Taming the Triskelion: Bacterial Manipulation of Clathrin

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SUMMARY The entry of pathogens into nonphagocytic host cells has received much attention in the past three decades, revealing a vast array of strategies employed by bacteria and viruses. A method of internalization that has been extensively studied in the context of viral infections is the use of the clathrin-mediated pathway. More recently, a role for clathrin in the entry of some intracellular bacterial pathogens was discovered. Classically, clathrin-mediated endocytosis was thought to accommodate internalization only of particles smaller than 150 nm; however, this was challenged upon the discovery that *Listeria monocytogenes* requires clathrin to enter eukaryotic cells. Now, with discoveries that clathrin is required during other stages of some bacterial infections, another paradigm shift is occurring. There is a more diverse impact of clathrin during infection than previously thought. Much of the recent data describing clathrin utilization in processes such as bacterial attachment, cell-to-cell spread and intracellular growth may be due to newly discovered

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divergent roles of clathrin in the cell. Not only does clathrin act to facilitate endocytosis from the plasma membrane, but it also participates in budding from endosomes and the Golgi apparatus and in mitosis. Here, the manipulation of clathrin processes by bacterial pathogens, including its traditional role during invasion and alternative ways in which clathrin supports bacterial infection, is discussed. Researching clathrin in the context of bacterial infections will reveal new insights that inform our understanding of host-pathogen interactions and allow researchers to fully appreciate the diverse roles of clathrin in the eukaryotic cell.

KEYWORDS *Coxiella burnetii*, EPEC, *Listeria monocytogenes*, *Shigella flexneri*, *Staphylococcus aureus*, adaptor proteins, autophagy, bacterial replication, clathrin, pathogen internalization

INTRODUCTION

Clathrin-coated vesicles, described as “vesicles in a basket,” were first observed 50 years ago (1, 2). In 1975, Barbara Pearse identified clathrin, an essential protein found in all eukaryotic cells, as the dominant constituent of these vesicles (3). Pearse isolated these vesicles and used SDS-PAGE to demonstrate that they were made up of one predominant protein of 180 kDa, which she termed “clathrin.” Today this protein is recognized as the heavy chain of the clathrin molecule. Since its discovery, clathrin has become widely known for its role in endocytosis, but additional functional connections in neuronal signaling, cell cycle, infection, and genetic disorders have also been made. In its canonical endocytic function, clathrin is a molecular scaffold for internalization of cargo and receptors from the plasma membrane into a clathrin-coated pit. The capacity of clathrin to oligomerize into a lattice also contributes to some of the less-studied functions of clathrin in the eukaryotic cell. However, recent reports indicate that clathrin may also make other contributions to cell function independent of scaffold formation.

Clathrin is one of many eukaryotic proteins that can be exploited by bacterial pathogens for their success. Evidence that clathrin is required for both extracellular and intracellular bacterial pathogens, both those that survive in the cytosol and those that survive in a membrane-bound vacuole, is mounting. In this review, the canonical role of clathrin in the context of bacterial infection is summarized and recent findings are highlighted to demonstrate emerging functions of clathrin and how diverse pathogens take advantage of these less-studied clathrin-related processes. Notably, recent research has demonstrated that clathrin is utilized not only for entry of bacteria but also for attachment, spread, and intracellular replication. The paradigm that clathrin was able to facilitate internalization of particles only of a certain size was changed in 2005, when the entry of *Listeria monocytogenes* was found to be dependent on clathrin (4). Recent findings implicating clathrin in a range of other bacterial virulence traits suggest that another change in the field is occurring and that investigating these host-pathogen interactions will further inform our understanding of important alternate functions of clathrin.

THE FUNCTIONAL DIVERSITY OF CLATHRIN

The plasma membrane is a dynamic structure that separates and protects a eukaryotic cell from the extracellular space while facilitating movement of particles in and out of the cell and acting as an important signaling platform for communication with neighboring cells. Endocytosis allows the cell to take up specific molecules from the environment and to recycle plasma membrane receptors. Various types of endocytosis have been described, including phagocytosis, pinocytosis, and receptor-mediated endocytosis, the last of which includes clathrin-mediated endocytosis (CME).

Clathrin-Mediated Endocytosis

CME is responsible for the internalization of a diverse range of molecules, such as growth factors, transferrin for transportation of iron, and low-density lipoprotein receptor bound to lipids (5). Such molecules and their receptors, termed cargo, are first engaged by early-arriving proteins during CME initiation, which then triggers the

FIG 1 Clathrin-mediated endocytosis. (A) During initiation of clathrin-mediated endocytosis (CME), proteins FCHO2, intersectin 1, and EPS15 form an early-arriving complex at phospholipid-rich regions of the plasma membrane. (B) Cytoplasmic tails of cargo molecules are selectively bound by adaptor protein AP-2 or DAB2. Adaptors also bind phospholipids on membranes in order to recruit clathrin molecules. Clathrin begins to oligomerize into a lattice structure around the clathrin-coated pit. (C) Once the clathrin-coated vesicle has reached its optimal size, the vesicle is pinched from the membrane by dynamin. Dynamin is recruited by proteins including endophilin and sorting nexin 9. Actin plays an important part in movement of the newly formed vesicle. (D) Once the vesicle is detached from the membrane, the clathrin lattice is rapidly disassembled by Hsc70.

assembly of a number of proteins (Fig. 1). The cargo is enclosed in a plasma membrane-derived vesicle of approximately 60 to 150 nm in diameter which pinches off the membrane during scission and enters the cytoplasm to be directed to endosomes (6). A group of over 50 proteins have been described to participate in CME from initiation and progressions to termination.

The most well-described of this cohort of CME-associated proteins is the multimeric protein clathrin, derived from the Latin word “clathratus,” which means lattice-like. Indeed, clathrin self-assembles into a lattice around the growing vesicle, and this process is facilitated by its unique triskelion shape. The triskelion is composed of three clathrin heavy chains (CHCs) (~180 kDa each) and three light chains (~25 kDa each), where the three CHCs interact at a central point and extend outwards in three directions. The light chains interact with each of the heavy chains and gather near the center of the structure. The CHC extensions interact with other triskelia, overlapping in their conformation to form a single-layer “coat” around the vesicle. Each CHC contains an N-terminal domain, extending inward from the lattice, which is responsible for binding numerous other assembly particles termed accessory proteins.

Cryo-electron microscopy studies, combined with crystal structures and atomic modeling, of *in vitro*-assembled clathrin coats have provided high-resolution understanding of these structures and have demonstrated that the curvature of the clathrin lattice depends on the ratio of hexagons to pentagons formed by the interacting triskelia (7, 8). Cryo-electron tomography of coated vesicles isolated from cells or tissues indicates that *in vivo*, clathrin-coated vesicles have great diversity in size and shape (9, 10).

CME initiation. CME is a highly regulated process that is initiated at phospholipid-rich areas of the plasma membrane. CME progresses based on the identity and timing of the recruited proteins; however, it is unclear whether the presence of cargo initiates CME or whether the cargo is recruited following a nucleation event. From initiation, and then through the stages of vesicle growth, budding, and scission from the plasma membrane, to clathrin uncoating, the process takes approximately 60 s. CME begins with a nucleation stage, where the membrane is selected and reshaped into a “pit.” Key to the nucleation stage is the involvement of phosphoinositides, especially phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂], which mediate membrane localization and recruitment of many accessory proteins (Fig. 1). Some studies indicate that the Fer/Cip4 homology domain only (FCHO) family of proteins, consisting of two homologs, FCHO1 and FCHO2, are the first to arrive at the plasma membrane to initiate endocytosis (11).

These proteins contain a membrane-binding F-BAR homodimerization domain at their N termini, which can sense less-extreme membrane curvature than the traditional BAR domain. Henne and colleagues determined that the abundance of clathrin-coated vesicles correlated with FCHO1/2 levels and that clathrin was not recruited to the membrane in the absence of FCHO1/2 (11). Furthermore, two additional proteins, intersectin 1 (ITSN) and epidermal growth factor receptor (EGFR) substrate 15 (EPS15), are recruited to the membrane early by FCHO1/2 and bound to the C-terminal μ HD domain of FCHO1/2. This FCHO-ITSN-EPS15 complex accumulates early in CME but is excluded from the mature clathrin-coated vesicle following budding (11). Subsequent studies have shown, however, that FCHO1/2 are dispensable for CME nucleation, and it was proposed that the adaptor protein complex AP-2 arrives at the plasma membrane jointly with a clathrin molecule to initiate endocytosis (12). In this model, FCHO1/2 are only responsible for sustained growth of the clathrin-coated vesicle before budding. Other studies highlight that there is redundancy among the accessory proteins of the clathrin machinery and that each of several proteins is capable of initiation (13, 14).

Adaptor proteins. Adaptor protein complexes are vital for membrane trafficking, and of the five different adaptor protein complexes, only AP-1 through -3 are able to bind clathrin (Fig. 2) (15–17). AP-4 and AP-5 are used for clathrin-independent transport of vesicles within the cell (18, 19). Of the clathrin-binding adaptor protein complexes, AP-2 is the most important for CME. Each adaptor protein complex is composed of two large subunits (one of γ , α , δ , ϵ , or ζ and a β subunit), a μ subunit, and a small σ subunit (20). AP-2 subunits assemble into a multimeric structure with four domains: the $\sigma 2$ core, the $\mu 2$ hinge, and two appendages, α and $\beta 2$ (21). The hinge and appendage domains protrude from the core and interact with clathrin and other accessory proteins involved in CME. AP-2 is responsible for recognizing and selecting transmembrane cargo receptors that are to be internalized, and it does so through recognition of signal sequences known as endocytic sorting motifs. Two such motifs, a dileucine motif ([DE]xxxL[L]) and tyrosine motif (Yxx $\emptyset$, where $\emptyset$ represents leucine, isoleucine, methionine, valine, or phenylalanine) are recognized by the α/σ domains and the $\mu 2$ subunits, respectively (22). In its cytosolic form, AP-2 adopts a locked conformation in which the cargo recognition sites are blocked by parts of the $\beta 2$ appendage. Upon binding to PI(4,5)P₂-rich membranes, AP-2 undergoes a conformational change to expose the cargo-binding domains and thus allow the progression of CME (23).

AP-2 is considered a “hub” during CME, acting as a linkage point from the membrane to the FCHO1/2-ITSN-EPS15 complex and then to clathrin itself. However, alternate adaptors exist, such as Dab2, enabling uptake of a selection of cargoes in the absence of AP-2 (24, 25). With adaptors in place, the coat is then assembled. The promotion of the growing coat may be facilitated by the type and specificity of the transmembrane cargo to be internalized, as well as the ubiquitylation status of this cargo (26, 27). As clathrin is recruited and is forming a lattice, the membrane undergoes significant deformation which is stimulated by membrane-bending molecules such as endophilin (28).

Scission and uncoating. Once the vesicle has reached its optimal size, it is excised from the plasma membrane in a scission step mediated by the GTPase dynamin (Fig. 1). Dynamin is recruited by BAR domain-containing proteins such as endophilin and sorting nexin 9, and over 20 dynamin molecules oligomerize around the neck of the vesicle to free it from the cell periphery (29). Following scission, the clathrin coat is rapidly disassembled and is recycled back to the plasma membrane. Hsc70 mediates dissociation of clathrin from vesicles and requires cofactor proteins such as auxilin or cyclin-G-associated kinase (30–34). AP-2 is then removed from the vesicle, and the cargo-carrying compartment is directed to fuse with endosomes for subsequent trafficking to the Golgi apparatus, recycling to the plasma membrane, or degradation in the lysosome.

FIG 2 Clathrin and adaptor proteins throughout the cell. AP-1 (purple) is located at the *trans*-Golgi network and at intermediate (recycling) endosomes and mediates vesicle budding and movement of cargo between these two organelles. This process is dependent on clathrin. At the plasma membrane, AP-2 (red) facilitates clathrin-mediated endocytosis, with the resulting internalized vesicles trafficked throughout the cell. AP-3 (green) binds clathrin on early endosomes. AP-4 (orange) is utilized in a clathrin-independent manner at the *trans*-Golgi network, and AP-5 (blue) is found on late endosomes with no known clathrin interaction.

Actin contribution to CME. Pioneering work in yeast demonstrated that the actin cytoskeleton is required for endocytosis, and this research was quickly followed by similar findings in mammalian cells (35, 36). However, some contradicting studies demonstrate no requirement for actin during canonical CME (37, 38). Rather, actin aids vesicle closure when cargo is larger than normal, for example, during uptake of vesicular stomatitis virus (VSV) particles (39), or when the cell membrane tension is high (38).

The interaction between clathrin and actin occurs via Hip1R, a light-chain-bound protein that can also interact with filamentous actin (F-actin) and cortactin (38, 40–42). F-actin has been shown to aid CME in multiple capacities, including membrane bending, scission force generation, and vesicle motility (43). Neural Wiskott-Aldrich syndrome protein (N-WASP), which activates Arp2/3 complexes that enable actin polymerization, is located at the site of CME and can propel clathrin-coated vesicles from the plasma membrane into the cytoplasm (44, 45). Furthermore, dynamin and several dynamin-binding partners regulate actin dynamics at the clathrin-coated vesicle during scission (46).

TABLE 1 Cellular processes involving clathrin and their unique properties

Process	Subcellular location(s)	Light chain involvement	Adaptor protein(s)	Clathrin conformation	Cargo type	Reference
CME	Plasma membrane	Yes	AP-2	Triskelial	Various	3
Golgi transport	<i>trans</i> -Golgi network, recycling endosome	Yes	AP-1	Triskelial	Lysosomal hydrolases	53
Endosomal	Early endosome, lysosome	Yes	AP-3	Triskelial	Lysosomal membrane proteins	54
G-clathrin	Endosome, plasma membrane	Yes	None	Triskelial	?	70
Mitosis	Spindle	Yes	?	?	No vesicle	73
Wnt pathway	Cell surface, LRP6 signalosomes	Yes	AP-2	?	?	79
Immunological synapse	Plasma membrane	Yes	AP-1	?	TCR-MHC complex, actin accumulation	81
Transcription factor	Nucleus	No	None	Monomer, unoligomerized	No vesicle	85
ALR	Autolysosomes	Yes	AP-2 + AP-4	?	Protolysosome contents	90
GLUT4 transport	Muscle cell, GLUT4 storage compartment	No	AP-1 + AP-3	Triskelial, CHC22 only	GLUT4	100

Unconventional Roles of Clathrin

As described above, the prototypical function of clathrin in eukaryotic cells is to facilitate endocytosis. This is not only for general uptake of typical cargo molecules but also for several specialized endocytic functions such as synaptic vesicle recycling in neurons, major histocompatibility complex class II (MHC-II) presentation in immune cells, and internalization of clusters of gap junction channels (47–51). However, the importance of clathrin in the human cell reaches beyond the plasma membrane. Just as COP-I and COP-II coats are involved in vesicular transport within the cell between organelles, clathrin and adaptor complexes can also provide a coated structure on vesicles budding from intracellular sites, such as those moving between the *trans*-Golgi network (TGN) and endosomes (Fig. 2) (52). In this context, clathrin retains its function as a vesicular coat. However, additional new discoveries also reveal that clathrin can act independently of a budding vesicle and even independently of the iconic triskelial conformation or an accompanying light chain. The research discussed in the following sections is summarized in Table 1.

Clathrin-mediated vesicular transport from internal organelles. Besides its role in endocytosis, clathrin can be recruited to intracellular organelles, such as the *trans*-Golgi network, and can facilitate vesicle budding from these sites (53). Clathrin also resides on specialized tubular endosomes (15, 54–57). The assembly of clathrin lattices on the Golgi apparatus requires the adaptor complex AP-1, which shares some domains of homology with AP-2 (58, 59). AP-1-mediated transport facilitates the movement of lysosomal hydrolases, such as those that bind to the mannose-6-phosphate receptor, in a bidirectional fashion from the Golgi apparatus to recycling endosomes, to be redirected to their appropriate resident vesicle (60–65).

The third clathrin adaptor, AP-3, facilitates coated budding from tubular endosomes, typically to carry lysosomal membrane proteins to lysosomes and associated organelles (66, 67). Both AP-1/clathrin and AP-3/clathrin vesicles do not utilize dynamin for vesicle scission (68). The last two adaptor protein complexes, AP-4, which was found to bind membrane of the *trans*-Golgi network, and AP-5, which mediates budding from sorting/late endosomes, do not recruit clathrin. AP-5 binds SPG11 and SPG15, which may form a similar coat-like complex (Fig. 2) (18, 19, 69).

A newly described transport route inside cells, where budding of vesicles takes place from extended tubules of early endosomes, recycles cargo straight toward the plasma membrane. The vesicles that carry out these feats utilize so-called gyrating clathrin (70). This clathrin is characterized by being fast moving in the cell, enabling rapid recycling of receptors, and is regulated by the GTP-binding protein ARF6 (71). Gyrating clathrin lacks any detectable associated adaptor protein complexes but is composed of both a heavy chain and a light chain (70, 72).

Clathrin and mitosis. A 1985 study published the first evidence that clathrin is localized to the mitotic spindle in the mouse embryo (73). It was subsequently shown

that clathrin is redistributed to mitotic spindles in tissue culture cells that had entered telophase (73, 74). These findings were confirmed almost two decades later, when CHC was one of four novel proteins identified to be associated with the mitotic spindle by gene-trapping experiments (75). Notably, the CHC located at the spindle does not appear to be associated with membranes and is not part of any discernible vesicle. It is hypothesized that CHC at the mitotic spindle acts to stabilize fibers and therefore aid chromosome alignment. In this context, CHC directly binds to kinetochore fibers, protein structures to which microtubules attach in the centrosome, and is therefore predicted to act as a scaffold support between multiple microtubules within a fiber (76). The triskelial structure and oligomerization capacity of CHC seem ideal to accomplish this task. In agreement with this idea, electron microscopic studies can observe “bridges” connecting spindle fibers (77). CHC normally involved in endocytosis is likely redirected to form part of the spindle, as indicated by observations that CME is shut down during mitosis (78). However, the exact mechanism for CHC recruitment to spindle fibers is not well understood.

Clathrin and intracellular signaling. Researchers have demonstrated that other cellular pathways depend on clathrin. For example, CME is important for Wnt signaling. Both clathrin and AP-2 are required for formation of low-density lipoprotein receptor 6 (LRP6) signalosomes at the plasma membrane (79). Treatment of cells with exogenous Wnt3a increases the number of clathrin plaques on the plasma membrane, and AP-2 recognizes endocytic sorting motifs on LRP6 to enable Wnt receptor activation and signal transduction (79, 80). Another unconventional function that CME plays is evident in the immunological synapse, where a lymphocyte interacts with an antigen-presenting cell. Internalization of the T cell receptor (TCR) in complex with MHC upon contact with an antigen-presenting cell is dependent on clathrin (81, 82). Similarly, clathrin is involved in the accumulation of actin for allowing the TCR to be internalized (83, 84).

CHC can also be detected in the nucleus, where it is thought to activate p53, a transcription factor which induces apoptosis and the arrest of cell growth (85, 86). CHC in this context is in its monomeric form and acts to stabilize the p53-p300 interaction to promote the transcription of p53-related genes (85, 86). Interestingly, mutating the residue involved in trimerization of CHC allows a proportion of clathrin molecules to enter the nucleus, while wild-type clathrin is diffuse throughout the cytoplasm only (87–89).

Clathrin and autophagy. Clathrin has recently emerged as a key player in autophagy, the process by which unwanted or damaged cellular components are enclosed in an autophagosome and targeted for destruction by fusion with the lysosome. The resulting compartment is known as the autolysosome. In a 2012 study, Rong and colleagues reported that clathrin controls autophagic lysosome reformation (ALR). ALR is the mechanism for recycling lysosomes from autolysosomes. The process involves the extension of thin tubes known as reformation tubules from the autolysosome, from which protolysosomes bud (90, 91). Proteomic analysis of purified reformation tubules demonstrated that CHC is a key constituent of the tubules. Not only was CHC shown to be required for the initiation of reformation tubule formation, but it also is required for protolysosome budding. Both AP-2 and AP-4 are also involved in ALR (90). Another role for clathrin in autophagy came from a report that upon autophagy initiation by starvation, clathrin is localized to autophagosomes (92). Clathrin may be present on autophagosomes due to the presence of clathrin on vesicles budding from the TGN which facilitate autophagosome biogenesis (92). The origin of membrane for the formation of autophagosomes therefore may include the TGN as a source.

An earlier study also highlighted the contribution of the plasma membrane to autophagosomes, by the discovery that plasma membrane-localized CHC interacts with ATG16L1, an essential protein in the progression of autophagy (93). ATG16L1 may localize to clathrin-coated vesicles before they are excised from the plasma membrane and thus help direct clathrin-associated membranes to autophagosomes. Furthermore, dynamin is required during autophagy to allow the double-membrane compartment

enclosing the lysosome-destined cytoplasmic components, known as the phagophore, to mature to an autophagosome and then to an autolysosome (93).

Differential Function of Clathrin Isoforms

There are two isoforms of clathrin heavy chain, CHC17 and CHC22, encoded by the *CLTC* and *CLTD* genes, respectively (94–96). They are approximately 85% homologous at the amino acid level but exhibit distinct localization patterns within the cell. CHC17 is most widely studied, as it is the isoform involved in endocytosis and mitosis. In comparison, CHC22 is not implicated in endocytosis (97). Both isoforms are able to form lattices made from triskelia, but they have distinct interaction profiles, as CHC22 binds sorting nexin 9 using a domain that is absent in CHC17 (98, 99). Moreover, while CHC17 interacts with and is regulated by clathrin light chain, CHC22 does not associate with a light chain. CHC22 does, however, associate with AP-1 and AP-3, but not with AP-2 (97, 99). In 2009, CHC22 was shown to participate in trafficking of the glucose transporter GLUT4 to insulin-responsive storage compartments in human muscle cells and thus was shown to be required for glucose uptake upon stimulation with insulin (100). Interestingly, the CHC22 coat is more resistant to uncoating by the Hsc70-auxilin complex and is more stable under pH changes (98).

CHCs from different organisms are highly conserved, and clathrin light chains display more amino acid sequence divergence. In humans, two distinct but related light chains, LCa and LCb, with approximately 60% identity, are encoded by *CLTA* and *CLTB*, respectively (101, 102). Each light-chain gene can also generate multiple isoforms through alternate splicing. Studies have indicated that each light chain incorporates into a triskelion or a clathrin coat with no preference as to the subtype and that different types can exist in the same triskelion (103). LCb seems to be predominant in cell types that maintain a strongly regulated pathway of protein secretion, and LCa is turned over more quickly than LCb (104). Studies on the importance of the light chain for CHC function and endocytosis have revealed that in the absence of a light chain, CHC shows decreased association with membranes (105, 106). Additionally, in yeast depleted of CHC, overexpression of the yeast light chain CLC1 restores growth of yeast, hinting at additional binding partners or activities of the light chain distinct from CHC (107).

These results collectively indicate that clathrin has far more diverse roles in the cell than initially thought, and there may be further different functions of distinct isoforms, either independent of or in conjunction with light-chain molecules. Very few research studies have distinctly discriminated between the clathrin isoforms, as commercially available antibodies readily recognize both isoforms. This makes it difficult to assess the significance of the two different CHCs and two different light chains and to what extent they may be utilized in the various pathways described above.

A TRADITIONAL ROLE FOR CLATHRIN DURING INFECTION: INTERNALIZATION OF INTRACELLULAR BACTERIA

Key to the life cycle of intracellular bacterial pathogens is the ability to enter the host cell, and during entry of nonphagocytic cells, this process is usually initiated by the bacteria themselves. Two methods of active bacterial entry into nonphagocytic cells have been described, namely, the “zipper” and “trigger” mechanisms. Bacteria that enter by zipper procedures, including *Listeria* spp., *Yersinia* spp., and *Neisseria* spp., do so by directly binding to host cellular receptors with their own bacterial surface proteins. This initiates a signaling cascade which culminates in the pathogen being engulfed in a phagosome. In contrast, “triggering” bacteria do not need to bind a cellular receptor and instead gain access through injected effector proteins (108). The activity of these proteins, for example, the SopE family of effectors from *Salmonella enterica* serovar Typhimurium, induces plasma membrane ruffling by activating key host cellular targets (109, 110). Like *S. Typhimurium*, *Shigella flexneri* injects effector proteins to gain entry; in particular, IpaC targets Cdc42 to induce actin polymerization and membrane ruffling and ultimately lead to bacterial internalization (111, 112).

The first report to show that clathrin was vital for a bacterial species to enter cells was published in 2005 (4); however, long before this discovery, it was established that clathrin is important for the entry of a variety of viruses. In the 1970s it was observed that viruses could be engulfed into endocytic compartments. The first report to link viral entry to clathrin came in 1981, when electron microscopy studies revealed that vesicular stomatitis virus (VSV) was taken up into clathrin-coated vesicles (113–115). Since then, many viruses, originating from over 10 different virus families, have been shown to use clathrin to enter eukaryotic cells (116).

The link between clathrin and bacterial pathogenesis was more recently discovered. A series of studies from the Cossart laboratory between 2005 and 2011 have revealed that clathrin is required for the internalization of *L. monocytogenes*, a microbe that is typically 0.5 to 2 μm in length (117). These studies demonstrated for the first time that clathrin not only is for internalization of small molecules but can facilitate internalization of objects larger than 150 nm (9, 118). As a consequence, a broad view of clathrin participation in bacterial entry was adopted, with researchers noting that clathrin contributes to the zippering mechanism for a range of pathogens (reviewed in reference 119). In contrast, clathrin is not involved in invasion through the triggering mechanism of pathogens such as *S. Typhimurium* and *S. flexneri* (120).

Listeria monocytogenes

L. monocytogenes is the causative agent of listeriosis and a foodborne opportunistic bacterial pathogen that promotes its own entry into nonphagocytic cells. The ability of this pathogen to cause disease requires entering epithelial cells, escaping the phagosome, and accessing the cytosol to replicate therein. *L. monocytogenes* has been extensively studied with respect to its adaptation to an intracellular niche, and this research has led to many important discoveries that impact biological sciences beyond bacteriology (121). Throughout its infection cycle, *L. monocytogenes* uses many virulence factors that are tightly controlled by the bacterium. For example, phagosome escape by *L. monocytogenes* involves enzymes including the pore-forming toxin listeriolysin O (LLO) and type C phospholipases (122–124). Various other roles have also been attributed to LLO (125). While unable to efficiently invade monocytes, *L. monocytogenes* is a master manipulator of epithelial cells, including enterocytes and endothelial cells (126, 127).

Receptors for entry of *L. monocytogenes*. *L. monocytogenes* enters nonphagocytic cells via the zipper mechanism, which involves bacterial interaction with receptors on the host cell. It is well established that to enter mammalian epithelial cells, two bacterial surface proteins, InlA and InlB, which belong to the internalin family, are essential (128, 129). The engagement of InlA or InlB with the mammalian cell receptors E-cadherin and Met, respectively, triggers engulfment of the bacterium (Fig. 3) (130, 131). The activation of either one of these receptors is sufficient to allow for bacterial entry into Caco-2 enterocytes; however, invasion of mouse hepatocytes exclusively requires InlB due to significant amino acid changes in E-cadherin. These mutations are in key regions of E-cadherin, abolishing the ability of InlA to engage this receptor (129, 132, 133). Following InlA/B engagement with E-cadherin or Met, the host receptors undergo posttranslational modifications. E-cadherin is phosphorylated and ubiquitinated by Src and Hakai, respectively, and Met is ubiquitinated via the ligase Cbl upon InlB engagement (4, 134). Once cellular receptors E-cadherin/Met are activated, a cascade of events allows the internalization of *L. monocytogenes* via the zipper mechanism into a phagosome containing the bacterium (Fig. 3).

Recruitment of clathrin. Endocytosis of the cellular receptors and the bacterium is ultimately mediated by actin polymerization at the site of entry. Clathrin recruitment is required to allow actin involvement. Both the heavy and light chains of clathrin are localized at the site of *L. monocytogenes* entry into cells. Upon silencing expression of CHC, bacterial entry is inhibited (4). Importantly, for clathrin to achieve its part in *L. monocytogenes* internalization, it is phosphorylated in a bacterially driven manner. This posttranslational modification is performed by Src at tyrosines in positions 1477 and

FIG 3 Site of *Listeria monocytogenes* entry into cells. A schematic of proteins involved in allowing *L. monocytogenes* uptake via the zipper mechanism is shown. The *L. monocytogenes* internalin proteins InlA and/or InlB engage the host proteins E-cadherin and Met, respectively, to begin recruitment of cellular components on the cytoplasmic side of the host cell. Clathrin is recruited to the site of entry and facilitates the eventual recruitment of F-actin through a signaling cascade that induces cytoskeletal rearrangements necessary for bacterial engulfment.

1487 of CHC and is triggered by InlA-mediated infection and to a lesser extent by InlB (135). Phosphorylation of CHC at these sites is normally necessary for uptake of cellular signaling receptors such as EGFR during CME (136). Recently it was shown that the alternative clathrin adaptor, Dab2, initiates clathrin recruitment during *L. monocytogenes* infection (135). Dab2 is an adaptor protein that can act independently of AP-2, and it is typically found at the largest of clathrin-coated pits, which can be over $3 \mu\text{m}^2$ (137, 138). Dab2 also acts in CME to aid movement of the clathrin-coated vesicle by interacting with the molecular motor protein myosin VI (139, 140). During *L. monocytogenes* infection, myosin VI is recruited downstream of clathrin, Dab2, and actin to be the last protein to act in the pathway. Myosin VI produces the force for movement of the cytoskeleton to direct the bacterium into the cell (135). AP-1 is important for *L. monocytogenes* invasion; however, it is recruited to the bacterial entry site at insignificant levels and thus may have an indirect function (120, 141). Neither AP-2 nor AP-3 is detected at the site of infection (120).

Interestingly, these studies with *L. monocytogenes* also led researchers to demonstrate that clathrin aids entry of the fungal pathogen *Candida albicans* (142). The *C. albicans* invasin Als3 shares structural similarity to InlA and also interacts with host E- or N-cadherin to facilitate pathogen entry in a clathrin-, dynamin-, and cortactin-dependent manner (142).

Internalization of *L. monocytogenes*. Following the recruitment of Dab2, clathrin, actin, and myosin VI, another round of actin rearrangement takes place during *L. monocytogenes* invasion, in which phosphatidylinositol 3-kinases (PI3-Ks) are crucial. Their activity is necessary for successful infection by either the InlA or InlB pathway (132). During InlA-mediated bacterial uptake, the Arp2/3 complex, which serves as an actin filament nucleator, is recruited by α - and β -catenins which associate with the entry site (Fig. 3) (143). A small interfering RNA (siRNA) screen identified 9 members of the PI3-K pathway that are involved in bacterial entry by the InlB pathway, including the

small GTPase Rab5c and other GTPases used in actin cytoskeleton formation, such as those from the Ras subfamily (144). Ras family proteins trigger a signaling cascade that leads to WAVE/N-WASP-mediated activation of the Arp2/3 complex (145, 146). Key to actin assembly during infection is the depolymerizing factor cofilin, which enables the turnover of actin monomers by severing F-actin monomers (147, 148). At the end of the bacterial internalization process, cofilin is inactivated by LIMK1-mediated phosphorylation, which halts depolymerization and slows actin dynamics (146). The pH drop of the phagosome during the entry and trafficking process then triggers LLO pore-forming activity and allows the progression of infection (149).

Staphylococcus aureus

Endothelial cells, along with various other nonprofessional phagocytes, internalize staphylococci (150–153). Under many conditions, these bacteria will replicate intracellularly (150, 154–156). *S. aureus* expresses fibronectin-binding proteins which engage with cellular fibronectin receptor $\alpha_5\beta_1$ -integrin to facilitate intracellular access (157–159). Treatment of cells with cytochalasin D severely interferes with *S. aureus* uptake, and polymerized actin is readily visible within minutes of *S. aureus* attachment to cells (160). As with *L. monocytogenes*, Src kinase performs a critical role during integrin-mediated *S. aureus* invasion.

The association of *S. aureus* with the clathrin machinery during the internalization process was first indicated in 1995 with electron microscopy studies visualizing what looked to be clathrin-coated pit formation during infection (161). Since then, systematic studies have revealed that, as a zippering bacterium, *S. aureus* recruits clathrin to facilitate invasion (120, 162). Cbl, a host ubiquitin ligase which is responsible for activating the endocytosis of integrins in the clathrin pathway, is also important for *S. aureus* infectivity (120).

During uptake of both *L. monocytogenes* and *S. aureus*, pathogen entry depends on binding receptors that would normally be endocytosed by the clathrin route. These bacterial pathogens are much larger than any typical cargo molecule or complex of molecules, at approximately 2 μm in length. Given the lack of micrograph evidence, it is not likely that these bacteria are actually engulfed inside a clathrin-coated vesicle. However, internalization still occurs in a clathrin-dependent manner. This hints at an alternative internalization mechanism supported by clathrin that may occur when many clathrin-coated pits form underneath a bacterium and act in unison to draw the membrane in (163).

CLATHRIN AND OTHER BACTERIA: ANOTHER PARADIGM SHIFT?

Studies defining bacterial pathogen internalization found that the endocytic role of clathrin was used to the advantage of some bacteria. However, considering the nonendocytic role of clathrin, further examples have emerged in which bacteria utilize clathrin in a nontraditional way (Fig. 4). Not only is clathrin at the plasma membrane for endocytosis, but, as described above, clathrin can act at distinct internal sites. Hence, there is precedence for clathrin involvement in bacterial infection which does not include uptake of bacteria from the plasma membrane. While the field is beginning to realize how clathrin influences nonendocytic processes, there are many unknowns, including the interaction repertoire of clathrin in each nonendocytic infection context. Additionally, the function of the clathrin molecule in these contexts is yet to be elucidated. Whether clathrin acts in a structural capacity, acts as a recruitment platform for other molecules, or provides key activity in activation of the process remains a mystery.

Escherichia coli

Enteropathogenic *E. coli* (EPEC) is a diarrheal pathogen normally affecting young children. Disease caused by EPEC is a result of the bacteria adhering to the mucosal layer of the gastrointestinal tract, disrupting the microvilli and causing substantial water loss (164). EPEC infection subverts host cell processes such as immune mechanisms and

FIG 4 Bacterial pathogens coopt clathrin for nonendocytic functions. In order to establish close attachment to host cells, enteropathogenic *Escherichia coli* (EPEC) induces formation of actin-rich pedestals. Clathrin accumulates on the cytoplasmic side of the pedestal in an EPEC-induced manner and is required for subsequent actin recruitment and bacterial virulence. *Coxiella burnetii* replicates within a large *Coxiella*-containing vacuole (CCV) which is surrounded by clathrin. Clathrin is thought to be redirected to the CCV by effectors of the type IV secretion system (T4SS), CvpA and Cig57, that hijack clathrin-coated vesicles. Clathrin may also be delivered to the CCV when clathrin-associated autolysosomes fuse with the CCV through the activity of Cig2. *Shigella flexneri* is taken up into host cells by the trigger mechanism, a clathrin-independent mechanism which depends on the activity of the pathogen's type III secretion system (T3SS). *S. flexneri* escapes into the cytosol and moves through the cell via actin-based motility until spreading to a neighboring cell at the tricellular junction. *S. flexneri* creates a protrusion which is engulfed by the neighboring cell using clathrin.

cell death, all while bacteria occupy the extracellular space (165, 166). A hallmark feature of EPEC infection is the formation of attaching and effacing (A/E) lesions on the intestinal epithelium, whereby the bacteria form an intimate attachment with the human cell and cause destruction of the brush border microvilli (167). Clathrin is important in formation of these distinctive A/E lesions, marking the first report of a nonendocytic role for clathrin in bacterial pathogenesis (120). Some of the important molecules that are recruited by clathrin for pedestal formation are known and are discussed below.

Pathogenesis of EPEC. EPEC is a pathogen that drives many host modifications from the extracellular space via an intimate attachment to enterocytes. The mechanism by which EPEC achieves these feats is largely through the use of effector proteins, which are translocated through a type III secretion system (T3SS) into the host cell (168). Manipulating the host cell cytoskeleton is a common strategy used to aid in the success of bacterial pathogens, and in the case of EPEC, the most well studied is hijacking of actin. The EPEC T3SS effector Tir (translocated intimin receptor) induces significant cytoskeletal changes and A/E lesion formation at the point of EPEC attachment (169). Tir is translocated into the host cell and inserted into the plasma membrane, where it functions as a membrane receptor for the EPEC outer membrane protein intimin. The strong interaction between Tir and intimin facilitates intimate attachment of EPEC at the host cell surface (170). Host cellular kinases are recruited to phosphorylate Tir at multiple residues. Phosphorylation of tyrosine 474 induces activation of the Arp2/3 complex, via N-WASP, inducing polymerization of F-actin (170–173). This culminates in the formation of an actin-rich pedestal from which EPEC disrupts microvilli and gains a closer, stable attachment to the cell (174). A diverse array of host proteins are enriched within the actin pedestal (175, 176). For example, cofilin helps turn over actin monomers to enable growth of the actin filament at the tip, and its presence is dependent on Tir translocation (177). The level of bacterial attachment to cells influences the efficiency of effector protein translocation by the T3SS (178). Since many effector proteins act as key virulence factors in their own right, the ability to form pedestals to allow optimal translocation is essential for the success of EPEC (179).

Pedestal formation. The first report to hint at a role for the endocytic machinery in actin filamentation and pedestal formation came in a 2007 publication with the discovery that dynamin is required for full virulence of EPEC (180). When dynamin expression is silenced, there is reduced actin polymerization at the site of EPEC attachment, and recruitment of N-WASP is inhibited. Importantly, dynamin recruitment is dependent on Tir and hence driven by the bacteria. Recruitment of dynamin also occurs in actin tail formation behind *L. monocytogenes* for movement throughout the cell, suggesting the possibility of a common mechanism for hijack of a similar set of cellular components by two different bacterial pathogens (181, 182).

Further investigation into the role of the endocytic pathway in EPEC pedestal formation led to the discovery that clathrin is essential for actin pedestal formation (120). This represented the first nonendocytic role for clathrin to be described in the context of bacterial infection. Clathrin localizes to EPEC-mediated actin-rich pedestals, and the phosphorylation of Tir at tyrosine 474, the same residue that is required for pedestal formation, is essential for the recruitment of clathrin (120, 173). This recruitment of clathrin occurs independently of the recruitment of actin monomers, but the presence of clathrin is required for subsequent actin polymerization (120). Furthermore, EPS15 and Epsin, two additional accessory components of clathrin-coated vesicles, are crucial for EPEC pedestal formation (183). This same study noted the lack of AP-2 at the pedestal sites, perhaps indicating that the clathrin involved in pedestal formation has no link to the plasma membrane (183). Instead, EPEC pedestals include the alternative clathrin adaptor Dab2 (135). Additionally, the recruitment of clathrin, EPS15, and Epsin is dependent upon the T3SS, indicating a putative role for an EPEC effector protein in clathrin recruitment (120, 183). As with the process of *L. monocytogenes* invasion, pedestal formation requires that the CHC be phosphorylated (135).

These studies provided the first evidence for a nonendocytic role for clathrin and highlighted the concept that clathrin can be an integral part of pathogen virulence. This opens the field to the possibility that clathrin is not only manipulated by bacteria in an endocytic capacity and that further investigation of the role of clathrin during bacterial infection could unveil important subtleties and complexities of clathrin function.

Shigella flexneri

As with *L. monocytogenes*, *Shigella flexneri* is a bacterium that thrives in the cytosol

of host epithelial cells, but in contrast, *S. flexneri* invades these host cells using the trigger mechanism in a bacterially active manner (184). *S. flexneri* triggers its engulfment through a family of T3SS effector proteins, termed invasins, which facilitate membrane and cytoskeletal changes to engulf the bacterium (185). This entry process is not affected by the treatment of cells with the dynamin inhibitor dynasore, nor does the reduction of clathrin levels by siRNA treatment interfere with initial *S. flexneri* invasion (120). Clathrin has previously been observed to accumulate at the site of *S. flexneri* entry, but there is no evidence for a functional role in this location (120, 186). Clathrin is, however, important for *S. flexneri* to spread from cell to cell (187).

***S. flexneri* intracellular life cycle.** *S. flexneri*, the causative agent of shigellosis, a self-limiting diarrheal disease, replicates within intestinal epithelial cells (188, 189). Upon ingestion of the bacteria, *S. flexneri* will preferentially enter the gut through M cells of the Peyer's patch and be delivered to macrophages in the deeper tissue. Within the macrophages, *S. flexneri* lyses the phagosome in which it was engulfed and replicates in the cytosol (190). The phagocytes are killed in great numbers due to induction of apoptosis by the bacteria (191–193). Proinflammatory cytokine release accompanies the macrophage death, and *S. flexneri* is released from the cells (194, 195). The bacteria are then able to enter, via the basolateral surface, the surrounding epithelium for subsequent rounds of infection (192, 196). *S. flexneri* utilizes its T3SS to gain access to the epithelial cells by actin rearrangement at the plasma membrane using T3SS effectors such as IpaA and IpaC (112, 197–199). The bacteria are taken up into a vacuole, and IpaB mediates escape to the *S. flexneri*-preferred niche of the cytosol (190). Once within the cytosol, *S. flexneri* again manipulates actin to form tail-like structures that propel the bacteria to move through the cell toward cell-to-cell junctions (200). This is driven by the bacterium via a bacterial surface protein, IcsA, which accumulates at the bacterial pole to recruit and activate the actin regulator N-WASP (201–206). Pushing into the plasma membrane of the cell, *S. flexneri* creates a pseudopodium protrusion into the neighboring cell. The neighboring cell engulfs the protrusion in a compartment containing membrane from both cells (184). Lysis of these membranes, again using IpaB, allows *S. flexneri* to continue its life cycle in other epithelial cells and spread throughout the host (Fig. 4). In spreading from cell to cell in this manner, *S. flexneri* avoids the extracellular space and the immune surveillance therein, such as complement and B cell recognition.

Clathrin mediates cell-to-cell spread of *S. flexneri*. Cell-to-cell spread has been studied in pathogens such as *L. monocytogenes*, *S. flexneri*, *Burkholderia* spp., and spotted fever group (SFG) *Rickettsia* species, all of which also use actin for movement in the cytosol (207). Despite it being a common virulence trait, the regulation and cellular components involved in cell-to-cell spread are unique for each pathogen (208). The *L. monocytogenes* effector InlC promotes formation of protrusions by inhibiting host proteins at apical junctions, and SFG *Rickettsia* spp. secrete Sca4 to reduce tension forces and allow protrusion engulfment (209, 210). *Burkholderia* spp. access neighboring cells by direct cell fusion without forming protrusions (211).

S. flexneri preferentially accesses neighboring cells in tricellular junctions of epithelial cells, and the ability of the neighboring cell to engulf the *S. flexneri*-mediated protrusion relies on clathrin (212). Chemical inhibition of clathrin-mediated endocytosis, caveolin-dependent endocytosis, and micropinocytosis during *S. flexneri* infection reveals that only the clathrin pathway is needed for *S. flexneri* to move throughout cellular monolayers (212). Clathrin, Epsin, and dynamin, which are key components of CME, are located at the plasma membrane of the receiving cell, and individually silencing expression of these genes by use of short hairpin RNA (shRNA) results in reduced cell-to-cell spread of *S. flexneri* (212). However, Fukumatsu and colleagues (212) did not observe an impairment of bacterial dissemination when treating cells with shRNA against AP-2, EPS15, or Dab2. Since AP-2 and Dab2 are major adaptors in CME, it was proposed that *S. flexneri* hijacks a noncanonical CME pathway. These authors also determined that *L. monocytogenes* protrusions were not affected by treatment with

phenylarsine oxide, which inhibits endocytosis by blocking tyrosine phosphatases, yet specific inhibition of clathrin in this case was not performed (212, 213).

Overall, *S. flexneri* remains to date the only example of a bacterium that uses clathrin to spread intracellularly. While other pathogens exhibit similar mechanisms of actin-based motility in the cytosol and also invade neighboring cells directly, the mechanisms and details of each process vary greatly. Whether clathrin is also involved in other methods for communication or transfer of other material from cell to cell is unknown but provides an exciting area of future research.

CAPTURING CLATHRIN FOR INTRACELLULAR GROWTH

The participation of clathrin as a structural component of the EPEC actin pedestal is not surprising given the known roles of clathrin in providing structural integrity of clathrin-coated vesicles and the lattice-forming capabilities of multiple clathrin triskelia during CME. Similarly, engulfment of cellular protrusions containing *S. flexneri* can also be linked back to known roles of clathrin in CME and internalization of some bacterial pathogens. However, a body of research is also developing that describes unexpected roles of clathrin in the intracellular success of some bacterial pathogens. The idea that a protein known to have structural roles also is integral for bacterial replication is novel and may inform our understanding of the full potential of clathrin in the eukaryotic cell.

Brucella abortus

Brucella spp. are Gram-negative bacterial pathogens which cause the zoonotic infection brucellosis (214). *B. abortus* replicates intracellularly within a *Brucella*-containing vacuole (BCV). The BCV interacts with endocytic compartments, reaching the late endosome/lysosome stage of maturation before the pathogen diverts the BCV to interact with the endoplasmic reticulum and establish a replicative niche (215, 216). Lipid rafts, which are specialized membrane microdomains that are rich in cholesterol, play an important part in *B. abortus* entry and in the endocytic trafficking of this bacterium (217, 218).

A 2013 study investigating the role of clathrin and Rab5 in the life cycle of *B. abortus* uncovered a dual role for clathrin in *B. abortus* pathogenesis (219). Entry of *B. abortus* into HeLa cells was inhibited by treatment with chlorpromazine, a CME inhibitor that acts by causing assembly of adaptor proteins and clathrin on endosomal membranes, rendering them unavailable for plasma membrane action. Silencing CHC expression with siRNA resulted in a similar phenotype (219). Furthermore, Lee and colleagues (219) showed that intracellular replication of *B. abortus* in HeLa cells was affected by siRNA silencing *CLTC*. Normal intracellular replication of *B. abortus* was observed in control knockdown cells, yet even accounting for the entry defect, bacterial numbers did not increase in the absence of CHC.

This study demonstrated that both bacterial uptake and intracellular replication of *B. abortus* depend on CHC. The authors also determined that by inhibiting clathrin, recruitment of the endosomal small GTPase Rab5 to the BCV was significantly perturbed. The *B. abortus* phagosome requires interaction with Rab5-positive endosomes to complete the maturation pathway of the BCV. It is hypothesized that clathrin is required for Rab5 recruitment to the BCV to allow subsequent vesicular interactions and ultimate bacterial replication (219).

Coxiella burnetii

Coxiella burnetii is the causative agent of a potentially life-threatening human infection termed Q fever. *C. burnetii* infects alveolar macrophages and establishes an intracellular niche which resembles an expanded autolysosome. This compartment is termed the *Coxiella*-containing vacuole (CCV). The establishment of the CCV is essential for *C. burnetii* to thrive, and recent research has uncovered that clathrin is required for CCV biogenesis (220, 221). Unlike that of *L. monocytogenes* and *S. aureus*, the entry of *C. burnetii* into nonphagocytic cells is not dependent upon clathrin. The exact mechanism of *C. burnetii* entry remains elusive; however, it is facilitated by interaction

between the bacterial outer membrane protein OmpA and an unidentified host receptor (222). Importantly, the proportion of *C. burnetii* organisms that are intracellular following a 4-hour infection is equivalent during siRNA treatment against *CLTC* and under nontargeting conditions as shown by genomic equivalent counts and microscopic analysis (220, 221). *C. burnetii* does not spread from cell to cell in a manner analogous to that for *S. flexneri*. Additionally, in the absence of clathrin, the CCV is composed of the expected markers such as LAMP1, indicating that clathrin does not contribute to the endocytic maturation of the pathogen-containing vacuole as it does during *B. abortus* infection. Recent findings regarding the role of clathrin during *C. burnetii* infection point to a different role for CHC in supporting pathogen intracellular replication.

A lysosomal lifestyle. *C. burnetii* is an intracellular pathogen which replicates within a lysosome-derived CCV. The CCV is formed through progressive endocytic maturation of the pathogen-containing phagosome from interaction with early endosomes to, finally, fusion with lysosomes. The environment provided by the lysosome is crucial for metabolic activation of *C. burnetii* and protein secretion through the Dot/Icm type IV secretion system (T4SS) (223). At least 130 bacterial effector proteins are translocated into the host cell via this T4SS, which collectively modify the CCV and control host behavior to enable *C. burnetii* replication (224). Multiple effectors perform antiapoptotic functions to keep the host alive, and others act to manipulate autophagy and vesicular traffic to keep the CCV in an autolysosomal state (225–227). The CCV expands to occupy much of the cytoplasm through constant fusion with other CCVs, autophagosomes, and endosomes, and it represents the only lysosomal compartment to permit growth of any known bacterium.

Even in the absence of infection, there are some functional links between clathrin and lysosomes that may point to a role for clathrin at the CCV. This is particularly evident in lysosomal storage diseases. For example, cells with mutations representative of Niemann-Pick disease display lower total and internal levels of transferrin and are less efficient at recruiting clathrin to the site of cargo uptake (228). Other studies observed clathrin coats on lysosomes and that clathrin is deposited on lysosomes upon autophagy induction (90, 229). Most obviously, the lysosomal environment is influenced by clathrin as lysosomal proteins are delivered by clathrin-coated vesicles from the Golgi apparatus (62, 67).

Coxiella effector proteins target clathrin-mediated traffic. The first link between *C. burnetii* and clathrin was made in 2013 when a *C. burnetii* T4SS effector, CvpA, was shown to interact with both clathrin and AP-2 via endocytic sorting motifs encoded on CvpA (220). It was also demonstrated, by immunofluorescence microscopy, that CHC surrounds the CCV. This is not observed when cells are infected with a CvpA-deficient *C. burnetii* strain (220). Interestingly, the absence of CvpA has a significant impact on the capacity of *C. burnetii* to replicate intracellularly. These data led to the hypothesis that CvpA may act to subvert clathrin-coated vesicles toward the CCV to undergo fusion with the bacterial replicative vacuole.

More recently, we have discovered an additional T4SS effector involved in *C. burnetii* subversion of clathrin (221). The effector Cig57 interacts with FCHO2, an early-arriving component of clathrin-coated vesicles. *C. burnetii* lacking Cig57 also has an intracellular replication defect and does not induce CHC recruitment to the CCV. Further mutational analysis demonstrates that efficient recruitment of CHC to the CCV requires Cig57 to interact with FCHO2 (221). Mutation of an endocytic sorting motif, one of three encoded by Cig57, abolishes FCHO2 binding, and upon infection with this mutant, CHC enrichment on the CCV is reduced.

Hijacking FCHO2 may allow bacterial control of clathrin-coated vesicles. Cargo molecules and luminal content from clathrin vesicles would benefit *C. burnetii* growth should these vesicles fuse with the CCV and deliver their contents, which are often nutrients. Additionally, clathrin-coated vesicles may be a source of membrane to help expansion of the CCV. In contradiction with this hypothesis, it is well established that during CME, clathrin is rapidly stripped from vesicles upon release into the cytoplasm,

making it unlikely that clathrin-coated vesicles could retain the clathrin coat through travel to the CCV and fusion with this membrane.

Given the large arsenal of T4SS effectors encoded by *C. burnetii*, it is hypothesized that CvpA and Cig57 are not the only effectors to target clathrin-mediated trafficking. Identifying additional effectors that modulate clathrin as well as mechanistically uncovering the actions of CvpA and Cig57 will aid development of an understanding of how and why *C. burnetii* manipulates CHC.

Clathrin as a bacterial replication factor. As summarized above, CHC is enriched on the CCV membrane and is manipulated in a bacterium-dependent manner. Most importantly, CHC also facilitates efficient intracellular *C. burnetii* replication. During siRNA silencing of *CLTC* in HeLa cells, CCVs expand to less than one-third of the area seen under control conditions (221). These CCVs harbor a bacterial growth increase of only approximately 50% of that quantified for control cells over 4 days of infection. The importance of CHC for intracellular replication of *C. burnetii* may be linked to clathrin localization on the CCV. When either CvpA or Cig57 is disrupted on the *C. burnetii* genome, the bacteria are no longer able to replicate efficiently, which phenocopies what is seen when clathrin is depleted from cells (220, 221). Similarly, during infection with *C. burnetii* expressing a form of Cig57 which cannot bind FCHO2 and hence does not allow for clathrin recruitment at the CCV, growth of *C. burnetii* is attenuated also (221). These results suggest that recruitment of CHC to the CCV by these effectors is necessary for intracellular survival and success of *C. burnetii*. The important function that clathrin performs to allow bacterial growth may take place on the CCV membrane, although this remains to be confirmed.

If indeed clathrin-coated vesicles fuse with the CCV during *C. burnetii* infection, the delivery of the cargo may be of upmost importance for replication and CCV expansion. Iron, for example, both is internalized by CME and is a requirement for *C. burnetii* growth, and it could serve as the necessary factor delivered by clathrin activity (230, 231). However, iron acquisition is not likely to be the sole determinant in causing the drastic lack of replication in the absence of CHC, given that lysosomes which have recently undergone fusion with an autophagosome are already rich in iron (232–234). A relationship between specific clathrin cargo molecules and *C. burnetii* growth remains to be determined. Regardless, the benefit of clathrin-coated vesicles for *C. burnetii* intracellular replication is clear and is supported by a study which used high-content imaging to show that levels of internalized transferrin are higher in infected cells than in uninfected counterparts (235). Thus, the bacteria actively increase the rate of clathrin-coated vesicles entering the cell, presumably to benefit their survival.

It remains possible that clathrin aids the *C. burnetii* intracellular life cycle through its canonical activity, that is, endocytosis and creation of cargo-carrying clathrin-coated vesicles for fusion with the CCV. However, given the diversity of clathrin functions, it is also likely that clathrin performs nonendocytic tasks to support intracellular replication of *C. burnetii*. Indeed, the fact that the CCV, to which clathrin is recruited, is a modified lysosome is enough to spark an alternative view. Clathrin is not associated with lysosomes under steady-state conditions; however, CHC does colocalize with LAMP1-positive lysosomes upon autophagy induction (90). This work sparked our recent research which examined the role of CHC in the context of autophagy during *C. burnetii* infection (236). We demonstrated that CHC is required for fusion of autophagosomes with the CCV. In the absence of CHC, LC3B, a key autophagy marker, is not delivered to the CCV. LC3B is normally found in the CCV lumen and is thought to be transported there when autophagosomes fuse with the CCV (227, 237). We observed that clathrin-positive autophagosomes are directed to the CCV to enable both normal homotypic fusion of CCVs and CCV expansion. Thus, clathrin has a benefit apart from existing in clathrin-coated vesicles from the plasma membrane.

Clathrin is key to enabling the delivery of lysosomal proteins to the lysosome itself. Through the AP-1 pathway, lysosomal hydrolases are transported to and from endosomes, and with the aid of AP-3, lysosomal membrane proteins are transported to the lysosome. Hence, the correct lysosomal CCV composition and activity may be deter-

mined by clathrin trafficking. However, cellular AP-1 silencing does not result in reduced *C. burnetii* growth, and elimination of AP-2 produces a growth defect similar to that when *CLTC* is silenced, emphasizing that CME is still important (220). Additionally, FCHO2 is not involved in the autophagic role of clathrin, but FCHO2, presumably from the plasma membrane, is needed for efficient *C. burnetii* replication and CCV formation (221).

It is worth noting that all the work performed so far on clathrin and *C. burnetii* has used only tools for CHC. As described above, the different CHC isoforms and clathrin that act independently of a light chain or of a triskelion conformation may provide additional approaches to further examine the intricacies of the capacity in which clathrin acts during infection. The experiments so far conducted have also relied on siRNA experiments, which eliminate expression throughout the cell. This makes it impossible to distinguish whether any effects of clathrin removal are as a result of disruption of CME, *trans*-Golgi trafficking, mitosis, lysosomal composition, or any other pathway examined in this review in combination or alone. It is highly plausible that clathrin acts with multiple complex purposes during infection.

CONCLUSIONS

The diverse and numerous activities of clathrin in eukaryotic cell biology, from the plasma membrane to endosomes, autophagosomes, and the Golgi apparatus, provide an important target that bacterial pathogens can manipulate to their advantage. Pursuing these interactions will facilitate knowledge of specific host-pathogen interactions and inform our understanding of clathrin beyond infection conditions. Internalization of molecules at the plasma membrane, via CME, is a well-studied consequence of clathrin oligomerization. The discovery that clathrin is required for the engulfment of *L. monocytogenes* sparked the realization that molecules larger than 150 nm could be internalized with the aid of clathrin. Since these early discoveries, clathrin has been implicated in the success of multiple bacterial pathogens through processes distinct from internalization from the extracellular space at the plasma membrane.

In reviewing these noncanonical roles of clathrin during infection, general themes begin to emerge. Such is the case when considering the role of adaptor proteins in clathrin function during infection. In three instances highlighted in this review, infection with either *L. monocytogenes*, *S. flexneri*, or EPEC, AP-2 is not associated with the clathrin found at the plasma membrane, cellular protrusions, or actin pedestals, respectively. Thus, it is possible that the properties of clathrin, or its interaction partners, that are utilized at sites such as budding from the Golgi apparatus or endosomes (which do not require AP-2) may also be exhibited at the sites of infection stated above. It is interesting to contemplate whether the CHC22 isoform plays a larger part in the infections described above, given that it does not associate with AP-2. A full elucidation of the adaptors used by clathrin during infection with each pathogen may prove helpful.

It is interesting to consider whether the way each bacterium manipulates clathrin is reflective of a different way in which the cell itself utilizes clathrin. As highlighted throughout this review, clathrin takes multiple forms, either with or without a corresponding light chain and in two distinct heavy-chain isoforms. Despite the recruitment of clathrin light chains to the site of entry of *L. monocytogenes*, it was not determined whether the light chain is equally as important as CHC for internalization. The light chain has also not been studied in the context of other pathogens mentioned in this article.

The ability of clathrin to facilitate replication of intracellular bacterial pathogens is a more recent addition to our knowledge of clathrin during infection. As is the case with the actin pedestal formation and cell-to-cell spread, clathrin may be seen in these contexts as playing a structural role through exploiting its lattice-like capabilities. However, whether this is the case in contexts that require clathrin for intracellular bacterial replication remains unknown. CHC is enriched on CCVs during *C. burnetii* infection and thus may provide essential structural support for the considerable size

and bacterial burden of this replicative niche. However, recent findings indicating the essential part clathrin plays in delivery of autophagosomes to the CCV may signal a deeper, more complex mechanism at play.

Further research is required to expand our understanding of clathrin during infection. Identification of other bacterial pathogens, and indeed more generally other pathogenic microbes, which utilize clathrin will no doubt follow in the coming years. Though a challenging prospect, it will be of much interest to both bacteriologists and cell biologists to learn how clathrin functions differentially to enable bacterial replication compared to bacterial cell entry. Thus, to conclude, the diversity among clathrin activity in the cell and the complex orchestration of clathrin conducted by bacteria together create an intricate puzzle to keep scientific minds active for years to come.

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