green channel. We found that on the green channel, even at long (4.7 s) exposures, no red fluorescence was observed. Cellular auto-fluorescence is present on the green channel, and virtually undetectable on the red channel. Very strong green fluorescence (saturated pixels at 100 ms exposure time on the green channel) could be detected at long (2.2 s) exposure times on the red channel.

ImageJ was used to make line profiles of cells imaged in phase contrast and fluorescence. Cell diameters were measured by estimating the pixel value where the intensity drop at the edge was half the value, and then multiplying by the pixel size (67 nm).