RESEARCH ARTICLE
International Microbiology 20(4):170-177 (2017)
doi:10.2436/20.1501.01.300. ISSN (print): 1139-6709. e-ISSN: 1618-1095
www.im.microbios.org

Fate of *Bacillus cereus* within phagocytic cells

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Received 13 June 2017 · Accepted 20 November 2017

Summary. In this study we assessed the interaction of different strains of *Bacillus cereus* with murine peritoneal macrophages and cultured phagocytic cells (Raw 264.7 cells). Association, internalization, intracellular survival, routing of bacteria to different compartments and expression of MHCII were assessed in cells infected with different strains of *B. cereus* in vegetative form. Association values (adhering + internalized bacteria) and phagocytosis were higher for strain B10502 than those for strains 2 and M2. However, after 90 min interaction, intracellular survival was higher for strain 2 than for strains M2 and B10502. Acquisition of lysosomal markers by *B. cereus* containing vacuoles (BcCV), assessed by LAMP1 and Lysotracker labelling occurred shortly after internalization. The highest ratio of LAMP1(+)-BcCV was found for strain M2. This strain was able to survive longer than strain B10502 which routes to LAMP1 containing vacuoles to a lesser extent. In addition, strain M2 stimulated expression of MHCII by infected cells. Confocal analyses 60 or 90 min post-infection showed different percentages of co-localization of bacteria with Lysotracker. Results suggest strain-dependent interaction and intracellular killing of *B. cereus* by phagocytic cells. These findings could be relevant for the pathogenic potential of *Bacillus cereus* strains.

Keywords: *Bacillus cereus* · LAMP1 · phagocytosis · virulence · endocytic pathway · intracellular trafficking.

Introduction

Bacillus cereus is a Gram-positive spore forming bacterium that leads to intestinal and non-intestinal pathologies [4, 31]. *Bacillus cereus* spores gain access to the digestive tract by oral ingestion and after germination, vegetative cells produce different extracellular factors with biological activity [2, 3, 24, 36]. In addition, it has been suggested that direct bacteria-cell interactions also play a role in *B. cereus* virulence [29] and it has been demonstrated that diversity of biological activity is related to the presence of specific sequences in the bacterial genome [27].

Macrophages are crucial players of the immune response through internalization of microorganisms and further antigen presentation [26]. Phagocytosis starts with attachment of bacteria to cell surface receptors. Next, a series of events determine the fate of the microorganism after internalization. Key steps include invagination of the host cell membrane, engulfment of the microorganism and maturation of the phagosome along the endocytic pathway that involves changes in the lipid and protein composition of its membrane [9, 26]. A hallmark in phagosome maturation is the acquisition of specific markers such as Lysosomal Associated Membrane Proteins (LAMP) that are related to the maintenance of a low intralysosomal pH as well as with the protection of lysosomes against autodigestion [9, 10].

Pathogens have evolved a variety of strategies to avoid uptake and/or processing by professional phagocytic cells [1, 18, 19]. These mechanisms could involve blockade of phagolysosome biogenesis, escape from the phagosome or survival in the intralysosomal milieu [20–22].

It is known that *Bacillus cereus* is able to produce several extracellular factors with biological activity on eukaryotic cells [2, 24, 27–29]. In addition, it has been demonstrated that vegetative cells of some strains, can invade cultured human entero-

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cytes [29, 30]. However, studies on the interaction between vegetative *B. cereus* and professional phagocytic cells are seldom found in scientific literature although the role of this interaction on virulence has been proposed [32, 38].

In the present work, we examine the interaction and intracellular lifestyle of different strains of *B. cereus* in two experimental models: freshly isolated resident murine peritoneal macrophages and cultured macrophage-like Raw 264.7 cells

Materials and Methods

Isolation of murine peritoneal macrophages. Specific pathogen free BALB/c mice (6-8 weeks old) were purchased from the Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata (Argentina). Mice were provided water and balanced diet *ad libitum*. All the experimental procedures were performed in accordance with the international guidelines for animal experimentation and approved by the Comité Institucional para el Cuidado y Uso de Animales de Laboratorio (CICUAL) of the Facultad de Ciencias Exactas, Universidad Nacional de La Plata (Argentina); (Protocol number 022-05-15). Mice were euthanatized by CO_2 inhalation and resident macrophages were recovered by peritoneal washing with 10 ml of ice-cold RPMI-1640 (Life Technologies, Cergy, France) tissue culture medium, supplemented with heparin (Rivero L.A.C.E. S.R.L., Argentina) (10 U ml^{-1}) and bovine serum albumin 0.1% (w/v) (PAA Laboratories, GmbH, Pasching, Austria). Cells were washed with phosphate buffered saline (PBS) containing 2% (v/v) fetal bovine serum (FBS) (PAA Laboratories, GmbH, Pasching, Austria), centrifuged at 600x*g* for 10 min (4°C) and suspended in PBS.

Culture of RAW 264.7 cells. RAW 264.7 cells (ATCC TIB-71), a macrophage-like Abelson leukaemia virus- transformed cell line derived from BALB/c mice, were maintained in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 2 g l^{-1} $NaHCO_3$, 10 mg l^{-1} streptomycin and 10 IU ml^{-1} penicillin G. Incubations were performed at 37°C in a 5% (v/v) CO_2 - 95% (v/v) air atmosphere.

Bacterial strains and growth conditions. Strains of *B. cereus* were selected on the basis of previous studies. They have different genetic backgrounds related to virulence markers and lead to different biological responses when tested on cultured cells [27–30]. *B. cereus* strains M2 and 2 were isolated from skim milk powder and infant formula respectively [28] whereas strain B10502 was isolated from a food poisoning outbreak [29]. Microorganisms were grown in BHI broth (BIOKAR Diagnostics, Beauvais, France) supplemented with 0.1% (w/v) glucose (BHIG) at 32ºC for 16 h under agitation. Next, bacteria were inoculated (4% v/v) in fresh BHIG and further incubated at 32ºC for 3 h to obtain mid-log cultures. Bacterial concentration was evaluated by optical density (OD) readings at 600 nm (Spectronic Helios Spectrophotometer UV-Vis**,** Thermo Electron Corporation, England). The correlation between plate counts and OD_{600} was previously established. Bacteria were harvested by centrifugation for 10 min at 900 x *g*. Pellets were washed twice with PBS and suspended in PBS before infection of macrophages.

Fluorescent labelling of *B. cereus* strains. *B. cereus* strains were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, Oregon, USA). Microorganisms, grown as described above, were washed 3 times with PBS and suspended in RPMI-1640 medium. Afterwards CFSE was added to a final concentration of 5 µmol l^{-1} [39]. Microorganisms were incubated for 30 min at 37°C in the dark, and then they were washed twice with PBS to eliminate CFSE excess. Labelling was evaluated by fluorescent microscopy (Leica Microscopy Systems Ltd., Microsystems, Germany) and flow cytometry by using blue-green excitation light (488 nm argon-ion laser, FACSCalibur™, CellQuest™ software). Unlabelled *B. cereus* was used as negative control.

Fluorescence microscopy. Peritoneal macrophages (3 x 10^5 cells), suspended in RPMI 1640 medium, were infected with *B. cereus* strains at a multiplicity of infection (MOI) of 20 and incubated for 30 min at 37°C in a 5% (v/v) CO_2 – 95% (v/v) air atmosphere. After incubation, samples were put on ice and non-attached bacteria were removed by careful washing with cold PBS. Infected cells were spun onto cover glasses by centrifugation. Cells were fixed in ice cold ethanol for 5 min and washed with PBS. Staining was performed with acridine orange (Aldrich CHEM. Co., USA) at a final concentration of 0.5 µg ml^{-1} for 5 min at 0ºC. After being washed three times with PBS, slides were mounted in PBS containing 50% (v/v) glycerol. A minimum of 100 cells per sample were analyzed by fluorescence microscopy using a Leica DMLB microscope coupled to a Leica DC 100 camera (Leica Microscopy Systems Ltd., Microsystems, Germany). Three independent experiments were performed. Cells associated to at least one microorganism were considered in the calculation of the percentage of association.

Flow cytometry. Infection of peritoneal macrophages was conducted as described above with CFSE-labelled bacteria. After incubation for 30 min at 37°C, samples were put on ice and non-attached bacteria were removed by carefully washing with cold PBS. Evaluation of cells with internalized bacteria was conducted after quenching of exocellular bacteria with 0.2 % trypan blue solution (GIBCO Invitrogen Corporation, USA) for 2 min and further washing with PBS [39]. After quenching, cells

with internalized bacteria were found in region R2 of the scatter plot as indicated in Figure 2. Macrophage population (region R1 in Figure 2) was localized by labelling with biotin conjugated anti-mouse F4/80 and PE-streptavidin (eBioscience, USA).

Survival of *B. cereus* within murine peritoneal macrophages. Peritoneal macrophages were infected with bacteria as described above. After 30 min incubation, cells were washed twice with warm PBS, and then incubated for different times in the presence of gentamicin 100 μg ml^{-1} (Parafarm, Argentina) to kill exocellular bacteria. After different incubation times (up to 180 min reckoned from the beginning of the infection), macrophages were washed twice with PBS, lysed by addition of sterile distilled water and homogenized by 3-6 passages through a 22G needle. Appropriate dilutions were plated on nutrient agar (BIOKAR Diagnostics, Beauvais, France) and incubated at 37°C to determine viable counts.

Analysis of *B. cereus*-containing vacuoles (BcCV). RAW 264.7 cells (2 x 10^5 ml^{-1}) were seeded in 75 cm^2 tissue culture flasks (Greiner Bio One, Frickenhausen, Germany) and incubated at 37°C for 72 h (80% confluence). Macrophages were infected with CFSE-labelled *B. cereus* (strains 2, B10502 or M2) at MOI 20 and incubated at 37°C in a 5% (v/v) CO_2 - 95% (v/v) air atmosphere for 30 min. Afterwards, cells were washed twice with warm PBS, and then incubated for 1h at 37°C in a 5% (v/v) CO_2 - 95% (v/v) air atmosphere. Chloramphenicol (100 μg ml^{-1}) (Parafarm, Argentina) was added to prevent bacterial growth. Next, cells were exhaustively washed with PBS containing 0.2% (v/v) FBS and centrifuged at 100 x*g* for 5 min. Infected cells were suspended in 2 ml of homogenization buffer (HB: 250 mM sucrose, 0.1% (v/v) gelatin, 0.5 mM EGTA, 1:1000 protease inhibitor cocktail pH 7.4 (Sigma-Aldrich Co. St Louis, USA) and then centrifuged at 1800x*g* for 5 min. Pellets were suspended in 400 μl of HB and gently homogenized by 3-6 passages through a 22G needle. Homogenates were diluted to 2 ml with HB and vacuoles were obtained from the supernatants after 3 centrifugations at 100 x *g* for 5 min. This fraction constitutes the post-nuclear supernatant (PNS) and contains the BcCV. Afterwards, samples were incubated with 100 μl of mAb PE anti-mouse LAMP1 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in PBS containing 10 % (v/v) FBS and incubated for 30 min on ice. Samples were analysed by flow cytometry (FACSCalibur and Cellquest software Becton Dickinson). Double labelled particles were considered as LAMP (+) vacuoles containing bacteria. Analysis was performed, after appropriate gating on 300000 events per sample [39].

Immunofluorescence labelling. To determine the intracellular localization of the different strains of *B. cereus* in acidic compartments, labelling with Lysotracker DND-99 (Molecular Probes, Eugene, OR) was conducted. This compound selectively accumulates in cellular compartments of low pH (e. g. late phagolysosome). RAW 264.7 cells were seeded in 24-well tissue culture plates (Greiner Bio One, Frickenhausen, Germany) on round glass coverslips (Assistant, Sondheim, Germany). After 30 minutes of incubation with CFSE-labelled *B. cereus* strains (MOI 20), macrophages were washed to remove non-attached bacteria, and then incubated for 30 or 60 minutes at 37°C in a 5% (v/v) CO_2 - 95% (v/v) air atmosphere. Chloramphenicol (100 μg ml^{-1}) was added to prevent bacterial growth. Cells were washed twice with cold PBS and then incubated for 5 minutes at 37ºC with 200 nM Lysotracker [21]. Afterwards, macrophages were washed twice and fixed with 3% (v/v) PFA for 1 h at 4ºC in the dark. Mounting medium (DakoCytomation, USA) was used as antifading reagent. Microscopic analyses were performed using confocal laser-scanning microscope (Leica TCS SP5 Leica Microsystems, Germany) (see below).

To assess trafficking to non lysosomal compartments, transferrin uptake was determined. After the phagocytosis experiment (30, 45, 60 and 90 min), macrophages were depleted of transferrin by incubation in RPMI containing 1 % (w/v) BSA (RPMI-BSA) for 1 h at 37 ºC. Next, cells were incubated for 10 min at 4 ºC with 10 μg/mL of transferrin Alexa Fluor-594 conjugate (Molecular Probes, Oregon, USA) in RPMI-BSA to saturate non-specific endocytosis. Following, incubation for 5 min at 37 ºC was performed to allow transferrin internalization and then cells were washed with RPMI-BSA and incubated in RPMI-BSA for 45 min at 37 ºC. Finally cells were fixed with 3% (v/v) paraformaldehyde for 1 h at 4ºC in the dark. Mounting medium (DakoCytomation, USA) was used as antifading reagent.

Image analysis. Microscopy images were captured using a Leica TCS SP5 confocal laser-scanning microscope (Leica Microsystems, Germany) at excitation wavelengths of 488 nm (argon laser) and 594 nm (helium-neon laser). A HCX PL APO CS 63.0x1.40 OIL UV objective (Leica Microsystems, Germany) was used. The resolution of the resulting images was 8 bits (1024 x 1024 pixels). For each microscope image of a random region, 25 Z-section images were collected and stacked to form a 2D image by using the accompanying software LAS AF Lite (Leica Microsystems, Germany). The interval between each Z-section was 0.3 μm. All experiments were performed at room temperature.

For quantification and analysis of internalized bacteria and co-localization with Lysotracker three independent experiments were performed (see below). For bacteria smaller than *B. cereus* (*Bordetella spp*, *Salmonella spp*, etc) co-localization is evidenced as yellow spots (merge of red and green fluorescence channels). However, because of the size of *Bacillus cereus* (around 1 x 4 μm) a conventional image analysis is not appropriate. Hence, co-localization was considered as positive when CFSE-labelled bacteria were in close contact with a red-labelled region situated around the bacteria (Fig 4B). Different focal planes (XY, XZ, YZ) on 50-100 intracellular compartments containing bacteria were analysed for co-localization with Lysotracker.

Expression of MHCII and CD86 in macrophages. RAW 264.7 cells were seeded in 24-well tissue culture plates (Greiner Bio One, Frickenhausen, Germany). Cells were infected with *B. cereus* strains at MOI 20 and incubated 24 or 48 h at 37°C in a 5% (v/v) CO_2 - 95% (v/v) air atmosphere. *E. coli* lipopol-

ysaccharide (LPS) (1 µg ml^{-1}) (Escherichia coli 0111:B4, Sigma-Aldrich, Saint Louis, Missouri, USA) was used as a positive control. Phagocytosis assay was performed in the presence of chloramphenicol (100 µg ml^{-1}) to prevent bacterial growth. Next, cells were washed twice with PBS containing 2% (v/v) FBS and incubated with the antibody 30 min at 4ºC in the dark. The expression of CD86 and MHCII was assessed by using a PE-conjugated Anti-mouse MHC class II, PE-conjugated Anti-mouse CD86 (B7-2) according to the manufacturer´s instructions (eBioscience, San Diego, USA). Appropriate isotype controls were used. After staining, cells were washed and analyzed by flow cytometry as described above. The expression of surface antigens was calculated as follows: Expression Index (EI): percent of positive cells x mean fluorescence intensity.

Data analysis. Results were analysed by means of two-tail Student's t test using the InfoStat software (InfoStat, Version 2008, Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina). In order to show an overall picture of the behaviour of each strain, a radial graph (Fig. 6) was constructed. To this end, normalized values (Nv) were calculated as follows: Nv=(Xi-Xmax)/(Xmax-Xmin); where Xi: value of the variable for a given strain; Xmin: minimum value for the variable; Xmax: maximal value for the variable.

Results

Association of *B. cereus* to peritoneal macrophages. In order to determine the interaction of *B. cereus* with peritoneal macrophages, association (adhered plus internalized bacteria) was assessed by fluorescence microscopy.

When the percentage of macrophages associated to bacteria was analysed a strain depended behaviour was observed (Fig. 1). Indeed, higher association values were found for strain B10502 (50.9 ± 0.8 %) whereas strains M2 and 2 showed significantly lower (P<0.05) association values (24.4 ± 2.7 % and 31.9 ± 4.3 % respectively) (Fig. 1).

Phagocytosis assays. Phagocytic activity of peritoneal macrophages was analysed by flow cytometry after incubation of cells with CFSE-labelled bacteria. Percentage of phagocytes interacting with bacteria was analysed in cells gated in R1 (Fig. 2A) that corresponds to F4/80 (+) cells (data not shown). Fig 2B is a representative scatterplot for cells incubated without bacteria whereas results for cells incubated with CFSE-labelled bacteria (Fig. 2C, D, E) and quenched with Trypan blue are shown in Fig 2C, D and E. Percentages of CFSE (+) cells are shown in the upper right corner of the figures. The highest percentage of cells with internalized bacteria was found for strain B10502 (36.3 ± 3.8; Fig. 2C). This value was significantly higher (P<0.05) than that found for strain 2 (15.0 ± 2.9; Fig. 2E) whereas values for strain M2 (22.0 ± 7.0) were between those of strains B10502 and 2 (Fig. 2D).

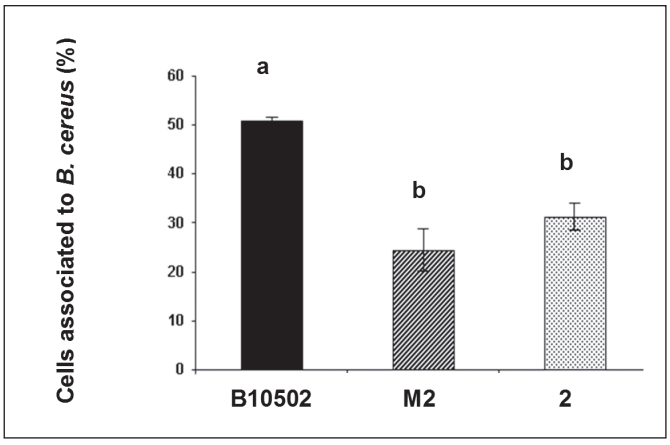


Fig. 1. Association of *B. cereus* to murine peritoneal macrophages. Peritoneal macrophages isolated from mice were inoculated with *B. cereus* B10502, 2 or M2 (MOI 20) and stained with orange acridine. Percentage of macrophages containing both attached and internalized microorganisms were determined. Different letters indicate significant differences (P<0.05).

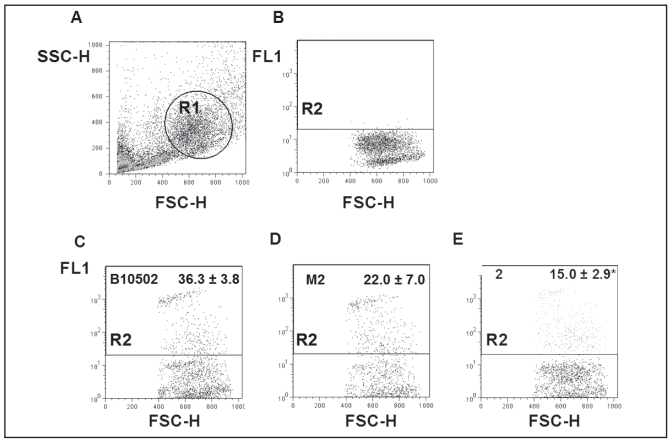


Fig. 2. Flow cytometry analysis of the interaction between *B. cereus* and murine peritoneal macrophages. Assays were conducted with CFSE-labelled *B. cereus* strains B10502, 2 or M2. A. FSC *vs* SSC plot of peritoneal macrophages showing selected R1 region corresponding to F4/80 (+) cells. B. Non-infected macrophages gated in R1.

C. D. E. Macrophages infected with CFSE-labelled *B. cereus* strains. Cells interacting with bacteria are shown in R2 (FL1+). Percentages of FL1(+) cells in the R1 population are shown in the upper right corner. Fluorescence of exocellular bacteria was quenched with Trypan blue. Results are representative from three independent experiments and are expressed as means ± standard error. Asterisks indicate significant differences as compared with *B. cereus* B10502 (P<0.05).

Survival of *B. cereus* in peritoneal macrophages. Results of association above mentioned prompted us to assess intracellular survival of microorganisms.

As shown in Figure 3, intracellular survival of *B. cereus* after 90 min infection (30 min infection + 60 min gentamicin) was strain-dependent. Values of viable counts for strain 2 were sig-

nificantly higher ($9.7 \pm 0.3 \times 10^2$ cfu/ml; $P<0.05$) than those for strains B10502 ($2.5 \pm 3.5 \times 10^1$ cfu/ml) and M2 ($2.8 \pm 0.2 \times 10^2$ cfu/ml). When survival was evaluated at longer incubation periods, viable counts were under detection limits (data not shown). It was not possible to evaluate survival at times shorter than 90 min since we had to perform 1 h incubation with gentamycin in order to kill exocellular bacteria. During this period, changes in intracellular compartments cannot be ruled out.

Fate of internalized bacteria. Flow cytometry was used to analyse large numbers of individual vacuola isolated from infected cells [34]. At 90 min post-infection (Table 1), BcCV acquired lysosomal associated membrane protein LAMP1 in a strain-dependent manner. Percentages of LAMP1 (+) vacuoles containing strain M2 (25.3 ± 1.2) were significantly higher ($P<0.05$) as compared with LAMP1 (+) vacuoles containing either strain B10502 or strain 2 (11.6 ± 1.4 and 10.9 ± 5.4 respectively).

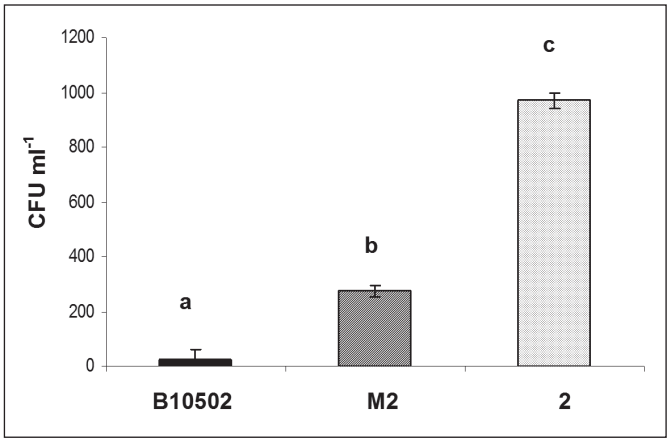


Fig. 3. Survival of *B. cereus* in peritoneal macrophages. Macrophages isolated from peritoneal cavity of BALB/c mice were infected with *B. cereus* B10502, 2 or M2 (MOI 20) and incubated for 90 min at 37°C. Viable bacteria in cell lysates were determined by plate counts. Results were expressed as means of CFU $ml^{-1} \pm$ standard error. Different letters indicate significant differences ($P<0.05$).

Table 1. Analysis of *B. cereus* containing vacuoles (BcCV) in Raw 264.7 cells incubated with different CFSE-labelled strains[1]

Strain	Percentage of LAMP1 (+) vacuola[2, 3]
B10502	11.6 ± 1.4^a
M2	25.3 ± 1.2^b
2	10.9 ± 5.4^a

[2]Results are expressed as means ± standard error from three independent experiments. Different letters indicate significant differences ($p<0.05$).
[3] Percentage of LAMP1 (+) was calculated as: double labelled events (FL1(+)-FL2(+))/total FL1 (+) events.

Localization of bacteria in acidic compartments was assessed by confocal microscopy. Figure 4B shows representative confocal fluorescence image of Lysotracker (red) co-localization with CFSE-labelled *B. cereus* strain B10502 (green). Lysosomal compartments were evidenced as well defined corpuscular regions within the cytoplasm. When bacteria were within a lysosome, red label (Lysotracker) was in close contact with green-labelled bacteria. In order to properly analyse confocal images, quantitative image analysis was performed. At 60 min post-infection, values of co-localization with Lysotracker were significantly higher ($P<0.05$) for strains B10502 (72.0 ± 7.4 %) and M2 (70.0 ± 7.0 %) as compared with strain 2 (53.5 ± 4.9 %). At 90 min post-infection, values for strains B10502 (75.8 ± 5.8%) and M2 (85.2 ± 8.7 %) were significantly higher ($P<0.005$) than for strain 2 (66.8 ± 7.6 %). Only for strain 2, co-localization with Lysotracker was higher at 90 min as compared with values obtained at 60 min post-infection ($P<0.05$; Fig. 4A). Interestingly at early stages of the interaction (30 and 45 min post infection), no co-localization of bacteria with Lysotracker was found (data not shown).

In order to assess routing to recycling endosomes, co-localization with transferrin was monitored by laser confocal microscopy. No co-localization of bacteria with transferrin was found at any of the timepoints assayed (data not shown).

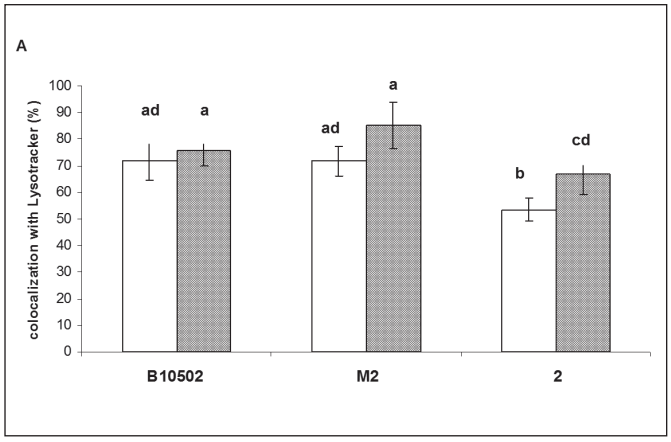
Expression of MHCII and CD86 after infection. The expression of MHCII and co-stimulatory molecules, such as CD86, was assessed in infected and uninfected RAW 264.7 cells. The expression index (EI) of MHCII significantly increases after 48 h of incubation with strain M2 (EI: 377.0 ± 89.1) as compared with strains B10502 and 2 that lead to low expression of this marker (EI: 104.2 ± 41.9 and 96.5 ± 50.8 respectively) (Fig. 5 $^{**}P< 0.005$). No changes in the expression of CD86 were detected (data not shown).

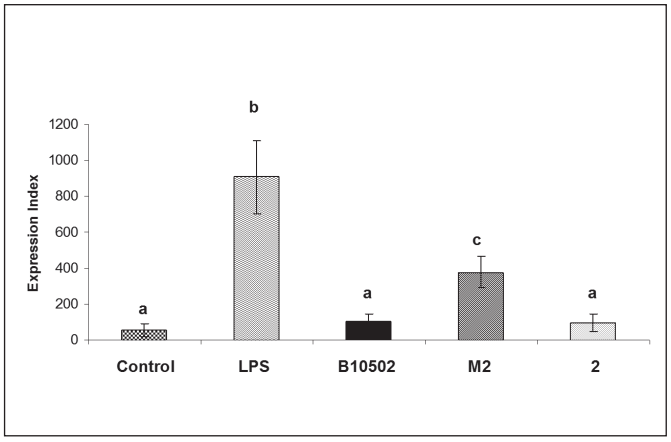
Multivariate analysis. In order to analyse together the studied variables, a multivariate analysis was conducted by means of a radial graph after data normalization as indicated in the Materials and Methods section (Fig. 6).

Strain B10502 showed the highest values of association/internalization. However, strain M2, that is internalized in a lower extent, route more efficiently to acidic and LAMP (+) intracellular compartments. Strain 2, on the other hand, showed lowest association and internalization values as well as lower values of routing to degradative compartments as compared with both B10502 and M2 strains.

Discussion

It is known that *B. cereus sensu stricto* belongs to a group of microorganisms that share many common characteristics but





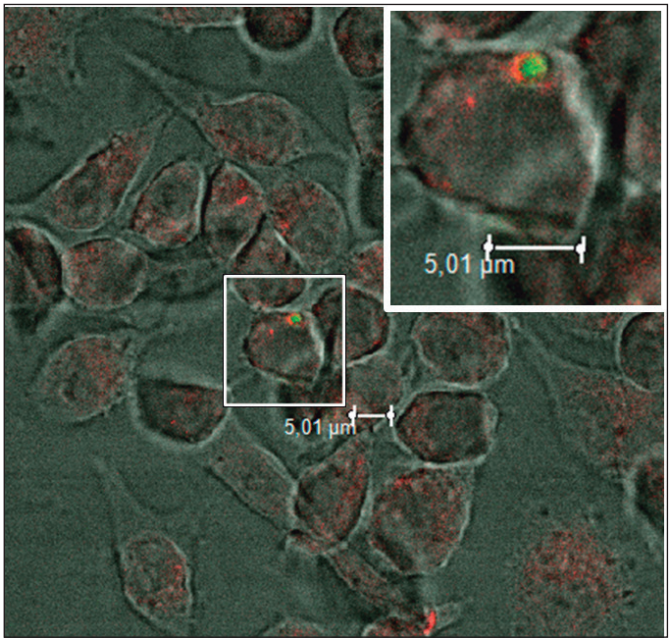


Fig. 4. A. Co-localization of CFSE-labelled *B. cereus* with Lysotracker in Raw 264.7 cells. After 60 (white bars) or 90 (grey bars) min infection, cells were labelled with Lysotracker and co-localization was analysed by confocal microscopy. Results were expressed as means ± standard error of the percentage of bacteria co-localizing with Lysotracker referred to the total of bacteria analysed. Different letters indicate significant differences (P<0.05). B. Macrophages incubated with *B. cereus* (MOI 20) at 37 ºC for 90 min. Confocal image showing CFSE-labelled *B. cereus* B10502 within acidic compartment (Lysotracker; red) in Raw 264.7 cells. Z-section images were collected and stacked to form a 2D image (XY). In the inset, there is an enlarged image of the selected region. Figure shows a representative image from three independent experiments (Logical size 1024 x 1024)

Fig. 5. Expression of MHCII in Raw 264.7 cells infected with *B. cereus* strains (MOI 20) after 48 h incubation. The expression of MHCII was assessed by using a PE-conjugated Anti-mouse MHC class II. LPS was used as a positive control. Expression index was calculated as EI = percentage of positive cells x mean fluorescence intensity. Different letters indicate significant differences (P< 0.005). Data were processed using the CellQuest software (BD Biosciences). Results are representative of three independent experiments.

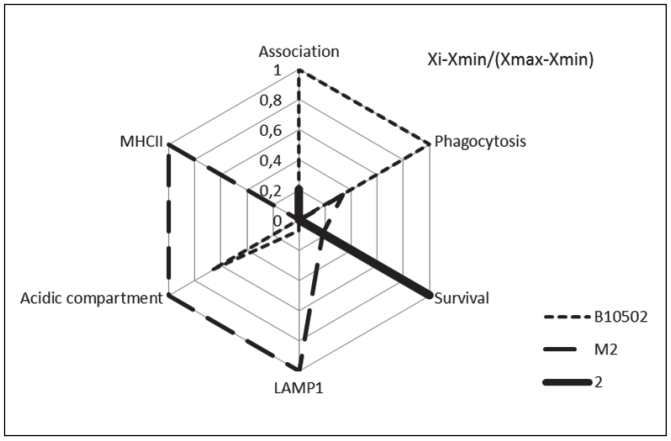


Fig. 6. Multivariate analysis of the interaction of strains 2, M2 and B10502 with phagocytic cells. Values were normalized as indicated in the Materials and Methods section.

show a wide range of virulence potential. This so-called *B. cereus sensu lato* group includes *B. cereus sensu stricto*, *B. anthracis*, *B. mycoides*, *B. thuringiensis*, *B. pseudomycoides, B. weihenstephanensis* and *B. cytotoxicus* [12, 13, 23]. This group show a very homogeneous genetic background and it has been proposed that some members (i. e. *B. cereus*, *B. anthracis* and *B. thuringiensis*) constitute single species [15]. Certainly, *B. anthracis* has the highest pathogenic potential and its ability for surviving within phagocytic cells is one of the hallmarks of the pathogenesis of this microorganism. Indeed, it has been demonstrated that spores of *B. anthracis* survive intracellular, germinate and scape from phagocytic cells. This ability, related to the transactivator AtxA located on the pXO1 plasmid, is a key event for systemic spreading [7]. Even though invasiveness of *B. cereus* is limited, there are reports on systemic dissemination in severe cases [14, 32, 37].

Macrophages are key players of the first line of defence against most bacterial pathogens. Concerning elimination of spore forming microorganisms such as *B. cereus*, it has been

demonstrated that macrophages can promote germination of intracellular spores that in turn determines killing of intracellular bacteria. These features correlate with the proposed protective role of macrophages in the infection with *Bacillus* spores [33].

In the present study we explore the ability of vegetative cells of different *B. cereus* strains to interact with professional phagocytic cells in an *in vitro* infection model in non-opsonic conditions. Our data reveal different patterns of association between macrophages and *B. cereus* strains.

Values of association and phagocytosis indicate that phagocytic cells efficiently interact with strain B10502 and, to a lesser extent, with strains 2 and M2 (Fig. 6). In addition, strain B10502 showed the lowest values of intracellular survival. Strain 2, on the other hand, showed the highest survival values although this strain routed to vesicles LAMP1(+) at similar levels of those for strain B10502. Even though results showed in table 1 are a snapshot of the distribution of bacteria within intracellular compartments after 90 min incubation, it is evident that there is a strain-dependent kinetics of routing to lysosomal compartments.

Quantitative analysis by flow cytometry of phagosomal markers provides a basis for investigating phagosome maturation [16]. Therefore, we applied this experimental procedure to examine the co-localization of *B. cereus* with the late endosomal/lysosomal glycoprotein LAMP1 which is a distinctive marker of late endosomal and lysosomal compartments. Its concentration has shown to increase in the phagosoma membranes during progression from early to late phagosome and phagolysosomal stages [17].

The highest values of co-localization in LAMP1(+) compartments were found for strain M2. Labelling with Lysotracker also demonstrated that routing to intracellular acidic compartments was higher for strain M2. These findings correlate with the high MHCII expression by cells infected with strain M2 and low intracellular survival (high antigen processing and presentation). Interestingly, strain M2 is the sole strain of this study that is positive for sequences or the piplC gene that encodes for phospholipase C [27]. This correlation could be relevant given the role of phosphorylated lipids in phagosome maturation [35].

Strains B10502 and 2 showed a particular behaviour, i. e. strain 2 lead to the highest values of intracellular survival and low values of MHCII expression. In contrast, with similar values of trafficking to LAMP1(+) compartments and induction of MHCII expression, strain B10502 showed the lowest ability to survive in intracellular compartments. It is worth to note that strains under study have demonstrated different levels of virulence *in vitro*. Whereas strains 2 and M2 were able to internalize in cultured human enterocytes [27, 29, 30] strain B10502 was not. Furthermore, in a multivariate analysis it has been demonstrated that these strains have different background related to the presence of virulence genes [27]. These characteristics probably impact on the kinetics of acidification of intracellular compartments as well as on the acquisition of lysosomal markers [9].

Interestingly, strains 2 and B10502 differ in genetic background related to virulence traits. Strain 2 is positive for the sequences of nheB and nheC genes (non-haemolytic enterotoxin) as well as for the sph gene (sphyngomielinase) [27]. These factors have demonstrated synergistic effect for cytotoxicity *in vitro* [8].

Concerning *B. cereus*, there is scientific evidence on the ability of spores to survive, germinate and escape from macrophages. This ability is related to InhA metalloproteases associated with both vegetative and sporulated forms [5, 33] that is a major component of the *B. cereus* exosporium [6] and it is also secreted during vegetative growth [11].

In the present work we demonstrate for the first time that vegetative *B. cereus* routed to phagolysosome shortly after internalization in a strain-dependent manner. We found that there are viable intracellular microorganisms after 90 min co-incubation bacteria-macrophages in non-opsonic conditions. After 2 h incubation, no viable microorganisms were found. These findings could be due to complete killing of intracellular microorganisms or to the escape from macrophages and subsequent killing by gentamycin. However, the lack of microorganisms co-localizing with transferrin is compatible with routing to lysosomal compartments.

It is worth noting that, intracellular survival is not only relevant for spreading of infections but also to shape host´s immune response by carrying intestinal bacteria to adjacent mesenteric lymph nodes [25]. Our results suggest strain-dependent kinetics of processing of *B. cereus* by professional phagocytic cells. Even though *B. cereus* is not an intracellular pathogen, some strains seem able to route slower than others to degradative compartments thus leading to both differential intracellular survival and stimulation of phagocytes.

We hypothesize that cell response (e. g. MHC II and CD86 expression) depends on the different kinetics of routing to intracellular compartments. As far as we know, our report is the first contribution on the relevance of a transient intracellular lifestyle of *B. cereus*. It has been previously demonstrated [27] that biological effects *in vitro* correlate with the presence of sequences of virulence genes. We are obviously aware that correlation does not indicate causation but our findings could give a clue for further research to elucidate the mechanisms involved in the virulence of *B. cereus*.

Even though the mechanisms involved in the differential processing of *B. cereus* strains by macrophages remain unknown, our findings suggest that the course of *B. cereus* infection could also depends on the kinetics of the routing of ingested microorganisms to lysosomal compartments. Further investigation is needed to elucidate the mechanisms behind differential intracellular fate of *B. cereus* in macrophages.

Acknowledgements. Strain B10502 was kindly provided by the Laboratorio Central de Salud Pública de la Provincia de Buenos Aires, Argentina. Current filiation for Ivanna Rolny is IIFP. Instituto de Estudios Inmunológicos y Fisiopatológicos.(CONICET-UNLP). Facultad de Ciencias Exactas Universidad Nacional de La Plata. Pablo F. Pérez is a member of the Carrera de Investigador Científico y Tecnológico of the CONICET. This work was supported by the Agencia Nacional de Investigaciones Científicas y Tecnológicas (ANPCyT), CONICET and Universidad Nacional de La Plata.

Competing interests. None declared

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