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Free-living Amoebae Serve as a Host for the *Chlamydia*-like Bacterium *Simkania negevensis*

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Summary. Members of the novel family Parachlamydiaceae are commonly observed in free-living amoebae (FLA) as host cells. Therefore, we examined the potential of 14 different species of free-living amoebae to serve as hosts of the *Chlamydia*-like bacterium, *Simkania negevensis*, previously isolated as a contaminant from a cell culture in Israel (Kahane *et al.* 1993, 1995). The inoculum of the obligate intracellular agent was prepared from Buffalo Green Monkey (BGM) cells. The infection of *Acanthamoeba* strain HLA and of *Naegleria clarki* (N-DMLGo) revealed typical morphological stages of a *Chlamydia*-like life cycle, including the presence of elementary and reticulate bodies, as could be shown by electron microscopy. Subsequent infection studies with an *Acanthamoeba*-adapted *Simkania* isolate showed that also *Balamuthia mandrillaris* and one of two *Hartmannella* strains supported the growth of *Simkania. Balamuthia* can be considered as an experimental host for mass production of elementary bodies. This is based on the finding that the host amoebae expelled great numbers of bacteria leading to a long-term survival of the infected trophozoites. The observation that *Simkania negevensis* can survive and replicate within at least four of tested FLA species suggests that various free-living amoebae may serve as survival and multiplication vehicles supporting the spread of these pathogens in aquatic environments. The concept that *Simkania* may fall into the group of environmentally preadapted pathogens is discussed as well.

Key words: *Acanthamoeba*, *Balamuthia*, *Chlamydia*, endoparasite, *Hartmannella*, host range, *Neochlamydia*, *Simkania negevensis*, ultrastructure, *Waddlia.*

INTRODUCTION

Chlamydiae are well known as important obligate intracellular pathogens. They are the causative agents of a variety of diseases in humans and animals, e.g. infections of the eye, as well as of the respiratory and genital tracts. The life cycle of chlamydiae is characterized by the development of reticulate bodies (RBs) that divide intracellularly by binary fission and the more resistant elementary bodies (EBs) that are specialized for transmission to another cell of the same host and to a new host (Moulder 1984).

Recent isolation of several novel *Chlamydia*-related bacteria from contaminated cell culture (Kahane *et al*. 1993, 1995), from an aborted bovine foetus (Dilbeck *et al*. 1990, Rurangirwa *et al.* 1999, Henning *et al.* 2002)

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and from free-living amoebae (Michel *et al*. 1994, Amann *et al.* 1997), has led to the reclassification of the order Chlamydiales and to the establishment of the families Simkaniaceae, Waddliaceae, and Parachlamydiaceae respectively (Everett *et al*. 1999, Poppert *et al*. 2002). Since the members of the Parachlamydiaceae, such as *Parachlamydia* (Amann *et al.* 1997), *Neochlamydia* (Horn *et al.* 2000), Halls Coccus (Birtles *et al.* 1997) and various hitherto unnamed strains isolated in the U.S. (Fritsche *et al*. 1993, 2000) multiply within free-living amoebae (FLA), the question arose as to whether other members of the Chlamydiales could also grow within FLA* This issue has been addressed in various host range studies i.e. for *Chlamydophila pneumoniae* or *Waddlia chondrophila* as the type species of *Waddliaceae*. While *C. pneumoniae* could be observed to survive and replicate within *Acanthamoeba polyphaga* for about 14 days (Essig *et al*. 1997), the *Waddlia* isolate was shown to be able to multiply permanently within various amoebal hosts (Michel *et al.* 2001b, 2004). Similarly, *A. polyphaga* could be successfully infected with *Simkania negevensis,* an organism which was originally isolated as a contaminant from a cell culture (Kahane *et al*. 1993, 1995; Lieberman *et al*. 1997) and formerly designated "Z"-organism. The infection resulted in a stable host-parasite association (Kahane *et al*. 2001). In the present study we aimed to confirm these results by evaluating a broader range of FLA as potential hosts as we had previously described for *Waddlia* (Michel *et al*. 2001b, 2004). The question of whether FLA - that are widely spread in nearly all known freshwater habitats - may serve as natural hosts and vehicles is of considerable interest since *S. negevensis* was shown to be the cause of bronchiolitis in infants (Kahane *et al*. 1998) and was also associated with community-acquired pneumonia (cap) in adults (Lieberman *et al*. 1997). It is widely spread in the Negev region of Israel, and seropositivity for *Simkania* has meanwhile been reported from Canada, Great Britain, and the Americas (cit. in Kahane *et al*. 2001). The findings presented in this article may help to identify the environmental reservoir and may therefore contribute to our knowledge on the epidemiology of this organism.

MATERIALS AND METHODS

Origin and maintenance of *Simkania negevensis*. Buffalo Green Monkey (BGM) cells were used for cultivation and maintenance of *Simkania* strain ATCC VR-1471, strain Z, Lot # 1171209. Cells were weekly passaged in serum-free medium (SF-3, CytoGen, 35764 Sinn / Germany) without antibiotics and with cycloheximide, were transferred in intervals of 6 weeks.

Protozoan strains and culture. The protozoa used in this study are listed in Tables 1 and 2. *Naegleria gruberi* (CCAP 1518/1e) and *N. lovaniensis* (Aq/9/45D) were kindly provided by Johan De Jonckheere/ Brussels, the *Balamuthia mandrillaris* strain CDC: VO39 was provided on Vero cells by courtesy of Klaus Janitschke/ Berlin. In order to replace the Vero cells as host cells, a cell-free medium that had been developed by Michel and Janitschke (1996) [a modification of SCGYE-medium (De Jonckheere 1977)] was used. The *Acanthamoeba* strain HLA was originally isolated from the cornea of a keratitis patient and has been kindly provided by Horst Aspöck, Vienna. The *Dictyostelium* strain Sö-P2 was isolated from soil by M. Steinert, Würzburg, who made it available to us. The *Naegleria* strain N-DMLGo isolated from a garden pond in Bad Hönningen has recently been sequenced and identified as *Naegleria clarki* by Julia Walochnik/Vienna (Walochnik *et.al*., in preparation). The protozoan cultures were maintained on non-nutrient agar (NNA) seeded with *Enterobacter cloacae* according to Page (1988). *N. gruberi, N. lovaniensis*, and *Hartmannella vermiformis* (OS 101) were grown axenically on SCGYE-medium according to De Jonckheere (1977).

Growth of bacteria within protozoa. The cell suspension of a 7-day-old infected BGM culture was passed through a membrane filter with a pore size of 1.2 µm. It was rich in elementary and reticular bodies representing a superabundance since these tiny stages are not countable, and since they do not produce colony forming units (cfu) on nutrient agar Alternatively, the bacterial inoculum was prepared from heavily infected acanthamoebae, strain HLA, grown axenically, by freeze-thawing and subsequent passage through a 1.2 µm filter. Bacterial elementary and reticulate bodies from 100 µl filtrate were added to 25 ml culture flasks containing trophozoites in SCGYE-medium forming a monolayer in a 24h log-phase culture. The same inoculum was added to agar plates with trophic forms preincubated for 24 h as well. After 48 h at 28°C, the infected amoebal cultures were inspected daily for a time period of 10 days. The only exceptions were the two *Dictyostelium* strains incubated at room temperature. Morphological alterations and symptoms of infection as seen by light microscopy were compared with uninfected host cells. In order to obtain protozoa-adapted *Simkaniae,* heavily infected *Acanthamoeba* trophozoites were submitted to freeze-thawing. The released EB's and RB's were filterpurified from amoebal debris and utilized for further cocultivation assays.

Electron microscopy. Infected amoebal host cells were harvested 5 days post infection. The cell suspension was centrifuged for 10 min at 600 g. The resulting pellet was fixed in 3% glutaraldehyde (1 h),

* The results had been presented at the 20th Annual Meeting of the German Protozoological Society at Bonn-Röttgen (see Michel *et al*. 2001a).

transferred to 0.1M cacodylate buffer, postfixed in 1% osmium tetroxide and embedded in Spurr resin. Thin sections were stained with 1% lead citrate and examined using a Zeiss EM 10 electron microscope.

RESULTS

Cocultivation of *S. negevensis* and protozoa. In order to evaluate representatives of the three important genera *Acanthamoeba, Naegleria* and *Hartmannella* as potential hosts for *Simkania negevensis*, we analysed the capability of the bacteria to multiply intracellularly in various strains (Table 1) by light microscopy, phase contrast microscopy and transmission electron microscopy. Bacterial inocula were prepared from infected BGM cells, and the results of the coincubations on both NN-agar plates and in axenic SCGYE-medium are summarized in Table 1. After 5 days of cocultivation, one of four *Naegleria* strains tested (*N. clarki*, N-DMLGo) and one of two *Acanthamoeba* strains (HLA) showed characteristic signs of infection, such as rounding up of cells, inhibition of cell migration and the presence of intracellular coccoid bacteria. Infected trophozoites of *Acanthamoeba* were not able to form cysts whereas the infected naegleriae still formed cysts which did not contain endocytobionts. These features resemble previous observations of *Neochlamydia- or Waddlia*-infected host cells (Horn *et al*. 2000, Michel *et. al*. 2004). Subsequent subcultures showed that the association of *Simkania* and its respective experimental host was stable during the time of observation. Since the degree of infection was highest with *Acanthamoeba* strain HLA, this host-parasite combination was maintained indefinitely for months and was chosen as an adaptation model of these bacteria to a protozoan host.

Subsequently, *Simkania* was isolated and filter-purified from axenically grown acanthamoebae which were jammed with intracellular bacteria after three serial transfers. Various other strains of FLA were cocultivated

Table.1. Cocultivation results of *Simkania negevensis* with various promising candidates of free-living amoebae. After three transfers on *Acanthamoeba*-strain HLA the protozoa-adapted *Simkania* strain "Z-P" was cocultivated with amoeba species shown in Table 2. Cysts - cysts were formed but did not harbour endocytobionts.

Amoeba species	Strain	Source	Intracellular growth
Naegleria gruberi	CCAP 1518/1e	Unknown	-
Naegleria lovaniensis	Aq/9/1/45D	Aquarium/Belgium	-
Naegleria sp.	N- Beck	Aquarium/Germany	-
Naegleria clarki	$N\text{-}DMLG_o$	Garden pond/Germany	+++ cysts: -
Acanthamoeba castellanii	C_3 ATCC 50739	Potable water reservoir	-
Acanthamoeba castellanii	HLA	Keratitis case/ Vienna	+++ → Z-P
Hartmannella vermiformis	A_1Hsp_o	Host of *Neochlamydia*	-

Table 2. Cocultivation assays of the protozoa-adapted strain "Z-P" of *Simkania* together with various further strains of free-living amoebae and two isolates of the slime mould *Dictyostelium discoideum.*

Amoeba species	Strain	Source	Intracellular growth
Naegleria lovaniensis	Aq/9/1/45D	Aquarium/Belgium	-
Willaertia magna	NI_4CL_1	Pond/India	-
Acanthamoeba castellanii	C_3 ATCC 50739	Potable water reservoir	-
Acanthamoeba lenticulata	45	Human nasal mucosa	-
Hartmannella vermiformis	Os 101	Physiotherap. bath	+++
Hartmannella vermiformis	C3/8	Surface water	-
Balamuthia mandrillaris	CDC: VO39	*Papio sphinx*. Brain	+++
Dictyostelium discoideum	$Berg_{25}$	Human nasal mucosa	-
Dictyostelium discoideum	$Sö\text{-}P_2$	Surface water	-

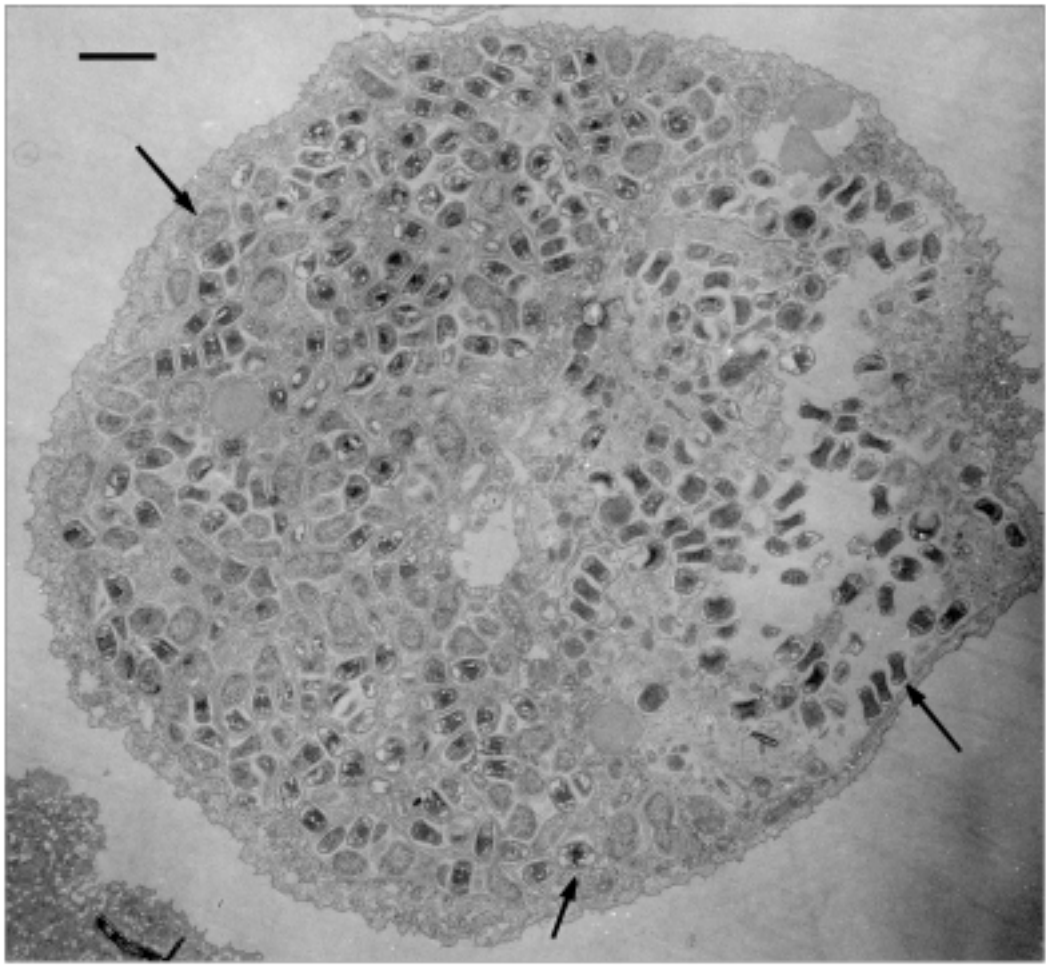

Fig. 1. Overview of an infected *Acanthamoeba*, strain HLA, jammed with numerous organisms of *Simkania negevensis (*arrows) as a result of multiplication of the endoparasite in a period of six days. Scale bar 1.0 µm.

with the bacteria-rich suspension (Table 2). As a result of the testing of 8 strains belonging to 6 different genera only *Hartmannella vermiformis*, strain Os101 and *Balamuthia mandrillaris* grown in SCGYE-medium proved positive within a period of 3-4 days. In addition to the confirmation of a cytoplasmatic infection with Simkaniae as seen by phase contrast microscopy, both strains of these unrelated amoebae lost their ability to form cysts, which proves that they were actually infected. These associations of amoebal hosts and endocytobionts turned out to be stable infections over many passages. Since the trophozoites of *Balamuthia* began shedding elementary bodies 3 to 5 days post infection, they survived because they could not be overgrown by the endoparasites. The number of expelled EBs in the medium increased to such an extent that this host-parasite combination was suited perfectly for mass production of EBs as the infectious stages of *Simkania*. In contrast, strains of *Willaertia magna*, a second *Hartmannella* strain designated C3/8, and two strains of the cellular slime mould *Dictyostelium* were resistant to infection. The same was true for *Naegleria lovaniensis* and *Acanthamoeba* strain C3, infection of which had been attempted by cocultivation with the protozoa-adapted *Simkania* cells.

Electron microscopy. Acanthamoebae of strain HLA were fixed on the 6th day after infection with a suspension of *Simkania* stages from BGM-cell culture. Electron microscopy revealed heavily infected trophozoites which were jammed with organisms belonging to different stages of the *Chlamydia*-like developmental cycle (Fig. 1). This reflects the high replication rate of these

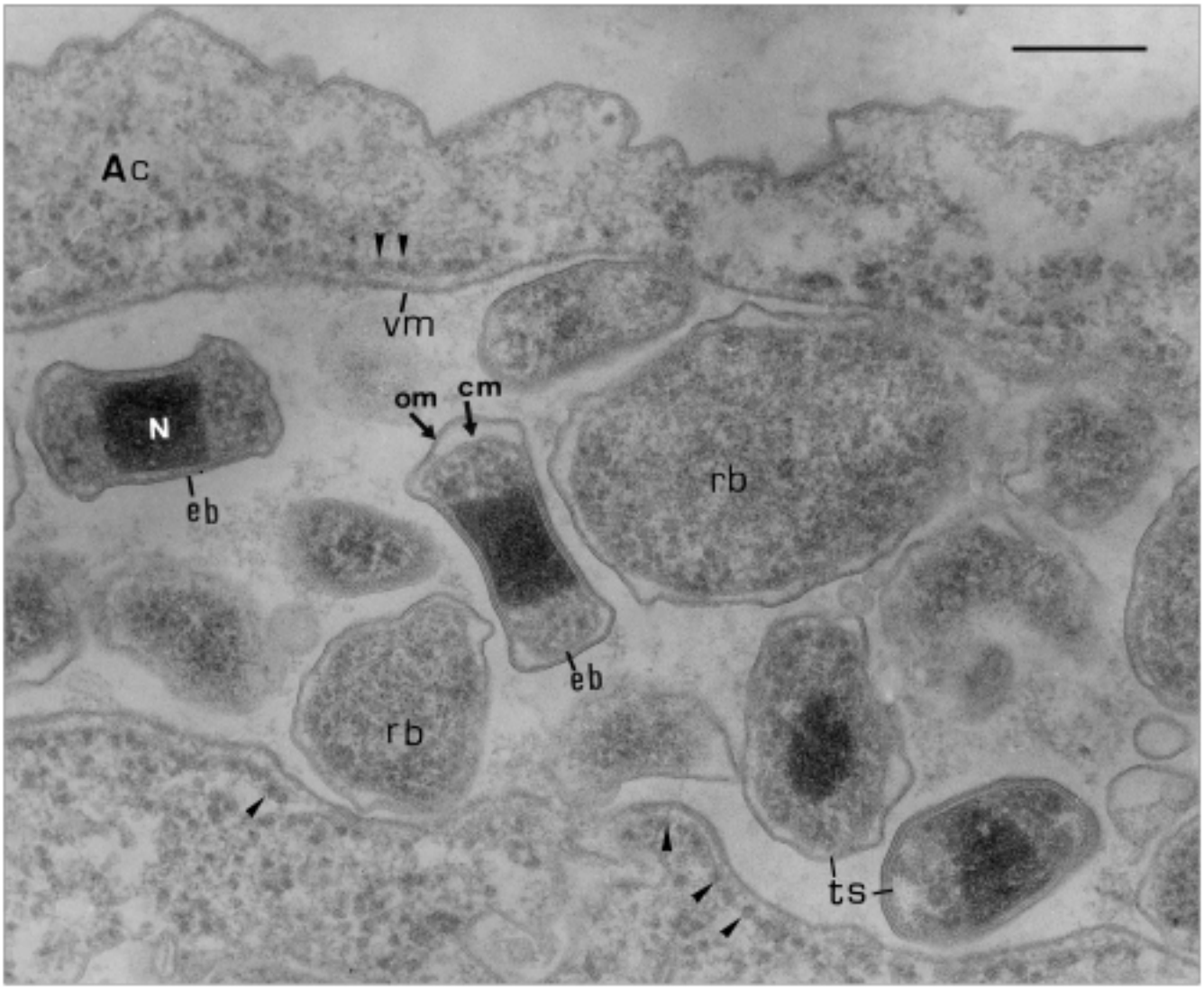

Fig. 2. Detail of an *Acanthamoeba* trophozoite (Ac) harbouring different developmental stages of *Simkania* replicating within a membrane-bound vacuole (vm). The elementary bodies (eb) contain electron-dense nuclear material (N) and ribosomes. They are surrounded by the outer membrane (om) at some places separated from the inner cytoplasmic membrane (cm). They are considered as Gram-negative. The significantly larger reticulate stages (rb) have less dense fibrillar nuclear material and more ribosomes. They are also surrounded by an obviously more flexible trilaminar envelope. Transitory stages (ts) show intermediate traits between EBs and RBs. Remarkable are distinct ribosomes within the host's cytoplasm which appear arranged like a string of pearls beneath the vacuolar membrane (arrowheads). Scale bar 0.25 µm.

intracellularly growing bacteria. Even at this low magnification, at least two kinds of developmental stages of the endocytobiont could be distinguished as exemplified in Fig. 2, which shows even three stages. They are located within an elongated, membrane-bound vacuole of the host amoeba. Replication is performed by the reticulate bodies, which finally transform *via* a transitory stage (ts) to the elementary bodies representing the infectious stage. The RBs containing numerous ribosomes appear pleomorphic with a flexible outer membrane. The characteristic shape of the mature EBs is quadrangular or dumbbell-shaped. As far as we know, this unique outline is different from the EBs of all other *Chlamydia*-like bacteria. Eb's contain electron-dense nuclear material (N) and ribosomes. They are enveloped by the outer membrane, which may be separated from the inner cytoplasmic membrane at some places. These stages appear Gram-negative. The transitory stages being still pleomorphic like the RBs already contain an increasing amount of electron-dense nuclear matter. In

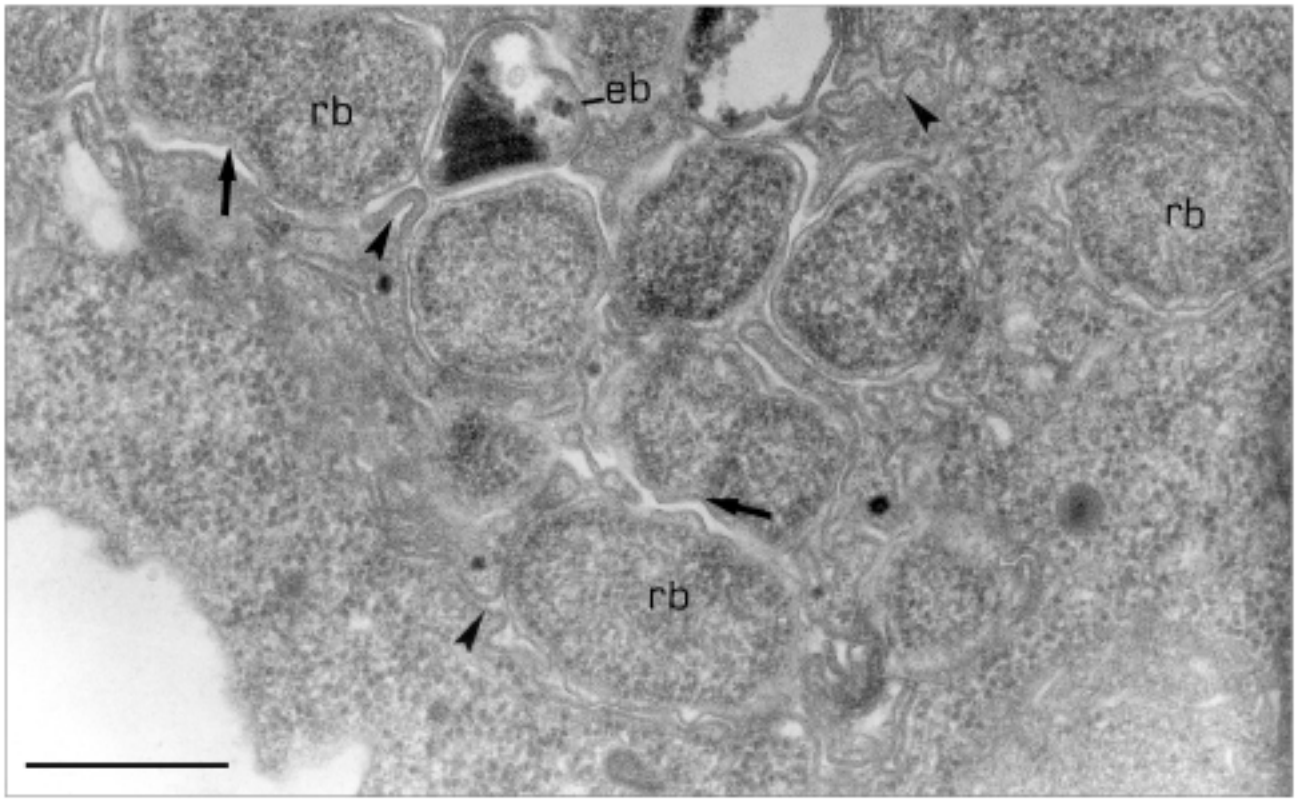

Fig. 3. *Naegleria*, strain DMLG, harbouring mainly reticulate bodies (rb) of *Simkania* replicating by binary fission (arrows). A single elementary body (eb) can also be distinguished. The endoparasites are closely surrounded by a membrane of the host amoeba. They have connections with wrinkled laminar structures found in the vicinity of the enclosed endocytobionts (arrowheads). Scale bar 0.5 μm.

the host amoeba a regular row of ribosomes was observed beneath the vacuolar membrane, appearing like a string of pearls. This unusual phenomenon could be seen in nearly all micrographs inspected so far (not shown).

Since in addition to this *Acanthamoeba* strain amoebae of the genera *Hartmannella, Naegleria*, and *Balamuthia mandrillaris* could be infected successfully with *Simkania*, at least one micrograph of a section of an infected *Naegleria* we have presented here. The only susceptible strain N-DMLG harboured numerous *Simkania* stages, mainly RBs and few EBs (Fig. 3). Since the host membrane surrounded the endoparasites closely, the bacteria appeared to be located within the cytoplasm as known from other endoparasites, such as *Legionella*-like amoebal pathogens (Llap's), for instance. RBs with signs of binary fission could be observed as indicated by a distinct division furrow. In the vicinity of the enclosed parasites an irregular system of laminar structures with unknown function could be observed as well. In contrast to infected acanthamoebae, the pearl-string-like assembly of ribosomes seen beneath the vacuolar membrane was not found within infected naegleriae.

DISCUSSION

Since most members of the family Parachlamydiaceae had been commonly observed in free-living amoebae before their taxonomic identification (Michel *et al.* 1994, Amann *et al*. 1997, Horn *et al.* 2000, Fritsche *et al*. 2000) they were placed into the newly established family Parachlamydiaceae which was reserved for this sister group of the Chlamydiaceae (Everett *et al*. 1999). The ability to grow intracellularly within protozoa could have preadapted these bacteria as pathogens of higher eukaryotes (Corsaro *et al.* 2002, Görtz and Michel 2003) in the same manner as assumed previously for *Legionella pneumophila* (Harb *et al*. 2000). This startling idea was the rationale for attempts to associate other members of the Chlamydiales primarily with acanthamoebae (Essig

et al. 1997, Kahane *et al*. 2001), but in the case of *Waddlia chondrophila* also with other species of FLA (Michel *et al*. 2001 a, b, 2004). Accordingly we started cocultivation assays of *S. negevensis* and various FLA of diverse taxonomic positions. As a result, not only could the findings of Kahane *et al*. (2001) be partially confirmed with acanthamoebae as experimental hosts, but also some different amoebal species (Tables 1, 2) could be successfully infected with long-term persistence and exponential growth of the endoparasitic simkaniae. On the other hand, Kahane's observation of *Acanthamoeba* cysts harbouring stages of *Simkania* could not be observed in the case of the susceptible strain HLA since it had lost its cyst-forming capacity as a result of infection. Similar observations have been made in the case of acanthamoebae naturally infected with *Parachlamydia* (Michel *et al*.1994). The fact that only one of four *Naegleria* strains was permissive to infection may be explained by the history of this host strain N-DMLGo - recently identified as *Naegleria clarki* (Walochnik *et. al.,* in preparation) which had originally harboured simultaneously two unidentified Gram-negative endocytobionts (Michel *et al*. 1999). Perhaps this isolate is a *Naegleria* strain susceptible to various endocytobionts. This original host strain was cured from its twofold infection step by step by a combined application of elevated temperatures and antibiotics and by utilisation of the flagellate transformation test as well (Michel *et al*., in preparation). As a result of these measures they resumed the ability to form cysts proving that they got rid of their cytoplasmatic fraction of endoparasites (Pcb), since infected trophic forms were unable to form cysts. The loss of the intranuclear population of parasites (Pn) could be observed by phase contrast microscopy without problems and was confirmed by electron microscopy. Interestingly, the parasite-free amoebae again lost their capability to form cysts if they were infected with simkaniae. The observation that only one of four *Naegleria* species and one of two *Acanthamoeba* strains were susceptible to infection underlines the need to expose more than one strain of a genus to these infectious agents. In the case of *Hartmannella vermiformis* it was even shown that only one strain of the same species was susceptible (OS101) whereas a second one was totally resistant to infection. Similar differences were obtained during recent investigations on the susceptibility of various FLA to *Waddlia chondrophila* (Michel *et al*. 2004). Only one of six *Acanthamoeba* strains tested proved positive and one of two *Dictyostelium dicoideum* strains could be infected permanently, consequently within the same species of slime mould. *Simkania* and *Waddlia* were both able to invade several unrelated species of FLA and to replicate in them, thus establishing a persistent host-parasite association. This is in contrast to *Chlamydophila pneumonia*e for instance, which survives no longer than a fortnight within its experimental host (Essig 1997). Consequently, it is justified to assume that the experimental hosts of *Simkania* and *Waddlia* identified in the present investigation or earlier (Michel *et al*. 2004), respectively, may play this role also in nature. This hypothesis would provide an explanation for the possible function of the susceptible host as a transportation vehicle and reservoir of these recently detected relatives of the Chlamydiaceae. Another important aspect is the assumption that the adaptation to intracellular survival and replication may have occurred in the course of evolution long before these bacteria were able to infect warm-blooded animals and humans (Harb *et al*. 2000, Corsaro *et al*. 2002). Comparing the host ranges of both bacteria, some significant differences can be emphasized. Four strains of FLA were susceptible to infection with *Simkania* - whereas *Waddlia* was found to be able to infect eight different strains. Both were infectious for at least one strain of *Acanthamoeba, Naegleria* and *Hartmannella*. The most remarkable differences were (a) the high susceptibility of *Balamuthia* for *Simkania* but not for *Waddlia* and (b) the ability of *Waddlia* to infect one of two strains of *Dictyostelium* whereas both tested strains of this cellular slime mould proved to be resistant to infection with *Simkania*. With *Balamuthia mandrillaris* a suitable host has been identified for laboratory mass production of *Simkania* elementary bodies as an alternative model for maintaining these endocytobionts in long-term cultures, providing high yields of bacteria for various scientific purposes. Thus *Balamuthia* was shown to serve as an experimental host of an obligate intracellular bacterium. Similar experience had been made earlier, when this pathogenic amoeba could be successfully infected with strain "Knic", a Gram-negative coccoid bacterium isolated from *Naegleria* sp. and resembling *Ehrlichia* species (Michel *et al*. 2000). Only recently, *Balamuthia* was also shown to be permissive to infection with *Legionella pneumophila* after in vitro cocultivation with this pathogenic agent of Legionellosis (Shadrach *et al*. 2004) well known to survive and replicate within acanthamoebae, amongst others (Rowbotham 1980). Although *Balamuthia* proved to be very susceptible to infection with *Simkania* in vitro, it does presumably not play an important epidemiological

role for the dispersal of *Simkania* and other intracellular bacteria since it has been isolated only once from the environment (Schuster *et al*. 2003) in contrast to frequently found FLA, such as acanthamoebae, hartmannellae or naegleriae. Each positive in vitro result obtained from cocultivation assays of FLA with certain pathogens is of great theoretical and practical value as a laboratory model. But their putative occurrence under natural conditions remains speculative unless infected host protozoa will be actually isolated from the environment. This was, for example, true for *Legionella pneumophila*, which first had been found to be able to replicate within *Acanthamoeba polyphaga* as a result of experimental cocultivation (Rowbotham1980).

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