INTRODUCTION

Bacterial secretion systems all tackle the challenge of secreting substrates from the cytoplasm out of the cell. Many unrelated secretion systems have evolved, strikingly diverse in their structures and mechanisms, from the small pump-like type II secretion systems to the colossal harpoon-like type VI secretion systems. What makes secretion systems intriguing also makes them difficult to study, however: they are cell envelope-associated, frequently contain moving or transiently associated components, and are very large.

Cryogenic electron microscopy (CryoEM) is ideally poised to study secretion systems. CryoEM excels at determining the structures of large protein complexes, can discern different conformational states, and can image structures in situ. CryoEM has come of age in the past half-decade following a spectacular resolution revolution that changed cryoEM from low-resolution “blobology” to a methodology that can compete with X-ray crystallography and NMR for structure determination of biological molecules (Kühlbrandt, 2014). Here, we provide a primer for microbiologists on these capabilities and limitations of two complementary cryoEM techniques for studying bacterial secretion systems. The first, single particle analysis, determines the structures of purified protein complexes to resolutions sufficient for molecular modeling, while the second, electron cryotomography and subtomogram averaging, tends to determine more modest resolution structures of protein complexes in intact cells. We illustrate these abilities with examples of insights provided into how secretion systems work by cryoEM, with a focus on type III secretion systems.

2 | CryoEM

CryoEM is a family of techniques that determine the three-dimensional (3-D) structures of biological molecules from two-dimensional (2-D) electron micrographs. In basic terms, this is achieved by imaging the specimen from many different angles to facilitate piecing together
its structure from disparate views (Penczek, 2010). The “cryo” refers to the cryogenic temperatures that the biological specimen is fixed and preserved at, which provides a near-native frozen-hydrated state and mitigates electron-mediated damage (discussed below).

The two major cryoEM techniques relevant to studying secretion systems are high-resolution structure determination of purified complexes using electron cryomicroscopy single particle analysis (SPA) (Cheng et al., 2015), and more modest-resolution structure determination of complexes in situ using electron cryotomography (cryoET) subtomogram averaging (STA) (Ferreira et al., 2018). SPA is the primary cryoEM method that takes hundreds of thousands of 2-D projection images of purified single particles in random orientations to determine their structure. CryoET, on the contrary, images a single unique object such as a cell from different angles to determine a 3-D volume of the cell’s ultrastructure, referred to as a tomogram. Similar to SPA, STA then takes hundreds or thousands of 3-D particles in situ from within tomograms to determine an average structure. Other specialized forms of cryoEM including helical analysis (Diaz et al., 2010; Egelman, 2010), 2-D electron crystallography (Schenk et al., 2010), and 3-D electron crystallography (MicroED) (Ubbink et al., 2018) are beyond the scope of this review.

2.1 | Sample preparation

As with all molecular biology, substantial sample optimization is crucial to increase purity, stability, and yield of purified protein complexes for SPA; or protein complex abundance and optimized cells for STA. All cryoEM approaches require that the user acquires electron micrographs of samples fixed by flash-freezing. Flash-freezing protects the specimen in the high vacuum of the microscope column and is sufficiently fast to freeze water as amorphous ice instead of the damaging expansion of crystalline ice. No stains are added, as contrast is achieved by manipulating the optics of the microscope to provide phase contrast. A central concern is that the sample must be no thicker than a few hundred nanometers to minimize noise from excessive electron scattering.

To flash-freeze the sample, the user pipettes microliters of sample suspension onto a cryoEM grid. CryoEM grids are typically a copper lattice that supports a holey, thin-carbon film. Ideally, the specimen accumulates as a suspension in a meniscus of solute within these holes. Because electrons are scattered readily by thick samples, excess liquid is wicked away using filter paper, and the grid plunged into a cryogen such as liquid ethane, cooling it sufficiently fast to vitrify the thin specimen suspension (Figure 1, panel 2) (Dobro et al., 2010; Dubochet & McDowall, 1981). Robotics aid reproducibility, enabling control of temperature, humidity, and blotting time. After freezing it is crucial to protect the sample from ambient humidity, as atmospheric water readily condenses and freezes on the sample.

Thick samples can be thinned for cryoET using genetic manipulation or cryo-focused ion beam (cryoFIB) milling. By manipulating cell division machinery, tiny minicells are ejected from bacteria, often containing the secretion system of interest for high-resolution imaging (Farley et al., 2016). Alternatively cryoFIB uses a focused beam of gallium ions to remove the top and bottom of a sample to leave a 100–300 nm-thick lamellum ideal for cryoET (Marko et al., 2006) and high-resolution subtomogram averaging (Figure 1, panel 2, right). While the milling process remains relatively low-throughput, a number of labs have now developed automated approaches for future uptake by the community (Buckley et al., 2020; Tacke et al., 2020; Zachs et al., 2020). CryoFIB holds particular promise for imaging secretion system-mediated infection of eukaryotic cells otherwise too thick for cryoET.

2.2 | Image acquisition

After freezing, the user inserts their sample into a transmission electron microscope (TEM) for image acquisition. Electrons are scattered by the specimen either elastically or inelastically. While elastically scattered electrons can be refocused by the electron microscope to form an image of the specimen, inelastically scattered electrons break covalent bonds and lose energy to contribute incoherently as noise to the final image. Images therefore have a low signal-to-noise ratio (SNR) because imaging is performed at low electron flux to minimize sample damage from inelastic scattering (Baker & Rubinstein, 2010). As a result, only low-resolution features are discernible in individual micrographs, while high-resolution features are present yet obscured by noise. At the heart of SPA and STA is the need to distill this high-resolution information from many low SNR images by collecting hundreds or thousands of images of the structure of interest for alignment of low-resolution features for subsequent averaging to reduce the noise.

As with visible light microscopes, electron microscopes use lenses (actually electromagnetic fields) to refocus electrons scattered by the specimen. Unlike the lenses of visible light microscopes, which can be precision ground to focus all photons of a given wavelength at the same image plane, electron lenses have considerable inherent “spherical aberration” which means that features of different resolutions are focused differently; the practical outcome being convolution of the image by a distinctive and unintuitive “contrast transfer function” (CTF) which alters the contrast in images of features at different resolutions. For example, the contrast of features at some resolutions becomes inverted, while the contrast of features at other resolutions disappears. The CTF is a blessing in disguise, however, as it can be harnessed to impose useful phase contrast on the image by modestly defocusing the objective lens. The pattern of contrast changes oscillates as a function of resolution, can be visualized with appropriate image processing, and can be digitally compensated for after acquiring data. The user can fill in features with zero contrast in one image by augmenting it with the images collected at different objective lens defocus that have different patterns of contrast at different resolutions.
2.3 | Microscope configuration

For cryoEM imaging, the TEM must be configured to reduce contamination from any residual gases in the microscope column that would otherwise condense on the sample. Image quality will depend upon the microscope configuration, which is invariably a function of purchase cost.

2.3.1 | Electron beam quality

A coherent beam from the electron emitter with little variation in electron energies will retain high resolution image details. Tungsten filaments and lanthanum hexaboride cathodes found in TEMs used for optimizing and screening samples and freezing
conditions are cheap but produce inadequately coherent electrons for structural studies. Field emission guns (FEGs) produce sufficiently coherent beams for structural studies; higher coherence can be achieved using a monochromator (Tiemeijer et al., 2012; Yip et al., 2020), or a cold FEG (Hamaguchi et al., 2019; Nakane et al., 2020), although these are very expensive (millions of dollars) and the benefits will only be apparent at very high (~2 Å) resolutions.

2.3.2 | Voltage

The voltage of the microscope determines the energy of the electron beam which dictates the average thickness of a sample before an electron is scattered. Data collection microscopes usually operate at 200 or 300 kV to produce a beam of sufficient energy to pass through samples a few hundred nanometers thick (Egerton, 2014; Henderson, 1995; Herzik et al., 2017). Contemporary cameras are optimized for 300 kV electrons.

2.3.3 | Camera

The rapid development of CryoEM has mainly been due to advances in detector technology. Contemporary direct electron detector (DED) cameras are optimized for higher voltage microscopes to collect significantly higher quality images than previous-generation cameras (Faruqi & McMullan, 2018; McMullan et al., 2014, 2016). Perhaps most significantly, these cameras are sufficiently fast to collect a “movie” of many short-exposure images instead of one long exposure, allowing for superimposition of movie frames of a specimen in motion induced by the electron beam that would otherwise blur high-resolution data. DEDs in recent years have further improved to allow quicker acquisition of larger, higher quality data sets, enabling cryoEM to become more accessible and higher throughput, as demonstrated by a corresponding increase in PDB depositions.

2.3.4 | Energy filter

Energy filters remove inelastically scattered electrons to increase the SNR. Energy filters are especially important in cryoET imaging of whole cells where noise from inelastic scattering can otherwise entirely obscure structures.

2.3.5 | User training and infrastructure

Although increasingly accessible, cryoEM still requires training to ensure users can operate the microscope effectively and safely, both for themselves and the (extremely) expensive microscopes. SPA is sufficiently developed that much of the process is automated for uncomplicated specimens, and the bottleneck remains adequate sample preparation; STA is still maturing, and the bottleneck remains adequate training, manipulation of the sample to maximize the number of particles per tomogram and the time taken to acquire data. Considerable computational power and data storage are also required for processing. CryoEM remains prohibitively expensive for many institutions, meaning that the synchrotron model is becoming more common, where expensive high-voltage microscopes, costing many millions of dollars, are provided by national facilities (Clare et al., 2017), and samples screened and optimized at home with 120 kV microscopes with tungsten filament or lanthanum hexaboride electron sources that cost less than a million dollars.

2.3.6 | Time and cost

In practical terms, SPA requires hundreds of thousands of particles from thousands of micrographs, taking one or a few days of microscope time on the order of ~$1,000 per day, while STA usually uses hundreds or thousands of particles extracted from tens or hundreds of tomograms, potentially requiring many days of microscope time. Although recent developments have accelerated data processing, this comes at a price: data from a single day are routinely of terabyte sizes,
requiring consideration of hardware for data processing, storage, and backup. The seemingly high daily costs are necessary to cover running costs, service contract, and facility staff salaries.

2.4 | Single particle analysis (SPA): High-resolution structures of purified proteins

The 2-D micrographs of purified proteins embedded in thin ice in random orientations are the input data for SPA. Individual particles are picked manually or semi-automatically. CTF artifacts are computationally corrected for, and the many short-exposure frames of the acquired movie are aligned and averaged to correct for motion during exposure. Particle images are then extracted and sorted into classes of 2-D images based on similar low-resolution features, which, when averaged, clarify high-resolution features. These 2-D classes group particles into homogenous subsets, discerning “junk” and providing information about heterogeneity and angular orientation distribution. These initial 2-D images are then used to infer a 3-D structure, and tease apart 2-D particles viewed from the same angle that have different conformations. This inference is achieved by iteratively refining the orientations of 2-D particles to an iteratively improving 3-D structure(s); the initial model may be a previous homologous structure or simply a suitably sized “blob.” This process is iterated until the resolution of the structure(s) converges to high resolution. Typically ~3.5 Å map resolution (so-called “near-atomic resolution”) is sufficient to build an atomic model, although recently true atomic resolution SPA structures have been achieved (Nakane et al., 2020; Yip et al., 2020). The theory behind these processes is reviewed fully by (Orlova & Saibil, 2011).

2.5 | Common challenges for SPA

2.5.1 | Protein complexes have poor or low contrast

Because SPA relies upon aligning particles based on their low-resolution features, the protein complex must be sufficiently large to be discerned in the vitreous ice. This limits typical SPA to complexes of ≥150 kDa. Recent developments of phase plates (see “Future prospects”), enable detection and alignment of smaller proteins enabling imaging, picking, and reconstructing proteins of 50 kDa (Fan et al., 2019), but phase plates remain somewhat experimental. Alternatively, the particle’s size can be increased by fusing additional domains or binding antibody fragments (Wu et al., 2012). Customizable protein scaffolds have recently enabled structural determination of a 26 kDa protein to sub-4 Å resolution (Liu et al., 2019). Nevertheless, X-ray crystallography or NMR remain more appropriate tools for structure determination of small protein complexes.

2.5.2 | Protein complexes behave poorly in ice

A pure protein preparation does not necessarily translate to excellent images. Dynamic or flexible complexes can be too heterogeneous to yield high resolution reconstructions. The user can stabilize their complex using gentle crosslinking methods to lock transient protein interactions for a more homogenous sample (Kastner et al., 2008). Another common issue is that the protein complex preferentially orients in the thin layer of vitreous ice, preventing collection of sufficient different views to determine its structure. Protein complexes may associate and denature at the air-water interface. Addition of a nanometers-thick layer of carbon, or addition of detergent, help prevent this (D’Imprima et al., 2019). Alternatively, elongated particles may “flatten” into the plane of the thin layer of vitreous ice, which may be tackled by manipulating freezing parameters to thicken the ice. Nevertheless, ice thickness is an important parameter to optimize for smaller complexes and achieving high resolution reconstructions.

2.5.3 | Protein complexes have different conformations

Dynamic features are challenging to resolve. Because a flexible density is in different orientations in different particles, it is blurred or invisible upon averaging. The most common approach to resolving heterogeneity is computational classification, which is widely implemented in SPA workflows to sort particles into homogenous subsets that are averaged individually to reveal different conformations (Elad et al., 2008; Orlova & Saibil, 2010; Scheres, 2010, 2012; Scheres et al., 2007; Sigworth et al., 2010). Where the complex has a continuum of conformation instead of discrete conformations, focused refinement of more rigid subcomplexes can build a structure of the full complex using a divide-and-conquer approach to discrete parts.

2.6 | Subtomogram averaging (STA): Medium-resolution structures of proteins in situ

While SPA provides near-atomic views of biological molecules, cryoET and STA can visualize individual components in context in the cell. STA shares much with SPA except that the input particles for SPA are 2-D while input particles for STA are 3-D subtomograms extracted from a tomogram, which is a 3-D reconstruction of a unique object such as a bacterial cell. To acquire a tomogram, the unique object is tilted over a range of angles in the microscope and images acquired at each angle to create a tilt series. Because the relative orientation of each image is known a priori, a tomogram can be reconstructed from the different images. As the total electron flux must be distributed between tens of images to minimize sample damage (Bharat et al., 2015), SNR of individual tilt series images is low. This potentially leads to inaccurate tomographic reconstruction and attenuation of high-resolution features, and limits specimens to ~500 nm thickness even for 300 kV microscopes before noise becomes overwhelming. Because the EM grid itself blocks the electron beam at high tilts, information about features perpendicular to the beam is always lacking, manifesting as blurring...
in that direction, most noticeable as failure to resolve membranes at the top and bottom of cells (Figure 1, Electron Cryo-Tomography panel). This missing information is termed the "missing wedge" due to its shape in Fourier space; for a more in-depth discussion, see (Ferreira et al., 2018).

Subtomogram averaging (STA) of subtomograms within the tomogram can then reveal the structure and composition of protein complexes in their native environments by extracting and averaging identical particles from tomograms. Because particles tend to be randomly oriented, the missing information of the missing wedge of one particle will be filled-in by information from another aligned particle. The alignment and averaging process is conceptually similar to alignment and averaging of SPA particles to yield a higher resolution structure. STA can distinguish features not discernible in individual particles in single tomograms (Figure 1, see panels Electron Cryo-Tomography and Sub-Tomogram averaging). STA structures bridge structural and cellular biology by retaining native contextual information, and structures regularly achieve resolutions of 15–50 Å. While such resolutions are inadequate to unequivocally assign densities to specific proteins, they provide invaluable in situ structures of many cellular components (Chen et al., 2011; Gold et al., 2015; McEwen et al., 2002) (Chang et al., 2016).

2.7 | Common challenges for STA

2.7.1 | The protein complex is rare

In many cases the structure of interest will be self-evident. Some structures, however, are unpredictably distributed and indiscernible until after tomogram reconstruction. This necessitates blindly targeting cells in the hope that they have the structure of interest, a time consuming and expensive process. Genetic approaches and the choice of organism may overcome these obstacles. Placing either the regulatory factors or the structural genes of a complex under inducible control can increase the number of complexes per cell. It may also be useful to change to a species in which the localization of the complex is determinate, such as polar.

If particle abundance cannot be increased, correlative light and electron microscopy (CLEM) can be used to locate rare structures by correlating fluorescence signal in a frozen hydrated specimen using a visible light microscope to a cell for cryoEM imaging (Figure 1, panel 2, middle) (Briegel et al., 2010; Sartori et al., 2007; Schwartz et al., 2007; van Driel et al., 2009; Wolff et al., 2016). Super resolution cryo-microscopy techniques can allow localization precision of ≤150 nm (Moser et al., 2019; Tuijtel et al., 2019).

2.7.2 | Resolution can be insufficient to identify proteins in complex

While resolution can be increased with the brute force acquisition of more data from higher specification microscopes, microscope time is limited and expensive, and subtomogram averages are typically obtained from between one hundred to a few thousand particles, often limiting achievable resolution. Resolution can also be increased by improving the SNR of individual tomograms by using thinner specimens. This includes changing to a thinner species, using genetic manipulation to disrupt cell division to form mini-cells, or deflating cells by gentle lysis, although lysis may rupture cells sufficiently to disrupt transiently associated components (Chen et al., 2011; Hu et al., 2015). FIB milling (discussed below) can make very thin samples but remains a relatively specialized approach. New automation protocols, however, promise to change this.

In many cases, even optimized samples and acquisition schemes will produce structures of insufficient resolution to identify discrete proteins. Imaging a mutant with a gene deletion can highlight absence of densities from the structure, a simple but powerful approach as long as deletion does not affect assembly of other components (Beeby et al., 2016; Chen et al., 2011; Zhu et al., 2017). Alternatively, additional domains can be fused to a component of interest to introduce additional density. Although fusion may be difficult to discern due to flexibility and heterogeneity, and may interfere with assembly or function, the approach has successfully identified several components of bacterial secretion systems (Chang et al., 2016; Hu et al., 2017; Tachiyama et al., 2019).

2.7.3 | The protein complex is dynamic or low-occupancy

STA is unique in being able to visualize transient binding components of a protein complex. Transient interactions or conformational states can be visualized by creating locked mutants or by overexpressing transient binding partners to increase occupancy, enabling visualization of the additional density in STA structures (Rossmann et al., 2020).

3 | EXAMPLES OF HOW CryoEM HAS CONTRIBUTED TO UNDERSTANDING MICROBIAL SECRETION SYSTEMS

3.1 | Type III secretion

Flagella and injectisomes use transmembrane type III secretion systems (T3SSs) to assemble and secrete proteins (Figure 2a). Multimicron-long flagella assemble at their tip with subunits exported through a central channel by their T3SS; injectisomes—degenerate flagella—use the same mechanism to assemble a shorter rigid needle and secrete effectors into host cells. As described in the MicroReview from Andreas Diepold and co-workers elsewhere in this issue, T3SSs feature five quasi-transmembrane proteins within a transmembrane chassis ring, cytoplasmic scaffolding proteins, and a cytoplasmic ATPase complex. Flagellar
FIGURE 2 Contributions of cryoEM single particle analysis (SPA) and subtomogram averaging (STA) to understanding type III secretion systems (T3SSs) (a) Schematic of the architecture of T3SSs from (left) injectisomes and (right) flagella, with insets of purified injectisomes (Kubori et al., 1998) and flagella (Khan et al., 1992). Scale bars, 50 nm; OM, outer membrane; PG, peptidoglycan; IM, inner membrane (b) SPA contributions to understanding T3SSs. (i) SctRST and FliPQR form comparable structures. SctR/FliP are blues, SctS/FliQ are reds, SctT/FliR is yellow, FlhBN is green (PDBs 6S3L and 6R6B). (ii) Cross-section through SctDJ structures shows their oligomeric state depends upon SctRST. Ring-building motif (RBM) symmetry elements that interact with SctRST/FliPQR are marked by red. (iii) Cross-sections through FlIF shows internal symmetry mismatches of rings comprised of 33, 21, and 9 domains (c) Timeline of resolution improvements with slices through injectisome subtomogram average structures from (L-R): Kawamoto et al. (2013), Kudryashev et al. (2013), Hu et al. (2015), Hu et al. (2017), Butan et al. (2019) (top) and Tachiyama et al. (2019) (bottom). Scale bar, 25 nm (d) biological insights from deletion analysis (i) wild type flagellar T3SS from Campylobacter jejuni; (ii) deletion confirms location of FliI (SctN); (iii) deletion confirms that FliH (SctL) is an anchor for FliI (SctN); (iv) truncation confirms location of FlhAC (SctVC); (v) location of transmembrane region of SctV by deletion (right: zoom of WT and ΔsctV SPI-1 transmembrane region for comparison). Same scale as panel C. (e) Biological insights from tagging analysis. (i) tagging SctV with GFP confirms deletion analysis; (ii) tagging SctO with PAFP confirms its location in the central channel of the ATPase. Same scale as panel C. (f) Insights into assembly (green), secretion (orange), and sensory transduction (red) from cryoEM. For assembly, (1) Five SctR assemble as a helix with a single SctT decorated by four SctS which (2) template assembly of 24-fold symmetric SctDJ, (3) a ring of nine SctV, and (4) correct formation of the six C-ring pods. For secretion, (1) cytoplasmic substrates are (2) delivered to the export gate by the ATPase and (3) into the export cage where solvent-accessible channels in the IM ring near SctV may power (4) delivery past the small cytoplasmic loop of SctVN to the SctRSTU for (5) secretion through the positively charged axial lumen. For sensory transduction, (1) conformational changes in the needle propagate (2) through SctDJ to (3) SctV where they may influence substrate specificity.
nomenclature is unified across species; injectisome nomenclature remains different in different species, and here, we standardize on the injectisome "Sct" nomenclature (Hueck, 1998). While X-ray crystallography has determined the structures of isolated T3SS components (Kudryashev et al., 2013; Lountos et al., 2012; Notti et al., 2015; Spreeter et al., 2009; Yip et al., 2005; Zarivach et al., 2007), their context within the assembled T3SS relied upon inference. SPA and STA have been key to providing the needed context. Chen et al. (2011), Kawamoto et al. (2013), Kudryashev et al. (2013), Marlovits et al. (2004), Murphy et al. (2006), among others, have built upon early EM studies of injectisomes (Kubori et al., 1998) and flagella (Khan et al., 1992) to reinforce that their core T3SSs have a conserved structure, function, and mechanism (Abby & Rocha, 2012) (Figure 2).

3.1.1 SPA delivers structures of large complexes intractable to other structural methods

SPA has yielded the structures and locations of core T3SS proteins (Figure 2b). The export apparatus is made of five proteins (SctRSTUV in injectisomes, FliPQR, FlhAB in flagella). Originally considered true membrane proteins, SPA of FliPQR (Kuhlen et al., 2018) showed an unusual membrane-peripheral complex with a pseudo-hexameric helical arrangement of FliP_5Q_4R_1 (Figure 2b(i)). FliR resembles a fusion of FliP and FliQ, meaning the complex is a helix with six quasi-repeats. SctRST has the same structure, cementing the evolutionary conservation between injectisome and flagellar T3SSs (Johnson et al., 2019). The auto-cleaving FlhB subunit forms the base of the FliPQR complex, where one of its interhelix loops forms a pore that may regulate the entrance of substrates into the SctRST cavity (Kuhlen et al., 2020).

3.1.2 SPA discerns the heterogeneity and symmetry mismatches often seen in secretion systems

One of the most exciting aspects of SPA is classification, in which heterogeneous particles and conformational variants can be "purified" in silico to tease apart differences between particles and even identify symmetry heterogeneity within individual particles. For example, classification of SctRST has revealed the presence of variants with two, three, or five SctS protomers instead of the normal four.

Classification has enabled characterization of the widespread symmetry mismatches within and between the inner and outer membrane chassis structures, SctDJ and SctC in injectisomes, and FliF in flagella. Unexpectedly, different symmetries are evident between SctDJ rings. Classification of manually picked data sets of over 100K purified SctDJ rings without imposed symmetry or focused refinement revealed mainly 23-fold symmetric rings with a minority with 24-fold symmetry (Figure 2b(iii)). Curiously, addition of SctRST enforces assembly of exclusively 24-fold symmetric SctDJ rings, suggesting SctRST templates correct SctDJ assembly (Butan et al., 2019).

The corresponding flagellar protein, FliF, has ring-building motifs (RBMs) in common with SctDJ, suggesting a common ancestor. FliF has even more diverse symmetries and internal symmetry mismatches than SctDJ (Johnson et al., 2020) (Figure 2b(iii)). The structure of purified FliF to 2.9 Å resolution was sufficient to reveal three different symmetries using focused refinement and appropriate symmetry imposition. The RBM domain in FliF that corresponds to SctJ’s export apparatus interface, RBM2, is only 21-fold or 22-fold symmetric when overexpressed, while a C-terminal domain forms a 32- to 36-fold symmetric ring, with a majority of 33. How can an oligomer formed from a single type of polypeptide chain assemble with different internal symmetries? Nine of the remaining RBM2s decorate the outer face of the ~21-fold RBM2 ring, filling 9 of the 12 spaces of a 12-fold symmetric ring, while the absence of some of the domains of the 33 subunits suggests they do not form regular structures, at least in vitro.

By imposition of symmetries during focused refinement, recent SPA and classification has revealed a surprising 15/16-fold internal symmetry mismatch in SctC (Worrall et al., 2016), previously thought to be entirely 15-fold symmetric (Schraidt & Marlovits, 2011) (Figure 2b(iv)). The 15-fold symmetry is disrupted by the 16-fold symmetric connector region (Hu, Worrall et al., 2019; Lunelli et al., 2020). Such unexpected findings can be missed during SPA map reconstruction, which often relies on the particle symmetry to increase resolution. Iterative signal subtraction and focused refinement resolved mismatched symmetries by temporarily removing parts of the structure that otherwise impeded correct alignment of mismatched symmetries.

3.1.3 STA locates proteins and reveals their in situ context and behavior

The challenges of T3SS structure determination have pushed the boundaries of what STA is capable of in "real world" samples that are scarcer and thicker than ideal, with rapid increase in resolution of injectisome and flagellar structures by STA (Figure 2c). Early STA revealed universally conserved transmembrane and cytoplasmic structures in injectisomes and flagella (Kawamoto et al., 2013; Kudryashev et al., 2013). These components are delicate and dynamic ATPase and “C-ring” (Cytoplasmic ring) structures often lost during purification: even the flagellar C-ring, the most stable cytoplasmic component, is difficult to retain during purification (Francis et al., 1994), making STA a well-suited approach.

A central challenge to imaging T3SSs by STA is that they are not abundant, requiring large, multi-day tomogram acquisition sessions to achieve high resolutions, and genetic approaches to increase particle yield and thin cells. Discovery that the injectisome sorting platform is a vestigial C-ring required unprecedented amounts of data at the time: over 4,500 particles from almost 2,000 tomograms, that is, 20 days of continuous data collection even with fast acquisition of 15 min per tilt series (Hu et al., 2015). Subsequent subtomogram averaging yielded an injectisome subtomogram average structure to
27 Å resolution. Surprisingly, the newly identified C-ring consisted of a discontinuous ring of six pods of SctQ instead of a continuous ring of FlhMN as in flagella, possibly the outcome of evolutionary loss of a continuous ring of FlIG in the ancestral flagellum (Beeby et al., 2020). The lower resolution and absence in previous studies of these pods (Kawamoto et al., 2013), is consistent with their being dynamic. Similarly high-throughput STA clarified the locations of all T3SS components using an optimized sample of over 6,500 injectisomes from minicells from just 326 tomograms (Butan et al., 2019). At the core of T3SSs is the transmembrane domain of the enigmatic SctV (FlhA), that poorly purifies with needle complexes and other export apparatus proteins (Wagner et al., 2010; Zilkenet et al., 2016). The first steps to locate the transmembrane domain, SctV\_\text{N}, therefore, necessitated an in situ approach (Butan et al., 2019). STA has resolved small densities projecting into the cytoplasm confirmed to be a cytoplasmic loop in SctV\_\text{N} (see below); determining the structure of SctV\_\text{N} may require an optimized STA approach.

By incorporating gene deletions (Figure 2d), STA deciphered the role of the cytoplasmic components as scaffolds to position the ATPase SctN (FliI). The ATPase, first located in the cytoplasm in a comparative STA study of Fli in diverse flagellar motors, was confirmed by deletion analysis (Chen et al., 2011). STA of both flagellar and injectisome T3SSs located a toroidal cytoplasmic density. This density disappeared after truncation of FlhA and injectisome T3SSs located a toroidal cytoplasmic density. This confirmed by deletion analysis (Chen et al., 2011). STA of both flagellar and injectisome T3SSs located a toroidal cytoplasmic density. This density disappeared after truncation of FlhA\_\text{C} (SctV\_2), thus, confirming its identity (Abrusci et al., 2013). Deleting all of SctV clarified the position of the membrane, which funnels to dock against a hydrophobic belt around the base of the conical SctRSTU/FlIPQR FlIB, and thins from 4 to 3 nm (Butan et al., 2019).

Surprisingly, Flil is not disrupted after removal of the FlhA cytoplasmic domain, instead remaining suspended in the cytoplasm ~20 nm from the membrane-distal rim of the C-ring. Together with FlilH being a homolog of F-ATPase peripheral stalk proteins, this observation prompted the proposal that six SctL\_2/FlilH 2 radial stalks beyond the resolution of the STA reconstruction attach SctN\_2/FlilH to the C-ring. Indeed, deletion of SctL/FlilH leads to loss of SctN/Flil, and destabilization of the C-ring leads to reduced SctN/Flil density (Henderson et al., 2019; Qin et al., 2018). Gene deletions indicated that the architecture of the injectisome’s cytoplasmic T3SS module is comparable to its flagellar homologs: a hexameric hub of SctN ATPase held in place by a basket of six spoke SctL that bridge to the “C-ring.” (Hu et al., 2015). Subsequent STA using over 7,000 particles of the *Borrelia burgdorferi* flagellar T3SS revealed a surprising 23-spike FlilH architecture. This 23-fold symmetry corresponds to half the 46-fold symmetry of the *B. burgdorferi* C-ring, echoing the match between sixfold symmetry of SctL\_2 with the sixfold symmetry of the injectisome C-ring pods (Qin et al., 2018).

While deletion analysis can be invaluable, its outcome can be ambiguous if it impacts the assembly of other components (Beeby et al., 2016). Fusing additional domains to components is an alternative approach, either confirming results from gene deletion, as with positive identification of SctVC by GFP tagging (Figure 2e). Elegant tagging approaches have allowed location of seven cytoplasmic T3SS components—SctV, SctD, SctK, SctQ, SctL, SctN, and SctO—and to orient some based on tag location (Hu et al., 2017; Tachiyama et al., 2019). Among other insights, these approaches revealed that SctD in situ has a different conformation than when it is purified, as purification leads to loss of the C-ring. The uniform 24-fold symmetry of the N-terminal domain of SctD changes to form a sixfold-symmetric structure of six SctD tetramers as platforms for the six C-ring pods.

### 3.1.4 Mechanistic insights into type III secretion

Taken together, these results provide insight into T3SS self-assembly, mechanism, control, and virulence due to T3SS (Figure 2f).

We now know more about how T3SSs self-assemble (Green numbers, Figure 2f). A helix of five SctR/Flip first assemblies, capped by a single SctT/Flir. 3-D classification suggests that the new SctR-SctT interface nucleates assembly of four SctS/Fliq, which in turn templates correct assembly of 24-fold symmetric SctDJ, a symmetry possibly needed in turn to template correct formation of the six C-ring pods (Johnson et al., 2019). SctL, and the ATPase.

CryoEM has also provided crucial information on the mechanism of secretion (Orange numbers, Figure 2f). Visualization of GFP exported by SPA and cryo-ET has confirmed substrate entry and exit points, cementing the model of the axial structure being a hollow molecular syringe (Radics et al., 2014). Alignment of SctN with the SctV torus suggests that substrates are first delivered by SctN through the SctV aperture into an "export cage" for substrates, bounded by the nine linker helices to the SctV transmembrane domains (Abrusci et al., 2013). Solvent-accessible channels in the IM arc adjacent to SctV suggest a route for proton flux to SctV, which energizes export through the SctRSTU pore (Hu, Worrall, et al., 2019). Within this export cage, the small cytoplasmic FHIEP loop of SctV\_N may guide the substrates up the funnel to the export gate for secretion through the axial lumen. The SctRST lumen is positively charged, corresponding to the positively charged lumen surfaces of the injectisome needle and the flagellar rod, hook, and filament. Notably, the helical parameters of the SctRST pseudo-helix match those of injectisome and flagellar axial components, implying that SctRST physically templates axial assembly. STA has also visualized intermediate needle assembly states by focusing refinement on the export apparatus (Worrall et al., 2020).

Finally, STA has provided glimpses of injectisome-driven infection. Injectisomes contacting host cell membranes have been visualized by cryo-ET (Hu et al., 2015; Nans et al., 2015), and recent STA studies have visualized the translocon in host cell membranes (Park, Lara-Tejero, et al., 2018). Early studies of *Yersinia enterocolitica* suggested axial injectisome expansion and contraction (Kudryashev et al., 2013), although such changes are not seen in the *Salmonella* SPI-1 injectisome (Hu et al., 2017). With the increasing speed and efficiency of FIB milling, it may become possible to image sufficient injectisomes interacting with host cells in situ to visualize this proposed allosteric signaling cascade through the injectisome by STA. Indeed, recent studies of the SPI-1 injectisome suggest a possible mechanism for modulation
of secretion specificity by signal transduction of detection of contact with the host (Red numbers, Figure 2f) (Guo et al., 2019). Mutant needles have key structural changes, that together with residues lining the lumen, affect T3SS secretion specificity. Polymorphic needle signaling to SctD through SctDJ (Park, Lara-Tejero, et al., 2018) may be communicated via a subdomain of SctV with the cytoplasmic SctD IR2. Early STA studies of flagella and injectisomes have suggested that SctN/FliI may be in an “off” state, 10 nm from the SctVc/FlhA c torus (Kawamoto et al., 2013). Into the future we can expect to see structural snapshots of injectisomes in action during infection.

3.2 | Other secretion systems

Although cryoEM has been applied most extensively to the determination of Type III secretion system structure and function, it has also been used to investigate other secretion systems.

3.2.1 | Type II secretion systems

Type II secretion systems (T2Ss) secrete fully folded proteins across the outer membrane (see the MicroReview from Harry Low and co-workers elsewhere in this issue). All T2Ss possess an inner membrane assembly platform (AP) with a cytoplasmic hexameric ATPase, a periplasmic pseudopilus that acts as a piston to eject T2S substrates, and a periplasm-spanning secretin complex—homologous to injectisome secretins—for exit from the cell. T2Ss are divided into the Klebsiella-type, exemplified by the T2SS used by Klebsiella oxytoca to secrete the starch-degrading enzyme pullulanase, and Vibrio-type, exemplified by the cholera toxin-secreting Vibrio cholerae T2SS (Cianciotto & White, 2017; Enfert et al., 1987; Johnson et al., 2006; McLaughlin et al., 2012).

While secretins are challenging X-ray crystallography subjects, their stability and ease of purification has enabled determination of their structures using SPA. A combination of SPA and STA showed striking architectural conservation between Klebsiella- and Vibrio-type secretins: all feature a 15-fold symmetric ring of PulD with a large outer-membrane pore (~180 Å diameter) (Chami et al., 2005; Nouwen et al., 1999) (Figure 3a,b). All feature a central gate to prevents leakage across the membrane, a periplasmic vestibule for cargo loading, and an N-terminal domain that interacts with the assembly platform (Chernyatina & Low, 2019; Ghosal et al., 2019; Reichow et al., 2010, 2011; Yan et al., 2017).

Two exciting contributions SPA has made to the study of the T2S has been the structural determination of secretin mutants to provide mechanistic insights into substrate secretion (Yan et al., 2017), and the implication of symmetry mismatch in substrate loading (Chernyatina & Low, 2019).

The recent SPA study by Chernyatina and Low suggests a mechanism for cargo loading into the secretin vestibule that depends on symmetry mismatch. T2S substrates are secreted into

![FIGURE 3](progress_towards_a_full_molecular_and_mechanistic_understanding_of_type_ii_secretion_t2s_using_cryoem_a_initial_cryoem_studies_of_the_t2ss_solved_the_structure_of_the_secretin_i_e_puld_to_~20Å_and_b_revealed_a_structure_with_possible_12-fold_symmetry_and_a_central_plug_chami_et_al_2005_nouwen_et_al_1999_c_subsequent_work_refined_the_structure_to_~3Å_and_clarified_that_the_puld_oligomer_is_15-fold_symmetric_and_that_the_central_gate_the_plug_has_a_hinge_that_allows_substrates_to_pass_through_yan_et_al_2017_d_the_most_recent_spa_of_the_t2ss_solved_the_structure_of_the_secretin_in_complex_with_several_components_of_the_inner_membrane_assembly_platform_ap-producing_a_model_for_substrate_loading_dependent_on_ap_secretin_symmetry_mismatch_chernyatina_and_low_2019)
the periplasm by the Sec secretion system and must enter the periplasmic vestibule of the secretin through a side-loading mechanism. Given that the N0 domain of the PulD secretin binds directly to the assembly platform in the periplasm via PulC, how substrates gain access to the vestibule was a mystery. By stabilizing entire OMC-AP complexes with glutaraldehyde, followed by focused refinement of the PulD N0 domain and docking of the preexisting PulC crystal structure, the structure and stoichiometry of the PulD-PulC interface was solved. Notably, PulC forms a hexamer of dimers, suggesting that symmetry mismatch with the 15-fold symmetric secretin provides a mechanism for substrate loading. In this model, 12 PulD protomers bind 12 PulC protomers, and a gap of three PulD N0 domains in the PulD-AP complex form windows through which secretion substrates enter the secretin vestibule (Chernyatina & Low, 2019) (Figure 3d).

Components of the T4SS beyond the core complex have been characterized to varying resolutions. Low et al. (Low et al., 2014) succeeded in purifying the entire T4SS of the conjugative T4aSS encoded on the R388 plasmid, and used NS-EM, SPA and biochemical approaches to determine its structure to ~20 Å resolution, revealing the topology of the IMC as well as the stoichiometry of the remaining VirB proteins in the T4aSS. The study by Low et al. was followed up by a NS-EM and biochemical study that characterized the protein-interaction network of the IMC component VirD4, which is

3.2.2 Type IV secretion systems

Type IV secretion systems (T4SSs) are found across Bacteria and Archaea where they are involved in effector translocation, conjugation, DNA uptake, adhesion, and motility (Alvarez-Martinez & Christie, 2009). T4SSs are grouped into archetypal T4aSSs, which include the Agrobacterium tumefaciens VirB system and the Escherichia coli F-type conjugative system; the more complex T4bSSs represented by the Legionella pneumophila Dot/Icm system; and the noncanonical Cag T4SS used by Helicobacter pylori to secrete the CagA oncoprotein into host cells.

Early structural work on the T4aSS focused on the periplasmic core complex, a ~ 1.1 MDa conduit for substrates across the outer membrane. The core complex is composed of 14 copies each of VirB7, VirB9, and VirB10, and can be divided into the outer membrane-spanning O-layer (VirB10) and the periplasmic I-layer (VirB7 and VirB9). While an early study combined X-ray crystallography and NS-EM (Chandran et al., 2009) to determine the structure of the O-layer, SPA later delivered the structure of the entire core complexes of the T4aSSs of Xanthomonas citri and the pKM101-encoded system, as well as that of the nonarchetypal Cag T4SS of H. pylori, at resolutions sufficient to build atomic models (Chung et al., 2019; Frick-Cheng et al., 2016; Fronzes et al., 2009; Sgro et al., 2018). In their SPA study of the H. pylori T4SS, Chung et al found that the periplasmic ring complex (PRC), which joins the 14-fold symmetric OMC to the inner membrane ATPase complex (IMC), has 17-fold symmetry. The symmetry mismatch between the PRC and IMC is postulated to be important in substrate translocation (Figure 4).
responsible for recruiting secretion substrates in the cytoplasm (Redzej et al., 2017).

Meanwhile the structure of the F-type pilus itself by helical reconstruction—a specialized form of SPA—has revealed phospholipids integrated into the wall of the extracellular pilus (Costa et al., 2016). Additionally, the structure of T4SS relaxase (TraI), a bifunctional enzyme responsible for nicking and unwinding DNA during conjugation, has been solved to near-atomic resolution by SPA (Ilangoivan et al., 2017); this provided mechanistic insight into TraI function by showing that two conformationally distinct monomers of TraI bind to target DNA, one with phosphatase (nicking) activity and the other with helicase activity.

Recent STA studies have provided in situ structures of the T4aSSs of E. coli and the Cag T4SS of H. pylori, as well as the T4BSS of Legionella pneumophila (Chang et al., 2018; Ghosal et al., 2017, 2019; Hu, Khara, Christie, et al., 2019; Hu, Khara, Song, et al., 2019). The H. pylori and L. pneumophila systems are larger and more complex than the canonical T4aSS, but all T4SSs share similar architectural features despite sharing little sequence homology. Intriguingly, however, the Legionella T4SS OMC was found to have 13-fold symmetry, rather than the 14-fold symmetry found in T4aSSs. STA combined with genetic approaches has also allowed models for T4SS assembly to be made for both the T4aSS and T4bSS systems, insights that at present have proven to be elusive by SPA alone. Looking forward, SPA and STA studies will be critical for gaining mechanistic insights into T4SS function, including pilus assembly, substrate recruitment at the IMC, and substrate translocation.

3.2.3 | Type VI secretion systems

SPA and STA have made substantial contributions to understanding T6SSs, used by bacteria to inject toxic effectors to kill Eukaryotes or other Bacteria (Figure 5). Cryo-ET of V. cholerae first identified T6SSs as micron-long sheathed cytoplasmic tubes attached to transmembrane baseplates (Basler & Mekalanos, 2012), and confirmed that the tube sheath resembles the contractile tails of bacteriophages such as T4 and lambda (Leiman et al., 2009; Pell et al., 2009; Pukatzki et al., 2007). Together with in vivo fluorescence microscopy, these findings provided a first model of T6SS assembly, structure, and function that has been reinforced through more recent cryoEM studies. On the contrary, STA has provided in situ structural information of the entire system, allowing determination of the membrane topology, sixfold symmetry of the baseplate complex, fivefold symmetry of the membrane complex, threefold symmetry of the spike, and a gated pore embedded in both membranes (Chang et al., 2017; Nazarov et al., 2018; Rapisarda et al., 2019). These studies demonstrated that the tube is driven through this pore during sheath contraction; in its closed conformation, the baseplate holds the sheath and tube inactive, preventing leakage across the outer membrane until contraction is triggered by unknown signals.

Structures of the sheathed tube and the baseplate have been determined using SPA (Nazarov et al., 2018; Park, Lara-Tejero, et al., 2018; Wang et al., 2017). Structures of the sheath in both its extended and contracted states have provided mechanistic insights into the translocation of the tube and spike into a target cell. The sheath is composed

![Figure 5](image-url)
of a helical assembly of hundreds of VipA/VipB dimers. Observation of sheaths in pre- and postcontractile states revealed that changes in contacts between VipA/B dimers causes the sheath as a whole to shorten and widen, propelling the tube through the cell envelope.

4 | FUTURE PROSPECTS

Building on the first cryoEM “resolution revolution,” a second revolution on the horizon promises full dynamic molecular models of bacterial secretion systems. Next-generation cameras are enabling faster acquisition of superior images for truly atomic-resolution structures (Nakane et al., 2020; Yip et al., 2020). Increasing throughput of STA to acquire more particles will enable higher resolutions, more nuanced classification, focused refinement, and multidbody refinement. Furthermore, the drive for cheaper atomic-resolution 100 kV microscopes may yield commercial products in the coming years an order of magnitude cheaper than a contemporary 300 kV microscope (Naydenova et al., 2019), enabling abundant access for larger data acquisition sessions. Tomography on such low-voltage microscopes would be enabled by fast automated FIB-milling and correlative light and electron microscopy to locate particles.

The most exciting future prospects are molecular models of protein complexes in situ. What will it take to routinely achieve sub-nanometer resolution STA structures required for this? High quality images from energy filters and next-generation cameras will feed into emerging software packages capable of high-resolution reconstructions from large tomographic data sets (Castaño-Díez et al., 2012; Himes & Zhang, 2018; Tegunov & Cramer, 2019) and the inherently low contrast of cryoEM images may be remedied by the development of robust phase contrast electron microscopy using phase plates (Danev et al., 2014; Fukuda et al., 2015; von Loeffelholz et al., 2018), although contemporary phase plates remain to be widely adopted. Many more particles will be essential, by both increasing the number of particles per tomogram and acquiring more tomograms than currently possible. Where FIB throughput is a bottleneck, more ingenious approaches to thinning and increasing sample abundance will be needed such as developing further optimized mutants. Improving quality of images will have multiplicative effects, improving both the final reconstruction as well as alignment accuracy of tilt series and subtomograms. Toward this, microscopes with stable stages and accelerated tilt series acquisition (Chreifi et al., 2019; Eisenstein et al., 2019) combined with fully automated image targeting can improve data quality and deliver potentially thousands of tilt series per day. Such optimization could make cryoET and STA the norm for structure determination, even for purified complexes, and the boundaries of SPA and STA are increasingly blurred with development of hybrid approaches (Song et al., 2020; Tegunov et al., 2020).

5 | CONCLUSIONS

Given the diverse evolutionary origins of the diverse secretion systems it is perhaps unsurprising that a common theme is a lack of common themes. Nevertheless, two recurring architectural motifs are worth highlighting. The first, of plugging or gating channels through the cell envelope is widespread across the secretins in the T2, T3, and T4SSs, open and closed SctRST T3SS export gate, and T6, T8, and T9SSs. Such plugs and gates avoid constitutively open secretion systems that would leak cell contents.

The second theme, symmetry mismatch, was first evoked in 1978 by Roger Hendrix as a mechanism for rotation between symmetry-mismatched rings in phages (Hendrix, 1978). In secretion systems, symmetry mismatch appears to be the rule rather than the exception, with examples including the 15/16-fold secretin symmetry to 33-fold symmetry of the upper region of FliF, FliF’s diverse internal symmetries and other interfaces, 24-fold SctDJ to sixfold SctRST, and fivefold T6SS membrane complex to sixfold baseplate complex. Nevertheless, it is not clear if any of these systems rotate, or whether they benefit from rotation if they do. Indeed, phages have now been shown not to rotate (Hugel et al., 2007), and feature sophisticated inter-symmetry interfaces that prevent interface slippage (Fang et al., 2020). Symmetry mismatch may be inevitable in cylindrical multiprotein complexes built of disparate cyclic or helical oligomers, with selective pressure driving evolution of assemblies that can cope with symmetry mismatch instead of driving evolution of proteins that all assemble with the same symmetry. Symmetry mismatch may have evolved as a spandrel, that is, an unbeneificial byproduct of evolution of other features. This spandrel may only occasionally have subsequently provided the selective benefit of rotation in, for example, mechanisms of passing periplasmic Sec substrates to the T2SS, or the first flagellar motor from an ancestral type III export apparatus.

CryoEM has delivered substantial insights on the molecular details of how bacterial secretion systems work and highlighted fundamental principles. The future looks just as bright as SPA and STA become increasingly vital tools in otherwise nonstructural studies.

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