



HAL
open science

Bacterial injection machines: Evolutionary diverse but functionally convergent

Sophie Bleves, Jorge E Galán, Matxalen Llosa

► **To cite this version:**

Sophie Bleves, Jorge E Galán, Matxalen Llosa. Bacterial injection machines: Evolutionary diverse but functionally convergent. Cellular Microbiology, 2020, pp.e13157. 10.1111/cmi.13157 . hal-02456027

HAL Id: hal-02456027

<https://amu.hal.science/hal-02456027>

Submitted on 27 Jan 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1

2

Bacterial injection machines:

3

Evolutionary diverse but functionally convergent

4

5

Authors: Sophie Bleves¹, Jorge E. Galán², Matxalen Llosa^{3*}

6

7

¹ LISM (Laboratoire d'Ingénierie des Systèmes Macromoléculaires-UMR7255),

8

IMM (Institut de Microbiologie de la Méditerranée), Aix-Marseille Univ and CNRS,

9

Marseille, France.

10

² Department of Microbial Pathogenesis, Yale University School of Medicine,

11

New Haven, USA.

12

³ Instituto de Biomedicina y Biotecnología de Cantabria (IBBTEC), Universidad

13

de Cantabria-CSIC-SODERCAN, Santander, Spain.

14

* Corresponding author. Phone +34 942201957. email llosam@unican.es

15

16

Running title: Bacterial injection machines

17

18 **Summary**

19

20 Many human pathogens use Type III, Type IV and Type VI secretion systems to
21 deliver effectors into their target cells. The contribution of these secretion systems to
22 microbial virulence was the main focus of a Workshop organized by the International
23 University of Andalusia in Spain. The meeting addressed structure-function, substrate
24 recruitment and translocation processes, which differ widely on the different secretion
25 machineries, as well as the nature of the translocated effectors and their roles in
26 subverting the host cell. An excellent panel of worldwide speakers presented the state
27 of the art of the field, highlighting the involvement of bacterial secretion in human
28 disease and discussing mechanistic aspects of bacterial pathogenicity, which can
29 provide the bases for the development of novel anti-virulence strategies.

30

31

32 **Keywords:** Bacterial secretion / Type III Secretion System / Type IV Secretion
33 System / Type VI Secretion System / machinery assembly/ protein translocation /
34 effector protein / toxin/ microbial virulence / host subversion / pathogenicity/
35 antibacterial

36

37 **Abbreviations:** cryo-EM, cryo-electron microscopy; cryo-ET, cryo-electron
38 tomography; IMCC, inner membrane – cytoplasmic complex; nsEM, negative staining
39 electron microscopy; OMC, outer membrane complex; T3/T4/T6SS, Type III / Type IV /
40 Type VI secretion system(s)

41 **Bacterial injection systems: all roads lead to Rome**

42

43 Bacterial secretion systems are trans-envelope multi-protein assemblies devoted
44 to the transport of specific macromolecules to the outside. These nanostructures
45 connect bacteria with the outer world, playing a pivotal role in essential bacterial life
46 processes such as finding nutrients, sharing genetic information, or communicating
47 with other organisms in the context of pathogenic or symbiotic interactions (Green *et*
48 *al.*, 2016). Among the many different families of secretion machineries, the Type III,
49 Type IV and Type VI protein secretion systems (T3/T4/T6SS) share the ability to inject
50 their substrates directly into human cells (Galan *et al.*, 2018), playing an important role
51 in pathogenesis. The transported substrates, collectively known as effectors, have the
52 capacity to modulate host cellular processes for the benefit of the bacterial pathogens
53 that encode them. The role of translocated effectors in virulence is well established for
54 an increasing number of human pathogens such as *Escherichia coli*, *Salmonella*
55 *enterica*, *Helicobacter pylori*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, or
56 *Brucella melitensis*. These three secretion machineries are also found within
57 phytopathogens whose effectors target plant cells. T4SS also play a role in horizontal
58 gene transfer, one of the main causes for spreading antibiotic resistance genes within
59 bacterial populations. This mechanism contributes to the generation of multi-resistant
60 bacteria, which pose a major threat to global health (Aslam *et al.*, 2018). T6SS are
61 mainly recognized as antibacterial injection machineries, able to deliver toxic effectors
62 in prey bacteria (Coulthurst, 2019).

63 Remarkably, the evolutionary origin of these protein secretion systems differs
64 significantly. T3SS are evolutionary related to the flagellar apparatus (Bhattacharya *et*

65 *al.*, 2019); T4SS were originally conceived as conjugative bridges for DNA transfer
66 (Christie, 2001), and T6SS are evolutionary related to contractile phage tails (Leiman *et*
67 *al.*, 2009). Thus, evolution has shaped multiprotein complexes with the capacity to
68 inject bacterially-encoded proteins into another cell multiple times. In spite of their
69 different evolutionary origins, these secretion machines converged mechanistically
70 into the ability to transport macromolecules across phospholipids bilayers, resulting in
71 the delivery of proteins synthesized by one bacterium into another cell target,
72 prokaryotic or eukaryotic. In the injection systems involved in pathogenicity, there is
73 also a functional convergence in the effectors delivered by the different machines,
74 which have the capacity to target similar cellular processes, such as signal transduction
75 pathways, vesicular trafficking, or the immune response (Personnic *et al.*, 2016,
76 Ratner *et al.*, 2017, Pisano *et al.*, 2018, Tsai *et al.*, 2019) (**Figure 1**).

77 The number of effector proteins transported by a given machine varies
78 significantly and can reach hundreds in some exceptional cases. As these effectors
79 have the capacity to modulate a vast range of cell physiology processes, their study are
80 not only providing insight into mechanisms of pathogenicity but also illuminating
81 fundamental principles of cell biology. Furthermore, the molecular knowledge of
82 these secretion machineries may pave the way to the design of new anti-infective
83 drugs. Thus, the search for inhibitors of the secretion machinery assembly or
84 functioning/operation or the effector themselves is an active field of research
85 (Fasciano *et al.*, 2019, Graf *et al.*, 2019). In addition, secretion systems can be
86 converted into intracellular delivery machines which could also have therapeutic
87 applications (Walker *et al.*, 2017).

88 Our knowledge on the architecture and function of these different secretion
89 machines has improved significantly over the last few years. Work during the 90's led
90 to the realization that T3SS had the capacity to deliver bacterially encoded proteins
91 into host cells, and in particular, virulence factors into human cells. The discovery of
92 the needle complex and associated structures led to the concept of the injectisome.
93 Combined with the description of a plethora of effector proteins and their biochemical
94 activities, these studies led to the emergence of novel paradigms in bacterial
95 pathogenesis, host pathogen interactions, protein secretion, and molecular machines
96 (Galan *et al.*, 2014). Furthermore, because of their high degree of conservation across
97 many important bacterial pathogens independently of their phylogeny and the
98 commonality of secretion mechanisms, T3SS emerge as a potential target for the
99 development of novel antimicrobial strategies (Lara-Tejero *et al.*, 2019). Overall, the
100 study of T3SS has serve as useful paradigm for research on other protein secretion
101 systems.

102 T4SSs stand out for their plasticity, since they can translocate both protein and
103 DNA molecules, and the destiny can be their secretion into the milieu or the delivery
104 into another cell, being either prokaryotic or eukaryotic (Li *et al.*, 2019). This versatility
105 allows T4SS to play a key role in many essential aspects of bacterial life. These
106 functions can be grossly classified in two: (i) contribution to the horizontal gene pool,
107 both in Gram-negative and positive bacteria, and (ii) modulation of eukaryotic host
108 cells. DNA transport is a particularity of T4SSs, which are an integral part of bacterial
109 conjugation machineries. A T4SS subfamily adapted to interact with eukaryotic cells
110 mediates secretion or direct cell-to-cell transfer of virulence factors to modulate host
111 cells (Hayek *et al.*, 2019). Members of this family contribute to the virulence of

112 significant human pathogens, such as *Helicobacter pylori*, *Legionella pneumophila*, or
113 *Brucella melitensis*. T4SS can also mediate interkingdom DNA transfer, as exemplified
114 by the prototypical T4SS VirB of *Agrobacterium tumefaciens*, responsible for the
115 transfer and integration of bacterial DNA into the plant cell genome (Li *et al.*, 2018).

116 Finally, T6SSs were described only a decade ago, but in spite of this, their
117 structure and mechanism have been deciphered in much detail quickly (Cherrak *et al.*,
118 2019). The T6SS functions as a dynamic contractile phage tail-like structure anchored
119 in the bacterial cell envelope to deliver effectors directly into the target cell. The T6SS
120 primary role is to target competitor bacteria in the environment or in the context of
121 host infection (Hood *et al.*, 2010, Coulthurst, 2019); however, interestingly some T6SS
122 effectors are also recognized as virulence factors that target eukaryotic cell
123 components (Hachani *et al.*, 2016). T6SSs are present among pathogens such as *P.*
124 *aeruginosa* or *Acinetobacter*, classified by the WHO as antibiotic-resistant "priority
125 pathogens".

126 The role of bacterial secretion in disease is an especially relevant research field
127 where to promote cooperation: the socio-economic importance of the issue raises
128 interests among scientists from different backgrounds, such as medicine, biochemistry,
129 structural, cellular and molecular biology, genetics, or microbiology, working on a
130 variety of organisms including microbes, plants, animals, and humans. A detailed
131 understanding of bacterial secretion systems requires a multidisciplinary approach that
132 addresses all structural, biochemical, microbiological, cellular and medical aspects:
133 from the elucidation of the molecular structure of the protein components to the cell
134 biology studies to elucidate the activities of the translocated effector proteins in
135 eukaryotic host cells. The ultimate goal of the Workshop "Contribution of bacterial

136 injection systems to human disease”, which took place at the International University
137 of Andalusia (UNIA, Spain) in November 2018, was to promote cross-talk between
138 scientists working in different secretion systems at different organization levels,
139 pursuing as goals a holistic comprehension of bacterial pathogenesis, and discussing
140 possible common strategies for anti-pathogen therapies.

141 Knowledge on the mechanism of secretion is essential to understand
142 pathogenicity and to design therapies that block the secretion process. The first
143 session of the workshop addressed the assembly of the multiprotein transmembrane
144 complex, the 3D structure of the individual protein components, and structure-
145 function analysis of the nanomachines. A second session dealt with the nature and
146 recruitment of substrates, and the active translocation process of both protein and
147 DNA molecules. The third session focused on the subversion of the human cell by
148 translocated effectors, including interaction of the delivered substrates with host
149 elements and modulation of the host response.

150

151

152 **Structure and function of T3, T4 and T6 Secretion Systems**

153

154 Structural biology provides an essential knowledge on which we base to great
155 extent our research for understanding multiprotein complexes. The size of these
156 complexes makes electron microscopy the ideal technology to elucidate their three-
157 dimensional architecture. In the last years, technologies requiring particle purification
158 have been complemented by other approaches, which allow the elucidation of
159 complex structures *in situ*. In this new context, cryo-electron tomography (cryo-ET) has

160 proven an excellent tool, and new exciting results argue for the importance of the
161 biological context on the elucidation of three-dimensional structures. María Lara-
162 Tejero (Yale University, New Haven, USA) presented the *in situ* structure of the
163 *Salmonella* T3SS obtained by cryo-ET. Comparison with the cryo-electron microscopy
164 (cryo-EM) structure of the isolated needle complexes revealed significant
165 conformational differences resulting from the docking of the sorting platform in the
166 needle complex, providing the symmetry adaptation required for the assembly of the
167 entire secretion within the bacterial envelope (Hu *et al.*, 2017). Expanding on the T3SS
168 structure, Samuel Wagner (University of Tübingen, Germany) presented work in
169 collaboration with Susan Lea (Oxford) on the T3SS core export apparatus components
170 SctR, SctS, and SctT, (Wagner *et al.*, 2010). A cryo-EM structure of the flagellar SctRST
171 homologs FlpQR showed that these proteins assemble into a unique helical complex
172 with a central pore, that is closed in the structure of the isolated complex, but that is
173 thought to open when located in the membrane. Despite their prediction as
174 membrane proteins, the helical SctRST complex resides within the bacterial inner
175 membrane during assembly, but it locates to a periplasmic supramembrane position in
176 the assembled injectisome. The details of its function in gating of and secretion
177 through the T3SS injectisome awaits further investigation.

178 There were a number of reports providing new, data on the structure of T4SS.
179 Until recently, our knowledge came from single particle negative staining electron
180 microscopy (nsEM) and reconstruction of the full transmembrane complex of the
181 conjugative plasmid R388 (Low *et al.*, 2014), and the nsEM structure of the core
182 complex of the Cag T4SS (Frick-Cheng *et al.*, 2016) involved in *H pylori* pathogenicity.
183 Although different in size and shape, both T4SS showed an outer membrane complex

184 (OMC) with a 14-fold symmetry. The atomic structure of the OMC of the VirB T4SS
185 from *Xanthomonas citrii*, which mediates bacterial killing, confirmed this 14-fold
186 symmetry, as reported Tiago R. Costa from Gabriel Waksman lab (Imperial College,
187 London, UK) (Sgro *et al.*, 2018). Intriguingly, the fine structure of two different T4SS,
188 solved *in situ* by cryo-ET, shed different results. Craig R Roy (Yale University, New
189 Haven, USA) presented a refined structure of the Dot-Icm T4SS from *L. pneumophila*
190 (Chetrit *et al.*, 2018), previously reported at lower resolution (Ghosal *et al.*, 2017); the
191 Dot-Icm T4SS is the prototype of so-called “Type B” T4SS, due to their partial homology
192 to the canonical “Type A” T4SS. Peter J. Christie (U Texas, Houston, USA) presented the
193 architecture of the T4SS of the conjugative plasmid F (Hu *et al.*, 2019a), which shows
194 little homology to that of R388. In both cases, a surprising 13-fold symmetry in the
195 OMC was evident.

196 The stoichiometry of the inner membrane / cytoplasmic complexes (IMCC)
197 received much focus. The reconstruction by Low et al (2014) proposed an inner
198 membrane complex formed by two side-by-side hexameric barrels of the cytoplasmic
199 ATPase VirB4; more recently, a five-barrel cytoplasmic structure was proposed for the
200 Cag T4SS (Chang *et al.*, 2018), although the level of resolution left open the question of
201 the structure/s of these subcomplexes. The new fine structures of the Dot-Icm and F
202 plasmid allowed detailed observation of the IMCC. Craig Roy reported a DotB-DotO
203 hexameric complex, involved in substrate recruitment, which creates a cytoplasmic
204 channel whose assembly opens up the T4SS, thus directing the translocation of
205 substrates through the T4SS (Chetrit *et al.*, 2018). A very similar structure is revealed in
206 the case of the system associated with the conjugative plasmid F, with a 13-fold
207 symmetry OMC linked to a 6-fold symmetry IMCC (Hu *et al.*, 2019a). In addition, Bo Hu

208 from University of Houston reported a refined cryo-ET structure of the Cag T4SS
209 confirming the previously reported 14-fold symmetry at the OMC, but showing a clear
210 6-fold symmetry in the cytoplasmic complex (Hu *et al.*, 2019b).

211 **Figure 2** shows a comparison of the features of the different T4SS structures
212 elucidated so far. Apart from the valuable insight into each particular T4SS, the set of
213 results opens up the question as to whether there are significant structural variations
214 among the T4SS, maybe adapted to their biological roles, and/or the variations are due
215 to the differences in the technology used to solve the structures. The fact that both the
216 14-fold and 13-fold symmetry OMC were solved in different systems from independent
217 labs argues for the co-existence of these alternative oligomeric structures in the T4SS
218 world. The newly solved structures provide strong evidence for a 6-fold symmetry
219 IMCC to which the 14-fold or 13-fold symmetric OMC has to engage. The apparent
220 stoichiometric mismatches among subcomplexes could be in fact an important
221 flexibility factor key to T4SS function. In this respect, it is interesting to note that the
222 recently reported near-atomic resolution structure of the Cag T4SS (Chung *et al.*, 2019)
223 shows that the 14-fold symmetric outer membrane core complex connects to a newly
224 described 17-fold symmetric periplasmic ring complex (PRC). This new structure
225 illustrates the structural diversity among T4SS.

226 Another significant difference concerns the dimensions of the OMC. While the
227 IMCC has a similar diameter in all the reported T4 structures (around 25-30 nm wide),
228 the OMC is about 20 nm wide in the case of the R388, F, and *Xanthomonas* T4SS, while
229 the OMC of Cag and Dot-Icm T4SS double this size. The obvious difference between
230 these two types of T4SS is the target cell (prokaryotic or eukaryotic), raising the

231 possibility that the extended OMC in the case of the *H. pylori* and *L. pneumophila* T4SS
232 is designed to target the human cell.

233 Structural studies not only shed light on the architecture of mature, functional
234 secretion machines. They can also illustrate the biogenesis pathway of the
235 transmembrane complexes. Peter Christie reported various structures of the F T4SS,
236 which may represent assembly intermediates or alternative biological states of the
237 T4SS, such as F pili associated with distinct basal platforms. Bo Hu showed the Cag
238 T4SS structures from mutants lacking the cytoplasmic ATPases, which do not affect the
239 formation of the outer membrane complex, but suggest a pathway for the assembly of
240 the inner membrane complex (Hu *et al.*, 2019b). Joseph Vogel (Washington University,
241 St Louis, USA) combined cryo-EM with immunofluorescence microscopy to address the
242 assembly process of the *L. pneumophila* Dot-Icm T4SS, revealing that early-stage
243 assembly process begins with the targeting of Dot/Icm components to the bacterial
244 poles. Interestingly, polar targeting is mediated by two T4 components, DotU and
245 IcmF, which have homologues in T6SS, raising the possibility that these elements may
246 have been recruited by a T6SS (Ghosal *et al.*, 2019).

247 Eric Cascales (CNRS, Marseille, France) reported the state of the art on T6SS
248 structure and assembly. T6SS includes a contractile sheath that covers a hollow tube
249 topped with a membrane-puncturing spike. The sheath can contract and inject the
250 arrow loaded with effectors into the prey cell. The cryo-EM structure of the wedge
251 complex of the enteroaggregative *Escherichia coli* T6SS and how the TssK wedge
252 subunit anchors the baseplate to the trans-membrane complex were briefly presented
253 at the workshop (Cherrak *et al.*, 2018). The group has also dissected T6SS biogenesis
254 using an original *in vivo* technique based on APEX2-dependent biotinylation to

255 determine proximity partners of TssA. TssA, by stabilizing the baseplate and
256 coordinating the polymerization of the tail plays a central role in the assembly of two
257 T6SS subcomplexes. The results revealed a new partner, TagA, which holds the distal
258 extremity of the sheath at the opposite site of the bacterial membrane (Santin *et al.*,
259 2018). In fact, these authors have recently reported that cell width determines the
260 length of the T6SS tail, since T6SS sheath polymerization is arrested upon contact with
261 the opposite membrane, likely by the TagA stopper (Santin *et al.*, 2019).

262 The intriguing relationships between T4SS and T6SS were also raised at the
263 Workshop. It was already known that the conjugative pilus triggers T6SS activity in the
264 recipient cell (Ho *et al.*, 2013), so T6SS-containing bacteria are unlike recipients of
265 conjugative plasmids. Mario Feldman (Washington University School of Medicine at St
266 Louis, USA) built further into this relationship. Coexistence of T4 and T6SS in donor
267 bacteria is functionally contradictory, since the former is intended to transfer DNA to
268 recipient bacteria, while the latter kills potential recipients. In *Acinetobacter baumannii*,
269 where most strains harbour a constitutively expressed T6SS, the solution is simple:
270 large conjugative plasmids repress co-existing T6SS in order to allow their spread and
271 the conjugation of other plasmids through their T4SS (Weber *et al.*, 2015). In fact, they
272 show that this repression is essential for conjugation to occur (Di Venanzio *et al.*,
273 2019). The molecular components involved in these interactions remain to be
274 determined.

275

276

277 **Translocated substrates**

278

279 The workshop devoted its attention also to different aspects of the substrates
280 translocated by T3, T4 and T6SS, from their evolutionary origin to their recruitment
281 and translocation by the secretion machineries, their detection and action in the target
282 cell, and even their possible biotechnological applications. The varied nature of the
283 substrates was represented in different talks, including the nucleoprotein complexes
284 transferred by T4SS, the toxins targeted to other bacteria through T6SS, or the protein
285 effectors injected into human cells by T3SS.

286 Christoph Dehio (University of Basel, Switzerland) addressed the evolution of
287 effectors, using the example of the *Bartonella* VirB/D4 T4SS substrates, collectively
288 known as Beps. Their group reported that Beps evolved from a single ancestral effector
289 that emerged by fusion of a bacterial toxin-antitoxin module and a T4 secretion signal
290 (Harms *et al.*, 2017). This ancestral Bep became the primordial interkingdom effector
291 when VirB/VirD4-T4SS was co-opted for host interaction. By parallel evolution, specific
292 sets of Bep repertoires have been adapted to three different *Bartonella* lineages, in a
293 remarkable example of host adaptation (Wagner *et al.*, 2019a).

294 Substrate recruitment and translocation are especially intertwined in the case of
295 T6SS. Luke Allsopp, from Alain Filloux lab (Imperial College London, UK), presented the
296 fine characterization of the secretion of a DNase toxin by the *P. aeruginosa* T6SS into
297 competitor bacteria (Pissaridou *et al.*, 2018). This evolved toxin, whose effector
298 domain is fused to a structural component of the T6 puncturing device, was shown to
299 fit exquisitely in the tip of the T6SS spike thanks to interactions by its PAAR domain. In
300 addition, they showed that immunity proteins are highly specific for their cognate
301 toxins, allowing interstrain competition. T6 effector genes can also be localized at the
302 vicinity of genes encoding the phage tail like structure. Like this, Sophie Bleves (Aix-

303 Marseille University, France) discovered a novel antibacterial phospholipase of *P.*
304 *aeruginosa* whose secretion requires an adaptor protein for the targeting to the T6
305 spike and an immunity protein to protect from sister cell attack (Berni *et al.*, 2019). The
306 existence of toxin-antitoxin pairs was the also the basis for the search for new
307 effectors associated with the *P. aeruginosa* H1-T6SS by David Albesa (CIC-Biogune,
308 Spain) in collaboration with Alain Filloux and Julian Parkhill. They developed a
309 methodology named TraDIS (Transposon directed insertion-site sequencing) and
310 mutagenized in parallel strains with active/inactive T6SS. By comparing the number of
311 transposon insertions in each gene, they identified putative immunity proteins (which
312 are essential genes), allowing the search for adjacent toxins. Following this approach
313 they discovered Tse8, an antibacterial toxin with an original mechanism of action, the
314 inhibition of protein synthesis, and its cognate immunity protein Tsi8 (Nolan *et al.*,
315 2019).

316 Access of the effectors to their host target requires different strategies depending
317 on the pathogen. In the case of the *Helicobacter pylori* Cag T4SS, the effector
318 oncoprotein CagA is secreted at the surface of the gastric cell and then translocated
319 into the target cell. Steffen Backert (Friedrich-Alexander University Erlangen-
320 Nuremberg, Germany) presented a novel basolateral T4 secretion model for CagA.
321 While the gastric pathogen is exposed only to the apical surface of epithelial cells, the
322 T4 needle-like pilus binds basolateral integrins to translocate CagA. The access to these
323 basolateral receptors is allowed by the secretion of the HtrA serine protease that
324 cleaves cell-to-cell junction factors as well as extracellular matrix proteins, disrupting
325 the epithelial barrier and allowing transmigration of the pathogen (Backert *et al.*,
326 2018). Exported HtrA proteases represent highly attractive targets for antibacterial

327 treatment by inhibiting their proteolytic activity or for its use in vaccine development.
328 In addition to integrins, *H. pylori* exploits at the basolateral surface the
329 carcinoembryonic antigen-related cell adhesion molecules (CEACAM) receptors via the
330 outer membrane adhesin HopQ (Moonens *et al.*, 2018). This binding is required for
331 CagA delivery into gastric epithelial cells. CEACAMs are also exploited by pathogenic
332 bacteria such as *Neisseria gonorrhoeae*, *N. meningitidis*, and *E. coli* during mucosal
333 colonization.

334 Bacterial secretion systems can target specifically a diversity of host cells and
335 translocate very different cargos, making them ideal systems to customize as substrate
336 delivery tools. Luis Angel Fernández (CNB, Spain) utilizes the ability of T3SS to
337 translocate proteins into human cells for biotechnological purposes. His group has
338 designed a non-pathogenic *E. coli* strain which produces a functional T3SS from
339 enteropathogenic *E. coli*, that injects the desired proteins into human cells (Ruano-
340 Gallego *et al.*, 2015). They also showed that *E. coli* can be targeted to specific cell
341 types, and in particular tumor cells, by expressing synthetic adhesins consisting of
342 nanobodies (Pinero-Lambea *et al.*, 2015). By joining both approaches, he presented
343 encouraging preliminary results of the engineered *E. coli* strain injecting customized
344 substrates specifically to tumor cells. Matxalen Llosa (Universidad de Cantabria, Spain)
345 aims to customize T4SS to deliver protein-DNA complexes for genomic editing
346 purposes. T4SS involved in effector translocation can also mediate DNA delivery into
347 human cells, when combined with a plasmid conjugation machinery (Llosa *et al.*,
348 2012). The translocated DNA is covalently attached to the conjugative relaxase. They
349 showed that certain relaxases promote random integration of the incoming DNA into
350 the recipient human genome (Gonzalez-Prieto *et al.*, 2017). Through protein

351 engineering, relaxase chimeric proteins can acquire new functions, including the ability
352 to integrate the attached DNA (Agundez *et al.*, 2018). Preliminary results suggest that a
353 site-specific nuclease fused to the relaxase can be translocated through the T4SS in
354 order to attain site-specificity in the integration of T4SS-delivered DNA.

355 Francisco Ramos-Morales (Universidad de Sevilla, Spain) presented a different
356 example of possible biotechnological/biomedical exploitation of the secreted
357 substrates, using *Salmonella* T3SS effectors as carriers in a live vaccine against *P.*
358 *aeruginosa*. A fusion between the *S. enterica* effector SseJ and the *P.*
359 *aeruginosa* antigen PcrV was translocated by *Salmonella* into host cells *in vitro*,
360 eliciting the generation of specific antibodies in mice, which showed enhanced survival
361 (Aguilera-Herce *et al.*, 2019).

362

363

364 **Effector-mediated subversion of host cells**

365

366 Although the T3, T4 and T6SS are evolutionary and structurally quite diverse, many
367 of the effector proteins that they deliver target common core eukaryotic functions.
368 Consequently, the study of these effectors share common experimental approaches.
369 One of the main limitations to study effector function in the host cell is their
370 visualization after translocation. Amy E. Palmer (University of Colorado, Boulder, USA)
371 reviewed the techniques they have developed to monitor T3SS effector injection in
372 human cells, which they also used to visualize *Listeria* protein secretion in infection,
373 and could ideally extend to imaging of T4 and T6 effectors. Effectors tagged with a

374 fluorescent peptide allow following the secretion process from the intracellular
375 bacteria by the loss of fluorescence. Another approach consists on an adaptation of
376 the split-GFP assay, fusing the FP11 GFP domain to the effector and expressing the rest
377 of the GFP protein (FP1-10) in the host cell (Young *et al.*, 2017, Batan *et al.*, 2018).

378 Ulla Bonas (Martin Luther University Halle-Wittenberg, Halle, Germany) discussed
379 how bacterial plant pathogens subvert their host by T3SS-mediated effector
380 translocation. Interestingly, effectors from human and plant pathogens share common
381 host targets. The repertoire of T3 effectors of *Xanthomonas campestris* pv. *vesicatoria*
382 comprises more than 35 proteins, among them the AvrBs3 transcription factor, cell
383 death inducers, plant immunity suppressors (Adlung *et al.*, 2017), or the XopL
384 ubiquitin ligase. The characterization of the latter reveals an unprecedented role of
385 microtubules in stromule extension and dynamics in *Nicotiana benthamiana* (Erickson
386 *et al.*, 2018).

387 Christoph Dehio addressed the functional versatility of the *Bartonella* VirB/D4
388 T4SS Bep substrates. The effectors are composed of only three basic domain types that
389 are highly versatile in function, allowing them to adapt their original function to host
390 cell subversion, by post-translation modification of target proteins, e.g. AMPylation by
391 FIC domains, interfering with signaling pathways mimicking eukaryotic protein pY
392 motifs, etc (Wagner *et al.*, 2019b). Another fine example of host cell subversion by a
393 T4SS effector was reported by Maria Lucas (University of Cantabria, Spain), who
394 showed that the effector RavN of the *L. pneumophila* Dot/Icm T4SS mimics host cell E3
395 ligases to exploit the ubiquitylation pathway (Lin *et al.*, 2018). Based on the structural
396 information obtained from this effector, they propose and validate other four effectors

397 with E3 ligase activity, underscoring the importance of manipulation of the host
398 ubiquitylation pathway for *Legionella* infection.

399 Since the main function of T6SS is to target other bacteria, there are few
400 examples of T6 anti-eukaryotic effectors (Hachani *et al.*, 2016). Some of them provide
401 unique cases of trans-kingdom toxins, which allow the search for antibacterial
402 compounds that may limit bacterial competition for the niche colonization and further
403 host subversion. While toxins are usually directed against eukaryotic cells (like AB
404 toxins) or against rival bacteria (like bacteriocins), three T6 phospholipases of *P.*
405 *aeruginosa* were previously shown to affect both, prokaryotic and eukaryotic target
406 cells (Jiang *et al.*, 2014, Jiang *et al.*, 2016). These T6 effectors disrupt membrane
407 integrity of their prey bacteria and promote internalization *P. aeruginosa* into
408 epithelial cells and autophagy. Sophie Bleves reported a fourth trans-kingdom effector
409 of a novel type, called VgrG2b, which acts as an antibacterial metallopeptidase
410 implicated in bacterial competition (Berni *et al.*, 2019). The bacterial target remains to
411 be discovered. VgrG2b was previously shown to facilitate the uptake of *P. aeruginosa*
412 into non-phagocytic cells by recruiting microtubules through an interaction with the
413 gamma-tubulin ring complex, the microtubule nucleating centre (Sana *et al.*, 2015). As
414 in the case of the *Xanthomonas* T3 effector XopL, the precise role on host microtubules
415 remains to be evaluated in future studies. This represents a novel internalization
416 strategy, since most pathogens manipulate the actin cytoskeleton to enter host cells.
417 One such example was described by Miguel Valvano (University of Belfast, UK), who
418 presented a T6 effector that targets the host cytoskeleton to survive in macrophages:
419 the protease TecA of *Burkholderia cenocepacia* (Aubert *et al.*, 2016) inactivates Rho
420 GTPases by deamidation, thus inducing actin disruption and caspase-1 inflammation

421 through activation of the Pysin inflammasome. Furthermore, his lab addressed the role
422 of T2, T4 and T6SS in the interactions between *B. cenocepacia* and macrophages by
423 constructing mutant strains lacking each secretion system. They appear to increase
424 pathogenicity in macrophages, while limiting the spread of infection.

425 Finally, three talks addressed the complex interplay between enteropathogens
426 and the host immune response. Jorge Galán (Yale University, New Haven, USA)
427 provided the demonstration that three well-characterized T3 effectors of *Salmonella*
428 Typhimurium, SopE, SopE2 and SopB, trigger intestinal inflammation bypassing innate
429 immune receptors. Instead, they do so through the activation of Cdc42 and Pak1.
430 These effectors conserve signalling pathways that operate downstream from canonical
431 innate immune receptors and involve Tak1 and TRAF6 (Sun *et al.*, 2018). These findings
432 illustrate the unique balance that emerges from the host/pathogen co-evolution, in
433 that pathogen-initiated responses that help its replication are also important to
434 prevent pathogen spread to deeper tissues. Furthermore, the mechanisms describe
435 here could help develop anti-pathogen therapeutic strategies by targeting specific
436 host-signaling pathways. Elisabeth L. Hartland (Monash University, Clayton, Australia)
437 reported on the suppression of host innate immunity pathways in enteropathogenic *E.*
438 *coli* and *Shigella* by T3SS effectors. EspL defines a family of cysteine protease effectors
439 which directly target RHIM-dependent inflammatory and necroptotic signalling
440 pathways (Pearson *et al.*, 2017). Using the proteomic approach ProtoMap (Fuhrman-
441 Luck *et al.*, 2017), they have defined the host interacting partners of the *Shigella* EspL
442 homologues, and found that they target components of the type I interferon signalling
443 pathway. This suggests that *Shigella* activity blocks the host type I interferon response
444 and that type I interferon induced proteins mediate host defense against *Shigella*

445 infection. Feng Shao (National Institute of Biological Sciences, Beijing, China) reported
446 the interaction of the T3 effector IpaH9.8 of *Shigella flexneri* with the host immune
447 response. IpaH9.8 suppresses host defense through ubiquitination and degradation of
448 guanylate-binding proteins (Li *et al.*, 2017). Lack of IpaH9.8 or its binding to GBPs
449 provoked translocation of GBPs such as hGBP1 and mGBP2 to intracellular *S. flexneri*,
450 where they inhibited bacterial replication, highlighting the functional importance of
451 GBPs in antibacterial defenses. The recently reported structure of the IpaH9.8:GBP
452 interaction reveals clues on how IpaH proteins discriminate among different GBP
453 targets through its LRR domain (Ji *et al.*, 2019). This same group recently reported that
454 the *Salmonella* SopF effector specifically blocks xenophagy by blocking V-ATPase
455 recruitment of ATG16L1 onto bacteria-containing vacuole, without affecting canonical
456 autophagy (Xu *et al.*, 2019). This is just the last example of how the study of bacterial
457 subversion of the human cell serves to elucidate basic aspects of cell biology.

458

459

460 **Conclusions and prospects**

461

462 The Workshop gathered an excellent panel of experts addressing different
463 aspects of the biology of T3, T4 and T6SS and their involvement in bacterial virulence.
464 The data presented reflected the exciting scientific moment in the field, and
465 underscored the fact that these evolutionary distant nanomachines converge
466 functionally in the host human cell, where they elicit pathogenic effects. This situation
467 calls for a joint action among experts in these fields to find common anti-virulence

468 strategies. In this context, the workshop constituted an excellent forum for merging
469 scientists from different backgrounds, which undoubtedly leads to fruitful synergies.
470 The authors hope similar meetings continue to take place in the future.

471 In the workshop, the reports on new 3D structures of the different secretion
472 machineries highlighted the importance of solving structures that are relevant *in vivo*.
473 The differences observed in the structures of the T3SS when it is embedded in the
474 membrane, or the different IMCC observed in T4SS structures solved *in situ* compared
475 to single particle reconstruction by cryo-EM (Fig 2), emphasizes the importance of the
476 physical context in biological structures. It is evident that structural biology of
477 secretion systems is moving towards the *in situ* characterization of the nanomachines
478 (Oikonomou *et al.*, 2019). Still, cryo-ET needs to reach a better level of resolution to
479 provide full answers to the structure-function relations.

480 Elucidation of the structures is essential to understand the secretion mechanism,
481 and from a practical point of view, to identify potential targets for blocking secretion,
482 and thus, microbial virulence. Such anti-virulence molecules would be an alternative to
483 classical bacteria-killing antibiotics. In addition, bacterial secretion systems offer
484 numerous possibilities to develop biotechnological and biomedical applications, and
485 some of them were illustrated here, such as customization of DNA- and protein-
486 delivery systems which access *in vivo* specific target cells for the purpose of human cell
487 modification, or even the construction of live vaccines based on secreted effectors.

488 Another highlight from the workshop is the increasing evidence in support of
489 convergent strategies for host cell subversion, regardless of the type of secretion
490 system involved in the translocation of the effectors. Different effectors, translocated
491 by different nanomachines, often end up interfering with the same host functions, key

492 for infection, survival and propagation of pathogens in the host cell, as illustrated in Fig
493 1. Typical examples are the manipulation of host cytoskeleton, subversion of host
494 immune response, interference with host signaling pathways, or alteration of vesicular
495 trafficking. This convergence implies that the identification of a novel host target for a
496 TxSS effector can pave the way to find the intracellular target of another, even if they
497 are not transported by the same machinery. Another important consequence is the
498 possibility of finding common effector motifs that could be targeted for broad-
499 spectrum anti-virulence strategies, which could block the action of effectors secreted
500 by different systems. Last, but not least, the study of host cell subversion through
501 bacterial secreted effectors keeps providing us with basic understanding of the human
502 cell physiology.

503

504

505 **Figure Legends**

506

507 **Figure 1.** Schematic representation of T3, T4 and T6SS showing their relation to
508 flagellum, conjugative DNA transfer and phage structures, and their common targets in
509 the host cell. Bacteria (in green) are shown hosting the secretion system and related
510 machineries: flagella for T3SS, conjugative DNA transfer complexes for T4SS, and phage
511 tails for T6SS. Their bacterial targets are also shown: recipient bacteria for DNA
512 transfer (T4SS), or bacteria killed (dotted lines) by T6SS-injected toxins. The three
513 secretion systems can translocate effectors to the human cell, shown to the right,
514 together with the most common subverted cellular functions.

515

516 **Figure 2.** Comparison of the 3D structure of T4SS from plasmid R388 (Low *et al.*,
517 2014), plasmid F (Hu *et al.*, 2019a), *L pneumophila* Dot-Icm (Chetrit *et al.*, 2018), and *H.*
518 *pylori* Cag (Hu *et al.*, 2019b). Below each T4SS, the nature of the translocated substrate
519 (Protein or protein-DNA complexes) and the target cell (bacteria or human) is
520 indicated. The R388 T4SS image is from (Galan *et al.*, 2018). The images for the F-Tra,
521 Dot-Icm and Cag T4SS were kindly provided by Bo Hu (University of Texas, USA). The
522 lanes below show the method for 3D structure elucidation, the stoichiometry of the
523 subcomplexes, and the size of the structure (in Armstrongs) at the outer membrane
524 (OM) side (the diameter of the pore is shown below) or the cytoplasmic (Cyt) side.

525

526

527 **Acknowledgements**

528

529 We wish to thank the UNIA staff, and in particular Joaquin Torreblanca, for
530 organization of the Workshop. We are grateful to the speakers for presenting
531 unpublished data and allowing their citation. We are especially indebted to Bo Hu for
532 kindly providing the Tra, Dot and Cag images shown in Fig 2.

533 Work in SB lab is supported by recurrent funding from the CNRS and Aix-Marseille
534 University and by a grant from the Excellence Initiative of Aix-Marseille University-
535 A*Midex, a French “Investissements d’Avenir” program (“Emergence & Innovation” A-
536 M-AAP-EI-17-139-170301-10.31-BLEVES-HLS). Work in ML lab is funded by grants
537 BIO2017 from the Spanish Ministry of (MINECO), and grant IDEASLLO17 from the
538 Spanish Association Against Cancer (AECC) to ML.

539

540

541 **References**

542

543 Adlung, N. and Bonas, U. (2017). Dissecting virulence function from recognition: cell
 544 death suppression in *Nicotiana benthamiana* by XopQ/HopQ1-family effectors
 545 relies on EDS1-dependent immunity. *Plant J* **91**, 430-442.

546 Aguilera-Herce, J., Garcia-Quintanilla, M., Romero-Flores, R., McConnell, M.J. and
 547 Ramos-Morales, F. (2019). A Live Salmonella Vaccine Delivering PcrV through
 548 the Type III Secretion System Protects against *Pseudomonas aeruginosa*.
 549 *mSphere* **4**.

550 Agundez, L., Zarate-Perez, F., Meier, A.F., Bardelli, M., Llosa, M., Escalante, C.R., *et al.*
 551 (2018). Exchange of functional domains between a bacterial conjugative
 552 relaxase and the integrase of the human adeno-associated virus. *PLoS One* **13**,
 553 e0200841.

554 Aslam, B., Wang, W., Arshad, M.I., Khurshid, M., Muzammil, S., Rasool, M.H., *et al.*
 555 (2018). Antibiotic resistance: a rundown of a global crisis. *Infect Drug Resist* **11**,
 556 1645-1658.

557 Aubert, D.F., Xu, H., Yang, J., Shi, X., Gao, W., Li, L., *et al.* (2016). A Burkholderia Type VI
 558 Effector Deamidates Rho GTPases to Activate the Pyrin Inflammasome and
 559 Trigger Inflammation. *Cell Host Microbe* **19**, 664-674.

560 Backert, S., Bernegger, S., Skorko-Glonek, J. and Wessler, S. (2018). Extracellular HtrA
 561 serine proteases: An emerging new strategy in bacterial pathogenesis. *Cell*
 562 *Microbiol* **20**, e12845.

563 Batan, D., Braselmann, E., Minson, M., Nguyen, D.M.T., Cossart, P. and Palmer, A.E.
 564 (2018). A Multicolor Split-Fluorescent Protein Approach to Visualize Listeria
 565 Protein Secretion in Infection. *Biophys J* **115**, 251-262.

- 566 Berni, B., Soscia, C., Djermoun, S., Ize, B. and Bleves, S. (2019). A Type VI Secretion
567 System Trans-Kingdom Effector Is Required for the Delivery of a Novel
568 Antibacterial Toxin in *Pseudomonas aeruginosa*. *Front Microbiol* **10**, 1218.
- 569 Bhattacharya, S., Baidya, A.K., Pal, R.R., Mamou, G., Gatt, Y.E., Margalit, H., *et al.*
570 (2019). A Ubiquitous Platform for Bacterial Nanotube Biogenesis. *Cell Rep* **27**,
571 334-342 e310.
- 572 Coulthurst, S. (2019). The Type VI secretion system: a versatile bacterial weapon.
573 *Microbiology* **165**, 503-515.
- 574 Chang, Y.W., Shaffer, C.L., Rettberg, L.A., Ghosal, D. and Jensen, G.J. (2018). In Vivo
575 Structures of the *Helicobacter pylori* cag Type IV Secretion System. *Cell Rep* **23**,
576 673-681.
- 577 Cherrak, Y., Flaugnatti, N., Durand, E., Journet, L. and Cascales, E. (2019). Structure and
578 Activity of the Type VI Secretion System. *Microbiol Spectr* **7**.
- 579 Cherrak, Y., Rapisarda, C., Pellarin, R., Bouvier, G., Bardiaux, B., Allain, F., *et al.* (2018).
580 Biogenesis and structure of a type VI secretion baseplate. *Nat Microbiol* **3**,
581 1404-1416.
- 582 Chetrit, D., Hu, B., Christie, P.J., Roy, C.R. and Liu, J. (2018). A unique cytoplasmic
583 ATPase complex defines the *Legionella pneumophila* type IV secretion channel.
584 *Nat Microbiol* **3**, 678-686.
- 585 Christie, P.J. (2001). Type IV secretion: intercellular transfer of macromolecules by
586 systems ancestrally related to conjugation machines. *Mol Microbiol* **40**, 294-
587 305.
- 588 Chung, J.M., Sheedlo, M.J., Campbell, A.M., Sawhney, N., Frick-Cheng, A.E., Lacy, D.B.,
589 *et al.* (2019). Structure of the *Helicobacter pylori* Cag type IV secretion system.
590 *Elife* **8**.
- 591 Di Venanzio, G., Moon, K.H., Weber, B.S., Lopez, J., Ly, P.M., Potter, R.F., *et al.* (2019).
592 Multidrug-resistant plasmids repress chromosomally encoded T6SS to enable
593 their dissemination. *Proc Natl Acad Sci U S A* **116**, 1378-1383.

- 594 Erickson, J.L., Adlung, N., Lampe, C., Bonas, U. and Schattat, M.H. (2018). The
 595 Xanthomonas effector XopL uncovers the role of microtubules in stromule
 596 extension and dynamics in *Nicotiana benthamiana*. *Plant J* **93**, 856-870.
- 597 Fasciano, A.C., Shaban, L. and Meccas, J. (2019). Promises and Challenges of the Type
 598 Three Secretion System Injectisome as an Antivirulence Target. *EcoSal Plus* **8**.
- 599 Frick-Cheng, A.E., Pyburn, T.M., Voss, B.J., McDonald, W.H., Ohi, M.D. and Cover, T.L.
 600 (2016). Molecular and Structural Analysis of the *Helicobacter pylori* cag Type IV
 601 Secretion System Core Complex. *MBio* **7**, e02001-02015.
- 602 Fuhrman-Luck, R.A., Silva, L.M., Hastie, M.L., Gorman, J.J. and Clements, J.A. (2017).
 603 Determining Protease Substrates Within a Complex Protein Background Using
 604 the PROtein TOpography and Migration Analysis Platform (PROTOMAP).
 605 *Methods Mol Biol* **1574**, 145-170.
- 606 Galan, J.E., Lara-Tejero, M., Marlovits, T.C. and Wagner, S. (2014). Bacterial type III
 607 secretion systems: specialized nanomachines for protein delivery into target
 608 cells. *Annual review of microbiology* **68**, 415-438.
- 609 Galan, J.E. and Waksman, G. (2018). Protein-Injection Machines in Bacteria. *Cell* **172**,
 610 1306-1318.
- 611 Ghosal, D., Chang, Y.W., Jeong, K.C., Vogel, J.P. and Jensen, G.J. (2017). In situ structure
 612 of the *Legionella* Dot/Icm type IV secretion system by electron
 613 cryotomography. *EMBO Rep* **18**, 726-732.
- 614 Ghosal, D., Jeong, K.C., Chang, Y.W., Gyore, J., Teng, L., Gardner, A., *et al.* (2019).
 615 Molecular architecture, polar targeting and biogenesis of the *Legionella*
 616 Dot/Icm T4SS. *Nat Microbiol* **4**, 1173-1182.
- 617 Gonzalez-Prieto, C., Gabriel, R., Dehio, C., Schmidt, M. and Llosa, M. (2017). The
 618 Conjugative Relaxase TrwC Promotes Integration of Foreign DNA in the Human
 619 Genome. *Appl Environ Microbiol* **83**.
- 620 Graf, F.E., Palm, M., Warringer, J. and Farewell, A. (2019). Inhibiting conjugation as a
 621 tool in the fight against antibiotic resistance. *Drug Dev Res* **80**, 19-23.

- 622 Green, E.R. and Meccas, J. (2016). Bacterial Secretion Systems: An Overview. *Microbiol*
623 *Spectr* **4**.
- 624 Hachani, A., Wood, T.E. and Filloux, A. (2016). Type VI secretion and anti-host
625 effectors. *Curr Opin Microbiol* **29**, 81-93.
- 626 Harms, A., Liesch, M., Korner, J., Quebatte, M., Engel, P. and Dehio, C. (2017). A
627 bacterial toxin-antitoxin module is the origin of inter-bacterial and inter-
628 kingdom effectors of Bartonella. *PLoS Genet* **13**, e1007077.
- 629 Hayek, I., Berens, C. and Luhrmann, A. (2019). Modulation of host cell metabolism by
630 T4SS-encoding intracellular pathogens. *Curr Opin Microbiol* **47**, 59-65.
- 631 Ho, B.T., Basler, M. and Mekalanos, J.J. (2013). Type 6 secretion system-mediated
632 immunity to type 4 secretion system-mediated gene transfer. *Science* **342**, 250-
633 253.
- 634 Hood, R.D., Singh, P., Hsu, F., Guvener, T., Carl, M.A., Trinidad, R.R., *et al.* (2010). A
635 type VI secretion system of *Pseudomonas aeruginosa* targets a toxin to
636 bacteria. *Cell Host Microbe* **7**, 25-37.
- 637 Hu, B., Khara, P. and Christie, P.J. (2019a). Structural bases for F plasmid conjugation
638 and F pilus biogenesis in *Escherichia coli*. *Proc Natl Acad Sci U S A* **116**, 14222-
639 14227.
- 640 Hu, B., Khara, P., Song, L., Lin, A.S., Frick-Cheng, A.E., Harvey, M.L., *et al.* (2019b). In
641 Situ Molecular Architecture of the *Helicobacter pylori* Cag Type IV Secretion
642 System. *MBio* **10**.
- 643 Hu, B., Lara-Tejero, M., Kong, Q., Galan, J.E. and Liu, J. (2017). In Situ Molecular
644 Architecture of the *Salmonella* Type III Secretion Machine. *Cell* **168**, 1065-1074
645 e1010.
- 646 Ji, C., Du, S., Li, P., Zhu, Q., Yang, X., Long, C., *et al.* (2019). Structural mechanism for
647 guanylate-binding proteins (GBPs) targeting by the *Shigella* E3 ligase IpaH9.8.
648 *PLoS Pathog* **15**, e1007876.

- 649 Jiang, F., Wang, X., Wang, B., Chen, L., Zhao, Z., Waterfield, N.R., *et al.* (2016). The
 650 *Pseudomonas aeruginosa* Type VI Secretion PGAP1-like Effector Induces Host
 651 Autophagy by Activating Endoplasmic Reticulum Stress. *Cell Rep* **16**, 1502-1509.
- 652 Jiang, F., Waterfield, N.R., Yang, J., Yang, G. and Jin, Q. (2014). A *Pseudomonas*
 653 *aeruginosa* type VI secretion phospholipase D effector targets both prokaryotic
 654 and eukaryotic cells. *Cell Host Microbe* **15**, 600-610.
- 655 Lara-Tejero, M. and Galan, J.E. (2019). The Injectisome, a Complex Nanomachine for
 656 Protein Injection into Mammalian Cells. *EcoSal Plus* **8**.
- 657 Leiman, P.G., Basler, M., Ramagopal, U.A., Bonanno, J.B., Sauder, J.M., Pukatzki, S., *et*
 658 *al.* (2009). Type VI secretion apparatus and phage tail-associated protein
 659 complexes share a common evolutionary origin. *Proc Natl Acad Sci U S A* **106**,
 660 4154-4159.
- 661 Li, P., Jiang, W., Yu, Q., Liu, W., Zhou, P., Li, J., *et al.* (2017). Ubiquitination and
 662 degradation of GBPs by a *Shigella* effector to suppress host defence. *Nature*
 663 **551**, 378-383.
- 664 Li, Y.G. and Christie, P.J. (2018). The *Agrobacterium* VirB/VirD4 T4SS: Mechanism and
 665 Architecture Defined Through In Vivo Mutagenesis and Chimeric Systems. *Curr*
 666 *Top Microbiol Immunol* **418**, 233-260.
- 667 Li, Y.G., Hu, B. and Christie, P.J. (2019). Biological and Structural Diversity of Type IV
 668 Secretion Systems. *Microbiol Spectr* **7**.
- 669 Lin, Y.H., Lucas, M., Evans, T.R., Abascal-Palacios, G., Doms, A.G., Beauchene, N.A., *et*
 670 *al.* (2018). RavN is a member of a previously unrecognized group of *Legionella*
 671 *pneumophila* E3 ubiquitin ligases. *PLoS Pathog* **14**, e1006897.
- 672 Low, H.H., Gubellini, F., Rivera-Calzada, A., Braun, N., Connery, S., Dujeancourt, A., *et*
 673 *al.* (2014). Structure of a type IV secretion system. *Nature* **508**, 550-553.
- 674 Llosa, M., Schroder, G. and Dehio, C. (2012). New perspectives into bacterial DNA
 675 transfer to human cells. *Trends in microbiology* **20**, 355-359.

- 676 Moonens, K., Hamway, Y., Neddermann, M., Reschke, M., Tegtmeyer, N., Kruse, T., et
 677 al. (2018). Helicobacter pylori adhesin HopQ disrupts trans dimerization in
 678 human CEACAMs. *EMBO J* **37**.
- 679 Nolan LM, Cain AK, Manoli E, Sainz-Polo MA, Dougan G, Mavridou DAE, et al. (2019).
 680 Discovery of a Pseudomonas aeruginosa type VI secretion system toxin
 681 targeting bacterial protein synthesis using a global genomics approach.
 682 [*Preprint*] Available from : <https://doi.org/10.1101/733030>
- 683 Oikonomou, C.M. and Jensen, G.J. (2019). Electron Cryotomography of Bacterial
 684 Secretion Systems. *Microbiol Spectr* **7**.
- 685 Pearson, J.S., Giogha, C., Muhlen, S., Nachbur, U., Pham, C.L., Zhang, Y., et al. (2017).
 686 EspL is a bacterial cysteine protease effector that cleaves RHIM proteins to
 687 block necroptosis and inflammation. *Nat Microbiol* **2**, 16258.
- 688 Personnic, N., Barlocher, K., Finsel, I. and Hilbi, H. (2016). Subversion of Retrograde
 689 Trafficking by Translocated Pathogen Effectors. *Trends Microbiol* **24**, 450-462.
- 690 Pinero-Lambea, C., Bodelon, G., Fernandez-Perianez, R., Cuesta, A.M., Alvarez-Vallina,
 691 L. and Fernandez, L.A. (2015). Programming controlled adhesion of E. coli to
 692 target surfaces, cells, and tumors with synthetic adhesins. *ACS Synth Biol* **4**,
 693 463-473.
- 694 Pisano, A., Albano, F., Vecchio, E., Renna, M., Scala, G., Quinto, I. and Fiume, G. (2018).
 695 Revisiting Bacterial Ubiquitin Ligase Effectors: Weapons for Host Exploitation.
 696 *Int J Mol Sci* **19**.
- 697 Pissaridou, P., Allsopp, L.P., Wettstadt, S., Howard, S.A., Mavridou, D.A.I. and Filloux, A.
 698 (2018). The Pseudomonas aeruginosa T6SS-VgrG1b spike is topped by a PAAR
 699 protein eliciting DNA damage to bacterial competitors. *Proc Natl Acad Sci U S A*
 700 **115**, 12519-12524.
- 701 Ratner, D., Orning, M.P. and Lien, E. (2017). Bacterial secretion systems and regulation
 702 of inflammasome activation. *J Leukoc Biol* **101**, 165-181.

- 703 Ruano-Gallego, D., Alvarez, B. and Fernandez, L.A. (2015). Engineering the Controlled
704 Assembly of Filamentous Injectisomes in *E. coli* K-12 for Protein Translocation
705 into Mammalian Cells. *ACS Synth Biol* **4**, 1030-1041.
- 706 Sana, T.G., Baumann, C., Merdes, A., Soscia, C., Rattei, T., Hachani, A., *et al.* (2015).
707 Internalization of *Pseudomonas aeruginosa* Strain PAO1 into Epithelial Cells Is
708 Promoted by Interaction of a T6SS Effector with the Microtubule Network.
709 *MBio* **6**, e00712.
- 710 Santin, Y.G., Doan, T., Journet, L. and Cascales, E. (2019). Cell Width Dictates Type VI
711 Secretion Tail Length. *Curr Biol* **29**, 3707-3713 e3703.
- 712 Santin, Y.G., Doan, T., Lebrun, R., Espinosa, L., Journet, L. and Cascales, E. (2018). In
713 vivo TssA proximity labelling during type VI secretion biogenesis reveals TagA as
714 a protein that stops and holds the sheath. *Nat Microbiol* **3**, 1304-1313.
- 715 Sgro, G.G., Costa, T.R.D., Cenens, W., Souza, D.P., Cassago, A., Coutinho de Oliveira, L.,
716 *et al.* (2018). Cryo-EM structure of the bacteria-killing type IV secretion system
717 core complex from *Xanthomonas citri*. *Nat Microbiol* **3**, 1429-1440.
- 718 Sun, H., Kamanova, J., Lara-Tejero, M. and Galan, J.E. (2018). Salmonella stimulates
719 pro-inflammatory signalling through p21-activated kinases bypassing innate
720 immune receptors. *Nat Microbiol* **3**, 1122-1130.
- 721 Tsai, A.Y., English, B.C. and Tsolis, R.M. (2019). Hostile Takeover: Hijacking of
722 Endoplasmic Reticulum Function by T4SS and T3SS Effectors Creates a Niche for
723 Intracellular Pathogens. *Microbiol Spectr* **7**.
- 724 Wagner, A. and Dehio, C. (2019a). Role of distinct type-IV-secretion systems and
725 secreted effector sets in host adaptation by pathogenic *Bartonella* species. *Cell*
726 *Microbiol* **21**, e13004.
- 727 Wagner, A., Tittes, C. and Dehio, C. (2019b). Versatility of the BID Domain: Conserved
728 Function as Type-IV-Secretion-Signal and Secondarily Evolved Effector
729 Functions Within *Bartonella*-Infected Host Cells. *Front Microbiol* **10**, 921.

- 730 Wagner, S., Konigsmair, L., Lara-Tejero, M., Lefebvre, M., Marlovits, T.C. and Galan, J.E.
731 (2010). Organization and coordinated assembly of the type III secretion export
732 apparatus. *Proc Natl Acad Sci U S A* **107**, 17745-17750.
- 733 Walker, B.J., Stan, G.V. and Polizzi, K.M. (2017). Intracellular delivery of biologic
734 therapeutics by bacterial secretion systems. *Expert reviews in molecular*
735 *medicine* **19**, e6.
- 736 Weber, B.S., Ly, P.M., Irwin, J.N., Pukatzki, S. and Feldman, M.F. (2015). A multidrug
737 resistance plasmid contains the molecular switch for type VI secretion in
738 *Acinetobacter baumannii*. *Proc Natl Acad Sci U S A* **112**, 9442-9447.
- 739 Xu, Y., Zhou, P., Cheng, S., Lu, Q., Nowak, K., Hopp, A.K., *et al.* (2019). A Bacterial
740 Effector Reveals the V-ATPase-ATG16L1 Axis that Initiates Xenophagy. *Cell* **178**,
741 552-566 e520.
- 742 Young, A.M., Minson, M., McQuate, S.E. and Palmer, A.E. (2017). Optimized
743 Fluorescence Complementation Platform for Visualizing Salmonella Effector
744 Proteins Reveals Distinctly Different Intracellular Niches in Different Cell Types.
745 *ACS Infect Dis* **3**, 575-584.
- 746