Spiroplasma eriocheiris Enters *Drosophila* Schneider 2 Cells and Relies on Clathrin-Mediated Endocytosis and Macropinocytosis

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ABSTRACT *Spiroplasma eriocheiris* causes great economic losses in the crustacean aquaculture industry. However, the mechanism of *S. eriocheiris* infecting host cells has been poorly studied. We established a *Spiroplasma*-infected *Drosophila* Schneider 2 (S2) cell model and investigated its pathogenic mechanism. First, *S. eriocheiris* induced S2 cell apoptosis and necrosis, seriously decreased cell viability, and increased the production of intracellular reactive oxygen species. Further research showed that *S. eriocheiris* can invade S2 cells, and the number of copies of intracellular spiroplasmas is sharply increased by 12 h postinfection. In addition, *S. eriocheiris* can cause S2 cells to form typical inclusion bodies and exhibit large vacuoles. Second, *S. eriocheiris* is internalized into S2 cells and strongly inhibited through blocking clathrin-mediated endocytosis using chlorpromazine and dynasore. Inhibitors of macropinocytosis, protein kinase C and myosin II, cause a significant reduction in *S. eriocheiris* in S2 cells. In contrast, disruption of cellular cholesterol by methyl- β -cyclodextrin and nystatin has no effect on *S. eriocheiris* infection. These results suggest that the entry of *S. eriocheiris* into S2 cells relies on clathrin-dependent endocytosis and macropinocytosis, but not via the caveola-mediated endocytic pathway. In addition, the intracellular numbers of *S. eriocheiris* are dramatically reduced after S2 cells are treated with cytoskeleton-depolymerizing agents, including nocodazole and cytochalasin B. Thus, cellular infection by *S. eriocheiris* is related to microtubules and actin filaments. This research successfully shows for the first time that *S. eriocheiris* can invade *Drosophila* S2 cells and provides a process for *S. eriocheiris* infection.

KEYWORDS *Spiroplasma eriocheiris*, *Drosophila* S2 cells, clathrin-dependent endocytosis, macropinocytosis

Spiroplasma consists of a large number of small, wall-less, helical, motile prokaryotes that radiate to occupy multiple habitats (1). In addition to insect and plant hosts, isolations from ticks (2) and crustaceans (3) are beginning to advance our understanding of the host range (4). *Spiroplasma eriocheiris*, as the first *Spiroplasma* pathogen found in crustaceans (5, 6), was previously identified as a causative agent of *Eriocheir sinensis* tremor disease (TD). The biochemical and biological properties (7), evolutionary analysis (8), taxonomic identification (9, 10), and antibacterial testing (11) of *S. eriocheiris* have been previously documented.

In addition to crustaceans, *S. eriocheiris* infects chicken embryos and newborn mice (5, 12). Furthermore, the cellular molecular pathogenesis of *S. eriocheiris* was elucidated using an infection model of a mouse 3T6-Swiss leukemia cell line (3T6 cells). *S. eriocheiris* infection of 3T6 cells was characterized by inclusion bodies and distinct vacuolization (12). These *S. eriocheiris* inclusion bodies formed due to the proliferation of *S. eriocheiris* in the cell. *S. eriocheiris* could lead to cell rupture, with the release of

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large numbers of inclusion bodies. Because mammalian cells are distantly related to crustacean cells, the infection model of 3T6 cells is somewhat inadequate to elucidate the mechanism of *S. eriocheiris* infection in invertebrate host cells. However, thus far, no cell lines have been established in crustaceans. Finding a cell line closer to those of crustaceans would be a better solution. As far as we know, the *Drosophila* Schneider 2 (S2) cell line has been widely used in the study of invertebrate resistance to pathogens (13). S2 cells have multiple advantages for studying the process of host infection by pathogenic bacteria. First, the S2 cell line has been developed as part of a plasmid-based nonlytic integration system for the construction of stably transfected cell lines. Second, many kinds of flies have been identified as a major reservoir for *Spiroplasma* maintenance and dispersal (14). For example, a *Spiroplasma* isolate naturally infected *Drosophila melanogaster* (15). *Spiroplasma poulsonii*, a *Drosophila willistoni* pathogen, has been shown to cause sex ratio disorders (16, 17). *S. poulsonii* is a maternally transmitted bacterial endosymbiont that is naturally associated with several *Drosophila* species, affecting host metabolism, reproduction, and defense against parasites (18). *D. melanogaster* can be used as a model to unravel aspects of the mechanistic basis of endosymbiont-host immune interactions, which shows that *S. poulsonii* does not activate an immune response in *Drosophila* (19). In contrast to *S. poulsonii*, *S. eriocheiris* is nonendosymbiotic and can cause crab TD. Phylogenetic analysis of the 16S ribosomal DNA gene sequences of *S. eriocheiris* showed that this spiroplasma strain had a close (98%) similarity to *Spiroplasma mirum* (7). *S. eriocheiris* also could infect newborn mice and caused cataract (12). Third, S2 cells have been used as *in vitro* models to study *Mycoplasma* infections (20). The *Chlamydia trachomatis* S2 cell infection model could provide a bona fide system to dissect host functions important in the pathogenesis of obligate intracellular pathogens (20). Thus, the study of *S. eriocheiris* infection of S2 cell holds significant promise for elucidating infection mechanisms. Examining the pathway by which *S. eriocheiris* enters cells can greatly enhance our understanding of the pathogenicity of *S. eriocheiris* in crabs.

Many pathogens have been reported to enter cells in three ways: by clathrin-dependent endocytosis, by lipid raft/cavity-dependent pathways, and by macropinocytosis (21, 22). *S. eriocheiris* belongs to the smallest prokaryotic microorganism that can be cultured; it differs from bacteria in that it lacks a cell wall and is 50 to 200 nm in diameter, similar to viruses (7). Singapore grouper iridovirus infection of host cells has been shown to occur by a cell-dependent clathrin-mediated phagocytic pathway and by macropinocytosis (23). In the present study, we introduce an *in vitro* infection model of *S. eriocheiris* using *Drosophila* S2 cells. To investigate the *S. eriocheiris* infection mechanism, we examined the process of *S. eriocheiris* infection using different pharmacological inhibitors. Our results not only contribute greatly to understanding the natural infection mechanisms of *S. eriocheiris* in invertebrates but also provide a useful tool for exploring *S. eriocheiris* pathogenesis.

RESULTS

***S. eriocheiris* induced S2 cell morphological changes.** S2 cells inoculated with *S. eriocheiris* showed cytopathic effects by 0, 24, 48, and 72 h postinoculation. There was no significant change in morphology between the heat-killed *S. eriocheiris* infected S2 cells and the control group. Initially, the reproductive ability of S2 cells was significantly weakened under the influence of *S. eriocheiris*. The infected cells gradually shrunk and were surrounded by exudates or cell debris. Also, the cell morphology changed significantly in the *S. eriocheiris*-infected group (Fig. 1).

***S. eriocheiris* infection affects the normal growth of S2 cells.** A cell viability assay was used to determine the S2 cell state after *S. eriocheiris* inoculation. As shown in Fig. 2A, after treatment with *S. eriocheiris* for 12 h the cell viability was significantly lower than at 0 h. There was no significant difference in cell viability between the heat-killed *S. eriocheiris* group and the control group. With increasing infection time, the cell viability gradually decreased, demonstrating that the number of surviving cells is also decreasing. After 48 h of *S. eriocheiris* infection, the cell viability was approximately only

FIG 1 Morphology of S2 cells after heat-killed *S. eriocheiris* and *S. eriocheiris* infection. S2 cells were either not infected (control) or infected with *S. eriocheiris* after treatment with the pathogen at 0, 24, 48, and 72 h. A black arrow indicates cells surrounded by debris. Scale bar, 100 μ m.

50% compared to the level for the control group. The S2 cells infected with *S. eriocheiris* not only undergo morphological changes but also gradually weaken cell viability.

To observe the S2 cell apoptosis or necrosis, the *S. eriocheiris*-treated cells were monitored with the fluorescent dye propidium iodide (PI). The cells were stained at 12 h after infection with *S. eriocheiris*. The results show that the apoptotic or necrosis cells were significantly stained red after *S. eriocheiris* infection from 24 to 72 h. The number of dead S2 cells at 72 h is significantly greater than at 48 h (Fig. 2B).

Flow cytometry (FCM) analysis was used to determine whether *S. eriocheiris* induced S2 cell apoptosis or necrosis by double staining with fluorescein isothiocyanate (FITC)-annexin V and PI. The results showed that the proportion of viable cells in the *S. eriocheiris*-induced group was significantly reduced. Most of the cells were broken and damaged, and a small number of cells were in advanced stages of apoptosis and necrosis. These results showed that S2 cell apoptosis and necrosis significantly increased in the *S. eriocheiris*-treated group (Fig. 2C).

The caspase-3 activity was determined at 24 and 48 h after infection with heat-killed *S. eriocheiris* (NC), viable *S. eriocheiris*, and a positive control. In order to test the GreenNuc live cell caspase-3 activity assay kit, the caspase-3 reversible inhibitor Ac-DEVD-CHO was also tested. The results showed that compared to the control, an obvious apoptotic signal was detected at 48 h after *S. eriocheiris* infection. The effect of the inhibitor proved the reliability of the experiment (Fig. 3A). As shown in Fig. 3B, apoptotic cells were observed clearly under a fluorescence microscope (TI-S; Nikon Japan).

To further confirm the presence of apoptosis, the level of reactive oxygen species (ROS) was determined. The cells were stained with 2,7-dichlorofluorescein diacetate (DCFH-DA) to examine ROS production in the S2 cells by FCM (24). When the heat-killed *S. eriocheiris* stimulated cells, the ROS was not significantly changed. The ROS in the S2 cells was significantly increased after 3 h with *S. eriocheiris* treatment and peaked at 9 h. After 12 h, the ROS significantly decreased (Fig. 3C). These results indicated that the

FIG 2 Cell viability assay and assessment of S2 cell status. (A) Cell viability assay of S2 cells. (a) Cell viability assay of S2 cells without stimulation. (b and c) Cell viability assays of S2 cells after heat-killed *S. eriocheiris* (b) and *S. eriocheiris* (c) inoculation for 0, 12, 24, 36, 48, and 72 h. Different letters indicate significant differences ($P < 0.05$). The data are means $\pm$ the standard deviations (SD) of three independent experiments. (B) *S. eriocheiris*-infected S2 cells at 0, 12, 24, 36, 48 and 72 h. Apoptotic or necrotic cells were stained red by PI, and normal cells were not stained. Scale bar, 10 μ m. (C) S2 cells were stimulated with PBS (left) or *S. eriocheiris* (right) for 48 h. The apoptotic cells were labeled with FITC-annexin V and PI for 30 min and analyzed by FCM.

early stage of *S. eriocheiris* infection can increase intracellular oxidative stress to resist *S. eriocheiris* infection.

Altogether, these results indicate that a large number of *S. eriocheiris*-infected cells resulted in increased cytotoxicity and induced apoptosis, ultimately leading to cell necrosis until death.

Dynamics of *S. eriocheiris* entry into S2 cells. To directly detect whether the *S. eriocheiris* can enter S2 cells, *S. eriocheiris* was labeled with the green fluorescent dye Alexa 488. The results, obtained at approximately 48 h postinfection, showed the presence of large numbers of *S. eriocheiris* organisms in the S2 cells. *S. eriocheiris* organisms were scattered within the S2 cell interior after 48 h, as determined by confocal laser scanning microscopy (CLSM) (Fig. 4A).

Using absolute real-time PCR, the copy numbers of *S. eriocheiris* were assessed. *S. eriocheiris* could be detected at 3 h postinfection of S2 cells. Over time, the numbers of *S. eriocheiris* organisms gradually increased. After the cells had been infected with *S. eriocheiris* for 24 h, the copy numbers of *S. eriocheiris* increased dramatically. The copy

FIG 3 Fluorescence and ROS detection of cells after infection by *S. eriocheiris*. (A) The relative fluorescence activity was determined at 24 and 48 h postinfection with *S. eriocheiris*. (B) Apoptotic cells were observed clearly under fluorescence microscope. Scale bar, 100 μ m. (C) Effects of *S. eriocheiris* on S2 cell ROS generation. FCM detects DCF fluorescence to indicate the production of ROS in the S2 cells. Statistical significance is indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). The data are means $\pm$ the SD for three independent experiments.

number of *S. eriocheiris* exceeded 1.0×10^6 copies/ng total DNA by 36 h after infection. By 72 h postinvasion, the *S. eriocheiris* copy number exceeded 4.0×10^6 copies/ng (Fig. 4B).

S. eriocheiris can enter cells and multiply, but its fate is unclear. To determine the cytopathologic effect of *S. eriocheiris* on S2 cells, transmission electron microscopy (TEM) was used to visualize intracellular inclusion bodies at 48 h postinfection. Compared to the control, an early-phase inclusion body was observed in the cytoplasm. Numerous *S. eriocheiris* organisms were found in each inclusion body of infected S2 cells (Fig. 4C). Subsequently, when the intracellular pathogens had proliferated further, the S2 cells showed obvious vacuolization (Fig. 4C). Therefore, *S. eriocheiris* infects cells to form inclusion bodies and vacuoles, leading to the eventual death of the cells. However, the detailed mechanism for this process is still unclear.

S. eriocheiris entry into S2 cells by passive endocytosis was further verified. As shown in Fig. 5, the heat-killed *S. eriocheiris* labeled with FITC was able to enter S2 cells, demonstrating that there is a passive internalization process in *S. eriocheiris* infections.

***S. eriocheiris* invasion of S2 cells required clathrin-dependent endocytosis and macropinocytosis.** To determine the role of clathrin-mediated endocytosis in *S. eriocheiris* entry, chlorpromazine (CPZ) and dynasore were utilized to block endocytosis. As indicated in Fig. 6, different concentrations of the drug were dissolved in dimethyl sulfoxide (DMSO). DMSO was used as a positive control. Cells added to DMSO were infected with *S. eriocheiris* as a negative control (mock-treated cells). For 5, 10, and 20 μ M CPZ-treated cells, the cell viability was significantly higher than for the control (NC) (Fig. 6). Moreover, when the concentration of CPZ was $>5 \mu$ M, the copy number for *S. eriocheiris* in cells was significantly lower than that for the mock-treated cells. Similar to the CPZ group, the activity of S2 cells in the dynasore treatment group was significantly greater than in the negative-control group, and the number of *S. eriocheiris* organisms was also greatly reduced. Therefore, after treatment of cells with different concentrations of drug, the disruption of the cell clathrin-mediated endocytic pathway inhibited *S. eriocheiris* entry into cells. In short, *S. eriocheiris* requires a clathrin-dependent pathway to internalize into host cells.

In addition, caveola-mediated endocytosis was investigated by pretreatment with various concentrations of methyl- β -cyclodextrin (M β CD) or nystatin. When S2 cells

FIG 4 Dynamics of *S. eriocheiris* entry into S2 cells. (A) *S. eriocheiris* was labeled with the green fluorescent dye Alexa 488 (green). CLSM images of the experimental group at 48 h are shown. Scale bar, 10 μ m. (B) Absolute real-time PCR was conducted to detect the copy number of *S. eriocheiris* in S2 cells. The data are means $\pm$ the SD for three independent experiments. (C) TEM images of S2 cells infected with *S. eriocheiris* for 48 h and control cells. Magnifications: left, $\times 700$ (scale bar, 5 μ m); right, $\times 1,200$ (scale bar, 2 μ m). High-magnification images of inclusion bodies and major intracellular vacuolization are shown. The arrow indicates the location of the inclusion body where the *S. eriocheiris* gathers. N, nucleus; S, *Spiroplasma* inclusion bodies; C, vacuolization.

were treated with M β CD or nystatin, there was no significant change in cell viability or the number of *S. eriocheiris* organisms in cells. There were no obvious differences in the internalization of *S. eriocheiris* into S2 cells among M β CD-treated, nystatin-treated, or mock-treated cells (Fig. 7). Furthermore, S2 cell viability was not affected by M β CD or nystatin treatment, even at the highest concentrations (Fig. 7). The results clearly showed that *S. eriocheiris* invasion of S2 cells was independent of caveolin-mediated endocytosis.

To further test whether *S. eriocheiris* used macropinocytosis as an entry route into S2 cells, cells were incubated with myosin light chain kinase inhibitor (ML-7) or rottlerin.

FIG 5 Passive endocytosis of S2 cells by *S. eriocheiris*. The nuclei of cells were stained blue by DAPI (4',6'-diamidino-2-phenylindole), and the heat-killed *S. eriocheiris* was labeled green with FITC. Scale bar, 10 μ m.

As shown in Fig. 8, when cells were treated with different concentrations of ML-7, the cell viability was significantly higher than that of the NC group. The number of *S. eriocheiris* organisms in cell decreased with the increase in ML-7 concentration (Fig. 8A). When the cells were treated with rottlerin, cell viability increased significantly compared to the NC group. Meanwhile, the number of *S. eriocheiris* organisms in S2 cells significantly decreased after treatment with rottlerin (Fig. 8B). These results suggest that the macropinocytosis pathway is necessary for *S. eriocheiris* entry into S2 cells.

Cytoskeleton dependence of *S. eriocheiris* entry. To demonstrate the roles of microtubules/actin filaments during the intracellular transport of *S. eriocheiris*, inhibitors of cytoskeletal dynamics, including nocodazole and cytochalasin B, were used (25, 26).

FIG 6 *S. eriocheiris* was internalized into the host cells through a clathrin-dependent pathway. (A) Cells treated with different concentrations of CPZ (5, 10, and 20 μ M). (a) Cell viability was assessed by using CCK-8 after *S. eriocheiris* infection. Only DMSO was used as a positive control. Cells added to DMSO were infected with *S. eriocheiris* as a negative control. (b) The copy numbers of *S. eriocheiris* were determined by real-time PCR. Cells added to DMSO were infected with *S. eriocheiris* as a negative control. (B) Cells treated with different concentrations of dynasore (5, 10, and 15 μ M). (a) Cell viability was assessed by using CCK-8 after *S. eriocheiris* infection. Only DMSO was used as a positive control. Cells added to DMSO were infected with *S. eriocheiris* as a negative control. (b) The copy numbers of *S. eriocheiris* were obtained by real-time PCR. Cells added to DMSO were infected with *S. eriocheiris* as a negative control. Different letters indicate significant differences ($P < 0.05$). The data are means $\pm$ the SD of three independent experiments.

FIG 7 *S. eriocheiris* invasion of S2 cells was independent of caveolin-mediated endocytosis. (A) Cells treated with different concentrations of MβCD (25, 50, and 100 μM). (a) Cell viability was assessed by using CCK-8 after *S. eriocheiris* infection. Only DMSO was used as a positive control. Cells added to DMSO were infected with *S. eriocheiris* as a negative control. (b) The copy numbers of *S. eriocheiris* were obtained by real-time PCR. Cells added to DMSO were infected with *S. eriocheiris* as a negative control. (B) Cells treated with different concentrations of nystatin (5, 10, and 15 μM). (a) Cell viability was assessed by using CCK-8 after *S. eriocheiris* infection. Only DMSO was used as a positive control. Cells added to DMSO were infected with *S. eriocheiris* as a negative control. (b) The copy numbers of *S. eriocheiris* were obtained by real-time PCR. Cells added to DMSO were infected with *S. eriocheiris* as a negative control. Different letters indicate significant differences ($P < 0.05$). The data are means $\pm$ the SD of three independent experiments.

The addition of DMSO alone did not affect *S. eriocheiris* entry into S2 cells, whereas nocodazole (Fig. 9A) and cytochalasin B (Fig. 9B) markedly inhibited *S. eriocheiris* invasion and further increased S2 cell viability. Although the effect of different nocodazole concentrations on cell viability was not significant, the difference in *S. eriocheiris* copy numbers between high and low concentrations was significant. The results of treatment with different actin drugs suggest that *S. eriocheiris* could be transported into the cytoplasm along actin filaments and microtubules.

DISCUSSION

To control TD of *E. sinensis* caused by *S. eriocheiris*, the infection mechanism should be studied as the initial step. Hemocytes are the most important immune tissue of crustaceans. *S. eriocheiris* infects and proliferates in the hemocytes of crabs first and then transfers to the connective tissues of various organs along with the circulation, leading to a systemic infection (6, 7). *S. eriocheiris* is a special bacterium whose infection and proliferation in host cells are particularly important for pathogenesis. However, at present, no cell lines have been established in crustaceans. In our quest to improve the methodology and develop new culture-based protocols, we began by choosing from among available cultured cell lines to study the infection mechanism of *S. eriocheiris*. The infection model based on 3T6 cells is somewhat inadequate to elucidate the infection mechanism of *S. eriocheiris*. Finding a cell line closer to that of crustaceans would be a better solution. In the present study, *Drosophila* S2 cells were chosen to experimentally study the infection process of *S. eriocheiris* using cell culture techniques.

After challenge with *S. eriocheiris*, the reproductive capacity of S2 cells was signifi-

FIG 8 The macropinocytosis pathway is necessary for *S. eriocheiris* entry into S2 cells. (A) Cells treated with different concentrations of ML-7 (5, 10, and 20 μ M). (a) Cell viability was assessed by using CCK-8 after *S. eriocheiris* infection. Only DMSO was used as a positive control. Cells added to DMSO were infected with *S. eriocheiris* as a negative control. (b) The copy numbers of *S. eriocheiris* were obtained by real-time PCR. Cells added to DMSO were infected with *S. eriocheiris* as a negative control. (B) Cells treated with different concentrations of rottlerin (5, 10, and 20 μ M). (a) Cell viability was assessed by using CCK-8 after *S. eriocheiris* infection. Only DMSO was used as a positive control. Cells added to DMSO were infected with *S. eriocheiris* as a negative control. (b) The copy numbers of *S. eriocheiris* were obtained by real-time PCR. Cells added to DMSO were infected with *S. eriocheiris* as a negative control. Different letters indicate significant differences ($P < 0.05$). The data are means $\pm$ the SD of three independent experiments.

cantly reduced. As the infection progresses, the pathological effects of S2 cells become more pronounced. The same results, interestingly, were obtained when *S. eriocheiris* infected 3T6 cells. 3T6 cell morphology changed from a spindle or an elliptical shape to a circular and irregular shape, and cell death was clearly recorded after challenge with *S. eriocheiris* (12). FCM and fluorescence analyses showed that *S. eriocheiris* could induce S2 cell apoptosis, ultimately leading to cell necrosis until death. CCK-8 analyses (EnoGene Cell Counting Kit-8; Beyotime, China) also showed that S2 cell viability was decreased significantly. Meanwhile, dichlorofluorescein (DCF) fluorescence showed that the level of ROS in S2 cells increased significantly at an initial stage of *S. eriocheiris* infection. Under external stimuli such as bacteria or viruses, cells produced oxidative stress and generated a large amount of ROS, which promoted cell apoptosis, leading to the ultimate death of cells (27). Pathogen-infected cells promoted apoptosis and necrosis by increased mitochondrial ROS production (28). ROS in the cells were significantly enhanced to combat the invasion of pathogens but also excessively mediated cell apoptosis. During the initial infection stages, *S. eriocheiris* invasion of S2 cells was verified by CLSM. After internalization, the *S. eriocheiris* organisms were confronted with the challenge of reaching the destined intracellular infective site. After *S. eriocheiris* entry into the S2 cell, the *S. eriocheiris* copy numbers increased, and early inclusion bodies were observed in the cytoplasm. Subsequently, the intracellular pathogen further proliferated, and the S2 cells showed significant vacuolation. These pathological features are similar to those reported for 3T6 cells infected with *S. eriocheiris*, namely, the presence of an inclusion body and vacuoles (12). *S. eriocheiris* infection of crusta-

FIG 9 Cytoskeleton dependence of *S. eriocheiris* entry. (A) Cells treated with different concentrations of nocodazole (5, 10, and 15 μ M). (a) Cell viability was assessed by using CCK-8 after *S. eriocheiris* infection. Only DMSO was used as a positive control. Cells added to DMSO were infected with *S. eriocheiris* as a negative control. (b) The copy numbers of *S. eriocheiris* were obtained by real-time PCR. Cells added to DMSO were infected with *S. eriocheiris* as a negative control. (B) Cells treated with different concentrations of cytochalasin B (Cyto B) (5, 10, and 15 μ M). (a) Cell viability was assessed by using CCK-8 after *S. eriocheiris* infection. Only DMSO was used as a positive control. Cells added to DMSO were infected with *S. eriocheiris* as a negative control. (b) The copy numbers of *S. eriocheiris* were obtained by real-time PCR. Cells added to DMSO were infected with *S. eriocheiris* as a negative control. Different letters indicated significant differences ($P < 0.05$). The data are means $\pm$ the SD of three independent experiments.

ceans and of chicken embryos could also cause large numbers of cell inclusion bodies and cell rupture (5). Our results indicated that after *S. eriocheiris* infection of invertebrate cells, *S. eriocheiris* could proliferate in large numbers and cause significant pathological and superstructural changes. Finally, *S. eriocheiris* infection eventually led to cell rupture and the release of large numbers of bacteria, which could lead to massive infections of other normal cells.

S. eriocheiris could enter the S2 cells and caused damage by mass reproduction. Knowledge of *S. eriocheiris* entry pathways was therefore important for understanding pathogenesis and the development of effective drugs to block *S. eriocheiris* infection before entry, i.e., prior to endocytic uptake. To determine whether *S. eriocheiris* could be endocytosed into cells, we noted that the FITC-labeled heat-killed *S. eriocheiris* can enter the cell by endocytosis. In clathrin-mediated endocytosis, clathrin is assembled on the inner surface of the plasma membrane to form coated grooves, which are then recessed to form clathrin-coated vesicles containing invaded pathogens, including *S. eriocheiris*. CPZ is known to disrupt the assembly of clathrin-coated pits on cell membranes, and dynasore is a commonly used inhibitor of dynein, an essential cellular GTPase involved in scission of the nascent vesicle from the plasma membrane during endocytic formation (29, 30).

It has been reported that treatment with CPZ in cases of human immunodeficiency virus (HIV)-dependent clathrin-mediated endocytosis could lead to the efficient entry of HIV into HeLa cells (31). In addition, the percentage of Singapore grouper iridovirus-infected host cells, and the amount of viral protein synthesis, was dose dependently

reduced by CPZ treatment. Likewise, the entry of this virus into the host cell was clathrin mediated (23). Using dynasore to inhibit kinetic protein studies, it has been reported that *Escherichia coli*, *Staphylococcus aureus*, and *Listeria innocua* utilize a protein-dependent clathrin-mediated pathway for entry into cells (32). In our study, the percentage of *S. eriocheiris*-infected cells was significantly reduced in the presence of CPZ or dynasore. Therefore, we hypothesized that *S. eriocheiris* enters *Drosophila* S2 cells via a clathrin-mediated endocytosis.

The function of the small-recess protein-mediated endocytic pathway in *S. eriocheiris* entry into S2 cell was studied. In contrast to clathrin-mediated endocytosis, during caveola-mediated uptake, caveolin moves with caveola-derived vesicles to multiple interior compartments. Because caveolae are rich in cholesterol and sphingolipids, caveola-mediated endocytosis is sensitive to cholesterol-depleting reagents such as M β CD or nystatin (33). M β CD disrupted cholesterol-rich microdomains, whereas nystatin was a sterol-binding drug (34). The drug could destroy the cell membrane lipid rafts with M β CD or nystatin to prevent the caveola internalization pathway (35, 36). However, our data revealed no reduction in *S. eriocheiris* infection by M β CD or nystatin, suggesting that lipid rafts and caveolae are not involved in *S. eriocheiris* entry and indicating that caveola-mediated endocytosis is not a universal requirement for *S. eriocheiris* entry into host cells.

In addition to clathrin-mediated lipid raft endocytosis, there has recently been a growing interest in the role of macropinocytosis in the invasion of pathogens. Inhibitor analysis showed that protein kinase C (PKC) and functional actomyosin networks are essential for macropinocytosis in regulating cytoskeletal dynamics and motility (37). Macropinocytosis has been shown to be associated with the entry of viruses into mammalian cells, including infection by respiratory syncytial virus and human cytomegalovirus (38, 39). The inhibition of PKC activity by ML-7 inhibitor or rottlerin prohibited the Singapore grouper iridovirus from entering host cells (23). ML-7 is an inhibitor of smooth-muscle myosin-light-chain kinase, and rottlerin is described as specifically inhibiting PKC δ (40). In our study, inhibition of PKC activity by ML-7 or rottlerin affected the entry of *S. eriocheiris* into S2 cells, and this inhibition was dose dependent. Based on these results, we propose that *S. eriocheiris* enters host cells through macropinocytosis and clathrin-mediated endocytic pathways.

It has been reported that infective pathogens hijack the host's cellular transport system, including microtubules and actin-dependent transport (41, 42). For example, microtubules and actin filaments provided the tracks for the movement of the hepatitis C virus RNA replication complex to other regions in the cell (43). The behavior of microtubules and actin filaments in *Drosophila* embryos had also been studied (44). To demonstrate the role of microtubules and actin filaments in the intracellular transport of *S. eriocheiris*, the correlations between *S. eriocheiris* and microtubules and actin filaments were experimentally examined using the cytoskeletal inhibitors nocodazole and cytochalasin B. In our study, cytoskeletal inhibitors significantly reduced the number of *S. eriocheiris* organisms in cells and enhanced cell viability. These findings suggest that *S. eriocheiris* can be transported into the cytoplasm along actin filaments and microtubules.

In conclusion, our studies have clearly shown that *S. eriocheiris* could invade *Drosophila* S2 cells. *S. eriocheiris* induced increased ROS levels in S2 cells, resulting in cell apoptosis and necrosis and significantly reducing cell viability. Thus, the *Drosophila* S2 cell is a successful model for use in experimentally studying *S. eriocheiris* infections in crustaceans, such as when a large number of *S. eriocheiris* organisms propagate, eventually forming typical inclusion bodies and exhibiting cellular vacuolization. Our results show that *S. eriocheiris* enters host cells via clathrin-mediated endocytosis and macropinocytosis. Internalized *S. eriocheiris* could be transported into the cytoplasm along actin filaments and microtubules (Fig. 10). The results reported here for the first time demonstrate that *S. eriocheiris* can invade *Drosophila* S2 cells, providing an ideal model for exploring the behavior of *S. eriocheiris*, as well as infection-dependent, clathrin-mediated endocytosis and macropinocytosis.

FIG 10 Model entry route of *S. eriocheiris* into S2 cells. Internalization of *S. eriocheiris* occurs via clathrin-mediated endocytosis and macropinocytosis.

MATERIALS AND METHODS

S2 cell and *S. eriocheiris* culture. S2 cells were grown at 28°C in Corning flasks in Schneider medium (Sigma, UK) supplemented with 10% fetal bovine serum that has been heat inactivated at 56°C for 30 min and antibiotics (100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin). The *S. eriocheiris* strain used here was the standard strain TDA-040725-5T (CCTCC M 207170T; DSM 21848T), which was isolated and purified from *Eriocheir sinensis* exhibiting symptoms of TD (7) and cultured at 30°C with R2 medium (6, 45).

S2 cell morphology. When cultured S2 cells grew to >60% confluence, the cells were infected with heat-killed *S. eriocheiris* and viable *S. eriocheiris* at 10⁸ *S. eriocheiris* organisms ml⁻¹ in each of three replicate wells of a six-well plate at a multiplicity of infection (MOI) of 30. The morphology of the S2 cells was observed at different time points after *S. eriocheiris* infection (0, 24, 48, and 72 h) using an inverted fluorescence microscope (Nikon TI-S).

Assessment of cell status. To determine whether *S. eriocheiris* influences S2 cells, the cell viability was assessed by using an EnoGene Cell Counting Kit-8 (CCK-8; Beyotime, China) according to the manufacturer's instructions; the findings indirectly reflect the presence of viable cells. *Drosophila* S2 cell was cultivated using a 96-well plate with a final approximate 60% confluence and then infected by heat-killed *S. eriocheiris* and viable *S. eriocheiris* (10 µl, 10⁸ cells/ml, MOI = 30). At 0, 12, 24, 36, 48, and 72 h postinfection, 10 µl of CCK-8 was added, followed by incubation for 4 h at 28°C. Estimates of cell abundance, based on the absorbance at 450 nm, were then determined using a microplate reader (Bio Tek).

Drosophila S2 cells were seeded onto coverslips and grown in culture dishes until they achieved 60% confluence. Then, the S2 cells were infected by 200 µl of *S. eriocheiris* (10⁸ cells/ml) as the experimental groups, and untreated S2 cells served as the control group. The treated S2 cells were fixed using an Immunol staining fix solution (Beyotime) for 30 min, washed with phosphate-buffered saline (PBS), and incubated with PI (Beyotime). At 0, 12, 24, 36, 48, and 72 h postinfection, the S2 cells were imaged using an inverted fluorescence microscope (Nikon TI-S). Apoptotic cells were assessed by FCM using an annexin V-FITC apoptosis detection kit (BD) according to the manufacturer's instructions. *Drosophila* S2 cells were seeded onto six-well plates (experimental group, *S. eriocheiris* infected for 48 h; control group, normal cells) to achieve 60% confluence. The cells were then collected by centrifugation (2,000 × *g*, 3 min). After gentle mixing with 5 ml of FITC-annexin V and 5 ml of PI, the cells were collected by using a 200-mesh filter cloth and incubated in the dark at 4°C for 30 min prior to FCM analysis.

Caspase (cysteine-requiring aspartate protease) is a family of proteases that play an important role in the process of apoptosis. Caspase-3 belongs to the CED-3 subfamily of the caspase family and is a key enzyme in the process of apoptosis. The caspase-3 activity was determined by using a GreenNuc live cell caspase-3 activity assay kit (Beyotime) according to the manufacturer's instructions. After growth to more than 60% confluence, S2 cells were infected with *S. eriocheiris* (200 µl, 10⁸ cells/ml, MOI = 30). At 0, 24, and 48 h postinfection, the green fluorescence of the apoptotic cells was detected by using a fluores-

cence microscope (Nikon TI-S). The fluorescence intensity was detected at an excitation wavelength of 485 nm and an emission wavelength of 515 nm with a microplate reader (Bio Tek).

Reactive oxygen species measurement. The intracellular ROS were detected using 2,7-dichlorofluorescein diacetate (DCFH-DA) as a probe (46). Then, S2 cells were treated with heat-killed *S. eriocheiris* and viable *S. eriocheiris* (200 μ l, 10^8 cells/ml, MOI = 30) for 0, 1, 3, 6, 9, 12, 24, 36, 48, and 72 h with three replicates. ROS were detected with a reactive oxygen species assay kit (Beyotime) according to the manufacturer's instructions. Briefly, the treated cells were collected, washed with PBS, and incubated with 10 μ M DCFH-DA for 0.5 h at 30°C in the dark. The formation of the fluorescence-oxidized derivative of dichlorofluorescein (DCF) was monitored using a flow cytometer (BDFACVerse) at an emission wavelength of 525 nm and an excitation wavelength of 488 nm. The measurement was repeated three times. Finally, ROS generation was quantified by determining the median fluorescence intensity of 10,000 cells.

Immunofluorescence experiment. After experimental infection with *S. eriocheiris* for 48 h, S2 cells were collected and washed three times with PBS. Then, the S2 cells were fixed with Immunol staining fix solution (Beyotime), permeabilized with PBS containing 0.1% Triton X-100 for 30 min, and blocked with 3% bovine serum albumin in PBS for 30 min. Next, the cells were incubated with the polyclonal antibody against *S. eriocheiris* (1:1,000) overnight at 4°C, briefly washed with PBS, and immunoreacted with fluorescence secondary antibodies. The cells were then imaged using an A1R confocal microscope system (Nikon). To further verify that *S. eriocheiris* can enter cells by passive phagocytosis, heat-killed *S. eriocheiris* was labeled with FITC and microscopically examined under a fluorescence microscope (Nikon TI-S). S2 cells were infected with the labeled, heat-killed *S. eriocheiris* and observed using the A1R confocal microscope system. Fluorescence emission was collected and imaged using a 100 $\times$ oil immersion objective with a numerical aperture of 1.4.

Kinetics of *S. eriocheiris* infection. S2 cells were incubated with *S. eriocheiris* for 0, 1, 3, 6, 9, 12, 24, 36, 48, and 72 h at 28°C. To evaluate the infection rate of *S. eriocheiris* and determine the number of intracellular spiroplasmas in the S2 cells, the infected cells were collected by centrifugation at $2,000 \times g$ and 4°C for 3 min and then washed with PBS to ensure that the culture medium was removed and free of external, nonattached *S. eriocheiris*. To examine *S. eriocheiris* copies, the total DNA was extracted with an Easy Pure Genomic DNA kit (TransGen, China) and detected by absolute real-time PCR with the primer pair Se-QF and Se-QR (47). Absolute real-time PCR was performed with the reaction mixture, which was added to a volume dose of 10 μ l, including 5 μ l of 2 $\times$ SYBR Premix Ex Taq (TaKaRa, Japan), 0.4 μ l of forward primer, 0.4 μ l of reverse primer, 1 μ l of DNA template, 3 μ l of RNase-free water, and 0.2 μ l of ROX reference dye (TaKaRa, Japan).

Transmission electron microscopy. The *S. eriocheiris*-infected S2 cells were pre-embedded with 1% agarose gel (1 g of agarose powder, 100 ml of deionized water [pH 7.2]) and fixed with 4% glutaraldehyde (diluted with phosphate buffer [pH 7.2]) as a primary fixative, followed by 1% osmium tetroxide (diluted with phosphate buffer [pH 7.2]) fixation at 4°C for 2 h. The fixed cells were dehydrated through an ethanol series, followed by absolute acetone, and then embedded in Epon 812. The samples were sectioned with a Reichert-Jung ultramicrotome using a diamond knife, collected on Formvar-coated grids, and double stained with uranyl acetate and lead citrate before they were examined using an H-7650TEM (Hitachi, Japan).

***S. eriocheiris* endocytosis assays.** To analyze *S. eriocheiris* uptake, S2 cells were pretreated with the inhibitors listed below in serum-free medium at 28°C for 2 h. *S. eriocheiris* cells were then added to the medium in the presence of the drugs for 12 h at 28°C. The cells were harvested either to detect cell viability or to determine *S. eriocheiris* copies. Different concentrations of the inhibitors were dissolved in DMSO. Only DMSO was used as a positive control (PC). Cells added to DMSO were infected with *S. eriocheiris* as a negative control (NC; mock-treated cells). Methyl- β -cyclodextrin (M β CD) was obtained from Macklin (catalog no. M812850). Chlorpromazine (CPZ; catalog no. B1993), dynasore (A1605), nystatin (B6803), ML-7 (B1480), rottlerin (A3626), cytochalasin B (C4939), and nocodazole (A8487) were purchased from ApexBio. All drugs were dissolved in DMSO according to the manufacturer's instructions. The drug treatment effect is obvious and reliable.

Statistical analysis. All of the experiments were performed three times. Statistical data were analyzed using one-way analysis of variance, and results are presented as means $\pm$ the standard errors. Statistical significance is defined as a *P* value of <0.05. The figures in this study were prepared using GraphPad Prism v5.0 software.

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REFERENCES

1. Weisburg WG, Tully JG, Rose DL, Petzel JP, Oyaizu H, Yang D, Mandelco L, Sechrest J, Lawrence TG, Van Etten J. 1989. A phylogenetic analysis of the mycoplasmas: basis for their classification. *J Bacteriol* 171:6455–6467. <https://doi.org/10.1128/jb.171.12.6455-6467.1989>.
2. Halos L, Mavris M, Vourc'h G, Maillard R, Barnouin J, Boulouis H-J, Vayssier-Taussat M. 2006. Vayssier-Taussat, broad-range PCR-TTGE for the first-line detection of bacterial pathogen DNA in ticks. *Vet Res* 37:245–253. <https://doi.org/10.1051/vetres:2005055>.

3. Nunan LM, Pantoja CR, Salazar M, Aranguren F, Lightner DV. 2004. Characterization and molecular methods for detection of a novel spiroplasma pathogenic to *Penaeus vannamei*. *Dis Aquat Org* 62:255–264. <https://doi.org/10.3354/dao062255>.
4. Regassa LB, Gasparich GE. 2006. Spiroplasmas: evolutionary relationships and biodiversity. *Front Biosci* 11:2983–3002. <https://doi.org/10.2741/2027>.
5. Wang W, Rong L, Gu W, Du K, Chen J. 2003. Study on experimental infections of *Spiroplasma* from the Chinese mitten crab in crayfish, mice, and embryonated chickens. *Res Microbiol* 154:677–680. <https://doi.org/10.1016/j.resmic.2003.08.004>.
6. Wang W, Gu W, Gasparich GE, Bi K, Ou J, Meng Q, Liang T, Feng Q, Zhang J, Zhang Y. 2011. *Spiroplasma eriocheiris* sp. nov. associated with mortality in the Chinese mitten crab, *Eriocheir sinensis*. *Int J Syst Evol Microbiol* 61:703–708. <https://doi.org/10.1099/ijs.0.020529-0>.
7. Wang W, Wen B, Gasparich GE, Zhu N, Rong L, Chen J. 2004. A spiroplasma associated with tremor disease in the Chinese mitten crab (*Eriocheir sinensis*). *Microbiology* 150:3035–3040. <https://doi.org/10.1099/mic.0.26664-0>.
8. Bi K, Huang H, Gu W, Wang JH, Wang W. 2008. Phylogenetic analysis of spiroplasmas from three freshwater crustaceans (*Eriocheir sinensis*, *Procambarus clarkii*, and *Penaeus vannamei*) in China. *J Invertebr Pathol* 99:57–65. <https://doi.org/10.1016/j.jip.2008.06.008>.
9. Meng Q, Ou J, Ji H, Jiang X, Gu W, Wang W. 2010. Identification and characterization of spiral-like protein SLP25 from *Spiroplasma eriocheiris*. *Vet Microbiol* 144:473–477. <https://doi.org/10.1016/j.vetmic.2010.01.005>.
10. Zhang Y, Bao H, Miao F, Shen Y, He Y, Gu W, Meng Q, Wang W, Zhang J. 2013. Characterization of a monoclonal antibody to *Spiroplasma eriocheiris* and identification of a motif expressed by the pathogen. *Vet Microbiol* 161:353–358. <https://doi.org/10.1016/j.vetmic.2012.07.042>.
11. Liang T, Feng Q, Wu T, Gu W, Wang W. 2009. Use of oxytetracycline for the treatment of tremor disease in the Chinese mitten crab *Eriocheir sinensis*. *Dis Aquat Org* 84:243–250. <https://doi.org/10.3354/dao02052>.
12. Hou L, Gu W, Zhu H, Yao W, Wang W, Meng Q. 2017. *Spiroplasma eriocheiris* induces mouse 3T6-Swiss albino cell apoptosis that associated with the infection mechanism. *Mol Immunol* 91:75–85. <https://doi.org/10.1016/j.molimm.2017.08.002>.
13. Cheng LW, Portnoy DA. 2003. *Drosophila* S2 cells: an alternative infection model for *Listeria monocytogenes*. *Cell Microbiol* 5:875–885. <https://doi.org/10.1046/j.1462-5822.2003.00327.x>.
14. Clark TB. 1982. Spiroplasmas: diversity of arthropod reservoirs and host-parasite relationships. *Science* 217:57–59. <https://doi.org/10.1126/science.217.4554.57>.
15. Montenegro H, Solferini VN, Klaczko LB, Hurst GD. 2005. Male-killing *Spiroplasma* naturally infecting *Drosophila melanogaster*. *Insect Mol Biol* 14:281–287. <https://doi.org/10.1111/j.1365-2583.2005.00558.x>.
16. Poulson DF, Sakaguchi B. 1961. Nature of “sex-ratio” agent in *Drosophila*. *Science* 133:1489–1490. <https://doi.org/10.1126/science.133.3463.1489>.
17. Williamson DL, Sakaguchi B, Hackett KJ, Whitcomb RF, Tully JG, Carle P, Bove JM, Adams JR, Konai M, Henegar RB. 1999. *Spiroplasma poulsonii* sp. nov., a new species associated with male-lethality in *Drosophila willistoni*, a neotropical species of fruit fly. *Int J Syst Bacteriol* 49:611–618. <https://doi.org/10.1099/00207113-49-2-611>.
18. Masson F, Copete CS, Schüpfer F, Garcia-Arreaz G, Lemaître B. 2018. *In vitro* culture of the insect endosymbiont *Spiroplasma poulsonii* highlights bacterial genes involved in host-symbiont interaction. *mBio* 9:e00024-18. <https://doi.org/10.1128/mBio.00024-18>.
19. Herren JK, Lemaître B. 2011. *Spiroplasma* and host immunity: activation of humoral immune responses increases endosymbiont load and susceptibility to certain Gram-negative bacterial pathogens in *Drosophila melanogaster*. *Cell Microbiol* 13:1385–1396. <https://doi.org/10.1111/j.1462-5822.2011.01627.x>.
20. Elwell C, Engel JN. 2005. *Drosophila melanogaster* S2 cells: a model system to study *Chlamydia* interaction with host cells. *Cell Microbiol* 7:725–739. <https://doi.org/10.1111/j.1462-5822.2005.00508.x>.
21. Cossart P, Helenius A. 2014. Endocytosis of viruses and bacteria. *Cold Spring Harb Perspect Biol* 8:6–8. <https://doi.org/10.1101/cshperspect.a016972>.
22. Seisenberger G, Ried MU, Endress T, Büning H, Hallek M, Bräuchle C. 2001. Real-time single-molecule imaging of the infection pathway of an adeno-associated virus. *Science* 294:1929–1932. <https://doi.org/10.1126/science.1064103>.
23. Wang S, Huang X, Huang Y, Hao X, Xu H, Cai M, Wang H, Qin Q. 2014. Entry of a novel marine DNA virus, Singapore grouper iridovirus, into host cells occurs via clathrin-mediated endocytosis and macropinocytosis in a pH-dependent manner. *J Virol* 88:13047–13063. <https://doi.org/10.1128/JVI.01744-14>.
24. Zhou Y-J, Zhang S-P, Liu C-W, Cai Y-Q. 2009. The protection of selenium on ROS mediated-apoptosis by mitochondria dysfunction in cadmium-induced LLC-PK(1) cells. *Toxicol In Vitro* 23:288–294. <https://doi.org/10.1016/j.tiv.2008.12.009>.
25. Luduena RF, Roach MC. 1991. Tubulin sulfhydryl groups as probes and targets for antimicrotubule agents. *Pharmacol Ther* 49:133–152. [https://doi.org/10.1016/0163-7258\(91\)90027-J](https://doi.org/10.1016/0163-7258(91)90027-J).
26. Cooper JA. 1987. Effects of cytochalasin and phalloidin on actin. *J Cell Biol* 105:1473–1478. <https://doi.org/10.1083/jcb.105.4.1473>.
27. Samali A, Nordgren H, Zhivotovsky B, Peterson E, Orrenius S. 1999. A comparative study of apoptosis and necrosis in HepG2 cells: oxidant-induced caspase inactivation leads to necrosis. *Biochem Biophys Res Commun* 255:6–11. <https://doi.org/10.1006/bbrc.1998.0139>.
28. Lee C-W, Yen F-L, Ko H-H, Li S-Y, Chiang Y-C, Lee M-H, Tsai M-H, Hsu L-F. 2017. Cudraflavone C induces apoptosis of A375.S2 melanoma cells through mitochondrial ROS production and MAPK activation. *Int J Mol Sci* 18:1508–1515. <https://doi.org/10.3390/ijms18071508>.
29. Wang H, Liu WS, Yu F, Lu LQ. 2016. Disruption of clathrin-dependent trafficking results in the failure of grass carp reovirus cellular entry. *J Virol* 90:13–10. <https://doi.org/10.1186/s12985-016-D485-7>.
30. Chen R-Y, Shen K-L, Chen Z, Fan W-W, Xie X-L, Meng C, Chang X-J, Zheng L-B, Jeswin J, Li C-H, Wang K-J, Liu H-P. 2016. White spot syndrome virus entry is dependent on multiple endocytic routes and strongly facilitated by Cq-GABARAP in a CME-dependent manner. *Sci Rep* 6:28694. <https://doi.org/10.1038/srep28694>.
31. Daecke J, Fackler OT, Dittmar MT, Kräusslich HG. 2005. Involvement of clathrin-mediated endocytosis in human immunodeficiency virus type 1 entry. *J Virol* 79:1581–1594. <https://doi.org/10.1128/JVI.79.3.1581-1594.2005>.
32. Veiga E, Guttman JA, Bonazzi M, Boucrot E, Toledo-Arana A, Lin AE, Enninga J, Pizarro-Cerdá J, Finlay BB, Kirchhausen T, Cossart P. 2007. Invasive and adherent bacterial pathogens co-opt host clathrin for infection. *Cell Host Microbe* 2:340–351. <https://doi.org/10.1016/j.chom.2007.10.001>.
33. Nabi IR, Le PU. 2003. Caveolae/raft-dependent endocytosis. *J Cell Biol* 161:673–677. <https://doi.org/10.1083/jcb.200302028>.
34. Pietiäinen V, Marjomäki V, Upla P, Pelkmans L, Helenius A, Hyypia T. 2004. Echovirus 1 endocytosis into caveosomes requires lipid rafts, dynamin II, and signaling events. *Mol Biol Cell* 15:4911–4925. <https://doi.org/10.1091/mbc.e04-01-0070>.
35. Bruno H, Covadonga A. 2010. Dynamin- and clathrin-dependent endocytosis in African swine fever virus entry. *J Virol* 84:2100–2109. <https://doi.org/10.1128/JVI.01557-09>.
36. Shao L, Zhao JR, Zhang HQ. 2016. Spring viraemia of carp virus enters grass carp ovary cells via clathrin-mediated endocytosis and macropinocytosis. *J Gen Virol* 97:2824–2836. <https://doi.org/10.1099/jgv.0.000595>.
37. Dharmawardhane S, Schürmann A, Sells MA, Chernoff J, Schmid SL, Bokoch GM. 2000. Regulation of macropinocytosis by p21-activated kinase-1. *Mol Biol Cell* 11:3341–3352. <https://doi.org/10.1091/mbc.11.10.3341>.
38. Krzyżaniak MA, Zumstein MT, Gerez JA, Picotti P, Helenius A. 2013. Host cell entry of respiratory syncytial virus involves macropinocytosis followed by proteolytic activation of the F protein. *PLoS Pathog* 9:e1003309. <https://doi.org/10.1371/journal.ppat.1003309>.
39. Haspot F, Lavault A, Sinzger C, Laib Sampaio K, Stierhof Y-D, Pilet P, Bressolette-Bodin C, Halary F. 2012. Human cytomegalovirus entry into dendritic cells occurs via a macropinocytosis-like pathway in a pH-independent and cholesterol-dependent manner. *PLoS One* 7:e34795. <https://doi.org/10.1371/journal.pone.0034795>.
40. Gschwendt M, Müller HJ, Kielbassa K, Zang R, Kittstein W, Rincke G, Marks F. 1994. Rottlerin, a novel protein kinase inhibitor. *Biochem Biophys Res Commun* 199:93–98. <https://doi.org/10.1006/bbrc.1994.1199>.
41. Sever S, Damke H, Schmid SL. 2000. Dynamin: GTP controls the formation of constricted coated pits, the rate limiting step in clathrin-mediated endocytosis. *J Cell Biol* 150:1137–1148. <https://doi.org/10.1083/jcb.150.5.1137>.
42. Brandenburg B, Lee LY, Lakadamyali M, Rust MJ, Zhuang X, Hogle JM. 2007. Imaging poliovirus entry in live cells. *PLoS Biol* 5:e183. <https://doi.org/10.1371/journal.pbio.0050183>.
43. Lai CK, Jeng KS, Machida K, Lai MM. 2008. Association of hepatitis C virus replication complexes with microtubules and actin filaments is dependent on

- dent on the interaction of NS3 and NS5A. *J Virol* 82:8838–8848. <https://doi.org/10.1128/JVI.00398-08>.
44. Kellogg DR, Mitchison TJ, Alberts BM. 1988. Behaviour of microtubules and actin filaments in living *Drosophila* embryos. *Development* 103: 675–686.
45. Moulder RW, French FE, Chang CJ. 2002. Simplified media for spiroplasmas associated with tabanid flies. *Can J Microbiol* 48:1–6. <https://doi.org/10.1139/w01-128>.
46. Wang H, Joseph JA. 1999. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radic Biol Med* 27: 612–616. [https://doi.org/10.1016/S0891-5849\(99\)00107-0](https://doi.org/10.1016/S0891-5849(99)00107-0).
47. Ding Z, Tang J, Xue H, Li J, Ren Q, Gu W, Meng Q, Wang W. 2014. Quantitative detection and proliferation dynamics of a novel *Spiroplasma eriocheiris* pathogen in the freshwater crayfish, *Procambarus clarkia*. *J Invertebr Pathol* 115:51–54. <https://doi.org/10.1016/j.jip.2013.10.012>.