Published in final edited form as:

Microbiol Spectr. 2019 March ; 7(2): . doi:10.1128/microbiolspec.PSIB-0019-2018.

Electron cryo-tomography of bacterial secretion systems

Catherine M. Oikonomou¹ and Grant J. Jensen^{1,2,*}

¹Department of Biology and Biological Engineering, California Institute of Technology, Pasadena, CA, USA

²Howard Hughes Medical Institute, Pasadena, CA, USA

SUMMARY

In biology, function arises from form. For bacterial secretion systems, which often span two membranes, avidly bind to the cell wall, and contain hundreds of individual proteins, studying form is a daunting task, made possible by electron cryo-tomography (ECT). ECT is the highest-resolution imaging technique currently available to visualize unique objects inside cells, providing a 3D view of the shapes and locations of large macromolecular complexes in their native environment. Over the past 15 years, ECT has contributed to the study of bacterial secretion systems in two main ways: by revealing intact forms for the first time, and by mapping components into these forms. Here we highlight some of these contributions, revealing: structural convergence in type II secretion systems, structural divergence in type III secretion systems, unexpected structures in type IV secretion systems, and unexpected mechanisms in types V and VI secretion systems. Together, they offer a glimpse into a world of fantastic forms—nanoscale rotors, needles, pumps and dart guns—much of which remains to be explored.

INTRODUCTION

The envelope of bacterial cells consists of at least one—and often two—membranes, a cell wall, and possibly a surface layer. This envelope allows cells to differentiate themselves from their environment and handle the resulting osmotic pressure, but it also presents a significant obstacle. Anything a cell wishes to export, from a motility appendage to a plasmid, needs to be ushered across this barrier. To accomplish this, bacteria have evolved a battery of secretion systems. Secretions systems are often constructed from dozens of protein building blocks embedded in the cell's envelope. The size, complexity, and location of these machines make them a particular challenge for structural characterization. High-resolution structure determination techniques such as X-ray crystallography and transmission electron microscopy (TEM)-based single particle reconstruction (SPR) require objects to be purified from their cellular environment. This is problematic for membrane-associated proteins, which embed in the lipid bilayer by means of exposed hydrophobic patches. These patches can be protected during purification by adding detergents to the solvent, but often structural alterations still occur. Secretion systems are also unusually large

*Correspondence: jensen@caltech.edu.

targets, and frequently lose peripheral or loosely-associated components during purification. In addition, they often cross two membranes and are avidly linked to the cell wall.

Before the advent of electron cryo-tomography (ECT), we knew an impressive amount about what secretion systems are made of; genetics revealed components, biochemistry revealed their properties, and X-ray crystallography and SPR revealed the structures of purified pieces. But we knew much less about how the pieces fit together into a functional whole. For that, we turned to a complementary technique: ECT. While other techniques reveal structural details of purified components, ECT can be applied directly to intact cells. Briefly, a small volume of bacterial culture is applied to an EM support grid. This grid is then plunged into a cryogen that freezes the sample so quickly that the water cannot crystallize, instead remaining amorphous. The cells are thereby immobilized in a fully-hydrated, native state. This frozen sample is then transferred to the TEM for cryo-EM imaging. A three-dimensional reconstruction of a cell is built up from two-dimensional projection images taken from different angles, accomplished by rotating the sample between images. This reconstruction, or tomogram, shows a snapshot of the contents of the cell without fixatives or stains, typically with a mid-range resolution (~5 nm) sufficient to see the shapes of large macromolecular complexes like secretion systems and their abundance and distribution in the cell. This can be particularly powerful for machines that exist in multiple states *in vivo*, only one of which may persist through purification. Images of different copies of the same structure, in the same cell or from many cells, can be merged by sub-tomogram averaging to generate a higher signal-to-noise view of the structure's inflexible regions (flexible or variable regions get washed out in the average, along with density from nearby but unassociated proteins) (1). While the resolution of these averages is still too low to unambiguously orient atomic structures, in ideal cases, we can combine the information ECT provides about the form(s) of a structure *in situ* with high-resolution knowledge about its isolated components from complementary structural biology techniques.

ECT has served the study of bacterial secretion systems in two main ways: first by simply showing what structures look like inside cells, and second by revealing the relative arrangement of building blocks within those structures. This has provided some spectacular glimpses into how form is transformed into function, which in some cases have dramatically advanced fields of research. Here we briefly highlight some examples of these insights into bacterial secretion systems provided by ECT; the biology of the systems is detailed elsewhere. For recent, in-depth reviews of the subject, including both ECT and SPR, we recommend (2, 3). In addition, (4) provides an excellent overview of the diversity of bacterial secretion systems.

Type II Secretion Systems (T2SS)

Capturing snapshots of cellular machinery in different states can often yield insights into how it works. For instance, ECT of the type IVa pilus (T4aP)—a T2SS-related machine that extends and retracts a long extracellular pilus for motility—showed that the structure comprises a series of stacked rings spanning the periplasm (Figure 1A) (5). Imaging structures with and without pili assembled revealed how these rings undergo an ~3 nm

rearrangement to un-gate the channel and allow the assembling filament to extend out of the cell (5, 6).

For the T4aP, the high-resolution structures of many components had been solved, and biochemical interactions between many components had been worked out (7–28). Still, putting the pieces together presented a puzzle. To solve it, we applied ECT to a series of mutant strains in which T4aP components were deleted or tagged with additional protein density. By looking for corresponding absences or additions in the structure, we deduced the approximate locations of the components. Guided by clues from biochemical studies, we could thus place each high-resolution 3D puzzle piece in its proper location (Figure 1A) (6). Again, knowing where different pieces lie in the finished form often gives clues about how they might function. For instance, we saw that the ATPase in the cytoplasm was leveraged by a clamp connected to the integrated system of rings. This likely allows the assembly ATPase to rotate an adapter protein in the inner membrane (IM) to scoop pilin subunits from the membrane into the assembling pilus. Switching to a retraction mode, the clamp recruits a different ATPase to release subunits back into the IM. The signal for this switch from extension to retraction is likely sensed by a ring in the periplasm and transmitted through coiled-coil domains to the ATPase clamp (6).

Often, knowing the map of one structure has a domino effect, triggering insights into other, related machines. ECT of the type IVb pilus (T4bP) revealed a structure strikingly similar to that of the T4aP despite the fact that only half its components have homologs in the T4aP (Figure 1B) (29). For instance, a ring in the periplasm thought to act as a sensor in the T4aP is also present in the T4bP, but is formed by an unrelated protein. Another member of the family, the pathogenic T2SS, does not assemble a long extracellular pilus filament but rather pumps out effector proteins (30). Again, ECT mapping found the same ring, again formed by a non-conserved protein (Figure 1C). In this case, the ring was flexible with respect to the rest of the machine, suggesting a possible role in loading cargo (31).

Type III Secretion Systems (T3SS)

The first secretion system studied by ECT was the T3SS-based flagellar motor, responsible for assembling, and subsequently rotating, the long flagellar filaments that propel the motility of many bacterial cells (32). The flagellar motor had been a subject of fascination for decades and elements of its mechanism (33) and components (34) were well understood. Numerous EM studies, including SPR, of purified motors had revealed its core structure (e.g. (35–40)). Still, key details remained unresolved, including the structure of the export complex in the cytoplasm responsible for assembling the machine, and the interaction between the rotating (rotor) and stationary (stator) portions of the motor. ECT studies were able to reveal the interactions between these components, as well as the structure and location of peripheral components lost upon purification, such as the export apparatus (32, 41–43).

One of the most striking observations that came from studying flagellar motors *in situ* was how their form is adapted to the lifestyles of different species (Figure 2A) (44). For instance, homologous components come together in different stoichiometries to form rings of different diameters, allowing the motor to gear up or down and generate different amounts of

torque (32, 45–47). Some species (particularly pathogens) have elaborated their motors with additional components, perhaps to stabilize them against the increased load of more viscous environments (48), or to keep their associated filaments sheathed in membrane (49).

Pathogenic bacteria like *Yersinia* and *Salmonella* use a different T3SS to assemble, instead of a motility apparatus, a needle-like injectisome to deliver effector proteins not only across the bacterium's envelope but further past the membrane of a target host cell (50). Injectisomes and flagellar motors are constructed from homologous components but serve strikingly different functions (Figure 2B) (51). In an example of how seeing the shapes of machines *in situ* can provide clues about how they work, ECT revealed how the export apparatus—also known as the sorting complex—differs from that of the flagellar motor, with subunits no longer forming a wide rotary ring, but rather radial spokes better suited to recognize and feed diverse effector proteins to the central export channel (52). Comparing ECT images of injectisomes *in vivo* with and without the sorting complex showed how the radial symmetry induces a dramatic rearrangement of a ring at the base of the needle (53). Even inside the cell, the sorting complex is fragile; deletion of individual components often disrupts the whole complex (53, 54). These pieces can, however, be functionally tagged with additional protein density, which allowed them to be mapped by ECT. Knowing where components are located, and in what orientation, often provides important clues about how the system works. For instance, seeing that the substrate/chaperone-binding domain of the ATPase sits adjacent to the export gate suggests that the cage of the sorting complex forms an antechamber in which substrates are prepared for secretion (53).

Recently, ECT was used to capture the interaction of *S. Typhimurium* injectisomes with host cells, revealing the translocon pore at the tip of the complex in the host membrane and supporting a direct injection model of substrates across the host membrane (55). The translocon was much smaller (~13.5 nm outer diameter) than a homologous structure from EPEC previously reconstituted in liposomes (~55–65 nm) (56), underscoring the importance of studying secretion systems *in situ*.

Type IV Secretion Systems (T4SS)

Attempting to purify structures out of the cell often leads to confounding alterations. For instance, SPR structures of purified T4SS showed a chamber adjacent to the outer membrane (OM), connected to the IM by a solid stalk, suggesting that substrates enter the outer chamber from the periplasm (57, 58). It was therefore a surprise when ECT structures of the Dot/Icm T4SS *in situ* revealed instead an open channel extending through the periplasm (Figure 3A) (59, 60). This feature, together with observations of complex assemblies of multiple ATPases in the cytoplasm and flexible “wings” in the periplasm (59–61), suggests that there might be two paths by which substrates could pass through the Dot/Icm T4SS: one through the central channel from the cytoplasm and one laterally into the outer chamber from the periplasm (60). This could help explain how the system can export such a spectacular number (~300) of discrete effectors (62).

Putting structures in context inside the cell can answer many questions, but it often raises more. ECT images of Cag T4SS in *Helicobacter pylori* showed periplasmic machines structurally very similar to Dot/Icm T4SS. Next to them, however, we sometimes saw wide

(~40 nm) OM tubes, studded with portals, extending outward up to 500 nm from the cell (Figure 3B) (61). The two structures appear to be related, but how, and what the resulting mechanism of effector secretion might be, remains puzzling.

Type V Secretion Systems (T5SS)

Sometimes, even seeing something as simple as a stick can be illuminating. Many bacteria kill nearby cells through the process of contact-dependent inhibition (CDI). In *E. coli* this process is mediated by a T5SS comprising two proteins; (1) CdiB, a β -barrel in the OM secretes (2) CdiA, a large multi-domain protein containing the toxin (63). It was known that toxin translocation is mediated by interaction of CdiA with a receptor, BamA, on the target cell (64), but the mechanism was unknown. Using ECT, we saw that CdiA forms rods extending out from the surface of the cell (Figure 4A), but the length of the rod was only half that predicted for the protein. This explained why the receptor recognition domain is located in the middle of the protein (65), and helped elucidate a novel mechanism whereby CdiB initially secretes only the N-terminal half of CdiA, which forms a structured rod. The rest of the protein, including the C-terminal toxin, remains sequestered in the periplasm; target binding at the end of the rod stimulates secretion to continue, bringing the toxin-laden tip out of the periplasm and, like a tetherball, into the target cell (Figure 4B) (66).

Type VI Secretion Systems (T6SS)

In 2005, our group observed tubular structures in cryotomograms of *Vibrio cholerae* (Figure 5A) but did not know what they were until, with John Mekalanos, we recognized them as T6SS, which allow some bacteria to kill others from different species (67). Our cryotomograms immediately revealed the basic mechanism: two nested tubes, a sheath and a rod, function as a nanoscale dart gun ((68); also recognized in (69)). Firing entails a constriction in the outer sheath, providing the energy to propel the inner rod, loaded with toxin, through the machine's baseplate in the cell's own envelope and then punching through the membrane of a neighboring target (68). Subsequent ECT studies revealed additional details, including the structure of the baseplate (Figure 5B) (70, 71) and higher-resolution details of the sheath that showed how its contraction may expose recycling domains, allowing it to be disassembled into the building blocks of another T6SS (70). We showed that T6SS assemble extensive batteries in some species (Figure 5C), and that other cells lyse to release micron-scale porcupine arrays of T6SS-related structures (Figure 5D) (72). Some details remain mysterious; could the extracellular fibers seen associated with T6SS *in vivo* (70) mediate target recognition?

OUTLOOK

Much work lies ahead. As described above, the machines we have managed to glimpse inside cells, or even map in detail, still hold many mysteries. In addition, systems studied in one species need to be compared across others; as the T3SS flagellar motor showed, they can be strikingly diverse. Some machines, including the T1SS, T7SS, T8SS and T9SS, have yet to be seen at all *in vivo*. Others, including the T2SS and T4SS, have only been observed in an inactive state and it is now of great interest to see their active forms. In some cases, this will require capturing pathogens interacting with their hosts, samples too thick for direct

imaging by ECT. The developing application of focused ion beam (FIB) milling to thin such biological samples should soon open up even these targets for observation (73).

The study of bacterial secretion systems has always been intricately linked to the development of ECT technology. ECT requires highly specialized, expensive equipment and considerable expertise. As a result, the studies we have described here come from only a handful of labs. Fortunately, secretion systems have proven to be wonderful test beds for pioneering new ECT technology. Our sub-tomogram average of the flagellar motor was one of the first sub-tomogram averages (32). Our subsequent comparison of flagellar motors in different species was the result of establishing a pipeline to collect many (~40) datasets per day (44). Our dissection of the T4aP by imaging strains with GFP tags was the first to demonstrate that approach (6). The Pilhofer lab's cryo-FIB milling of bacterial cells to study the T6SS was one of the first examples of that application (74). No doubt more discoveries about these fascinating nanomachines will come from continuing development of ECT.

ACKNOWLEDGMENTS

We apologize to our colleagues whose work we could not discuss due to limited space and thank members of the Jensen lab for helpful discussions. Work on bacterial secretion systems in the lab is supported by the NIH (grant R01 AI127401 to G.J.J.).

REFERENCES

1. Oikonomou CM, Jensen GJ. 2017 Cellular Electron Cryotomography: Toward Structural Biology In Situ. *Annu Rev Biochem* 86:873–896. [PubMed: 28426242]
2. Rapisarda C, Tassinari M, Gubellini F, Fronzes R. 2018 Using Cryo-EM to Investigate Bacterial Secretion Systems. *Annu Rev Microbiol* 72:231–254. [PubMed: 30004822]
3. Kooger R, Szwedziak P, Bock D, Pilhofer M. 2018 CryoEM of bacterial secretion systems. *Curr Opin Struct Biol* 52:64–70. [PubMed: 30223223]
4. Costa TR, Felisberto-Rodrigues C, Meir A, Prevost MS, Redzej A, Trokter M, Waksman G. 2015 Secretion systems in Gram-negative bacteria: structural and mechanistic insights. *Nat Rev Microbiol* 13:343–359. [PubMed: 25978706]
5. Gold VA, Salzer R, Averhoff B, Kuhlbrandt W. 2015 Structure of a type IV pilus machinery in the open and closed state. *Elife* 4.
6. Chang YW, Rettberg LA, Treuner-Lange A, Iwasa J, Sogaard-Andersen L, Jensen GJ. 2016 Architecture of the type IVa pilus machine. *Science* 351:aad2001. [PubMed: 26965631]
7. Abendroth J, Murphy P, Sandkvist M, Bagdasarian M, Hol WG. 2005 The X-ray structure of the type II secretion system complex formed by the N-terminal domain of EpsE and the cytoplasmic domain of EpsL of *Vibrio cholerae*. *J Mol Biol* 348:845–855. [PubMed: 15843017]
8. Craig L, Volkmann N, Arvai AS, Pique ME, Yeager M, Egelman EH, Tainer JA. 2006 Type IV pilus structure by cryo-electron microscopy and crystallography: implications for pilus assembly and functions. *Mol Cell* 23:651–662. [PubMed: 16949362]
9. Balasingham SV, Collins RF, Assalkhou R, Homberset H, Frye SA, Derrick JP, Tonjum T. 2007 Interactions between the lipoprotein PilP and the secretin PilQ in *Neisseria meningitidis*. *J Bacteriol* 189:5716–5727. [PubMed: 17526700]
10. Yamagata A, Tainer JA. 2007 Hexameric structures of the archaeal secretion ATPase GspE and implications for a universal secretion mechanism. *EMBO J* 26:878–890. [PubMed: 17255937]
11. Satyshur KA, Worzalla GA, Meyer LS, Heiniger EK, Aukema KG, Misic AM, Forest KT. 2007 Crystal structures of the pilus retraction motor PilT suggest large domain movements and subunit cooperation drive motility. *Structure* 15:363–376. [PubMed: 17355871]

12. Ayers M, Sampaleanu LM, Tammam S, Koo J, Harvey H, Howell PL, Burrows LL. 2009 PilM/N/O/P proteins form an inner membrane complex that affects the stability of the *Pseudomonas aeruginosa* type IV pilus secretin. *J Mol Biol* 394:128–142. [PubMed: 19857645]
13. Abendroth J, Mitchell DD, Korotkov KV, Johnson TL, Kreger A, Sandkvist M, Hol WG. 2009 The three-dimensional structure of the cytoplasmic domains of EpsF from the type 2 secretion system of *Vibrio cholerae*. *J Struct Biol* 166:303–315. [PubMed: 19324092]
14. Bulyha I, Schmidt C, Lenz P, Jakovljevic V, Hone A, Maier B, Hoppert M, Sogaard-Andersen L. 2009 Regulation of the type IV pili molecular machine by dynamic localization of two motor proteins. *Mol Microbiol* 74:691–706. [PubMed: 19775250]
15. Sampaleanu LM, Bonanno JB, Ayers M, Koo J, Tammam S, Burley SK, Almo SC, Burrows LL, Howell PL. 2009 Periplasmic domains of *Pseudomonas aeruginosa* PilN and PilO form a stable heterodimeric complex. *J Mol Biol* 394:143–159. [PubMed: 19857646]
16. Mistic AM, Satyshur KA, Forest KT. 2010 *P. aeruginosa* PilT structures with and without nucleotide reveal a dynamic type IV pilus retraction motor. *J Mol Biol* 400:1011–1021. [PubMed: 20595000]
17. Karuppiiah V, Hassan D, Saleem M, Derrick JP. 2010 Structure and oligomerization of the PilC type IV pilus biogenesis protein from *Thermus thermophilus*. *Proteins* 78:2049–2057. [PubMed: 20455262]
18. Karuppiiah V, Derrick JP. 2011 Structure of the PilM-PilN inner membrane type IV pilus biogenesis complex from *Thermus thermophilus*. *J Biol Chem* 286:24434–24442. [PubMed: 21596754]
19. Korotkov KV, Johnson TL, Jobling MG, Pruneda J, Pardon E, Heroux A, Turley S, Steyaert J, Holmes RK, Sandkvist M, Hol WG. 2011 Structural and functional studies on the interaction of GspC and GspD in the type II secretion system. *PLoS Pathog* 7:e1002228. [PubMed: 21931548]
20. Tammam S, Sampaleanu LM, Koo J, Sundaram P, Ayers M, Chong PA, Forman-Kay JD, Burrows LL, Howell PL. 2011 Characterization of the PilN, PilO and PilP type IV pilus subcomplex. *Mol Microbiol* 82:1496–1514. [PubMed: 22053789]
21. Gu S, Kelly G, Wang X, Frenkiel T, Shevchik VE, Pickersgill RW. 2012 Solution structure of homology region (HR) domain of type II secretion system. *J Biol Chem* 287:9072–9080. [PubMed: 22253442]
22. Georgiadou M, Castagnini M, Karimova G, Ladant D, Pelicic V. 2012 Large-scale study of the interactions between proteins involved in type IV pilus biology in *Neisseria meningitidis*: characterization of a subcomplex involved in pilus assembly. *Mol Microbiol* 84:857–873. [PubMed: 22486968]
23. Karuppiiah V, Collins RF, Thistlethwaite A, Gao Y, Derrick JP. 2013 Structure and assembly of an inner membrane platform for initiation of type IV pilus biogenesis. *Proc Natl Acad Sci U S A* 110:E4638–4647. [PubMed: 24218553]
24. Li C, Wallace RA, Black WP, Li YZ, Yang Z. 2013 Type IV pilus proteins form an integrated structure extending from the cytoplasm to the outer membrane. *PLoS One* 8:e70144. [PubMed: 23922942]
25. Lu C, Turley S, Marionni ST, Park YJ, Lee KK, Patrick M, Shah R, Sandkvist M, Bush MF, Hol WG. 2013 Hexamers of the type II secretion ATPase GspE from *Vibrio cholerae* with increased ATPase activity. *Structure* 21:1707–1717. [PubMed: 23954505]
26. Tammam S, Sampaleanu LM, Koo J, Manoharan K, Daubaras M, Burrows LL, Howell PL. 2013 PilMNOPQ from the *Pseudomonas aeruginosa* type IV pilus system form a transenvelope protein interaction network that interacts with PilA. *J Bacteriol* 195:2126–2135. [PubMed: 23457250]
27. Friedrich C, Bulyha I, Sogaard-Andersen L. 2014 Outside-in assembly pathway of the type IV pilus system in *Myxococcus xanthus*. *J Bacteriol* 196:378–390. [PubMed: 24187092]
28. Siewering K, Jain S, Friedrich C, Webber-Birungi MT, Semchonok DA, Binzen I, Wagner A, Huntley S, Kahnt J, Klingl A, Boekema EJ, Sogaard-Andersen L, van der Does C. 2014 Peptidoglycan-binding protein TsaP functions in surface assembly of type IV pili. *Proc Natl Acad Sci U S A* 111:E953–961. [PubMed: 24556993]
29. Chang YW, Kjaer A, Ortega DR, Kovacicova G, Sutherland JA, Rettberg LA, Taylor RK, Jensen GJ. 2017 Architecture of the *Vibrio cholerae* toxin-coregulated pilus machine revealed by electron cryotomography. *Nat Microbiol* 2:16269. [PubMed: 28165453]

30. Thomassin JL, Santos Moreno J, Guilvout I, Tran Van Nhieu G, Francetic O. 2017 The trans-envelope architecture and function of the type 2 secretion system: new insights raising new questions. *Mol Microbiol* 105:211–226. [PubMed: 28486768]
31. Ghosal D, Kim KW, Zheng H, Kaplan M, Vogel JP, Cianciotto NP, Jensen GJ. 2019 In vivo structure of the *Legionella* type II secretion system by electron cryotomography. *bioRxiv* doi: 10.1101/525063.
32. Murphy GE, Leadbetter JR, Jensen GJ. 2006 In situ structure of the complete *Treponema primitia* flagellar motor. *Nature* 442:1062–1064. [PubMed: 16885937]
33. Berg HC. 2003 The rotary motor of bacterial flagella. *Annu Rev Biochem* 72:19–54. [PubMed: 12500982]
34. Macnab RM. 2003 How bacteria assemble flagella. *Annu Rev Microbiol* 57:77–100. [PubMed: 12730325]
35. DePamphilis ML, Adler J. 1971 Fine structure and isolation of the hook-basal body complex of flagella from *Escherichia coli* and *Bacillus subtilis*. *J Bacteriol* 105:384–395. [PubMed: 4993325]
36. Stallmeyer MJ, Aizawa S, Macnab RM, DeRosier DJ. 1989 Image reconstruction of the flagellar basal body of *Salmonella typhimurium*. *J Mol Biol* 205:519–528. [PubMed: 2648007]
37. Francis NR, Sosinsky GE, Thomas D, DeRosier DJ. 1994 Isolation, characterization and structure of bacterial flagellar motors containing the switch complex. *J Mol Biol* 235:1261–1270. [PubMed: 8308888]
38. Thomas DR, Morgan DG, DeRosier DJ. 1999 Rotational symmetry of the C ring and a mechanism for the flagellar rotary motor. *Proc Natl Acad Sci U S A* 96:10134–10139. [PubMed: 10468575]
39. Suzuki H, Yonekura K, Namba K. 2004 Structure of the rotor of the bacterial flagellar motor revealed by electron cryomicroscopy and single-particle image analysis. *J Mol Biol* 337:105–113. [PubMed: 15001355]
40. Thomas DR, Francis NR, Xu C, DeRosier DJ. 2006 The three-dimensional structure of the flagellar rotor from a clockwise-locked mutant of *Salmonella enterica* serovar Typhimurium. *J Bacteriol* 188:7039–7048. [PubMed: 17015643]
41. Liu J, Lin T, Botkin DJ, McCrum E, Winkler H, Norris SJ. 2009 Intact flagellar motor of *Borrelia burgdorferi* revealed by cryo-electron tomography: evidence for stator ring curvature and rotor/C-ring assembly flexion. *J Bacteriol* 191:5026–5036. [PubMed: 19429612]
42. Kudryashev M, Cyrklaff M, Wallich R, Baumeister W, Frischknecht F. 2010 Distinct in situ structures of the *Borrelia* flagellar motor. *J Struct Biol* 169:54–61. [PubMed: 19699799]
43. Abrusci P, Vergara-Irigaray M, Johnson S, Beeby MD, Hendrixson DR, Roversi P, Friede ME, Deane JE, Jensen GJ, Tang CM, Lea SM. 2013 Architecture of the major component of the type III secretion system export apparatus. *Nat Struct Mol Biol* 20:99–104. [PubMed: 23222644]
44. Chen S, Beeby M, Murphy GE, Leadbetter JR, Hendrixson DR, Briegel A, Li Z, Shi J, Tocheva EI, Muller A, Dobro MJ, Jensen GJ. 2011 Structural diversity of bacterial flagellar motors. *EMBO J* 30:2972–2981. [PubMed: 21673657]
45. Beeby M, Ribardo DA, Brennan CA, Ruby EG, Jensen GJ, Hendrixson DR. 2016 Diverse high-torque bacterial flagellar motors assemble wider stator rings using a conserved protein scaffold. *Proc Natl Acad Sci U S A* 113:E1917–1926. [PubMed: 26976588]
46. Qin Z, Lin WT, Zhu S, Franco AT, Liu J. 2016 Imaging the motility and chemotaxis machineries in *Helicobacter pylori* by cryo-electron tomography. *J Bacteriol* doi:10.1128/JB.00695-16.
47. Chaban B, Coleman I, Beeby M. 2018 Evolution of higher torque in *Campylobacter*-type bacterial flagellar motors. *Sci Rep* 8:97. [PubMed: 29311627]
48. Kaplan M, Ghosal D, Subramanian P, Oikonomou CM, Kjaer A, Pirbadian S, Ortega DR, Briegel A, El-Naggar MY, Jensen GJ. 2019 The presence and absence of periplasmic rings in bacterial flagellar motors correlates with stator type. *Elife* 8.
49. Zhu S, Nishikino T, Hu B, Kojima S, Homma M, Liu J. 2017 Molecular architecture of the sheathed polar flagellum in *Vibrio alginolyticus*. *Proc Natl Acad Sci U S A* 114:10966–10971. [PubMed: 28973904]
50. Galan JE, Lara-Tejero M, Marlovits TC, Wagner S. 2014 Bacterial type III secretion systems: specialized nanomachines for protein delivery into target cells. *Annu Rev Microbiol* 68:415–438. [PubMed: 25002086]

51. Diepold A, Armitage JP. 2015 Type III secretion systems: the bacterial flagellum and the injectisome. *Philos Trans R Soc Lond B Biol Sci* 370.
52. Hu B, Morado DR, Margolin W, Rohde JR, Arizmendi O, Picking WL, Picking WD, Liu J. 2015 Visualization of the type III secretion sorting platform of *Shigella flexneri*. *Proc Natl Acad Sci U S A* 112:1047–1052. [PubMed: 25583506]
53. Hu B, Lara-Tejero M, Kong Q, Galan JE, Liu J. 2017 In Situ Molecular Architecture of the *Salmonella* Type III Secretion Machine. *Cell* 168:1065–1074 e1010. [PubMed: 28283062]
54. Wagner S, Konigsmaier L, Lara-Tejero M, Lefebvre M, Marlovits TC, Galan JE. 2010 Organization and coordinated assembly of the type III secretion export apparatus. *Proc Natl Acad Sci U S A* 107:17745–17750. [PubMed: 20876096]
55. Park D, Lara-Tejero M, Waxham MN, Li W, Hu B, Galan JE, Liu J. 2018 Visualization of the type III secretion mediated *Salmonella*-host cell interface using cryo-electron tomography. *Elife* 7.
56. Ide T, Laarmann S, Greune L, Schillers H, Oberleithner H, Schmidt MA. 2001 Characterization of translocation pores inserted into plasma membranes by type III-secreted Esp proteins of enteropathogenic *Escherichia coli*. *Cell Microbiol* 3:669–679. [PubMed: 11580752]
57. Low HH, Gubellini F, Rivera-Calzada A, Braun N, Connery S, Dujeancourt A, Lu F, Redzej A, Fronzes R, Orlova EV, Waksman G. 2014 Structure of a type IV secretion system. *Nature* 508:550–553. [PubMed: 24670658]
58. Frick-Cheng AE, Pyburn TM, Voss BJ, McDonald WH, Ohi MD, Cover TL. 2016 Molecular and Structural Analysis of the *Helicobacter pylori* cag Type IV Secretion System Core Complex. *MBio* 7:e02001–02015. [PubMed: 26758182]
59. Chetrit D, Hu B, Christie PJ, Roy CR, Liu J. 2018 A unique cytoplasmic ATPase complex defines the *Legionella pneumophila* type IV secretion channel. *Nat Microbiol* 3:678–686. [PubMed: 29784975]
60. Ghosal D, Chang YW, Jeong KC, Vogel JP, Jensen GJ. 2018 Molecular architecture of the *Legionella* Dot/Icm type IV secretion system. *bioRxiv* doi:10.1101/312009.
61. Chang YW, Shaffer CL, Rettberg LA, Ghosal D, Jensen GJ. 2018 In Vivo Structures of the *Helicobacter pylori* cag Type IV Secretion System. *Cell Rep* 23:673–681. [PubMed: 29669273]
62. Ensminger AW. 2016 *Legionella pneumophila*, armed to the hilt: justifying the largest arsenal of effectors in the bacterial world. *Curr Opin Microbiol* 29:74–80. [PubMed: 26709975]
63. Aoki SK, Pamma R, Hernday AD, Bickham JE, Braaten BA, Low DA. 2005 Contact-dependent inhibition of growth in *Escherichia coli*. *Science* 309:1245–1248. [PubMed: 16109881]
64. Aoki SK, Malinverni JC, Jacoby K, Thomas B, Pamma R, Trinh BN, Remers S, Braaten BA, Silhavy TJ, Low DA. 2008 Contact-dependent growth inhibition requires the essential outer membrane protein BamA (YaeT) as the receptor and the inner membrane transport protein AcrB. *Mol Microbiol* 70:323–340. [PubMed: 18761695]
65. Ruhe ZC, Nguyen JY, Xiong J, Koskiniemi S, Beck CM, Perkins BR, Low DA, Hayes CS. 2017 CdiA Effectors Use Modular Receptor-Binding Domains To Recognize Target Bacteria. *MBio* 8.
66. Ruhe ZC, Subramanian P, Song K, Nguyen JY, Stevens TA, Low DA, Jensen GJ, Hayes CS. 2018 Programmed secretion arrest and receptor-triggered toxin export during antibacterial contact-dependent growth inhibition. *Cell* in press.
67. MacIntyre DL, Miyata ST, Kitaoka M, Pukatzki S. 2010 The *Vibrio cholerae* type VI secretion system displays antimicrobial properties. *Proc Natl Acad Sci U S A* 107:19520 [PubMed: 20974937]
68. Basler M, Pilhofer M, Henderson GP, Jensen GJ, Mekalanos JJ. 2012 Type VI secretion requires a dynamic contractile phage tail-like structure. *Nature* 483:182–186. [PubMed: 22367545]
69. Bonemann G, Pietrosiuk A, Mogk A. 2010 Tubules and donuts: a type VI secretion story. *Mol Microbiol* 76:815–821. [PubMed: 20444095]
70. Chang YW, Rettberg LA, Ortega DR, Jensen GJ. 2017 In vivo structures of an intact type VI secretion system revealed by electron cryotomography. *EMBO Rep* 18:1090–1099. [PubMed: 28487352]
71. Rapisarda C, Cherrak Y, Kooger R, Schmidt V, Pellarin R, Logger L, Cascales E, Pilhofer M, Durand E, Fronzes R. 2018 In situ and high-resolution cryo-EM structure of the type VI secretion membrane complex. *bioRxiv* doi:10.1101/441683.

72. Shikuma NJ, Pilhofer M, Weiss GL, Hadfield MG, Jensen GJ, Newman DK. 2014 Marine tubeworm metamorphosis induced by arrays of bacterial phage tail-like structures. *Science* 343:529–533. [PubMed: 24407482]
73. Medeiros JM, Bock D, Pilhofer M. 2018 Imaging bacteria inside their host by cryo-focused ion beam milling and electron cryotomography. *Curr Opin Microbiol* 43:62–68. [PubMed: 29291450]
74. Bock D, Medeiros JM, Tsao HF, Penz T, Weiss GL, Aistleitner K, Horn M, Pilhofer M. 2017 In situ architecture, function, and evolution of a contractile injection system. *Science* 357:713–717. [PubMed: 28818949]

Figure 1. Type II Secretion Systems.

(A) ECT revealed the structures of the T4aP in *Myxococcus xanthus* and *Thermus thermophilus*, enabling high-resolution structures to be placed into the map to produce a hypothetical model of the machine. Further studies revealed structural convergence of the related (B) T4bP in *Vibrio cholerae* and (C) T2SS in *Legionella pneumophila*, with a similar periplasmic ring (arrows) formed by non-homologous proteins in each system. Note in these and subsequent figures how bacterial secretion systems often locally distort the cell envelope *in vivo*. Images: (A left, middle) reprinted with permission from (6); (A right) (5); (B) (29); (C) (31).

Figure 2. Type III Secretion Systems.

ECT and sub-tomogram averaging revealed the *in situ* structures of diverse T3SS, including the flagellar motors of many bacteria (three examples, from *Salmonella enterica*, *Vibrio fischeri* and *Campylobacter jejuni*, shown in **A**) and the injectisome from *Salmonella Typhimurium* (**B**). Structures shown from left to right: EMD-3154, EMD-3155 and EBD-3150 from (45) and EMD-2667 from (52).

Figure 3. Type IV Secretion Systems.

(A) ECT revealed the *in situ* structure of the *Legionella pneumophila* Dot/Icm T4SS, including a central channel extending from the inner membrane, and enabled components to be placed in the map. (B) It also revealed the similar structure of the *Helicobacter pylori* Cag T4SS (inset), as well as novel, related outer membrane tubes. Images: (A) reprinted with permission from (60), see also (59); (B) from (61).

Figure 4. Type V Secretion System.

(A) ECT revealed the stick-like form of the *Escherichia coli* CDI T5SS (arrows) and (B) helped elucidate its mechanism, in which half of CdiA extends out from the cell surface. Upon target binding, the remaining half of CdiA is exported to deliver the toxin to the target cell. Images reprinted with permission from (66).

Figure 5. Type VI Secretion Systems.

ECT revealed (A) the contractile mechanism of the T6SS and (B) its structure *in situ* in *Myxococcus xanthus*. It also revealed (C) higher-order arrays of T6SS in *Amoebophilus asiaticus* and (D) a T6SS-related metamorphosis-associated contractile structure (MAC) in *Pseudoalteromonas luteoviolacea*. Images: (A, B) reprinted with permission from (70), (D) from (72).