

## Resuscitation of Viable but Nonculturable *Legionella pneumophila* Philadelphia JR32 by *Acanthamoeba castellanii*

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*Legionella pneumophila* is an aquatic bacterium and is responsible for Legionnaires' disease in humans. Free-living amoebae are parasitized by legionellae and provide the intracellular environment required for the replication of this bacterium. In low-nutrient environments, however, *L. pneumophila* is able to enter a non-replicative viable but nonculturable (VBNC) state. In this study, *L. pneumophila* Philadelphia I JR 32 was suspended in sterilized tap water at  $10^4$  cells/ml. The decreasing number of bacteria was monitored by CFU measurements, acridine orange direct count (AODC), and hybridization with 16S rRNA-targeted oligonucleotide probes. After 125 days of incubation in water, the cells were no longer culturable on routine plating media; however, they were still detectable by AODC and by in situ hybridization. The addition of *Acanthamoeba castellanii* to the dormant bacteria resulted in the resuscitation of *L. pneumophila* JR 32 to a culturable state. A comparison of plate-grown legionellae and reactivated cells showed that the capacity for intracellular survival in human monocytes and intraperitoneally infected guinea pigs, which is considered a parameter for virulence, was not reduced in the reactivated cells. However, reactivation of dormant legionellae was not observed in the animal model.

Natural freshwater biotopes are sources for the dissemination of legionellae to man-made water systems, a habitat which often provides intensive amplification (14). *Legionella pneumophila* is the causative agent of Legionnaires' disease, a common life-threatening atypical pneumonia in humans (12, 44). The infection mainly affects immunocompromised patients and occurs by inhalation of contaminated aerosols produced by showers and air conditioning systems (44). In natural and man-made environments, free-living amoebae serve as host organisms in which legionellae multiply intracellularly in organelle-studded phagosomes (5, 12). Several protozoan species support the intracellular growth of *L. pneumophila*. *Acanthamoeba*, *Hartmannella*, and *Naegleria* are the genera isolated most commonly from *Legionella*-contaminated plumbing systems (24, 26, 40, 42). The amoebae are thought to provide the intracellular environment required for the replication of this bacterium, and the host-parasite interaction is known to be affected by temperature, the nutritional condition of the host, and the availability of ferrous and ferric ions (32, 41).

Multiplication within certain amoebae has been shown to be linked to pathogenicity, and the processing of *L. pneumophila* by *Acanthamoeba castellanii* shows many similarities to what is observed in human phagocytic cells including monocytes (11, 41). These similarities include the confinement of *Legionella pneumophila* in ribosome-studded phagosomes and the inhibition of phagosome-lysosome fusion (5, 15). However, to date, only little is known about the gene products of *L. pneumophila* required for optimal intracellular infection. The *Legionella* Mip (macrophage infectivity potentiator) protein potentiates

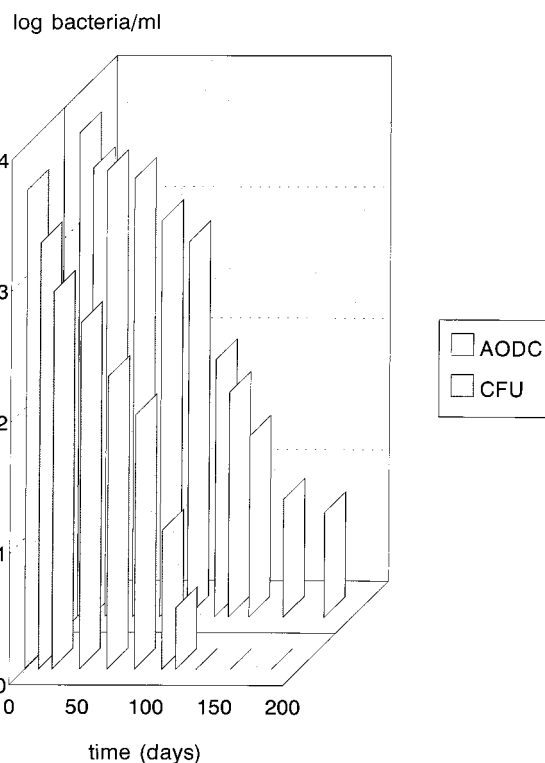


FIG. 1. Entry of *L. pneumophila* JR 32 into the VBNC state in sterilized tap water at 20°C. Shown are results of plate counts (CFU per milliliter) on BCYE agar and AODC per milliliter.

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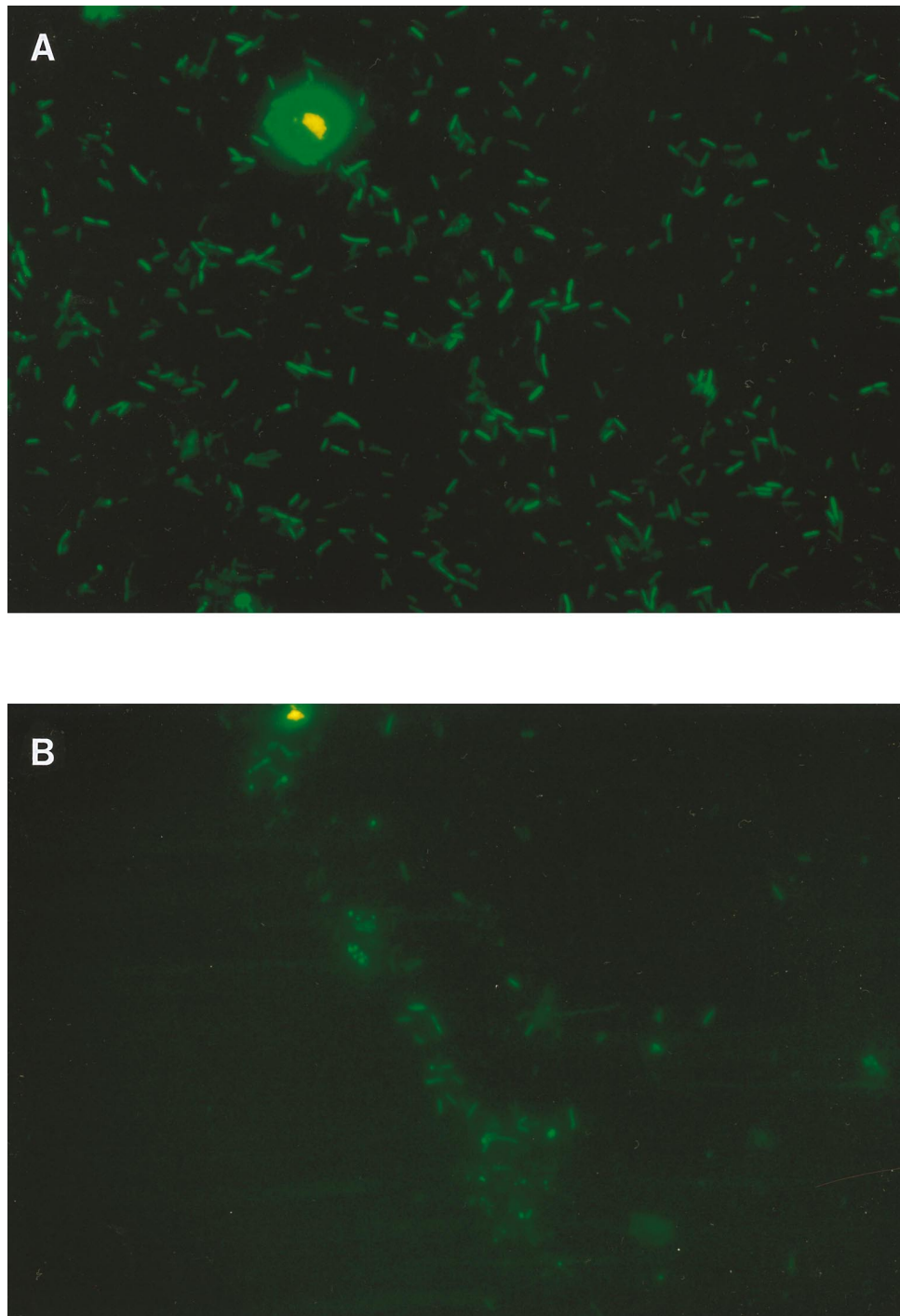


FIG. 2. 16S rRNA specific in situ hybridization of *L. pneumophila* with the FLUOS-labelled probe LEG705. Epifluorescence micrographs show freshly inoculated tap water microcosms (A) and *L. pneumophila* in the VBNC state after 180 days (B). The samples were concentrated by centrifugation as described in the text.

the survival of the bacteria in amoebae and monocytes in the early stage of infection (7–9, 46). Whether the *icm* (intracellular multiplication) and the *dot* (defect in organelle trafficking) loci are essential for intracellular replication in amoebae, as they are in human macrophages, remains to be determined (3, 4, 6).

Although amoebae are the key factors in the *Legionella* amplification process, this pathogen is able to survive as a free

organism for long periods. Its persistence has been attributed to survival within biofilms. Detection of legionellae for extended periods is also possible in a low-nutrient environment under appropriate conditions (e.g., low temperature) (17, 21, 36). Several authors have reported that *L. pneumophila* cells are able to enter a viable but nonculturable (VBNC) state, which may account for the fact that *L. pneumophila* often cannot be cultured from cooling towers suspected to be the

source of infecting bacteria (10, 17, 31). Since earlier epidemiological investigations reported an improved detection of legionellae following incubation with amoebae (40), the present study focused on the resuscitation of VBNC cells of *L. pneumophila* in the natural host *A. castellanii*. The data provided here demonstrate that VBNC cells regain pathogenic potential with reactivation in amoebae and are therefore relevant in the risk assessments of implicated reservoirs.

#### MATERIALS AND METHODS

**Inoculation of microcosms and determination of culturability.** *L. pneumophila* Philadelphia I JR 32 (23, 45, 46) was grown on buffered charcoal yeast extract agar (BCYE agar; Mast Diagnostica, Rheinfeld, Germany). The plates were incubated at 37°C under a 5% CO<sub>2</sub> atmosphere for 3 days. The *L. pneumophila* cells were harvested with sterile tap water and adjusted to a concentration of 10<sup>8</sup> cells/ml by measuring the optical density at 600 nm with a Unicam 8625 spectrophotometer until it reached 0.1. The bacterial cells were then resuspended in 500 ml of autoclaved, filter-sterilized tap water to an initial cell concentration of 10<sup>4</sup> CFU/ml. Microcosms were incubated at room temperature (20°C) in the dark without shaking. They were sampled after various periods in duplicate for culturability on BCYE agar. When the concentration reached 50 CFU/ml, 1-ml inocula of the microcosm sample (in duplicate) were used for plating until no colonies were found on BCYE agar. Nonculturability was confirmed by filtering 20 ml of the microcosm sample and placing the polycarbonate filters (pore size, 0.2 µm) onto BCYE plates.

**Viability assays and enumeration of *L. pneumophila*.** The viability of the nonculturable legionellae was tested by *in vivo* resuscitation in *A. castellanii*. The presence of VBNC cells was determined by 16S rRNA-targeted oligonucleotide hybridization as well as by acridine orange direct counts (AODC). Samples for AODC were filtered onto Nuclepore 0.2-µm-pore-size membranes, stained with 0.01% acridine orange for 2 min, and then washed twice with filtered distilled water as described previously (13, 33). The cells were then counted by epifluorescence microscopy.

***In vivo* resuscitation of *L. pneumophila* by *A. castellanii*.** *A. castellanii* ATCC 30234 was obtained from the American Type Culture Collection. Axenic cultures of acanthamoebae were prepared in 10 ml of PYG 712 medium (American Type Culture Collection) (2% proteose peptone, 0.1% yeast extract, 0.1 M glucose, 4 mM MgSO<sub>4</sub>, 0.4 M CaCl<sub>2</sub>, 0.1% sodium citrate dihydrate, 0.05 mM Fe (NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub> · 6H<sub>2</sub>O, 2.5 mM NaH<sub>2</sub>PO<sub>3</sub>, 2.5 mM K<sub>2</sub>HPO<sub>3</sub>) at room temperature. The amoebae were subcultured at intervals of 10 days. For resuscitation of *L. pneumophila*, axenic cultures of *A. castellanii* were adjusted to a titer of 10<sup>5</sup> cells per ml. Then 1 ml of culture was pipetted into 24-well infection plates (Nunclon, Wiesbaden, Germany). Following overnight incubation, the medium was replaced with "Acanthamoeba buffer" (i.e., PYG 712 medium without proteose peptone, glucose, and yeast extract). In case culturable *Legionella* cells were not detectable in the microcosms, the Acanthamoeba buffer was replaced by a 1-ml aliquot from the microcosm. Following this inoculation, the infection plates were centrifuged for 3 min at 1,200 rpm (Sorvall RT 6000 centrifuge) and incubated at 37°C. After 0, 3, 24, 48, and 72 h, the protozoa were removed from the bottom of the wells by vigorous agitation. The number of CFU of culturable legionellae was determined by the spread plate technique on BCYE agar plates.

**rRNA-targeted oligonucleotide probes.** Probe LEG705 has been described by Manz et al. (22). This probe is complementary to positions 705 to 722 of the 16S rRNA of *L. pneumophila*. The sequence 5'-CTG GTG TTC CTT CCG ATC-3' was synthesized with a C6-TFA amino-linker [6-trifluoroacetylaminohexyl-(2-cyanoethyl)-(N,N-diisopropyl)phosphoramidite] at the 5'-end (MWG Biotech, Ebersberg, Germany). Labeling with FLUOS (Boehringer, Mannheim, Germany) was performed as described previously (1).

***In situ* hybridization with *L. pneumophila* and with *L. pneumophila* within *A. castellanii*.** For *in situ* hybridization with *L. pneumophila*, 50-ml (freshly inoculated microcosm) and 200-ml (microcosm with VBNC cells) aliquots of the microcosms were concentrated by centrifugation (5,000 × g for 25 min) and resuspended in 100 µl of the supernatant. The collected cells were transferred to sterile glass slides and air dried. The *in situ* hybridization of *L. pneumophila* within fixed cells of *A. castellanii* was performed with amoebae from the *in vivo* resuscitation assay (see above). The infected amoeba cells were chilled on ice, transferred to sterile glass slides, and air dried. For the *in situ* hybridization, the air-dried preparations were overlaid with a 4% (wt/vol) paraformaldehyde solution for 3 h, washed twice with phosphate-buffered saline, and then dehydrated in 50, 80, and 96% (vol/vol) ethanol (3 min each). A 20-µl aliquot of hybridization buffer (20% [vol/vol] formamide, 0.9 M NaCl, 0.01% sodium dodecyl sulfate, 20 mM Tris HCl [pH 7.6]) containing 50 ng of the labelled probe was spotted onto the cells, which were then incubated for 1.5 h at 46°C in a moisture chamber. The glass slides were quickly transferred to washing jars containing washing buffer at pH 7.2 (20 mM Tris HCl, 180 mM NaCl, 0.01% sodium dodecyl sulfate), and incubated for 15 min at 46°C. Finally, the slides were washed with distilled water, air dried, and mounted with Citifluor. The probe-conferred fluorescence was detected with a Zeiss Axioplan microscope (22).

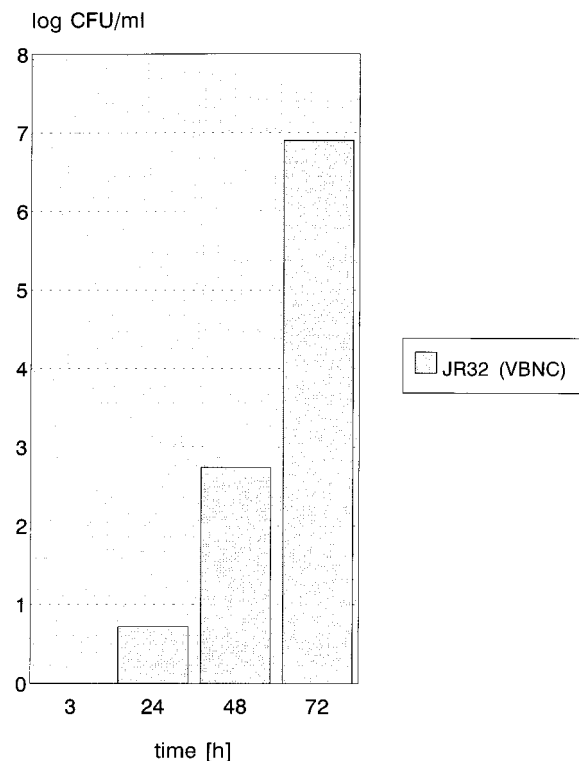


FIG. 3. Resuscitation of VBNC cells of *L. pneumophila* JR 32 by coinubation with *A. castellanii*. Samples (1 ml) of a 180-day-old tap water microcosm with *L. pneumophila* JR 32 cells that had not been culturable for 55 days were used to infect 10<sup>5</sup> amoeba cells/ml. After various incubation periods, the formation of CFU was determined in triplicate.

**Isolation of *L. pneumophila* and infection of human blood monocytes.** Blood mononuclear cells were isolated from the blood of adult donors who had never been diagnosed with Legionnaires' disease. Furthermore, the donor serum had an anti-*L. pneumophila* titer below 1:64 as determined by the indirect fluorescent-antibody assay. The blood mononuclear cell fraction was obtained by centrifugation over a polysucrose sodium diatrizoate gradient (Histopaque 1119 and 1077) as specified by the manufacturer (Sigma, Deisenhofen, Germany). Human monocytes were grown at 37°C in RPMI 1640 medium (Gibco BRL, Eggenstein, Germany) supplemented with 20% inactivated fetal calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, and 1% minimal essential medium plus nonessential amino acids (Gibco BRL). Mononuclear cells were allowed to adhere and to mature for 4 days before they were infected with *L. pneumophila*. The infection was carried out as described previously (45). Briefly, 5 × 10<sup>5</sup> monocytes per ml were infected with 10<sup>6</sup> legionellae, resulting in a multiplicity of infection of 2. Following 2 h of incubation at 37°C, the supernatant was removed and substituted with 1 ml of medium containing 80 µg of gentamicin to kill the remaining extracellular bacteria. Following a 1-h treatment, the medium was replaced by antibiotic-free medium. This was defined as the zero hour value. Following another 3, 24, and 48 h of incubation, CFU were determined by the spread plate technique on BCYE agar plates. As a control in the determination of intracellular multiplication, the avirulent derivative of *L. pneumophila* Philadelphia I XXXV was used (46).

**Intraperitoneal infection of guinea pigs with *L. pneumophila*.** Hartley guinea pigs weighing approximately 300 to 350 g were used for the infection studies. *L. pneumophila* JR 32 cells which had been cultured exclusively on BCYE agar, as well as *L. pneumophila* JR 32 cells which had been reactivated by coinubation with *A. castellanii* and *L. pneumophila* JR 32 VBNC cells, were adjusted to a final concentration of 10<sup>5</sup> cells/ml. Then 2-ml samples were injected into the peritoneal cavity of the guinea pigs. The body temperature of the guinea pigs was recorded daily with a thermometer introduced into the colon. All the animals were sacrificed by the day 6 postinfection. Homogenized lung suspensions were plated in a series of dilutions on BCYE agar to determine the CFU.

#### RESULTS

**Entry into the VBNC state.** Plate counts (BCYE agar) of *L. pneumophila* from tap water microcosms decreased from 10<sup>4</sup>

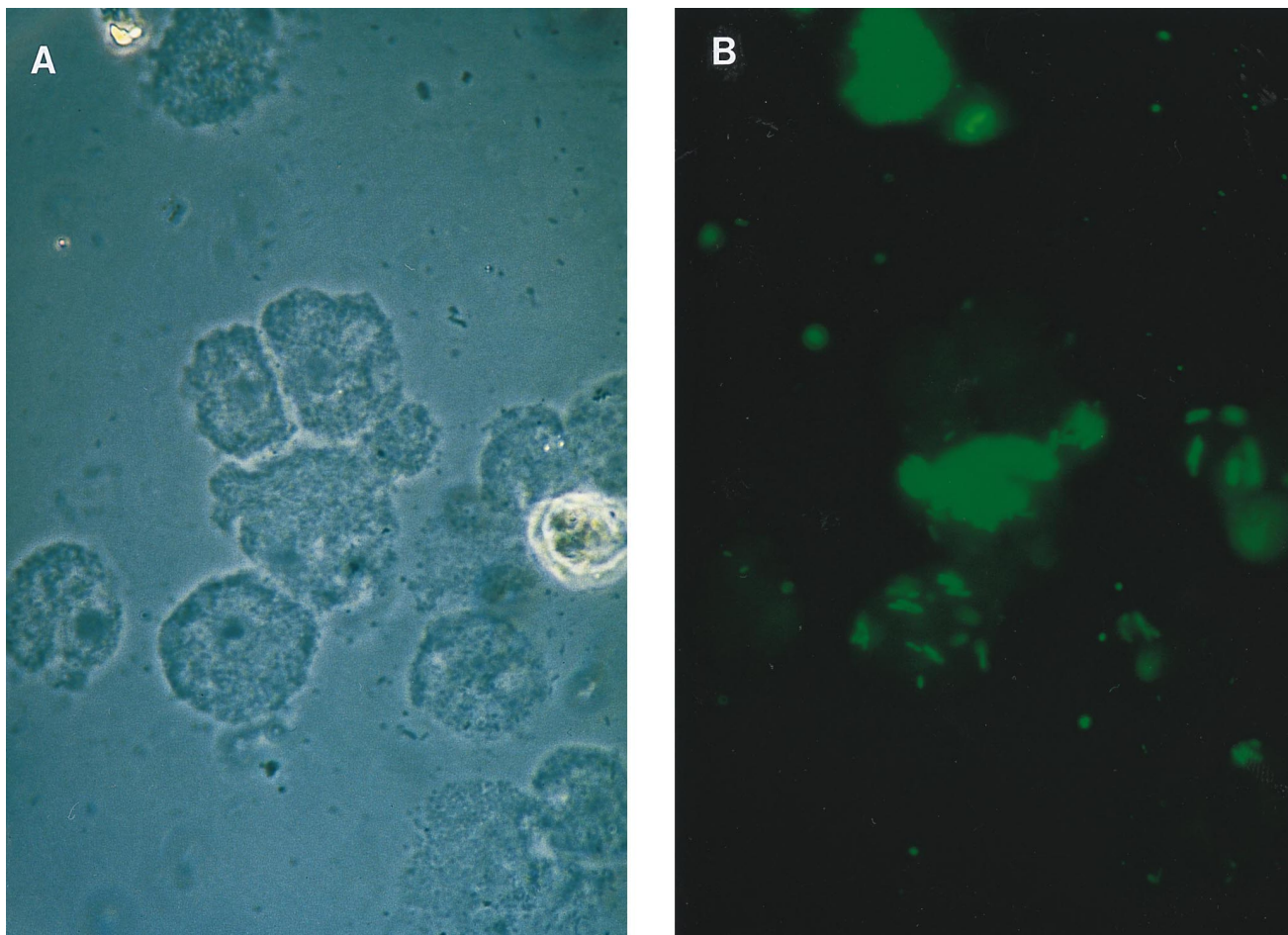


FIG. 4. In situ hybridization of infected *A. castellanii* cells with the FLUOS-labelled probe LEG705. Phase-contrast (left) and epifluorescence (right) micrographs of identical microscopic fields are shown. Cells of *L. pneumophila* reside within vacuoles of *A. castellanii*.

cells/ml to levels below detection after 125 days whereas the AODC showed a much smaller decline (Fig. 1). Even after 55 days of nonculturability, a significant *L. pneumophila* population remained. The viability of *L. pneumophila* after this prolonged time of dormancy is indicated by the 16S rRNA probing, since probe-conferred fluorescence after 180 days was seen at detectable levels (Fig. 2). In agreement with the studies of Hussong et al. (17), the application of the direct viable count with yeast extract enrichment and nalidixic acid treatment to prevent cell division was unsuccessful. However, the results of the acridine orange stain, the successful hybridization with the rRNA-targeted oligonucleotide probe, and the resuscitation studies with *A. castellanii* demonstrated the presence of viable bacteria.

**Resuscitation studies.** To examine whether nonculturable cells of *L. pneumophila* are able to regain the culturable state, VBNC cells were coincubated with axenic cultures of *A. castellanii* at 37°C. *A. castellanii* at titers of  $10^5$  cells/well were inoculated with 1-ml samples of the 180-day-old tap water microcosm. To improve the contact between host and parasite, the infection plates were centrifuged. After 0, 24, 48 and 72 h of coincubation, the protozoa were resuspended vigorously, and CFU of *L. pneumophila* per milliliter were determined on BCYE agar plates. As shown in Fig. 3, culturable cells were detected after 1 day of coincubation. Multiplication of *L. pneumophila* occurred since the number of bacteria after 3 days of

coincubation with amoebae was larger ( $8 \times 10^6$  cells/ml) than in the freshly inoculated microcosm ( $10^4$  cells/ml). Oligonucleotide probe hybridization with infected amoebal cells from the resuscitation assay clearly visualized dense clusters of intracellular legionellae, thereby proving that multiplication had occurred (Fig. 4). The bright probe-conferred fluorescence due to a large number of rRNA molecules (constituent of ribosomes) also indicates the high metabolic activity of *L. pneumophila* in the amoeba vacuole.

**Intracellular multiplication of *L. pneumophila* in human monocytes.** The virulence of reactivated *L. pneumophila* cells was evaluated by determining the rate of intracellular multiplication in human monocytes relative to log-phase grown legionellae of the same strain. The blood mononuclear cells used in this assay were isolated from blood of adult donors, who were not known to have ever contracted Legionnaires' disease. The results of the infection assay are shown in Fig. 5. The infectivity of both strains was not significantly different, and approximately  $10^5$  CFU/ml were obtained after 1 day. As the bacteria were unable to grow in RPMI medium, their increasing number was due to intracellular replication. In contrast, intracellular multiplication was not observed with the avirulent Philadelphia I variant (XXXV), obtained by passage over Mueller-Hinton agar (41). These data suggest that crucial parameters of virulence of *L. pneumophila* are maintained after resuscitation in *A. castellanii*.

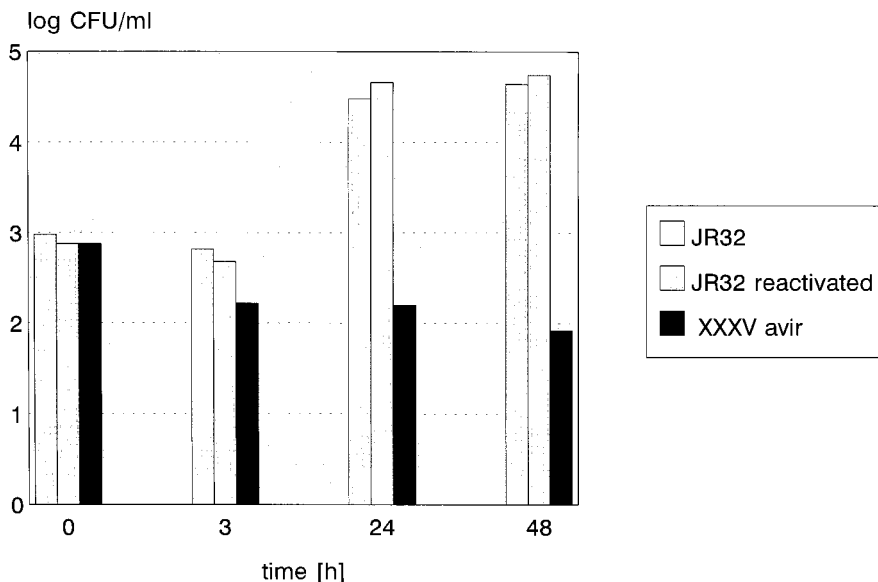


FIG. 5. Intracellular multiplication of *L. pneumophila* JR 32 in human blood monocytes. The monocytes were infected with *L. pneumophila*, resulting in a multiplicity of infection of 10. After various incubation periods, the formation of CFU was determined in duplicate for three independent experiments. The standard derivation was below 0.5 log unit. Shown is *L. pneumophila* JR 32 grown to log phase and *Acanthamoeba*-reactivated formerly VBNC cells of the same strain. *L. pneumophila* XXXV is derived from the Philadelphia I strain and serves as an avirulent control.

**Intraperitoneal infection of guinea pigs with *L. pneumophila*.** *L. pneumophila* JR 32 which had been cultured exclusively on BCYE agar, *L. pneumophila* reactivated by *A. castellanii*, and dormant VBNC cells were inoculated intraperitoneally into three guinea pigs each. Virulence was defined when the animals died or when they showed a temperature of 1.5°C above the established norm for three consecutive days. All animals infected either with the original or reactivated strain showed symptoms of disease and developed a body temperature above 40°C after infection (Table 1). In these cases, *L. pneumophila* JR 32 was reisolated from homogenized lung suspensions. In contrast, no significant febrile symptoms were detected in guinea pigs which had been inoculated with VBNC cells. Furthermore, no growth was obtained by plating lung suspensions of guinea pigs infected with VBNC cells in these concentrations.

## DISCUSSION

The aquatic habitats of *L. pneumophila* are characterized by discontinuity in nutrient concentrations. Especially in tap water, where the nutrient content is low, suboptimal growth conditions seem to be the norm. One strategy of the fastidious pathogen *L. pneumophila* is to obtain growth-supporting conditions by parasitizing amoebae (2, 9, 24, 26, 41, 42). This intracellular residence also provides a reservoir for legionellae when environmental conditions become unfavorable (20). Particularly inside *Acanthamoeba* cysts, intracellular legionellae may be transported to more favorable environments, since this vehicle provides a means to survive disinfection procedures and drying (19). However, *L. pneumophila* is also able to survive without host organisms for extended periods. In addition to its ability to survive within biofilms and its general high resistance to environmental stress, the pathogen is able to enter a VBNC state (17, 31). Similar to the situation for other nonsporulating gram-negative bacteria, this state may promote survival under adverse environmental conditions (30). The loss of culturability of *L. pneumophila* is known to be induced

under low-nutrient conditions and appears to be accelerated at higher temperatures (37 versus 4°C) (17).

In this study, we compared the decline of culturability as measured by plating viable *L. pneumophila* JR 32 cells in a sterilized tap water microcosm. After 125 days in the tap water microcosm, no bacteria were found when tested for by their ability to grow on BCYE agar. However, a significant *Legionella* population could be observed by the AODC technique. In addition, rRNA-specific in situ hybridization with the fluorescently labelled probe LEG705 demonstrated the presence of *Legionella* cells. The value of this technique for detecting non-culturable legionellae has been demonstrated recently (22).

The reentry into the culturable state appears to be unique for each organism and is influenced by a multitude of physical and chemical parameters (34). Evidence for the resuscitation of VBNC *L. pneumophila* cells following inoculations of embryonated eggs has been presented. Microcosm samples caused a far greater chicken embryo mortality than could be accounted for by the number of culturable cells. Moreover, *L. pneumo-*

TABLE 1. Intraperitoneal infection of guinea pigs with *L. pneumophila* JR 32

<i>Legionella</i> strain	No. of dead guinea pigs/no. infected <sup>a</sup>	Mean temp elevation (°C) ± SD	Mean recultivation from lung (CFU/organ) <sup>c</sup> ± SD
JR 32	1/3	2.06 ± 0.21	1.4 × 10 <sup>4</sup> ± 2.3 × 10 <sup>3</sup>
JR 32 reactivated	0/3	2.1 ± 0.61	4.0 × 10 <sup>4</sup> ± 8.4 × 10 <sup>3</sup>
JR 32 VBNC	0/3	0.56 ± 0.15	0
JR 32 in H <sub>2</sub> O	0/3	0.86 ± 0.2	0

<sup>a</sup> Guinea pigs were infected intraperitoneally with 2 × 10<sup>5</sup> *Legionella* cells.

<sup>b</sup> Temperatures were taken with a thermometer introduced into the colon. The reference temperature is the mean of the temperatures taken on three consecutive days before infection. The values shown are the peak difference above or below the reference temperature on the first 3 days after infection. Values given represent the mean determined in three animals.

<sup>c</sup> CFU/organ was determined by plating several dilutions on BCYE agar. Values given represent the means of three organ homogenates.

*phila* was recoverable from yolk sac tissue of lethally infected embryos by culture (17). However, to our knowledge, other procedures to reactivate VBNC legionellae have failed. In contrast to *Vibrio vulnificus*, which was reported to be resuscitated by heat shock, this stimulus had a detrimental effect on the culturability of legionellae (27, 29, 43). The results provided in the present study suggest that resuscitation from the VBNC state can also occur in the natural host *A. castellanii*. To minimize the possibility that the growth of a few residual culturable cells accounts for the resuscitation of *Legionella*, the amoebae were infected only after 55 days of nonculturability. In situ hybridization of infected *Acanthamoeba* demonstrated dense clusters of intracellular *L. pneumophila* cells by bright fluorescence. Thus, previously nonculturable *Legionella* cells were taken up by the host, multiplied intracellularly, and became culturable on BCYE agar. Apparently, the intracellular residence within the host organism effectively triggered resuscitation. The influence of intra-amoebic conditions on the surface properties of *L. pneumophila* has been described previously (2).

Maintenance of virulence by nonculturable pathogens varies in different species and is influenced by the conditions applied. Nonculturable cells of *Campylobacter jejuni* and *Vibrio vulnificus* retain infectivity for mice (30, 37). The virulence of *Aeromonas salmonicida* in the VBNC state is controversial (25, 28). VBNC cells of *Shigella dysenteriae* were found to maintain active Shiga toxin and the capability of adhering to Henle 407 cells. However, the ability to invade these eucaryotic cells was lost (35). In the course of human infection, *L. pneumophila* is phagocytosed by monocytes and macrophages, and it has been demonstrated that the host-directed uptake occurs with heat-killed, glutaraldehyde-killed, formalin-killed, and avirulent *L. pneumophila* cells (15, 16). Thus metabolic activities, which are reduced in dormant bacteria, do not seem to be required for invasion.

Although there is a correlation of *Legionella* infection of amoebae, human cell lines, and animal models (41, 45, 46), our studies suggest that the VBNC cells of *Legionella* cannot be resuscitated in the guinea pig. This might be attributed to the low infection dose that was used in our experiments or to the action of the immune system in the animal model. Since the infection of guinea pigs as a result of intraperitoneal inoculation shows pathological discrepancies with human disease (18), it cannot be excluded that VBNC forms are virulent for humans. Although this is highly speculative, VBNC forms of legionellae might cause a mild, self-limiting disease called Pontiac fever. However, for reactivated, formerly nonculturable legionellae, the infection studies in human monocytes and in the guinea pig model provided clear evidence that these cells are fully virulent. Since VBNC legionellae probably represent a large portion of the population in natural and man-made environments, the VBNC state may constitute an unrecognized reservoir for disease. In addition to the presence of faster-growing bacteria, the VBNC state could account for the failure to culture the pathogen from suspected sources. Experimental evidence of this and previous studies shows that the incubation of water samples with amoebae (38–40) and the application of rRNA-targeted oligonucleotide probing provide important tools in risk assessment and in monitoring of disinfection procedures.

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