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Nienke Buddelmeijer, Olivera Francetic

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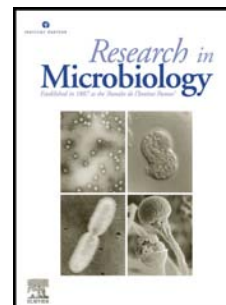
Nienke Buddelmeijer, Olivera Francetic

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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₋₁₁ 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

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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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326 Nienke Buddelmeijer*, Olivera Francetic

327 *Institut Pasteur, Unité de Génétique moléculaire, CNRS ERL3526,*
328 *25 rue du Dr Roux F-75724 Paris Cedex , France*

329 * Corresponding author

330 E-mail address: niebud@pasteur.fr

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