The growth cycle of *Simkania negevensis*

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***Simkania negevensis*, a bacterium formerly referred to as 'the micro-organism Z' or 'Simkania Z', belongs to the order *Chlamydiales*, assigned to the family *Simkaniaceae*. The purpose of this study was to investigate the production of *Simkania negevensis* progeny in infected cells in comparison with the well-documented *Chlamydiaceae* developmental cycle. It was found that replicating *Simkania negevensis* in Vero cells resembled the reticulate bodies of all known chlamydial species: in electron micrographs they were reticulated, homogeneously staining, and often caught in the process of binary division. These replicative forms were found in low abundance shortly after infection, but by 3 days post-infection they were the most prevalent particles in host cells. Electron-dense forms of *Simkania negevensis* began to appear on the third day post-infection, but quantitatively did not account for the high titre of infectivity in extracts from these host cells. These had both electron-dense and electron-lucent areas, a characteristic seen only in a few chlamydial species. *Simkania negevensis* infectivity did not appreciably change during the ensuing 12 days required for host cell lysis, despite an eightfold increase in the proportion of electron-dense bacteria over this time. The emergence of electron-dense bodies, increase in infectivity and host-cell lysis were not synchronized developmental events. This is a novel finding in *Chlamydiales* spp. and suggests that *Simkania negevensis* will provide new perspectives in the mechanisms of chlamydial intracellular growth.**

Keywords: *Chlamydia*-like micro-organism, infectivity, *Simkania*

INTRODUCTION

Simkania negevensis, formerly referred to as 'the micro-organism Z' or 'Simkania Z', has been associated with community-acquired pneumonia in adults (Lieberman *et al.*, 1997) and with bronchiolitis in infants (Kahane *et al.*, 1998). Exposure to the organism is widespread in the Negev region of Israel (Friedman *et al.*, 1999), in Vancouver, BC, Canada, in Brooklyn, NY, and in Lima, Peru (unpublished data). Members of two other families in the *Chlamydiales*, the well-studied *Chlamydiaceae* and the family of *Parachlamydiaceae*, have also been associated with respiratory disease (Schachter, 1999; Birtles *et al.*, 1997). Knowledge of the *in vitro* growth cycle and molecular traits of novel intracellular bacteria may shed light on pathological and epidemiological processes associated with infection by the org-

anisms, and may have implications for the development of effective treatment and prevention strategies.

It has been proposed that *Simkania negevensis* be assigned to the new family *Simkaniaceae*, in the order *Chlamydiales*, based on its intracellular bimorphic parasitic growth in cultured cells and on rDNA sequence comparisons (Kahane *et al.*, 1999; Everett *et al.*, 1999). Its genome length is 1.7 Mbp, compared to 1.0–1.2 Mbp for members of the family *Chlamydiaceae* (Kahane *et al.*, 1999); its full-length 16S and 23S rDNA sequences are each 80–87% identical to those of members of the *Chlamydiaceae*, whereas all members of the *Chlamydiaceae* have > 90% identity with each other. [Members of the genus *Chlamydia* have > 95% identity with each other; members of the genus *Chlamydophila* have ≥ 95% identity with each other (Everett *et al.*, 1999).]

Parachlamydiaceae, *Chlamydiaceae*, *Simkania negevensis* and other *Chlamydia*-like bacteria placed in the order *Chlamydiales* exhibit some variation in their developmental morphology. In *Chlamydiaceae*, the infectious elementary body (EB) is an electron-dense, coccoid

Abbreviations: EB, elementary body; EM, electron microscopy; IFU, infectious-centre-forming unit; p.i., post-infection; PIPA, plate immunoperoxidase assay; RB, reticulate body.

body, 0.2–0.35 µm in size (Rake, 1957). It changes into a replicating form that is 0.8 µm in size and can be seen 7–9 h after infection of a host cell (Rake, 1957). This irregularly shaped replicative form is called the ‘reticulate body’ (RB), because it is ‘composed of amorphous or reticulated material with moderate density’ (Higashi *et al.*, 1962; Higashi, 1965). For reasons that are not well-understood, the *Chlamydiaceae* RB is not infective and does not survive long outside of the host cell. One to three days after infection [depending on the strain and the multiplicity of infection (m.o.i.)], most RBs have reorganized back into EBs and are released either by host-cell lysis or by fusion of the phagocytic inclusion with the cell wall. A method that has been invaluable in elucidating the replication cycle and in characterizing the developmental forms in *Chlamydiaceae* is the purification of EBs and RBs using Renografin density gradients (Caldwell *et al.*, 1981; Barbour *et al.*, 1982). Pleomorphic *Simkania negevensis* forms, some of which are reticulated and large (0.3–0.7 µm) and some of which have electron-dense and electron-lucent areas, can be selectively fractionated on density gradients in much the same way as *Chlamydiaceae* spp. bodies (Kahane *et al.*, 1999). The purpose of this study was to describe the *Simkania negevensis* growth cycle *in vitro* and compare it with that of its close relatives, the *Chlamydiaceae*. In keeping with the degree of rDNA sequence divergence observed between members of the two families, it was expected that both similarities and differences would be found.

METHODS

Growth of organisms and standard purification procedure.

Vero cells were grown in RPMI medium supplemented to 15% with fetal calf serum, 1% glucose, 100 U penicillin ml⁻¹, 100 µg streptomycin ml⁻¹, 1.2 µg nystatin ml⁻¹, 8 µg gentamicin ml⁻¹ and 50 µg vancomycin ml⁻¹. *Simkania negevensis* ATCC VR-1471^T was grown in Vero-cell cultures in the presence of 1 µg cycloheximide ml⁻¹. For the standard purification procedure cultures were harvested with glass beads between 7 and 15 days after infection and mildly sonicated; cell debris was removed by centrifugation for 10 min at 1000 g. *Simkania negevensis* organisms were purified on Urografin gradients as follows. After removal of cell debris by low-speed centrifugation, the bacterial suspension was centrifuged through a 35% (v/v) Urografin ‘cushion’ (30 min at 54000 g) in an SW 28 Beckman rotor. The pellet was resuspended in HEPES (25 mM) saline buffer and layered onto a discontinuous (40/44/52%, v/v) Urografin gradient in HEPES saline buffer. After centrifugation for 45 min at 54000 g, interface bands were removed by puncturing the gradient tube and were diluted in HEPES-saline; particles were then sedimented by further centrifugation (30 min at 54000 g). Control studies were carried out separately under identical conditions using *Chlamydia trachomatis* L2/434/BU.

Fixation and staining of infected cell monolayers for observation under light microscopy. Vero cell cultures were grown in Nunclon SlideFlasks and infected with *Simkania negevensis* (the 44/52% Urografin gradient fraction) at an m.o.i. of 0.3. At various times post-infection (p.i.) monolayers were washed with PBS (NaCl, 0.137 M; KCl, 0.0027 M;

Na₂PO₄, 0.008 M; KH₂PO₄, 0.00147 M; pH 7.2), fixed for 3 min in May–Grünwald solution (MG-500, Sigma), washed three times with distilled water and further stained for 25 min with Giemsa stain (GS-500, Sigma) diluted to 27% (v/v) with distilled water. After washing with tap water, the Nunclon flasks were taken apart, and the slide portion was examined by light microscopy and the image photographed.

Infectivity assays for *Simkania negevensis*. For growth curves, monolayers of Vero cells in replicate 25 cm² flasks were inoculated with *Simkania negevensis*. At various times after infection cells were scraped into the medium with glass beads; the suspensions were frozen at –70 °C with 50% fetal calf serum. The number of infectious-centre-forming units (IFUs) for each sample was determined by 10-fold dilution and infection of Vero cells cultured in 96-well plates. Three days p.i. the plates were fixed in 95% ethanol for 10 min at room temperature and examined for IFUs by the microtitre ‘plate immunoperoxidase assay’ (PIPA), performed as described previously (Kahane *et al.*, 1998). Briefly, after fixation, plates were incubated for 1 h at 37 °C with *Simkania negevensis*-specific antisera raised in rabbits, washed and re-incubated (1 h, 37 °C) with swine anti-rabbit horseradish-peroxidase-conjugated antibodies, and after another wash, stained with diaminobenzidine as substrate. Infectious centres were counted under a magnification of ×200 with an inverted microscope, and the counts for three wells were averaged; the IFU ml⁻¹ count for the original sample was then calculated.

Titration of infectivity. *Simkania negevensis* particles that banded at the 40/44% interface and at the 44/52% interface of a Urografin density gradient upon centrifugation were directly examined in two ways: (1) by titration on Vero cells to determine the number of IFUs in each fraction, and (2) by enumeration of aliquots mixed with known concentrations of polystyrene latex beads by negative-stain electron microscopy (EM). Replicative forms and electron-dense forms of the bacteria present in both fractions were determined by photography of random EM thin-section fields and by counting samples of several hundred particles. The relative infectivity of the constituents of each fraction was determined.

EM

Negative staining. Purified *Simkania negevensis* particles suspended in 25 mM HEPES saline buffer were homogeneously mixed with polystyrene latex particles (mean diameter 0.175 µm, at 3.51 × 10¹¹ particles ml⁻¹; Agar Scientific) and applied to a 400-mesh copper Formvar/carbon-coated grid which was fixed and stained as described by Glauret (1975). Briefly, the grids were dipped for 15 s in paraformaldehyde/glutaraldehyde fixative and then three times in distilled water, followed by staining in 2% ammonium molybdate solution, pH 7.4. Negative staining was used to count total particle numbers, without discrimination as to the type of the particle.

Thin sections of cultured cells or gradient-fractionated bacteria. Infected cells were scraped from flasks and pelleted, or bacteria were fractionated on discontinuous Urografin gradients. These preparations were fixed, embedded in Araldite, and stained for EM as described by Biberfeld (1971). The larger morphological forms, 0.3–0.7 µm, were designated ‘replicative’ forms for the following reasons: (i) they were the first forms to appear after infection; (ii) they multiplied rapidly and were often captured apparently in the process of binary division; and (iii) they contained reticulated, homogeneously stained material and no white, unstained or electron-dense areas. The smaller particles, 0.2–0.3 µm, were designated ‘electron-dense’ forms because they contained at least some

Fig. 1. Vero-cell monolayers fixed and stained with May-Grünwald/Giemsa at various times after infection with *Simkania negevensis*. (a) Uninfected cells; (b) 2 days; (c) 5 days; (d) 13 days. Bar, 50 µm.

electron-dense material with or without an electron-lucent area and no reticulated material (Kahane *et al.*, 1993). Typically, 8–10 randomly chosen EM fields of sectioned infected cells were photographed, and several hundred particles were counted from the photographs of each Urografin fraction to determine the relative proportion of the morphological forms.

***Chlamydia trachomatis* EB and RB infectivity.** The infectivity of *Chlamydia trachomatis* EBs and RBs prepared on discontinuous Urografin gradients was assessed as a control for the *Simkania negevensis* experiments. *Chlamydia trachomatis*-infected cells were identified in the PIPA by using polyclonal rabbit antisera raised against *Chlamydia trachomatis* (Gonen *et al.*, 1993).

RESULTS

Light microscopy studies

It was previously reported (Kahane *et al.*, 1999) that the replication cycle of *Simkania negevensis*, as observed by phase-contrast microscopy, takes about 12–15 days, whereas *Chlamydia trachomatis*-infected (and other *Chlamydiaceae*-infected) cells detach from the monolayer and lyse as early as 2–3 days after infection. To observe in detail the growth of *Simkania negevensis* in cell culture, Vero cells were infected at an m.o.i. of 0.3, then fixed and stained at various times. Fig. 1(a) shows uninfected cells and Fig. 1(b–d) shows cells infected for 2, 5 and 13 days, respectively. It appeared that infection did not readily extend to neighbouring cells within the experimental time window. Infected cells could be distinguished from uninfected cells by the presence of

dark (mostly perinuclear) patches in the cytoplasm. With increasing time after infection, these patches built up into masses which occupied a large proportion of the cytoplasm of the cell (Fig. 1c), and at later times large vacuoles appeared which seemed to be empty (Fig. 1d). However, when such vacuoles were seen by phase-contrast microscopy, they often contained fine, rapidly moving particles (not shown). These observations were in agreement with studies carried out at higher m.o.i. values (Kahane *et al.*, 1999). In control studies, carried out under identical conditions using *Chlamydia trachomatis*, infected cells detached from the monolayer and lysed 2–3 days p.i.

Morphology of particles and inclusions

The electron-dense area in *Simkania negevensis* particles began to appear on day 3 p.i. and was located to only one side of each coccoid or slightly elongated 0.2–0.3 µm particle. Each particle almost always contained some amorphous electron-lucent areas as well. Replicative forms of *Simkania negevensis* resembled RBs of other chlamydiae in size (0.3–0.7 µm), in homogeneity and in light staining (Fig. 2b–f). They were somewhat pleomorphic in shape and were the only form that was ever seen undergoing binary fission. Particles whose appearance was consistent with being in transition between these two morphological types were also seen in EM thin sections; in Urografin gradients these intermediate forms banded at both the 40/44% and the 44/52% Urografin gradient interfaces. In contrast to *Chlamydia trachomatis*, where only one inclusion body is observed

Fig. 2. Electron micrographs of thin sections of Vero cells infected with purified *Simkania negevensis* 40/44% fraction, fixed and embedded at various times after infection. (a) 1 day; (b) 2 days; (c) 3 days; (d) 6 days; (e) 9 days; (f) 12 days. Bars, 1 μ m.

in light microscopy or electron micrographs, electron micrographs of *Simkania negevensis*-infected cells showed many small inclusions (Fig. 2e, f) close and intertwining, especially late in infection.

Kinetics of production and release of progeny infectious organisms

It had been shown previously that when the majority of cells in a culture were infected with *Simkania negevensis*, the growth curve of progeny IFUs had an exponential phase and a plateau (Kahane *et al.*, 1999). Since high titres of infectious particles were detectable already at 2–3 days p.i., it was of interest to determine whether in cultures infected at very low input m.o.i. values, particles produced at early times would initiate further cycles of infection. Fig. 3 shows that a somewhat longer lag period and a lower plateau level were found with decreasing input m.o.i., and that further cycles of infection were apparently not initiated. Infection of other cell lines (e.g. HEp-2) with *Simkania negevensis* resulted in similar growth kinetics, namely a plateau stage following relatively brief exponential growth. No evidence of cell lysis was visible in culture bottles, even 12–15 days p.i. When supernatants of cultures infected at an m.o.i. of 0.1 were tested for the presence of

infectious particles as a function of time, to find evidence for the release of infectious particles from cells, no infectious particles were found in the culture medium during the first 3 days p.i.; thereafter the level of particles in the culture medium was, at each time point, less than one-thousandth of the total level of infectious particles in the culture (measured in parallel in replicate cultures). Thus, either less than one infected cell in a thousand lysed or any given infected cell released less than one one-thousandth of its progeny infectious particles into the culture medium.

Growth curves of density-gradient-fractionated *Simkania negevensis*

Separation of *Simkania negevensis* morphological forms on Urografin gradients produced bands at the 40/44% interface and at the 44/52% interface; exactly the same was observed for *Chlamydia trachomatis* EBs and RBs under identical conditions in control studies. Material from both fractions was examined by transmission EM, titrated by PIPA to determine the number of IFU ml⁻¹, and used immediately to initiate growth in fresh monolayers. Particles in transition between the electron-dense and replicative form were evident in both bands, according to EM. EM also indicated that the flasks

Fig. 3. Growth curves of *Simkania negevensis* on Vero cells at various m.o.i. values. Cells were infected with gradient-fractionated electron-dense bacteria at the m.o.i. values indicated and harvested at the time points indicated. Each culture was titrated on Vero-cell monolayers by PIPA. m.o.i. values: ■, 0.5; ▲, 0.05; ●, 0.005; △, 0.0005; ○, 0.00005. Bars indicate the standard deviation from the mean for triplicate wells at each time point. The upper curve was previously published as Fig. 1 in Kahane *et al.* (1999).

infected with the 44/52% fraction received 8.4 times as many electron-dense particles as the flasks infected with the 40/44% fraction. The m.o.i., however, as determined in parallel by PIPA titration (IFU ml⁻¹), was 2.8 for the 44/52% inoculum and 1.2 for the 40/44% inoculum (Fig. 4). The growth curves of the 44/52% inoculum and the 40/44% inoculum were practically superimposable, which would not be expected if one fraction had eight times as many infectious particles as the other (cf. Fig. 3).

To investigate the infectivity of *Simkania negevensis* particles in Vero-cell cultures, fresh preparations of the 40/44% or the 44/52% Urografin fractions were used to inoculate cell monolayers at an m.o.i. of 0.1. When cultures were infected with the 44/52% inoculum, electron-dense particles could be seen at 17–24 h p.i. (not shown). The growth cycle resulting from the 40/44% inoculum was followed by both EM and by infectivity assays (Figs 2 and 5). EM of cells infected with the 40/44% Urografin fraction showed only one inclusion body early in infection (17 and 20 h, and up to 1–2 days) that typically contained one or, infrequently, two replicating bacteria. By day 3, replicative forms were numerous and only an occasional electron-dense body was present (Fig. 2c). Subsequently, inclusions could be found that contained diverse particles in various stages of development. By 6, 9 and 12 days p.i., the total number of intracellular particles had increased

Fig. 4. Growth curves of *Simkania negevensis* in Vero cells infected with 44/52% (●) and 40/44% (○) Urografin fractions at an m.o.i. of 2.8 and 1.2, respectively. IFU values were determined by PIPA. Bars indicate the standard deviation from the mean for triplicate wells at each time point.

Fig. 5. Kinetics of the appearance of infectious particles (●) after inoculation of Vero-cell monolayers with purified *Simkania negevensis* 40/44% inoculum and the proportion of electron-dense particles to total cellular particles as counted from electron micrographs of thin sections of the infected cells (○). Bars (most are not visible) indicate the standard deviation from the mean for triplicate wells at each time point.

greatly, with the proportion of electron-dense bacteria rising rapidly (Fig. 5, right vertical axis). Some replicative forms with irregular morphology were present at 12 days p.i. (Fig. 2f). The proportion of electron-dense to replicative forms in several electron micrographs photographed at random for each time point is represented in Fig. 5. Total particle counts ranged from 500 to 1000 for

Table 1. Data used to calculate infectivity not explained by electron-dense bacteria in 40/44 % Urografin fractions of *Simkania negevensis* or by EBs in 40/44 % Urografin fractions of *Chlamydia trachomatis* (representative experiments)

Organism	Urografin fraction (%)	Total no. particles	IFU	Particle proportions*		Infectivity accounted for by dense bacteria (%)†	Unexplained infectivity§
				Dense	Replicative		
<i>Simkania negevensis</i>	44/52	5.7×10^9	3.13×10^9	90.1	9.9	100‡	—
	40/44	6.9×10^9	2.5×10^9	25.2	74.8	42	58 %
<i>Chlamydia trachomatis</i>	44/52	5×10^{10}	3.4×10^8	92.0	8.0	100‡	—
	40/44	3.8×10^{10}	2.3×10^6	9.0	91.0	100	—

* As determined by EM.

† The ratio of electron-dense particles : IFUs determined for the 44/52 % Urografin fractions were assumed to be valid for electron-dense bacteria in the 40/44 % Urografin band as well.

‡ Per definition.

§ —, No unexplained infectivity, either by calculation or by definition.

each time point between 6 and 12 days. For days 1, 2 and 3, total particle counts were 15, 32 and 104, respectively.

Simkania negevensis particles in the Urografin fractionated inocula were enumerated by EM. As shown in Table 1, not all of the infectivity in the 40/44 % fraction could be accounted for by electron-dense bacteria, as enumerated by EM. In four repetitions of the experiment, the level of infectivity not accounted for ranged from 48 to 94 %. Identical control studies were carried out in several replicate experiments using *Chlamydia trachomatis*. In these experiments all of the infectivity in the 40/44 % fraction was accounted for by the number of EBs present, whether the proportion of EBs in the fraction was minimal or substantial.

DISCUSSION

This is the first detailed account of the developmental cycle of a *Chlamydia*-like bacterium that does not belong to the family *Chlamydiaceae*. The growth cycle of *Simkania negevensis* was found to be similar to that of members of the well-known family of the *Chlamydiaceae*, in that compact electron-dense and larger replicative forms were seen in EM thin sections and in that electron-dense forms were never observed dividing. However, given the larger genome size of *Simkania negevensis* and its phylogenetic distance from the *Chlamydiaceae*, it was not surprising that differences were also observed. The growth cycle, as observed by phase-contrast microscopy, was longer (Kahane *et al.*, 1999) and the morphology of electron-dense particles, as shown by EM, was somewhat different from chlamydial EBs. These particles were 0.2–0.3 µm in diameter and usually contained some electron-lucent areas in addition to areas of condensed DNA; replicative forms were more pleomorphic than *Chlamydiaceae* RBs, usually being elongated with dimensions of 0.3–0.7 µm, and without areas of condensed DNA (Fig. 2). The multiple, small, intertwining inclusions seen by EM in cells infected with *Simkania negevensis* (Fig. 2e, f) contrasted

with the single inclusions which are seen in *Chlamydia trachomatis*-infected cells, and which are generated by the fusion of multiple inclusions (Ridderhof & Barnes, 1989). The *Simkania negevensis* inclusions were more similar to the multiple inclusions seen in cells infected with *Chlamydia psittaci* (Hodinka & Wyrick, 1986).

In the present study, we extended our original observations that the phenotypic replication cycle of *Simkania negevensis* takes about 12–15 days, while the number of IFUs produced increases exponentially for only 2–4 days, after which time a plateau is reached (Kahane *et al.*, 1999). The photomicrographs of fixed and stained infected cells obtained in this study (Fig. 1) clearly show the accumulation of bacterial particles with time in the infected cells, as well as the relative lack of spread of the infection to neighbouring uninfected cells. Growth curves obtained at lower m.o.i. values (Fig. 3) also suggested that the infection does not spread to neighbouring cells, and that infectious particles are not released in significant amounts into the culture medium. The significance of the long plateau stage of the growth cycle during which cytopathic effects increased, but the organisms appeared to be ‘trapped’ in their host cells, is not understood. We found that four sequential ‘rapid’ passages of organisms present at 3–4 days p.i. did not result in a decrease of infectious yields, as compared to four ‘long’ sequential passages of 10 days duration (data not shown). This would seem to indicate that at least under *in vitro* conditions, there is no need for ‘maturation’, which might be postulated to be taking place during the long plateau period. However, the stationary phase may result in some survival advantage for the organisms during transmission in nature. Cells infected with *Chlamydia trachomatis* L2 detach from the monolayer or lyse by 2 days after infection; uninfected neighbouring cells are rapidly infected (data not shown). On the other hand, cells infected with the more distantly related intracellular bacterium *Coxiella burnetii* show minimal cytopathic effects and it has been suggested that this organism may lack an efficient membranolytic

system (Heinzen *et al.*, 1999); however, infectious forms can be mechanically released from infected cells early in the developmental cycle (Howe & Mallavia, 2000).

The first hint that *Simkania negevensis* replicative forms may be infectious was the similar kinetics of growth of particles from the 44/52% and 40/44% Urografin fractions (Fig. 4). This possibility was lent more credibility by the fact that in growth curves, progeny infectivity increased not simultaneously with the appearance of electron-dense forms in EM thin sections, but earlier (Fig. 5), and received further support from the results of infectivity experiments done with purified 44/52% and 40/44% fractions (Table 1). In control experiments, using *Chlamydia trachomatis*, EBs totally accounted for the infectivity found in the 40/44% fraction, but this was not the case for *Simkania negevensis*. The infectivity of particles appearing to be in transition between the larger replicative form and the more compact electron-dense form is not known. For the calculations, transition particles showing either a dense area or an unstained area were counted as dense forms and presumed to be infectious. Counting these particles as dense forms may have resulted in an overestimation of the level of such forms in the 40/44% fraction and, therefore, may have led to an overestimation of their relative contribution to the infectivity in the 40/44% fraction. Nevertheless, the data indicate that in the case of *Simkania negevensis*, infectivity was apparently not limited to the dense forms.

Since the genome of *Simkania negevensis* is nearly twice the size of the *Chlamydia trachomatis* genome (Kahane *et al.*, 1999), it is possible that additional genes permitting infectivity of all morphological forms are present in *Simkania negevensis*. If *Simkania negevensis* replicative forms are indeed infectious it may be predicted that these particles might be characterized by a physical stability comparable to that of the electron-dense forms, in contrast to *Chlamydiaceae* RBs which are osmotically fragile and incapable of infecting cells (Moulder, 1985; Tamura *et al.*, 1967). Preliminary evidence suggests that *Simkania negevensis* replicative forms have a survival curve similar to that of electron-dense forms after exposure to various temperatures and to osmotic shock (unpublished data). More extensive studies comparing the morphological forms of *Simkania negevensis* are necessary; examination of the similarities and differences in membrane composition is of special interest.

It is notable that the growth cycle of *Coxiella burnetii*, which belongs to the order *Rickettsiales* (family *Rickettsiaceae*), includes two forms, a 'small cell variant' and a 'large cell variant', both of which are infectious (Wiebe *et al.*, 1972). Our results raise questions as to the role played by electron-dense bodies and larger bodies in the growth cycles of newly described *Chlamydia*-like micro-organisms, some of which have not yet been grown in cell culture (Fritsche *et al.*, 2000; Osseward & Meijer, 1999), including members of the *Parachlamydiaceae* and *Waddliaceae*, in the order *Chlamydiales*

(Everett *et al.*, 1999). Also, more general questions are raised as to the potential significance of *Simkania negevensis* morphological forms in the pathogenesis or epidemiology of infection by this novel micro-organism.

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