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

Verhoeven, G.S.

IV

Chapter 4: Domain fusions to the C-terminus of cell division protein FtsQ

Abstract

With the aim of sub-localizing the OmpA transmembrane (TM) domain to the site of constriction (mid-cell) in dividing *Escherichia coli*, we explored the possibility of using cell division protein FtsQ as mid-cell localization factor. To establish whether GFP-FtsQ could be used to target a complete protein domain to mid-cell, we constructed fusion proteins between FtsQ and AcrA (a periplasmic efflux pump component) and between FtsQ and ALBP (D-allose binding protein).

A GFP-FtsQ-AcrA protein showed reduced affinity for mid-cell in the presence of wild-type FtsQ, but complemented an FtsQ(Ts) mutant. Further extension with either a HSV or a 3xFLAG epitope to co-localize the C-terminus with the N-terminal GFP *in situ*, caused an "exclusion" from mid-cell. Also, extension with the OmpA TM domain interfered and caused mid-cell exclusion. Surprisingly, when a 3xFLAG epitope was present in the OmpA TM domain, dominant negative effects were reduced and mid-cell localization was somewhat restored in TY medium.

We speculate that for the AcrA extensions, only partially degraded fusion can localize to mid-cell, perhaps because intact AcrA interacts with the efflux pumps present in the lateral cell envelope. If further C-terminal extension with HSV, 3xFLAG or the OmpA TM domain makes the fusions more resistant to degradation, mid-cell localization of degraded fusion would be reduced, explaining the observed mid-cell exclusion.

During the course of this research, it was found that AcrA folds as a coiled-coil with its N- and C-terminus in close proximity. Folded AcrA is therefore not able to function as spacer domain. AcrA was replaced by ALBP, with in between FtsQ and ALBP a myc epitope to act as a spacer and to enable detection. Localization in the presence of wild-type FtsQ was good, and degradation on immunoblot was less pronounced compared to AcrA. This suggests that FtsQ can bring the ALBP domain to mid-cell.

Introduction

Our aim is to construct a fusion protein containing that spatially constrains the OmpA β-barrel to mid-cell during cell division. (see also **Figure 1.4** in **Chapter 1**). In this work, we explore the possibility to use the mid-cell affinity of cell division protein FtsQ to accomplish this. As FtsQ is an inner membrane protein with an extended periplasmic domain (van den Ent et al. 2008), an additional spacer domain is required that bridges the periplasm. During inward growth of the septum, this spacer domain has to connect the FtsQ domain with the OmpA β-barrel domain in the outer membrane. As FtsQ is an essential protein, the fusion either has to be functional, or has to be able to co-localize with functional wild-type FtsQ.

Previous work in our group has shown that the C-terminus of FtsQ could be extended with a small HSV epitope (13 residues) without interfering with mid-cell localization (see Chapter 4, Figure 4 of (Vinkenvleugel 2006)). In this chapter, we have fused complete spacer domains to the C-terminus of FtsQ and characterized their localization and functionality.

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.

Initially, the efflux pump component AcrA was chosen as spacer domain. At that time, AcrA was described as a elongated protein (~10-20 nm), thought to contain long α-helices and span the periplasm, with its hydrophobic (lipidated) N-terminus anchored in the IM, and its (slightly) hydrophobic C-terminus interacting with the OM (Elkins and Nikaido 2003). Although primary sequence analysis indicated that the N- and C-terminal regions flanking the central α-helical region could come together to form a single lipoyl-like domain (Johnson and Church 1999), the possibility of AcrA folding back on itself was considered unlikely, mostly based on dynamic light scattering experiments that predicted a highly elongated protein (Zgurskaya and Nikaido 1999). Furthermore, as a member of the "membrane fusion" protein (MFP) family (Dinh et al. 1994), AcrA shares homology to a paramyxovirus MFP (Baker et al. 1999). Based on this homology, AcrA's function was thought similar to that of a viral fusion protein (Baker et al. 1999), and it was speculated that coiled-coil formation could bring together the inner and outer membranes (Johnson and Church 1999). These properties made AcrA a suitable spacer domain to bridge the

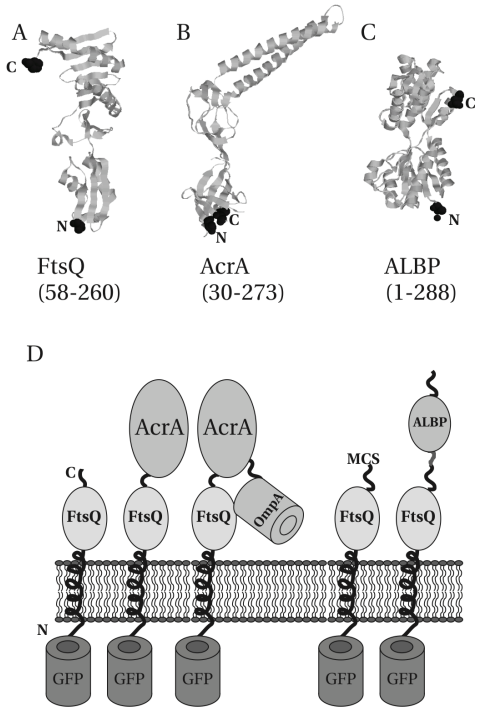

Figure 4.1: **(A-C)** structures with their N- and C-terminus indicated. For AcrA and ALBP, residue numbering is that of the mature protein. Note that for FtsQ, only the periplasmic domain is shown (with the last 16 residues missing) and for AcrA (397 residues, mature protein 373 residues) the first 29 residues and the last 100 residues of the mature protein are missing. ALBP is complete (311 residues, mature protein 288 residues). Figures were created by Rasmol, PDB files used were 2vh1 (FtsQ) (van den Ent et al. 2008), 2F1M (AcrA) (Mikolosko et al. 2006) and 1gub (ALBP) (Magnusson et al. 2002). **(D)** Cartoon of the main fusion proteins described in this chapter. From left to right: GFP-FtsQ, GFP-FtsQ-AcrA, GFP-FtsQ-AcrA-(OmpA-177), GFP-FtsQ-MCS and GFP-FtsQ-myc-ALBP.

periplasm.

Later work on AcrA, in particular its crystal structure (first of its homologue MexA (Akama et al. 2004; Higgins et al. 2004), later also for AcrA (Mikolosko et al. 2006)) showed that it folds back on itself to form three separate domains: a β-barrel, a lipoyl domain and a coiled-coil α-helical hairpin (**Figure 4.1B**). It was concluded that it did not span the periplasm and functioned more like an adaptor protein connecting/ regulating/stabilizing the IM (AcrB) and OM protein (TolC) components of the drug efflux pump (Touze et al.

2004). Thus, AcrA is not suitable to act as a periplasmic bridging domain.

An alternative spacer domain was needed. We subsequently opted for an approach in which the periplasm is bridged through an artificial "string" of folded spacer domains connected with flexible linkers. 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, as already mentioned, 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 (**Figure 4.1C**). 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 (**Figure 4.1C**, (Magnusson et al. 2002)). 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.

In *E. coli*, temperature-sensitive (Ts) mutants are routinely used to check if particular fusions/mutants are functional in the absence of (essential) wild-type protein. E.g. if a particular FtsQ mutant is expressed in a FtsQ(Ts) strain, it is said to complement (i.e. to be functional) if the cells continue to divide normally at the restrictive temperature. This is the temperature at which the Ts strain cannot divide and forms filaments because the endogenous gene product is not functional.

As in this work, a GFP-FtsQ fusion is extended with additional periplasmic domains, this approach is not suitable to establish unequivocally the functionality of a fusion, because extensions can be proteolytically removed without loss of the GFP (localization) or FtsQ (complementation) domains. Therefore, to check if fusion proteins localize to mid-cell intact, *in situ* immunolabeling experiments are performed which are directed against small epitopes attached at the C-terminus of every fusion protein. This allows co-localization of the C-terminus with the N-terminal GFP *in situ*.

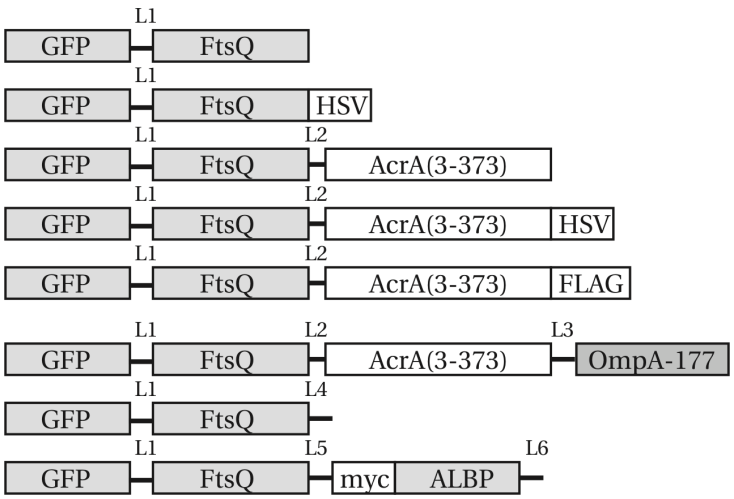

Figure 4.2: Schematic diagram showing all GFP-FtsQ fusion proteins described in this chapter. Linkers used were L1 (EFNNN, between GFP and FtsQ), L2 (RS, between FtsQ and AcrA), L3 (RAQQ, between AcrA and OmpA) L4 (GSSSSRG, MCS for ALBP fusions), L5 (GSST, L4 after addition of myc-ALBP) and L6 (GSSSRG, present at the C-terminus of a myc-ALBP building block). Epitopes used are HSV (QPELAPEDPED), 3xFLAG (DYKDHDG-DYKDHDI-DYKDDDDK), and 2xmyc $(EQKLISEEDL)_2$.

In this chapter, we describe the characterization of a series of C-terminal GFP-FtsQ-based fusion proteins (**Figure 4.1D, Figure 4.2**) containing either an AcrA or alternatively, an ABLP spacer domain. Although the fusions based on the AcrA domain are not suitable to bridge the periplasm, they are potentially informative on the tolerance of FtsQ towards C-terminal extension with bulky protein domains, on which the alternative strategy is based.

We found that GFP-FtsQ-AcrA mid-cell localization is less pronounced compared to GFP-FtsQ, but that it complemented the FtsQ(Ts) strain. However, we observed that the fusion is partially degraded to GFP-FtsQ*, and this degradation product alone is sufficient to explain the mid-cell localization and complementation. Further extension of AcrA with epitopes (HSV, 3xFLAG) or with the OmpA TM domain completely abolishes mid-cell localization and causes dominant-negative effects. Surprisingly, introducing a 3xFLAG epitope in the OmpA TM domain partially restored mid-cell localization and reduced the dominant-negative effects. We now know that this epitope makes the OmpA TM domain

susceptible to degradation (see **Chapter 3**). Therefore, this suggests that intact GFP-FtsQ-AcrA-X fusion protein cannot localize, but partially degraded (e.g. GFP-FtsQ*, or GFP-FtsQ-AcrA*) fusion protein can. Whether GFP-FtsQ-AcrA is able to localize remains an open question.

We designed a new series of fusion proteins based on ALBP domains connected with myc spacers. We show that GFP-FtsQ-myc-ALBP localizes to mid-cell. However, at this time, a more promising mid-cell localization candidate was found (see next chapter), and FtsQ was put on hold.

Results

We have used immunoblotting to check for correct expression and possible degradation of the fusions, and used fluorescence microscopy to visualize the (mid-cell) localization of the GFP-fusions. Because it is possible that a fusion will have a reduced affinity for mid-cell compared to wild-type FtsQ, GFP localization was studied in two strains, a common laboratory wild-type strain (LMC500) and an otherwise isogenic FtsQ(Ts) temperature-sensitive strain (LMC531). Functionality or the ability to not interfere with functional wild-type FtsQ is quantified by cell length measurements under various conditions as explained below.

Detection of fusion proteins on immunoblot

In **Figure 4.2**, a schematic overview of the main fusion proteins is shown (C-terminal epitope extensions are not depicted). To simplify the interpretation of results later on, we first discuss the expression and degradation results obtained from immunoblots.

Simultaneously with harvesting cells for microscopy (see below), samples for immunoblotting were taken. The immunoblots (**Figure 4.3**) were probed with a polyclonal anti-GFP antibody that (weakly) cross-reacted with several proteins in *E. coli* whole cell lysates. Open circles indicate full-length proteins at their expected height (GFP-FtsQ: 58.8 kDa, GFP-FtsQ-MCS: 59.4 kDa, GFP-FtsQ-myc-ALBP: 91.2 kDa, GFP-FtsQ-AcrA: 98.7 kDa). Degradation products are marked with asterisks. The first lane of each blot contains cells that do not express GFP.

In our experience, when grown in TY medium without glucose (**Figure 4.3 left panel**), expression levels from the pTrc99A-down plasmid (the expression vector used for all constructs in this chapter) are higher (~2-3 fold) compared to growth in GB1 medium and

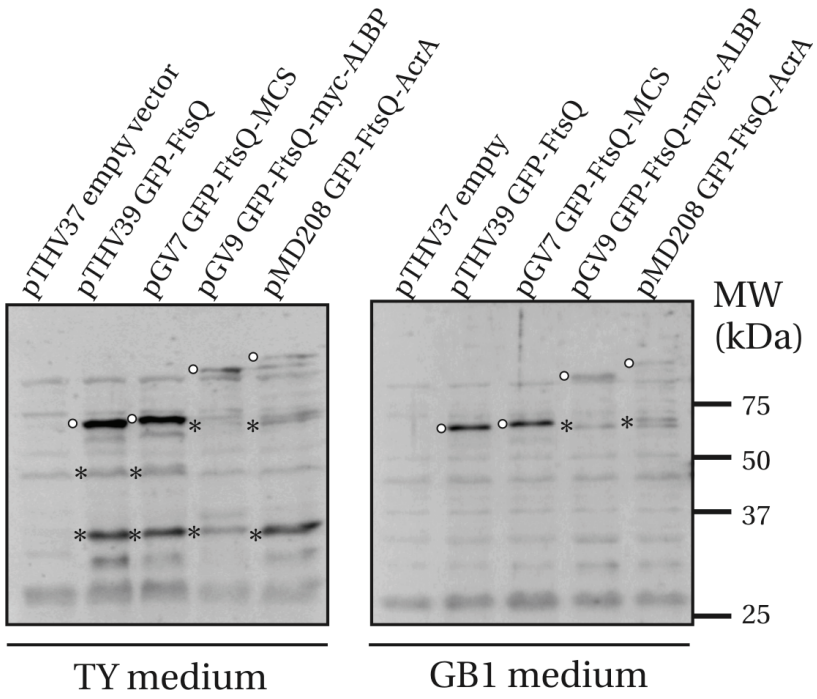

Figure 4.3. Immunoblots of GFP-FtsQ variants probed against the GFP domain. Cells were cultured as described in the caption of Figure 1. Samples were resuspended in sample buffer and boiled. The immunoblot was probed with a polyclonal anti-GFP antibody that (weakly) cross-reacted with several proteins in *E. coli* whole cell lysates. Open circles indicate full-length proteins at their expected height (GFP-FtsQ: 58.8 kDa, GFP-FtsQ-MCS: 59.4 kDa, GFP-FtsQ-myc-ALBP: 91.2 kDa, GFP-FtsQ-AcrA: 98.7 kDa). Degradation products are marked with asterisks. The first lane of each blot contains cells that do not express GFP. When grown in TY medium (left panel), expression levels were higher, and more degradation occurred. When grown in GB1 medium (right panel), both GFP-FtsQ and GFP-FtsQ-MCS have no detectable GFP degradation products. As expected from its 7 extra residues, the GFP-FtsQ-MCS band is slightly retarded with respect to GFP-FtsQ. Both GFP-FtsQ-myc-ALBP and GFP-FtsQ-AcrA are detected at their expected height. In addition, for both GFP-FtsQ-myc-ALBP and GFP-FtsQ-AcrA, a breakdown product GFP-FtsQ* is present. The degradation appears more pronounced for GFP-FtsQ-AcrA.

more degradation occurred. In particular, GFP-FtsQ and GFP-FtsQ-MCS were degraded to what is most likely GFP*.

When grown in GB1 medium (**Figure 4.3 right panel**), both GFP-FtsQ and GFP-FtsQ-MCS have no detectable GFP degradation products. As expected from its 7 extra residues, the GFP-FtsQ-MCS band is slightly retarded with respect to GFP-FtsQ. Both GFP-FtsQ-

myc-ALBP and GFP-FtsQ-AcrA are detected at their expected height. In addition, for both GFP-FtsQ-myc-ALBP and GFP-FtsQ-AcrA, a breakdown product GFP-FtsQ* is present that runs at roughly the same height as GFP-FtsQ, suggesting the proteolytic removal of the C-terminal extension. The degradation appears more pronounced for GFP-FtsQ-AcrA.

Localization and complementation of GFP-FtsQ and GFP-FtsQ-HSV

In this chapter, length measurements of cells containing GFP-FtsQ-based fusion proteins over-expressed from plasmid and grown to steady state are used to make statements about the functionality of the particular fusion. GFP fluorescence at mid-cell, visible as a bright "band" or spot in the middle of a bacterium is a measure for mid-cell affinity. In this thesis, when statements are made about mid-cell localization, this is always judged by visual inspection of fluorescence microscopy images. Furthermore, mid-cell is judged relative to signal elsewhere in the cell. Thus, strong mid-cell localization is when signal is present only at mid-cell and absent from elsewhere in the cell: it is weak when signal is present throughout the cell with only a slight enrichment at mid-cell. For the data presented in e.g. **Table I** (the percentage of "Q-rings" at mid-cell), weak mid-cell localization is scored as mid-cell localization. For the moment, these qualitative judgements are sufficient for our purpose. We use a wild-type strain (LMC500) to study the fusions in the presence of endogenous wild-type FtsQ as well as a (isogenic) temperature-sensitive FtsQ strain (LMC531) to study the fusions in the absence of wild-type FtsQ.

Wild-type FtsQ has a copy number of 25-50 (Carson et al. 1991). Using quantitative immunoblotting, it has been determined that GFP-FtsQ is over-expressed ~70 fold when expressed from the plasmid (pTrc99A-down) used throughout this chapter (Vinkenvleugel 2006). At these expression levels, GFP-FtsQ is present along the complete IM cell perimeter (**Figure 4.5A**), with only a 2-3 fold increase at the division site. However, if expression levels are reduced to ~5 fold over-expression by e.g. integrating GFP-FtsQ in the chromosome, only mid-cell localization remains (Vinkenvleugel 2006).

In the wild-type background, the average cell lengths of the fusions relative to GFP-FtsQ(wt) provide information on how a fusion functions together with wild-type FtsQ. An increase in cell length is interpreted as interference with the functioning of wild-type FtsQ by the GFP-fusion under study (that fusion is then said to be "dominant-negative"). The over-expressed GFP-FtsQ itself is already slightly dominant-negative, as average cell length

is increased compared to cells bearing empty plasmid (3.0 μm compared to 2.4 μm). When GFP-FtsQ is replaced by GFP-FtsQ(E125K), average length is increased further (to 3.7 μm). As no GFP mid-cell localization was observed for this fusion, it can interfere with wild-type FtsQ "from a distance", possibly by titration of FtsQ binding partners. It is likely that a fusion that interferes with wild-type FtsQ will be non-functional (see below).

As mentioned in the introduction, preliminary experiments showed that the C-terminus of GFP-FtsQ could be extended with a small epitope (HSV, 11 residues, Novagen) without loss of mid-cell localization (Vinkenvleugel 2006). In the wild-type background, cells expressing GFP-FtsQ-HSV were slightly longer compared to cells expressing GFP-FtsQ (3.3 instead of 3.0 μm, **Table I**). This indicates that the GFP-FtsQ-HSV fusion is less functional than GFP-FtsQ.

In the FtsQ(Ts) temperature-sensitive background, the fusions can be studied in the absence of wild-type FtsQ. The FtsQ(Ts) strain used has a chromosomal mutation in the *FtsQ* gene (*FtsQ1*, encoding FtsQ(E125K) (Taschner et al. 1988)) which makes FtsQ temperature-sensitive for mid-cell localization (Aarsman et al. 2005). At the permissive temperature (28°C), it localizes to mid-cell (inferred from the average cell length), but at the restrictive temperature (42°C) it does not (inferred from a GFP-FtsQ(E125K) fusion), and the cells form filaments as length growth continues (Hirota et al. 1968; Ricard and Hirota 1973). It was found that GFP-FtsQ expressed from plasmid localizes to mid-cell and could rescue the FtsQ(Ts) filamentation phenotype (average cell length after two mass doublings at 42°C was 4.7 μm (GFP-FtsQ) instead of 8.8 μm (empty plasmid), LMC500 (empty plasmid) at 42°C is 2.6 μm (Aarsman et al. 2005) and **Table I**). From these average length values, it is clear that complementation (the ability to divide at the restrictive temperature in the Ts background) is not a simple yes or no, but a gradual scale set by the average cell length at 42°C.

Table I Morphological parameters of strains LMC500 and LMC531 expressing FtsQ fusion proteins from plasmid. The cells were grown to steady state at 28°C in GB1 medium and subsequently shifted to 42°C and grown for a further 2 mass doublings. Partly reproduced from (Vinkenvleugel 2006). For FtsQ(Ts) at 42°C, three length classes are indicated with white (functional), light gray (partially functional) and gray (non-functional). For each sample, at least 300 cells were scored. An indication of the width of each length distribution is given by the S.D.

Strain/mutant	wild-type (LMC500) / 28°C			wild-type (LMC500) / 42°C			FtsQ(Ts) (LMC531) / 28°C			FtsQ(Ts) (LMC531) / 42°C		
	length (m)	T(%)	Q(%)	length (m)	T(%)	Q(%)	length (m)	T(%)	Q(%)	length (m)	T(%)	Q(%)
empty plasmid	2.39±0.50	ND	NA	2.59±0.58	ND	NA	4.68±2.40	ND	NA	8.77±3.39	ND	NA
GFP-FtsQ	3.04±0.62	16	23	3.97±1.45	23	31	3.67±0.92	26	28	4.70±1.93	24	46
GFP-FtsQ-HSV	3.34±0.73	39	33	5.09±2.15	D	45	3.26±0.78	33	30	6.28±2.74	D	39
GFP-FtsQ-AcrA	2.98±0.90	26	8	3.43±0.96	38	4	3.20±0.86	38	23	4.28±0.90	47	27
GFP-FtsQ-AcrA-HSV	4.08±1.60	45	4	5.72±3.05	50	2	4.29±1.95	35	8	9.45±4.12	35	5
GFP-FtsQ-AcrA-3xFLAG	4.18±1.44	37	0	5.30±2.83	38	0	4.47±2.10	43	0	7.86±4.31	37/D/B	0
GFP-FtsQ-AcrA-(OmpA-177)	4.45±1.98	ND	0	8.31±4.67	ND	0	7.51±5.59	ND	0	9.18±5.70	ND	0
GFP-FtsQ-AcrA-(OmpA-177)3xFLAG	3.33±1.02	ND	ND	4.88±2.26	ND	ND	4.25±2.04	ND	ND	6.15±2.21	ND	ND

Length indicates average length with standard deviation, T indicates percentage of visually constricting cells, Q indicates percentage of cells with a GFP signal at mid-cell, NA non-applicable, ND not determined, B blunt filaments, D deeply constricted filaments.

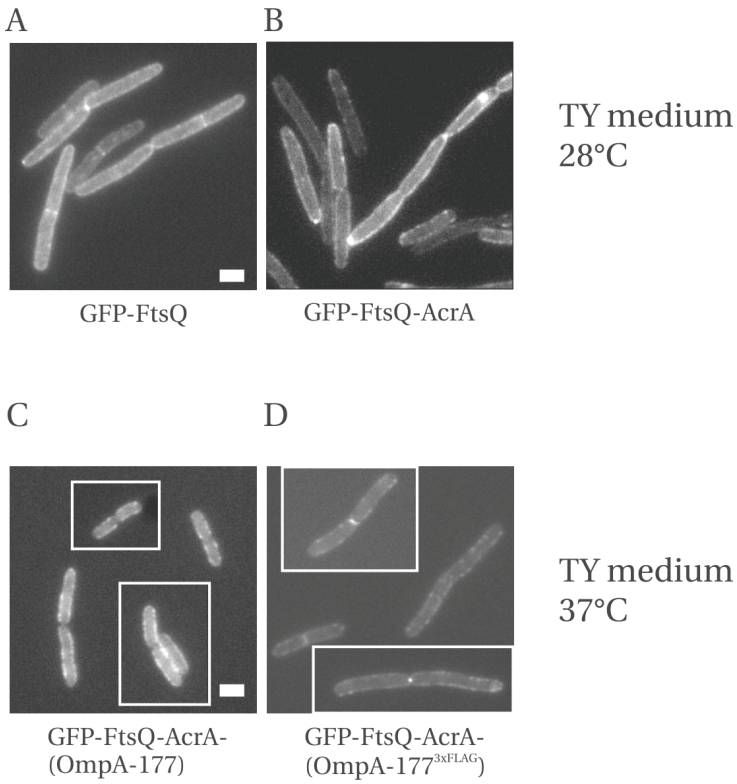

Figure 4.4. Mid-cell localization of FtsQ C-terminal domain fusions grown in TY medium (either at 28°C or, as indicated, at 37°C) in the presence of wild-type FtsQ. **(A, B)** Cells grown in TY medium at 28°C, fixed in growth medium and resuspended in PBS. **(C, D)** Cells grown in TY medium at 37°C, imaged directly, in the presence of growth medium (a collection composed of different raw images is shown). Scale bar has dimensions 1 x 2 μm.

At the restrictive background, GFP-FtsQ-HSV localized to mid-cell, but cell division was partially impaired (6.3 μm filaments). As immunoblotting indicated that the fusion was largely intact (data not shown), we speculate that, as the C-terminus is required for binding downstream division proteins FtsL and FtsB, possibly the presence of the HSV epitope interfered with this process.

For our purpose of localizing C-terminal extensions to mid-cell however, the fact that GFP-FtsQ-HSV not fully functional is not a strict requirement. Since it localizes to mid-cell in the presence of wild-type FtsQ seemingly as good as GFP-FtsQ, it will be expected that the 10-fold reduction in expression levels needed to obtain exclusively mid-cell

localization of GFP-FtsQ will also be possible with GFP-FtsQ-HSV, and thus, we continued the step-by-step assembly of the fusion by addition of the AcrA domain to the C-terminus of FtsQ.

Localization of GFP-FtsQ-AcrA-X fusions in the presence of wild-type FtsQ

First, we compared the localization of all fusions in the presence of wild-type (endogenous) FtsQ (LMC500: MC4100 *LysA*). Initially, the cells were grown in TY medium at 28°C (t_{MD}~50 minutes, for details see Materials & Methods). As mentioned, the fusions are over-expressed from plasmid about 100 fold, and as overproduction of FtsQ is known to impair division (Carson et al. 1991), it was not surprising that the cells grew as filaments (length ~7-14 μm, control with empty plasmid 3-6 μm). However, such extreme conditions can amplify minor differences between the constructs. The cells were fixed in growth medium and imaged.

GFP-FtsQ (**Figure 4.4A**) showed clear mid-cell localization. In contrast, localization of GFP-FtsQ-AcrA (**Figure 4.4B**) was mostly in the membrane, without any clear enrichment at mid-cell in most cells (and some cells had polar spots). Addition of the OmpA β-barrel resulted in exclusion (absence) of GFP from mid-cell (**Figure 4.4C**), but unexpectedly, when the 3xFLAG was present in the β-barrel (**Figure 4.4D**), mid-cell localization was restored in a sizeable number of cells (Note: temperature is 37°C).

Next, the cells were grown in GB1 medium. The cells grow much slower in this medium (t_{MD}~90-95 minutes). Cells were fixed in growth medium, resuspended in PBS and imaged. Phase-contrast and fluorescence images of the fusions in the presence of wild-type FtsQ are shown in **Figure 4.5**. As expected from a reduction in expression levels due to growth in GB1 medium, we find that cells expressing GFP-FtsQ no longer grow as filaments, although average cell lengths remain higher compared to cells with empty plasmid (Aarsman et al. 2005). Both cells expressing GFP-FtsQ and GFP-FtsQ-MCS show a clear mid-cell localization ("Q-ring"), as well as membrane localization (due to over-expression).

In LMC500 (wild-type background), GFP-FtsQ-AcrA showed reduced affinity for/GFP intensity at mid-cell, (the percentage of cells with Q-rings decreases from 23% to 8% upon addition of AcrA to GFP-FtsQ, **Table I**) and brighter patches along the perimeter of the cell and towards the poles can be observed. Clearly, addition of AcrA to the C-terminus of FtsQ

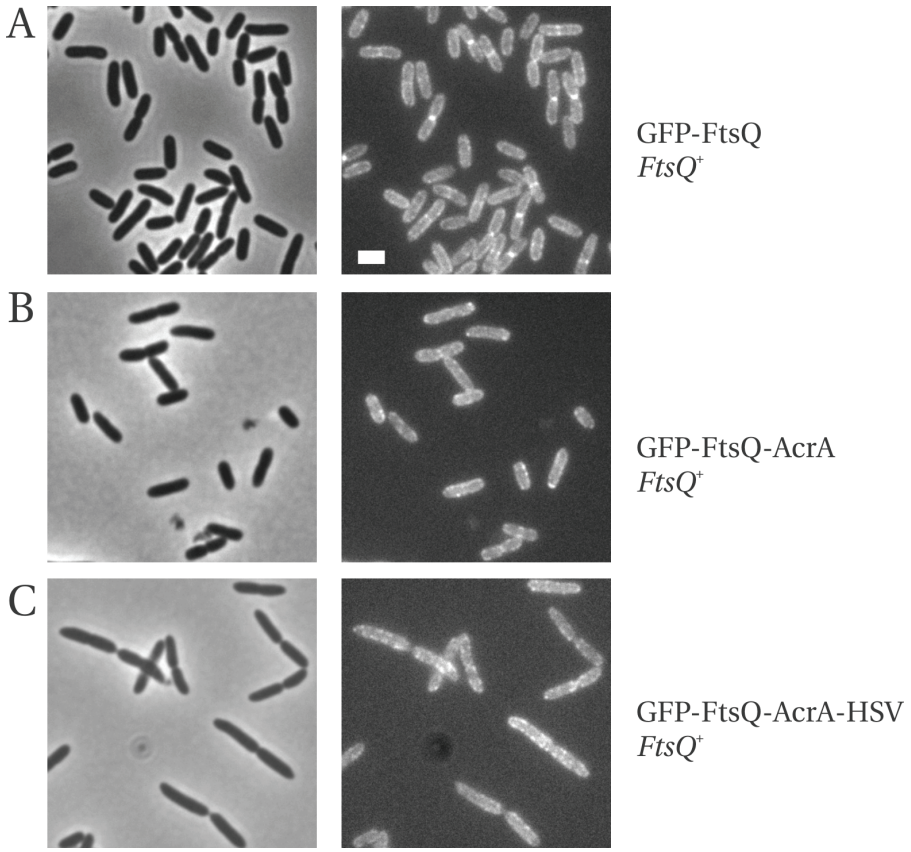

Figure 4.5. Mid-cell localization of FtsQ C-terminal domain fusions in the presence of wild-type FtsQ. Cells grown in GB1 medium were fixed in growth medium, resuspended in PBS and imaged. **(A)**GFP-FtsQ localizes efficiently to mid-cell. **(B)** Cells expressing GFP-FtsQ-AcrA had reduced GFP intensity at mid-cell, and brighter patches along the perimeter of the cell and towards the poles can be observed. **(C)** Further extension of GFP-FtsQ-AcrA with either a HSV (shown) or a 3xFLAG epitope (not shown) abolished mid-cell localization almost completely. For the fluorescent images, exposure times of 1-3 s were used. Scale bar has dimensions 1 x 2 μm.

affects its localization.

Further extension of GFP-FtsQ-AcrA with a HSV (**Figure 4.5C**), a 3xFLAG epitope (not shown) or the OmpA β-barrel (not shown) abolished mid-cell localization almost completely, creating dark bands at mid-cell (mid-cell exclusion), with GFP fluorescence mostly in the cylindrical part of the cell membrane. This suggests that little degradation to

GFP-FtsQ* occurs, since such a truncate would be expected to localize to mid-cell. Also, the fusions exerted dominant-negative effects on cell division, as inferred from the increased cell lengths (**Table I**).

In the previous chapter, it has been shown that insertion of a 3xFLAG epitope into OmpA caused a ~10 fold reduction in expression levels. As already mentioned, when the 3xFLAG epitope is present in the OmpA β-barrel, mid-cell localization is somewhat restored in TY medium (**Figure 4.4D**), although not so much in steady state GB1 medium (data not shown). Nevertheless, in GB1 medium, introduction of 3xFLAG in OmpA caused a dramatic reduction in division impairment, i.e. a reduction of the fusion's dominant-negative effect (**Table I**). (However, no complementation occurred, see below). If we assume that the increased degradation caused by the 3xFLAG insertion also holds true when the barrel is part of an FtsQ fusion protein, then this suggests that degradation of the fusion has a positive effect on the division process.

Localization of GFP-FtsQ-AcrA-X fusions in the presence of FtsQ(E125K)

For the GFP-FtsQ-AcrA based extensions, we also tested their functionality and localization in the absence of wild-type FtsQ, using the temperature-sensitive mutant strain LMC531, as described above. LMC531 cells were grown to steady state at 28°C in GB1 medium. Then, part of the cells was diluted in pre-warmed GB1 medium at 42°C and grown for an additional two mass-doublings. As before, the cells were imaged, their lengths were measured and GFP mid-cell localization was scored. All the relevant parameters can be found in **Table I**. The average length at the restrictive temperature was used to define three complementation classes: (i) Full complementation (Average length similar to GFP-FtsQ: ~5 μm), (ii) No complementation (Average length similar to empty plasmid: ~ 9 μm) and (iii) Partial complementation (Average length in between: ~7 μm). In **Table I**, the length classes are indicated with different gray scales.

In the presence of FtsQ(E125K), GFP-FtsQ-AcrA localized at mid-cell in 23% of the cells (**Figure 4.6A**). Thus, replacing wild-type FtsQ by FtsQ(E125K) increases the mid-cell localization of GFP-FtsQ-AcrA. For GFP-FtsQ-AcrA-HSV, which did not localize in the presence of wild-type FtsQ, no such improvement was found. Localization patterns with either endogenous FtsQ or FtsQ (E125K) were similar. This is expected when the fusion has lost its capacity for mid-cell localization.

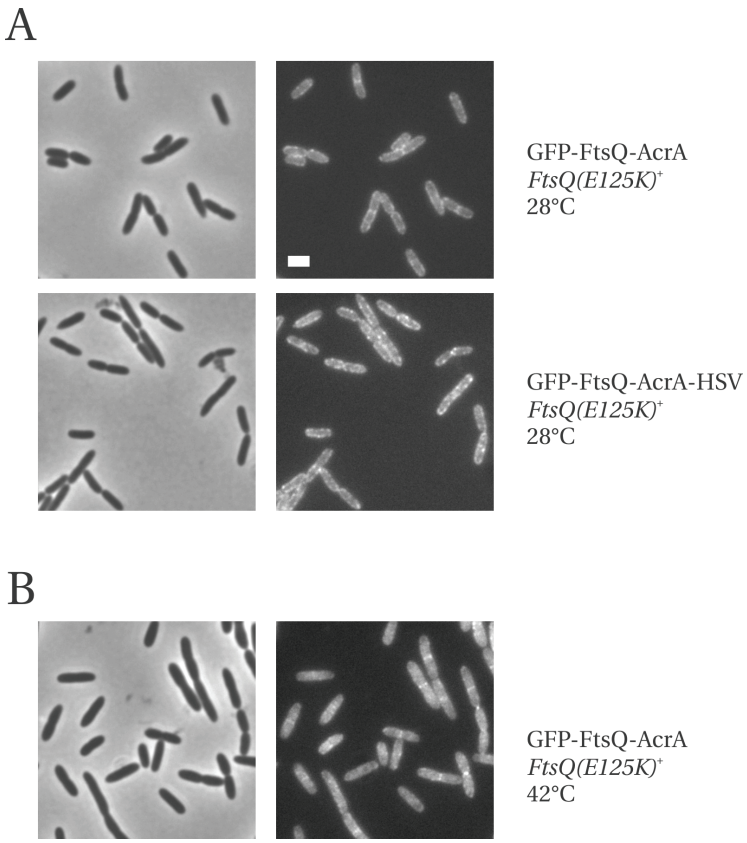

Figure 4.6. Localization of GFP-FtsQ-AcrA and GFP-FtsQ-AcrA^{HSV} in the presence of temperature sensitive FtsQ(E125K) at both **(A)** permissive (28°C) and **(B)** restrictive (42°C) temperatures. LMC531 cells were grown to steady state at 28°C in GB1 medium. Part of the cells was diluted in pre-warmed GB1 medium at 42°C and grown for an additional two mass-doublings. (A) In the presence of FtsQ(E125K), GFP-FtsQ-AcrA is localized at mid-cell in 23% of the cells (Table III). (B) At 42°C, a temperature at which FtsQ(E125K) does not localize to mid-cell, mid-cell localization increased (to 27% of all cells) and cells continue to divide normally. Thus, GFP-FtsQ-AcrA complements the temperature-sensitive LMC531 strain. In contrast, cells expressing GFP-FtsQ-AcrA^{HSV} formed long filaments, and mid-cell localization did not improve (data not shown). Similar results were obtained for cells expressing a GFP-FtsQ-AcrA^{FLAG} fusion (data not shown). Scale bar has dimensions 1 x 2 μm.

At the restrictive temperature (42°C) (**Figure 4.6B**), 27% of the cells showed mid-cell localization and cells continue to divide normally. Thus, GFP-FtsQ-AcrA complements the

temperature-sensitive LMC531 strain. In contrast, cells expressing GFP-FtsQ-AcrA-HSV formed long filaments (~10 μm), and mid-cell localization was not restored (data not shown). Similar results were obtained for cells expressing a GFP-FtsQ-AcrA-3xFLAG fusion and a GFP-FtsQ-AcrA-(OmpA-177) fusion (data not shown). Surprisingly, the GFP-FtsQ-AcrA-(OmpA-177)^{FLAG} fusion not only reduced toxic effects at the permissive temperature, but also exhibited partial complementation. Again, apparently the presence of 3xFLAG inside OmpA-177 improves the functionality of the fusion. We speculate this is due to an increased degradation of the fusion.

Extending GFP-FtsQ with a myc linker and the ALBP domain

Thus AcrA turned out not suitable to act as spacer domain, because of its unfavorable fold as a coiled-coil, and because we found experimentally that further extension destroyed its mid-cell localization capacity. As mentioned in the introduction, AcrA was replaced as spacer domain by ALBP, and myc epitopes were to be used as linkers.

On the DNA level, this required the design of a new multiple cloning site (MCS). The design had to meet several criteria: (i) the complete C-terminal extension downstream FtsQ can be swapped to a different mid-cell localizing factor (e.g. FtsN) via unique restriction sites (this required a unique site upstream of the extension (SacI) and a replacement (PstI) for the downstream HindIII as ALBP contains two HindIII sites), (ii) variable integer numbers of the (–myc-ALBP) spacer building block can be inserted (solved by XhoI/SalI-XmaI compatible ends cloning, see Materials & Methods, and **Figure 4.8** for details) (iii) at the very C-terminus a XmaI site allowed sub-cloning of the HSV/3xFLAG epitopes (iv) GFP could be exchanged easily with other FPs in our lab and (v) linker residues should be preferentially be either {GST} (small) and/or the myc epitope.

When grown in TY medium, GFP-FtsQ-MCS shows weak mid-cell localization but even membrane localization (**Figure 4.7A**). GFP-FtsQ-myc-ALBP had weak membrane localization and lots of bright fluorescent spots (**Figure 4.7B**). When grown in GB1 medium, GFP-FtsQ-MCS showed strong mid-cell localization, similar to GFP-FtsQ(wt) (**Figure 4.7C**). Finally, the GFP-FtsQ-myc-ALBP localized to mid-cell in most dividing cells, but not in all dividing cells, and not as well (i.e. as bright) as the GFP-FtsQ or GFP-FtsQ-MCS (**Figure 4.7D**). As we know from immunoblotting against GFP that the majority of the fusion is intact under these conditions, this suggests that it is possible to fuse a complete protein domain (ALBP) to the C-terminus FtsQ that subsequently localizes it to mid-cell.

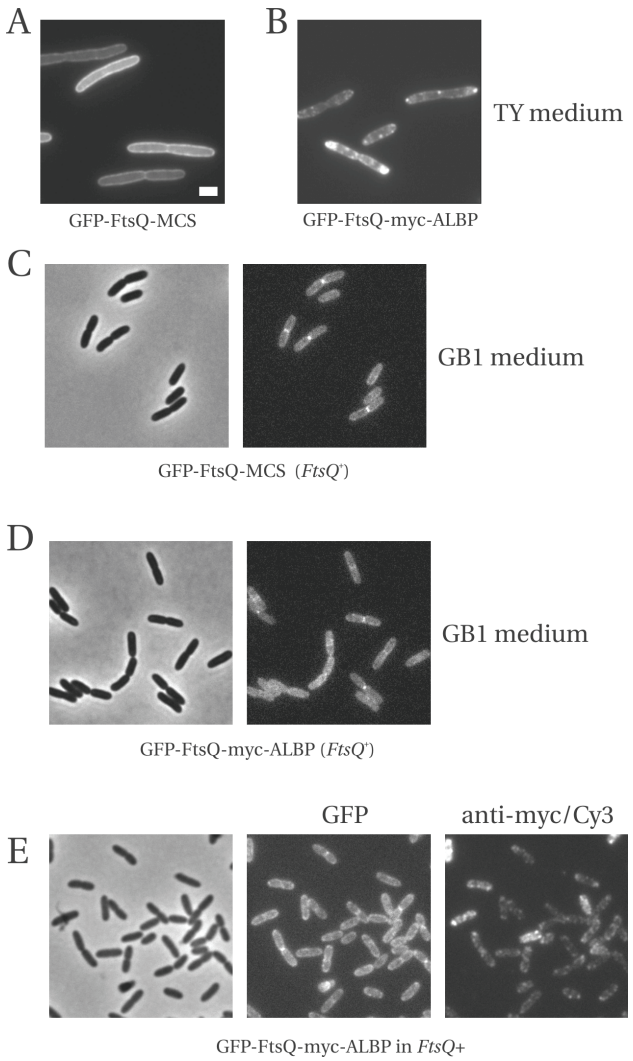

Figure 4.7. GFP-FtsQ-myc-ALBP. **(A, B)** Cells expressing either GFP-FtsQ-MCS or GFP-FtsQ-myc-ALBP were grown in TY medium at 28°C, fixed and imaged. **(C, D)** Same strains as in (A,B) now grown in GB1 medium at 28°C. **(E)** Cells were grown to pseudo-steady state in GB1 medium at 28°C, fixed and permeabilized with Triton X-100, EDTA and lysozyme. Cells were labeled with an anti-myc monoclonal antibody (50 μg/ml). This primary antibody was detected with a Donkey-anti-mouse Cy3-conjugated secondary antibody. Although several cells show a strong GFP mid-cell localization in the green channel, no corresponding myc spot was observed in the red channel (Cy3). Possibly, the signal from a single myc epitope is not sufficient to stand out in the labeling background. Alternatively/additionally, localized in the divisome, in between two presumably folded domains, the myc epitope might no be accessible for antibodies and therefore escape detection. Scale bar has dimensions 1 x 2 μm.

In an attempt to co-localize mid-cell localized GFP with the myc-epitope present at the C-terminus of FtsQ in GFP-FtsQ-myc-ALBP, we permeabilized fixed cells and immunolabeled them with anti-myc antibodies (**Figure 4.7E**). Although this does not prove the presence of ALBP (downstream of the myc epitope) at mid-cell, it can provide information on degradation at mid-cell.

Although several cells show a strong GFP mid-cell localization in the green channel, no corresponding myc spot was observed in the red channel (Cy3). On the whole, no co-localization was apparent, also not for the membrane (cylindrical) localized GFP. Possibly, the signal from a single myc epitope is not sufficient to stand out in the labeling background of the anti-myc antibody. A separate control experiment confirmed that indeed, anti-myc has a high labeling background in permeabilized cells (data not shown).

Alternatively/additionally, localized in the divisome, in between two presumably folded domains, the myc epitope might no be accessible for antibodies and therefore escape detection.

Discussion

First we discuss some implications of the observed mid-cell localization of an FtsQ fusion that is not fully functional (GFP-FtsQ-HSV), yet does not seem to interfere with wild-type FtsQ. Then we try to rationalize the observed behavior of the AcrA fusions, in particular the surprising mid-cell exclusion of the FtsQ-AcrA extensions. We conclude with two strategies to continue work on the GFP-FtsQ-myc-ALBP fusion, which localizes to mid-cell and is not dominant-negative, and therefore suitable for further extension.

FtsQ as part of the divisome appears dynamic

At first sight, it is surprising that GFP-FtsQ-HSV localized to mid-cell as efficiently as GFP-FtsQ(wt) in the presence of a low amount of endogenous FtsQ, but is not fully functional. Why can wild-type FtsQ function normally (the cells are slightly longer) in the presence of a large excess of less-functional FtsQ-HSV at mid-cell? After all, the amounts of wild-type FtsQ (25-50 copies) and plasmid-encoded GFP-FtsQ-HSV protein (2500-5000 copies) differ by two orders of magnitude. And because GFP-FtsQ-HSV localization appears similar to GFP-FtsQ, it is reasonable to expect that both will have equal affinity for the divisome.

If during execution of its (unknown) function FtsQ would be embedded in a static complex that is stable during division, then one expects that during the actual division,

FtsQ-HSV would no longer be able to localize, as all complexes containing FtsQ binding sites would have found their "rare" wild-type FtsQ copy. Since this is not observed, i.e. GFP-FtsQ-HSV stays localized during active division, this suggests that FtsQ localization is dynamic. For a long time, FtsZ was the only divisome component that has experimentally been shown to be highly dynamic (Stricker et al. 2002), but recent FRAP experiments in *Caulobacter* show that also FtsI (PBP3) is dynamic (Costa et al. 2008). Our results indicate that this might hold true also for FtsQ.

Why are GFP-FtsQ-AcrA-X fusions excluded from mid-cell?

The most striking result obtained with the GFP-FtsQ-AcrA fusion series is the mid-cell exclusion and toxicity that occurs after extending GFP-FtsQ-AcrA either with small epitopes or with the OmpA β-barrel. Subsequent insertion of the FLAG epitope into the OmpA barrel then reduces toxicity and partially restores mid-cell localization in TY medium.

Let us try to rationalize these observations. Could the mid-cell exclusion be caused by a reduced affinity for mid-cell (e.g. for binding to FtsK)? This is not likely, because then one would expect a (partial) restoration of mid-cell localization in the absence of endogenous FtsQ (i.e. at the restrictive temperature), which is not observed. Could the mid-cell exclusion be caused by a steric hindrance after extension of AcrA? In this scenario, the extended fusion no longer would fit in the protein complex at mid-cell that FtsQ is part of. However, AcrA contains a N-terminal linker of ~30 residues and a long 90-100 residue flexible C-terminus (Mikolosko et al. 2006). With this in mind, it is not likely that extending FtsQ with a large elongated domain such as AcrA does *not* cause mid-cell exclusion, but a small C-terminal extension to AcrA would suddenly abolish this capacity.

A possible explanation for the observed (weak) mid-cell localization of GFP-FtsQ-AcrA and the subsequent mid-cell exclusion of e.g. GFP-FtsQ-AcrA-HSV could be that addition of e.g. HSV causes stabilization against (occurring) degradation of the GFP-FtsQ-AcrA. In this scenario, the mid-cell localization of GFP-FtsQ-AcrA is then predominantly caused by GFP-FtsQ* that has AcrA proteolytically removed.

Is an increased stability of the GFP-FtsQ-AcrA-X fusions sufficient to explain all the observations? It provides a natural way to explain their toxicity: since their are excluded from mid-cell and more stable, they can titrate away division factors important for cell division (such as FtsL or FtsB, which form a complex with FtsQ (Buddelmeijer and

Beckwith 2004)).

However, to explain the mid-cell exclusion, further assumptions are required. Although, as discussed above, (small) extensions to AcrA are not expected to block mid-cell localization, extension of FtsQ with AcrA itself could already block mid-cell localization. Then it follows that all mid-cell localization observed for GFP-FtsQ-AcrA must be due to GFP-FtsQ*. However, this cannot explain the mid-cell exclusion of the stabilized AcrA extensions. In the absence of diffusion barriers or a dense protein packing in the IM at mid-cell, this is expected to result in a homogeneous distribution of GFP signal over the membrane, and not specifically in the cylindrical part of the cell, as observed. Thus, this scenario is not so likely.

Alternatively, GFP-FtsQ-AcrA is unstable but can in principle localize to mid-cell (i.e. it would do so if it was stable). Then stabilization after extension is not enough to explain the mid-cell exclusion. A further assumption is required; E.g. the existence of AcrA/AcrB/TolC efflux pumps along the cylindrical part of the cell, but not at mid-cell. Based on the relative copy numbers (Tikhonova and Zgurskaya 2004) and a proposed stochiometry for a single efflux pump (AcrA:AcrB:TolC 9:3:3 (Higgins et al. 2004)), we estimate the presence of a few hundred efflux pumps per cell. Interaction of stabilized AcrA-X with these efflux pumps would explain the observed mid-cell exclusion. This scenario requires that most GFP-FtsQ-AcrA is degraded to GFP-FtsQ*, since otherwise the same mid-cell exclusion would be expected.

Finally, introducing the 3xFLAG epitope in the OmpA barrel four-domain fusion protein reduced toxicity of the construct and partially restored mid-cell localization in TY medium. As such behavior resembles that of GFP-FtsQ-AcrA, this suggests that the stabilization through extension is in part lost. We know that introducing the FLAG epitope into OmpA reduces OmpA expression levels through increased degradation (see previous Chapter). Increased degradation of the fusion GFP-FtsQ-AcrA-(OmpA-177)^{FLAG} and formation of GFP-FtsQ* then explains the observed reduced toxicity and partially restored mid-cell localization.

Thus, the degradation of GFP-FtsQ-AcrA, together with stabilization upon further extension with e.g. HSV or the OmpA barrel, can explain all observations, if it is further assumed that either FtsQ-AcrA is sterically excluded from mid-cell or efflux pumps are present in the cylindrical part of the cell but not at mid-cell.

How to explain our "explanation"? I.e. why would the addition of the HSV or 3xFLAG or the OmpA β-barrel extension make the fusions more resistant to degradation? It has been shown that the flexible C-terminus of AcrA is proteolytically sensitive *in vitro* (Mikolosko et al. 2006). Furthermore, the C-terminus is required for interaction with AcrB, and possibly also with TolC (Elkins and Nikaido 2003; Touze et al. 2004). It is possible that outside of its natural complex, the AcrA C-terminus is susceptible to proteolytic degradation. Addition of an epitope tag could protect the C-terminus from degradation. As popular epitope tags, it is expected for HSV and 3xFLAG that they are resistant to degradation. Likewise, chaperones might protect the β-barrel from degradation.

Finally, results on two GFP-FtsQ-AcrA fusions not discussed here (GFP-FtsQ-AcrA-(PGLSLAVLADERRFSA) and GFP-FtsQ-AcrA-(PGPA), pMD008 and pMD009, see Materials & Methods) provide some additional evidence for the link between degradation and complementation, as GFP-FtsQ-AcrA-(PGLSLAVLADERRFSA) was found to be largely degraded to GFP-FtsQ* at 42°C as determined using immunoblotting with anti-FtsQ (data not shown), and complemented, whereas GFP-FtsQ-AcrA-(PGPA) was found to be largely intact using immunoblotting with anti-FtsQ and did not complement (8.8 μm filaments) (Vinkenvleugel 2006).

Recommendations for future work on GFP-FtsQ-myc-ALBP

As mentioned before, to check if fusion proteins localize to mid-cell intact, *in situ* immunolabeling experiments must be performed which are directed against small epitopes attached at the C-terminus of every fusion protein. This allows co-localization of the C-terminus with the N-terminal GFP *in situ.* Unfortunately, addition of either a HSV or a 3xFLAG epitope to GFP-FtsQ-AcrA interfered with mid-cell localization.

Because of the proximity of its N- and C-termini and the poor performance of the AcrA fusion proteins, AcrA was deemed unsuitable to function as a spacer domain. Therefore, AcrA was replaced by ALBP, with in between FtsQ and ALBP a myc epitope to act as a linker and to enable detection. Localization in the presence of wild-type FtsQ was good, and degradation on immunoblot was less pronounced compared to AcrA.

To verify that the myc-ALBP fusion is intact, the next step therefore should be the addition of a small epitope tag downstream of ALBP. As the HSV tag has proven itself for the co-localization of GFP and HSV in the GFP-FtsQ-HSV fusion (Vinkenvleugel 2006), this epitope would be first choice to clone downstream the myc-ALBP fusion.

Alternatively, to avoid the laborious immunolabeling procedure and possible problems with aspecific labeling and epitope accessibility, a fluorescent protein (mCherry) could be fused to the very C-terminus of the fusion constructs. mCherry could also be used as a spacer domain itself instead of ALBP, but if multiple copies are placed in series, the presence of mCherry at mid-cell cannot be used as proof for the absence of degradation anymore. A combination of both ABLP and mCherry would be best: With mCherry attached at the very C-terminus of each fusion, more and more ALBP spacer domains can be inserted between FtsQ and mCherry.

Acknowledgements

I like to thank Thessa Vinkenvleugel for all her work on FtsQ, which formed the starting point of this work. In particular I like to thank her for imaging and measuring some of the data points in Table I. In addition, I like to thank Reinier Boon for providing the pMD015 construct, Roland Dries for help with cloning of pGV9 and Svetlana Alexeeva and Frank Poelwijk for useful discussions on cloning strategies. Finally, I like to thank the people at the Plant Pathology/ Plant Physiology departments at the University of Amsterdam (Gert-Jan de Boer, Caroline Michielse, Ringo van Wijk and Eleni Spyropulou) for their generous help with DNA sequencing.

Materials and Methods

Strains and growth conditions

Strains used are listed in **Table II**. LMC500 (MC4100 *lysA*) and LMC531 were made chemically competent using the calcium chloride method.

For experiments performed in TY medium (1% Bacto trypton, 0.5% Bacto yeast extract, 0.5% NaCl and 3 mM NaOH) at 28°C, overnight cultures were prepared by culturing in TY at 28°C. In the morning, cells were diluted 500x into 10 ml fresh TY and grown in 3.5-4 hours to exponential phase (OD600~0.2) before harvesting. Under these conditions t_{MD}~50 minutes, so we expect ~5 mass doublings, and possible stationary phase accumulations have been diluted a factor 32.

Alternatively, strains were grown at 28°C in glucose minimal medium (GB1 medium, (Taschner et al. 1988). It has the following composition: 6.33 g/l $K_2HPO_4.3H_2O$, 2.95 g/l KH_2PO_4 (50 mM phosphate buffer pH 7.0) 1.05 g/l $(NH_4)_2SO_4$ (0.4 mM), 0.10 g/l $MgSO_4.7H_2O$ (8 mM), 0.28 mg/l $FeSO_4.7H_2O$ (1 μM), 7.1 mg/l $Ca(NO_3)_2.4H_2O$ (30 μM), 4 mg/l thiamine (vitamin B1), 4 g/l glucose (20 mM) and 50 g/l lysine (LMC500 is *LysA*) (Monod et al. 1951; Howard-Flanders et al. 1964). For the experiments described in this chapter, overnight cultures were grown in GB1 medium with ampicillin (100 μg/ml) to an OD450~1-3. In the morning, the cells were diluted 100x into 10 ml fresh GB1 medium +

Strains	Genotype	Reference
LMC500 (MC4100 lysA)	*F⁻, araD139, Δ(argF-lac)U169, deoC1, flbB5301, ptsF25, rbsR, relA1, rpslL150, lysA1*	(Taschner et al. 1988)
LMC531 (LMC500 *FtsQ*(Ts)	LMC500 *FtsQ1*	(Taschner et al. 1988)
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 II. Strains used in this study.

Amp and grown in 7-8 hours to an OD450~0.3-0.6. Under these conditions, t_{MD}~90 minutes, so we again expect ~5 mass doublings. We compared cells expressing GFP-FtsQ-AcrA not grown to steady state with earlier images that were grown to steady state by continuous growth and dilution in GB1 for 2-3 days, and found that cell morphology and GFP localization are similar. For the results shown in **Table I**, cells were used that were grown to steady state, here defined as the moment when the average cell mass/optical density does not change anymore (Fishov et al. 1995).

DNA Constructs

All DNA manipulation, analysis and bacterial transformations were performed according to standard protocols (Sambrook and Russel 2001). 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. All plasmids relevant to this chapter are listed in **Table III**.

To facilitate sub-cloning of C-terminal peptides/protein domains, a BglII site and a PstI site were introduced at the C-terminus of FtsQ (resulting in 4 extra residues RSLQ) using a PCR on pIB3 (containing GFP-FtsQ) using FtsQEcoRIsense and FtsQBglIIHindIIIRev primers. The fragment was ligated in pTHV022 via EcoRI/HindIII to form pMD001. As pTHV022 (pTrc99A-two-down) contained an in-active promoter, the fragment was subsequently sub-cloned in pTHV039 to form pMD003. pTHV039 is GFPmut2 fluorescent protein (Cormack et al. 1996) fused via linker EFNNN to FtsQ (excluding the start codon) cloned into the empty expression vector pTHV37. pTHV37 is identical to pTrc99A (Pharmacia), except for a “down” mutation in the –35 promoter region, which weakens the promoter, thereby reducing expression (Weiss et al. 1999). All further DNA constructs in this chapter are constructed in this plasmid.

Subsequently, the HSV-tag (FtsQHSVBglIIFw and FtsQHSVtagHindIIIRev) was ligated in the BglII-HindIII sites of pMD003 to yield pMD007 that expressed GFP-FtsQ-HSV. Residues 27-397 of AcrA (i.e. excluding the signal sequence and the lipidated cysteine) were PCR-ed from the chromosome of LMC500 using the AcrA27BglIIFw and AcrAXmaIRev primers and ligated via BglII-HindIII in pMD003 to yield pMD008. An additional bp in the XmaI site (CCCCGGGG) caused a frame-shift resulting in an additional 24 c-terminal residues. Then, either a HSV or a 3xFLAG epitope was introduced

at the C-terminus of AcrA via this XmaI site. This was done by annealing partially complementary oligos (AcrAHSVXmaIFw and AcrAHSVHindIIIRev) or (AcrA3xFLAGFw and AcrA3xFLAGRev) and ligating them into pMD008 via XmaI/HindIII to form pMD009 and pMD015, respectively. pMD017 was constructed by ligation of a PCR product on pMD005 (using primers OmpAXmaIFW and OmpAHindIIIRV) and subsequent ligation of the fragment into pMD008 via XmaI/HindIII.

A DNA fragment of the C-terminus of AcrA, obtained by PCR using the BsiWI-AcrAFw and XmaI-AcrARev primers on pMD008, was swapped via BsiWI/HindIII into pMD008 and corrected the XmaI site resulting into pMD208. Subsequently, pMD009, pMD015, and pMD017 were corrected to pMD209, pMD215 and pMD217 via the sub-cloning of an NcoI/XmaI fragment from pMD208. PCR fragments of pGV2 and pGV3 (see **Chapter 3**) using primers OmpAXmaIFW and OmpAHindIIIRV were ligated into pMD208 via XmaI/HindIII to form pGV5 and pGV6 respectively.

The myc-ALBP extension was constructed as follows: A PCR on pMD208 with primers

Plasmid	Protein	Molecular Weight (kDa)	Reference
pTHV039	GFP-(EFNNN)-FtsQ; GFP-FtsQ	58.7	(Vinkenvleugel 2006)
pMD003	GFP-FtsQ-(RSLQ)	59.2	(Vinkenvleugel 2006)
pMD007	GFP-FtsQ-HSV	60.2	(Vinkenvleugel 2006)
pMD208	GFP-FtsQ-AcrA	98.7	(Vinkenvleugel 2006)
pMD209	GFP-FtsQ-AcrA-HSV	100.0	(Vinkenvleugel 2006)
pMD215	GFP-FtsQ-AcrA-3xFLAG	101.7	(Vinkenvleugel 2006)
pMD217	GFP-FtsQ-AcrA-OmpA	118.2	This study
pGV5	GFP-FtsQ-AcrA-OmpA^{FLAG}	121.0	This study
pGV6	GFP-FtsQ-AcrA-OmpA^{myc}	120.6	This study
pGV7	GFP-FtsQ-(GSSSSRG); GFP-FtsQ-MCS	59.4	This study
pGV9	GFP-FtsQ-myc-ALBP	91.2	This study

Table III: Plasmids used in this study. All vectors are based on pTHV037 (Den Blaauwen et al. 2003). Predicted molecular weight is indicated in kDa.

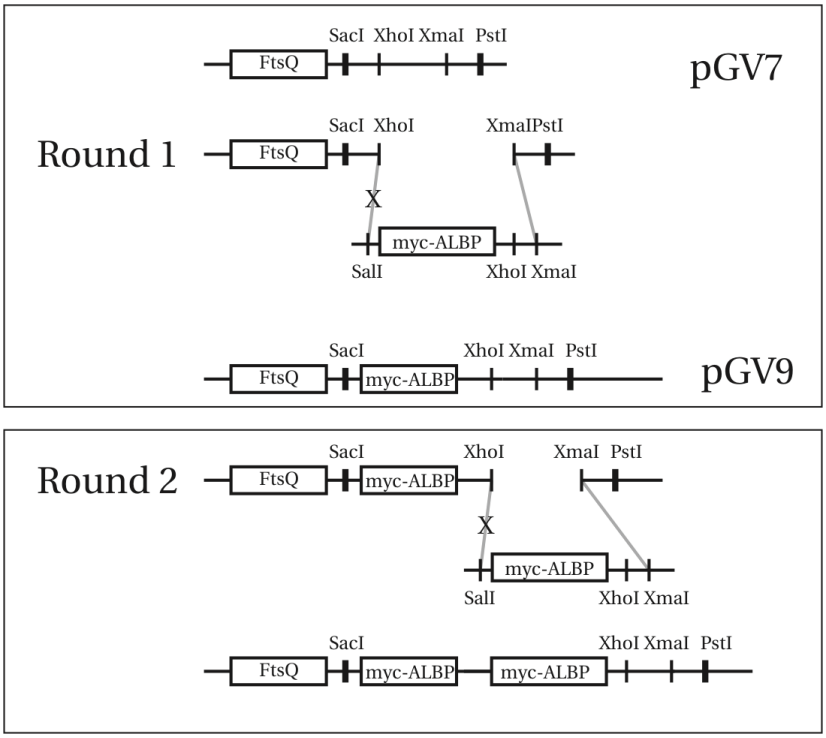

Figure 4.8. Compatible cohesive-ends cloning strategy to clone a series of identical repeats. The insert containing the myc-ALBP spacer fragment contains three restriction sites of which two have compatible cohesive-ends that annihilate each other (SalI/XhoI). Cutting the vector with XhoI/XmaI allows directional cloning of the insert, and after ligation, the existing XhoI site in the vector has disappeared and a new XhoI site (present in the insert) has appeared downstream of the added DNA fragment. "Round 2" shows how repeating the steps in "Round 1" would allow insertion of the next myc-ALBP fragment.

KpnIFtsQFW and FtsQSacIXhoIXmaIPstIHindIIIRV was cloned into pGEM-T, sequenced and subsequently subcloned via KpnI/HindIII into pMD208 resulting in pGV7. The ALBP gene (excluding the signal sequence) was PCR-ed from the LMC500 genome using primers SalImycALBPFW and ALBPXhoIXmaIRV, cloned into pGEM-T and sequenced. Then pGV7 was digested with XhoI/XmaI and ligated with SalI/XmaI digested pGEM-T-(myc-ALBP) to form pGV9. In this process XhoI/SalI annihilate each other and only one XhoI is left behind at the very C-terminus, allowing additional myc-ALBP building blocks to be ligated in (see **Figure 4.8**).

To add additional myc-ALBP fragments, one of two strategies can be followed. Either

by step-by-step addition of single fragments (repeating the procedure that lead to pGV9), or alternatively, as follows:

Adding ligase to purified SalI/XhoI myc-ALBP DNA generates potential inserts containing 1,2,3... myc-ALBP fragments, with each fragment in either a forward or reverse orientation. Only if all fragments are ligated in the proper orientation will XhoI annihilate all internal SalI sites. Therefore, by subsequently digesting with SalI/XhoI, only fragments that are in the correct orientation will remain, and the desired band (e.g. myc-ALBP-myc-ALBP) can be isolated from gel and ligated to XhoI-digested, dephosphorylated pGV9 vector, forming, after screening for the proper orientation, pGV13 (however, after initial attempts to create pGV13, the FtsQ project was put on hold). A list of primer sequences is available on request.

SDS-PAGE 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) heated to 99°C for 5 min and electrophoresed on 15% polyacrylamide slabs. The bio-rad semi-dry blotting apparatus was used for immunoblotting. Anti-GFP polyclonal antibody (Molecular probes) was used at 1:1000. The bands were detected using the ECL+ chemiluminescence kit (Amersham) and scanning with the STORM 860 fluorescence imager.

Immunolabeling of permeabilized cells

Cells were grown to steady state in GB1 medium at 28°C, fixed in 2.8% formaldehyde and 0.04% glutaraldehyde for 15 min and resuspended in PBS. Membranes were (partially?) dissolved by incubation for 45 min at RT with 0.1% Triton X-100 in PBS. The cells were permeabilized by incubation for 45 min at RT with a fresh solution of lysozyme (100 μg/ml) and EDTA (5 mM e.c.). Next, cells were washed three times by pelleting (7000 rpm for 5 min in a Eppendorf tabletop centrifuge) and resuspension in PBS. Cells were blocked for aspecific binding sites by incubation for 30 min at 37°C in a fresh solution of 5 mg/ml "Blocking reagent for DNA hybridization" (#11096176, Roche) in PBS. Then, primary antibody (mouse monoclonal anti-myc, Roche) was added (end concentration 50 μg/ml) and the cells were incubated for 2 h at 37°C. Then, the cells were washed with PBS/0.05% (v/v) Tween20 and resuspended in blocking reagent. Then, Donkey-anti-mouse Cy3-

conjugated secondary antibody (Jackson ImmunoResearch, USA) was added at 5 μg/ml and the cells were incubated for 30 min at 37°C. Finally, the cells were washed with PBS/0.05% Tween20, resuspended in PBS and stored at 4°C before imaging.

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). Images were processed in ImageJ.