CURRENT MICROBIOLOGY Vol. 49 (2004), pp. 136–140
DOI: 10.1007/s00284-004-4298-3

Current Microbiology
An International Journal

News & Notes

Intracellular Location and Survival of *Mycoplasma penetrans* Within HeLa Cells

M. Tarshis,[1] A. Yavlovich,[1] A. Katzenell,[1] I. Ginsburg,[2] S. Rottem[1]

[1]Department of Membrane and Ultrastructure Researh, The Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel
[2]Department of Oral Biology, The Hebrew University-Hadassah School of Dental Medicine, POB 12272, Jerusalem 91120, Israel

Received: 17 December 2003 / Accepted: 18 February 2004

Abstract. *Mycoplasma penetrans* invades HeLa cells and survives within them for prolonged periods of time. The intracellular distribution of *M. penetrans* within HeLa cells was studied utilizing the acidotropic dye LysoTracker (green), which permeates cell membranes and upon protonation remains trapped in acidic compartments. The excitation and emission spectra of the green LysoTracker are suitable for colocalization studies with rabbit anti-*M. penetrans* antibodies and red Cy5 goat anti-rabbit IgG. The images collected by confocal laser scanning microscopy revealed that in the infected HeLa cells almost all Cy5 fluorescent foci (red) were located within the LysoTrack-labelled intracellular compartments, apparently endosomes. Viable mycoplasmas were detected within endosomes for prolonged periods of time, apparently due to a potent antioxidant activity detected in *M. penetrans.*

Invasive bacterial pathogens are capable of entering non-phagocytic eukaryotic host cells where the host cytoplasm serves as a permissive environment for intracellular bacterial persistence and growth [19, 22, 23]. The invasion of the pathogen is initiated by the binding of the pathogen to the host cell surface, followed by internalization. The intracellular fate of invading bacteria can vary greatly. Some invasive bacteria appear to be able to survive intracellularly for extended periods of time [1, 19, 22]. Other bacteria are degraded intracellularly via endosome-lysosome fusion.

Mycoplasmas are the smallest and simplest self-replicating bacteria. These organisms lack a rigid cell wall and most of them are parasites, exhibiting strict host and tissue specificities [21]. In some cases mycoplasmas are associated with diseases in the urogenital or respiratory tracts, but in most cases mycoplasmas enter an appropriate host in which they multiply and survive for extended periods of time without causing major harm [4, 20, 25]. Current theory holds that mycoplasmas remain attached to the surface of epithelial cells, although some mycoplasmas have evolved mechanisms for entering non-phagocytic host cells [21]. The intracellular location is obviously a privileged niche, well protected from the immune system and from the action of many antibiotics.

Mycoplasma penetrans strain GTU was isolated from the urogenital tracts of an AIDS patient [17]. This mycoplasma has invasive properties and was detected within a variety of host cells [2, 11, 17]. In the present study, we utilized the fluorescent pH-sensitive LysoTracker (LyT) stain and confocal microscopy to study the intracellular fate *M. penetrans* internalized by HeLa cells.

Materials and Methods

M. penetrans strain GTU was grown for 24 h at 37°C in a modified Chanock medium [2] supplemented with 5% horse serum. The bacteria were collected by centrifugation at 12,000 *g* for 20 min, washed twice, and resuspended in a cold solution of 10 mM Tris-HCl in 250 mM NaCl (pH 7.5; TN buffer) to a protein concentration of 1 mg/mL ($\sim 10^{11}$ CFU/mL). The human adenocarcinoma cells originating from the cervix, HeLa-229 (ATCC#CCL-42.1), were grown at 37°C in humidified atmosphere of 5% CO_2 and 95% air in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% glutamine and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) and with 10% fetal calf serum (FCS, Biological Industries, Israel).

Invasion of *M. penetrans* into host cells was determined by the

Correspondence to: S. Rottem; *email:* rottem@cc.huji.ac.il

gentamicin resistance assay [2, 8] and by confocal laser scanning microscopy (CLSM) of immunofluorescently stained preparations [6]. For measuring the oxidative potential of *M. penetrans,* the cyclic voltametry (CV) assay [14, 15] and the luminol-enhanced chemiluminescence (CL) assay [10] were utilized. For cyclic voltametry, *M. penetrans* (1 mg cell protein/mL) was dissolved in 2% Triton X-100 and the antioxidant activity was measured in a cyclic voltametry apparatus (Model CV-1B, BAS, West Lafayette, IN). The antioxidant activity of *M. penetrans* was also measured by the CL assay. Intact *M. penetrans* (10 μg cell protein/mL) were added to a reaction mixture containing urea-hydrogen peroxide (1 mM) and Na_2SeO_3 (2 mM), and chemiluminescence was measured in a LUMAC/3M Biocounter M2010 connected to a linear recorder.

To locate the adherent and ingested *M. penetrans* in infected HeLa cells, a polyclonal monospecific antibody against *M. penetrans* was used [6]. The infected HeLa cells were harvested with trypsin-EDTA solution and seeded onto 12-mm glass coverslips, at a density of 3–5 $\times$ 10^4 cells per coverslip, placed in 24-well tissue culture plates with 1 mL of fresh DMEM medium in each well and further incubated under the same growth conditions to the confluent level of 60% (5 $\times$ 10^5 HeLa cells per coverslip). The culture medium was then replaced with 1 mL of fresh DMEM medium without antibiotics and infected with *M. penetrans* at a multiplicity of infection (MOI) of 10 for 4 h. The infected HeLa cell cultures were incubated for various periods of time, washed twice with phosphate-buffered saline (PBS) and stained for 30 min at room temperature with the acidotropic vital fluorescent probe, LysoTracker Green DND-26 (50 nM, Molecular Probes, Eugene, OR). Cells were washed two times with PBS, fixed and permeabilized for 5 min at −20°C with cold absolute methanol and washed twice with PBS. Nonspecific staining was prevented by blocking with 2% normal horse serum for 20 min. Then the serum was removed and HeLa cells were immunostained with primary anti-*M. penetrans* antibody at a 1:200 dilution for 50 min. The coverslips were washed three times with PBS and then incubated for 40 min with 1:200-fold diluted goat anti-rabbit Cy5-conjugated IgG (Jackson, USA). Coverslips were finally washed four times with PBS and mounted onto glass slides with antifading buffered medium (3% DABCO (Sigma), 25% glycerol, 10% Mowiol (Calbiochem), and 0.1 NaN_3, pH 8.0). Nonimmune sera were used for negative controls.

A Zeiss LSM 410 confocal system attached to an Axiovert 135 M inverted microscope and C-Apochromat 63 $\times$ 1.2 water immersion lens was utilized. The fluorescence of LysoTracker (488 nm line of argon ion laser with 515 nm emission filter) and Cy5 (633 nm line of He-Ne laser with 665 nm emission filter) were monitored simultaneously with differential interference contrast (DIC) bright field images according to Nomarsky using dual detectors and filter block combinations with dichroic beam splitters and emission filters. The small pinhole aperture was set to give the best signal-to-noise ratio and image resolution, thus optimizing optical sectioning. Confocal images were converted to a TIFF format and transferred to a Zeiss imaging workstation for pseudo-color representation. Regions where *M. penetrans* antibody (red) and LysoTracker (green) are co-localized after superimposition of the two images are shown in yellow. Z-series of optical sections were acquired at spacing steps of 0.6 μm from the surface through the vertical axis of the specimen by a computer-controlled motor drive. Entire series of confocal images were assembled in an integral image processor and projected into a single in-focus three-dimensional image using the image analysis software (Zeiss).

Results and Discussion

In studying cell invasion it is necessary to discriminate between external and internal bacteria. Among the various techniques used to determine internalization [12, 23] confocal laser scanning microscopy (CLSM) is an invaluable technique available to modern biology for examining single living cells in real time, using the growing arsenal of vital fluorescent probes [12]. We have adapted CLSM for studying the intracellular distribution of *M. penetrans* within HeLa cells. This approach provides a high degree of three-dimensional resolution and allows distinction between intracellular and extracellular mycoplasmas by simultaneous visualization of both populations. The difference between HeLa cells fixed with formaldehyde and those fixed and permeabilized with Triton X-100 represents intracellularly located *M. penetrans.* Using these methods it was previously established that *M. penetrans* invades HeLa cells in a time-, concentration-, and temperature-dependent fashion [2, 6]. The cytoplasmic pool of ingested mycoplasmas is organized in small and confluent bright fluorescent aggregates within vesicles [6, 24], supporting the transmission electron microscopy observations showing that intracellularly located mycoplasmas are embedded within well-defined vesicles [17, 24]. Accumulating data suggest that *M. penetrans* entry into HeLa cells involves a series of discrete events including membrane-assisted binding, recruitment of cytoskeleton components, internalization and intracellular transport via a microtubule-dependent process [20, 21]. The intracellular environment of the internalized *M. penetrans* is still unclear. It is reasonable to assume that *M. penetrans*-containing endosomes subsequently fuse with lysosomes, the terminal degradative compartment in the endocytic routes of animal cells [16]. Within the endolysosomes formed the endocytosed macromolecules and foreign components are digested [3, 16, 18]. To examine this possibility we utilized the acidotropic dye LysoTracker Green, a weak base conjugated to a green fluorochrome, as a marker of endolysosome acidification. The LysoTracker freely permeates cell membranes, and upon protonation remains trapped in the acidic compartment such as lysosomal organelles. The excitation and emission spectra of the green LysoTracker are suitable for colocalization studies with rabbit anti-*M. penetrans* antibodies and red Cy5 goat anti-rabbit IgG. LSCM collects images that, unlike standard fluorescence microscopy, are free from out-of-focus blur, thus greatly improving in-plane resolution along the *z*-axis and analysis of the surface of the cells and the fine intracellular fluorescent structures [6]. Images collected on LSCM depicted the general pattern of lysosomes stained by LysoTracker (green) and Cy5 (red) representing intracellular mycoplasmas (Fig. 1). When the intracellular distribution of *M. penetrans* in intracellular compartments was investigated utilizing this double staining method, it was observed that in infected HeLa

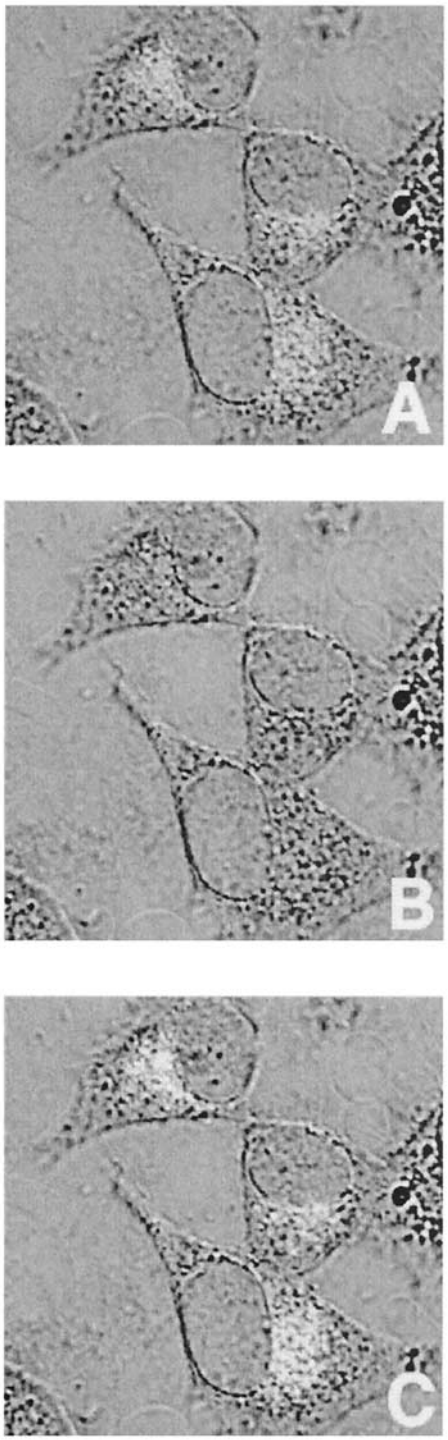

Fig. 1. Laser scanning confocal images of lysosomes and bacteria within HeLa cells. HeLa cells were infected with *M. penetrans* for 4 h, and double-stained with LysoTracker (green) for lysosomes and with the Cy5 fluorophore (red) for mycoplasmas as described in Materials and Methods. Panels A, B, and C show one *xy* optical section taken of the same field of cells. LysoTracker fluorescence is depicted in green (A) and Cy5-labeled *M. penetrans* antigens are depicted in red (B). The co-localized regions (the combination of red and green colors) are depicted in yellow (C).

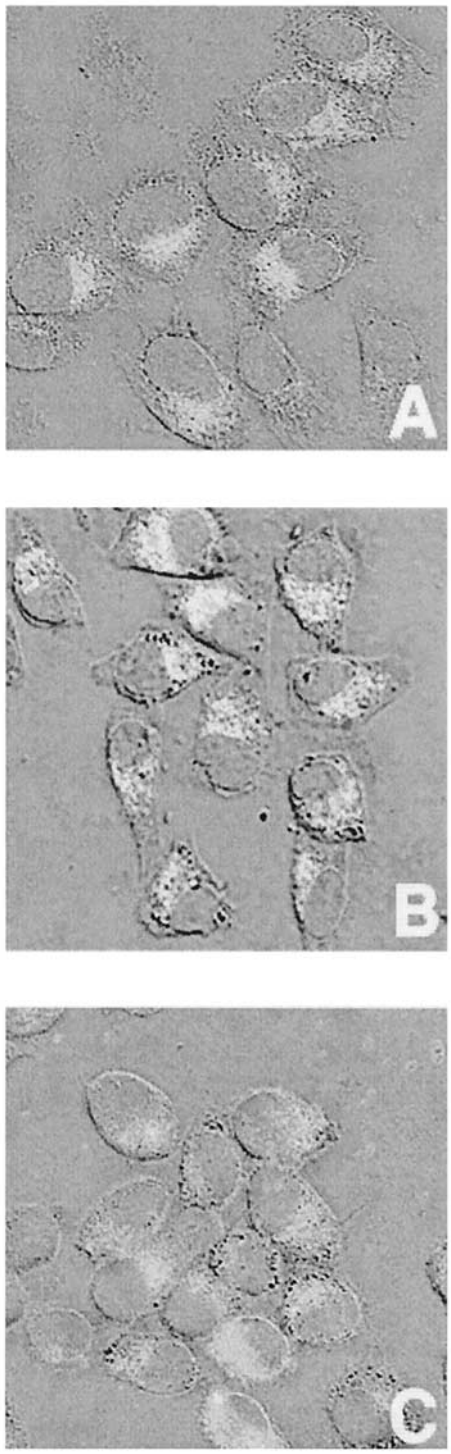

Fig. 2. Confocal micrographs of *M. penetrans*-infected HeLa cells. HeLa cells infected with *M. penetrans* were double-stained for lysosomes (green) and mycoplasmas (red) as described in Materials and Methods. (A) Uninfected cells; (B) 4 h postinfection; (C) 4 h postinfection of cells pretreated for 10 min with 4% formaldehyde.

Table 1. Persistence of *M. penetrans* within HeLa cells

Time postinfection (h)	Viability of HeLa cells (%)	Intracellular *M. penetrans* (CEU/10^6 HeLa cells)	Fluorescence intensity (arb. units)
0	98.7	$3.1 \pm 0.3\times10^4$	23.8
4	96.0	$2.5 \pm 0.3\times10^4$	26.2
6	91.9	$2.1 \pm 0.2\times10^4$	27.4
10	85.3	$2.2 \pm 0.2\times10^4$	26.0
24	81.1	$3.0 \pm 0.2\times10^3$	27.1
48	74.0	$<10^3$	19.9
168	31.2	$<10^3$	10.1

M. penetrans were incubated with HeLa cells for 2 h (MOI = 10) in an antibiotic-free DMEM medium, washed and further incubated in DMEM containing 200 μg/mL of gentamicin. Viability of HeLa cells was assessed with the trypan blue cell exclusion test, and of *M. penetrans* by plating. HeLa cells were washed, fixed with methanol, and immunostained with anti-*M. penetrans* antibodies with Cy5-conjugated goat anti-rabbit serum. For each time period, fluorescence intensity of 10 random fields (about 20 cells per area) was taken and scored with confocal microscopy and then averaged.

cells almost all Cy5 fluorescent foci (red) were located within the LysoTracker-labeled intracellular compartments (green), resulting in a yellow staining (Fig. 2B). The relative extent of co-localization with the LysoTracker was over 90% and remained stable for the duration of the experiment. As can be seen from Fig. 2C, pretreatment of HeLa cells with 4% formaldehyde completely prevents the invasion of *M. penetrans* and all cell-associated mycoplasmas were found outside the lysosome-stained areas.

To check whether the cell cytoplasm can serve as a permissive environment for intracellular bacterial persistence and growth, HeLa cells were infected with *M. penetrans.* Two hours postinfection, *M. penetrans* was internalized without causing obvious damage to the host cells, as shown by staining with the Live-Dead Viability kit (Molecular Probes; data not shown). When the infected cells were washed and further incubated for up to 7 days in a gentamicin-containing growth medium to kill the extracellular mycoplasmas, mycoplasmal material reacting with anti-*M. penetrans* antiserum was found to persist within the host cells throughout the incubation period (Table 1), supporting previous data and suggesting that the invading *M. penetrans* could be detected within host cells for prolonged periods of time [11, 17]. Nonetheless, viable mycoplasmas could be detected within HeLa cells up to 24 h postinfection (Table 1). The duration of viable microorganism recovery depends on the type of host cell. Thus, viable *Yersinia enterocolitica* may reside within primary culture of synovial cells for 6 weeks [13], whereas in HeLa cells these organisms reside for only a few hours, apparently due to the high proliferation rate of these cells [7]. Similar results were obtained with *Salmonella typhimurium* [9].

Although *M. penetrans* is unable to circumvent the host endocytic pathway and the organism is continuously exposed within the endolysosomes to oxidative stress, viable *M. penetrans* were detected within HeLa cells for as long as 24 h postinfection, suggesting that *M. penetrans* possesses a mechanism capable of scavenging oxygen radicals. Reactive oxygen species (ROS) have

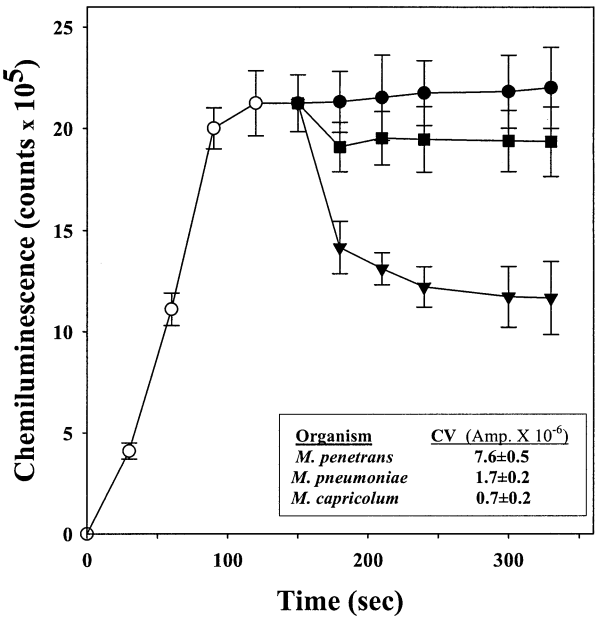

Fig. 3. Antioxidant activity in *M. penetrans.* Antioxidant activity was determined by cyclic voltametry (CV) and by the luminol-enhanced chemiluminescence assay (CL). For CL, mycoplasmas (10 μg cell protein) were added to a radical-generating reaction mixture containing 1 mM urea, 1 mM hydrogen peroxide and 2 mM Na_2SeO_3, and chemiluminescence was measured: triangles, *M. penetrans*; squares, *M. capricolum*; circles, control without mycoplasmas. For CV measurements (insert), mycoplasmas (1 mg protein/mL) were treated with 2% Triton X-100 and analyzed as described in Materials and Methods.

been increasingly implicated as playing a central role in the pathophysiology of clinical infection [18]. ROS exhibit a broad spectrum of biotoxicity and are crucial to host defense and the optimal microbicidal activity of neutrophils and other phagocytes. In response, microorganisms have developed complex strategies to defend themselves against the injury, once oxidants are encountered [5, 18]. These defense mechanisms appear to be particularly important for intracellular pathogens as they aid in survival within the host cell. Indeed, we detected a potent antioxidant activity within *M. penetrans* using cyclic voltametry [14, 15] and the luminol-enhanced chemiluminescence assays [10] (Fig. 3). Preliminary results suggest that the enhanced antioxidant activity of mycoplasmas is due, in part, to a low-molecular-weight component detected in the cytoplasm (A. Yavlovich, unpublished data).

Literature Cited

1. Almeida RA, Fang W, Oliver SP (1999) Adherence and internalization of *Streptococcus uberis* to bovine mammary epithelial cells are mediated by host cell proteoglycans. FEMS Microbiol Lett 177:313–317
2. Andreev Y, Borovsky Z, Rosenshine I, Rottem S (1995) Invasion of HeLa cells by *Mycoplasma penetrans* and the induction of tyrosine phosphorylation of a 145-kDa host cell protein. FEMS Microbiol Lett 132:189–194
3. Ayala P, Lin L, Hopper S, Fukuda M, So M (1998) Infection of epithelial cells by pathogenic *Neisseria* reduces the levels of multiple lysosomal constituents. Infect Immun 66:5001–5007
4. Baseman JB, Lange M, Criscimagna NL, Giron JA, Thomas CA (1995) Interplay between mycoplasmas and host target cells. Microb Pathogen 19:105–116
5. Ben Menachem G, Himmelreich R, Herrmann R, Aharonowitz Y, Rottem S (1997) The thioredoxin reductase system of mycoplasmas. Microbiology 143:1933–1940
6. Borovsky Z, Tarshis M, Zhang MP, Rottem S (1998) Protein kinase C activation and vacuolation in HeLa cells invaded by *Mycoplasma penetrans.* J Med Microbiol 47:915–922
7. Devenish JA, Schiemann DA (1981) HeLa cell infection by *Yersinia enterocolitica*: Evidence for lack of intracellular multiplication and development of a new procedure for quantitative expression of infectivity. Infect Immun 32:48–55
8. Elsinghorst EA (1994) Measurements of invasion by gentamicin resistance. Methods Enzymol 236:405–420
9. Garcia-del Portillo F, Finlay BB (1995) Targeting of *Salmonella typhimurium* to vesicles containing lysosomal membrane glycoproteins bypasses compartments with mannose 6-phosphate receptors. J Cell Biol 129:81–97
10. Ginsburg I, Kohen R (1995) Cell damage in inflammatory and infectious sites might involve a coordinated "cross-talk" among oxidants, microbial haemolysins and amphiphiles, cationic proteins, phospholipases, fatty acids, proteinases and cytokines (an overview). Free Radic Res 22:489–517
11. Giron JA, Lange M, Baseman JB (1996) Adherence, fibronectin binding, and induction of cytoskeleton reorganization in cultural human cells by *Mycoplasma penetrans.* Infect Immun 64:197–208
12. Heesemann J, Laufs R (1985) Double immunofluorescence microscopic technique for accurate differentiation of extracellularly and intracellularly located bacteria in cell culture. J Clin Microbiol 22: 168–175
13. Huppertz H-I, Heesemann J (1996) Experimental *Yersinia* infection of human synovial cells: persistence of live bacteria and generation of bacterial antigen deposits including "ghosts", nucleic acid-free bacterial rods. Infect Immun 64:1484–1487
14. Kohen R, Beit-Yannai E, Berry EM, Tirosh O (1999) Overall low molecular weight antioxidant activity of biological fluids and tissues by cyclic voltammetry. Methods Enzymol 300:285–296
15. Kohen R, Nyska A (2002) Oxidation of biological systems: Oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. Toxicol Pathol 30:620–650
16. Kornfeld S, Mellman I (1989) The biogenesis of lysosomes. Annu Rev Cell Biol 5:483–525
17. Lo S-C, Hayes MM, Kotani H, Pierce PF, Wear DJ, Newton PB, Tully JG, Shih JW-K (1993) Adhesion onto and invasion mammalian cells by *Mycoplasma penetrans*: A newly isolated mycoplasma from patients with AIDS. Mod Pathol 6:276–280
18. Miller RA, Britigan BE (1997) Role of oxidants in microbial pathophysiology. Clin Microbiol Rev 1:1–17
19. Niebuhr K, Sansonetti PJ (2000) Invasion of epithelial cells by bacterial pathogen: The paradigm of *Shigella.* In: Oelschlaeger TA, Hacker J (eds) Bacterial invasion into eukaryotic cells. Subcellular biochemistry, vol. 33. New York: Kluwer Academic/Plenum Press, pp 251–287
20. Rottem S (2002) Invasion of mycoplasmas into and fusion with host cells. In: Razin S, Herrmann R (eds) Mycoplasmas: Molecular biology and pathogenicity. New York: Kluwer Academic/Plenum Press, pp 391–401
21. Rottem S (2003) Interaction of mycoplasmas with host cells. Physiol Rev 83:417–432
22. Shaw JH, Falkow S (1988) Model for invasion of human tissue culture cells by *Neisseria gonorrhoeae.* Infect Immun 56:1625–1632
23. Tang P, Foubister V, Pucciarelli MG, Finlay BB (1993) Methods to study bacterial invasion. J Microbiol Methods 18:227–240
24. Taylor-Robinson D, Davies HA, Sarathchandra P, Furr PM (1991) Intracellular location of mycoplasmas in cultured cells demonstrated by immunocytochemistry and electron microscopy. Int J Exp Pathol 72:705–714
25. Winner F, Rosengarten R, Citti C (2000) In vitro cell invasion of *Mycoplasma gallisepticum.* Infect Immun 68:4238–4244