

Bordetella parapertussis Survives inside Human Macrophages in Lipid Raft-Enriched Phagosomes

Juan Gorgojo,^a Eric T. Harvill,^b Maria Eugenia Rodríguez^a

CINDEFI (UNLP CONICET La Plata), Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina^a; Department of Veterinary and Biomedical Science, The Pennsylvania State University, University Park, Pennsylvania, USA^b

***Bordetella parapertussis* is a human pathogen that causes whooping cough. The increasing incidence of *B. parapertussis* has been attributed to the lack of cross protection induced by pertussis vaccines. It was previously shown that *B. parapertussis* is able to avoid bacterial killing by polymorphonuclear leukocytes (PMN) if specific opsonic antibodies are not present at the site of interaction. Here, we evaluated the outcome of *B. parapertussis* innate interaction with human macrophages, a less aggressive type of cell and a known reservoir of many persistent pathogens. The results showed that in the absence of opsonins, O antigen allows *B. parapertussis* to inhibit phagolysosomal fusion and to remain alive inside macrophages. The O antigen targets *B. parapertussis* to lipid rafts that are retained in the membrane of phagosomes that do not undergo lysosomal maturation. Forty-eight hours after infection, wild-type *B. parapertussis* bacteria but not the O antigen-deficient mutants were found colocalizing with lipid rafts and alive in nonacidic compartments. Taken together, our data suggest that in the absence of opsonic antibodies, *B. parapertussis* survives inside macrophages by preventing phagolysosomal maturation in a lipid raft- and O antigen-dependent manner. Two days after infection, about 15% of macrophages were found loaded with live bacteria inside flotillin-enriched phagosomes that had access to nutrients provided by the host cell recycling pathway, suggesting the development of an intracellular infection. IgG opsonization drastically changed this interaction, inducing efficient bacterial killing. These results highlight the need for *B. parapertussis* opsonic antibodies to induce bacterial clearance and prevent the eventual establishment of cellular reservoirs of this pathogen.**

B*ordetella parapertussis* and *Bordetella pertussis* are human pathogens that cause whooping cough, a reemerging disease that remains a threat to human health. Despite high vaccination coverage, whooping cough is still endemic. Current clinical surveys indicate that *B. parapertussis* is responsible for a significant number of cases of whooping cough, particularly in vaccinated populations (1–5). The switch from whole-cell to acellular vaccines is associated with a significant increase in the prevalence of *B. parapertussis* in the epidemiology of the disease (6, 7). Several studies have demonstrated that pertussis acellular vaccines fail to protect against *B. parapertussis* (6, 8). The lack of cross protection was mainly attributed to the presence of the O antigen on the surface of *B. parapertussis*, which blocks antibody access to the vaccine antigens common to both species (9, 10). *In vitro* studies confirmed that pertussis acellular vaccines induce antibodies that opsonize *B. pertussis* but not *B. parapertussis* (9, 10). In the absence of opsonic antibodies, *B. parapertussis* survives neutrophil phagocytosis by preventing lysosomal maturation in a lipid raft-dependent manner (11). O antigen is involved in this nonbactericidal interaction, mediating the targeting of host cell lipid rafts. Several intracellular pathogens hijack host rafts to create sheltered environments that prevent bactericidal activity. In particular, persistent bacteria, such as *Mycobacteria* spp., *Brucella* spp., and *Legionella pneumophila*, were found to generate phagosomes enriched in raft components which avoid the degradative pathway, allowing long-term bacterial survival inside the host cell (12–18). The establishment of an intracellular niche provides a significant survival advantage to these pathogens.

The ability of *B. parapertussis* to avoid the bactericidal activity of polymorphonuclear leukocytes (PMN) in the nonimmune host is likely to contribute to the infectious process, but PMN are unlikely cells for the establishment of intracellular infections. Many

facultative intracellular bacteria, *B. pertussis* among them (19), survive inside macrophages, immune cells that are less aggressive and live longer than PMN in the human body. Macrophages have both a primary role in innate immunity and a role in adaptive immunity. Their ability to influence the immune response makes them a central determinant of the course of an infection. Intracellular microbes are poised to affect macrophage functions that can profoundly influence host immune response (20). In the present study, we examined the interaction between human macrophages and *B. parapertussis* in order to investigate whether this pathogen is also able to survive encountering this other cell type and persist in a viable state for an extended period of time. We identified a critical role of the O antigen in *B. parapertussis* survival against macrophage phagocytosis and a critical role of the Fc receptor (FcR) in the promotion of cellular bactericidal activity against *B. parapertussis*. We also observed a novel association between flotillin and intracellular *B. parapertussis* cells at times long after infection and presumably linked to bacterial intracellular survival. These findings have important implications for our understanding of how this pathogen avoids immune clearance to persist within the infected host.

Received 25 August 2014 Returned for modification 16 September 2014

Accepted 23 September 2014

Published ahead of print 29 September 2014

Editor: R. P. Morrison

Address correspondence to Maria Eugenia Rodríguez, mer@quimica.unlp.edu.ar.

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doi:10.1128/IAI.02553-14

MATERIALS AND METHODS

Bacterial strains and growth. *B. parapertussis* strain CN2591 and a previously described isogenic *B. parapertussis* mutant strain lacking the O antigen, CN2591 Δ wbm (21, 22), were used in this study. For phagocytosis experiments, these strains were transformed with plasmid pCW505 (kindly supplied by Alison Weiss, Cincinnati, OH), which induces cytoplasmic expression of green fluorescent protein (GFP) without affecting growth or antigen expression (23). Bacteria were stored at -70°C and recovered by growth on Bordet-Gengou agar (BGA) plates supplemented with 15% defibrinated sheep blood (bBGA) at 36°C . Bacteria were subsequently plated on bBGA, cultured for 20 h at 36°C , and used in all experiments.

Cells. Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated by Ficoll-Paque (GE Healthcare, Uppsala, Sweden) gradient centrifugation as previously described (24). The mononuclear cell layer was washed and suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% inactivated autologous normal human serum, added to tissue culture plates (2×10^6 cells/ml), and incubated for 2 h at 37°C in 5% CO_2 . Nonadherent cells were then removed by gentle washing (three times with DMEM plus 10% inactivated autologous normal human serum), and adherent cells were cultured for 6 more days in DMEM plus 10% inactivated autologous normal human serum, as previously described (19), prior to infection.

Antibodies. The following antibodies were used: polyclonal rabbit antibody against human flotillin-1 (Santa Cruz Biotechnology, Santa Cruz, CA), Cy3-conjugated goat F(ab')₂ fragments of anti-rabbit immunoglobulin and fluorescein isothiocyanate (FITC)-conjugated goat F(ab')₂ fragments of anti-rabbit immunoglobulin (both from Jackson ImmunoResearch, West Grove, PA), and Cy3-conjugated goat F(ab')₂ fragments of anti-mouse immunoglobulin and FITC-conjugated goat F(ab')₂ fragments of anti-mouse immunoglobulin (from Southern Biotechnology). Immunoglobulin G (IgG) fractions from pooled sera of whooping cough patients with high titers of anti-*B. parapertussis* antibodies, as measured by enzyme-linked immunosorbent assay (25), were isolated as described previously (26). Polyclonal rabbit anti-*B. parapertussis* antiserum was obtained as described elsewhere (27).

Macrophage cholesterol sequestration. Before infection, cholesterol sequestration was achieved by incubating macrophages with 1, 3.5, or 10 mg/ml of methyl- β -cyclodextrin (15 min at 37°C) or 8, 17.5, or 35 $\mu\text{g}/\text{ml}$ of nystatin (30 min at 37°C) in serum-free DMEM plus 0.2% bovine serum albumin and 5 $\mu\text{g}/\text{ml}$ lovastatin (DMEM-BSA-L) (methyl- β -cyclodextrin, nystatin, BSA, and lovastatin were all from Sigma). Cells were then washed and used immediately. Infection was performed in DMEM-BSA-L to avoid restoration of membrane cholesterol levels due to *de novo* synthesis. No decrease in macrophage viability was detected after treatment.

Macrophage treatment with vacuolar-pH-neutralizing reagent. Neutralization of vacuolar pH was performed by incubating macrophages with bafilomycin A1 (BAF) (Sigma), an inhibitor of vacuolar v-ATPase. One hour before infection, 100 nM BAF was added to adherent differentiated macrophages. BAF was maintained during the assay. In control experiments, the effect of BAF on phagosomal pH was investigated by incubating *B. parapertussis*-infected macrophages treated with or without BAF with LysoTracker red DND-99 (Molecular Probes). No LysoTracker-positive organelles were observed in BAF-treated macrophages, as assessed by confocal microscopy. There was no toxic effect of BAF on *B. parapertussis* or macrophages, as determined by CFU counts and trypan blue dye exclusion, respectively.

Quantification of *B. parapertussis* attachment and phagocytosis. Bacterial attachment to and phagocytosis by macrophages were evaluated by double staining and fluorescence microscopy. Macrophages were infected with opsonized or nonopsonized GFP-expressing *B. parapertussis* bacteria suspended in DMEM plus 0.2% BSA at a multiplicity of infection (MOI) of 30 bacteria per cell. The bacterial inoculum was quantified by CFU counts. To facilitate bacterial interaction with macrophages, plates

were centrifuged for 5 min at $640 \times g$. After 40 min of incubation at 37°C with 5% CO_2 , nonadherent bacteria were removed by three washing steps prior to fixation with 4% paraformaldehyde for 10 min. After fixation, macrophages were washed once with phosphate-buffered saline (PBS) and incubated for 10 min at room temperature with PBS containing 50 mM NH_4Cl . Macrophage surface-bound and phagocytosed bacteria were discriminated by using a two-step labeling procedure and fluorescence microscopy. For this purpose, surface-bound bacteria were evidenced by incubation with polyclonal rabbit anti-*B. parapertussis* antiserum (30 min at 4°C), followed by incubation with Cy3-conjugated goat F(ab')₂ fragments of anti-rabbit immunoglobulin (30 min at 4°C). In order to determine the number of intracellular bacteria, after two washing steps, the cells were permeabilized by incubation with PBS containing 0.1% saponin (Sigma) and 0.2% BSA (30 min at 25°C) and further incubated with rabbit anti-*B. parapertussis* antiserum in the presence of 0.1% saponin and 0.2% BSA (30 min at 25°C). After washing three times, the macrophages were incubated with FITC-conjugated F(ab')₂ fragments of goat anti-rabbit immunoglobulin (30 min at 25°C). Labeling of the bacteria with FITC-conjugated antibodies was performed to minimize the loss of read-out sensitivity due to the quenching of GFP fluorescence after internalization. After washing with distilled water, coverslips were mounted on microscope slides and analyzed by fluorescence microscopy using a DMLB microscope coupled to a DC100 camera (Leica Microscopy Systems Ltd., Heerbrugg, Switzerland). The numbers of extracellular (red and green) and intracellular (green) bacteria were evaluated by examination of at least 100 macrophages. All experiments were carried out at least three times in triplicate.

Confocal microscopy analysis. Colocalization studies were performed as described before (19), with minor modifications. Briefly, macrophages incubated with GFP-expressing *B. parapertussis* at 37°C for 40 min were washed to remove nonattached bacteria and further incubated with 100 $\mu\text{g}/\text{ml}$ polymyxin B for 1 h at 37°C to kill extracellular non-phagocytosed bacteria. Next, the concentration of polymyxin B was reduced to 5 $\mu\text{g}/\text{ml}$. At 2 and 48 h postinfection, macrophage samples were incubated with or without 200 nM LysoTracker DND-99 (5 min at 37°C) prior to fixation with paraformaldehyde. Those samples that were incubated with LysoTracker stain were washed twice with phosphate-buffered saline (PBS) and incubated for 10 min at room temperature with PBS containing 50 mM NH_4Cl . After two washing steps, the cells were incubated for 30 min with PBS containing 0.1% saponin and 0.2% BSA, followed by incubation with polyclonal rabbit anti-*B. parapertussis* antiserum (30 min at 25°C) in the presence of 0.1% saponin and 0.2% BSA. After three washing steps, macrophages were incubated with FITC-conjugated goat F(ab')₂ fragments of anti-rabbit immunoglobulin (30 min at 25°C). Samples of infected cells that were not incubated with LysoTracker stain were washed twice with PBS and incubated for 10 min at room temperature with PBS containing 50 mM NH_4Cl . After two washing steps, the cells were incubated for 30 min with PBS containing 0.1% saponin and 0.2% BSA. Next, the cells were incubated with rabbit anti-human flotillin polyclonal antibodies plus polyclonal mouse anti-*B. parapertussis* antiserum (30 min at 4°C) in the presence of 0.1% saponin and 0.2% BSA. After three washing steps, macrophages were incubated with Cy3-conjugated F(ab')₂ fragments of goat anti-rabbit antibodies plus FITC-conjugated goat F(ab')₂ fragments of anti-mouse immunoglobulin for another 30 min at 4°C . To avoid cytophilic binding of antibodies to Fc γ R, all incubations were done in the presence of 25% heat-inactivated human serum. Additionally, isotype controls were run in parallel. Microscopic analyses were performed using a confocal laser scanning microscope (Leica TCS SP5; Leica, Germany). The percentage of phagosomes containing bacteria that colocalized with a given marker was calculated by analyzing at least 50 phagosomes per sample.

Killing assay. Macrophages were infected with *B. parapertussis* at an MOI of 30 as described above. The bacterial inoculum was quantified by plating appropriate dilutions on bBGA. After 40 min of incubation at 37°C with 5% CO_2 , nonadherent bacteria were removed through three

washing steps. Then, 100 $\mu\text{g/ml}$ polymyxin B sulfate (Sigma) was added for 1 h to kill the extracellular bacteria. Thereafter, the antibiotic concentration was decreased to 5 $\mu\text{g/ml}$. Control experiments showed that in the presence of 5 $\mu\text{g/ml}$ polymyxin B, *B. paraptentussis* bacteria were neither able to replicate nor to remain alive in the culture medium. The numbers of CFU in the cell culture supernatants were examined, and no viable bacteria were detected at any time postinfection.

The macrophages were washed, and intracellular survival of *B. paraptentussis* was determined at 2 and 48 h postinfection as follows. After washing with PBS, monolayers were incubated for 15 min at 37°C with PBS containing 5 mM EDTA and 4 mg/ml lidocaine to promote macrophage detachment, and cells were scraped immediately. The number of viable eukaryotic cells was determined by trypan blue dye exclusion. Next, macrophages were lysed with 0.1% saponin in sterile water and serial dilutions were rapidly plated onto bBGA plates to enumerate CFU. Control experiments to determine the numbers of phagocytosed bacteria 40 min postinfection were run in parallel to be used to calculate the percentages of phagocytosed bacteria that were still alive 2 and 48 h after phagocytosis. Bacterial phagocytosis was determined by fluorescence microscopy as previously described. Control experiments were run in parallel to assess the efficacy of the bactericidal activity of polymyxin B (100 $\mu\text{g/ml}$). Briefly, samples of 5×10^8 bacteria were incubated with the antibiotic for 1 h at 37°C and plated on bBGA. This resulted in a 99.999% decrease in the CFU. No significant differences in bacterial sensitivity to the antibiotics were detected among the strains used in this study.

In selected experiments, GFP-expressing *B. paraptentussis* was opsonized with human IgG (200 $\mu\text{g/ml}$) for 30 min at 37°C prior to incubation with macrophages.

Fluorescent *in situ* hybridization staining of intracellular live bacteria. Infected macrophages were fixed with 4% paraformaldehyde. Hybridization with fluorescently labeled oligonucleotides was done as described previously (28). Alexa Fluor 488-conjugated DNA probes BET42a (5'-GCCTTCCACTTCGTTT-3') and EUB338 (5'-GCTGCCCTCCGT AGGAGT-3') were designed for rRNA labeling of the betaproteobacteria and eubacteria, respectively (29). As a negative control, an Alexa Fluor 488-conjugated non-EUB338 DNA probe complementary to EUB338 was used. Bacterial detectability depends on the presence of enough ribosomes per cell, thus providing information on the viability of the bacteria based on the amount of ribosomes per cell.

BET42a and EUB338 were used together to get a stronger signal. Both probes were added to a final concentration of 10 nM in the hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, pH 7.4, 0.01% SDS, and 35% formamide). Next, the hybridization was allowed to occur for 1.5 h at 46°C in a humid chamber. The cells were then washed for 30 min with buffer (80 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.01% SDS, and 5 mM EDTA, pH 8) at 48°C. The total number of intracellular bacteria (both live and dead) was determined by immune staining using polyclonal mouse anti-*B. paraptentussis* antiserum followed by Cy3-conjugated F(ab')₂ fragments of goat anti-mouse antibodies as described above. Samples were mounted onto glass slides. Fluorescent *in situ* hybridization labeling of free bacteria incubated with polymyxin B did not give any detectable signal.

Transferrin uptake. Transferrin uptake by macrophages was assayed as described before (30), with minor modifications. Briefly, infected macrophages were depleted of transferrin by incubation in DMEM containing 1% BSA for 1 h at 37°C and further incubated for 10 min at 4°C with 10 $\mu\text{g/ml}$ Alexa Fluor 594-labeled transferrin (Molecular Probes) in the presence of BSA (1%) to saturate the capacity for nonspecific endocytosis. Next, cells were incubated for 5 min at 37°C to allow internalization of the ligand, washed with DMEM containing 1% BSA, and further incubated for another 45 min at 37°C. Finally, the cells were fixed and microscopic analyses were performed using a confocal laser scanning microscope (Leica TCS SP5; Leica, Germany). At least 50 bacteria per sample were analyzed for colocalization with transferrin in each experiment.

Statistical analysis. Student's *t* test (95% confidence level) or analysis of variance (ANOVA) was used for statistical evaluation of data. The significance of the differences between the mean values of the data evaluated by ANOVA was determined with the least-significant-difference (LSD) test at a 95% confidence level. Results are shown as means and standard deviations (SD).

RESULTS

Previous studies have shown that *B. paraptentussis* survives innate interaction with human PMN (11). Here, we investigated the outcome of *B. paraptentussis* encounters with macrophages either in the presence or the absence of opsonic antibodies. IgG-opsonized or nonopsonized *B. paraptentussis* bacteria were added to adherent human macrophages at an MOI of 30. After 40 min of incubation, the number of macrophage-associated bacteria was determined by fluorescence microscopy. The results in Fig. 1A show that IgG opsonization significantly increased bacterial association with the immune cells. In order to quantify bacterial phagocytosis under each condition, the numbers of intracellular and extracellular bacteria were determined by double immunofluorescence staining. As can be seen from the results in Fig. 1B, in the absence of opsonins, around 59% of the macrophage-associated bacteria were phagocytosed, while under opsonic conditions, this percentage went up to 80%. These results indicate that IgG opsonization increased both the attachment and phagocytosis of *B. paraptentussis*.

Phagocytosis does not necessarily result in bacterial killing. We therefore investigated bactericidal activity upon phagocytosis. After an initial infection period of 40 min, macrophages were extensively washed and split into two samples. One sample was used to determine the number of phagocytosed bacteria by two-color staining and fluorescence microscopy. The other sample was further incubated with polymyxin B to kill extracellular bacteria and determine the counts of viable intracellular bacteria at different time points. The results in Fig. 1C show the percentages of phagocytosed bacteria that were still alive 2 and 48 h postinfection under the different conditions tested. In the absence of opsonic antibodies, a substantial proportion of *B. paraptentussis* bacteria remained alive within macrophages 48 h after infection. In the presence of antibodies, bacterial survival decreased significantly within the first 2 h and was almost absent by 48 h (>99% decrease) (Fig. 1C), suggesting that the intracellular fate of *B. paraptentussis* depends on the macrophage receptor(s) targeted during the initial bacterium-host cell interaction. The O antigen was previously implicated in the survival of *B. paraptentussis* upon interaction with human PMN (11). In order to determine the relevance of O antigen to *B. paraptentussis* survival in its innate interaction with macrophages, we evaluated the outcome of macrophage interaction with an O antigen-deficient mutant of *B. paraptentussis*. The lack of the O antigen resulted in a significant decrease in bacterial survival immediately after phagocytosis, and no viable bacteria were found 48 h postinfection (Fig. 1C), suggesting that O antigen plays a critical role in *B. paraptentussis* intracellular survival within macrophages.

In order to evaluate bacterial distribution within macrophages and the proportion of infected cells, double staining and fluorescence microscopy were used to determine both the number of intracellular bacteria per cell and the proportion of macrophages containing intracellular bacteria. Nonopsonized *B. paraptentussis* bacteria were still visible within macrophages 48 h after infection (Fig. 2). Moreover, 14.2% of the macrophages contained more than 10 intracellular bacteria at this time point. Fluorescent *in situ*

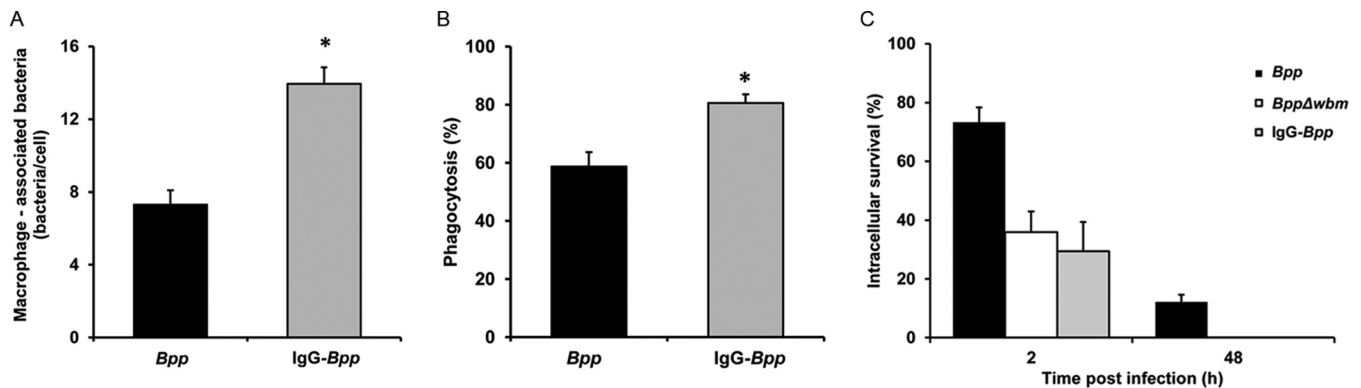


FIG 1 *Bordetella paraptussis* phagocytosis by human macrophages and intracellular survival. (A) Wild-type *B. paraptussis* (*Bpp*) and IgG-opsonized *B. paraptussis* (IgG-*Bpp*) bacteria were incubated with macrophages (MOI of 30) for 40 min at 37°C. The cells were washed, fixed, and permeabilized, and extracellular and intracellular bacteria were quantified by immunofluorescence staining (green fluorescent dye). The numbers of macrophage-associated bacteria were determined by fluorescence microscopy. At least 100 cells were counted per slide. The data represent the means \pm SD of the results of three independent experiments. The number of IgG-opsonized *B. paraptussis* bacteria associated with macrophages was significantly different from the number of nonopsonized *B. paraptussis* bacteria (*, $P < 0.05$). (B) Wild-type *B. paraptussis* (*Bpp*) and IgG-opsonized *B. paraptussis* (IgG-*Bpp*) bacteria were incubated with macrophages (MOI of 30) for 40 min at 37°C. The cells were fixed and permeabilized prior to labeling the intracellular bacteria with green fluorescent dye and the extracellular bacteria with both green and red fluorescent dyes. The numbers of phagocytosed bacteria were determined by fluorescence microscopy. At least 100 cells were counted per slide. Phagocytosis was expressed as the percentage of associated bacteria that were internalized. The data represent the means \pm SD of the results of three experiments with macrophages from different donors. The level of phagocytosis of nonopsonized *B. paraptussis* bacteria by macrophages was significantly different from the macrophage phagocytosis of IgG-opsonized *B. paraptussis* bacteria (*, $P < 0.05$). (C) Nonopsonized *B. paraptussis* (*Bpp*), nonopsonized *B. paraptussis* O antigen-deficient mutant (*Bpp* Δ *wbm*), or IgG-opsonized *B. paraptussis* (IgG-*Bpp*) bacteria were incubated with human macrophages (MOI of 30) for 40 min at 37°C, washed extensively, and further incubated with polymyxin B to kill the extracellular bacteria. The numbers of CFU of *B. paraptussis* per cell were determined at 2 and 48 h postinfection. Bacterial survival was expressed as the percentages of intracellular bacteria at 40 min postinfection that were still alive 2 and 48 h after phagocytosis. The data represent the means \pm SD of the results of three independent experiments. The numbers of viable intracellular nonopsonized *B. paraptussis* bacteria per cell at 2 and 48 h postphagocytosis were significantly different from the numbers of viable intracellular opsonized *B. paraptussis* and O antigen-deficient *B. paraptussis* mutant bacteria at the respective time points ($P < 0.05$).

hybridization for live bacteria revealed that around 78% of the nonopsonized intracellular bacteria were alive 48 h after infection (Fig. 2). Conversely, the O antigen-deficient mutant was found alive intracellularly only at early time points. Importantly, *B. paraptussis* intracellular infections did not affect macrophage viability as assessed by propidium iodide staining of the cells (31).

Since our results revealed an involvement of the O side chain in bacterial survival upon macrophage phagocytosis, we next investigated whether the O antigen affects intracellular trafficking to avoid bactericidal mechanisms, as found before for other bacterial pathogens (32–34). To this end, bacterial colocalization with the acidotropic dye LysoTracker was investigated at different time points postinfection. The results in Fig. 3 show that 2 h after infection, around 65% of bacteria of the nonopsonized wild-type strain of *B. paraptussis* were in nonacidic compartments. Bacteria of the *B. paraptussis* O antigen-deficient mutant strain showed a significantly ($P < 0.05$) higher level of colocalization with the acidotropic dye by this early time point. By 48 h, the different intracellular trafficking of these two strains was evidenced by the difference in the percentages of bacteria found in LysoTracker-negative phagosomes. While around 73% of the wild-type bacteria were found in nonacidic compartments, no O antigen-deficient *B. paraptussis* bacteria were found in LysoTracker-negative compartments, suggesting that the O antigen is involved in the ability of *B. paraptussis* to prevent phagolysosome maturation. The intracellular fate of *B. paraptussis* was modified by bacterial opsonization with specific IgG antibodies. The images in Fig. 3A show that opsonized bacteria were already mainly transported to acidic compartments by 2 h postinfection, indicating that the ability to prevent phagolysosome maturation was abrogated by targeting the bacteria to Fc γ receptors.

In order to investigate the importance of the acidification of *B. paraptussis*-containing phagosomes in the bactericidal activity of the macrophages against this pathogen, bafilomycin A1 (BAF), an inhibitor of vacuolar ATPase that blocks the acidification of the late endosomal/lysosomal compartments, was used. BAF had no toxic effect on *B. paraptussis*, as assessed by CFU counts (data not shown). One hour before infection, the macrophages were treated with or without BAF. The drug was present during the whole experimental period. The numbers of viable intracellular bacteria 2 and 48 h after infection were determined by fluorescent *in situ* hybridization of live bacteria. In control experiments, the inhibition of vacuolar acidification was confirmed at every time point by the lack of accumulation of the acidotropic dye LysoTracker in the intracellular compartments (data not shown). The number of viable intracellular bacteria increased in BAF-treated macrophages both at early and late time points (3.1- and 6.8-fold at 2 and 48 h postinfection, respectively), demonstrating that the phagosome acidification is required for macrophages to efficiently kill *B. paraptussis*.

Taken together, our results suggest that *B. paraptussis* bacteria remain alive inside the macrophages by preventing phagolysosomal maturation. In order to further investigate this issue, the localization of live bacteria inside the macrophages was determined by fluorescent *in situ* hybridization staining for viable bacteria. Colocalization studies with LysoTracker dye demonstrated that 48 h after infection, the viable intracellular bacteria were those that did not colocalize with the LysoTracker dye (Fig. 4).

Previous studies have demonstrated that *B. paraptussis* O antigen interaction with cholesterol-rich domains of PMN is critical to avoid the lysosomal pathway and bacterial killing (11). Since *B. paraptussis* O antigen was involved in the nonbactericidal traf-

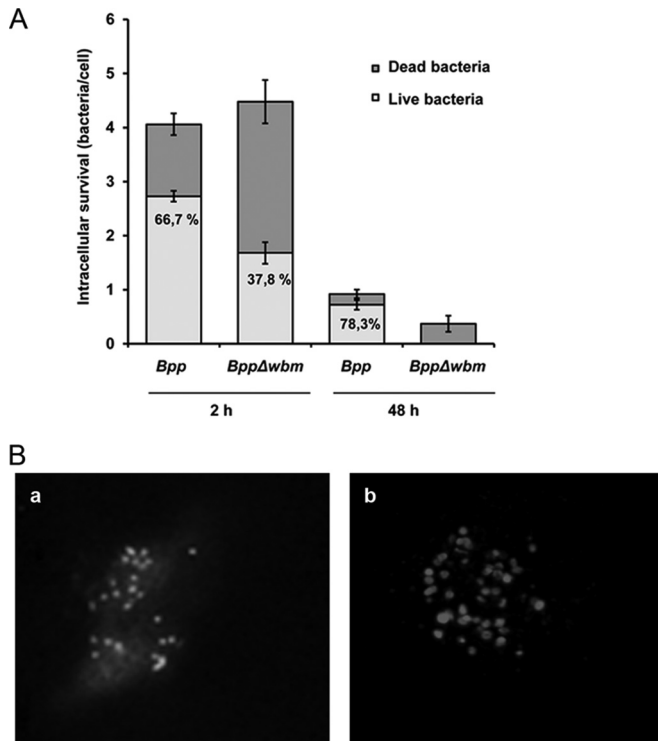


FIG 2 Distribution of intracellular live and dead *B. paraptentussis* bacteria at different times during infection. (A) Nonopsonized *B. paraptentussis* (*Bpp*) and nonopsonized *B. paraptentussis* O antigen-deficient mutant (*BppΔwbm*) bacteria were incubated with human macrophages (MOI of 30) for 40 min at 37°C, washed extensively, and further incubated with polymyxin B to kill the extracellular bacteria. After fixation, the numbers of live and dead *B. paraptentussis* bacteria per cell were determined at 2 and 48 h postinfection by fluorescent *in situ* hybridization for live bacteria and immune staining for live and dead bacteria. The intracellular bacteria were quantified by confocal fluorescence microscopy. At least 100 cells were counted per slide. The data represent the means \pm SD of the results of three experiments with macrophages from different donors. The percentages of live intracellular bacteria are indicated in the bars. (B) Representative confocal microscopy images of macrophages 48 h postinfection. Live intracellular bacteria are seen in panel a, and both live and dead bacteria are seen in panel b. Representative confocal microscopy images from one out of three independent experiments are shown.

ficking inside macrophages, we next investigated the role of cholesterol-rich domains in the innate interaction of *B. paraptentussis* with these immune cells. To this end, before bacterial infection, macrophages were treated with methyl- β -cyclodextrin, a compound that disrupts cholesterol-rich domains by removing cholesterol from the plasma membrane, and lovastatin, a pharmacological inhibitor of cholesterol synthesis. The nonopsonized wild type and an O antigen-deficient mutant of *B. paraptentussis* were used to infect treated and untreated macrophages at an MOI of 30. After 40 min of infection, the cells were washed and the numbers of intracellular and extracellular bacteria were determined by double immunofluorescence staining. Depletion of plasma membrane cholesterol led to a significant ($P < 0.05$) decrease in the phagocytosis level of the nonopsonized *B. paraptentussis* (Fig. 5). Conversely, phagocytosis of nonopsonized *B. paraptentussis* lacking the expression of the O antigen was not changed significantly by cholesterol depletion of the immune cells. Together, these data indicate that the O antigen is involved in *B. paraptentussis* uptake by macrophages via a mechanism involving cholesterol-rich do-

main. Despite its different mode of action, similar results were obtained with nystatin, another lipid raft-disrupting drug that binds cholesterol (data not shown). Importantly, none of these molecules had cytotoxic effects on macrophages, as demonstrated by propidium iodide staining of the cells (data not shown). The ability of cholesterol-depleted macrophages to phagocytose opsonized *B. paraptentussis* was analyzed in parallel. Opsonization was previously found to direct *B. paraptentussis* through lipid raft-independent pathways in PMN. The results in Fig. 5 show that the cholesterol-depleting drug had no effect on bacterial uptake by macrophages when *B. paraptentussis* was opsonized, indicating that lipid rafts are not involved in macrophage phagocytosis of antibody-opsonized bacteria and that macrophage phagocytosis of *B. paraptentussis* through docking sites other than lipid rafts was not affected by cholesterol depletion.

To further investigate the involvement of the O antigen in directing *B. paraptentussis* bacteria to macrophage lipid rafts, we used a lipid raft marker, flotillin, and confocal microscopy. Whereas in uninfected macrophages, flotillin could be seen distributed between the plasma membrane and the intracellular stores, infection of macrophages with the wild type but not with the O antigen-deficient mutant of *B. paraptentussis* resulted in the relocation and accumulation of flotillin around the bacteria (Fig. 6). As can be seen from the results in Fig. 6, at 2 h postinfection, around 60% of the wild-type bacteria but not of the O antigen-deficient *B. paraptentussis* mutants were found colocalizing with flotillin-enriched areas. Importantly, this percentage did not vary significantly 48 h postinfection, indicating that *B. paraptentussis* not only hijacked host lipid rafts but also retained them in the phagosome membrane inside the cell over time, which might be important in the intracellular fate of these bacteria.

Since our results suggested that *B. paraptentussis* can avoid bactericidal intracellular trafficking and remain alive inside the host cell, we next investigated whether *B. paraptentussis*-containing phagosomes have access to essential nutrients, a prerequisite for prolonged intracellular survival. To that end, at 48 h postinfection, macrophages were pulsed with Alexa Fluor 594-transferrin to assess the access of *B. paraptentussis*-containing phagosomes to extracellular nutrients. The image in Fig. 7A shows that *B. paraptentussis* O antigen-deficient mutants do not colocalize with Alexa Fluor 594-transferrin. At this time postinfection, only a few O-antigen-deficient mutant bacteria were still seen inside macrophages and none of them were found colocalizing with transferrin. Conversely, the image in Fig. 7B clearly shows colocalization of wild-type *B. paraptentussis* with exogenous transferrin at 48 h after infection, suggesting that intracellular bacteria have access to essential nutrients, as expected for a pathogen with long-term intracellular persistence.

DISCUSSION

Many persistent pathogens that have historically been considered extracellular, such as *Staphylococcus aureus*, *Helicobacter pylori*, *Campylobacter jejuni*, and group A streptococci, among others, were found to have one or more intracellular phases of different characteristics that play a role in the induction of disease, immune evasion, and persistence (35–38). Some of them were found to be able to duplicate within host cells, establishing significant intracellular infections, while others, like *Campylobacter jejuni*, may not duplicate inside the cells but still critically depend on their intracellular phases to establish persistent infections. *B. paraper-*

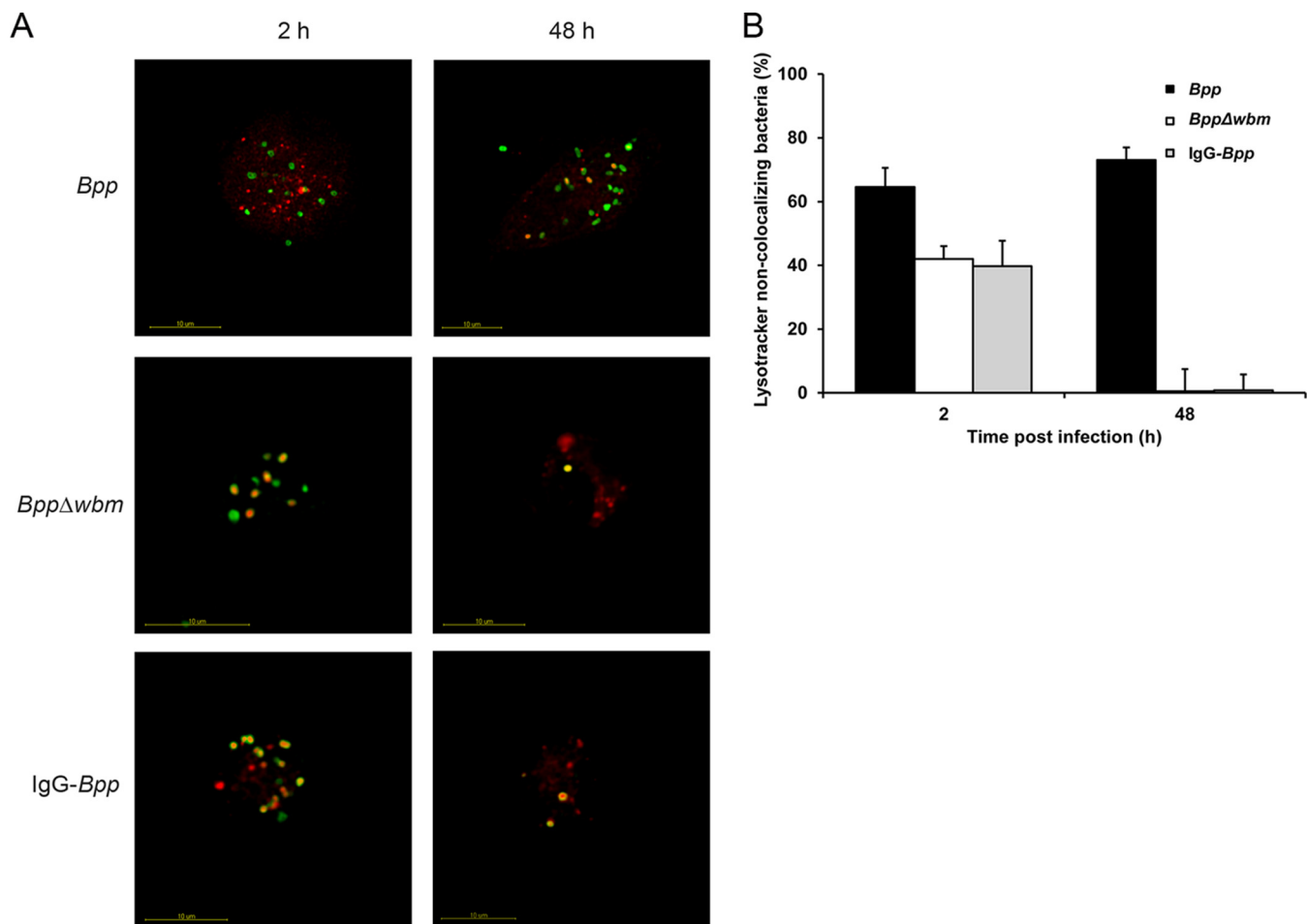


FIG 3 Time course of *B. parapertussis* colocalization with the acidotropic dye LysoTracker. Nonopsonized *B. parapertussis* (*Bpp*), nonopsonized *B. parapertussis* O antigen-deficient mutant (*Bpp* Δ *wbm*), or IgG-opsonized *B. parapertussis* (IgG-*Bpp*) bacteria were incubated with human macrophages (MOI of 30) for 40 min at 37°C. After washing, cells were incubated with polymyxin B to kill the extracellular bacteria. Samples were taken at 2 and 48 h postinfection, incubated with LysoTracker, and fixed. Intracellular bacteria were green fluorescently labeled prior to confocal microscopic analysis. (A) Confocal microscopy images of macrophages at 2 and 48 h postinfection. Colocalization is reflected by the yellow areas. Representative images from one out of three independent experiments are shown. (B) The bars indicate percentages of bacteria that are not colocalized with LysoTracker. The data represent the means \pm SD of the results of three independent experiments.

tussis, as well as *B. pertussis*, were generally considered extracellular bacteria. Both species are strictly human and persistent pathogens that could not be eradicated despite decades of vaccination. Recent studies support the hypothesis that *B. pertussis* is indeed a facultative intracellular bacterium (19). Early studies have suggested that *B. parapertussis* might also hide inside host cells (11, 39), eventually establishing asymptomatic infections. Clinical data have also challenged the assumption that *B. parapertussis* is strictly extracellular. The source of infection in isolated cases in which there is no obvious contact with symptomatic individuals raised questions about how *B. parapertussis* persists within the population. Reported cases of bacteremia and death (40–42) caused by *B. parapertussis* both in immunocompromised and immunocompetent human hosts also argue against a purely extracellular behavior. Also pointing in this direction, recent studies have shown the importance of adaptive immunity to restrain *B. parapertussis* to the lung lumen and the role of the O antigen in *B. parapertussis* invasive behavior in the mouse model (43).

Current whooping cough vaccines do not induce antibodies

against *B. parapertussis* (6, 8, 9, 44, 45). The lipopolysaccharide (LPS) O antigen was found to be implicated in the lack of protection of pertussis vaccines against this pathogen by interfering with the binding of antibodies induced by vaccine antigens common to both species (9, 10). The O antigen is a key molecule in *B. parapertussis* host defense evasion. It not only precludes opsonization by antibodies induced by pertussis vaccines (9) but is also involved in the nonbactericidal interaction of *B. parapertussis* with PMN. In the absence of opsonic antibodies, *B. parapertussis* survives the encounter with neutrophils by impairing both cell activation and phagolysosomal maturation, which eventually leads to bacterial survival (11). PMN constitute the first line of host defense against bacterial infections. Bacteria that avoid this defensive barrier can still be cleared by other host defense cells, such as macrophages. Macrophages, which exhibit a different set of bactericidal mechanisms than PMN, are also key factors of the innate immune response against invading microorganisms. Several pathogens, however, have evolved strategies to subvert the bactericidal activity of the macrophages, establishing persistent intracellular infec-

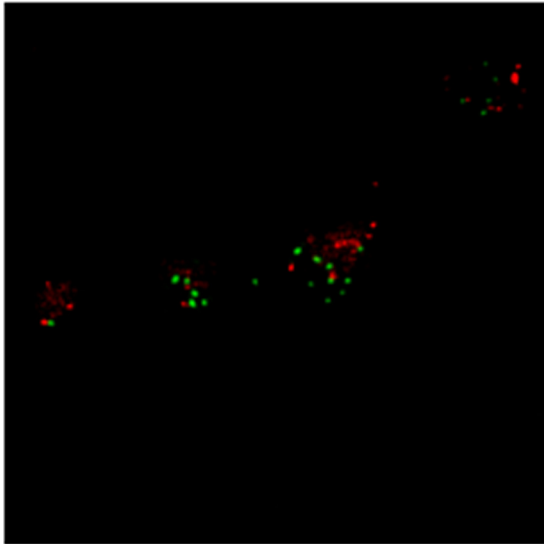


FIG 4 Intracellular live *B. paraptentis* bacteria do not colocalize with the acidotropic dye LysoTracker. Nonopsonized *B. paraptentis* bacteria were incubated with human macrophages (MOI of 30) for 40 min at 37°C, washed extensively, and further incubated with polymyxin B to kill the extracellular bacteria. Samples were taken at 48 h and further incubated with LysoTracker (red), fixed, stained for live *B. paraptentis* bacteria by fluorescent *in situ* hybridization (green), and subjected to confocal microscopic analysis. Colocalization is reflected by yellow areas. A representative image from one of three independent experiments is shown.

tions (46). Bacteria that remain alive in these types of long-lived cells might hide from the host antimicrobial defense system, reach other areas or other hosts, or even repopulate the extracellular medium once the environmental conditions are favorable. Moreover, they might manipulate the host immune response.

We investigated whether *B. paraptentis* is able to survive encounters with these kinds of cells. We examined intracellular survival over time postphagocytosis, bacterial trafficking inside the cell, and the importance of specific antibodies in the outcome of

this interaction. To this end, we used macrophages differentiated from primary human blood monocytes, which are reported to be a good surrogate for human alveolar macrophages (47, 48), cells that are likely to interact with *B. paraptentis* at the site of infection. Although alveolar macrophages do not derive from blood monocytes in nature, macrophages differentiated from monocytes have a number of characteristics that makes them resemble these tissue macrophages. Using this experimental setup, we found that in the absence of specific antibodies, this pathogen is able to remain alive for days inside human macrophages. Unlike what was observed in PMN, we found that macrophages managed to kill a high proportion of bacteria right after phagocytosis but a significant number of internalized bacteria remained viable within the macrophages. Considering that most of the population lacks antibodies against this pathogen, this observation becomes rather significant. In this study, we used a fluorescent probe specific for viable bacteria to be able to image their intracellular location. By means of this experimental setup, we could not only determine that live bacteria were outside the lysosomes long after infection but also gain insight into the distribution of viable bacteria among the macrophages. Although many macrophages had cleared intracellular *B. paraptentis* 2 days after infection, around 14.2% of the macrophages showed high numbers of viable intracellular bacteria residing in nonacidic compartments. Since the extracellular medium was bactericidal, the high numbers of live bacteria in these macrophages cannot be explained by newly phagocytosed bacteria. These results might, then, be consistent with intracellular replication of *B. paraptentis*. The number of intracellular viable bacteria might indeed be a balance between entry, intracellular growth, bacterial death, and exit from cells. Bacterial survival and eventual duplication within a cell rely on the ability both to avoid the degradative pathway and to obtain nutrients inside the cell. We found in the work presented here that 48 h postinfection, *B. paraptentis*-containing phagosomes acquired exogenously added transferrin, indicating that *B. paraptentis* resides in compartments with early endosomal characteristics in which it has

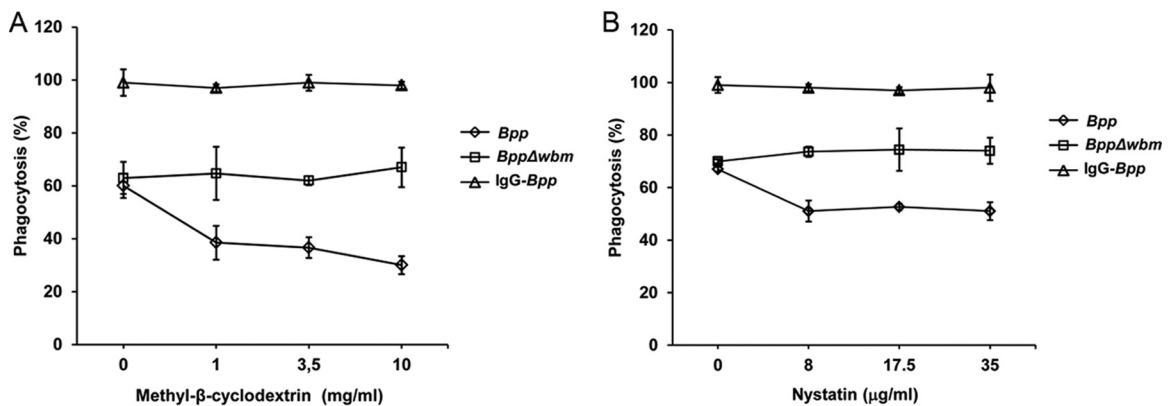


FIG 5 Effects of methyl-β-cyclodextrin and nystatin on macrophage phagocytosis of *B. paraptentis*. Human macrophages were treated with or without methyl-β-cyclodextrin (1, 3.5, or 10 mg/ml) (A) or nystatin (8, 17.5, or 35 mg/ml) (B) prior to incubation with IgG-opsonized *B. paraptentis* (IgG-Bpp), nonopsonized *B. paraptentis* (Bpp), or nonopsonized *B. paraptentis* O antigen-deficient mutant (*BppΔwbm*) bacteria (MOI of 30) for 40 min at 37°C to allow phagocytosis. The cells were fixed and permeabilized prior to labeling the intracellular bacteria with green fluorescent dye and the extracellular bacteria with both green and red fluorescent dyes. To assess the number of phagocytosed bacteria, at least 100 cells per slide were counted. Phagocytosis was expressed as the percentage of associated bacteria that were internalized. The data represent the means \pm SD of the results of four experiments with macrophages from different donors. Phagocytosis of nonopsonized *B. paraptentis* by methyl-β-cyclodextrin-treated macrophages was significantly different from phagocytosis by untreated macrophages ($P < 0.05$).

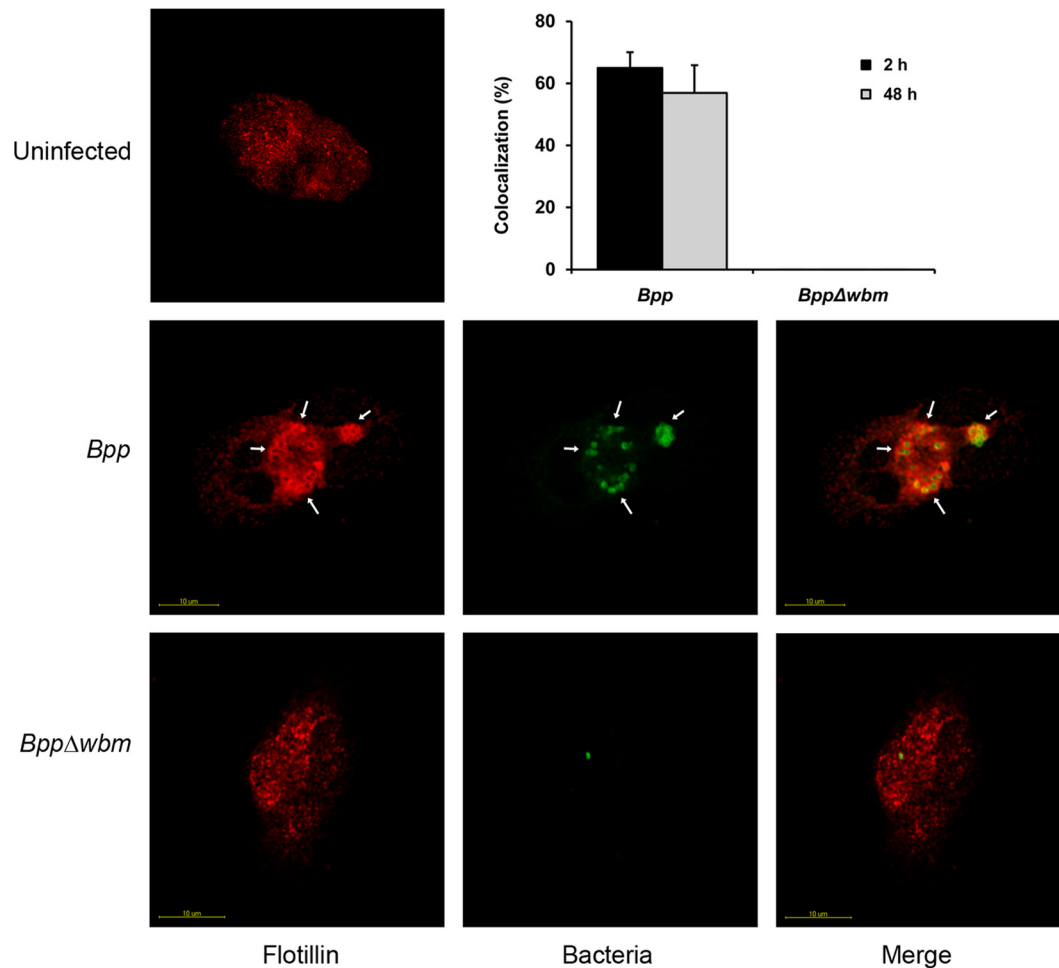


FIG 6 *B. paraptussis* colocalization with flotillin. Nonopsonized *B. paraptussis* (*Bpp*) or nonopsonized *B. paraptussis* O antigen-deficient mutant (*BppΔwbm*) bacteria were incubated with human macrophages (MOI of 30) for 40 min at 37°C. After washing, cells were incubated with polymyxin B to kill the extracellular bacteria. Samples were taken at different time points (2 or 48 h postinfection). Macrophages were fixed and permeabilized prior to incubation with antibodies against flotillin-1 (red) or bacteria (green). Colocalization of bacteria and flotillin was investigated using confocal microscopy. The bars and error bars show the mean percentages of flotillin-positive phagosomes \pm SD for three independent experiments. Confocal microscopy images of macrophages 48 h postinfection are shown. Colocalization is reflected by green fluorescent *B. paraptussis* surrounded by Cy3-labeled flotillin (indicated by arrows). While both uninfected and *B. paraptussis* Δ *wbm*-infected macrophages show a uniform pattern of distribution of flotillin, macrophages infected with nonopsonized *B. paraptussis* do not. Representative images from one of three independent experiments are shown.

access to the recycling compartment, eventually getting essential nutrients.

As observed for other O antigen-presenting pathogens, the LPS O antigen of *B. paraptussis* was found to be involved in this long-term intracellular survival. *B. paraptussis* mutants lacking the expression of this molecule were killed shortly after macrophage phagocytosis. Previous studies have shown that the O antigen is involved in directing other bacteria through an endocytic pathway that avoids phagosomal maturation into lysosomes by recruiting lipid rafts in the phagosomal membrane (32). In the present work, we observed that the *B. paraptussis* bacteria lacking the LPS O chain entered macrophages in a lipid raft-independent manner and their phagosomes fused rapidly with lysosomes. Colocalization studies showed that wild-type bacteria but not the O antigen-deficient mutants colocalized with the lipid raft marker flotillin at early time points postinfection. Accordingly, the phagocytosis levels of the wild-type bacteria but not the O antigen-deficient mutants were drastically reduced by macrophage treatment

with a cholesterol-sequestering drug, methyl- β -cyclodextrin. Similar results were obtained with nystatin, another lipid raft-disrupting drug that binds cholesterol. Importantly, colocalization studies further showed that 2 days after infection, *B. paraptussis*-containing phagosomes were still enriched in lipid rafts, which might have a role in bacterial survival. Accordingly, the percentage of intracellular bacteria colocalizing with lipid raft markers was similar to both the percentage of viable bacteria and the percentage of bacteria residing in nonacidic compartments 48 h after infection. The nature of the interaction between O antigen and lipid rafts, which are microdomains enriched in cholesterol, glycosphingolipids, and glycosylphosphatidylinositol-anchored molecules, remains to be investigated. Very little of this interaction can be concluded from our results. Cholesterol itself might be involved since nystatin, a drug that interferes with cholesterol binding, decreases *B. paraptussis* attachment to macrophages. However, O antigen interaction with other molecules embedded in lipid rafts cannot be excluded. Likewise, a contribution of GM1,

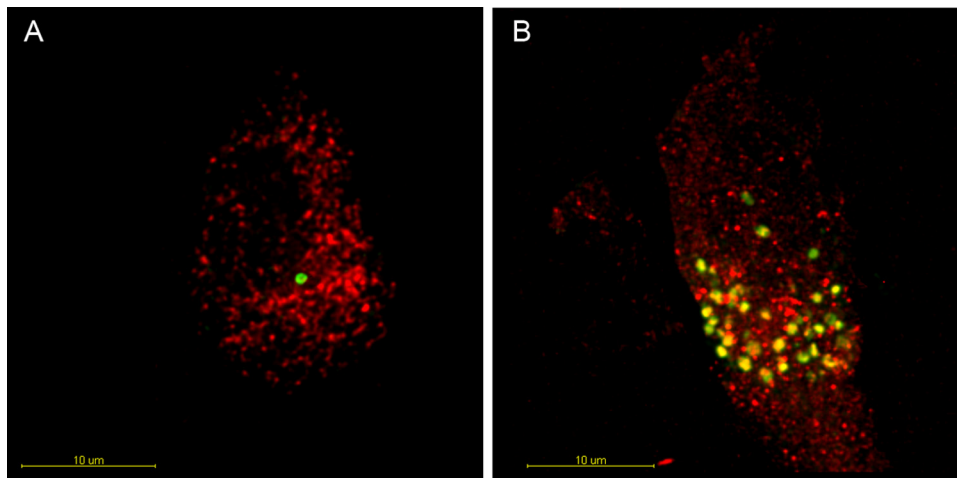


FIG 7 *B. paraptussis*-containing phagosome has access to recycling vesicles. Macrophages incubated with nonopsonized *B. paraptussis* O antigen-deficient mutants (A) or nonopsonized *B. paraptussis* (B) (MOI of 30) at 37°C for 40 min were washed and further incubated with polymyxin B to kill extracellular bacteria. At 48 h after infection, macrophages were incubated with Alexa Fluor 594-transferrin, fixed, and analyzed by confocal microscopy. Alexa Fluor 594-transferrin uptake is seen in red, and *B. paraptussis* bacteria are green. (B) The image shows colocalization of *B. paraptussis* and Alexa Fluor 594-labeled transferrin, reflected by yellow areas. Representative images from one out of three independent experiments are shown.

as found for other pathogens (32, 49), or even CR3, which is known to interact with the filamentous hemagglutinin of *B. pertussis*, cannot be ruled out. Nevertheless, the results obtained with the O antigen-deficient mutant of *B. paraptussis* seem to argue against the latter possibility. Further studies are needed to dissect this issue.

The data presented here identify macrophages as a possible niche of persistence of *B. paraptussis*. Our results show that the O antigen protects *B. paraptussis* from immune functions, allowing the bacteria to eventually hide inside the immune cells. This seems to be related to the ability of the O antigen to generate a lipid raft-enriched phagosome, which alters its trafficking to the degradative pathway. The data obtained in this study will have to be integrated with further clinical observations before we can conclude that *B. paraptussis* intracellular survival plays a role in bacterial pathogenicity and whooping cough epidemiology. However, the finding that the presence of opsonic antibodies is critically required to induce cellular bactericidal activity against *B. paraptussis* to promote clearance and to avoid the development of eventual intracellular infections is already of particular importance, since current pertussis acellular vaccines do not induce these kinds of antibodies against *B. paraptussis* and the innate immunity is not enough to control this pathogen. The need for a new generation of vaccines that induce immunity against *B. paraptussis* should be considered in order to properly control whooping cough.

ACKNOWLEDGMENTS

This work was partially supported by ANPCyT PICT413 (M.E.R.). M.E.R. is a member of the Scientific Career of CONICET. J.G. is a doctoral fellow of CONICET. E.T.H. is supported by NIH (GM083113).

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