Institut Pasteur Minisymposium on Bacterial Membranes 2013

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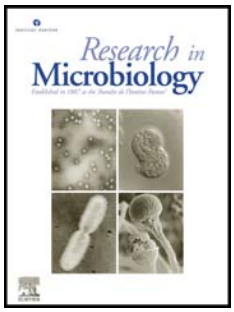

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For publication

Meeting report

Institut Pasteur Minisymposium on Bacterial Membranes 2013

The first Minisymposium on Bacterial Membranes was organized more than ten years ago and has become a successful tradition, with the 2013 edition taking place last March. This edition covered a variety of topics, including advances in the structural analysis of membrane proteins by solid-state NMR, the structure and function of components of several extracellular protein secretion systems, lipid modification and lipid-protein interactions, insertion of proteins into the outer membrane and uptake of DNA and nutrients. Over fifty young scientists from Germany, United Kingdom, Belgium and France gathered at the Institut Pasteur in Paris to share recent findings on these topics.

1. Structural analysis of membrane proteins by solid-state NMR

Structural analysis of membrane proteins is a prerequisite for understanding their function and biogenesis. Although membrane proteins form 30% of the proteome, they still represent only 1% of the structures in the Protein Data Bank. X-ray crystallography has been the main method used for structure determination. Typically produced in small quantities, requiring specific detergents for membrane extraction, flexible and dynamic, membrane proteins have been far fewer for yielding crystals of sufficient quality for X-ray diffraction analysis. Among alternative methods for structure determination, such as nuclear magnetic resonance (NMR) and electron microscopy (EM), solid-state NMR (ssNMR) has proven particularly powerful, providing structures of soluble and membrane proteins with equal success. Importantly, ssNMR is the only high-resolution technique that allows structural

determination of protein polymers or aggregates, membrane proteins in native-like environments or protein filaments / fibrils, without limitations in size and solubility. Thanks to a number of recent technical improvements, the limitations linked to the size of polymer subunits are progressively being reduced.

Antoine Loquet (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany) described some of the most recently developed labeling techniques that greatly improve the resolution and information content of ssNMR data, using combinations of sparsely labeled carbon sources (Loquet et al., 2011). These approaches have proven extremely powerful when applied to structural analysis of the type III secretion needle from *Salmonella typhimurium*, revealing the molecular bases of stable polymer formation from flexible building blocks. Typically, polymer structures are deduced by rigid docking of isolated subunit structures into the electron density maps of multimers obtained by cryo-EM. These approaches are not only limited by the resolution of EM structures, but also by the extent of conformational changes that monomers undergo upon assembly within the polymer. Contrary to previous needle models obtained using rigid docking (Fujii et al., 2012), which placed the N-terminus of the monomer in the lumen of the needle, ssNMR analysis showed that these domains, disordered and invisible in X-ray monomer structures, adopt a rigid and defined conformation within the needle. An impressively detailed interaction network could be deduced from ssNMR data with over 150 intersubunit distance restraints. In the new model, the N-terminus of the needle subunit PrgI is stabilized by specific contacts with protomer P_{-11} and exposed on the needle surface (Loquet et al., 2012). This model was confirmed by immunogold labeling of the N-terminally tagged protein PrgI, not only on needles assembled in *Salmonella,* but also on needles of the *Shigella* T3SS formed by MxiH (Demers et al., 2013).

Important components of NMR analysis are powerful computational methods, such as ARIA or ISD, that allow structural calculations based on the distance data (Rieping et al., 2008, Bardiaux et al., 2012). Since not only biological polymers, but also membrane proteins typically exist as symmetric assemblies, the use of symmetry to calculate structures can reduce calculation complexity and cost (Bardiaux et al., 2012). Benjamin Bardiaux (Institut Pasteur, CNRS UMR3528, Paris, France) described the power of combining ssNMR and computational methods based on ambiguous distance restraints to solve the structure of the *Yersinia* adhesin A (YadA), secreted via the type V pathway (Leyton et al., 2012). YadA belongs to a class of type V secretion systems called autotransporters, which cross the inner membrane of Gram-negative bacteria via the Sec system. Their N-terminal part forms the passenger domain that is secreted via a channel in the outer membrane formed by the C-terminal beta-barrel domain. Cellular Bam machinery facilitates insertion of the C-terminal domain into the outer membrane. Autotransporters are widespread and come in many flavors. *Yersinia* YadA is a member of the trimeric autotransporter family, with the outer membrane barrel formed by beta sheets derived from three polypeptides. The passenger domain of YadA has been analyzed by X-ray crystallography of its different fragments. Interestingly, poorly diffracting crystals of the outer membrane domain were used as material for ssNMR analysis, successfully providing the de novo structure of the trimer (Shahid et al., 2012). NMR analysis provided crucial information on the flexible nature of a short conserved tetrapeptide ASSA between the passenger and the beta-barrel domains. The results support the model where this sequence forms an essential hairpin that initiates secretion of the passenger domain, which is subsequently driven by folding of the passenger domain into a beta-helix structure.

Autotransporter family adhesins play important functions in bacterial colonization. In *Helicobacter pylori,* outer membrane adhesins SabA and BabA are crucial for bacterial

establishment in the hostile niche of human gastric mucosa. Gaetano Castaldo (Vrije Universiteit Brussels, Brussels, Belgium) uses X-ray crystallography to study the structure of BabA, a 721 aa protein that binds to the Lewis b antigen (Yamaoka, 2008). Using a series of constructs facilitating BabA fragment production and purification, they revealed that these adhesins have an unusual fold. Although structural analysis is required to confirm this, preliminary biochemical studies strongly suggest that the BabA passenger domain is "inserted" into the beta-barrel-encoding polypeptide, thus defining a novel class of auto-transporters.

2. Type VI secretion: killing (or not) by secreted toxins

Type VI secretion system (T6SS) analysis is currently one of the most dynamic areas of research in the field of bacterial protein transport. Although T6SSs are implicated in bacterial pathogenesis and interactions with eukaryotic host cells, their role in interspecies competition in the context of microbial communities and biofilms has been the focus of recent attention. Contact-dependent killing, discrimination between sister cells and members of different species and killing and resistance mediated by T6SSs are novel factors that need to be taken into account when considering seemingly peaceful communities of bacteria growing in close proximity on various surfaces. Yannick Brunet (University Aix-Marseille, Marseille, France) illustrated the role of T6SS in bacterial competition between two strains of the same species, the domesticated *Escherichia coli* K-12 and a pathogenic enteroaggregative *E. coli* (EAEC). Co-cultivation of these strains results in killing of the laboratory workhorse in a contact-dependent manner. The "secret weapon" of the EAEC strains, the T6S apparatus, looks like an inverted phage tail with its piercing device pointing outwards. Assembled in the bacterial envelope, it is connected to a cytoplasmic shaft harboring a hollow tube filled with

the panoply of "bullets", effector proteins with various enzymatic activities. Using live fluorescence imaging and the GFP-labeled shaft component TssB, one can observe the assembly and contraction of the shaft in a predator cell. This contraction in an EAEC cell that is in direct contact with *E. coli* K-12 leads to the lysis of prey within minutes (Brunet et al., 2012). The tube is built as a stack of Hcp subunit hexamers, which can be assembled in vitro both in a head-to-tail and tail-to-tail orientation. However, the preferred orientation in vivo is head-to-tail, suggesting a mechanism that regulates tube assembly.

Serratia marcescens strain Db10 is a significant cause of hospital-acquired infections, including in neonates. Using *S. marcescens* strain Db10 as model to study the T6SS function, Grant English (University of Dundee, UK) focused on effector proteins secreted by this system. Genome analysis identified genes encoding small highly acidic and highly basic proteins that seem to come in pairs. They are non-essential for T6SS function and the basic proteins Ssp1 and Ssp2 are indeed secreted by the T6SS. Interestingly, deletion of genes encoding their acidic partners Rap1a and, in particular, Rap2a led to severe toxicity. Bacterial filamentation caused by *rap2a* deletion was alleviated by T6SS inactivation or by deleting the *ssp2* gene, but not *ssp1*, strongly suggesting that these proteins act as toxin and immunity protein pairs. Consistent with this model, purified Ssp1 and Rap1a form a stoichiometric 2:2 complex with nanomolar affinity. Remarkably, despite similar charge properties of these pairs, binding is highly specific, since Ssp1 does not form a complex with Rap2a (English et al., 2012). While structural analysis of other members of this class Rap1b and Rap2b provides possible clues for this specificity, co-crystal structures of the cognate pairs should shed more light on the mechanism by which antitoxins block the active sites as well as on toxin activities. Recent in vitro studies show that Ssp1 is a member of cell-wall-degrading amidases. Its production in the bacterial cytoplasm is tolerated, but its periplasmic production leads to cell death, which is prevented by co-expression of its partner gene *rap1a*. Similar

results were obtained with the Ssp2 and Rap2a pair and several other toxin-antitoxin modules were found not only in the Db10 genome, but also in other species, including *Agrobacterium* and *Salmonella*. These results have interesting implications for the evolution of strains in populations, with continuous acquisition of toxin/antitoxin pairs with different specificities and enzymatic activities, contributing to rapid target killing, but also possibly propagating the same weapons and resistance proteins across species.

3. Lipid modification and lipid-protein interactions

Bacterial membranes are composed of phospholipids, proteins and modified lipids such as lipoteichoic acids (LTA), lipopolysaccharides (LPS) and lipoarabinomannan (LAM). Peter Bond (University of Cambridge, Cambridge, UK) provided structural and thermodynamic insights into the interaction between the lipid moiety of LPS and the MD-2 co-receptor of Toll-like receptor 4 (TLR4) using a molecular dynamics simulation approach (Paramo et al., 2013). Molecular simulation is based on descriptions of energy states of protein structures obtained from X-ray and NMR data (i.e. the TLR4 co-receptor MD-2) in a specific environment, here an asymmetric lipid bilayer composed of phospholipids and LPS. These energy descriptions are translated into forces and then into motion, resulting in a dynamic structural movie of a protein in a membrane. MD-2 binds the lipid A moiety of LPS (Park et al., 2009). The simulation data presented by Bond showed that MD-2 induces stronger binding of the agonist (LPS) by TLR4 and that the conformational changes predicted in MD-2 depend on the fatty acids of LPS. Sabina Chalabaev (Institut Pasteur, Paris, France) discussed the regulation of lipid A modification in biofilm-grown bacteria. Different growth conditions have been shown to lead to alterations in the composition of the bacterial cell envelope. For example, bacteria in biofilms are more resistant to antimicrobial agents and

their surface exposed polysaccharides differ in structure compared to free-living planktonic bacteria (Ciornei et al., 2010). In *E. coli* biofilms, the lipid A moiety of LPS is modified by addition of palmitate, a reaction catalyzed by PagP. This phenomenon is also observed in other biofilm-forming enterobacteria. Activation of *pagP* expression occurs via SlyA, which releases the inhibitory effect of H-NS on expression of the *pagP* gene. The PhoPQ and EvgAS pathways are not involved in induction of *pagP* expression. Muriel Masi (University Paris XI, Orsay, France) presented results of her work on the molecular basis of recognition of sugar entities within the cell wall of *Corynebacterium glutamicum* by mycoyltransferase A (MytA). The cell envelope of *Corynebacterineae* is a complex structure containing lipids, peptidoglycan and arabinogalactan that is itself covalently linked to mycolic acids, the so-called mAGPG. Preliminary structural data on the N-terminus of MytA indicates the presence of an esterase fold, while its C-terminus contains several unique repeats involved in mycolic acid transfer to the sugars of the cell envelope. Masi et al. reported a surprising observation that MytA interacts with peptidoglycan and not arabinogalactan, and that arabinogalactan inhibits binding of peptidoglycan by MytA.

4. Transport of folded proteins across membranes

Proteins are synthesized in the cytoplasm as linear polypeptides and are typically exported across the inner membrane in an unfolded state through narrow membrane channels, minimizing chances of causing proton leakage that would be detrimental to the energetic state of the cell. An example is the inner membrane channel formed by the essential protein SecY with a constriction and a plug, or the more complex trans-envelope channels in type I, type III and type IV protein secretion systems. However, bacteria have also met the challenge of transporting folded proteins across the inner or outer membrane. In the late 1990s, one of the

first Symposia on Bacterial Membranes witnessed the discovery of a novel transport system allowing export of folded proteins, typically those that acquire various cofactors upon synthesis in the cytoplasm (Santini et al., 1998). Such proteins are synthesized with N-terminal signal sequences containing a double arginine sequence in a conserved signature motif, lending the name to this export pathway-- the twin arginine translocation (Tat) pathway. Three essential protein components of this export system, TatA, TatB and TatC, have been extensively characterized (Palmer and Berks, 2012). TatA and TatB are single-span membrane proteins with cytoplasmic membrane associated alpha-helical domains. TatC is a polytopic membrane protein that is involved in specific interactions with Tat signal peptides. Sarah Rollauer (University of Oxford, Oxford, UK) described the 3-D structure of TatC from a themophillic bacterium *Aquifex aeolicus*, recently determined by X-ray crystallography (Rollauer et al., 2012).

The TatC structure is a product of extensive groundwork employed to optimize the expression and solubilization of the protein using an elegant TatC-GFP fusion tool to facilitate the screening process. TatC has 6 transmembrane segments and a large surface area with a concave face oriented towards the cytoplasm. To assess its binding to other Tat components, cysteine cross-linking data provided information on putative interaction sites with TatA and TatB. The dynamic nature of these interactions, however, makes it difficult to provide a clear model of this complex in action or to understand how the translocation proceeds without compromising the membrane impermeability. Nevertheless, together with in vitro binding assays and a vast collection of mutations of *E. coli* TatC, the structure provides molecular insight into the mechanism of signal sequence recognition and binding. Clearly, this structure is an essential and crucial step forward in structure-function analysis of this fascinating transport system.

Type II secretion systems (T2SSs) provide a unique route for prefolded proteins to the bacterial cell surface or to the surrounding medium, through an outer membrane pore formed by the protein secretin. This unique class of membrane proteins forms multimeric channels that are also present in bacteria producing filamentous phage and type III secretion systems. One of the best studied is the family of T2SS secretins exemplified by the *E. coli* GspD and *Klebsiella oxytoca* PulD. Domains N0, N1 and N2 residing in the periplasm have been crystallized, providing insight into the molecular basis of their flexible nature. However, little is known about the highly conserved module composed of domains N3 and C that seems to contain all information required for formation of multimeric channels, their gating and insertion into the outer membrane (Korotkov et al., 2011). While cryoEM has provided a global view of secretins, a high-resolution structure of the channel is still lacking. Even if it were available, structural information is unlikely to be sufficient to allow us to understand the biogenesis of this multimer and its membrane insertion. Gerard Huysmans (Institut Pasteur, Paris, France) described new biophysical approaches used to characterize the folding pathway of PulD from the pullulanase type II secretion system. PulD is a 65 kDa protein that forms multimers in an in vitro transcription-translation system and inserts into liposomes apparently without any accessory factors. Adding liposomes to the in vitro reaction allows one to monitor the kinetics of multimer formation. Assays of resistance to SDS, urea and trypsin are used to discriminate between the formation of different states along the folding pathway. This analysis suggests that multimer formation precedes insertion of PulD into membranes. Changing the composition of the bilayer by variation in the acyl chain length and saturation and the reaction temperature allows one to manipulate the rate-limiting steps. Specific PulD mutations that influence some of these steps have also been very useful tools for monitoring the folding pathway of PulD. Interesting future directions of this work include structural or

biophysical analysis of PulD variants blocked at different steps of this unique folding pathway.

5. Uptake of DNA and nutrients

Although bacteria lack the ability to carry out endocytosis, a variety of molecules of different size and composition can enter bacterial cells utilizing specific transport machineries in the cell envelope. The Com system required for DNA uptake by the Gram-positive organism *Streptococcus pneumoniae* includes putative surface pili, a DNA translocation system in the membrane and an endonuclease EndA peripherally attached to the external side of the membrane. In this bacterium, competence for DNA transformation occurs during a narrow time span at the beginning of the exponential phase of growth. Using a fusion protein between EndA and YFP, Nathalie Campo (CNRS-LMGM, P. Sabatier University, Toulouse, France) showed that EndA-YFP is uniformly distributed in the membrane of non-competent cells and concentrates into one or two foci located at mid-cell during competence. Relocalization is dependent on membrane-bound DNA-receptor ComEA. They developed an elegant method based on binding of fluorescently labelled (Cy3) PCR products to demonstrate that DNA binding also takes place at midcell. Furthermore, a synthetic fluorescent DNA fragment composed of cell division gene *ftsZ* and *gfp* was shown to bind at midcell, was taken up by the cell, recombined into the chromosome at the *ftsZ* locus and expressed with detection of the resulting functional FtsZ-GFP fusion protein. The process was completed 70 minutes after addition of DNA.

Laurent Guillon (University of Strasbourg, Illkirch, France) also used fluorescence microscopy to localize the biosynthetic machinery for the *Pseudomonas aeruginosa* pyoverdine and to identify the site where this siderophore enters the cell following iron

binding. Using fluorescent proteins fused to pyoverdine biosynthesis enzymes PvdA and PvdQ, Guillon showed different localization sites for pyoverdine synthesis: at the old cell pole and throughout the periplasm. Recent findings suggest that uptake of iron-bound pyoverdine most likely occurs over the entire cell surface and not at a specific cellular location.

6. Concluding remarks

Bacterial membranes, membrane proteins and cell surface structures involved in bacterial interactions with the environment and their hosts have been an important and dynamic area of research. To understand how bacteria acquire their shape, communicate with their environment and impact the ecosystems, whether in an infected host or in a particular environmental niche, an ever-increasing number of tools are available for biochemical, genetic, structural, visual, biophysical and bioinformatic analysis. Increasingly inter-disciplinary and structure-oriented, the studies of membrane phenomena have provided mechanistic insights, but also novel biological concepts. Initiated in the late 1990s, the Minisymposia on Bacterial Membranes continue to foster informal exchanges in these areas and build ties between European groups mastering diverse experimental tools but sharing common interests. Over the years, these meetings have witnessed the substantial development of a dynamic scientific community and have favored its renewal.

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