***Simkania negevensis* strain Z^T: growth, antigenic and genome characteristics**

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***Simkania negevensis* is the type species of *Simkaniaceae*, a recently proposed family in the order *Chlamydiales*. In the current study, growth, antigenic and genomic characteristics of this intracellular bacterium were investigated and compared to those of members of the family *Chlamydiaceae*. Growth of the organism, as assessed by infectivity assays, reached a plateau in 2–3 d although by light microscopy the cytopathic effect on the host cells increased for 12 or more days after infection. *S. negevensis* growth was unaffected by sulfadiazine. Cells infected by *S. negevensis* strain Z^T were not recognized by either of two monoclonal antibodies specific for *Chlamydiaceae* LPS and several specific *Chlamydiaceae ompA* primers were unable to PCR amplify a *S. negevensis* gene. The *S. negevensis* genome contained one copy of the ribosomal operon. The genome size of *S. negevensis* strain Z^T was determined by PFGE to be 1.7 Mbp, and the G+C content was 42.5 mol%. These data, taken together with other published data, are consistent with the proposal that *S. negevensis* belongs to a distinct family in the order *Chlamydiales*.**

Keywords: *Chlamydiales*, *Simkaniaceae*, rDNA copy number, genome size, *Chlamydia*

INTRODUCTION

Simkania negevensis, previously characterized as 'the chlamydia-like micro-organism Z,' is related to other species in the order *Chlamydiales* based on its rDNA sequence and intracellular, chlamydia-like developmental cycle (Kahane *et al.*, 1993, 1995; Everett *et al.*, 1999). The family *Simkaniaceae* currently includes only strain Z^T, which is the type strain of *Simkania negevensis*, and the description of the family is derived from current information about the Z^T strain as compared with other families in the *Chlamydiales*. The full-length 16S and 23S rRNA sequences of *S. negevensis* strain Z^T are 80–87% identical to those of other members of the *Chlamydiales* (Everett *et al.*, 1999). Phylogenetic analyses of the ribosomal sequences have been published previously (Kahane *et al.*, 1995;

Amann *et al.*, 1997; Everett & Andersen, 1997; Pettersson *et al.*, 1997; Everett *et al.*, 1999).

While it is evident that *S. negevensis* shares a common ancestor with the *Chlamydiaceae*, a well-studied family in the *Chlamydiales*, *S. negevensis* differs from members of the *Chlamydiaceae* in that it is resistant to ampicillin, penicillin G and cyclosporin (Kahane *et al.*, 1993). Antibody prepared against *Chlamydia* GroEL cross-reacts with *S. negevensis* GroEL, but no other serological cross-reaction between these bacteria has been documented (Kahane *et al.*, 1993). In addition, the extrachromosomal plasmid that is present in some *Chlamydiaceae* strains is absent from *S. negevensis* (Kahane *et al.*, 1993). In contrast to *Chlamydiaceae* and all known bacteria, *S. negevensis* has a group I intron in the 23S rRNA (K. D. E. Everett, S. Kahane, R. M. Bush & M. G. Friedman, unpublished results).

S. negevensis was isolated as a culture contaminant from human and simian cells. *S. negevensis* is similar to other chlamydial species in that it is a probable aetiological agent of human disease. It has been implicated in cases of bronchiolitis in infants (Kahane *et al.*, 1998) and is associated serologically with community-acquired pneumonia in adults (Lieberman *et al.*, 1997).

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Abbreviations: EB, elementary body; IFU, infectious-centre-forming unit.

The GenBank accession number for the 23S rRNA sequence of *Simkania negevensis* strain Z^T is U68460

There is evidence that human exposure to *S. negevensis* is widespread (Kahane *et al.*, 1996; Friedman *et al.*, 1999). The purpose of this study was to provide an extended description of the family *Simkaniaceae* by determining additional growth, antigenic and genomic characteristics of the type strain of *S. negevensis*.

METHODS

Bacterial strains. The bacterial strains used in these studies were *S. negevensis* strain Z^T (ATCC VR 1471^T) (Kahane *et al.*, 1993), *Chlamydophila pneumoniae* strain TW-183^T (Washington Research Foundation), *C. trachomatis* strain D/UW-3/CX (H. D. Caldwell, NIAID Rocky Mountain Laboratory, Hamilton, MT, USA) and strain L2/434/BU (Dr. R. Cevenini, Department of Microbiology, University of Bologna, Italy), *Chlamydia muridarum* strain MoPn^T (H. D. Caldwell), *Chlamydia suis* strain R22 (NADC), and *Chlamydophila psittaci* strain NJ1 and strain GD (from NADC) (Everett & Andersen, 1997).

Growth characteristics of *S. negevensis*. *S. negevensis* strain Z^T was grown in Vero cells (ATCC CCL 81) obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) in RPMI medium (Biological Industries) containing 15% foetal bovine serum (FBS), 1% glucose, 100 units penicillin ml⁻¹, 100 µg gentamicin ml⁻¹, 160 µg vancomycin ml⁻¹ and 1 µg cycloheximide ml⁻¹. To test for sulfadiazine resistance, some experiments were duplicated in the presence of 2–100 µg sulfadiazine ml⁻¹ (Sigma). In some experiments, glucose-free medium was used for the growth of cells infected by strain Z^T. Glucose-free medium consisted of special order preparations of RPMI containing no glucose (Biological Industries) that were supplemented with dialysed FBS (15%) and with antibiotics as above. Whole cells were removed from the flasks with glass beads, and after mild sonication, elementary bodies (EBs) that served as inoculum for growth curves and DNA preparation were purified using Renografin (Solvay Animal Health) by the method of Caldwell *et al.* (1981). For growth curves, monolayers of Vero cells in 25 cm² flasks were inoculated with *S. negevensis* EBs at an m.o.i. of 0.5. At various times after infection, cells were scraped into the medium with 1 mm diameter glass beads, and the suspension was frozen at –70 °C with 50% FBS. Infectious-centre-forming units (IFUs) were determined for each time point by titration in 10-fold dilutions on Vero cell monolayers plated in 96-well cell culture plates. Detection of IFUs was done with an immunoperoxidase assay using rabbit antisera raised against *S. negevensis* (Kahane *et al.*, 1998).

Reaction of *S. negevensis* with *Chlamydiaceae* group-specific mAbs. Monolayers of Vero cells were infected with *S. negevensis* strain Z^T, *Chlamydia trachomatis* strain L2/434/BU, or *C. pneumoniae* strain TW-183^T. Fully intact monolayers showing clear evidence of chlamydial inclusions were released by light trypsinization after 5 d (*S. negevensis*) or after 1 d (*C. trachomatis* and *C. pneumoniae*), and cell suspensions were spotted onto glass slides and air-dried (Kahane *et al.*, 1998). After fixation with cold acetone, slides were incubated for 1 h at 37 °C with monoclonal antibodies (mAbs) to chlamydial LPS (NJ1/D3; Andersen & Van Deusen, 1988) in PBS pH 7.4, rinsed several times with PBS, and incubated for 1 h at 37 °C with either peroxidase-conjugated affinity-purified goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) or FITC-conjugated rabbit anti-mouse immunoglobulins (Dakopatts) in PBS. Colour development for the peroxidase-conjugated antibody was

with chromogen (Savyon Diagnostics), and slides were examined for stained cells by light microscopy. FITC-stained slides were examined with a Zeiss 9901 fluorescence microscope.

DNA base composition. The DNA G + C content of the *S. negevensis* genome was determined by the thermal denaturation method (Stanton *et al.*, 1997). DNA was prepared carefully from EBs to minimize shearing. Renografin-purified EBs were suspended in 30 mM Tris, 10 mM EDTA, 50 mM DTT, pH 9.0, and lysed for 1 h at 37 °C. Nonidet P-40 was then added to 1%, along with 0.5 mg DNase-free RNase ml⁻¹ (Boehringer Mannheim), and incubation was continued for 1 h at 37 °C. Proteinase K (GibcoBRL Life Technologies) was added to 0.2 mg ml⁻¹ and the lysate was incubated overnight at 37 °C. The DNA was then sequentially extracted with TE buffer (10 mM Tris, 10 mM EDTA, pH 8.0) saturated with phenol, phenol/chloroform/isoamyl alcohol (24:24:1, by vol.) and chloroform, and finally dialysed extensively at 4 °C against 1 × SSC buffer (0.15 M NaCl, 15 mM sodium citrate, pH 7.0). Control DNA was prepared from Renografin-purified EBs of *C. psittaci* strains NJ1 and GD using CsCl gradient isolation and ultracentrifugation. Melting temperatures were determined using the Beckman DU 650 Spectrometer, the Beckman High Performance Temperature Controller, and software that calculated the first derivative and the 2-point mean (Beckman). The *S. negevensis* G + C content was the mean of three experiments.

Analysis of the genome size of *S. negevensis* strain Z^T, by PFGE. *S. negevensis* was grown in Vero cells and EBs were purified by the Renografin method as described above. Prior to the Renografin gradient step, cellular DNA was removed by treatment with bovine pancreatic DNase (Amersham; 300 U ml⁻¹ containing 5 mM MgSO₄, 30 min, 37 °C). Agarose blocks for pulsed field gel electrophoresis experiments were prepared by mixing equal volumes of 2% low-melting-point (50 °C) agarose (Bio-Rad) and purified *S. negevensis* EBs (approx. 1 µg in each plug). After overnight incubation of the agarose blocks at room temperature with 30 mM Tris, 10 mM EDTA, 50 mM DTT, pH 9.0, RNA was digested with DNase-free RNase in TE buffer. Proteinase K digestion was carried out overnight in TE buffer and was followed by extensive washing of the plugs in TE buffer at room temperature for several days. Control plugs containing DNA from *C. muridarum* MoPn^T, *C. suis* R22 and *C. trachomatis* D/UW-3/CX were prepared similarly, except that pellets of *Chlamydia* and *Chlamydia*-infected cells were prepared as described in Everett *et al.* (1999).

The plugs were inserted into a 1% (w/v) agarose gel (12.5 × 12.5 × 1.2 cm) and electrophoresed at 150 V, 14 °C using the CHEF-DR Drive Module, Pulsewave 760, and power supply model 200/2.0 (Bio-Rad) with recirculating buffer containing 22.5 mM Tris-borate, 1 mM EDTA (TBE). A pulse time of 2 min was applied for 24 h, the buffer was replaced with fresh buffer, then a pulse time of 4 min was applied for 30 h (Bergthorsson & Ochman, 1995). The bands of DNA were stained with ethidium bromide. PFGE II size markers (Boehringer Mannheim) were used.

Restriction enzyme analysis of whole *S. negevensis* genomic DNA. Agarose plugs containing whole-genomic DNA of *S. negevensis* strain Z^T were prepared as described above. The rare-cutting restriction enzymes *NotI*, *AscI* and *SfiI* were obtained from BioLabs. Individual pre-incubation of the

plugs was carried out in the appropriate buffer for 1 h at 37 °C in 4-well culture dishes. Buffer was removed, and the restriction enzyme reaction mixture was added. Digestion was performed overnight at 37 °C, and plugs were washed for 30 min at room temperature with TBE electrophoresis buffer. If storage was necessary, digested DNA was kept in a minimal amount of buffer to prevent diffusion of small DNA fragments. Electrophoresis was carried out using the CHEF-DR II PFGE apparatus (Bio-Rad) at 14 °C at 6 V cm⁻¹. Small fragments were resolved in 1.2% agarose gels, switch time 12–2 s, for 18 h; intermediate size fragments in 1% agarose gels, switch time 30–15 s, for 20 h; and fragments ≥300 kbp in 0.9% agarose gels, switch time 80–20 s, for 24 h.

Determination of the number of rRNA operons. Southern blotting was performed according to Sambrook *et al.* (1989) to determine the number of rRNA operons in the genome of *S. negevensis* strain Z^T. Genomic DNA of *S. negevensis* strain Z^T was digested overnight at 37 °C with *Sal*I, *Cla*I, *Eco*RV and *Sac*I (Promega) and fragments were separated on a 1% agarose gel by electrophoresis. Nucleotide sequence analysis of the 5546 bp long rRNA operon of *S. negevensis* strain Z^T indicated that *Sac*I would cut at position 621 in the 16S rDNA, *Sal*I at position 1441 in the 16S rDNA, *Cla*I at position 1801 in the 16S rDNA, just before the start of the 23S rDNA, and *Eco*RV at position 2700. There is a final *Sal*I site at position 5057. The separated DNA fragments were transferred by capillary action to a Hybond-H+ membrane (Amersham) and fixed by heating for 2 h at 80 °C. A segment of the 23S rRNA gene was amplified by PCR and used as the hybridization probe. Primers AF (5' CACAGGTAGGCA-TGATGA 3') and BR (5' CTAGCTGCGGGTAAACG 3') were used to amplify a 1099 bp segment of rDNA from *S. negevensis* genomic template. This segment corresponds to the positions 3506–4604 in the rRNA operon of *S. negevensis* strain Z^T (GenBank accession no. for the 23S rRNA gene U68460) and includes the 658 bp long intron in the 23S rRNA gene and 441 bp of the 23S rRNA gene. The amplified DNA was purified from the reaction mixture with the Wizard PCR Preps (Promega) and the probe was labelled with the AlkPhos Direct Labelling kit (Amersham). Hybridization was performed according to the instructions accompanying the kit. Detection was done with the chemiluminescent reagent CDP star (Amersham).

Amplification of the *ompA* gene by PCR. Primers previously described by Denamur *et al.* (1991), which have been used to PCR amplify a portion of the *ompA* gene from all species of chlamydiae, were employed to PCR amplify a homologue in *S. negevensis*. Primer 1260 [5' CGCTTAATC(a/g)A(t/c)-GAAAGAGCTGCTCA 3'], which matched or closely matched a segment in all *Chlamydiaceae* just upstream of the *ompA* stop codon, along with primers that complemented a perfectly conserved tRNA_{gly}, located approximately 300 bases downstream of the *ompA* stop codon, were used in an attempt to amplify an *ompA* homologue in *S. negevensis*. In addition, several combinations of the Denamur and tRNA_{gly} primers were used in further attempts to amplify an *ompA* homologue in *S. negevensis*.

RESULTS AND DISCUSSION

Growth characteristics of *S. negevensis*

Vero cells were infected with *S. negevensis* strain Z^T at an m.o.i. of 0.5. Infectivity assays showed a 2–3-d period of very rapid growth, which is typical of other

Fig. 1. Growth curve of *S. negevensis* strain Z^T in Vero cells. Cells were inoculated at a multiplicity of 0.5, and cultures were harvested at the time points indicated. Each culture was titrated on Vero cell monolayers by immunoperoxidase assay. The curve is a representative one of five different experiments. Bars indicate standard deviation from the mean of triplicate wells for each time point.

chlamydial species, followed by a stationary phase of 8 or more days during which infectivity increased and then decreased only marginally (Fig. 1). During the 3 d period of exponential growth, the cytoplasm of the infected cells showed tarry patches which appeared to be masses of tiny vacuoles. Subsequently, the cells became laced with angular vacuoles which at first appeared to be empty, but by 7–12 d had filled with dancing flickering particles, finally taking up most of the cell. By 12–14 d, monolayers were largely disrupted. Fig. 2 shows live cells at various times after infection. Growth was unaffected by the presence of sulfadiazine in the culture medium. The replication cycle of *S. negevensis* strain Z^T differed somewhat from the typical pattern of inclusion development exhibited by *C. trachomatis*. *C. trachomatis* replication typically takes up to several days, and the chlamydial inclusion is bounded by a clearly defined membrane that is apparent less than 20 h post-infection. Some species of *Chlamydiaceae* form a single inclusion that pushes the host nucleus out of the way as it fills with progeny;

Fig. 2. Phase-contrast microscopy of living Vero cells (a) 2 d, (b) 6 d, (c) 9 d and (d) 13 d post-infection; (e) uninfected control cells at 13 d. Bar, 10 µm.

others form multiple foci of infection in a cell, and these small inclusions enlarge and may merge to eventually fill the cell. Two or three days post-infection, chlamydial growth will have progressed to the stage where the host cell has been utterly usurped and the cell ruptures or the inclusion membrane fuses with the plasma membrane, and chlamydiae are released (Moulder, 1991). The *Chlamydiaceae* do not have a stationary phase. The nature of the organisms that accumulate in the intracellular inclusions during the stationary phase of *S. negevensis* strain Z^T development (Fig. 1) needs to be further examined.

Previously it was reported that *S. negevensis* was dependent on glucose for productive infection (Kahane *et al.*, 1993). At a high m.o.i. in the absence of glucose, *S. negevensis* strain Z^T infections produced tarry patches in the host cell cytoplasm and empty vacuoles, but the latter did not become filled with 'dancing' particles. To define the glucose requirement more thoroughly, growth curves were carried out (at an m.o.i. of 0.8) in parallel in 'complete' medium (described above), in RPMI lacking glucose and supplemented with dialysed FBS ('glucose-free' medium), and in 'glucose-free' medium supplemented with 10 mM glucose. The progress of infection as observed by light microscopy was similar under all conditions, and growth curves were almost identical (data not shown). Thus in the present study, when infection was carried out at a lower m.o.i., productive infections developed even when glucose was entirely absent from the medium. These data also indicated that high titres of *S. negevensis* strain Z^T inoculum had an effect on host cells that was different from that of the *Chlamydiaceae*, which at high m.o.i. exhibit toxicity for host cells (Moulder, 1991). The extended intracellular stationary phase, after maximal infectious titres were reached, suggests that the release of infectious EBs may be dependent on host-associated factors that are not currently understood. The changes observed in the appearance of the inclusions and cells by light microscopy during this period suggest that some *Simkania*-associated processes may also be taking place. It should be noted that currently the

natural host of *S. negevensis* strain Z^T is not known; it is possible that if the organism was grown in its natural host, the stationary phase might be greatly reduced.

Genome analysis

DNA base composition. The G + C content of the genome of *S. negevensis* strain Z^T was 42.5 ± 1.8 mol %. Under the same conditions, *C. psittaci* DNA from strains NJ1 and GD had a G + C content of 39.7 ± 0.7 mol %. With a relative mean difference of 2.8 mol % between *S. negevensis* and *C. psittaci*, these data were at the extreme ends of G + C ranges typically reported for the *Chlamydiaceae*: 42.7 ± 1.4 mol % (Moulder *et al.*, 1984); 40.5 ± 1.5 mol % (Cox *et al.*, 1988); 39.5 ± 0.3 mol % (Fukushi & Hirai, 1992). The G + C data were consistent with the proposition that *Simkaniaceae* and *Chlamydiaceae* descended from a common ancestor.

Genome length. Restriction analysis and PFGE of the *S. negevensis* strain Z^T chromosome produced *NotI* fragment lengths that added up to 1.734 Mbp, *AscI* fragment lengths that added up to 1.772 Mbp and *SfiI* fragment lengths that added up to 1.725 Mbp (Table 1). These sums were averaged and indicated that *S. negevensis* had a genome size of 1.744 ± 0.028 Mbp. PFGE analysis of whole, uncut genome was also carried out, comparing the chromosome of *S. negevensis* with those of strains *C. muridarum*, *C. suis* and *C. trachomatis*. The uncut *S. negevensis* genome migrated at an approximate size of 1.7 Mbp and the *Chlamydia* genomes migrated at approximately 1.1 Mbp (data not shown). The complete genome sequence has been determined for *Chlamydia trachomatis* L2/434/BU to be 1 038 680 bp (GENSET, 1997), so there is a reasonable certainty that the *S. negevensis* strain Z^T genome is significantly larger than genomes of strains in the *Chlamydiaceae*.

Number of ribosomal operons. Southern blotting of *S. negevensis* strain Z^T DNA cut with *SacI*, *SalI*, *EcoRV* and *ClaI*, followed by probing for 23S rDNA fragments showed only a single band of hybridization for

Table 1. Genome size of *S. negevensis* strain Z^T as determined by restriction enzyme analysis of chromosomal DNA

Restriction enzyme	Fragment size (kbp)	Estimated length (kbp)
<i>NotI</i>	980, 388, 160, 140, 66	1734
<i>AscI</i>	582, 540, 300, 150, 120, 80	1772
<i>SfiI</i>	500, 470, 270, 205, 175, 105	1725
Mean estimated length		1744

Fig. 3. Southern blotting of *S. negevensis* strain Z^T DNA cut with restriction enzymes *SacI* (lane 1), *EcoRV* (lane 2), *ClaI* (lane 3) and *SalI* (lane 4). The DNA fragments were hybridized with a 1.1 kbp probe from the 23S rRNA gene.

each enzyme (Fig. 3). Because multiple copies of the rRNA operon in various locations on the bacterial genome would give bands of different sizes upon hybridization with the labelled probe, our results indicated that *S. negevensis* strain Z^T contained only a single copy of the rRNA operon. Members of the *Chlamydiaceae* that have been examined have only one or two ribosomal operons, while the number of rRNA operons that are present in members of other families in the order *Chlamydiales* is not yet known. Characterization of the number of rRNA genes in the other chlamydial families will indicate whether this restricted number of ribosomal operons is a characteristic of the order *Chlamydiales*. The presence of only one operon in *S. negevensis* strain Z^T was of particular significance because *S. negevensis* strain Z^T encodes the only bacterial 23S rRNA intron ever discovered. Other chlamydial 23S genes do not have an intron (Everett *et al.*, 1999).

Chlamydial genes/products in *S. negevensis* strain Z^T

Primers matching highly conserved DNA segments in or downstream of the *Chlamydiaceae* *ompA* gene were able to amplify an *ompA* PCR product from all known chlamydial species, but these primers failed to amplify a detectable PCR product from *S. negevensis* strain Z^T. These data supported our previous observation that mAbs against chlamydial OMP1 and OMP2 proteins do not react with *S. negevensis* strain Z^T in immunoblots (Kahane *et al.*, 1993). The mAb NJ1/D3, which is specific for the trisaccharide antigen of *Chlamydiaceae*, α Kdo-(2 $\rightarrow$ 8)- α Kdo-(2 $\rightarrow$ 4)- α Kdo, was tested

on Vero cells infected with *S. negevensis*, *C. trachomatis* or *C. pneumoniae*. The mAb clearly stained inclusions in cells infected with *C. trachomatis* and *C. pneumoniae* using either peroxidase-conjugated second antibody or FITC-conjugated second antibody. Under these conditions, the inclusions of cells infected with *S. negevensis* did not stain. Furthermore, the S25-23 mAb, specific for chlamydial LPS (Fu *et al.*, 1992) did not bind *S. negevensis* strain Z^T lysates in immunoblots (W. Brabetz, personal communication). Since all species of *Chlamydiaceae* encode the OmpA major outer-membrane protein and are recognized by the mAb NJ1/D3 specific for an LPS epitope (Andersen & Van Deusen, 1988), our results support the distinction of *S. negevensis* strain Z^T from known species of *Chlamydiaceae*. Moreover, since there is now clear evidence for the definition of known chlamydial species as belonging to more than one genus (Everett *et al.*, 1999), the distinction between *S. negevensis* strain Z^T and the *Chlamydiaceae* must be on the family level.

Conclusions

In this study we have characterized growth, antigenic and genetic characteristics of *S. negevensis*. Many features of the intracellular developmental cycle of *S. negevensis* in eukaryotic host cells were similar to those described for members of the *Chlamydiaceae*, including the infection of host cells by electron-dense infectious EBs that were 0.4–0.6 μ m in diameter, intracellular development of less dense replicating reticulate bodies that were 0.4–1.0 μ m in diameter, and inclusion morphology similar to that of some *C. psittaci* strains. The replicative phase lasted at least 2–3 d and was followed by an intracellular stationary phase lasting up to 12 d, prior to host cell lysis. *S. negevensis* growth was unaffected by the presence of sulfadiazine in the culture medium. *S. negevensis* did not contain the LPS antigen common to all members of the *Chlamydiaceae*, neither was it recognized by mAbs to the chlamydial major outer-membrane proteins nor by primers to the *ompA* gene. The size of the *S. negevensis* strain Z^T genome was 1.7 Mbp, its G + C content was 42.5 ± 1.8 mol %, and it only contained one ribosomal operon. These data were consistent with the observation that *S. negevensis* strain Z^T and other species in the order *Chlamydiales* share a common ancestor (Everett & Andersen, 1997; Everett *et al.*, 1999; Amann *et al.*, 1997; Kahane *et al.*, 1995;

Pettersson *et al.*, 1997). The full-length 16S rRNA gene and the full-length 23S rRNA gene are each 18.6% different from those of the *Chlamydiaceae*, fitting into the 80–90% identity range that makes this organism a member of the order *Chlamydiales*, but not a member of *Chlamydiaceae*. These data support the proposal to place *Simkania negevensis* strain Z^T in a new family, *Simkaniaceae*, in the order *Chlamydiales* (Everett *et al.*, 1999). Although the exact relationships between *Simkaniaceae*, *Chlamydiaceae* and the other *Chlamydiales* families are still not fully elucidated, such relationships will become clearer as new members of the family *Simkaniaceae* are discovered and characterized.

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