

RESEARCH ARTICLE

Simkania negevensis may produce long-lasting infections in human pneumocytes and endometrial cells

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One sentence summary: *Simkania negevensis*, an emerging *Chlamydia*-related bacterium, efficiently grows in human cells, suggesting a potential implication in human diseases, and harbors growth characteristics that differ from other known *Chlamydiales*.

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ABSTRACT

Simkania negevensis is a novel *Chlamydia*-related bacterium and the founding member of the *Simkaniaceae* family within the *Chlamydiales* order. Little is known about the biology and pathogenesis of this bacterium. So far, *S. negevensis* has been considered as an amoebal symbiont, but its natural host remains unknown. Moreover, evidence of human exposition has been reported worldwide and an association with pneumonia and bronchiolitis is suspected. Here, we evaluated the ability of *S. negevensis* to replicate in potential environmental reservoirs, namely amoebae and arthropods, as well as in mammalian cells (Vero cells, pneumocytes and endometrial cells) and further evaluated the characteristics of its replicative vacuole. We demonstrated that *S. negevensis* efficiently replicates in all cell lines tested, with the shortest doubling time and an increased adhesion observed in pneumocytes. Our work highlights the specificities of the *Simkania*-containing vacuole compared to other *Chlamydiales*; contrarily to *Chlamydia trachomatis*, *S. negevensis* does not disrupt the Golgi apparatus. Importantly, our work suggests that *S. negevensis* infection is associated with few cytopathic effects and might persist for a prolonged time in infected cells. Further evaluation of its implication in human diseases is required; an implication in chronic or subacute respiratory infections might be suspected.

Keywords: intracellular bacteria; *Chlamydia*-related bacteria; free-living amoebae; *Chlamydiales* order; *Simkaniaceae*; pneumonia

INTRODUCTION

Simkania negevensis is a novel *Chlamydia*-related bacterium discovered in 1993 (Kahane et al. 1993). Similarly to *Chlamydia* spp., this bacterium exhibits a strict intracellular biphasic cy-

cle and was therefore classified within the *Chlamydiales* order as the founding member of the *Simkaniaceae* family (Kahane et al. 1993). In the past decades, various *Chlamydia*-related bacteria have been discovered, such as *Parachlamydia acanthamoebae* (Amann et al. 1997), *Waddlia chondrophila* (Rurangirwa et al. 1999),

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Criblamydia sequanensis (Thomas, Casson and Greub 2006) and *Estrella lausannensis* (Lienard et al. 2011b). A growing interest has developed toward these bacteria as they offer some interesting perspectives in the understanding of the biology of the *Chlamydiales*. In addition, some of them represent potential emerging pathogens; *W. chondrophila* has been associated with miscarriages (Baud et al. 2007, 2014) and evidence of *P. acanthamoebae* infection was found in cases of lower respiratory tract infections (Birtles et al. 1997; Marrie et al. 2001; Greub et al. 2003).

So far, little is known about the biology and pathogenesis of *S. negevensis*. Various studies performed in the early 2000s suggest the ability to replicate in various hosts ranging from mammalian cells to arthropods and amoebae (Kahane et al. 2001, 2007; Kahane, Dvoskin and Friedman 2008; Sixt et al. 2012). However, the natural host of *S. negevensis* remains unknown, though it is highly suspected that it may be a symbiont of amoebae (Kahane et al. 2001, 2004; Lamoth and Greub 2010). In various studies, *Simkania* infection has been associated with respiratory diseases such as pneumonia and bronchiolitis (Kahane et al. 1998; Greenberg et al. 2003; Friedman et al. 2006; Fasoli et al. 2008; Heiskanen-Kosma, Paldanius and Korppi 2008; Nascimento-Carvalho et al. 2009; Donati et al. 2013). Its similarities with adverse pregnancy outcomes-related pathogens such as *C. trachomatis* and *W. chondrophila* make it a putative candidate for gynecological infections and obstetrical complications, such as premature labor, stillbirths and miscarriages (Baud and Greub 2011).

In order to precise its natural host and mode of transmission, we characterized the intracellular growth cycle of *S. negevensis* in various cell lines representing potential environmental reservoirs, namely amoebae and arthropods. We also investigated its growth within mammalian hosts such as endometrial cells and pneumocytes in order to evaluate its potential implications in human diseases such as pneumonia and miscarriages. Finally, we further investigated its intracellular traffic and characteristics of its replicative vacuole.

MATERIALS AND METHODS

Cell cultures and bacterial strains

Vero cells, A549 cells and Ishikawa cells were maintained, as previously described (Kebbi-Beghdadi, Cisse and Greub 2011), at 37°C with 5% CO₂, either in Dulbecco's modified essential medium (DMEM; PAN Biotech, Aidenbach, Germany) supplemented with 10% fetal calf serum (FCS) for Vero cells and A549 cells or in a Roswell Park medium (RPMI; PAN Biotech, Aidenbach, Germany) supplemented with 5% FCS for Ishikawa cells. Similarly, *Spodoptera frugiperda* ovarian epithelial cells (Sf9) (ATCC CRL-1711) were maintained at 27°C in Grace Insect Medium (GIM; Gibco, Invitrogen, ThermoFisher Scientific, Waltham, USA) supplemented with 10% FCS. The amoeba *Acanthamoeba castellanii* (ATCC 30010) was grown in PYG medium at 25°C.

Simkania negevensis strain Z (ATCC VR-1471) was grown within Vero cells in 25 cm² cell culture flasks (Corning, Corning, USA) in DMEM supplemented with 10% FCS at 37°C with 5% CO₂, according to the ATCC recommendations.

Chlamydia trachomatis serovar D strain UW-3/Cx was grown within McCoy cells in 25 cm² cell culture flasks (Corning) in DMEM supplemented with 10% FCS and cycloheximide at 37°C with 5% CO₂, following a 45-min centrifugation at 750 × g.

Waddlia chondrophila strain WSU 86-1044 (ATCC VR-1470) was grown at 32°C within *A. castellanii*, as previously described (Kebbi-Beghdadi, Cisse and Greub 2011).

Infection procedure

Cells were seeded 16 h prior to infection to obtain confluence (2–4 × 10⁵ cells ml⁻¹) either on a 24-wells plate (Corning) for growth kinetics and immunofluorescence analysis or on a 96-wells plate for mortality assay. Infection was performed with a 7-day-old *S. negevensis* co-culture. Briefly, cell cultures were harvested using a cell scraper and bacteria were released from cells using glass beads. The medium was then filtered through a 5-μm filter (Millipore, Merck & Cie, Schaffhausen, Switzerland) to eliminate cellular debris. Recovered bacteria were diluted in fresh medium and used to infect cells. A quantitative analysis of the bacterial particles produced at day 7 was performed using a specific quantitative polymerase chain reaction (qPCR). Dilution was adjusted to correspond to an inoculum of 25 or 5 bacteria per cell according to the various experiments (see results). Plates were centrifugated at 1790 × g for 10 min and incubated for 15 min at 37°C for mammalian cells, 27°C for insect cells or 32°C for amoebae. Then, infected cells were washed with phosphate-buffered saline (PBS) to remove non-internalized bacteria prior to the addition of fresh media. This was considered as time 0 post-infection (p.i.). Cells were then incubated for various periods of time at 37°C in presence of 5% of CO₂ for epithelial cells and at 32°C or 27°C without CO₂ for amoebae and Sf9 cells, respectively. Medium was not changed during the procedure if not otherwise specified.

C. trachomatis infection was performed using a 5-day-old co-culture and processed as described above for *S. negevensis*. Plates were centrifugated at 750 × g for 45 min, prior to media exchange. Quantification of the inoculum was performed using a pan-*Chlamydiales* PCR as described earlier (Lienard et al. 2011a).

W. chondrophila infection was performed as previously described with a dilution of 1:2000 of the amoebal co-culture (Kebbi-Beghdadi, Cisse and Greub 2011).

Quantitative polymerase chain reaction

Cells were harvested at different time points after infection for DNA extraction and qPCR. Briefly, the wells were scraped off using a 1000 μl micropipette and the recovered cells (1 ml in total) were stored at -20°C until DNA extraction; 50 μl were used for genomic DNA extraction, performed according to the manufacturer's instructions using the Wizard SV Genomic DNA purification kit (Promega, Fitchburg, USA). Quantification of *S. negevensis* DNA was performed using an in-house specific qPCR targeting the 16S rRNA gene. The following primers, amplifying a 125 bp fragment, were used: 16S forward primer (5'-ACC-TCT-TAC-CTG-GGG-ATA-ACG-GTT-GG-3'), 16S reverse primer (5'-CCA-TGA-GCC-TCT-CTA-CCG-CA) and the probe (5'-FAM [6-carboxyfluorescein]-GA*G-AGC-T*GG-GGT-AGC-CTG*-GTT-TCT- BHQ1[Black Hole Quencher 1]-3'). Nucleic acids marked by an * are locked nucleic acids, which are used to ensure a higher specificity. PCR reactions were performed as previously described for *W. chondrophila* (Goy et al. 2009). The PCR products, tested in duplicate, were detected with a StepOne Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). Water was used as a negative PCR control.

Fluorescence staining for confocal laser scanning microscopy

To perform immunofluorescence analysis, cells were grown on glass coverslips. At various time points, coverslips were fixed with ice-cold methanol 100% for 5 min. Coverslips were

processed as previously described (Kebbi-Beghdadi, Fatton and Greub 2015). *S. negevensis* was detected using an in house mouse (1:2000) or rabbit (1:2500) polyclonal anti-*S. negevensis* (antibodies that were obtained through the classical polyclonal antibody production program of Eurogentec (Liège, Belgium)). *C. trachomatis* was detected using a mouse anti-MOMP antibody (1:50) (ab20881, Abcam, Cambridge, UK), followed by a secondary antibody (Alexa Fluor 488 goat anti-mouse antibody (1:1000); Molecular Probes, Thermo Fisher Scientific). *W. chondrophila* staining was performed as reported earlier (Kebbi-Beghdadi, Cisse and Greub 2011).

To stain the organelles, coverslips were fixed according to the manufacturer protocol either in paraformaldehyde 4% (Electron Microscopy Sciences, Hatfield, USA) for 15 min at room temperature or 100% ice-cold methanol for 10 min. Both steps were subsequently used for protein disulfide isomerase (PDI) and mitochondria staining; coverslips were washed three times in PBS between blocking steps. Following a 1-h permeabilization in a 0.3% Triton X-100 5% FCS PBS solution, the Golgi apparatus and the endoplasmic reticulum were stained using the organelle localization kit containing specific markers, RCAS1 and PDI, respectively (Cell Signaling Technology Inc., Danvers, USA) or the mouse anti-GM130 (Molecular Probes, Thermo Fisher Scientific) according to the manufacturer's instructions. Subsequent staining with mouse or rabbit polyclonal anti-*S. negevensis* antibodies was performed as described earlier, prior to incubation with secondary antibodies (Alexa Fluor 488 goat anti-mouse (1:1000) and Alexa Fluor 594 donkey anti-rabbit (1:1000) or Alexa Fluor 488 donkey anti-rabbit (1:1000) and Alexa Fluor 594 goat anti-mouse (1:1000); Molecular Probes, Thermo Fisher Scientific) and 4',6-diamidino-2-phénylindole (DAPI) (1:1000) performed as previously described (Rusconi, Kebbi-Beghdadi and Greub 2015). Coverslips were washed three times in PBS between different steps. Mitochondria were stained using the MitoTracker Red CMXRos (Molecular Probes, Thermo Fisher Scientific). Briefly, cells were incubated with a 250 nM concentration for 30 min at 37°C prior to blocking. Blocking, permeabilization, *S. negevensis* detection and DAPI staining were performed as described above.

Cells were observed by confocal microscopy (Zeiss LSM 780 Quasar Confocal Microscope) and images were analyzed using the Zen Blue program (Zeiss, Feldbach, Switzerland) and the Image J program (US National Institutes of Health).

Adhesion and internalization assay

To assess adhesion and internalization of the bacteria, cells were fixed at various time points with paraformaldehyde 4% for 15 min at room temperature without permeabilization. After a 1-h saturation step in PBS supplemented with 5% FCS, external bacteria were detected, as described above, using a mouse polyclonal anti-*S. negevensis* antibody and a secondary antibody (Alexa Fluor 594 goat anti-mouse). Subsequently, cells were permeabilized in a PBS supplemented with 5% FCS and 0.1% saponin solution for 1 h, after which internal and external bacteria were detected using a rabbit polyclonal anti-*S. negevensis* antibody and a secondary antibody (Alexa Fluor 488 donkey anti-rabbit). DAPI was used to mark DNA.

Electron microscopy

Two 25 cm² cell culture flasks (Corning) containing confluent Vero cells were infected as described above with an inoculum of 25 bacteria per cell. After 18 and 48 h of infection, cells were harvested using a cell scraper. Cells were centrifuged for 10

min at 1000 rpm, resuspended in a phosphate buffer (19 ml of 0.2 M NaH₂PO₄ + 81 ml of 0.2 M Na₂HPO₄, pH 7.4) containing 0.2% glutaraldehyde (Fluka Biochemika, Buchs, Switzerland) and 4% paraformaldehyde (Electron Microscopy Sciences) and incubated 4 h at 4°C. After two additional washing steps with PBS, cells were prepared as described previously (Casson et al. 2006). Thin sections on grids were examined with a transmission electron microscope Philips CM 100 (Philips, Eindhoven, The Netherlands). Images were analyzed using the Image J program (US National Institutes of Health).

Cellular mortality assay

Cellular mortality was performed using a propidium iodide incorporation assay as previously described (de Barys, Bottinelli and Greub 2014). Briefly, propidium iodide (Sigma-Aldrich, Buchs) was diluted at 6.6 µg ml⁻¹ concentration in RPMI or DMEM without red phenol and added immediately after infection. Uninfected cells were used as a negative control. Uninfected cells treated with ice-cold methanol 100% served as a positive control and considered as a 100% mortality. A positive control was performed for each time point. After a 5-min exposure to methanol, cells were washed three times with PBS prior to the addition of propidium iodide.

Infectivity of released bacterial particles

Two 25 cm² cell culture flasks (Corning) of Vero cells were infected as described above with an inoculum of 25 bacteria per cell. At various time points, 2 ml of the supernatant of the *S. negevensis*-Vero cells co-culture were taken to inoculate fresh Vero cells cultures, seeded 6–8 h earlier on a 24-wells plate (2–4 × 10⁵ cells per well). After inoculation, plates were centrifugated as described above. Infected cells were, then, washed with PBS to remove non-internalized bacteria prior to addition of fresh media. Wells were harvested for DNA extraction at 0 h and day 6 post-inoculation with the supernatant to quantify *S. negevensis* DNA by qPCR, as described above.

Statistical analysis

Statistical analyses were performed using Prism 6.00 for Windows (GraphPad software, La Jolla, USA).

RESULTS

Growth kinetics of *Simkania negevensis* in mammalian epithelial cells, insect cells and amoebae

Simkania negevensis is traditionally described as an amoebal symbiont (Kahane et al. 2001; Lamoth and Greub 2010). Nevertheless, efficient growth has been described in mammalian cells or arthropods, and its natural host remains unknown (Kahane et al. 2007; Sixt et al. 2012). Using a specific qPCR, we compared the ability of *S. negevensis* to grow in Vero cells, endometrial cells (Ishikawa cell line), pneumocytes (A549 cells), insect cells (*Spodoptera frugiperda* cells (Sf9)) and the *A. castellanii* amoeba. As shown in Fig. 1A, we confirmed that *S. negevensis* efficiently replicates in all cell lines tested; bacterial replication started after approximately 24 h and a plateau was reached at day 6. These qPCR results were confirmed in pneumocytes, endometrial cells and Vero cells by monitoring the number of bacteria per cell observed through immunofluorescence and confocal microscopy (Fig. 1B). Interestingly, *S. negevensis* replication seemed more

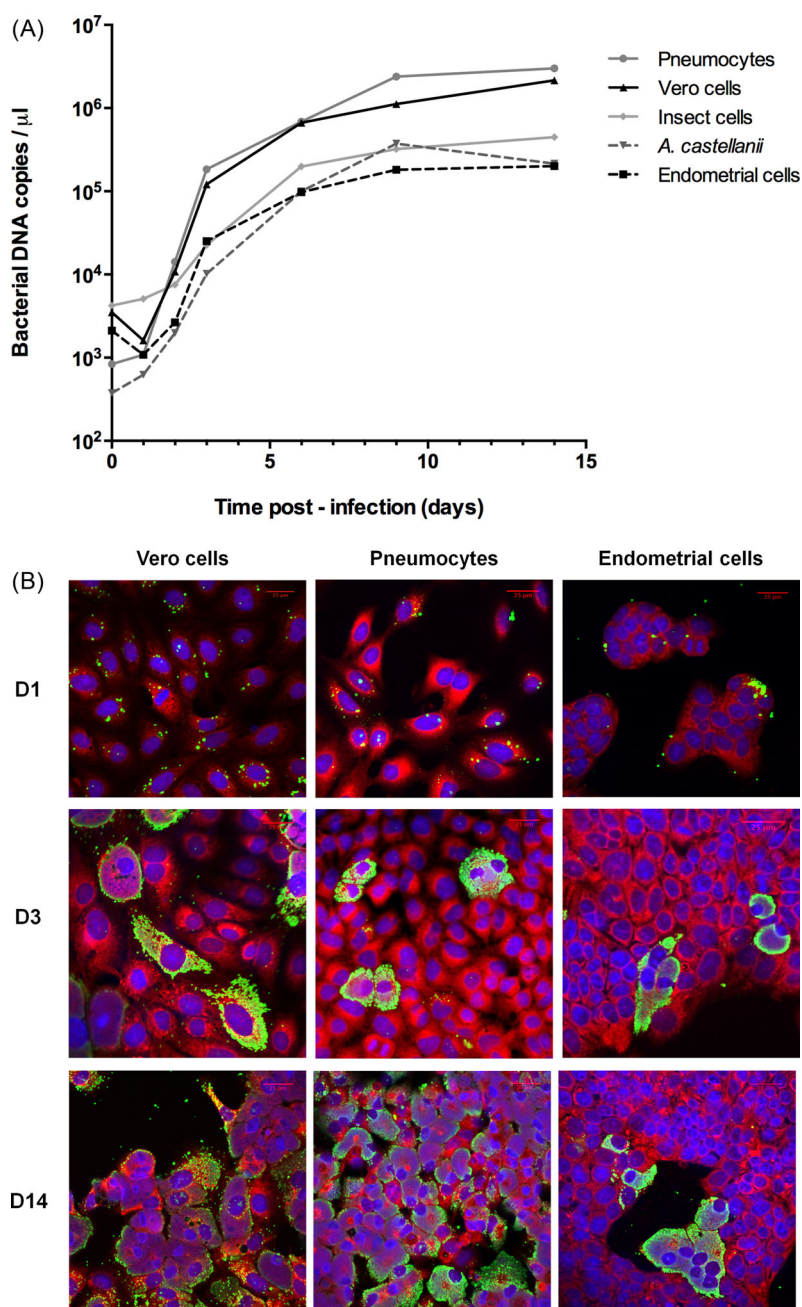


Figure 1. Growth of *S. negevensis* within mammalian cells, insect cells and amoebae. (A) Replication in Vero cells—inoculum 25 bacteria per cell, endometrial cells (Ishikawa cell line)—inoculum 25 bacteria per cell, pneumocytes (A549 cell line)—inoculum 5 bacteria per cell, *Spodoptera frugiperda* ovarian epithelial cells (Sf9 cells)—inoculum 25 bacteria per cell, and amoebae (*A. castellanii*)—inoculum 25 bacteria per cell, measured by quantitative PCR. Results are the mean of one representative experiment performed in duplicate. In total, three independent experiments in duplicate were performed for each cell line. (B) Growth of *S. negevensis* observed by immunofluorescence—*S. negevensis* particles are detected with a rabbit polyclonal anti-*S. negevensis* antibody (1:2500), followed by a secondary antibody Alexa Fluor 488-conjugated goat anti-rabbit IgG (green), mammalian cells (red) are stained with texas red-conjugated Concanavalin A (1:50) and nucleic acids (blue) are stained with DAPI (1:1000). Abbreviation: D, day.

efficient in Vero cells and pneumocytes compared to endometrial cells, insect cells or amoebae in which doubling times were prolonged (see Table 1). More importantly, we observed a higher adhesion of *S. negevensis* to pneumocytes compared to Vero cells or endometrial cells. As shown in Table 2, a mean of 8.09 bacteria per cell was observed in pneumocytes, compared to 2.01 in Vero cells and 1.22 in endometrial cells, under the same conditions. As presented in Fig. 2, at 0 h p.i., most bacteria remained extracellular (83%). However, by 24 h p.i., almost all bacteria were

found inside pneumocytes and only few extracellular bacteria remained, as illustrated in panel C, thus demonstrating an efficient internalization.

Cytopathic effect on epithelial cells and bacterial particles release

Waddlia chondrophila has been shown to rapidly induce the lysis of host cells (Kebbi-Beghdadi, Cisse and Greub 2011), a

Table 1. Doubling time of three *Chlamydiales* members in various cell lines (in hours).

Cell lines	<i>Simkania negevensis</i> (this study)	<i>Waddlia chondrophila</i> (Kebbi-Beghdadi, Fatton and Greub 2015)	<i>Chlamydia trachomatis</i> (this study)
Vero cells	13.81	1.19	11.42
Endometrial cells (Ishikawa)	18.48	2.13	22.45
Pneumocytes (A549)	12.89	1.9	nd
Amoebae (<i>A. castellanii</i>)	16.38	1.95	nd
Insect cells (Sf9)	21.07	3.78	nd

This table shows the doubling time of *S. negevensis*, strain Z (ATCC VR-1471) in various cell lines, compared to *C. trachomatis*, serovar D, strain UW-3/Cx and *W. chondrophila* strain WSU 86-1044 (ATCC VR-1470). Doubling time was calculated using the doubling-time calculator (Roth V. 2006 Doubling Time Computing, Available from <http://www.doubling-time.com/compute.php>). Doubling time was calculated between mean concentration at day 1 and day 5 or 6, for *C. trachomatis* and *S. negevensis*, respectively. Results are in hours.

Table 2. Adhesion and entry of *S. negevensis*.

	Vero cells		Pneumocytes		Endometrial cells	
	Inoculum 5	Inoculum 25	Inoculum 5	Inoculum 25	Inoculum 5	Inoculum 25
% Infected cells (1–3)	47	57	48	27	17	50
% Infected cells (>3)	2	26	12	61	2	9
Average bacteria per cell	0.72	2.01	1.05	8.09	0.32	1.22
% Intracellular bacteria	24	13	29	17	28	23

This table represents the adhesion and internalization of *S. negevensis* at time 0 h p.i. Quantification was performed under an epifluorescence microscope. The number of cells stained by DAPI was determined in each field with a $\times 100$ magnification, as well as the number of external bacteria, stained in red and green, and the number of internal bacteria, stained in green.

phenomenon also observed following *C. trachomatis* infection, though to a lesser extent (Hybiske and Stephens 2007). Previous culture observations suggest that *S. negevensis* does not induce host lysis, though quantification has never been performed (Kahane, Kimmel and Friedman 2002). In order to better characterize the effect of *S. negevensis* on host cells, we developed a mortality assay based on iodide propidium incorporation. As shown in Fig. 3, a slight increase in mortality was observed in infected pneumocytes in early time points. Nevertheless, despite high mortality rates in endometrial cells and pneumocytes at late time points, associated with significant bacterial production, similar results were obtained in infected and uninfected cells; the addition of fresh media every 3 days to prevent starvation did not change the results (data not shown) and suggests the absence of significant specific *S. negevensis*-induced mortality in these cells. Congruently, the observation of infected cells under confocal microscopy did not reveal massive lysis, as shown in Fig. 1B. On the other hand, mortality was significantly higher in *S. negevensis*-infected Vero cells, especially at day 9, suggesting an effect of the bacteria on susceptible cells.

For intracellular bacteria, lysis is an important mechanism of particles release. We monitored *S. negevensis* particles released over time and their ability to infect. As presented in Fig. 4, we observed a significant increase in bacterial particles in the supernatant starting from day 3, with a 2-log increase from day 2 to day 3, despite the absence of significant lysis observed at that time. Newly released particles were efficiently capable of producing subsequent cycles of infection, as shown in Fig. 4. Due to the high number of bacteria present at days 6, 9 and 14, in the supernatant inoculum, no specific increase could be highlighted, probably related to the saturation of the cell culture system; when diluted to 1/1000, the day 14 inoculum grew efficiently by 2 log as represented by the black and gray dashed columns in Fig. 4.

Morphology of the *Simkania negevensis* vacuole in epithelial cells

Recent works have proposed that the *S. negevensis*-containing vacuole (SnCV) was strongly associated with the endoplasmic reticulum (ER) in amoebae and epithelial cells; some association with mitochondria was also observed in epithelial cells (Mehlitz et al. 2014; Pilhofer et al. 2014). To extend these observations, we evaluated the morphology of the SnCV as well as its interactions with host organelles in the cell lines used in this work. Using the PDI as specific marker of the ER, we observed some colocalization with the ER at 48 h p.i. Interestingly, at this time point, the vacuole was completely surrounded by a double membrane as revealed by electron microscopy (Fig. 6B and C). One of these membranes most certainly corresponds to the ER membrane, as it appeared covered with ribosomes. However, a specific recruitment of the ER in early time points, as seen during *W. chondrophila* infection, could not be observed, as shown in Figs 5A and 6A. In contrast to later findings, at this early time point, the vacuole was surrounded by a single membrane (Fig. 6A). Moreover, *S. negevensis* did not seem to replicate in a single vacuole, but possibly formed a vacuolar network inside the ER, as large areas containing only bacterial particles, as seen during *W. chondrophila* infection, were not observed. Congruently, at early time points, bacterial particles were diffusely seen in the perinuclear region, which differed from the defined inclusions observed during *W. chondrophila* or *C. trachomatis* infections (see Figs 2B and 5).

Golgi apparatus fragmentation and subsequent fragments' recruitment to the vacuole are observed during *C. trachomatis* infection (Heuer et al. 2009). The morphology of the Golgi apparatus has never been studied during *S. negevensis* infection. We therefore evaluated its integrity and putative recruitment to the SnCV. In comparison to what we observed in *C. trachomatis* infection, the Golgi apparatus remained intact at 48 h p.i. in case

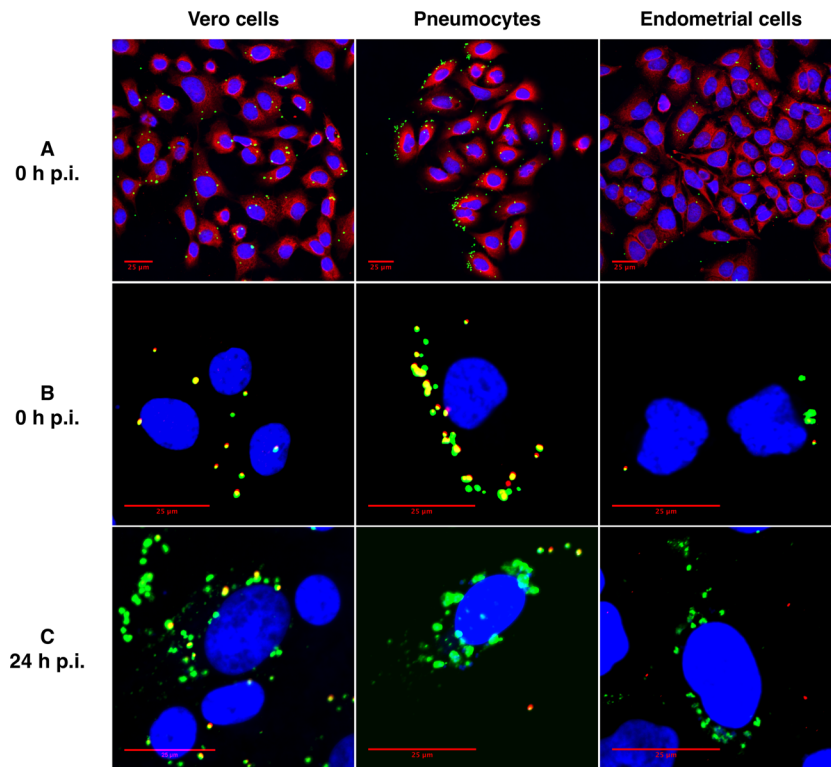


Figure 2. A higher bacterial adherence is observed in pneumocytes. Cells were infected with the same inoculum of 25 bacteria per cell and examined by immunofluorescence at 0 and 24 h p.i. (A) *Simkania negevensis* particles are detected with a rabbit polyclonal anti-*S. negevensis* antibody (green) (1:2500), cells are stained with texas red-conjugated Concanavalin A (red) (1:50) and nucleic acids with DAPI (blue) (1:1000). (B and C) Unpermeabilized fixed cells were incubated with a mouse anti-*S. negevensis* antibody (red) (1:2000), followed by a permeabilization step and a second incubation immunofluorescence with a rabbit anti-*S. negevensis* antibody (green) (1:2500) to allow discrimination between internalized and non-internalized bacteria. Bacteria were considered external when marked in red or in red and green (yellow), while particles stained only in green were considered internal.

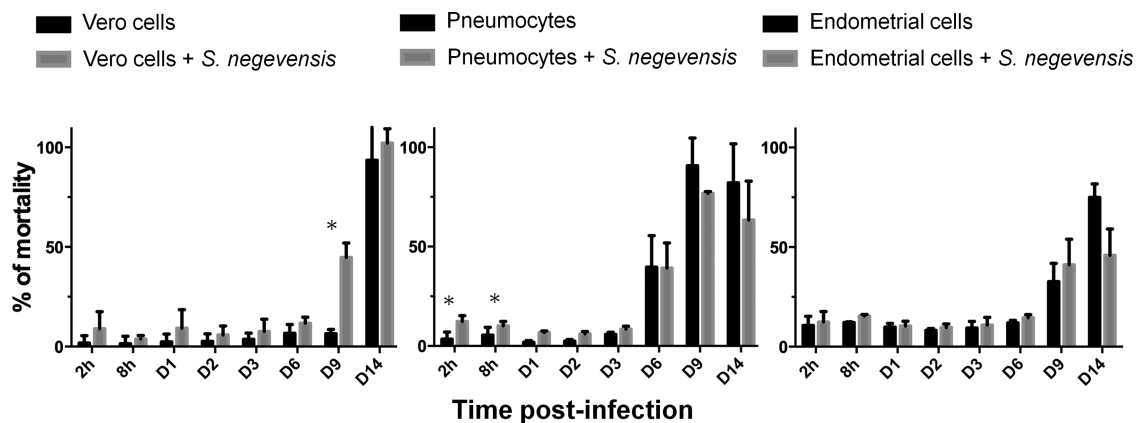


Figure 3. Cytopathic effect of *S. negevensis* on mammalian cells using the propidium iodide-based cellular mortality assay. The values correspond to the means \pm SD of three different experiments made in triplicate. Percentage of mortality was calculated compared to the value obtained in non-infected cells treated with methanol 100% for 5 min and considered as 100% mortality. * = $P < 0.01$ when comparing infected and uninfected cells using Student's t-test. Abbreviation: D, day.

of *S. negevensis* infection under the same conditions, as demonstrated in Fig. 5B. A similar pattern was observed at day 6 p.i. (data not shown). No specific association between the SnCV and the Golgi apparatus was observed.

DISCUSSION

We provided, here, the first comparative quantitative analysis of *S. negevensis* growth in various cell types. We confirmed the efficient replication in Vero cells, amoebae, insect cells and, impor-

tantly, in human endometrial cells and pneumocytes. Moreover, we demonstrated that *S. negevensis* does not induce significant lysis of host cells, in comparison to what is observed for other *Chlamydiales* (Kebbi-Beghdadi, Cisse and Greub 2011).

Our results confirm the large range of hosts harbored by *S. negevensis* that include mammalian cells, amoebae and arthropods reviewed in (Vouga, Baud and Greub 2016). This dramatically contrasts with the limited host range observed for *Chlamydiaceae*. The significantly larger genome of *S. negevensis* and its extended metabolic abilities may explain this broad host range

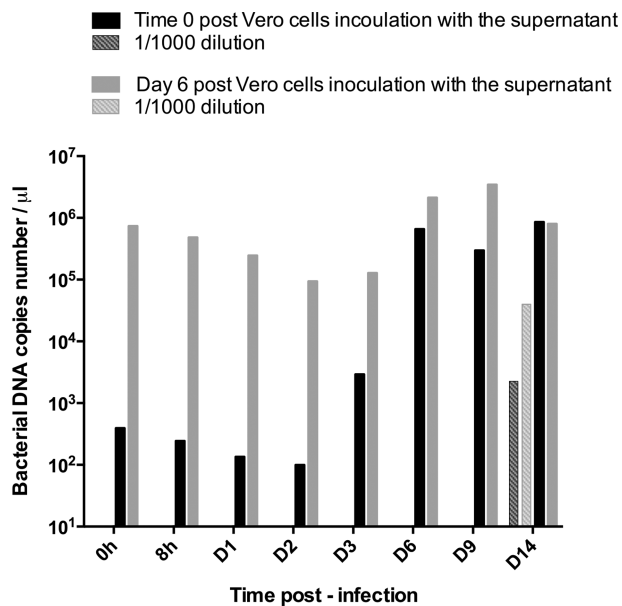


Figure 4. Infectivity of bacterial particles released upon Vero cells infection. Two milliliters of the supernatant of a Vero cells co-culture were taken at various time points to inoculate fresh Vero cells. Cells were harvested for DNA extraction at 0 h and day 6 post-inoculation with the supernatant to quantify *S. negevensis* DNA by qPCR and monitor the amount of bacteria present in the supernatant and their ability to produce an efficient infection, as assessed at day 6. Dashed lines at day 14 represent the results of a similar experiment performed with a 1/1000 dilution of the supernatant prior to inoculation of fresh Vero cells. Results are representative of one experiment. In total, two independent experiments were performed. Abbreviation: D, day.

by reducing dependency on its host for nutrients and energy sources (Collingro et al. 2011). Interestingly, infection seemed more efficient in mammalian cells, especially in Vero cells and pneumocytes, than in amoebae. This questions the role of amoebae as the natural host and main vector of transmission of *S. negevensis* (Kahane et al. 2001) and may indirectly suggest some pathogenic role toward permissive mammals (see below). On the other hand, *S. negevensis* genome possess a type I intron localized in the 23S rRNA gene, a molecular pattern normally absent in other prokaryotes (Everett et al. 1999). This structure is also present in the genome of the *A. castellanii* amoeba (Everett et al. 1999), suggesting their co-evolution. Adaptation to human hosts and possibly to other mammals may have been acquired later.

We observed a relatively slow cycle of replication compared to other *Chlamydiales* such as *W. chondrophila* or *P. acanthamoebae* (Casson et al. 2006; Kebbi-Beghdadi, Cisse and Greub 2011). It has been suggested that the particularly long cycle might be necessary for bacterial particles' maturation (Kahane, Kimmel and Friedman 2002). However, as we could obtain an efficient cycle of infection with newly released particles as early as day 3 (Fig. 4), the plateau phase does not seem to be necessary to gain infectivity. This observation may reflect the extreme adaptation of *S. negevensis* to its host as well as the few induced cytopathic effects, which provide the optimal conditions for a prolonged persistence in infected cells.

Despite the low rate of cellular mortality at day 2, we observed significant release of newly formed bacterial particles. It has recently been proposed that *C. trachomatis* particles were released by two mechanisms: (i) cell lysis and (ii) extrusion of a membrane-bound compartment containing bacterial particles (Hybiske and Stephens 2007). A similar mechanism might take

place for *S. negevensis* particles release. Such a mechanism would offer the advantage to allow protected particles release, without stimulating the host immune system, further promoting *S. negevensis* persistence.

We observed significant differences regarding the morphology and interactions with host cells' organelles of the SnCV as compared to others *Chlamydiales*. Previous reports suggested that the SnCV was associated with the ER and mitochondria in some cell lines (Mehlitz et al. 2014; Pilhofer et al. 2014). In a recent work, Mehlitz et al. (2014) proposed that the association with the ER was the strongest at 48 h p.i. using PDI as a specific marker of the ER. Using the same technique, we also observed a strong association of the SnCV with the ER and some colocalization with mitochondria at 48 h p.i. in the cell lines tested in this study. Furthermore, the ribosome covered membrane surrounding the SnCV at 48 h p.i., observed in this work, highly resembles observations performed in previous reports (Mehlitz et al. 2014; Pilhofer et al. 2014). Interestingly, such structure was not observed at early time points suggesting that subsequent maturation processes are required. Nevertheless, no active recruitment of the ER or the mitochondria seemed to occur. In contrast, *C. trachomatis*-containing vacuole forms single contacts with the ER, referred to as pathogen synapses, and *W. chondrophila* actively recruits the mitochondria and the ER by a redundant mechanism of both microtubules and actin polymerization (Croxatto and Greub 2010; Derré 2015). The observed association of the SnCV with the ER might be due to space constraints or reflect the fact that the SnCV can only develop inside a specific ER network. This is further supported by the fact that *S. negevensis* has been shown to actively inhibit ER stress (Mehlitz et al. 2014). The retrograde transport has been demonstrated to be necessary for the maturation of the SnCV (Herweg et al. 2015), but further studies regarding the SnCV maturation and its interaction with the ER are needed. Interestingly, the double membrane surrounding the SnCV highly resembles nascent autophagosomes (Campoy and Colombo 2009). A similar vacuolar organization has been described for *Legionella pneumophila*, which replication is increased in case of autophagy induction (Swanson and Isberg 1995). Potential interactions of the SnCV with autophagosomal structures need further evaluation.

We demonstrated, here, the integrity of the Golgi apparatus during *S. negevensis* infection, despite being considered as an important source of lipids through the retrograde transport in *S. negevensis* infection (Herweg et al. 2015). This further emphasizes the specificities of host cell nutrients and energy parasitism of each *Chlamydiales* family level lineage, since in opposition to *C. trachomatis*, which disrupts the host cell Golgi to obtain sphingolipids (Heuer et al. 2009), *S. negevensis* has developed a mechanism to obtain lipids less detrimental to the host cell.

On a clinical perspective, we demonstrated efficient growth in human endometrial cells and pneumocytes. This suggests that *S. negevensis* could colonize human respiratory or genital tracts. Congruently, serological evidence of human exposition has been reported worldwide (reviewed in Vouga, Baud and Greub 2016) and an association with lower respiratory tract infections, such as pneumonia and bronchiolitis, is highly suspected (Kahane et al. 1998; Friedman et al. 2006; Fasoli et al. 2008; Heiskanen-Kosma, Paldanius and Korppi 2008; Nascimento-Carvalho et al. 2009). As adhesion is a key step in the pathogenesis of intracellular bacteria, the preferential adherence to pneumocytes observed in this study further supports a specific pulmonary tropism of *S. negevensis* infection. The presence of *S. negevensis* in the genital tract has, to our knowledge, never

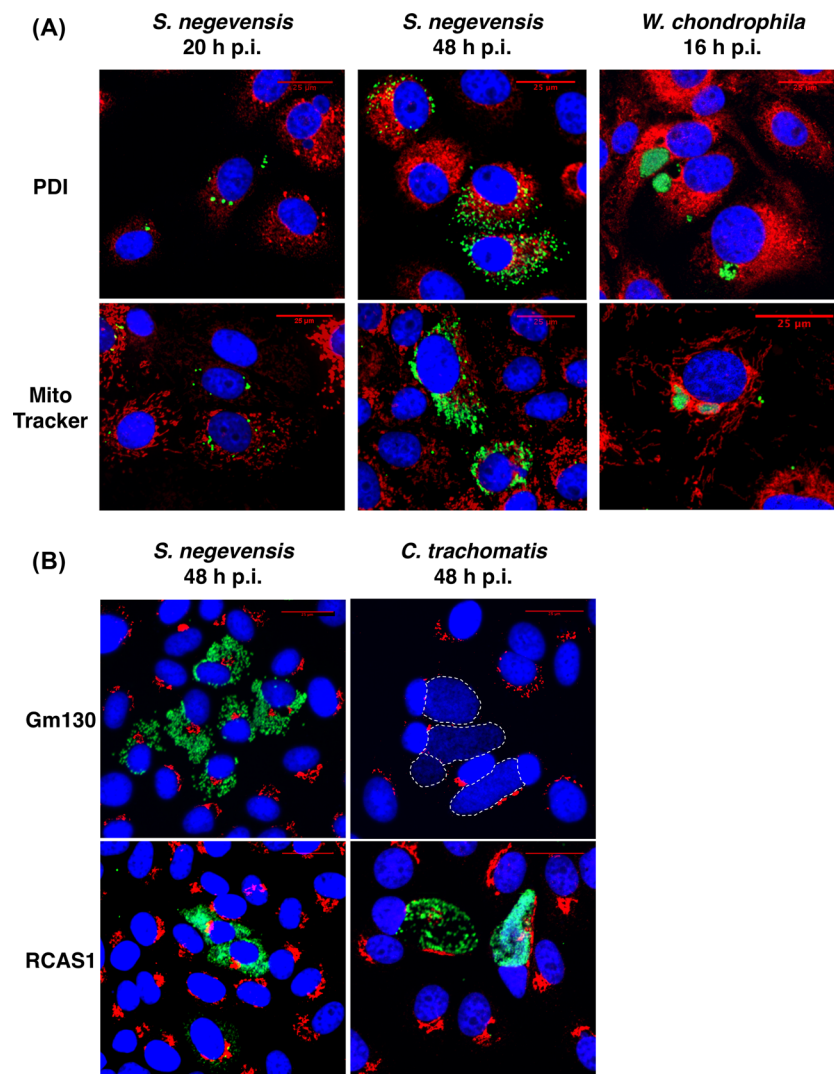


Figure 5. Assessment of the interactions of the SnCV with different host organelles. (A) ER and mitochondria as observed in Vero cells. Bacteria (*S. negevensis* or *W. chondrophila*) were detected with a species-specific polyclonal antibody (green), PDI was used to label the ER (red) and MitoTracker was used to stain mitochondria (red); nucleic acids were stained with DAPI (blue). (B) Golgi apparatus as observed in Vero cells. *Simkania negevensis* particles were detected with a specific polyclonal antibody (green); *C. trachomatis* was either detected with a specific polyclonal antibody (green) or DAPI to stain the nucleic acids (blue); for clarity, dashed lines highlight the DAPI-positive *C. trachomatis* inclusion. Gm130 and RCAS1 specific markers were used to mark the Golgi apparatus (red); nucleic acids in all experiments were stained with DAPI (blue).

been evaluated. As *C. trachomatis* and *W. chondrophila* are often detected in the female genital tract (Baud et al. 2007, 2014), investigations regarding *S. negevensis* colonization should be performed. As suggested, it is possible that *S. negevensis* infection may persist for a prolonged time in infected cells. Such persistence in respiratory or genital tracts could be associated with chronic inflammation, which may (i) lead to scarring as described for *C. trachomatis* (Hornung et al. 2014; Menon et al. 2015) or (ii) induce hyperreactive state, implicated in pulmonary pathologies such as asthma. Though an association with asthma or exacerbation of chronic obstructive pulmonary diseases was not confirmed in previous studies (Lieberman et al. 2002; Kumar et al. 2005; Korppi et al. 2006), definite conclusions are difficult to draw due to controversies regarding diagnostic tools' specificity and risks of PCR contaminations (Vouga, Baud and Greub 2016). Finally, the inhibition of the ER stress by *S. negevensis*, as well as

the presence of the bacterium itself, could act as promoting factors for other infections. The frequent association of *S. negevensis* with other pathogens, especially respiratory syncytial virus, *Mycoplasma pneumoniae*, *Streptococcus pneumoniae* and *Chlamydia pneumoniae* (Lieberman et al. 1997; Fasoli et al. 2008; Nascimento-Carvalho et al. 2009), further supports this hypothesis.

In conclusion, our work provides significant information regarding the growth cycle of this emerging bacterium and highlights significant differences with other members of the *Chlamydiales* order. Further studies are needed to better characterize the SnCV, as well as the mechanisms of host cell entry and particles release. Importantly, our work suggests that *S. negevensis* might persist for a prolonged time in infected cells. Its presence in human, either as part of the normal flora or as a pathogen associated with subacute or chronic infections should be further evaluated.

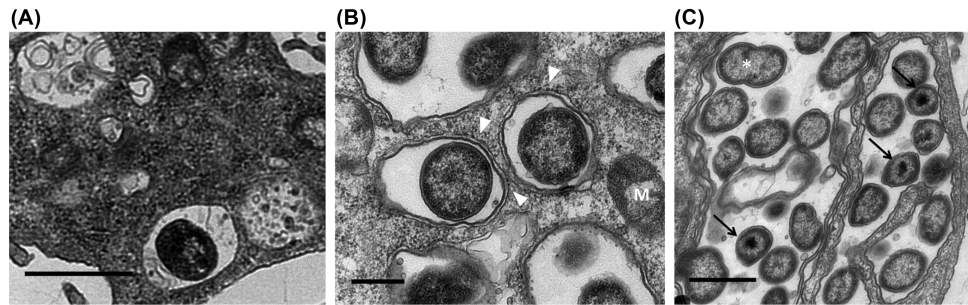


Figure 6. Morphology of the SnCV observed under electron microscopy. (A) Vero cells at 18 h p.i. with one elementary body (EB). (B) Vero cells at 48 h p.i.; various reticulate bodies (RBs) are observed. White arrowheads highlight some of the ribosomes covering the double membrane surrounding the SnCV. M, mitochondria. (C) Vero cells at 48 h p.i.; various RBs are observed. Intermediate bodies marked by a central dark spot representing condensed nucleic acids are also seen (black arrows). The white star indicates a dividing RB. Scale bars in black = 1 μ m.

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