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Come out and play

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Document Version

Publisher's PDF, also known as Version of record

Publication date:

2017

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

de Sousa Borges, A. (2017). *Come out and play: Exploring bacterial cell wall synthesis and cell division*. University of Groningen.

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CHAPTER 2

The *Escherichia coli* membrane protein insertase YidC assists in the biogenesis of Penicillin Binding Proteins

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Journal of Bacteriology **2015** 197(8):1444-50

Experiments presented in Figure 1, Figure 2, Figure 4, Table 1 and Figure S1 were performed by A. de Sousa Borges. Experiment presented in Figure 3 was performed by J. de Keyzer.

ABSTRACT

Membrane proteins need to be properly inserted and folded in the membrane in order to perform a range of activities that are essential for the survival of bacteria. The Sec translocon and the YidC insertase are responsible for the insertion of the majority of proteins into the cytoplasmic membrane. YidC can act in combination with the Sec translocon in the insertion and folding of membrane proteins. However, YidC also functions as an insertase independently of the Sec translocon for so-called YidC-only substrates. In addition, YidC can act as a foldase and promote the proper assembly of membrane protein complexes. Here, we investigate the effect of *Escherichia coli* YidC depletion on the assembly of Penicillin Binding Proteins (PBPs), which are involved in cell wall synthesis. YidC depletion hardly affects the total amount of the specific cell division PBP3 (FtsI) in the membrane, but the amount of active PBP3, as assessed by substrate binding, is reduced 2-fold. A similar reduction in the amount of active PBP2 was observed, while the levels of active PBP1A/1B and PBP5 were essentially similar. PBP1B and PBP3 disappeared from higher Mw bands upon YidC depletion, indicating that YidC might play a role in PBP complex formation. Taken together, our results suggest that the foldase activity of YidC can extend to the periplasmic domains of membrane proteins.

INTRODUCTION

Membrane proteins need to be properly inserted and folded in the membrane in order to be functional. The *Escherichia coli* Sec translocon and the YidC insertase are involved in the insertion of the majority of membrane proteins into the membrane. YidC can act in combination with the Sec translocon to facilitate the insertion and folding of membrane proteins, but can also function on its own as an insertase for so-called YidC-only substrates (1). Although YidC was discovered more than thirteen years ago (2, 3), only a few YidC-only substrates are known at present. YidC-only substrates have short translocated regions and include the F_1F_0 -ATPase subunit c (4-7), the M13 phage procoat protein (8), the mechano-sensing MscL protein (9), the Sci-1 type VI secretion system subunit TssL (10) and the Pf3 coat protein (3).

In addition to its role as an insertase, YidC can also act as a foldase for some proteins such as the sugar transporter LacY (11, 12) and mediate the proper assembly of membrane protein complexes such as the MalFGK₂ maltose transporter (13) and the MscL homopentameric pore (14). This feature might be related to the capacity of YidC to interact with the transmembrane domains of proteins that are released by the Sec translocon, whereupon YidC would facilitate the correct assembly and interaction of the transmembrane helices (15, 16). A major step in understanding the mode of action of YidC was accomplished with the elucidation of its crystal structure, providing insights on how single-spanning proteins may be inserted in the membrane involving a positively charged hydrophilic groove of YidC (17).

To identify novel YidC substrates, and to assess the role of YidC in the assembly of membrane protein complexes, inner membrane proteomes of *E. coli* cells with and without YidC have been analyzed by various groups. Depletion of YidC results in an increased chaperone response, a reduction in the levels of protein complexes and a lower level of membrane proteins with polar domains smaller than 100 amino acids (18, 19). In addition, YidC depletion results in changes in overall gene expression and increased cell length (20). The latter, also known as filamentation, is often associated with defects in cell division or cell wall synthesis. Most proteins involved in cell division and cell wall synthesis are present in low amounts and are generally not identified in membrane proteomic studies, and yet many contain at least one transmembrane segment and are thought to be part of large protein complexes (21). Nevertheless, an earlier study on YidC-

depleted cells revealed a decrease of the cell division associated ATP-binding cassette transporter-like proteins FtsEX that activates cell wall hydrolysis during division (18, 22). In contrast, the levels of penicillin binding protein 5 (PBP5) and PBP6 increased upon YidC depletion (18). The cell division protein FtsQ was not identified as a YidC substrate in proteomic studies but is often used as a substrate for *in vitro* protein translocation studies (18, 23). *In vitro*, the activities of SecYEG, the SecA translocation ATPase and the proton motive force are sufficient for the correct and complete membrane insertion of FtsQ (24). However, a kinetic effect of YidC cannot be excluded as YidC was found to interact with the transmembrane segment of FtsQ when the FtsQ nascent chain bound to ribosomes was used as a substrate in *in vitro* insertion experiments (25, 26).

In the present study, we focused on PBPs involved in cell division and cell wall synthesis. PBPs play a crucial role in cell growth by adding new peptidoglycan (PG) to the cell wall and in cell division by synthesizing new PG between two daughter cells. PBPs can be divided into two main groups, the high molecular weight (Mw) and the low Mw PBPs. *E. coli* high Mw PBPs are subdivided into class A PBPs (1A/1B/1C) that have both transglycosylase and transpeptidase activity and class B PBPs (2/3) that only have transpeptidase activity. Class B PBPs define the directionality of peptidoglycan (PG) growth, with PBP2 being required for cell elongation and PBP3 (or FtsI) required for cell division (27). Low Mw PBPs include type 4 (PBP4) and type 5 (PBP5/6/6b) carboxypeptidases, that are involved in PG modification, recycling and separation (28). Several high Mw PBPs have been found to interact with either themselves or other PBPs, forming complexes involved in cell division or cell elongation (21). PBP1B is known to dimerize (29) and to interact with PBP3 (30). PBP3 also forms dimers (31, 32), and interacts with PBP2 (33). PBPs can collectively be labeled and visualized using Bocillin-FL, a fluorescent derivative of penicillin that covalently binds to their active site (34). Importantly, binding of Bocillin-FL is also an indication for correct folding of the active site present in the (trans)peptidase domain of a PBP (34). It has to be noted that not all PBPs are detected by Bocillin-FL, since the affinity for Bocillin-FL varies per PBP and per organism. In *E. coli*, PBP1A/B, 2, 3, 4 and 5 are readily labeled and detected. Therefore, these PBPs were the focus of our study.

We show here that YidC depletion does not affect the total level of PBP3 in the membrane but that the amount of correctly folded PBP3 is reduced. A similar reduction in the level of folded PBP2 was observed, while the levels of other PBPs

remained essentially unaltered. Our results suggest that the foldase activity of YidC can extend to the periplasmic domains of membrane proteins.

METHODS

Strains and Media

E. coli strains FTL10 (MC4100-A, $\Delta yidC$, *attB::(araC P_{BAD} yidC⁺ kan^R)* (35) and JOE417 (MC4100 *araD+ftsQE14::kan/pBAD33-ftsQ*) (36) were used for depletion experiments. *E. coli* DH5 α was used for cloning. Strains were grown at 37°C on solid medium (LB Lennox plus agar) and liquid medium (LB Lennox), with 1% NaCl when required. When necessary, ampicillin (100 μ g/ml), kanamycin (25 μ g/ml) or chloramphenicol (25 μ g/ml) were added. When appropriate, LB was supplemented with 0.2% (w/v) of glucose or arabinose.

Cloning

The coding sequence for *ftsI* was amplified from *E. coli* DH5 α chromosomal DNA using the primer pair (5' CGGCGGCATATGAAAGCAGCGGCGAAAAC 3'/5' GCCGCCGGATCCTTACGATCTGCCACCTGTC 3') and cloned into the pGEM-T vector (Promega). An internal *NdeI* restriction site was removed from *ftsI* by Quiksite site-directed mutagenesis with the primer pair (5' GCACCGTGGTGCACATGATGGAAAGCG 3'/5' CGCTTCCATCATGTGCACCACGGTGC 3') according to manufacturer's protocol (Stratagene), resulting in a silent replacement of thymine 1406 for a cytosine (*ftsI**). After *NdeI/BamHI* digestion, *ftsI** was cloned into *NdeI/BamHI* digested pET-20b(+) (Novagen), resulting in plasmid pDJ125. The final construct was confirmed by sequencing.

YidC and FtsQ depletion

To generate cells depleted of YidC or FtsQ, the *E. coli* bacterial strains FTL10 (35) and JOE417 (36) were used, in which the expression of either *yidC* or *ftsQ* is under control of the arabinose promoter. YidC and FtsQ depletions were carried out as previously described (18, 37) with minor alterations. A single colony of FTL10 or JOE417 was pre-cultured in 5 ml LB with 0.2% arabinose and 25 mg/ml of kanamycin, plus 25 mg/ml of chloramphenicol for JOE417, at 37°C during the day. Pre-culture was diluted 100-fold with fresh LB containing the

same supplements and growth was continued overnight at 37°C. The overnight culture was then diluted 100 times into warm LB with 0.2% arabinose and grown at 37°C until an A_{600} of 0.6-0.8 was reached. Cells were washed in warm LB, and concentrated 3.3 fold with warm LB. For control cells, the concentrated suspension was diluted 200 fold in warm LB containing 0.2% arabinose, grown to A_{600} of 0.8 and collected. For depleted cells, the concentrated suspension was diluted 25 fold in warm LB containing 0.2% glucose (depleted cells), grown until A_{600} of 0.6-0.8, at which point the depleted cells were diluted 2 times with warm LB containing 0.2% of glucose so that the culture remained in the exponential phase. This process was repeated until the depleted cells ceased to grow, at which moment the cells were collected. After collection (8,950 x *g*, 15 min., 4°C), cells were resuspended in 50 mM Tris/HCl pH 8.0 with 20% sucrose, flash frozen in liquid nitrogen and stored at -80°C. Three independent depletions were performed with each strain.

Inner Membrane Vesicle isolation

Inner membrane vesicles (IMVs) were obtained as previously described (38), with some alterations. Frozen cells were thawed and passed through a cell disruptor (Constant systems One Shot, LA Biosystems) twice at 13 kPsi. Centrifugation was used to remove unbroken cells (2,095 x *g*, 10 min., 4°C) and debris (7,649 x *g*, 5 min., 4°C). Membranes were collected from the supernatant at 186,010 x *g* for 60 min. at 4°C and were resuspended in 50 mM Tris/HCl pH 8.0. The membrane suspension was loaded on top of a sucrose gradient composed of 1 ml of 55% sucrose, 1.8 ml of 51% sucrose, 0.8 ml of 45% sucrose and 0.8 ml of 36% sucrose (w/v) in 50 mM Tris/HCl pH 8.0. After centrifugation (444,000 x *g*, 30 min., 4°C) the IMVs, visible as a brown band in the 45% sucrose fraction of the gradient, were collected and diluted into 50 mM Tris/HCl pH 8.0. The IMVs were harvested after centrifugation (186,010 x *g*, 60 min., 4°C) and resuspended in 50 mM Tris/HCl pH 8.0 with 20% glycerol. Purified IMVs were flash frozen and stored at -80°C. Total protein concentration was determined using the Bio-Rad DC protein assay kit with BSA as standard.

Bocillin labeling

IMVs were equalized for protein concentration and incubated with 7.5 μ M of Bocillin-FL (Life Technologies) for 15 min. at 23°C with shaking. To stop

the labeling reaction, SDS-PAGE loading buffer was added and samples were incubated for 5 min. at 95°C. Samples were analyzed on 10% SDS-PAGE. Fluorescent bands were visualized using a Typhoon Trio (GE Healthcare) scanner with 488 and 526 nm as excitation and emission wavelengths, respectively. The Pre-stained Protein ladder (Thermo Scientific) was visualized with 532 and 670 nm as excitation and emission wavelengths, respectively. The experiment was performed for each set (3 in total) of depleted IMVs. Fluorescent bands were quantified with ImageQuant TL 2003.v3 (Amersham Biosciences) and the ratio between PBP band intensity in nondepleted and depleted samples within the same gel was determined.

Immunoblotting

Equal amounts of protein were loaded and separated on 10% SDS-PAGE, and transferred to a PVDF membrane according to standard protocols. Primary antibodies for protein detection were α -YidC, α -FtsZ (lab collection), α -PBP3, α -FtsQ, α -LepB (gift from J.W. de Gier), α -PBP1a, α -PBP1B (gift from W. Vollmer) and α -PBP5 (gift from T. den Blaauwen). Alkaline-phosphatase-conjugated secondary antibodies were from Sigma Aldrich. Blots were developed using the chemiluminescent substrate CDP-Star (Roche) and chemiluminescence was detected using a Fujifilm LAS 4000 imager (GE Healthcare). Images were analyzed and band intensities were quantified using the software AIDA 4.22.034 (Raytest). The ratios of band intensities between nondepleted and depleted sample were determined. The experiment was performed for each set (3 in total) of depleted IMVs.

In vitro PBP3 insertion

PBP3 was expressed from plasmid pDJ125 and synthesized as described (39) using a S135 lysate prepared from the RNase I-deficient strain *E. coli* A19 (40), T7 polymerase (Fermentas) and Easytag express ³⁵S protein labeling mix (PerkinElmer Life Sciences). When indicated, reactions were supplemented with 50 μ g/ml IMVs. After 30 min. a 10% synthesis control was collected and the remainder of the reaction mixture was treated with 6 M urea (final concentration) for 30 min. on ice to assay membrane integration (41). Vesicles were sedimented by ultra-centrifugation at 150.000 x g for 30 min. after which the supernatant was removed and the pellet resuspended in SDS-PAGE loading

buffer. Samples were analyzed by SDS-PAGE and ^{35}S -labeled proteins were detected by phosphorimaging. The experiment was performed twice using independent IMV preparations.

Microscopy

Samples for microscopy analysis were collected during YidC depletion. Samples of 1 ml culture were taken from the control and YidC-depleted cells and fixed with 1 ml of 8% formaldehyde for 30 min. at RT. After incubation, cells were washed 2 times in Phosphate Buffered Saline (PBS, 58 mM Na_2HPO_4 ; 17 mM NaH_2PO_4 ; 68 mM NaCl, pH 7.3) and resuspended in a final volume of 100 μl of PBS. Fixed cells were mounted on an agarose pad (1% w/v in PBS) and were visualized using a Nikon Ti-E microscope (Nikon Instruments, Tokyo, Japan) equipped with a Hamamatsu Orca Flash4.0 camera. Image analysis was performed using the software ImageJ (<http://rsb.info.nih.gov/ij/>).

RESULTS AND DISCUSSION

YidC depletion influences cell morphology

The effects of YidC depletion on cell morphology were analyzed using bright-field microscopy. YidC depletion resulted in cells that were both longer and wider compared to the control (Fig. 1. A, B), as previously described for a different YidC depletion strain (20). This aberrant shape is generally associated with defects in cell wall synthesis and impaired cell division. YidC depletion affects the levels of the ABC transporter FtsEX that is involved in cell wall hydrolysis during division

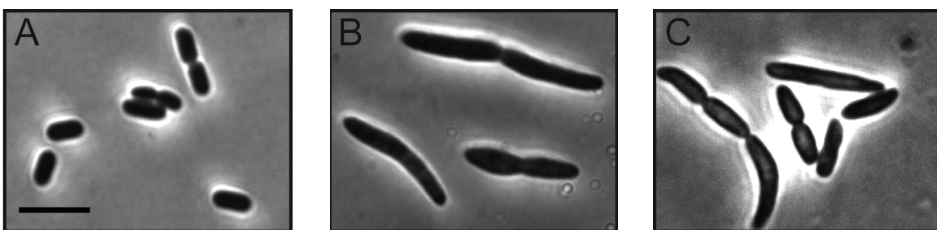


Figure 1. Cell morphology is altered upon YidC depletion. Bright-field microscopy images of fixed FTL10 cells after growth in LB with 0.2% arabinose to express *yidC* (A); fixed FTL10 cells after growth in LB with 0.2% glucose to repress the expression of *yidC* (B), and fixed FTL10 cells after growth in LB with 1% NaCl and 0.2% glucose (C). Scale bar: 5 μm .

(18, 22). To test whether the observed phenotype can be attributed to FtsEX, YidC depletion was also carried out in medium containing 1% NaCl since a high salt concentration recovers the elongated cell phenotype of *ftsEX* knockout cells (42). Since cells depleted in the presence of 1% NaCl remained both longer and wider, it is unlikely that the phenotype is caused by reduced levels of FtsEX (Fig. 1C). Therefore, we decided to analyze the effect of YidC depletion on the assembly of PBPs in the membrane.

Class B PBPs require YidC for proper folding

PBPs are a group of inner membrane proteins involved in cell wall synthesis during growth and cell division. In order to investigate the effect of YidC depletion on the PBPs, we labeled IMVs with and without YidC with the fluorescent penicillin analogue Bocillin-FL and detected the patterns of fluorescently labeled PBPs by SDS-PAGE (Fig. 2A). Efficient YidC depletion was assessed by immunoblotting

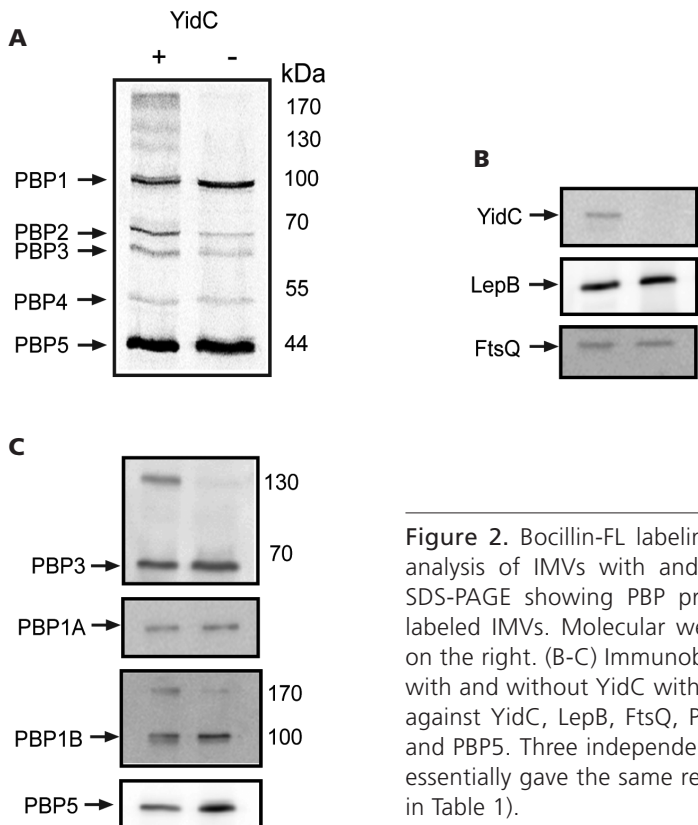


Figure 2. Bocillin-FL labeling and immunoblot analysis of IMVs with and without YidC. (A) SDS-PAGE showing PBP profiles of Bocillin-FL labeled IMVs. Molecular weights are indicated on the right. (B-C) Immunoblot analysis of IMVs with and without YidC with antibodies directed against YidC, LepB, FtsQ, PBP3, PBP1A, PBP1B and PBP5. Three independent IMV preparations essentially gave the same results (quantification in Table 1).

Table 1. Ratios of bocillin-labeled or immunodetected proteins present in IMVs upon YidC or FtsQ depletion^a

Protein	Avg ratio (nondepleted/depleted IMVs) \pm SEM ^b			
	YidC ⁺ /YidC ⁻		FtsQ ⁺ /FtsQ ⁻	
	Bocillin labelling	Immunoblotting	Bocillin labelling	Immunoblotting
YidC	ND	ND	ND	1.02 \pm 0.02
FtsQ	ND	1.16 \pm 0.18	ND	ND
LepB	ND	0.97 \pm 0.03	ND	1.1 \pm 0.1
PBP1A	1.13 \pm 0.18	1.19 \pm 0.18	0.94 \pm 0.09	0.99 \pm 0.17
PBP1B		1.07 \pm 0.09		0.81 \pm 0.13
PBP2	2.43 \pm 0.21*	ND	0.89 \pm 0.14	ND
PBP3	2.06 \pm 0.35*	1.17 \pm 0.11	1.17 \pm 0.19	1.2 \pm 0.11
PBP4	1.86 \pm 0.34	ND	1.12 \pm 0.09	ND
PBP5	1.15 \pm 0.23	0.88 \pm 0.07	1.36 \pm 0.34	1.00 \pm 0.17

^a YidC⁺/YidC⁻ IMVs (three independent preparations, in duplicate) and FtsQ⁺/FtsQ⁻ IMVs (three independent preparations) were tested, and the band intensities were quantified. The ratio of proteins in nondepleted IMVs to proteins in depleted IMVs was determined. The resulting average ratios and standard error of the mean are shown.

^b*, samples in which the levels of detected protein were significantly different; ND, not determined.

(Fig. 2B) and equal amounts of loaded protein were confirmed by comparison of the levels of the inner membrane protein leader peptidase (LepB) (Fig. 2B, Table 1), which remained constant upon YidC depletion (43). The absence of YidC resulted in the disappearance of some fluorescent bands running at a molecular mass higher than 100 kDa, which is indicative of complexes of PBPs (with themselves or other proteins) that have not been completely disintegrated upon treatment with heat and sample buffer (Fig. 2A). Quantification of the fluorescence associated with the bands revealed that Bocillin-FL labeled class B PBPs 2 and 3 were over 2-fold reduced in the absence of YidC and that the difference between the samples from nondepleted and depleted cells was significant (Table 1). Also, PBP4 was nearly 2-fold reduced. Although the reduction was robust in the gels, this difference was not significant. The levels of PBP1 and PBP5 were essentially not affected by YidC depletion (Table 1).

The decrease in the levels of labeled PBP2 and PBP3 can be caused by either a decrease of the total amounts of these proteins in the membrane or by a reduced folding of the protein, resulting in a misfolded transpeptidase domain that no longer binds Bocillin-FL. Therefore, we assessed the levels of various PBPs in the IMVs by immunoblotting. Levels of PBP1A, 1B and 3 were essentially similar (Fig. 1C and Table 1). PBP5 levels appeared to be slightly increased upon YidC depletion (Fig 1C), in agreement with an early study (18); however, this difference was not significant (Table 1). Both PBP3 and PBP1B antibodies cross-reacted with two bands, one corresponding to a PBP3 or PBP1B monomer and one at a higher Mw that was reduced upon YidC depletion. The absence of specific antibodies prevented us from performing the similar experiments for PBP2 and PBP4.

PBP3 and PBP1B are known to interact (30). The presence of both PBP3 and PBP1B in higher Mw complexes and the disappearance of these bands upon YidC depletion suggested that YidC might facilitate this interaction. Careful analysis of the high Mw bands found with antibodies against PBP1B and PBP3 revealed that these bands do not overlap each other (although they overlap the bocillin-labeled high Mw bands), suggesting that they do not correspond to a putative PBP3/PBP1B complex (see Fig S1 in the supplemental material). The absence of the PBP3/PBP1B complex is probably caused by complex disruption during sample preparation as the nondepleted sample also did not show overlapping bands of PBP3 and PBP1B (see Fig. S1 in the supplemental material).

The high Mw bands may correspond to PBP1B and PBP3 homodimers or to complexes of PBP1B and PBP3 involving other, as-yet-unknown proteins. PBP3 was shown to homodimerize *in vivo* (32) and the crystal structure of the periplasmic domain of PBP3 was recently determined, supporting the evidence of PBP3 dimers (31). PBP1B, a bifunctional transglycosylase enzyme, is known to form dimers *in vivo* (44) that are capable to synthesize glycan chains and cross-link the peptide bridges *in vitro* (29). As outlined above, we note that our findings do not exclude the possibility that the high Mw bands correspond to complexes containing PBP1B and PBP3 with other proteins. However, given that both proteins form dimers *in vivo* we think that the high Mw bands containing PBP3 and PBP1B correspond to homodimers and that YidC is involved in formation or stabilization of PBP dimers.

Together, these results indicate that in the absence of YidC the total levels of PBPs in the membrane are hardly affected but that at least a considerable fraction of the class B PBPs PBP2 and PBP3 no longer bind a fluorescent substrate analog, an observation indicative of misfolding of the periplasmic peptidase domain. The absence of high Mw forms of PBP1B and PBP3 suggests that their capacity for complex formation is disturbed. This suggests that the YidC foldase activity (11-14) can extend to the periplasmic domains of membrane-anchored proteins.

PBP3 insertion is not dependent on YidC

The presence of PBP3 in YidC-depleted IMVs (Fig. 2C) suggested that, similar to other monotopic membrane proteins with periplasmic domains (24), PBP3 does not require YidC for membrane insertion. We performed an *in vitro* synthesis and membrane insertion assay for PBP3 using YidC-depleted IMVs. *In vitro*-synthesized PBP3 inserted into the membrane of IMVs independent of the presence of YidC (Fig. 3). This experiment confirms that YidC is not required for membrane insertion of PBP3.

PBP2 and PBP3 folding does not depend on FtsQ

PBP3 is an essential cell division protein that requires FtsQ for localization to the cell division site (45). Since FtsQ has been reported to interact with YidC

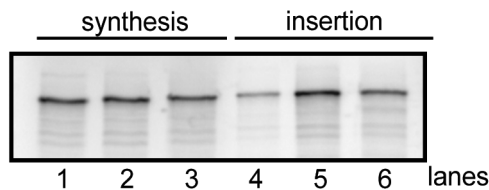


Figure 3. PBP3 membrane insertion is independent of YidC. *In vitro* membrane insertion assay using YidC depleted and control IMVs. ^{35}S -labeled PBP3 was synthesized in the absence of IMVs (lanes 1,4) or in the presence of IMVs with (lanes 3, 6) or without YidC (lanes 2,5). *In vitro* synthesis of ^{35}S -labeled PBP3 was similar irrespective of the presence of IMVs (lane 1-3, 10% of total synthesis for each reaction). Subsequently, IMVs were incubated with 6M urea followed by recovery of the IMVs by ultracentrifugation. The presence of membrane inserted ^{35}S -labeled PBP3 in the pellet fractions was analyzed (lane 4-6). The experiment was performed twice using independent IMV preparation, and a representative result is shown.

(24-26), we considered the possibility that the effect on PBP3 folding upon YidC depletion is indirect, resulting from improper assembly of FtsQ. To exclude this possibility, we first determined the levels of FtsQ upon YidC depletion and observed similar levels of FtsQ in YidC-depleted cells (Fig. 2B, Table 1). This suggests that FtsQ does not require YidC for its assembly, in agreement with previous findings that showed that membrane insertion of FtsQ is not affected in the absence of YidC (23) and that FtsQ requires only SecYEG and the proton motive force for proper insertion (24). Second, the effects of FtsQ depletion on PBPs were investigated by Bocillin-FL labeling and immunoblotting of IMVs isolated from cells depleted for FtsQ. Controls showed depletion of FtsQ, with similar levels for LepB and YidC, indicating equal loading (Fig. 4B, Table 1). Bocillin-FL labeling of IMVs of FtsQ-depleted cells did not show substantial differences in the levels of labeled PBPs. Fluorescent bands above 100 kDa were not absent but appeared to be reduced upon FtsQ depletion. Immunoblotting confirmed that the levels

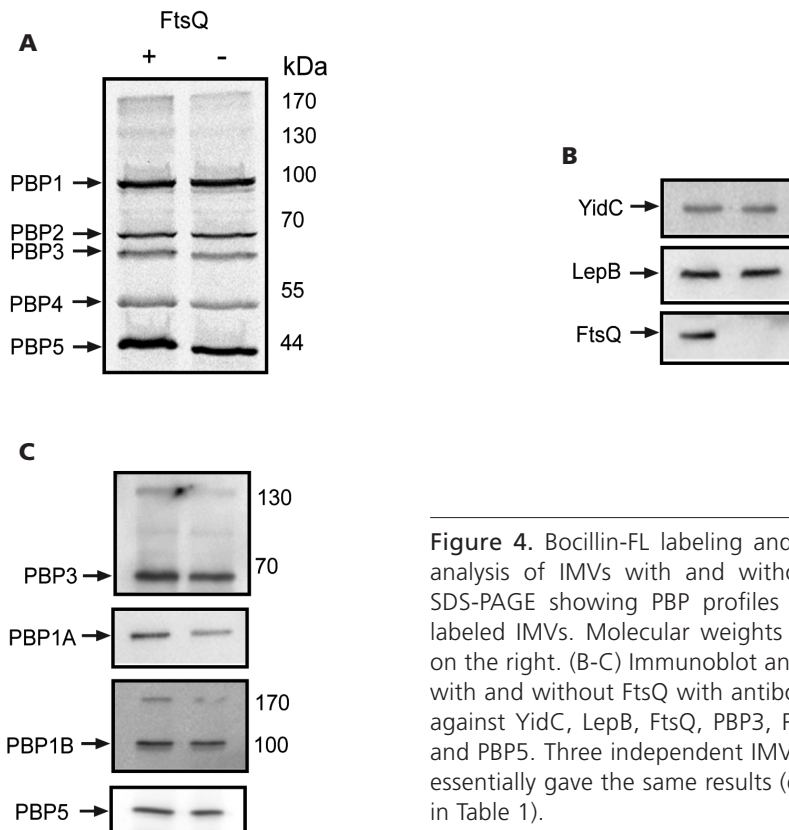


Figure 4. Bocillin-FL labeling and immunoblot analysis of IMVs with and without FtsQ. (A) SDS-PAGE showing PBP profiles of Bocillin-FL labeled IMVs. Molecular weights are indicated on the right. (B-C) Immunoblot analysis of IMVs with and without FtsQ with antibodies directed against YidC, LepB, FtsQ, PBP3, PBP1A, PBP1B and PBP5. Three independent IMV preparations essentially gave the same results (quantification in Table 1).

of PBP1A and PBP5 were similar in these samples (Fig. 4C, Table 1). The slight disappearance of the high Mw bands, corresponding to complexes, in the absence of FtsQ could be due to a reduction of FtsQ-PBP3 complexes or other membrane protein complexes involved in cell division (46). Our results show that the folding defects in PBP3 (and other PBPs) upon YidC depletion are not an indirect effect of an FtsQ assembly defect.

CONCLUSION

Here, we report that the correct folding of the class B transpeptidases PBP2 and PBP3 is dependent on the presence of the membrane protein insertase/foldase YidC. Depletion of YidC leads to wider cells that have a division defect, which is in accordance with a reduction in function of the elongation and division specific class B transpeptidases PBP2 and PBP3. Importantly, whereas earlier reports already identified YidC as a foldase/chaperone for proteins with multiple transmembrane segments (11-14), we report here a role for YidC in the correct assembly of periplasmic domains of membrane proteins. We observed that the absence of YidC hardly affects the total amount of PBP3 in the membrane but more than halves the amount of correctly folded PBP3, as determined by substrate binding. The amount of correctly folded PBP5 is also slightly decreased even though the total amount of PBP5 increases upon YidC depletion, as noted earlier (18). Transcriptome analysis showed a 2.45-fold upregulation of *dacA* (which codes for PBP5) transcription upon YidC depletion (20) but it is not clear whether this is a direct effect of the diminished amount of correctly folded PBP5 in the cells or caused by another regulatory mechanism. PBP6 levels have also been found to increase upon YidC depletion (18), but as PBP6 is not labeled with Bocillin-FL, we have not been able to determine whether YidC depletion affects PBP6 folding.

We also noted that various PBPs are no longer present in higher Mw complexes in the absence of YidC. *Bacillus subtilis* class B PBPs also require a chaperone, the lipoprotein PrsA (47), for correct folding. PrsA is a peptidyl-prolyl *cis-trans* isomerase that is conserved in Gram-positive organisms. It may be that the effect of YidC on PBP folding in *E. coli* is indirect, with YidC required for folding of a factor that aids in PBP biogenesis. Nevertheless, such a factor would also (predominantly) be located in the periplasm, and thus this would still extend

the role of YidC to the assembly of periplasmic protein domains. It remains to be determined how exactly YidC exerts its function as a foldase.

ACKNOWLEDGEMENTS

We are grateful to Prof. Waldemar Vollmer (The Centre for Bacterial Cell Biology, Newcastle University, UK), Dr. Jan-Willem de Gier (Department of Biochemistry and Biophysics, Stockholm University, SE), Dr. Tanneke den Blaauwen (Department of Bacterial Cell Biology, University of Amsterdam, NL) and Dr. Mohammed Terrak (Centre for Protein Engineering, University of Liège, Belgium) for the generous gift of antibodies.

This work was funded by a doctoral grant to ASB (SFRH/BD/78061/2011) from POPH/FSE and FCT (Fundação para a Ciência e Tecnologia) from Portugal and a VIDI grant, to DJS, from the Netherlands Organisation for Scientific Research (NWO).

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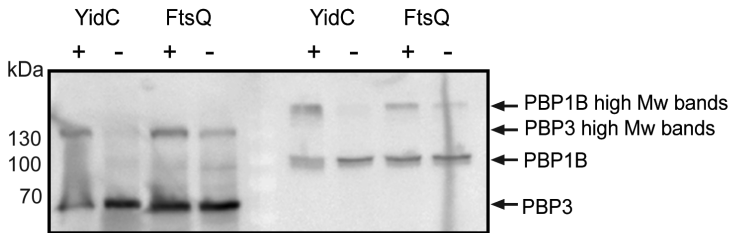
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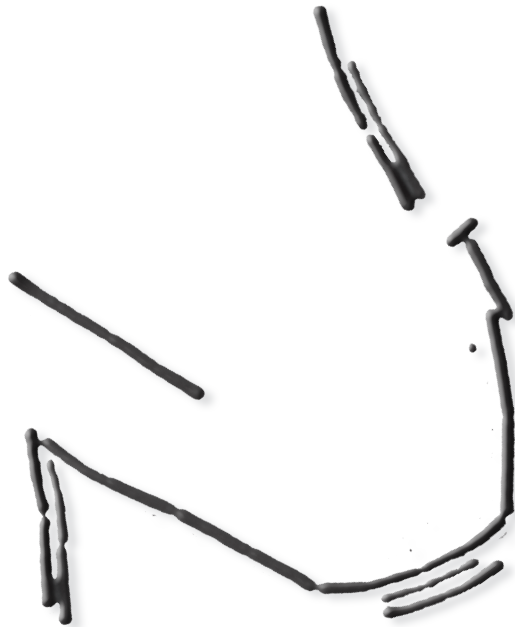
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SUPPLEMENTAL MATERIAL



Supplemental Figure 1. Immunoblot analysis of PBP3 and PBP1B. IMVs with and without YidC or FtsQ were loaded in duplicate on a gel and blotted onto a single membrane. The membrane was divided in the middle (arrows) and the left part was developed with anti-PBP3 and the right part with anti-PBP1B. Both monomer and higher Mw forms are detectable for both proteins. The higher Mw bands run at different heights and might correspond to dimers or complexes with other proteins, but not to a PBP3/PBP1B complex.



Bacillus subtilis cells forming a smiling face
Figure 6B, Chapter 4