

## Institut Pasteur Minisymposium on Bacterial Membranes 2013

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1 **For publication**

2 **Meeting report**

3 **Institut Pasteur Minisymposium on Bacterial Membranes 2013**

4  
5 The first Minisymposium on Bacterial Membranes was organized more than ten years  
6 ago and has become a successful tradition, with the 2013 edition taking place last March. This  
7 edition covered a variety of topics, including advances in the structural analysis of membrane  
8 proteins by solid-state NMR, the structure and function of components of several extracellular  
9 protein secretion systems, lipid modification and lipid-protein interactions, insertion of  
10 proteins into the outer membrane and uptake of DNA and nutrients. Over fifty young  
11 scientists from Germany, United Kingdom, Belgium and France gathered at the Institut  
12 Pasteur in Paris to share recent findings on these topics.

13

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

15

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

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

30

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

51

52           Important components of NMR analysis are powerful computational methods, such as  
53 ARIA or ISD, that allow structural calculations based on the distance data (Rieping et al.,  
54 2008, Bardiaux et al., 2012). Since not only biological polymers, but also membrane proteins  
55 typically exist as symmetric assemblies, the use of symmetry to calculate structures can  
56 reduce calculation complexity and cost (Bardiaux et al., 2012). Benjamin Bardiaux (Institut  
57 Pasteur, CNRS UMR3528, Paris, France) described the power of combining ssNMR and  
58 computational methods based on ambiguous distance restraints to solve the structure of the  
59 *Yersinia* adhesin A (YadA), secreted via the type V pathway (Leyton et al., 2012). YadA  
60 belongs to a class of type V secretion systems called autotransporters, which cross the inner  
61 membrane of Gram-negative bacteria via the Sec system. Their N-terminal part forms the  
62 passenger domain that is secreted via a channel in the outer membrane formed by the C-  
63 terminal beta-barrel domain. Cellular Bam machinery facilitates insertion of the C-terminal  
64 domain into the outer membrane. Autotransporters are widespread and come in many flavors.  
65 *Yersinia* YadA is a member of the trimeric autotransporter family, with the outer membrane  
66 barrel formed by beta sheets derived from three polypeptides. The passenger domain of YadA  
67 has been analyzed by X-ray crystallography of its different fragments. Interestingly, poorly  
68 diffracting crystals of the outer membrane domain were used as material for ssNMR analysis,  
69 successfully providing the de novo structure of the trimer (Shahid et al., 2012). NMR analysis  
70 provided crucial information on the flexible nature of a short conserved tetrapeptide ASSA  
71 between the passenger and the beta-barrel domains. The results support the model where this  
72 sequence forms an essential hairpin that initiates secretion of the passenger domain, which is  
73 subsequently driven by folding of the passenger domain into a beta-helix structure.

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

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

84

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

86

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

101 the panoply of "bullets", effector proteins with various enzymatic activities. Using live  
102 fluorescence imaging and the GFP-labeled shaft component TssB, one can observe the  
103 assembly and contraction of the shaft in a predator cell. This contraction in an EAEC cell that  
104 is in direct contact with *E. coli* K-12 leads to the lysis of prey within minutes (Brunet et al.,  
105 2012). The tube is built as a stack of Hcp subunit hexamers, which can be assembled in vitro  
106 both in a head-to-tail and tail-to-tail orientation. However, the preferred orientation in vivo is  
107 head-to-tail, suggesting a mechanism that regulates tube assembly.

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

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

132

### 133 **3. Lipid modification and lipid-protein interactions**

134

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



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

166

#### 167 **4. Transport of folded proteins across membranes**

168

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

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

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

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

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

226

## 227 **5. Uptake of DNA and nutrients**

228

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

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

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

254

## 255 **6. Concluding remarks**

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

268

## 269 **Acknowledgements**

270 We thank Tony Pugsley for initiating the Minisymposia on Bacterial Membranes and  
271 for organizing this event for many years.

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