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A method to purify bacteria-containing phagosomes from infected macrophages

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Abstract. When small particles, such as microorganisms, are taken up by macrophages, they are wrapped with a portion of the host cell plasma membrane and ingested, creating a new organelle, the phagosome. This phagosome matures stepwise as newly formed endosomes do, finally forming a phagolysosome, a process that contributes to killing of ingested microbes and to the presentation of microbial antigens on the surface of the phagocyte. Some pathogenic bacteria, however, reprogramme the phagocytic cell in such a way that the phagosome will either be arrested in an early stage of maturation or will be diverted and create an unusual, novel phagosomal compartment. To study the molecular processes that underly biogenesis of bacteria-containing phagosomes, we have established a method to isolate and to biochemically analyse bacteria-containing phagosomes. This method consists of mechanical lysis of infected macrophages, production of a postnuclear supernatant followed by fractionation in a discontinuous sucrose density gradient, separation through a Ficoll cushion, and by a final concentration step. These phagosome preparations contain very little endosomal or lysosomal contamination (the organelles of most concern when studying phagosome biogenesis) and very little Golgi- and plasma membrane-derived contamination, but do contain some mitochondrial and ER contamination. This method could also be used to study bacterial factors (proteins, RNA) produced while in phagosomes.

Key words: Macrophage, Organelle, Pathogenic bacteria, Phagocytosis, Phagosome

Abbreviations: HRP = horseradish peroxidase; LAMP-1 = lysosome-associated membrane protein 1; OD_{600} = optical density at 600 nm

1. Introduction

When a foreign particle, usually a microorganism, enters the sterile section of the human body, it is quickly taken up and digested by so-called professional phagocytic cells (i.e., in particular, polymorphonuclear lymphocytes and macrophages). The particle is surrounded by a portion of the host cell plasma membrane, followed by membrane fusion on the tip of the nascent membrane bag to form a new cytoplasmic organelle, the 'phagosome'. Recent evidence shows that phagosomes 'mature' after formation and that they are compatible for fusion with lysosomes only after many changes of their composition, opposed to previous interpretations that phagosome-lysosome fusion was a one-step-process in macrophages. Soon after phagosome formation, the phagosome lumen is flushed with protons (to a pH of 5.0 and below) by a newly incorporated phagosomal proton-pumping ATPase complex [29]. Acquisition of lysosomal hydrolases by the phagosome through biosynthetic trafficking into the phagosome and, particularly, by fusion with late endocytic compartments exposes the microbe to various acidic hydrolases, such as the cathepsin proteases. In addition, toxic oxygen derivatives are produced on the plasma and phagosome membranes by a newly assembled NADPH-oxidase complex and released into the phagosomal lumen, further contributing to microbial killing [27, 41]. Antibacterial peptides (defensins) and compounds related to nitric oxide are released into the phagosome lumen, further contributing to the elimination of microbes.

We now know that phagosomes possess a complex protein pattern, that they lose and acquire various compartmental markers during their coordinate transformation into phagolysosomes [13, 14, 25, 26, 31, 38], that phagosomal membranes acquire typical lysosomal features over time [26] concommitantly with changes in the phosphorylation pattern of phagosomal proteins [16], and that phagosomes are transported along microtubules within the host cell [4, 5, 13, 14] and interact with host cell actin [12]. Maturation also leads to the selective removal of

some proteins (particularly cell surface receptors) from the phagosome membrane within three to five minutes after uptake. These proteins are then recycled to the cell surface for reuse [3, 4, 34, 35, 37, 42]. All these molecular changes are due to the vectorial trafficking of phagosomes along (or in parallel with) the endosomal pathway.

Most interestingly, several species of pathogenic, 'intracellular' bacteria manage to reprogram their host macrophages for their purpose: *Mycobacterium tuberculosis* (causing tuberculosis) inhibits acidification of its phagosomes [43] and arrests phagosome maturation at an early stage with several characteristics of early recycling endosomes [39]. This compartment also contains immature cathepsin D, acquired from a biosynthetic pool rather than from lysosomes [44]. *Legionella pneumophila* (causing legionaire's disease) diverts maturation of its phagosome so that a novel compartment is produced that contains neither endocytic nor lysosomal marker proteins but which associates closely with elements of the rough endoplasmic reticulum and mitochondria [23]. Similarly, *Chlamydia trachomatis*, a bacterium that can only grow within eukaryotic cells (maybe, even only in mammalian cells) causes the generation of a phagosomal compartment that is disconnected from the endosome-lysosome continuum, but it does intersect with the secretory pathway, causing the fusion of sphingomyelin-containing vesicles with the phagosome [21].

A clear technical progress for phagosome analysis was the use of latex bead-containing phagosomes (LBCPs) whose low density greatly allows the one-step floatation of phagosomes on discontinuous sucrose gradients. Using this method, phagosomes from human and murine monocyte cell lines were shown to contain more than 200 different proteins over all maturation stages ([8, 13, 14, 37]; for recent comprehensive lists of proteins identified on phagosomes in various systems, see [18, 20]. Also, their phospholipid composition varies over the time of maturation [13, 14].

Despite all the merit of the latex bead phagosome system (and, similarly, of phagosomes containing synthetic, paramagnetic beads; [9, 30]), the study of these phagosomes also has its drawbacks: Several pieces of evidence suggest that the content of a phagosome critically contributes to its maturation and therefore that the phagosome is not really just a membrane bag around a passive particle [6, 19, 33, 45]. Therefore, latex bead-containing phagosomes are one interesting model system which, however, probably describes events during maturation of these particular phagosomes and possibly not so much of phagosomes in general. Many more studies are needed to understand phagosome maturation after ingestion of living material such as non-pathogenic and pathogenic microorganisms, i.e., physiological substrates for these phagocytes. This line of research, too, would greatly profit from the isolation of the corresponding phagosomes which is not readily accomplished due to variations in size, shape, and density of microorganisms and also due to the fact that bacteria migrate very similar to lysosomes and mitochondria in various density gradient systems.

Although a few protocols for purification and analysis of phagosomes containing bacteria are now available [1, 9, 32, 38, 43], each of them has its (dis)advantages and there may actually be no protocol that can be used for all bacteria-containing phagosomes with the same chance of success. Here, we present a purification protocol which we have, so far, successfully applied to the isolation of phagosomes containing differently sized and differently shaped bacteria belonging to the genera *Listeria* and *Rhodococcus* (Gram positive bacteria) as well as *Afipia* and *Bordetella* (Gram negative bacteria) and, hence, seems to be a versatile tool. Our hope is that, as intracellular trafficking of phagosomes has become a very active branch of the recently defined field 'Cellular Microbiology' [11], methods like this will find widespread use, and will contribute to a better understanding of the cell biology of infection.

2. Materials

A. Chemicals
- ABTS [2,2′-Azinobis(3-ethylbenzthiazoline 6-sulfonic acid)] (Sigma, A-1888)
- Benzonase (Merck, 101653)
- BioRad DC Protein Assay (Bio Rad, 500-0116)
- Brain Heart Infusion (Difco, 237300)
- Bromphenol blue (Merck, 8122)
- Cacodylic acid sodium salt trihydrate (Roth, 5169.2)
- Citric acid (Sigma, C-7129)
- di-Sodium hydrogen phosphate (Merck, 1065855000)
- Dodecenylsuccinic anhydride (Serva, 2075502)
- E64 [trabs-Epoxysuccinyl-L-leucylamido-(4-guanidino)butane] (Sigma, E-3132)
- ECL chemiluminiscence kit (Amersham Pharmacia Biotech, RPN-2106)
- EGTA [Ethylene Glycol-bis(β-Aminoethyl Ether)-N,N,N′,N′-Tetraacetic Acid] (Sigma, E-4378)
- Ficoll – 70,000 Mw (Sigma, F-2878)
- Formaldehyde (Sigma, F-1268)
- Glutardialdehyde (Merck, 1042391000)
- Glycerol (87%) (Merck, 1040922500)
- Glycid ether (Serva, 2104502)
- Hepes (Serva, 25245)
- Horseradish peroxidase (Sigma, P-8375)
- Hydrogen peroxide (Fluka, 95313)
- Leupeptin hydrochloride (Sigma, L-0649)

- β-mercaptoethanol (Roth, 42271)
- Methylnadic anhydride (Serva, 2945203)
- *p*-nitrophenyl-β-D-galactopyranoside (Sigma, N-1252)
- Osmium tetroxide (Roth, 83713)
- Pefabloc (Boehringer Mannheim, 1429868)
- Pepstatin A (Sigma, P-5318)
- 1,10-phenanthroline monohydrate (Sigma, P-9375)
- Potassium chloride (Merck, 104935)
- Potassium dihydrogen phosphate (Merck, 4871)
- Potassium hydroxide (Merck, 5033)
- Propylene oxide (Merck, 1124921000)
- Sodium acetate (Merck, 106268)
- Sodium carbonate (Merck, 1063925000)
- Sodium chloride (Roth, 39572)
- Sodium dodecyl sulfate (Roth, 23262)
- Sucrose (Sigma, S-0389)
- Tris (Roth, 54292)
- 2,4,6-Tris(dimethylaminomethyl)phenol (Serva, 3697503)
- Triton X-100 (Sigma, T-9284)
- Uranyl acetate dihydrate (Merck, 1084730100)

B. Plastic tubes and similar consumables
- For ultracentrifugation: SW40 tubes (Beckman; through Konrad Beranek, 7031)
- For centrifugation and handling in 1.5 ml tubes: sialinized tubes (Sarstedt, 72.690.550)
- X-ray films 13 × 18 cm, 100NIF (Noras, LAB-30102)
- Cultivation plates for macrophages, 9 cm diameter (Falcon; Laborbedarf Schubert, 353003)
- PVDF membranes for protein blotting (Pharmacia Biotech, RPN-2020F)

C. Cell growth media
- Brain Heart Infusion Broth (BHI)
- BCYE-Agar (as described in [15])
- RPMI 1640/2 mM glutamine (Gibco, 21875-034)
- Fetal calf serum (FCS) (Gibco, 10270-106). The complement contained in each preparation was heat-inactivated by a 30 min incubation at 56 °C before addition to RPMI 1640.

D. Additional equipment
- Dura Grind stainless-steel homogenizer (Wheaton Scientific; through Zinsser Analytic, 357572)
- ELISA-Reader (Dynatech Laboratories, MR-600)
- Refrigerated table-top centrifuge (Eppendorf, 5412)
- Rubber policeman (Hartenstein, GWSP)
- Spectrophotometer (Pharmacia Biotech, Ultraspec III)
- Ultracentrifuge (Beckman, L8-55M)

E. Antibodies
1. Primary polyclonal rabbit antibodies
   A. . . . to calnexin (A. Helenius, ETH, Zürich; [22])
   B. . . . to GM-130 ('anti-N73-pep' from M. Lowe, University of Manchester; [36]);
   C. . . . to TOM-20 (Drs. K. Mihara and H. Suzuki, Kyushu University Graduate School of Medicine; [24]).
2. Primary monoclonal rat antibody
   A. . . . monoclonal rat antibody to murine LAMP-1 (1D4B) was donated by U. Schaible (MPI for Infection Biology, Berlin) and also obtained from the Developmental Study Hybridoma Bank (University of Iowa; http://www.uiowa.edu/~dshbwww/list.html).
3. Secondary horseradish peroxidase-labeled antibodies
   A. Goat anti-rabbit IgG (Dianova, 111035003), 1:4,000
   B. Goat anti-rat IgG (Dianova, 112035003), 1:4,000

F. Bacteria and mammalian cells
1. Bacterial strains
   A. *Listeria innocua* serotype 6b (strain collection, Lehrstuhl für Mikrobiologie, Universität Würzburg)
   B. *Afipia felis*, ATCC 53690
2. Mammalian cell line
   A. Murine macrophage-like cell line J774E [37, 38] from P.D. Stahl (Washington University, St. Louis, USA). This cell line strongly expresses the macrophage plasma membrane mannose receptor.

3. Procedures

A. Preparation of solutions and buffers
1. Cacodylate buffer (0.2 M)
   - 42.8 g cacodylic acid sodium trihydrate
   - fill up to 1 l with aqua dest
   - adjust to pH 7.2
2. Epon 812
   prepare fresh; mix the following:
   - 12.30 g Solution A (stock prepared as follows)
     - 37.20 g Glycid ether
     - 50.10 g Dodecenylsuccinic anhydride
   - + 10.40 g Solution B (stock prepared as follows)
     - 54.25 g Methylnadic anhydride
     - 60.00 g Glycid ether
   - + 0.40 g 2,4,6-Tris(dimethylaminomethyl) phenol
3. Ficoll cushion
   - 15% (w/v) Ficoll (Mw 70,000)
   - 5% sucrose [w/v]

- 0.5 mM EGTA (pH 8.0)
- 20 mM Hepes/KOH (pH 7.2)

4. Homogenization buffer (HB)
   - 250 mM sucrose
   - 0.5 mM EGTA
   - 20 mM Hepes/KOH (pH 7.2)
5. HRP activity determination mixture
   - 250 µl Triton X-100 (20% v/v in aqua dest)
   - 2.5 ml 1 M sodium acetate (pH 5.0)
   - 7.5 ml deion water
   - 5 mg ABTS
   - 3.4 µl H_2O_2 (add last, just before starting the reaction!)
6. Karnovsky buffer
   - 2.5 ml formaldehyde (8% in deion. water)
   - 1.0 ml glutardialdehyde (25%)
   - 5.0 ml cacodylate buffer, 200 mM, pH 7.2
   - 1.5 ml aqua dest
7. Laemmli buffer
   - 5 ml 1M Tris/Cl (pH 6.8)
   - 0.8 g sodium dodecyl sulfate
   - 4.6 ml glycerin (87%)
   - 0.4 ml β-mercaptoethanol
   - 0.01% bromphenol blue
   - fill up to 25 ml with aqua dest.
8. PBS (phosphate-buffered saline)
   - 8 g NaCl
   - 0.2 g KCl
   - 0.2 g KH_2PO_4
   - 2.5 g Na_2HPO
   - fill up to 1 l with aqua dest
   - adjust to pH 7.2
9. Protease inhibitor cocktail (final concentrations)
   - [stock solutions were kept at –20 °C]
   - leupeptin, 1 µM (stock solution 1 mg/ml deion. water)
   - 1,10-phenanthroline, 0.75 mM (stock solution 99.1 mg/ml ethanol)
   - Pepstatin A, 1 µM (stock solution 1 mg/ml deion. water)
   - E64, 2 µM (stock solution 1 mg/ml deion. water)
   - Pefabloc, 1 mM (stock solution 50 mg/ml deion. water)
10. Sucrose solutions for density gradients
    - 65% (w/v) or 55% or 32.5% or 10% sucrose
    - 0.5 mM EGTA (pH 8.0)
    - 20 mM Hepes/KOH (pH 7.2)

    As an example, 65 g sucrose are weighed into a measuring zylinder, 1 ml 50 mM EGTA (pH 8.0) and 10 ml 200 mM Hepes/KOH (pH 7.2) are added, and deion water is added up to 90 ml volume. Once the sugar has dissolved, the total volume is adjusted to 100 ml with deion water. Keep these solutions at 4 °C.

B. Cultivation of bacteria
   1. *Listeria innocua*: Grow in BHI broth shaking at 190 rpm overnight at 37 °C and harvest by centrifugation in a table top minifuge at 8,000 rpm (5,600 ×*g*) and room temperature for 5 min. Determine OD_{600} as a measure for indirect cell count. 1 OD_{600} corresponds to 2×10^8 live bacteria.
   2. *Afipia felis*: Grow fresh streaks of *Afipia felis* on BCYE agar plates [7] for three days at 30 °C, collect from these plates with a sterile inoculation loop and resuspend in 1 ml PBS. Determine OD_{600} as a measure for indirect cell count. 1 OD_{600} corresponds to 2.8×10^8 live bacteria.

C. Cultivation of macrophages
   - Macrophages are grown with RPMI1640/2 mM glutamine/10% FCS in cell culture dishes with 9 cm diameter to semiconfluence and splitted (1:2) every 2 days after changing the media carefully scraping the cells off the dish with a rubber policeman. Splitted cultures are grown at 37 °C/5% CO_2 in a water steam-saturated atmosphere.

D. Infection of macrophages
   - Split macrophages (1:2) into fresh media into new cultivation dishes two days before the experiment. On the day of the experiment, 12 cultivation plates (9 cm diameter each) with approximately 80% confluent macrophages (corresponding to a total of $1–2 \times 10^8$ macrophages for each phagosome preparartion) are used. Incubate cultures for 60 min at 37 °C/5% CO_2 with the bacteria at a multiplicity of infection (MOI = bacteria/macrophage) of 10. Discard medium with contained bacteria and wash the macrophage monolayers three times with 10 ml 37 °C PBS per dish each to remove non-ingested bacteria. Add 5 ml fresh warm medium per plate and incubate the infected macrophages for another 2 hrs at 37 °C/5% CO_2 to complete maturation. After this chase, discard the warm medium and add 2 ml ice-cold PBS (for each plate) in which the infected macrophages are carefully scraped off using a rubber policeman. Collect macrophages by centrifugation in a Hettich centrifuge at 700 rpm (55 *g*) for 7 min at 4 °C. Wash the resulting pellet once in 30 ml ice-cold PBS, spin the suspension as above, and suspend the resulting pellet in 20 ml ice-cold homogenization buffer. Spin at 1,000 rpm (110 *g*) at 4 °C for 7 min, discard the supernatant, and resuspend the pellet in 2 ml HP without EGTA, but containing the protease inhibitor cocktail.

E. Breaking of infected cells
   - Lyse the infected macrophages by 10 strong

strokes in a Dura Grind stainless-steel homogenizer. Make every effort to avoid the development of air bubbles and foam in the preparation as this can lead to membrane and protein denaturation. Use light microscopy to determine the optimal level of lysis: When about 70–80% of all macrophages are lysed, the samples should not be sheared any further, as otherwise phagosome damage would be exceedingly high and the overall yield in intact phagosomes would be decreased. Also, the number of strokes necessary depends on the cell type used. Primary murine macrophages, e.g., are usually much easier to lyse than corresponding immortalized cells. To remove unbroken macrophages and whole nuclei, the lysate is spun for 3 min at 440 *g* and and 4 °C in a table-top centrifuge. We have confirmed the virtual absence of a nuclear marker (lamin) from this postnuclear supernatant (PNS).

F. Treatment of PNS with Benzonase
   - Treatment of the postnuclear supernatant with nuclease-digesting enzymes. Polymeric nucleotides (DNA in particular) can produce a sticky, gel-like consistency of the PNS. Therefore, as one must fear that phagosomes will stick to other (unrelated) organelles, we treat with Benzonase at this point (50 U for each 2 ml PNS, corresponding to 2 µl of the commercially available enzyme solution). Benzonase is a protease-free enzyme mixture which degrades all types of polynucleotides. By warming the samples to 37 °C for 5 min in the continued presence of the protease inhibitor cocktail (see above), a much more liquid consistency of the samples is reached.

G. Production of a postnuclear supernatant (PNS)
   - Clear the resulting cell homogenate from unlysed macrophages and intact nuclei by a centrifugation step in a Hettich centrifuge at 2,000 rpm (440 *g*), 4 °C, for 3 min. Carefully remove this 'postnuclear supernatant', either discard the pellet or carefully resuspend in homogenization buffer (including protease inhibitor cocktail), followed by a repeated centrifugation step and combining the resulting PNS with the first. It should be noted, however, that this procedure increases the total volume of PNS-requiring modified centrifugation conditions below. For all steps from here on, we advise that the phagosome material should be dealt with very carefully. Phagosomes are labile organelles and any shearing can (and usually will) result in mechanic damage. We use swing out-rotors whenever possible (i.e., even with mircoliter tubes) so that phagosomes do not 'smear' along the tube walls, but rather move directly towards the conical bottom of the tube to be collected in a small pellet. For the same reason, we usually employ sialinized tubes. Furthermore, we cut the tips from pipetting tips before use, as this treatment, too, minimizes shearing of the phagosome-containing samples.

H. The first ultracentrifugation step: A discontinuous sucrose gradient
   - Adjust the resulting approximately 2 ml solution to [39% (w/v) sucrose/0.5 mM EGTA/20 mM Hepes/KOH, pH 7.2] using the 65% sucrose solution described above (to a total volume of approximately 4.4 ml), and load it in a SW40Ti tube on top of a prepipetted 1 ml of 65% sucrose solution/2 ml of a 55% sucrose solution steps. On top of the sample, add 2 more steps (32.5% and 10% sucrose solution) and spin the resulting 5-step gradient (see Figures 1 and 2) for 1 hr at 100,000 *g* (SW40Ti rotor in a Beckman centrifuge, 28,400 rpm) at 4 °C. Most of the phagosomes will migrate into the 55–65% sucrose space and are collected (approximately 2 ml). For routine analysis, we combine the fractions 8–10 (Figure 2) for further analysis. To evaluate the usefulness of this technique for a particular phagocytic probe, all fractions are collected and numbered as indicated in Figure 2 and further analysed for their contents in various subcellular organelle marker proteins or enzymatic activities.

I. The second ultracentrifugation step: A Ficoll cushion
   - Adjust the collected fractions (8–10) very slowly to a final sucrose concentration of 11% with HB without sucrose and place on a 15% Ficoll (in 5% sucrose [w/v]/0.5 mM EGT/20 mM Hepes/KOH, pH 7.2) cushion in a SW40Ti tube. Spin the samples at 18,000 *g* for 20 min in a Beckman SW40Ti rotor (10,000 rpm), discard the supernatant and carefully resuspend the resulting loose pellet in 11 ml HB.

J. The third centrifugation step: Phagosome concentration
   - Spin the tube at 18,000 *g* for 10 min (SW40Ti; 10,000 rpm). The resulting pellet is considered the product, contains approximately 40% of the bacteria that were present in the postnuclear supernatant (as judged by life cell count), take up in 0.1 ml homogenization buffer, and transfer to a sialinized 1.5 ml tube.

K. Electron microscopical analysis of purified phagosomes
   - Phagosome fraction 'F' is taken up in 200 µl PBS. 150 µl of these are centrifuged

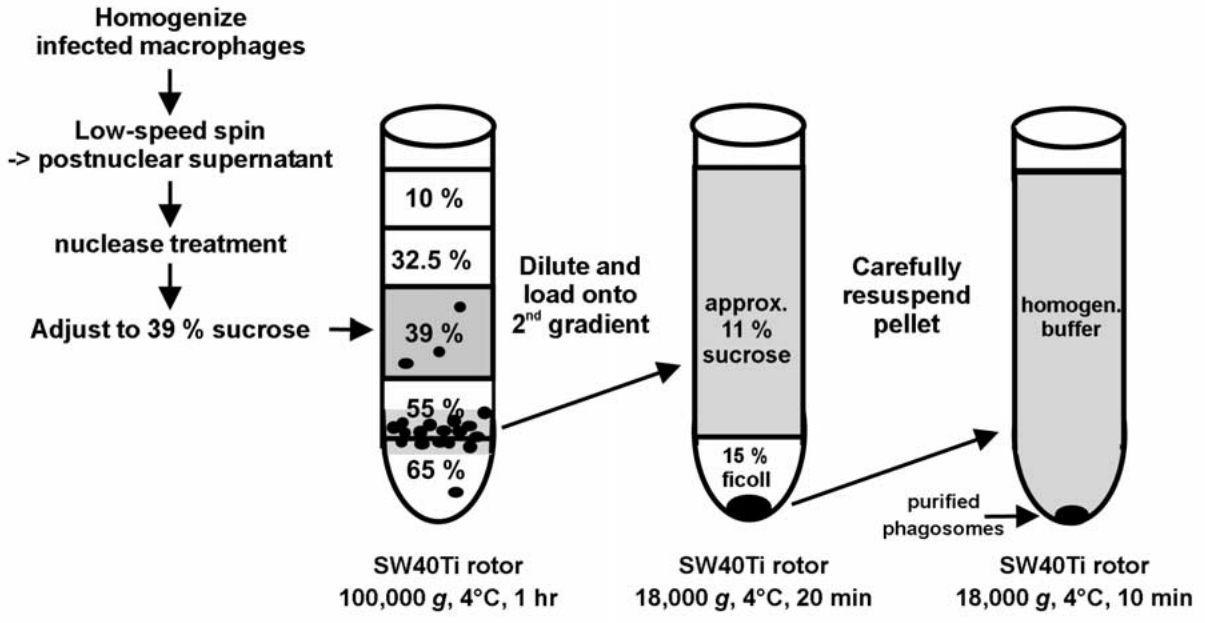

Figure 1. Schematic presentation of the phagosome purification method. For details, see text.

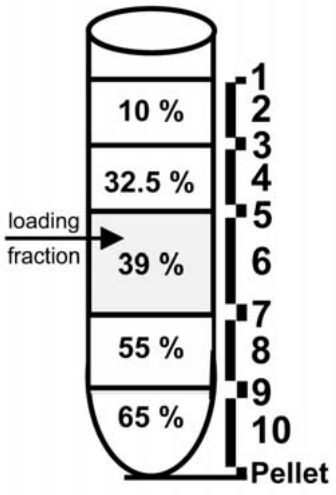

Figure 2. Schematic presentation of the fraction selection in the discontinuous sucrose density gradient analysis. Numbers of fractions are the same as used in Table 1.

in a 1.5 ml tube for 10 min at 4,000 rpm (1,780 ×*g*) and 4 °C. The pellet is carefully resuspended in 1 ml Karnovsky buffer and is left overnight on ice. Wash 3 times with 300 µl 0.1 M cacodylate buffer (centrifugation steps for 5 min at 4,000 rpm (1,780 ×*g*) at 4 °C. Resuspend in 1% (w/v) osmium tetroxide in 50 mM cacodylate buffer and leave on ice for 1 hr. Wash 3 times with 300 µl aqua dest as above. Resuspend pellet carefully in 0.5% (w/v) uranyl acetate in aqua dest and leave overnight at 4 °C. Remove the supernatant carefully. The phagosomal fraction is dehydrated with ethanol (successively with 30%, 50%, 70%, 90%, 96% ethanol in deion water for 30 min at 4 °C, respectively, and finally two times 100% ethanol for 30 min at room temperature). Do this by carefully overlaying the phagosome pellet with 300 µl of the respective solution without resuspending the pellet. Add propylene oxide (300 µl) to the pellet in the same way, leave it on ice for 5 min at room temperature and then the supernatant is carefully removed. Repeat this twice. The sample is now dehydrated. Add Epon-propylene oxide overnight (150 µl Epon prepared as described above + 150 µl propylene oxide). Discard Epon-propylene oxide carefully and add 300 µl Epon to the phagosome pellet after incubation for some 3–4 hrs at room temperature. Finally, add 300 µl fresh Epon 812. Leave for 3 to 5 days at 60 °C for the Epon to harden. All further steps (cutting the slices and preparation of grids) follow standard electron microscopic methods.

L. Labeling of the plasma membrane with HRP and determination of the cross-contamination

– After scraping of macrophages from the dishes, wash the cells directly once with cold PBS, centrifuge at 700 rpm (55 ×*g*) and 4 °C, and resuspend in a PBS/horseradish peroxidase (HRP; 0.25 mg/ml) solution. Cells remain in there for 30 min at 4 °C, allowing the HRP to bind to the host cell membrane, but inhibiting the intracellular uptake of HRP. Then centrifuge at 700 rpm (55 ×*g*) at 4 °C, wash once with ice-cold PBS and once with ice-cold homogenization buffer. Homogenization and phagosome isolation is performed as above.

M. Labeling of early endosomes with HRP and determination of the cross-contamination
   – After scraping of macrophages from the dishes, wash the cells directly once with cold PBS, centrifuge at 700 rpm and 4 °C, and resuspend in a RPMI/horseradish peroxidase (HRP; 0.25 mg/ml) solution. Place cells on ice for 10 min, allowing the HRP to bind to the host cell plasma membrane. Shift this suspension then for 4 min to 37 °C with light shaking by hand, allowing the HRP to be taken up by the macrophages and to accumulate in early endocytic structures. Wash cells once in ice-cold PBS and once in ice-cold homogenization buffer. Homogenization and phagosome isolation is performed as above.

N. Assay for horseradish peroxidase activities
   – Samples to be tested are diluted to 50 µl with homogenization buffer and 100 µl HRP activity mixture is added. Reactions are allowed to progress for 20 min at room temperature in the dark. Samples are diluted with ice-cold aqua dest (150 µl) and absorbance at 405 nm is determined immediately in an ELISA reader.

O. Colorimetric assay for lysosomal β-galactosidase
   – β-galactosidase activity measurements are in a volume of 150 µl 10 mM *p*-nitrophenyl-β-D-galactopyranoside/0.7% Triton X-100/ 150 mM citrate buffer (pH3.5) (including sample). Samples, in a microtiter plate, are incubated at 37 °C for 2 hrs, the reaction is stopped with 150 µl 0.5 M $NaCO_3$, and absorbance at 405 nm (A_{405}) is determined in an ELISA reader. Activity is indicated as U (A_{405}/mg protein/hr). The high stability of lysosomal enzymes such as β-galactosidase would allow longer incubation periods without loss in activity and, hence, require less precious product. In fact, some protocols call for overnight enzyme assays. We prefer, however, relatively short incubation times (up to 2 hours) to minimize misinterpretations due to potential enzyme instabilities and to always keep the reaction in the linear range. When we use 20 vol. % of a final phagosome preparation product, we usually obtain an optical reading of approximately 0.020–0.300 A_{405} under standard assay conditions described above (see also Table 1).

P. Separation of the (host cell) proteins of interest from bacterial proteins
   – To separate bacterial from host cell (phagosome) protein (which is the focus of this kind of protocol), 50 µl of all samples are treated with Triton X-100 detergent (5.5 µl of a 2% solution is added, equal to a final concentration of 0.2%). Samples are incubated for 30 min at 4 °C and then spun for 5 min at 25,000 *g* at 4 °C in a minifuge to pellet the bacteria. The resulting supernatants are transfered to fresh tubes. Protein concentrations are determined with the BioRad DC Protein Assay, using bovine serum albumin as a standard.

Q. SDS-PAGE and Western blot-analysis
   – The detergent extracts are diluted with 2× Laemmli sample buffer and heated for 5 min at 95 °C, followed by a run in a 10% standard SDS polyacrylamide gel. After transfer to PVDF membranes, Western blots are decorated with the indicated antibodies and antibody binding is detected using chemiluminiscence with HRP-coupled secondary antibodies and exposure to X-ray films.

R. Note of caution
   – With *Afipia felis* ('risk level II organism') and any other pathogens, exercise the necessary caution and follow the local legal regulations. It is also recommended to avoid the generation of any aerosols containing bacteria or bacteria-infected cells whenever working with infectious material. All work involving free pathogens must be done in a safety cabinet.

4. Results and discussion

The method described in detail above (for a scheme, see Figure 1) allows the purification of bacteria-containing phagosomes from macrophages and therefore, the biochemical analysis of this complex organelle. Our method combines and modifies previously described methods [1, 2, 32, 43] with the most important goal being to remove as much of the endocytic organelles (other than phagosomes) as possible. This is particularly important in the case of pathogen-containing phagosomes suspected to evade the endocytic pathway or to induce arrest at a certain maturation step. Relatively minor contaminations of the product with endosomes or lysosomes could drastically alter the interpretation while these contaminations are not particularly disturbing when analysing the maturation of normally developing phagosomes. Table 1 shows the distributions of protein (as % of the protein concentration in the starting postnuclear supernatant, PNS), the distribution of early endosomes (as % activity of that in PNS and determined by defining early endosomes by a short pulse of horseradish peroxidase), and of lysosomes (again, as % of PNS, determined by measuring lysosomal acid β-galactosidase activities; Figure 3). Data are shown for a series of experiments using *L. innocua* (does not interfere with phagosome maturation) and non-infected macrophages ('pseudophagosomes') as a control. The differences between early endosomal

Table 1. Analysis of fractions from the discontinuous sucrose gradient and the Ficoll cushion centrifugation. Samples from fractions 1–10, the pellet (for definition of the fractions, see Figure 2) of the discontinuous sucrose gradient centrifugations step, and the final product (marked 'F') after the Ficoll cushion centrifugation were analysed for: (1) their protein contents; (2) their contamination with early endosomes (i.e., by determining HRP activity in samples that had been fed with HRP for 4 min – conditions that label preferentially early endosomes); (3) their lysosome contents (as determined by measuring lysosome acid β-galactosidase activities); (4) or for bacterial life cell counts (to determine yield; note that very little bacteria killing occurs within two hours, i.e., the time range of the experiment). Data are indicated as percentages of total, with 'total' being the activity in the starting homogenate (= postnuclear supernatant). Data are from three independent experiments and are expressed in per cent of total as means +/– standard deviations. Relevant differences in the lysosome contents between the pseudophagosome preparation and *Listeria*-containing phagosomes are marked in bold

Fraction	Protein		Early Endosomes		Lysosomes		Bacteria
	not infected	Listeria innocua	not infected	Listeria innocua	not infected	Listeria innocua	Listeria innocua
1	0.39 +/– 0.16	0.37 +/– 0.02	0.06 +/– 0.06	0.03 +/– 0.02	0.09 +/– 0.02	0.09 +/– 0.07	< 0.1
2	0.86 +/– 0.20	0.70 +/– 0.25	0.30 +/– 0.04	0.19 +/– 0.11	1.79 +/– 1.06	0.71 +/– 0.31	< 0.1
3	1.14 +/– 0.12	1.15 +/– 0.37	5.40 +/– 1.62	5.04 +/– 2.81	21.52 +/– 10.10	17.79 +/– 9.77	< 0.1
4	2.30 +/– 0.31	1.46 +/– 0.28	8.02 +/– 3.31	4.44 +/– 0.41	14.18 +/– 2.36	7.71 +/– 1.79	< 0.1
5	10.71 +/– 2.96	10.14 +/– 2.09	15.26 +/– 2.64	15.88 +/– 4.37	18.50 +/– 2.29	15.94 +/– 2.18	0.6 +/– 0.4
6 (load.)	61.81 +/– 4.66	65.26 +/– 1.55	55.68 +/– 7.42	57.71 +/– 6.21	27.56 +/– 7.12	31.12 +/– 7.57	1.0 +/– 0.7
7	17.35 +/– 1.55	14.53 +/– 1.70	11.81 +/– 0.28	9.96 +/– 1.24	12.90 +/– 3.48	18.20 +/– 4.33	11.5 +/– 6.0
8	1.58 +/– 0.59	1.80 +/– 0.34	0.84 +/– 0.23	1.18 +/– 0.22	**0.91 +/– 0.38**	**2.40 +/– 0.98**	24.0 +/– 4.4
9	1.10 +/– 0.49	1.37 +/– 0.25	0.53 +/– 0.30	0.85 +/– 0.17	**0.68 +/– 0.43**	**2.60 +/– 1.32**	34.3 +/– 14.6
10	1.48 +/– 0.59	1.91 +/– 0.22	1.05 +/– 0.17	1.08 +/– 0.13	**1.07 +/– 0.30**	**2.15 +/– 0.48**	21.5 +/– 8.8
Pellet	1.28 +/– 0.03	1.32 +/– 0.19	1.03 +/– 0.08	1.02 +/– 0.05	0.80 +/– 0.06	1.29 +/– 0.30	7.7 +/– 3.4
8–10	4.17 +/– 1.49	5.08 +/– 0.50	2.43 +/– 0.47	2.96 +/– 0.30	**2.66 +/– 1.10**	**7.15 +/– 2.78**	79.8 +/– 3.4
F	0.32 +/– 0.05	0.45 +/– 0.10	0.13 +/– 0.07	0.20 +/– 0.02	**0.19 +/– 0.11**	**0.89 +/– 0.31**	39.0 +/– 10.2

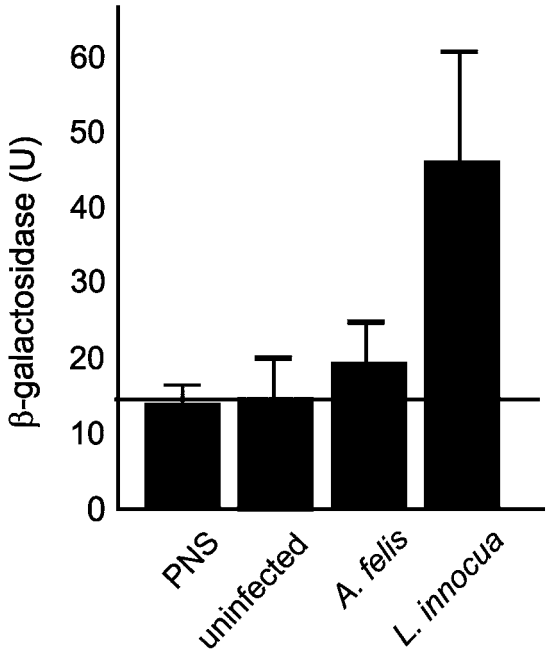

Figure 3. Acid β-galactosidase activities in purified phagosome fractions. Specific enzyme activities are shown as means from six different experiments and their standard deviation. Enzyme activities in PNS are shown for comparison. Note that *A. felis*-containing phagosomes which do not significantly mature to phagolysosomes [28] have β-galactosidase activities which are only slightly higher than those of the background (either pseudophagosome preparation = 'phagosomes from uninfected cells' = enzymatic level marked by the horizontal line, or relative to the same protein concentration of postnuclear supernatant). Opposed to this, maturing *Listeria innocua*-containing phagosomes accumulate the lysosomal enzyme.

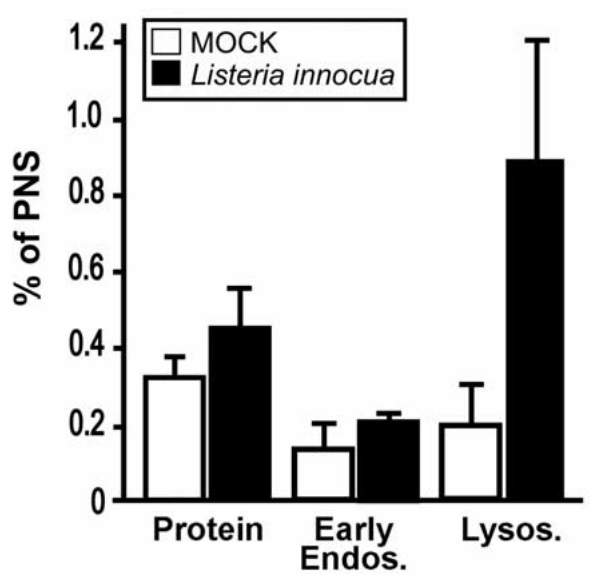

Figure 4. Characterization and comparison of the product fractions. Contents of the purified phagosomes in protein (mg/ml), in early endosomes (in HRP enzymatic units per mg protein), or lysosomes (expressed as acid β-galactosidase enzymatic activities per mg protein) were determined and are presented as percentage of the starting material (PNS). 'mock' = 'pseudophagosome preparation' from uninfected macrophages, '*Listeria innocua*-' phagosomes from macrophages infected with *L. innocua*. Please note that these fractions do not contain bacteria protein, as bacteria were removed before the protein concentration determination (see *Methods*). 'Early Endos.' = presence of early endosomes as determined by HRP-labeling (see *Methods*), 'Lysoso.' = presence of lysosomes as determined by β-galactosidase activities (see *Methods*).

and lysosomal enzyme activities and the protein distribution across the gradient are very similar between the pseudophagosome and the *Listeria*-containing phagosome preparations (and other phagosome preparations; data not shown). The overall differences between the various fractions are only marginal. Clear differences, however, exist between the lysosomal enzyme activities in fraction 8 through 10 and in the resulting purified phagosome fractions, although the protein contents and the early endosomal enzyme activity are almost identical in the same fractions (Table 1, bold characters; Figure 4). This demonstrates a specific enrichment of phago(lyso)somes in these fraction, in agreement with our finding that approximately 80% of all bacteria in the starting PNS can later be found in fractions 8 through 10 (Table 1).

Note that the important advantage of incorporating a centrifugation step through a Ficoll cushion is that the activities of early endosome and lysosome markers after Ficoll cushion-mediated separation are only some 5–10% compared to the material loaded onto the cushion (i.e., the united fractions 8–10), whereas the yield in bacteria, as determined by life cell counts after cultivation on agar plates, is equal to or more than 50% during this step. This indicates that we remove a substantial proportion of early and late endosomal organelles with a moderate decrease in bacteria-containing phagosomes yield. This is also true for other phagosomes such as *Afipia felis*-containing phagosomes (Please note that the life cell counts of intracellular bacteria are largely constant over this 2 to 3 hours incubation). Table 1 also shows a number of mean standard deviations and demonstrates the reproducibility of this method when the same or even different kinds of phagocytic probes are used.

Reproducibility is further demonstrated when analyzing the purification products by Western blot analysis (Figure 5). We have probed the blots with an antibody to the late endosomal/lysosomal marker lamp-1 [10]. As can clearly be seen, the concentration of lamp-1 is very low in the pseudophagosome preparation from non-infected macrophages (i.e., contaminating material) compared to normally maturing phagosomes containing *Listeria innocua* [40] as expected. On the other hand, phagosomes containing pathogenic *Afipia felis* accumulate only minor quantities of this marker, as expected from our

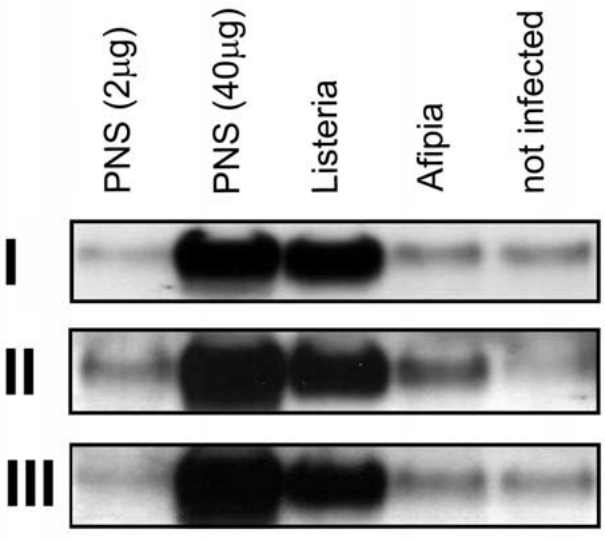

Figure 5. Reproducibility of LAMP-1 contents in three phagosome purification experiments. Phagosomes were isolated in three independent experiments (I–III) as described and phagosome protein was run on a 10% polyacrylamide gel and transferred onto PVDF membranes, followed by decoration with rat monoclonal LAMP1-antibody and a secondary HRP-coupled antibody to visualize binding in chemiluminscence. Note the high degree of similarity between the blots containing proteins from independent isolations, and containing different phagocytic probes. Either 2 or 40 μg of protein from postnuclear supernatant (PNS) or 2 μg protein of a *Listeria*- or *Afipia*-containing phagosome preparation were loaded per lane.

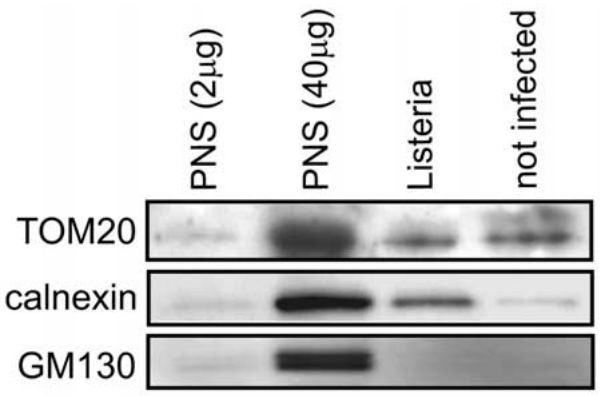

Figure 6. Western blot analysis of the distribution of diagnostic markers for the endoplasmic reticulum, the Golgi, or mitochondria in final phagosome fractions. Phagosomes were isolated as described, and phagosome protein was run on a 10% polyacrylamide gel and transfered onto PVDF membranes, followed by decoration with antibodies to an ER (calnexin), a mitochondrial (TOM20), or a Golgi (GM130) protein. A secondary HRP-coupled antibody was used to visualize binding using chemiluminscence chemistry. Either 2 or 40 μg of protein from postnuclear supernatant (PNS) or 2 μg protein of a phagosome preparation from *Listeria*-infected or uninfected macrophages ('pseudophagosome preparation') were loaded per lane.

data which demonstrate that a substantial portion of these phagosomes is disconnected from the endocytic system [28]. Also, note that the three Western blots, done using three independent phagosome preparations are virtually identical, further substantiating our claim of a high level of reproducibility of this method. This is in excellent agreement with our determinations of lysosomal β-galactosidase activities in product fractions (Figure 3).

It should be noted that the control samples containing a pseudophagosome preparation from uninfected macrophages are stringent controls for cellular contaminations and that we have included them in every single experiment. We also include PNS samples (2 and 40 μg per lane, respectively) in each blot to be able to evaluate the quality of the antibody and to visualize the acquisition or loss of subcellular markers in phagosome fractions relative to the starting homogenate (i.e., PNS). Furthermore, these two lanes in each blot allow an estimate of the factor of enrichment or depletion. As for contaminations with host cell material other than endosomal or lysosomal material, we have found a clear contamination with material from the endoplasmic reticulum (determined using an antibody to calnexin; Figure 6) and from mitochondria (as probed using an antibody to the mitochondrial outer membrane protein TOM-20; Figure 6) both of which are approximately twice as concentrated in the final fraction compared to the starting postnuclear supernatant. Nevertheless, the bulk of these organelles is separated from phagosomes during the purification procedure. Endoplasmic reticulum, as a contamination, is present in virtually every organelle preparation, regardless of the technique used for purification [17] and is possibly even a 'meaningful' constituent of many phagosomes [18]. Mitochondria were clearly expected to be present, as their density and shape approximates that of bacteria.

Our data show that the final phagosome product contains only 0.13 +/– 0.07% (pseudophagosomes) or 0.20 +/– 0.02% (*Listeria*-containing phagosomes) or 0.21 +/– 0.13% (*Afipia*-containing phagosomes; not shown) of the total (PNS) early endosomal activities. In this protocol, HRP is bound in the cold (no endocytic trafficking), and the HRP is chased into the early endocytic system during a 4 min warm-up at 37 °C. Cells are cooled down immediately, washed twice, and the lysates are produced and processed 'as usual'. The yield of bacteria in the final phagosome product is between 30–50% (Table 1; Figure 4). The fact that early endosomal activity is not enriched in the bacteria-containing phagosome preparations was expected, as the bacteria had more than plenty of time (120 min) to reach a late phagosomal or an unusual compartment, depending on their final destination. These findings agree very well with our immunoblotting experiments in which very low levels of early endosomal rab5, EEA1 or transferrin receptor were

detected in purified phagosomes ([28] and data not shown).

Similarly, low levels of plasma membrane contaminations were found in the purified products. We defined plasma membranes operationally as the compartment that copurifies with horseradish peroxidase (HRP) bound to the cell surface in the cold when all endocytic trafficking is blocked. After HRP exposure, macrophages were washed thoroughly and were immediately lysed. HRP activities were determined from all subcellular fractions. The pseudophagosome preparation contained only 0.06 +/– 0.02% of the HRP activity in the PNS, whereas the *Listeria* phagosome preparation contained 0.11 +/– 0.02%, and the *Afipia* phagosomes 0.08 +/– 0.01%. As all HRP was added *after* the infection and as it is not possible that under these conditions the HRP trafficked endocytically into the various phagosomal compartments, these numbers really reflect directly plasma membrane contamination.

Finally, it should be noted that we could not at all detect the Golgi compartment marker GM130 [36] in the final phagosome fractions of infected or uninfected macrophages, indicating efficient removal of this organelle (Figure 6).

We have determined by life cell counts that approximately 40% of the live bacteria that were present in the postnuclear supernatant were then present in the phagosome purification product, indicating an excellent yield. The distribution of live bacteria cell counts over the various fractions can be taken from Table 1. Similar data were obtained when fluorescently labeled bacteria were used as infectious material and the number of bacteria in various fractions was estimated microscopically (not shown).

When interpreting the biochemical data obtained with purified phagosomes, it should be taken into account that each preparation consists of the proper phagosome product but also some co-purifying organelle material (see above). This means that indicating specific marker enzyme activities or interpreting immunoblot signals of preparations containing the same protein amount (as done in this study) can only be meaningful, if the background activities or protein amounts are highly constant. In Figures 3, 5, 6 and Table 1 we demonstrate that this is indeed the case. This potential problem becomes more important with increasing concentrations of co-purifying host cell protein. In fact, as the amount of such protein is relatively high compared to the amount of 'true' phagosomal protein (Figure 4, compare the two 'Protein' bars), the true specific lysosomal enzyme activities in *phagosomes* (vs the complete product) are underestimated.

In addition to the biochemical analysis of our phagosome product fractions, we have also done transmission electron micrscopy (Figure 7) as an independent means of determining product purity. As can be clearly seen, *Listeria* bacteria (one being marked by a cross) are surrounded by loosely fitting phagosomal membranes (arrowheads) with possibly decomposition of one bacterium (marked by an asterisk) in progress. A mitochondrion is seen under the bar scale. The relative enrichment of phagosomes over subcellular debris is clearly visible and, to our opinion, the level of contamination is much lower than with most other comparable methods.

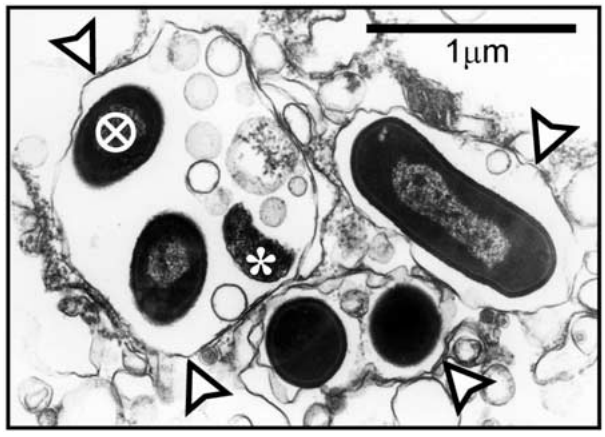

Figure 7. Analysis of purified *Listeria innocua*-containing phagosomes using transmission electron microscopy. *Listeria* bacteria (one being marked by a cross) are surrounded by loosely fitting phagosomal membranes (arrowheads) with possibly decomposition of one bacterium (marked by an asterisk) in progress. A mitochondrion is seen directly under the bar scale. Bar scale = 1 μm.

To conclude, we present here a method for the purification of bacteria-containing phagosomes that seems to be widely useful. Although the products still contain some minor subcellular contaminations with material from other organelles, we found only some enrichment for markers of the endoplasmic reticulum and mitochondria. As mitochondria are present only in very low numbers in the phagosome products (as judged from electron microscopy analysis of purified phagosomes), but as numerous 'empty' and smooth vesicular structures are visible which resemble breakdown vesicles of the ER, we assume that parts of the ER must be the major contaminants in our system. Using Western blot analysis in dozens of experiments we could often not detect any early endosomal background (rab5, TfR, EEA1), or late endosomal – lysosomal background (β-glucuronidase) in the pseudophagosome preparation (not shown). We hope that this method will become useful for many applications.

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Notes on suppliers

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