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ORIGINAL PAPER

Mechanisms associated with phagocytosis of *Arcobacter butzleri* by *Acanthamoeba castellanii*

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Abstract *Acanthamoeba castellanii* is a free-living amoeba widely found in environmental matrices such as soil and water. *Arcobacter butzleri* is an emerging potential zoonotic pathogen that can be isolated from environmental water sources, where they can establish endosymbiotic relationships with amoebas. The aim of this study was to describe the implication of mannose-binding proteins and membrane-associated receptors of glucose and galactose present in the amoebic membrane, during the attachment of *Arcobacter butzleri* by blocking with different saccharides. Another objective was to describe the signaling pathways involved in phagocytosis of these bacteria using specific inhibitors and analyze the implication of phagolysosome formation on the survival of *Arcobacter butzleri* inside the amoeba. We infer that the attachment of *Arcobacter butzleri* to the amoeba is a process which involves the participation of mannose-binding proteins and membrane-associated receptors of glucose and galactose present in the amoeba. We also demonstrated an active role of protozoan actin polymerization in the phagocytosis of *Arcobacter butzleri* and a critical involvement of PI3K and RhoA pathways. Further, we demonstrated that the tyrosine kinase-induced actin polymerization signal is essential in *Acanthamoeba*-mediated bacterial uptake. Through phagolysosomal formation analysis, we conclude that the survival of *Arcobacter butzleri* inside the amoeba could be related with the ability to remain inside vacuoles not fused with lysosomes, or with the ability to retard the fusion between these structures. All these results help the understanding of the bacterial uptake mechanisms used by *Acanthamoeba castellanii* and contribute to evidence of the survival mechanisms of *Arcobacter butzleri*.

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Introduction

Acanthamoeba castellanii is a free-living amoeba (FLA) recognized as an opportunistic parasite that can cause fatal granulomatous amebic encephalitis and eye keratitis in humans (Marciano-Cabral and Cabral 2003). Moreover, *Acanthamoeba castellanii* is recognized as an environmental host for several intracellular pathogenic bacteria. FLA and bacteria can be widely found in environmental matrices such as soil and water, where they can establish different types of interactions (Marciano-Cabral 2004). These interactions vary from the simple use of bacteria by FLA as food sources to symbiotic relationships that allow long-term intra-amoeba survival of bacteria, enhancing bacterial permanence in the environment and favoring their dissemination (Weekers et al. 1993; Greub and Raoult 2004; Marciano-Cabral 2004; Tezcan-Merdol et al. 2004). Recently, *Acanthamoeba* spp. has been described as a reservoir and/or vector of pathogenic bacteria like *Salmonella typhimurium*, *Mycobacterium avium*, *Chlamydia pneumonia*, *Legionella pneumophila*, and *Burkholderia cepacia* (Marolda et al. 1999; Tezcan-Merdol et al. 2004; Molmeret et al. 2005; Iskandar et al. 2010).

Arcobacter butzleri is the most common specie of the genus *Arcobacter*, which causes diarrhea and occasional systemic infections in humans. Currently, *Arcobacter butzleri* has been recognized as an emerging potential zoonotic pathogen and considered a serious hazard to human health by the International Commission on Microbiological Specification for Foods (Collado and Figueras 2011; Fernández et al. 2013; ICMSF 2002). *Arcobacter butzleri* has been isolated from different environmental water sources, where these bacteria could enter in contact with *Acanthamoeba castellanii*, establishing endosymbiotic relationships (Collado and Figueras 2011). Recently, we demonstrated that *Arcobacter butzleri* has the

ability to enter and settle within vacuoles of *Acanthamoeba castellanii*, surviving inside the amoeba for at least 10 days. This result indicated that *Acanthamoeba castellanii* could be a potential environmental reservoir and vehicle of *Arcobacter butzleri* (Fernández et al. 2012).

It is known that polysaccharide-binding proteins especially mannose-binding proteins (MBP)—also called membrane-associated mannose receptors or mannose-binding lectins (MBL)—present in the surface of *Acanthamoeba* spp. play a role in the attachment and internalization of different bacteria. After attachment, the uptake of bacteria occurs through the phagocytosis process, involving actin polymerization in a similar way described in non-professional phagocytes. Once inside, some bacteria can inhibit the phagolysosomal formation, allowing them to survive inside vacuoles in amoeba cells. These processes have been described in *Acanthamoeba* spp. for different bacterial species, but not in *Acanthamoeba castellanii* for *Arcobacter butzleri* (Alsam et al. 2005; Harb et al. 1998; Declerck et al. 2007; Thomas and Brooks 2004; Akya et al. 2009).

Considering the ecological and medical relevance of *Acanthamoeba castellanii* and *Arcobacter butzleri*, and emphasizing that these organisms share similar ecosystems, the purposes of this study were to investigate the role of MBP, membrane-associated glucose receptors, and membrane-associated galactose receptors present in the amoeba during attachment of *Arcobacter butzleri* and to describe the signaling pathways involved in internalization of the bacteria and analyze the implication of phagolysosome formation on the survival of *Arcobacter butzleri* inside of *Acanthamoeba castellanii*.

Materials and methods

Amoebae and culture conditions

Acanthamoeba castellanii T4 genotype strain was used in all the experiments. Trophozoites were grown at 25 °C in T-25 tissue culture flasks with 10 mL of peptone-yeast-glucose (PYG) medium (Bozue and Johnson 1996), modified as follows: 2 % protease peptone, 0.2 % yeast extract, 0.1 M glucose, 4 mM $MgSO_4$, 0.4 M $CaCl_2$, 0.1 % sodium citrate dihydrate, 0.05 mM $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$, 2.5 mM NaH_2PO_3, and 2.5 mM K_2HPO_3 at pH 6.5–7.0. Fresh PYG medium was used 17–20 h prior to experiments to harvest more than 95 % of the amoeba in the trophozoite form. All procedures were performed with trophozoites in logarithmic phase.

Bacterial strains and culture conditions

Arcobacter butzleri NAV 16-4 strain isolated from an environment source and ATCC 49616 strain were used. Routine cultures were performed in Blood Agar Base No.2 supplemented with sheep blood 5 %. Plates were incubated for 24 h at 30 °C in aerobiosis. All procedures were performed with bacterial cultures with less than 24 h of incubation.

Attachment inhibitory assay

The assay was performed according to the protocol described by Akya et al. (2009), modified as follows. Monolayers of amoebae (2.5×10^5 cell/mL) contained in six-well trays were pretreated with glucose, galactose, or mannose (Sigma-Aldrich® Co., MO, USA) dissolved in PBS at different concentrations (50, 100, and 150 mM) for 1 h at 25 °C. After washing with PBS buffer, the monolayers were inoculated with *Arcobacter butzleri* at multiplicity of infection (MOI) 100 (2.5×10^7 bacteria/mL) and incubated at 25 °C for 1 h in PBS. Unattached bacteria were removed by three washes with PBS buffer. To produce amoebal lysis, 1 mL of PBS buffer containing sodium desoxicholate (final concentration 0.5 %) was added and incubated at room temperature for 20 min. Enumeration of viable bacteria was performed according to Chen et al. (2003) as previously described.

Phagocytosis inhibitory assay

Inhibitor to block the actin-dependent phagocytic process cytochalasin D, PI3K inhibitor (LY294002), Rho kinase inhibitor (Y27632), tyrosine protein kinase inhibitor (genistein), and tyrosine protein phosphatase inhibitor (Na-orthovanadate) (Sigma-Aldrich® Co., MO, USA) were used. The assay was performed as described by Alsam et al. (2005), with the following modifications: amoebae (2.5×10^5 cell/mL) were incubated for 1 h prior to bacterial infection in the presence of the inhibitor (concentrations used are shown in Table 1) in a total volume of 1 mL PBS buffer. Infection was performed at MOI 100 (2.5×10^7 bacteria/mL) for 1 h under constant slow agitation at 25 °C, followed by the addition of 50 μg/mL gentamicin for 1 h to kill extracellular bacteria. Amoebae were washed with PBS for three times (2,000 rpm×10 min) to remove gentamicin and extracellular bacteria. Amoeba lysis and bacterial enumeration were performed as mentioned above.

Phagolysosome formation and survival of *Arcobacter butzleri*

Acid phosphatase staining and transmission electron microscopy Amoebae were infected with *Arcobacter butzleri* ATCC 49616 at MOI 100 as mentioned above for 2, 4, and 8 h at 25 °C and then exposed to 50 μg/mL gentamicin during 1 h and washed three times (2,000 rpm×10 min) with PBS to remove the extracellular bacteria. Amoebae were fixed in 1 % Karnovsky solution (2.5 % glutaraldehyde, 2 % paraformoldehyde, and 0.1 M sodium cacodylate buffer pH 7.2) and stained for cytochemical detection of acid

Table 1 Phagocytosis inhibitory assay results

Inhibitors	Relative bacterial uptake (%)	
	ATCC 49616	NAV16-4
Cytochalasin D (2 μM)	80±3.2[a]	13.6±1***
Cytochalasin D (5 μM)	40±5.4*	11.1±0.7***
Cytochalasin D (8 μM)	26.6±4.3**	6.6±0.4***
LY294002 (10 μM)	18.2±1.1**	1.4±0.07***
LY294002 (50 μM)	13±0.7**	1±0.1***
LY294002 (100 μM)	6.6±1**	0.5±0.04***
Y27632 (10 μM)	71±4.6[a]	69±1.7**
Y27632 (50 μM)	40.3±2.8**	52.6±3.1***
Y27632 (100 μM)	23.2±1.2***	44.5±1***
Genistein (10 μM)	97±7.5[a]	92.7±4.3[a]
Genistein (50 μM)	65.3±6.6*	77.5±4.2*
Genistein (100 μM)	43.5±4.5***	64.5±2.4***
Na-orthovanadate (10 μM)	106.6±8.6[a]	97.3±4.6[a]
Na-orthovanadate (50 μM)	174±10.8*	110.7±5.8[a]
Na-orthovanadate (100 μM)	174.1±18.5*	130.7±5.4**

Results are presented as relative percent of viable intra-amoebic bacteria over control (internalized bacteria by amoeba without inhibitory treatment). Data were analyzed using Student's *t* test through Graph Pad software

*$p<0.05$; **$p<0.01$;***$p<0.001$

[a] Not significant

phosphatase according to the protocol described by Worth et al. (2009) modified as follows: After fixation, samples were kept in sodium cacodylate buffer for 24 h, followed by an incubation period of 1 h in modified Gomori solution (13.9 mM β-glycerophosphate, 1 mM lead nitrate, 0.05 M sodium cacodylate buffer pH 5, 0.08 % calcium chloride, 5 % sucrose). Samples were washed in sodium cacodylate buffer (0.2 M) for 6 h. The material was dehydrated in alcohol, and the samples were subsequently processed in LR White resin. Ultrathin sections were contrasted by uranyl acetate and lead nitrate on nickel small screens. The small screens were analyzed in a transmission electron microscope Zeiss EM-109 coupled to the image capture software MegaviewG2/Olympus Soft Imaging Solutions.

Inhibition phagolysosome fusion assay This experiment was performed according to the protocol described by Akya et al. (2009) with the following modifications: The infection was made in the same conditions as the phagocytosis inhibitory assay, but *Acanthamoeba castellanii* were previously incubated in the presence of 20 and 40 mM of NH_4Cl in 1 mL PBS for 1 h. The infection was performed at two different times, during 1 and 4 h. The elimination of extra-amoebic bacteria, release, and counting of viable intra-amoebic bacteria were performed as in the phagocytosis inhibitory assay.

Statistical analysis

All experimental units were done in duplicate (biological duplicate), and each duplicate was performed in triplicate. Data were analyzed using Student's *t* test through Graph Pad software. Values of $p<0.05$ were considered statistically significant. For the attachment inhibitory assay, results are shown as graphs indicating the count of attached bacteria to the amoeba (log CFU/mL) with and without monosaccharide treatment. For the phagocytosis inhibitor assay, results are presented as relative percent of viable intra-amoebic bacteria over control (internalized bacteria by amoeba without inhibitory treatment). For the phagolysosomal formation inhibitory assay, results are presented as graphs indicating the count of viable intra-amoebic bacteria (log CFU/mL) with and without NH_4Cl treatment.

Results

Attachment inhibitory assay

Our results showed that the presence of glucose, galactose, and mannose significantly inhibited bacterial attachment in a concentration-dependent manner, indicating the active role of these monosaccharide receptors in the attachment process between both organisms (Fig. 1a, b).

Even though the amoebal exposition to saturated solutions of these saccharides caused a significant decrease in bacterial attachment, the galactose treatment at 100 mM had a stronger blocking effect on the attachment process of ATCC 49616 and NAV16-4, obtaining a 73 %±1.1 and 88.7 %±2.5 of bacterial attachment inhibition, respectively. Glucose treatment at 100 mM inhibited bacterial attachment in 44 %±0.7 for ATCC 49616 and 47 %±0.9 for NAV16-4, while mannose treatment at 100 mM caused the smallest, however significant, reduction of bacterial attachment (38 %±1.1) for NAV16-4 strain and 66 %±2.5 for ATCC 49616. In addition, significant differences in the attachment inhibition with mannose solution were observed in the two *Arcobacter butzleri* strains used.

Phagocytosis inhibitory assay

To demonstrate the involvement of actin filaments in the internalization of *Arcobacter butzleri* by *Acanthamoeba castellanii*, we tested different concentrations of the actin polymerization inhibitor cytochalasin D. Bacterial uptake was blocked by cytochalasin D treatment, suggesting an active role of protozoan actin polymerization dynamic in the internalization of *Arcobacter butzleri* by amoeba.

As shown in Table 1, cytochalasin D significantly reduced bacterial uptake by *Acanthamoeba castellanii* of the NAV16-4 strain in a concentration-dependent manner. Similarly, the

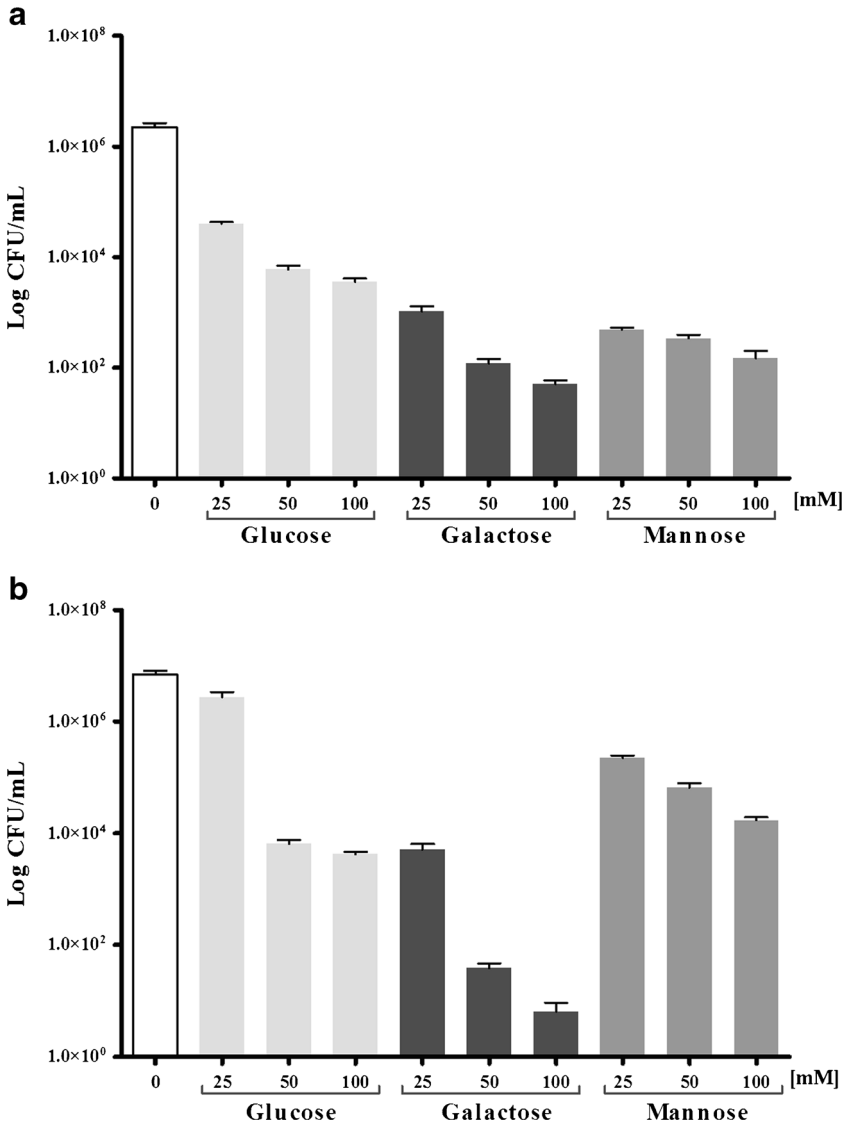

Fig. 1 **a**, **b** Attachment inhibitory assay results. Graphics represent our results indicating the count of attached bacteria to the amoeba expressed as log CFU/mL with and without monosaccharide treatment at different concentrations. We observed statistical significance at all the monosaccharide concentrations tested with respect to the control ($p<0.001$ in all cases). Data were analyzed using Student's *t* test through Graph Pad software

bacterial uptake of the ATCC 49616 strain decreased significantly at 5 and 8 μM of cytochalasin D treatment.

To determine the role of PI3K in the internalization of *Arcobacter butzleri* by *Acanthamoeba castellanii*, amoeba cultures were treated with a selective inhibitor of PI3K referred to as LY294002. Bacterial phagocytosis (ATCC 49616 and NAV16-4 strains) by amoeba were significantly inhibited by LY294002 in a concentration-dependent way, indicating the participation of PI3K pathways in the internalization process (Table 1).

The Rho kinase inhibitor Y27632, which partially blocks RhoA pathway, significantly reduced bacterial uptake of the NAV16-4 strain in a concentration-dependent manner. Similarly, the bacterial uptake of the ATCC 49616 strain decreased significantly at 50 and 100 μM of Y27632 (Table 1). These results indicate that downstream signaling pathways involved in the actin cytoskeleton remodeling are essential for internalization of *Arcobacter butzleri* by *Acanthamoeba castellanii*.

To study the involvement of intracellular signaling pathways, phagocytosis assays were performed in the presence of genistein and sodium orthovanadate, a protein tyrosine kinase inhibitor and a protein tyrosine phosphatase inhibitor, respectively. The treatment with 50 and 100 μM of genistein significantly inhibited the uptake of both bacterial strains by amoeba. However, the treatment of sodium orthovanadate at 100 μM significantly increased the bacterial uptake of both bacterial strains by *Acanthamoeba castellanii* (Table 1). These results indicate that protein tyrosine kinases, but not phosphatase, play an important role in the internalization process of *Arcobacter butzleri* by *Acanthamoeba castellanii*.

Phagolysosome formation and survival of *Arcobacter butzleri*

We observed active phagolysosomes containing previously internalized bacteria at all times of infection. However, we

also observed *Arcobacter butzleri* cells with preserved morphology inside amoeba vacuoles not associated or fused with lysosomes (Fig. 2). These results indicated that *Arcobacter butzleri* remains viable within *Acanthamoeba castellanii* inside intracytoplasmic vacuoles not associated or fused with lysosomes, at least during the first 8 h of infection.

The results obtained through TEM were complemented by an inhibition phagolysosome fusion assay using NH_4Cl, obtaining results that show a clear trend according to the infection time and NH_4Cl concentration. After 1 and 4 h of infection, at all concentrations used, NH_4Cl significantly increased the intra-amoebic bacterial count of strain ATCC 49616 (Fig. 3a). Meanwhile, after 1 h of infection, the intra-amoebic bacterial count of strain NAV16-4 increases significantly only at the highest concentration of NH_4Cl (40 mM). Finally, after 4 h of infection, NH_4Cl significantly increased

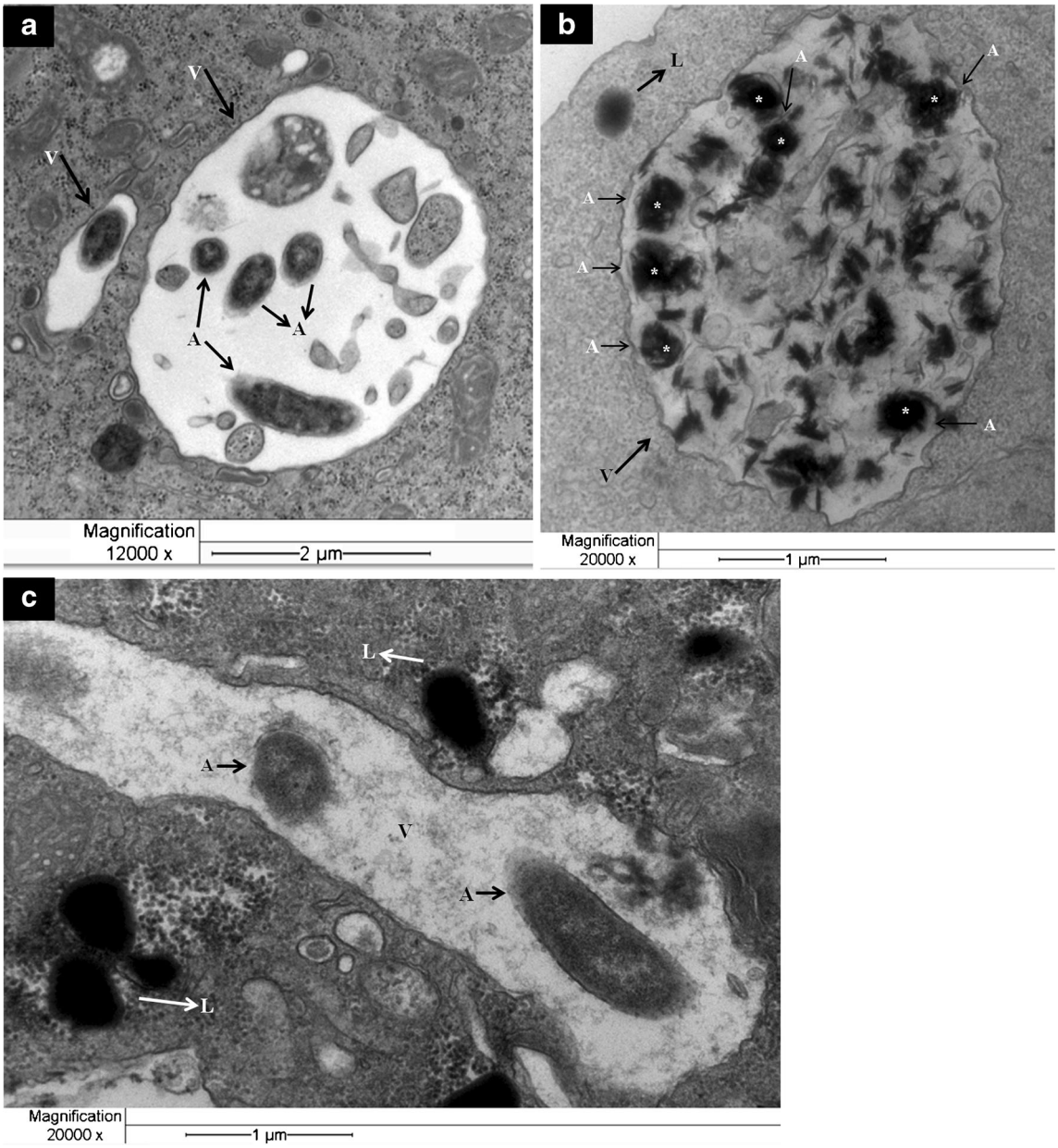

Fig. 2 Association between *Arcobacter butzleri* and lysosomes of *Acanthamoeba castellanii.* The samples were processed post-infection through acid phosphatase staining for TEM according to protocol described by Worth et al. (2009). The samples were analyzed in a transmission electron microscope Zeiss EM-109 coupled to the image capture software MegaviewG2/Olympus Soft Imaging Solutions. *V*, vacuole; *A*, *Arcobacter butzleri*; *L*, lysosome; *asterisk*, positive acid phosphatase staining. **a** Control without acid phosphatase staining 2 h post-infection. It is possible to observe the presence of bacteria with preserved morphology within amoebal vacuoles. **b** Acid phosphatase staining 2 h post-infection. It is possible to observe the presence of bacteria within active phagolysosomes. **c** Acid phosphatase staining 8 h post-infection. It is possible to observe the presence of bacteria with preserved morphology within amoebal vacuoles not associated or fused with lysosomes

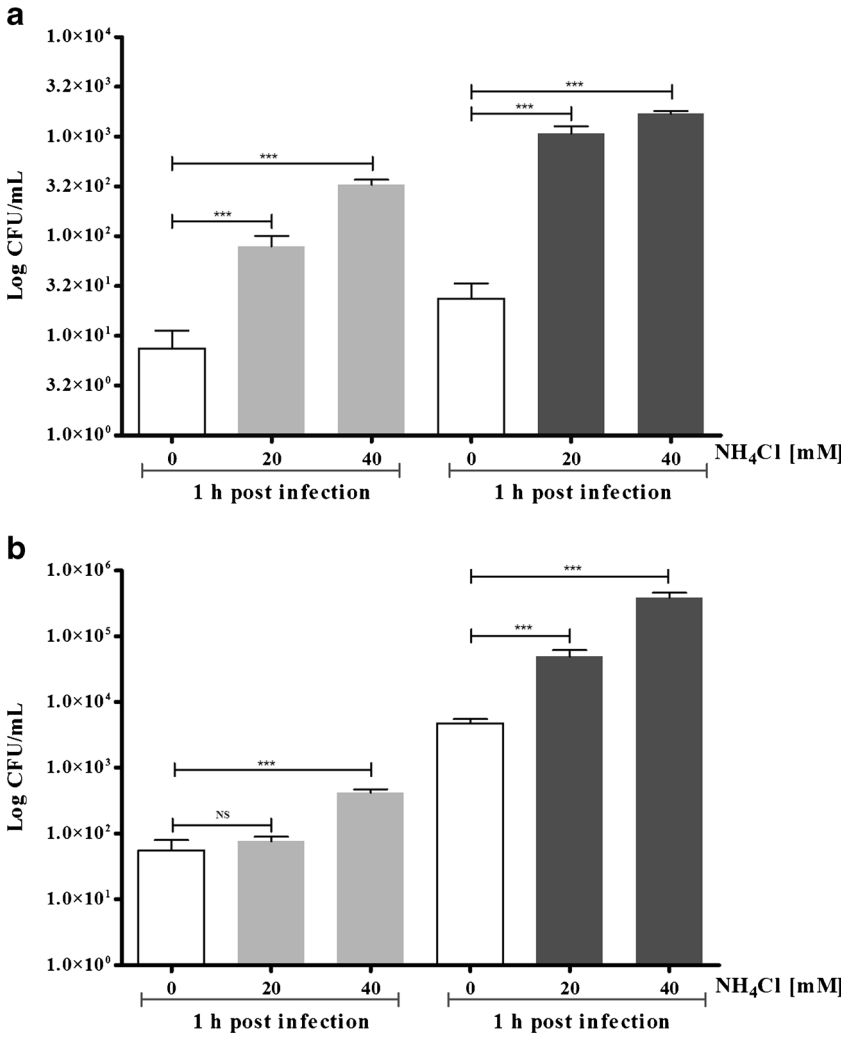

Fig. 3 **a**, **b** Inhibition phagolysosome fusion assay. Graphics represent our results indicating the count of viable intra-amoebic bacteria expressed as log CFU/mL with and without NH_4Cl treatment at 1 and 4 h post-infection. Data were analyzed using Student's *t* test through Graph Pad software. ***$p<0.001$, *ns* not significant

the intra-amoebic bacteria of this strain at all concentrations used (Fig. 3b). These results showed that prevention of phagolysosome fusion significantly increases the survival of *Arcobacter butzleri* in endosymbiosis with *Acanthamoeba castellanii*.

Discussion

Bacterial attachment is a complex and multifactorial process involving a large number of bacterial and host cell components (Samrakandi et al. 2002). In order to determine the role of mannose-binding protein (MBP) and other membrane-associated polysaccharide receptors (glucose and galactose) in *Arcobacter butzleri* attachment to *Acanthamoeba castellanii*, an attachment inhibitory assay was performed with solutions of glucose, galactose, and mannose. Our results suggest that attachment of *Arcobacter butzleri* to amoeba occurred via interactions of oligosaccharides located on the bacterial surface with membrane-associated galactose receptors, MBP, and membrane-associated glucose receptors present in the membrane of *Acanthamoeba castellanii*. These results are consistent with previous studies, where the ability of exogenous mannose to block the bacterial attachment to amoebae was demonstrated. Allen and Dawidowicz (1990) demonstrated that yeast uptake by *Acanthamoeba castellanii* is a receptor-dependent process mediated by MBP. Additionally, Alsam et al. (2005) confirmed the ability of exogenous mannose to block the phagocytosis of *Escherichia coli* by *Acanthamoeba castellanii*, and Declerck et al. (2007) observed that mannose highly inhibited the uptake of *Legionella pneumophila* by *Acanthamoeba castellanii* in a dose-dependent manner. In contrast, Harb et al. (1998) reported that mannose had no detectable effect on the uptake of *Legionella pneumophila* by *Hartmanella vermiformis* and *Acanthamoeba polyphaga*. On the other hand, different studies have shown that MBP is involved in the early events of *Acanthamoeba* binding to host cells, suggesting its role in the infection process (Alsam et al. 2003; Morton et al. 1991; Yang et al. 1997). Considering these data, we can infer that MBP plays an important role in the specific binding to host cells, as well in the attachment of *Arcobacter butzleri* to the surface of *Acanthamoeba castellanii*.

The exposure of amoebae to different solutions of galactose and glucose significantly blocked the attachment of *Arcobacter butzleri* to *Acanthamoeba castellanii*, results that are consistent with previous studies. Harb et al. (1998) concluded that uptake of *Legionella pneumophila* by *H. vermiformis* can be completely blocked by galactose. The same study indicates that galactose partially blocked the uptake of *Legionella pneumophila* by *A. polyphaga*. However, the treatment with glucose had no detectable effect in the uptake of *Legionella pneumophila* by *H. vermiformis* and *A. polyphaga*. Later, Declerck et al. (2007) observed that galactose partially inhibited the uptake of *Legionella pneumophila* by *Acanthamoeba castellanii* and *Naegleria lovaniensis*. On the other hand, Alsam et al. (2005) reported that treatment of *Acanthamoeba castellanii* with galactose and glucose exhibited minimal effects in the phagocytosis of *E. coli*, and the uptake of *Listeria monocytogenes* by *A. polyphaga* pretreated with galactose and glucose was not significantly different from untreated amoebae cultures (Akya et al. 2009). These data allows us to infer that polysaccharide-binding proteins present in the amoebic surface have a role in the bacterial attachment. This role can vary depending of the amoebic and bacterial species. In our case, the results showed an active role of membrane-associated galactose receptors in the attachment of *Arcobacter butzleri* to the surface of *Acanthamoeba castellanii*, compared to glucose and mannose receptors. This may be due to the presence of galactose /*N*-acetyl-D-galactosamine lectin receptor, which has been implicated in the attachment of *Legionella pneumophila* to *H. vermiformis* and to *A. polyphaga* (Harb et al. 1998; Venkataraman et al. 1997). This receptor activated several signal transduction processes that are manifested together with de novo synthesis of amoeba host proteins, essential for bacterial uptake (Venkataraman et al. 1997). In addition, significant differences in the attachment inhibition with mannose solution were observed in the two *Arcobacter butzleri* strains used. We do not investigate this phenomenon, but we believe it may be attributed to the fact that strains are from different sources which we consider relevant for future studies.

The role of microfilaments and cytoskeleton rearrangement in phagocytosis by mammalian cells and other eukaryotes, such as amoebae, has been documented (Akya et al. 2009; Cossart and Sansonetti 2004; Elliott and Winn 1986). These cellular processes are normally dependent on actin polymerization dynamic and PI3K kinase activity, which are sensitive to cytochalasin D and LY294002, respectively. Cytochalasin D has been extensively used to study actin polymerization inhibition in mammalian cells and amoebae (Alsam et al. 2005; Elliott and Winn 1986; Weihing 1978; King et al. 1991). LY294002 binds to the ATP binding site of PI3K, thereby inhibiting their catalytic activity (Alsam et al. 2005). PI3K are versatile signaling molecules that play crucial roles in receptor-mediated signal transduction, such as actin remodeling and membrane trafficking processes (Downes et al. 2005). Our results indicated an active role of protozoan actin polymerization in the internalization of *Arcobacter butzleri* and a critical involvement of PI3K pathways in this process. These results are consistent with previous studies in which cytochalasin D and wortmannin, another PI3K inhibitor, reduced the ability of *A. polyphaga* to phagocyte *Listeria monocytogenes* (Akya et al. 2009). In addition, it was observed that cytochalasin D and LY294002 inhibited *E. coli* uptake by *Acanthamoeba castellanii* (Alsam et al. 2005), and it has been reported that cytochalasin D reduced uptake of *Legionella pneumophila* by *Acanthamoeba castellanii* and *N. lovaniensis* (Declerck et al. 2007; Moffat and Tompkins 1992). However, this inhibitor produced no detectable effect on the uptake of *Legionella pneumophila* by *A. polyphaga*. Similar results were obtained from *H. vermiformis* infected with these bacteria (Harb et al. 1998). Previous studies identified that actin polymerization plays an important role in bacterial internalization into the host mammalian cell as shown in *Legionella pneumophila* entry into human monocytes and *S. typhimurium* invasion of epithelial cells (Coxon et al. 1998; Finlay et al. 1991). These studies indicate that, despite different bacteria and distinct cell types, actin-mediated cytoskeletal rearrangements play an important role in bacterial internalization into the eukaryotic cells. In view of the data present, we can infer that uptake of *Arcobacter butzleri* by *Acanthamoeba castellanii* occurs through a *phagocytosis process* similar to that of mammalian cells, in which the cytoskeletal rearrangement and the involvement PI3K are essential.

Rho GTPases such as RhoA, Cdc42, and Rac1 are the known regulators of actin polymerizations and are key regulators of actin cytoskeleton in all eukaryotic cells. These Rho GTPases involve three major pathways resulting in specific cytoskeletal rearrangements: RhoA pathway leading to stress fiber formation, Rac1 activation triggers lamellipodia formation, and Cdc42 activation promotes filopodia formation (Mackay and Hall 1998). To demonstrate the involvement of Rho GTPases in the uptake of *Arcobacter butzleri* by *Acanthamoeba castellanii*, the phagocytosis assays were performed in the presence of Y27632, an inhibitor that partially blocks RhoA pathway. Our results demonstrated that Y27632 treatment of *Acanthamoeba castellanii* decreased significantly the phagocytosis of both *Arcobacter butzleri* strains. These results indicate that downstream signaling pathways involved in the actin cytoskeleton remodeling, mainly RhoA pathway, are essential for phagocytosis of *Arcobacter butzleri* by *Acanthamoeba castellanii* and suggest that other Rho GTPases such as Cdc42 and Rac1 could also be involved in this process. Our results are consistent with those reported previously by Alsam et al. (2005), who observed that Y27632 reduced the *E. coli* uptake by *Acanthamoeba castellanii*, concluding that Rho GTPases are involved in the pathway that activates the phagocytosis mechanism in *Acanthamoeba castellanii*.

To study the involvement of intracellular signaling pathways in the internalization of *Arcobacter butzleri* by *Acanthamoeba castellanii*, we used genistein, a tyrosine protein kinase inhibitor, and sodium orthovanadate, a tyrosine protein phosphatase inhibitor. Genistein significantly inhibited the uptake of both bacterial strains, indicating that phosphorylation of tyrosine residues are critical in the phagocytosis process. In contrast, sodium orthovanadate increased the bacterial uptake, confirming that phagocytosis of *Arcobacter butzleri* is dependent on intracellular signaling pathways. These results are consistent with those reported previously by Alsam et al. (2005) demonstrating that genistein significantly inhibited *E. coli* uptake by *Acanthamoeba castellanii*, whereas sodium orthovanadate increased bacterial uptake. Based on these data and considering that tyrosine phosphorylation of host proteins has been shown to be important in the uptake of several intracellular pathogens (Finlay and Cossart 1997; Rosenshine et al. 1992, 1994), we infer that protein tyrosine kinases play an important role in the process of *Arcobacter butzleri* phagocytosis by *Acanthamoeba castellanii*, showing that tyrosine kinase-induced actin polymerization signal is important in *Acanthamoeba*-mediated bacterial uptake.

In our studies, although the levels of adherence and internalization of *Arcobacter butzleri* by *Acanthamoeba castellanii* showed a clear tendency, we note that these levels were strain specific and we consider that the bases of these differences need further investigations.

Recently, we demonstrated that *Arcobacter butzleri* has the ability to enter and settle within vacuoles of *Acanthamoeba castellanii*, surviving inside the amoeba at least for 10 days (Fernández et al. 2012). In order to assess the implication of phagolysosome formation on the survival of these bacteria, we analyzed the association between *Arcobacter butzleri* ATCC 49616 and amoebal lysosomes using acid phosphatase staining and TEM at different infection times. We observed active phagolysosomes containing previously internalized bacteria and identify *Arcobacter butzleri* cells with preserved morphology inside vacuoles not associated or fused with lysosomes. Therefore, we can conclude that *Arcobacter butzleri* remains viable within *Acanthamoeba castellanii* inside intracytoplasmic vacuoles not associated or fused with lysosomes, at least during the first 8 h of infection.

Phagosomal acidification has a key role in the degradation of phagocytosed bacterial cells by mammalian macrophages. This process can be effectively inhibited by NH_4Cl, which is a weak base that accumulates in acidic compartments within the cell cytoplasm and neutralizes the pH. The neutralization of acidic compartments interferes with the maturation of the phagosome and inhibits phagosome-lysosome fusion (Hart and Young 1991). In order to quantify the impact of the fusion of lysosomes with internalized bacteria, we performed an inhibition phagolysosome fusion assay using NH_4Cl. Depending on NH_4Cl concentration and infection time, NH_4Cl increased the intra-amoebic bacterial count of both strains in a concentration-dependent way, except at the lowest concentration (20 mM) and 1 h post-infection of the NAV 16-4 strain. Our results showed that the prevention of phagolysosome fusion increases significantly the survival of *Arcobacter butzleri* in endosymbiosis with *Acanthamoeba castellanii*, and thereby, a percentage of *Arcobacter butzleri* phagocytosed by *Acanthamoeba castellanii* is destroyed (killed) inside the phagolysosome. These results are consistent with those reported previously by Akya et al. (2009). They obtained that NH_4Cl significantly downregulated the mechanism utilized by *A. polyphaga* trophozoites to kill *Listeria monocytogenes* cells.

These results allow us to infer that survival of *Arcobacter butzleri* inside amoeba might occur through one or more of the mechanisms previously described for *Salmonella* (Ibarra and Steele-Mortimer 2009) that are localized in lysosomal vacuoles and included regulation of effector proteins, participation of type I secretion system, and regulation of virulence factors including ion transporters, superoxide dismutase, flagella, and fimbriae. Survival mechanisms similar to those have been described in *M. avium* and *Legionella pneumophila* (Frehel et al. 1986; Bozue and Johnson 1996). Therefore, the survival of *Arcobacter butzleri* in endosymbiosis with *Acanthamoeba castellanii* may be related with the same ability to remain inside vacuoles not fused with lysosomes, or with the ability to retard the fusion between these structures.

In conclusion, our data indicate that polysaccharide-binding proteins (glucose, galactose, and mannose), actin polymerization, and cytoskeletal rearrangement are involved in phagocytosis of *Arcobacter butzleri* by *Acanthamoeba castellanii* trophozoites. This study reveals similar molecular mechanisms used by different types of eukaryotes in uptake of bacteria. However, much remains to be uncovered of the molecular mechanisms used by *Acanthamoeba* to uptake *Arcobacter butzleri*. Further, we suggest that survival of *Arcobacter butzleri* depends mostly on the capacity to remain inside vacuoles not fused with lysosomes; nevertheless, it is necessary to analyze the molecular mechanisms of survival. Thus, understanding the mechanisms of uptake by protozoa and the bacterial survival mechanisms will facilitate the design of measures to prevent uptake and survival of *Arcobacter butzleri*, providing effective preventive approaches for controlling the transmission of this pathogen.

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