

Final Report Return Grant, Adeline Derouaux.

Interactions and dynamic of the main *E. coli* divisome proteins in membranes.

Introduction and Objectives.

The bacterial cell wall peptidoglycan (PG) is essential to prevent lysis due to the internal osmotic pressure and determines the shape of the cell. PG synthesis is the target of the most important antibiotics as its unique structure is found only in bacteria. PG need to be synthesized in coordination with cell growth and division.

During *E. coli* cell division, a membrane protein complex called the divisome synthesizes the PG of the septum that will become the two new cell poles. Localization of the division site depends on the cytoskeletal FtsZ ring. The divisome complex contains a septal PG synthesis machinery which is composed of the main PG synthase, the class A **PBP1B** which is a periplasmic protein with a transmembrane segment responsible for both glycan chain synthesis and peptide crosslinks of the PG, the class B **PBP3** which is a periplasmic protein with a transmembrane segment essential for cell division but for which no activity of PG peptide crosslinking has been observed in vitro so far, **FtsW** which has 10 transmembrane segments and plays essential role as a lipid II flippase that brings the substrate to PBP1B, **FtsN** which is a periplasmic protein with a transmembrane segment that activates PBP1B synthase activity and could stabilize the cell division ring, and the lipoprotein **LpoB** which activates the crosslinking activity of PBP1B from the outer membrane. Interactions between those 5 proteins have been studied in vivo and in vitro. PBP1B interacts directly with PBP3, FtsN and LpoB. FtsW and PBP3 form a sub-complex. PBP3 interacts directly with FtsN (Figure 1).

The aim of my project was to analyze the interactions and the orientation of the *E. coli* divisome proteins cited above anchored to the membrane in order to have a dynamic picture of that complex. Then, we would like to show if these interactions are essential for cell division. Our study should provide a better understanding of the dynamical process of bacterial cell division that is a fundamental biological process. Furthermore, detailed knowledge of the essential interactions between bacterial cell division proteins and better understanding of PG morphogenesis might lead to the identification of novel targets and strategies for antimicrobial therapy.

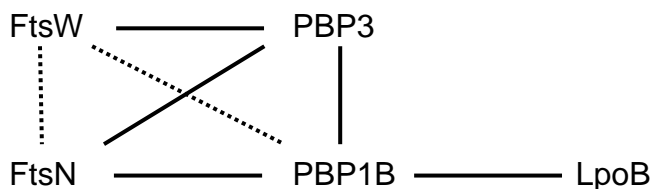


Figure 1. Direct interactions between proteins of the septal PG synthesis machinery studied in this project (straight lines), interaction by the two-hybrid technique (dotted lines).

Methodologies and results.

We planned to produce and purify the full length versions of 4 proteins from the *E. coli* septal PG synthesis machinery (PBP1B, PBP3, FtsW and FtsN). Then, we planned to reconstitute them in lipidic vesicles for structural studies. The outer membrane protein LpoB and its interactions has been studied by Dr W. Vollmer (Newcastle University, UK), with whom we collaborate.

1. Protein purifications

The membrane proteins PBP1B and FtsN were purified as described previously by our laboratory.

Concerning the purification of integral membrane protein His-FtsW, we had to optimize the protocol of expression and purification. The key steps were the production in TB medium, in *E. coli* C43(DE3) at 37°C, induction at an OD₆₀₀ of 0.8-1 with 1 mM IPTG for 4h, classical membrane preparation and extraction at room temperature using a Potter and 2% dodecylmaltoside (DDM) as detergent added after homogenization. After HisTrap purification, we obtained good quantity of FtsW with some degradations that we could remove by ion exchange. But with time, the degradations tended to form again.

The membrane protein His-PBP3 was produced with many degradation products, our option was to improve its quality by overproducing it together with its partner FtsW. The same strategy was used to try to reduce the degradations of FtsW (see point 3).

2. Reconstitution of purified proteins in lipidic vesicles

2.1. PBP1B and FtsN vesicles

We reconstituted PBP1B and FtsN in lipidic vesicles alone or together. We encountered a technical problem to form vesicles in sufficient quantity and with the good ratio protein/lipid in order to do our structural studies. The salt concentration essential for the solubility of our proteins was responsible for the low efficiency of our reconstitution. We solved the problem by adding 25% sucrose to our proteins so that we could reduce the salt concentration to 300 mM and obtain more vesicles of good quality. We mixed 5 mg of dried *E. coli* polar lipids with 0.5 mg of membrane proteins in DDM. We removed the DDM by SDR Hyper D (Pall Life Sciences) or Biobeads (Biorad), and purified multilamellar vesicles (MLV) on a sucrose gradient (40 to 5%). We put a sample of the vesicles on SDS-PAGE (Figure 2A), digested a sample by proteinase K. As the protein bands disappeared we supposed that all the proteins are oriented outside the vesicles (data not shown). We showed that the PBP1B is able to synthesize PG from the lipid II substrate in vesicles (Figure 2B). We verified the presence and the quality of the vesicles in an electron microscope (Figure 3).

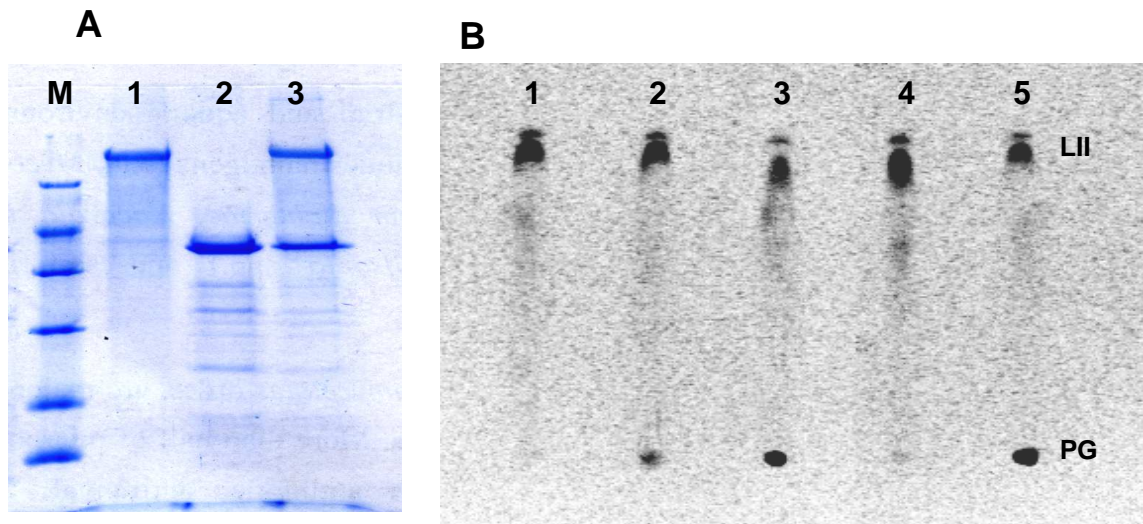


Figure 2. A. SDS-PAGE of vesicles containing PBP1B (1), FtsN (2) and both proteins (3). B. Activity test for PBP1B in vesicles. Thin liquid chromatography of radioactive lipid II (LII) polymerised by PBP1B in PG. Lipid II alone (1), PBP1B in detergent (2), vesicles with PBP1B (3), vesicles with FtsN (4), vesicles with PBP1B and FtsN (5).

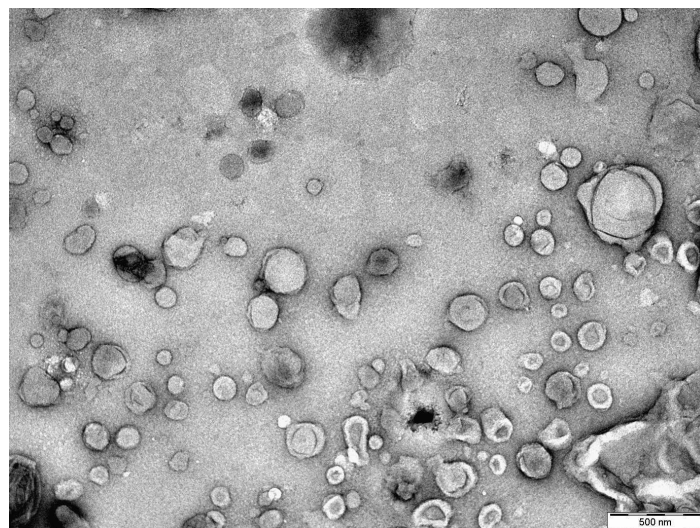


Figure 3. TEM micrograph of vesicles containing PBP1B and FtsN.

2.1.1. ATR-FTIR measurement of PBP1B and FtsN in vesicles

Attenuated Reflection Fourier Transformed Infrared spectroscopy (ATR-FTIR) is a useful technique to study the structure of proteins in membranes. Proteins in membranes are dried on a trapezoidal plate of Germanium transparent for IR to form oriented multilayer arrangements (Figure 4A). It allows the evaluation of secondary structures in presence of lipids, the detection of secondary structure changes, the determination of the orientation of secondary structures by linear dichroism and the measurement of hydrogen/deuterium exchange kinetics. The interesting regions of the spectrum for proteins are between 1800 and 1400 cm^{-1} (Figure 4B). The absorbances of the amide I ($1600\text{--}1700\text{ cm}^{-1}$, a carbonyl stretching vibration) are correlated to the secondary structure of proteins.

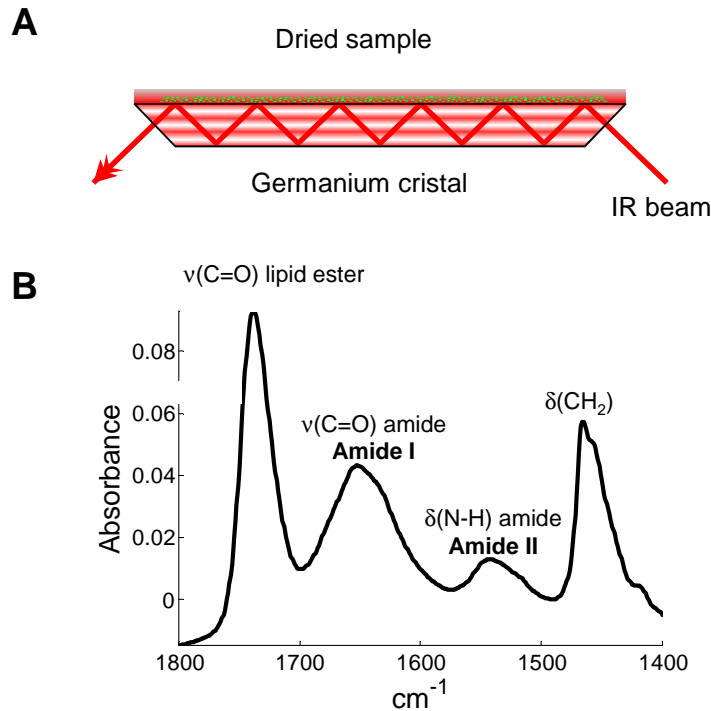


Figure 4. A. Schematic representation of a Germanium crystal used in ATR-FTIR experiment with the dried sample and the IR beam. B. Example of an ATR-FTIR spectrum of PBP1B in lipids.

We dried the MLV containing the reconstituted proteins on the Germanium crystal and did ATR-FTIR measurements in Prof. E. Goormaghtigh lab (Free University of Brussels, Belgium). Concerning the vesicles containing FtsN alone, the secondary structure content for FtsN was: 57.5% α -helices, 0.3% β -sheet, 12.3% turns, 32.8% random. The experiments of linear dichroism did not allow us to assign an orientation to FtsN maybe because of its high flexibility. Hydrogen/Deuterium (H/D) exchange kinetics during 75 min. showed a reduction of the amide II band (Figure 5A). The area of this band is plotted in function of time (Figure 5B).

The analysis of PBP1B vesicles showed a slower rate of exchange of the hydrogens than in FtsN vesicles and the vesicles containing both PBP1B and FtsN didn't give reproducible results. We probably need more controls on the vesicles to be sure that we work each time with the same material (same ratio protein/lipid, same orientation of the proteins, same size of vesicles,...). We tried also others protocols for vesicles formation in order to obtain reproducible results when more than one protein is reconstituted without more success.

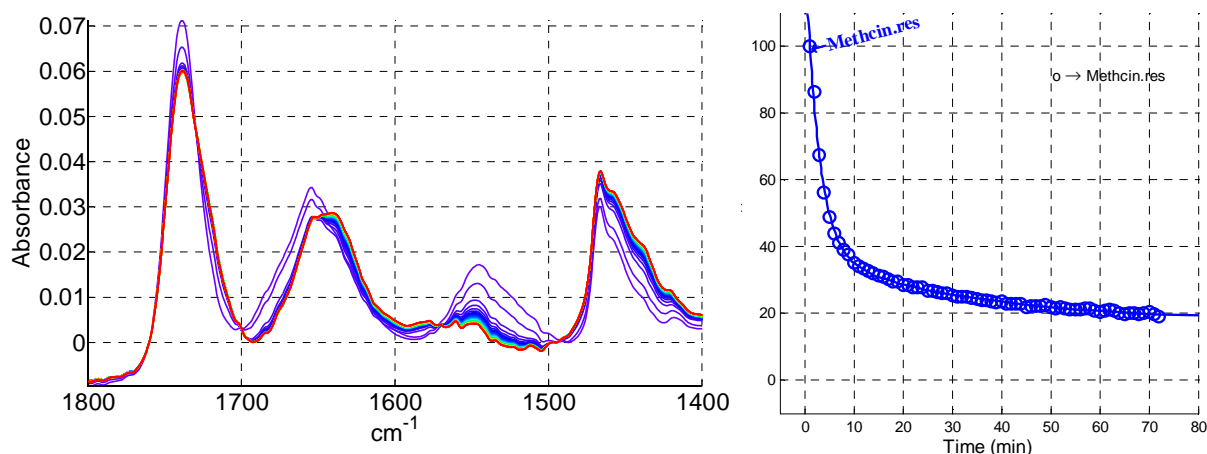


Figure 5. A. ATR-FTIR spectra of FtsN vesicles during an H/D exchange of 75 min. (from blue to red, 1 scan/min.). B. Decrease of the amide II band area of FtsN vesicles in function of time.

3. Complex purifications

To increase the quality of our membrane proteins and in the aim to isolate complexes of 2 or more protein partners, we cloned the genes of PBP1B, PBP3, FtsW and FtsN in Duet vectors that allow to co-produce 2 proteins by vector and we used 2 compatible vectors (pETDuet1 and pACYCDuet) to produce our 4 proteins, 2 of them with a purification tag (see Table 1).

Table 1. List of the co-production plasmids (*ftsI* is the PBP3 gene).

pDML2040	pETDuet1-HisftsI-ftsW	copurification of the complex HisPBP3-FtsW
pDML2041	pETDuet1-HisftsW-ftsI	copurification of the complex HisFtsW-PBP3
pDML2043	pETDuet1-HisftsI-ftsWHA	purification of HisPBP3, no complex formed
pACYCDuet-PBP1B-FtsNStrep		copurification of the complex PBP1B-FtsNStrep

3.1. Complex between FtsW and PBP3

The pDML2040 and pDML2041 (Table 1) were used to co-produce and co-purify FtsW and PBP3. With both plasmids, we obtained a complex of both proteins after HisTrap purification, the His-tag being fused to PBP3 (pDML2040) or to FtsW (pDML2041) (Figure 6). In contrary, with the pDML2043, we didn't obtain a complex between HisPBP3 and FtsW, but we obtained very good quality HisPBP3 alone with very little degradation that we used for others experiments (Figure 7). The only difference between pDML2040 and pDML2043 is the presence of the HA epitope in the loop between transmembrane segments 7 and 8 of FtsW. This change in the loop seems to destabilize the interaction between the 2 proteins. This loop might be in direct interaction with PBP3.

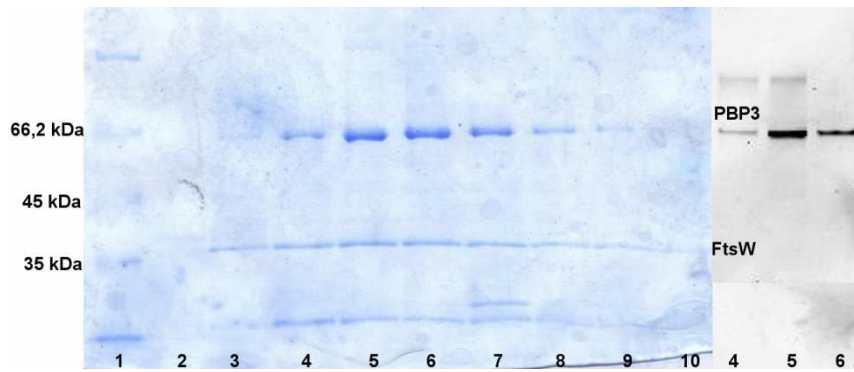


Figure 6. SDS-PAGE of the complex HisFtsW-PBP3 produced from pDML2041 after HisTrap purification. On the right, fluorescent ampicillin labelling of PBP3. Molecular weight ladder (1), elution from HisTrap column (2-10). On the right, ampicillin fluorescent labelling of PBP3.

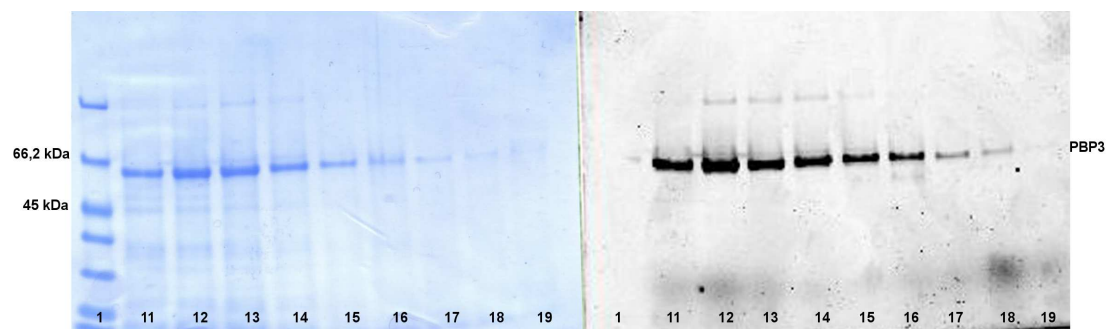


Figure 7. SDS-PAGE of PBP3 produced from pDML2043 after HisTrap purification. Molecular weight ladder (1), elution from HisTrap column (11-19). On the right, fluorescent ampicillin labelling of PBP3.

3.2. Complex between PBP1B and FtsN

The pACYCDuet-PBP1B-FtsNStrep has been used to produce the complex PBP1B-FtsNStrep. After StrepTactin purification, we eluted a complex containing FtsN and PBP1B. FtsN is present in larger quantity than PBP1B, so we might have purified some FtsN alone in addition to the complex (Figure 8).

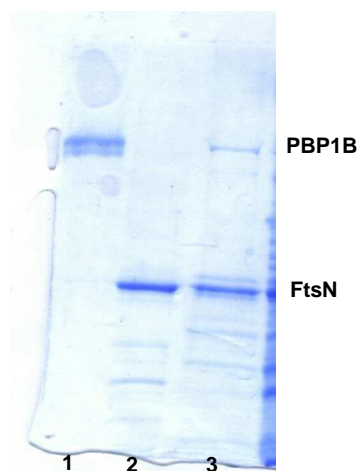


Figure 8. SDS-PAGE of PBP1B-FtsNStrep complex produced from pACYCDuet-PBP1B-FtsNStrep. Purified PBP1B (1), purified HisFtsN (2), purified complex PBP1B-FtsNStrep (3).

3.3. Complex with the 4 proteins PBP1B, FtsN, FtsW and PBP3.

E. coli C43(DE3) were transformed by both pDML2041 and pACYCDuet-PBP1B-FtsNStrep. Purification on StrepTactin didn't give any protein at the elution step. FtsNStrep was in the unbound fraction. Purification on HisTrap gave mainly FtsW with degradations. We couldn't obtain a complex with the 4 partners, not even reproduce the complexes with 2 of them that we obtained previously with one plasmid at a time in the cells.

We tried then to produce the proteins 2 by 2 in 2 different cultures and to mix the cells before membrane preparation. None of the proteins bound on the StrepTactin. But with the HisTrap, we purified a complex containing 3 of the 4 proteins : PBP1B, FtsW and PBP3 (Figure 9). The degradation of FtsW was still present and FtsN was found in the unbound fraction.

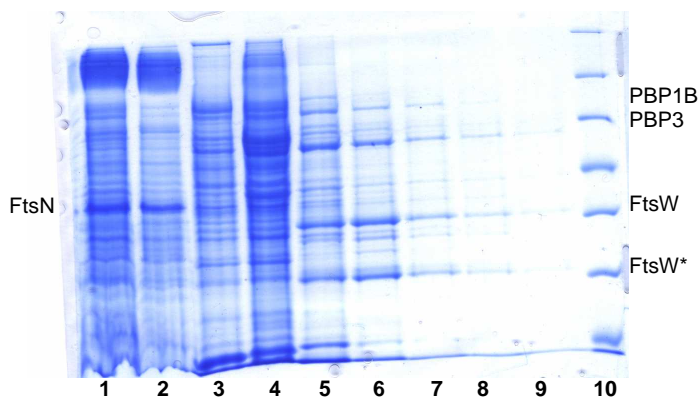


Figure 9. SDS-PAGE of PBP1B-HisFtsW-PBP3 complex produced from pDML2041 and pACYCDuet-PBP1B-FtsNStrep in separate cultures and purified on a HisTrap column. Solubilized membrane extract (1), unbound fraction (2), washes (3-4), elution (5-9), molecular weight ladder (10). FtsW* is the degradation product of FtsW.

The complex containing PBP1B, PBP3 and HisFtsW was concentrated on a 100 KDa cut off Amicon and loaded on a gel filtration column (Superdex 200). The complex was stable enough to be concentrated on high cut off and to elute from the gel filtration in one peak (Figure 10).

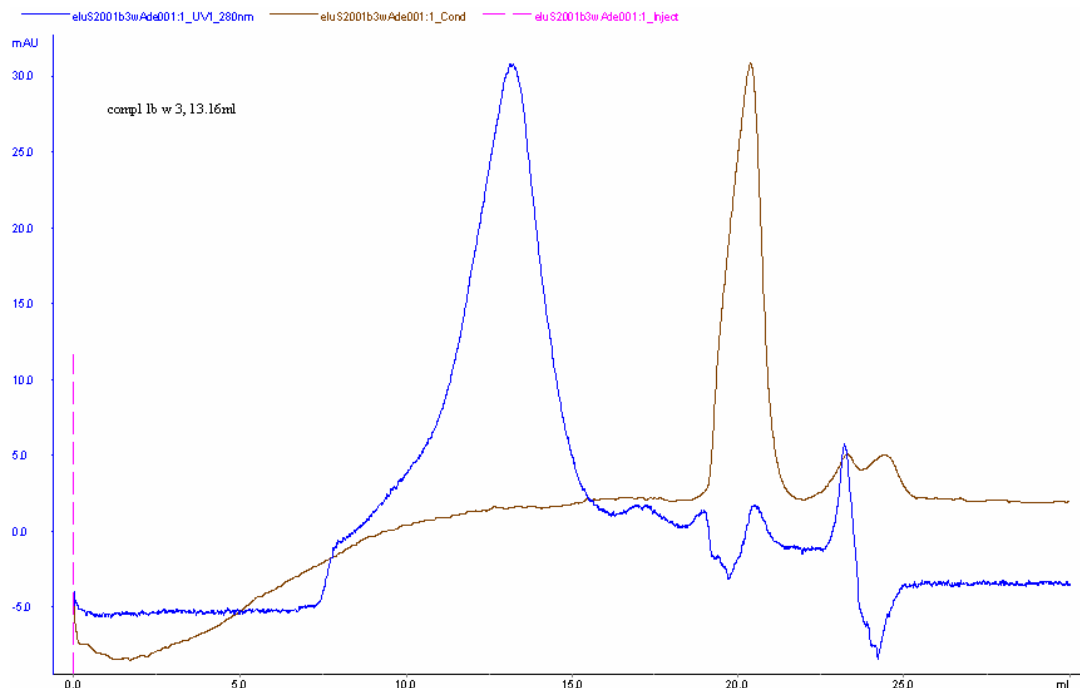


Figure 10. Chromatogram of the gel filtration Superdex 200 of the complex PBP1B-PBP3-HisFtsW. Blue: absorbance at 280 nm, brown: conductivity.

4. Influence of the PBP1B partners on its activity

PBP1B synthesizes the PG at the division site. As PBP3, FtsW and FtsN interact with PBP1B, they might modulate its activity. The impact of these proteins on glycan chain production has been tested using the radioactive PG precursor, lipid II. The reactions were loaded on a TrisTricine SDS-PAGE. The lipid II migrates at the bottom of the gel, the glycan chains are distributed in the gel in function of their length, and the crosslinked chains stay in the well. Our data showed an increase in the quantity of lipid II used in the presence of the complex FtsW-PBP3. This increase is not observed if we add the 2 proteins purified separately. FtsW alone seems to inhibit glycosyltransferase activity of PBP1B and to delay the migration of lipid II in the gel probably by interacting with it (Figure 11). In our assay, PBP3 was unable to crosslink the glycan chains as do the PBP1B wt (well 12, Figure 11).

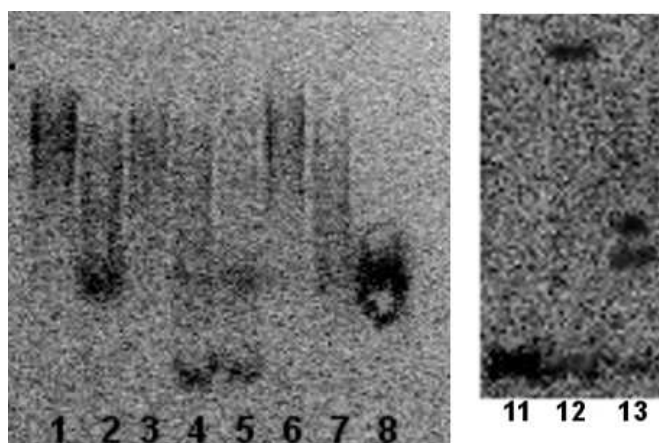


Figure 11. TrisTricine SDS-PAGE of the influence of FtsW, PBP3 and FtsN on PBP1B activity. (1) Lipid II, PBP1B*, complex FtsW-PBP3 ;(2) Lipid II , PBP1B*, PBP3 ; (3) Lipid II , PBP1B*, buffer ; (4) Lipid II, PBP1B*, FtsN ; (5) Lipid II, PBP1B* ,PBP3, FtsN ; (6) Lipid II, PBP1B* ; (7) Lipid II, PBP1B*, complex FtsW-PBP3, FtsN ; (8) Lipid II, PBP1B*, FtsW. (11) Lipid II ; (12) Lipid II, PBP1B wt; (13) Lipid II, PBP1B*, PBP3, FtsW. PBP1B* has an inactive transpeptidase domain.

5. Reconstitution of the proteins of interest alone or in complex in lipidic vesicles

FtsW, PBP3 or the complex FtsW-PBP3 were reconstituted in large unilamellar vesicles (LUV). After sucrose gradient, we observed easily vesicles containing FtsW. For vesicles containing PBP3 or the complex FtsW-PBP3, we could see some protein in the vesicles but the efficiency was very low (Figure 12). We would need to produce more protein and to improve the protocol of reconstitution for further use.

We tested the reconstitution of PBP1B together with FtsW and/or PBP3 without any success. The protein precipitated when we remove the detergent.

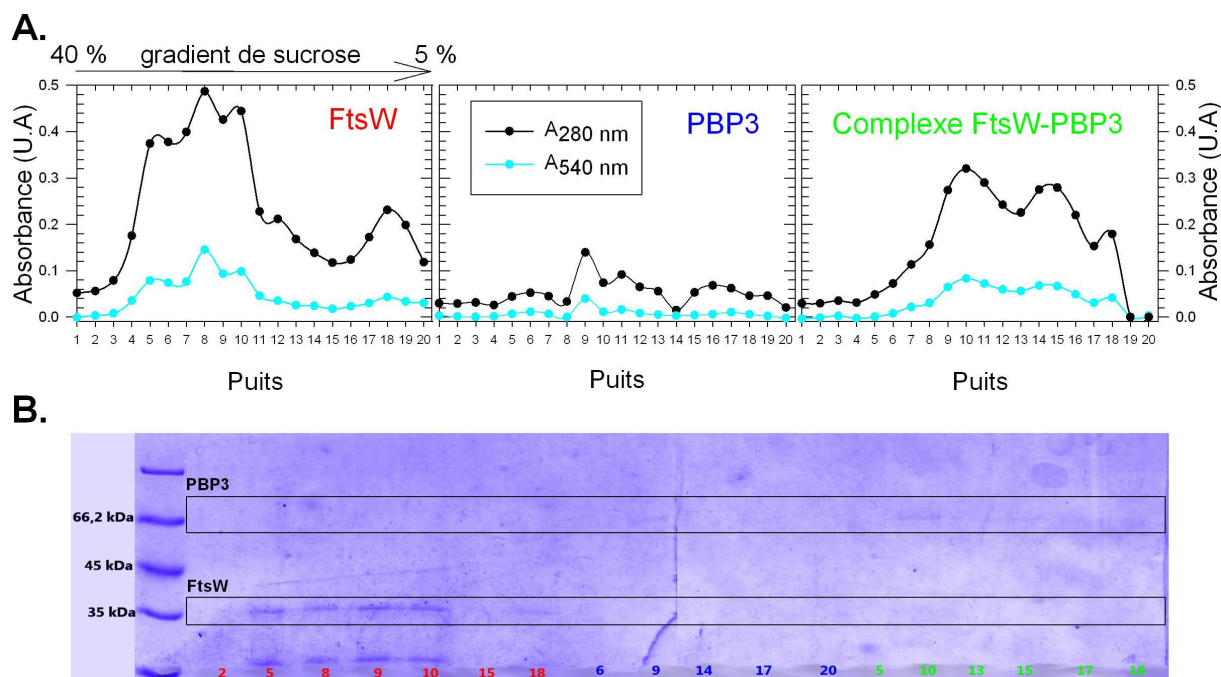


Figure 12. Measurement of the turbidity and of the protein concentration in sucrose gradient fractions (A), and SDS-PAGE of the fractions where we measured turbidity (B).

6. Conclusion and Perspectives

Concerning the production and the purification of the 4 membrane proteins, we established a protocol for all of them. The co-production of FtsW and PBP3 improved the quality of PBP3, but we still have a degradation of FtsW. We planned to add a His-tag in C-terminal to avoid the purification of the degradation.

We obtained lipidic vesicles containing each of our proteins separately. We obtained vesicles containing PBP1B and FtsN purified separately and mixed together in the lipids. PBP1B is active in vesicles. The assay of ATR-FTIR experiments on those vesicles didn't give reproducible results. More experiments are needed to reconstitute the 4 proteins together in lipidic vesicles. Tests starting from separately purified proteins didn't give much success. We planned to start now from complexes purified from the cells.

The most important result of the project has been the purification of membrane protein complexes from cells co-producing 2 partners. We could purify the complex PBP1B-FtsN, FtsW-PBP3 and even the complex PBP1B-FtsW-PBP3. The interactions of FtsN with the other partners seemed to be too weak to obtain a complex with the 4 proteins. The complex PBP1B-FtsW-PBP3 was stable enough to elute in one peak in gel filtration.

The idea is now to stabilise the complexes *in vivo* by crosslinking and to analyse the position of the crosslinks by mass spectrometry in order to identify the interfaces of interaction.

7. Side project

In parallel to my main project, I was involved in the study of glycosyltransferase that synthesize PG and their inhibition. PG synthesis inhibition leads to bacterial cell lysis, making it an important target for many antibiotics. The

final two reactions in PG synthesis are performed by the membrane inserted penicillin-binding proteins (PBPs). Their glycosyltransferase (GT) activity uses the lipid II precursor to synthesize glycan chains and their transpeptidase (TP) activity catalyzes the cross-linking of two glycan chains via the peptide side chains. Inhibition of either of these two reactions leads to bacterial cell death. β -Lactam antibiotics target the transpeptidation reaction while antibiotic therapy based on inhibition of the GTs remains to be developed.

Our main objective was to develop tests for high throughput screening of compounds that inhibit GT independently of the use of the substrate lipid II that is difficult to synthesize and expensive. We chose the MtgA from *Staphylococcus aureus* as model enzyme because it is a monofunctional GT (without any TP domain) active without its transmembrane segment and its structure is known.

One tryptophan was added at different position in the active site of the MtgA (Figure 13). The fluorescence of the tryptophan was measured in presence of increasing concentration of moenomycin, a known inhibitor of GT (Figure 14). We observed a decrease in the fluorescence intensity when moenomycin binds to the active site up to a ratio moenomycin/MtgA of ~ 1 , indicating a specific binding of one molecule of moenomycin to one molecule of MtgA with a high affinity. This binding test will be done in microtiterate plates with libraries of compounds in order to identify potential inhibitory compounds.

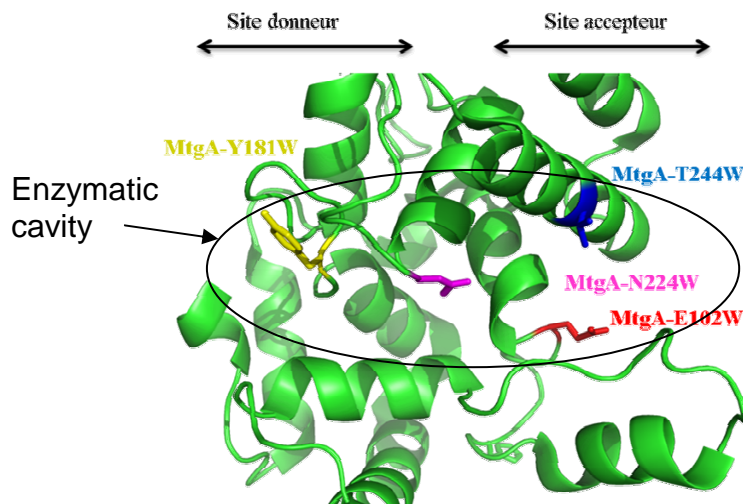


Figure 13. Positions of the different tryptophans inserted in the MtgA from *S. aureus*.

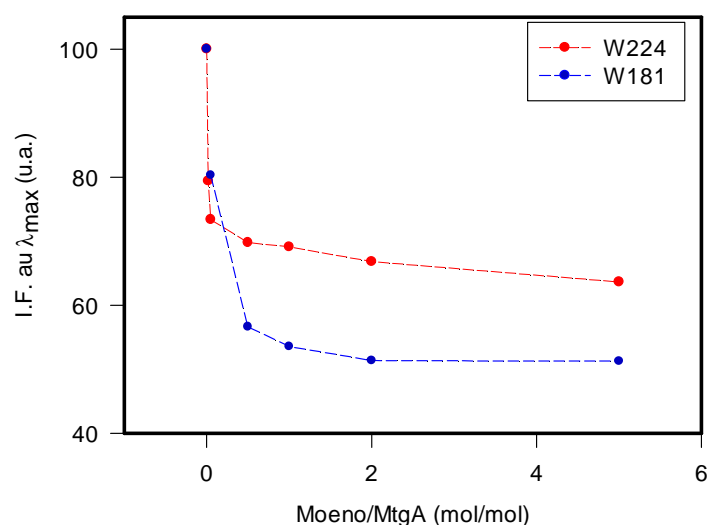


Figure 14. Fluorescence intensity at the maximum wavelength for MtgA-N224W (red) and MtgA-Y181W (blue) with increasing concentration of moenomycin.

Diffusion and valorisation.

Publications:

The outer-membrane activator of peptidoglycan synthesis LpoA has an N-terminal TPR domain and an elongated molecular shape. Jean N, Bougault C, Lodge A, Derouaux A, Callens G, Egan A, Lewis R, Vollmer W, Simorre JP. in preparation (work done during my PostDoc in UK, not related to BELSPO return grant)

The Crystal structure of *Escherichia coli* Penicillin-Binding Protein 3, a key player of the divisome. Sauvage E, Fraipont C, Joris M, Derouaux A, Herman R, Rocaboy M, Schloesser M, Dumas J, Lampilas M, Aszodi J, Kerff F, Nguyen-Distèche M and Charlier P. in preparation. (related to BELSPO return grant).

Study of the activities of PBP3/PBP1b from *Escherichia coli* and the effect of the immediate divisome partners. Leclercq S, Derouaux A, Terrak M. Poster presented at the Great Wall Conference, Paris, France, 23-25 September 2013. (related to BELSPO return grant).

Study of the membrane binding region of the monofunctional glycosyltransferase MtgA from *Staphylococcus aureus*. Dahmane I, Derouaux A, Terrak M. Poster presented at the Biomedica summit, 2013 Jun 19, Aachen, Germany. (not related to BELSPO return grant).

Synthesis of modified peptidoglycan precursor analogues for the inhibition of glycosyltransferase. Dumbre S, Derouaux A, Lescrinier E, Piette A, Joris B, Terrak M, Herdewijn P., J Am Chem Soc. 2012 Jun 6;134(22):9343-51. (not related to BELSPO return grant).

Peptidoglycan glycosyltransferase substrate mimics as template for the design of new antibacterial drugs. Derouaux A, Sauvage E and Terrak M., *Frontiers in Immunology* 2013;4:78 (not related to BELSPO return grant).

Bacterial Cell Wall Growth, Shape and Division. Derouaux A, Terrak M, den Blaauwen T and Vollmer W. Chapter of the book *Bacterial Membranes: Structural and Molecular Biology*, Horizon Scientific Press. 2013; 3-52. (work done during my PostDoc in UK, related to BELSPO return grant).

Study of structural changes when major *E. coli* divisome proteins interact in membrane. Derouaux A, Goormaghtigh E, Joris B and Terrak M., poster presented at the EMBO workshop on « Reconstructing the essential bacterial cell cycle machinery », 16-19 September 2012, Real Sitio de San Ildefonso, Spain. (related to BELSPO return grant).

Participation to missions or conferences:

EMBO workshop on « Reconstructing the essential bacterial cell cycle machinery », 16-19 September 2012, Real Sitio de San Ildefonso (Segovia), Spain. The objective was to meet and discuss with scientifics in my field of research and present them my work.

EMBO laboratory management course for Post-docs by Leadership sculptor, 13-15 November 2012, Leimen (Heidelberg), Germany. EMBO invited me to that course. The objectives were to think about how to be a good team leader and to give us tools to improve our leadership.

Biomedica summit, 19 June 2013, Aachen, Germany.