ELSEVIER

FEMS Microbiology Ecology 31 (2000) 217–224

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Francisella tularensis does not manifest virulence in viable but non-culturable state

Mats Forsman [a,*], Eva W. Henningson [b,1], Eva Larsson [a], Thorsten Johansson [a], Gunnar Sandström [c,d]

[a] Department of Microbiology, National Defence Research Establishment, S-901 82 Umeå, Sweden
[b] Department of Environment and Protection, National Defence Research Establishment, S-901 82 Umeå, Sweden
[c] Division of NBC, National Defence Research Establishment, S-901 82 Umeå, Sweden
[d] Department of Clinical Microbiology, Infectious Diseases, University of Umeå, S-901 87 Umeå, Sweden

Received 30 March 1999; received in revised form 5 November 1999; accepted 7 December 1999

Downloaded from https://academic.oup.com/femsec/article/31/3/217/461898 by guest on 24 April 2024

Abstract

Francisella tularensis is a small Gram-negative bacterium that causes tularemia in animals and man. The disease can be transmitted by handling of infected animals, by contaminated dust, by insect vectors, or by drinking contaminated water. In the present study cells of *F. tularensis* were subjected to extended storage in cold water devoid of carbon sources. Total cell counts remained constant throughout a 70-day period and beyond, while plate counts decreased to an undetectable level after 70 days. Attempts to resuscitate the cells were unsuccessful. Quantitative PCR targeting the 16S rDNA of *F. tularensis* showed an increase in variability after 25 days and the signal was lost after 45 days. Metabolic activity, measured by accumulation of rhodamine 123, declined to approximately 35% after a 140-day period. Analyses of substrate responsiveness of cells stored for 140 days in cold water showed that approximately 30% of the population increased in size after incubation in rich medium in the presence of nalidixic acid. Approximately 10^5 of these cells were injected intraperitoneally into mice. No signs or symptoms of tularemia were observed during 3 weeks. In addition, there was no evidence of stimulation of lymphocytes with *F. tularensis* as recall antigen. In conclusion, viable but non-culturable cells of *F. tularensis* are avirulent in mice, giving new insight into the ecological niche of this bacterium. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Francisella tularensis*; Tularemia; Survival; Water; Rhodamine 123; Nalidixic acid; Monoclonal antibody

1. Introduction

Tularemia is a zoonosis caused by the small, Gram-negative, rod-shaped bacterium *Francisella tularensis*. It occurs naturally in logomorphs (rabbits and hares), rodents, especially microtine rodents such as voles, vole rats, and muskrats, as well as beavers. In addition, a wide variety of other mammals, and several species of birds, have been reported to be infected [1].

The bacterium occurs as two main types [2]. Type A, also designated *F. tularensis* var. *tularensis*, is predominately found in North America and is highly virulent for humans and frequently transmitted by ticks. The other main type is type B, *F. tularensis* var. *palaearctica*, which can be found in all epizootic areas where tularemia occurs [2,3,4]. This type is less virulent than type A in humans and is often seen as a disease in man attributed to contact with muskrats and/or beavers [5]. Type B has been isolated from ponds and streams [6].

Studies of *F. tularensis* type A in natural waters and mud in North America have shown that water and mud contamination with the organisms and occurrence of tularemia in muskrats and beavers is widespread. Water and mud contamination may persist for at least 16 years [7]. Available information suggests that factors governing persistence are present in water or mud or both which indicates that the organism might multiply in water-mud suspensions [8,9].

In several countries the type B variant of *F. tularensis* has been isolated from water samples [2,4,10–12]. Isolates of the bacterium have been obtained from domestic water supplies [4,10–12] as well as from natural water systems [4,6]. However, isolation of *F. tularensis* from water sam-

* Corresponding author. Tel.: +46 (90) 106669; Fax: +46 (90) 106800; E-mail: forsman@ume.foa.se

[1] Present address: Biopool AB, Box 7133, S-907 04 Umeå, Sweden.

0168-6496/00/$20.00 © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.
PII: S0168-6496(99)00100-2

ples in Sweden has never been reported, although it is endemic in the northern part of the country [13,14]. One reason for this could be that *F. tularensis* is a fastidious bacterium which requires special medium supplements for its growth in vitro and thus has not been trapped by ordinary diagnostic methods. Furthermore, isolation of the bacterium is hampered since other bacteria may be favored on most media used for growth of *F. tularensis* [15]. Another possibility, supported by the data herein presented, is that *F. tularensis* can reside in a viable but non-culturable (VBNC) form in the environment.

2. Materials and methods

2.1. Bacteria and reagents

F. tularensis LVS, live vaccine strain (type B), was obtained from the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD, USA. Monoclonal antibodies specific for *F. tularensis*, designated FT14, were kindly provided by the Defence Establishment and Research Agency, Porton Down, Salisbury, UK.

2.2. Growth and viable counts of F. tularensis

For growth of *F. tularensis* LVS in liquid, Scharer medium [16] or Chamberlain medium [17] was used. Modified Thayer-Martin agar plates containing Gc medium base (36 $g\ l^{-1}$; Difco Laboratories, Detroit, MI, USA), hemoglobin (10 $g\ l^{-1}$; Difco), and IsoVitaleX (10 $mg\ l^{-1}$; BBL Microbiology Systems, Cockeysville, MD, USA) were used for viable counts. After spreading of the inoculum, the agar plates were incubated at 37°C in 5% CO_2 for at least 2 days if not otherwise stated.

2.3. Microcosms of F. tularensis

After inoculation of approximately 10^6 cells ml^{-1} in Scharer medium, determined by viable count, the cultures were agitated (150 rpm) at 37°C for 18 h. To remove nutrients originating from the medium, the cells were then washed twice in sterile tap water and finally resuspended in 10 ml sterile tap water to form a 10-ml microcosm. The microcosms were immediately transferred to 8°C, and aliquots were periodically subjected to viable counts, total count analyses and direct viability assays.

2.4. Total cell count analyses

Three different methods were used to estimate the total count. Analysis by Coulter counter (CC_{TOT}) was according to a previously described method [18]. Determination of total number of *F. tularensis* cells by specific targeting with monoclonal antibodies and detection by phycoerythrin-conjugated secondary antibodies in a flow cytometer (FC_{TOT}) has also been described previously [19]. In addition, the amount of PCR-amplified *F. tularensis* 16S rDNA was used as a relative measure of the total amount of *F. tularensis* cells. To quantify the PCR-generated 16S rDNA, a heterologous internal standard DNA fragment was constructed. A 673-bp DNA fragment was generated from the 2,3-dioxygenase-encoding gene (*dmpB* gene) of the phenol-catabolizing *Pseudomonas* CF600 [20] using the composite primers F11dmpF and F5dmpR (Table 1). The composite primers consisted of primers F11 and F5 (Table 1) appended to the 5′ end of sequences corresponding to bases 259–278 (dmpF) and 874–892 (dmpR), respectively, of the *dmpB* gene. PCR using composite primer F11dmpF as the 5′ primer and primer F5dmpR as the 3′ primer amplified a 673-bp DNA fragment containing the binding sequence for primer F11 at its 5′ end and the binding sequence for primer F5 at its 3′ end. Serial dilutions of this internal standard were added to a constant amount of a sample and the resulting PCR products were separated on 1.0% agarose gels. The intensity of the bands was quantified by densitometric scanning. At the start of the experiments the intensity of the target bands from the *F. tularensis* cells and the intensity of the internal standards (corrected for the shorter length) were plotted against the amount of added internal standard. The point at which the sample and standard lines intersected was taken as a measure of the amount initially present in the sample. This point was set to 1.0 unit. To verify that the target (16S rDNA) and the internal standard amplified with similar efficiencies, approximately equal molar quantities of the *F. tularensis* 16S rDNA gene and the internal standard

Table 1
PCR primers used in this study

Primer	Sequence (5′-3′)	Location
F5	CCTTTTTGAGTTTCGCTCC	1257–1275[a]
F11	TACCAGTTGGAAACGACTGT	142–161[a]
dmpF	ATCGACGAGGACTGCCTGAA	259–278[b]
dmpR	CCAGCCAGGTCACGGGCTT	874–892[b]
F11dmpF	TACCAGTTGGAAACGACTGTATCGACGAGGACTGCCTGAA	259–278[b]
F5dmpR	CCTTTTTGAGTTTCGCTCCCCAGCCAGGTCACGGGCTT	874–892[b]

[a]Location relative to the start site of the *F. tularensis* 16S rRNA gene.
[b]Location relative to the start site of the *Pseudomonas* CF600 *dmpB* gene.

Downloaded from https://academic.oup.com/femsec/article/31/3/217/461898 by guest on 24 April 2024

DNA fragment were added to single PCR tubes. Aliquots were removed after each cycle for a total of nine cycles, starting when PCR products were first visualized on an agarose gel. The intensity of the bands was then quantified by densitometric scanning as described above. The amounts of amplified *F. tularensis* 16S rDNA product and amplified internal standard were plotted as a function of cycle number. The slope for the linear portion of the two curves was calculated in three independent experiments and no significant difference ($P = 0.05$) was found.

2.5. Direct viable count analyses

Two different methods were used for direct viable count analyses. Metabolic activity was quantified by accumulation of rhodamine 123 according to [19]. In addition, substrate responsiveness in the presence of nalidixic acid [21] was analyzed by flow cytometry. The *F. tularensis* microcosms were diluted 10 times in Chamberlain medium containing 0.5 μg ml^{-1} of nalidixic acid (Sigma) and incubated at 37°C for 7 days. An identical culture containing formalin-killed [19] *F. tularensis* cells was prepared in parallel. The cell population sizes were analyzed before and after incubation in Scharer medium containing nalidixic acid by the FC_{TOT} method, specific for *F. tularensis*, methods described previously [19].

2.6. Resuscitation studies

Several procedures were tried for resuscitation of VBNC *F. tularensis* from 100-day or older 8°C microcosms. Microcosms (10 ml) were initially transferred to room temperature for 24 h, followed by centrifugation at $5000 \times g$ for 10 min prior to resuspension in equal volumes of Sharer medium and Chamberlain medium. The resulting cultures were incubated at room temperature without agitating 7 days prior to plating on modified Thayer-Martin agar plates. In parallel, the same procedure was followed with the difference that cells from microcosms, after centrifugation, were directly resuspended in Sharer and Chamberlain medium and incubated at 8°C and 37°C, respectively, for 7 days without agitation, prior to plating. After centrifugation 10-ml microcosms were also resuspended in 10 ml of spent Sharer medium and incubated at 26°C or 37°C without agitation for 3 days prior to plating on modified Thayer-Martin agar plates. Spent Sharer medium was the supernatant obtained by centrifugation at $5000 \times g$ of a *F. tularensis* culture, with a density of approximately 10^9 bacterial cells ml^{-1}, grown for 2 days at 37°C with shaking at 250 rpm.

2.7. Flow cytometer analyses

Measurements of total counts, metabolic activity and cell population size were done using a flow cytometer (FACSort, Becton Dickinson Immuno Cytometry Systems, San Jose, CA, USA) equipped with an argon laser with a 488-nm primary emission line.

The instrument was calibrated (Calibrite™ Beads, Becton Dickinson) prior to each set of analyses. A negative sample was then analyzed to adjust forward scatter and side scatter correctly. Thereafter, fluorescence was adjusted by compensation. The measuring time was 30 s with a medium flow rate of 35 μl s^{-1}. From each sample 50 000 events were collected.

2.8. Assay of virulence and proliferative T-lymphocyte response

Four groups of female BALB/c mice were used. The mice were 3 months old and weighed 25–30 g. Five mice were used in each immunization group. Mice was injected intraperitoneally with 10^5 VBNC *F. tularensis* cells starved for 140 days, 10^5 formalin-killed cells, 10^5 cells grown for 2 days, or with 0.9% NaCl. Three weeks after injection lymphocytes were prepared from spleens according to a previously described method [22]. The spleens were initially homogenized in sterile saline and prior to purification of lymphocytes aliquots were removed for subsequent viable counts on modified Thayer-Martin agar plates. After dilution of the purified lymphocytes to a concentration of 3×10^6 ml^{-1}, 100 μl was seeded in each well in a microtiter plate. Five wells were used for each antigen. Specific positive antigen was heat-killed bacteria of a *F. tularensis* strain deficient in the ability to produce capsule [23], negative control was RPMI 1640 medium and the mitogen concanavalin A was used as positive control. To estimate the proliferative response cultures were incubated at 37°C in 5% CO_2 for 4 days and pulsed for 6 h with $[^3H]$thymidine and harvested [24].

3. Results

3.1. Storage of F. tularensis in water

Cells of *F. tularensis* were subjected to extended storage in cold water devoid of carbon sources. Total counts and plate counts were measured over time. Fig. 1 shows the response of *F. tularensis* following incubation in water at 8°C. Plate counts decreased to undetectable levels after 70 days. These results are typical of several independently prepared microcosms.

Total cell counts were estimated with three different methods. (i) FC_{TOT} [19]: the total cell counts of *F. tularensis* cells were determined by specific targeting with monoclonal antibodies and detection by phycoerythrin-conjugated secondary antibodies in a flow cytometer. (ii) CC_{TOT} [18]: the total cell counts were determined with Coulter counter analyses. (iii) Quantitative PCR: the total amount of cells was determined by quantitative PCR on the *F. tularensis* 16S rRNA with a heterologous internal

Downloaded from https://academic.oup.com/femsec/article/31/3/217/461898 by guest on 24 April 2024

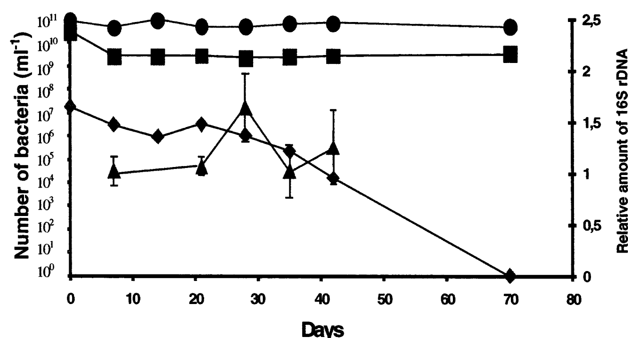

Fig. 1. Storage of *F. tularensis* in water at 8°C. Filled circles show the total number of *F. tularensis* cells determined by specific targeting with monoclonal antibodies and detected by phycoerythrin-conjugated secondary antibodies in a flow cytometer. Filled boxes show the total number of *F. tularensis* cells determined by Coulter counter analyses. Filled diamonds show colony-forming units obtained on modified Thayer-Martin agar plates. Filled triangles indicate the results obtained from quantitative PCR analyses specifically targeting the 16S rDNA of *F. tularensis*. Error bars show standard deviation ($n = 3$).

standard. Precision and accuracy for FC_{TOT} and CC_{TOT} have been reported previously [19], whereas the variation observed for quantitative PCR is indicated in Fig. 1. No amplified target DNA was obtained from samples taken on day 70. Taken together, the results showed that total cell counts remained constant throughout the 70-day period and longer (data not shown),

Cells from the microcosm placed in Sharer or Chamberlain medium at room temperature for 7 days did not produce any increased turbidity, nor did such treatment give rise to colonies when subsequently plated on modified Thayer-Martin agar plates. Preincubation for 3 days of cells from the microcosm in spent medium at 26°C and 37°C prior to plating did not result in any observable colonies on modified Thayer-Martin agar plates after 5 days incubation at 37°C in 5% CO_2.

3.2. Direct viable count on starved F. tularensis

The metabolic activity of *F. tularensis* starved for 140 days in cold water devoid of carbon sources was compared with overnight grown and formalin-killed *F. tularensis*. The metabolic activity was quantified by staining with rhodamine 123 as previously described [19]. The fluorescence resulting from uptake of rhodamine 123 was measured in a flow cytometer. A comparison of fluorescence between formalin-killed, starved and overnight grown *F. tularensis* is shown in Fig. 2. An approximately 35% decrease in metabolic activity was found after a 140-day starvation period compared with an overnight culture of *F. tularensis*. Thus, 65% of the cells were VBNC after a starvation period of 140 days as judged from rhodamine accumulation. Next, substrate responsiveness [21] of the cells was analyzed. Here the size of the formalin-killed and 140-day starved cells before and after incubation in rich medium in the presence of nalidixic acid was compared. The size distribution analysis was performed by forward scatter analysis in a flow cytometer. This analysis showed that approximately 30% of the cells stored for 140

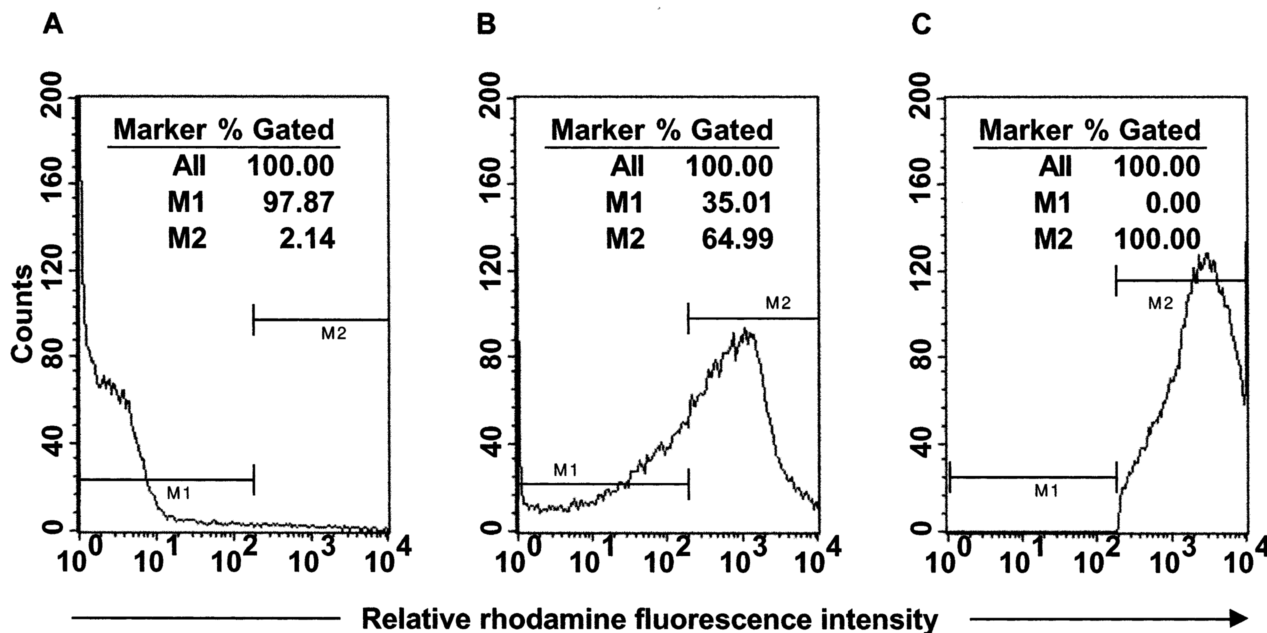

Fig. 2. Metabolic activity quantified by staining with rhodamine 123. Fluorescence emitted from cell-accumulated rhodamine was measured in the interval 512–545 nm on a flow cytometer equipped with a 488 nm laser. Formalin-killed *F. tularensis* cells (A). *F. tularensis* cells starved for 140 days in water (B). *F. tularensis* cells grown for 2 days on a modified Thayer-Martin agar plate (C). Marker 1 (M1) defines fluorescence emitted from formalin-killed rhodamine-stained cells (A). Marker 2 (M2) defines fluorescence emitted from fully viable rhodamine-stained cells (C).

Downloaded from https://academic.oup.com/femsec/article/31/3/217/461898 by guest on 24 April 2024

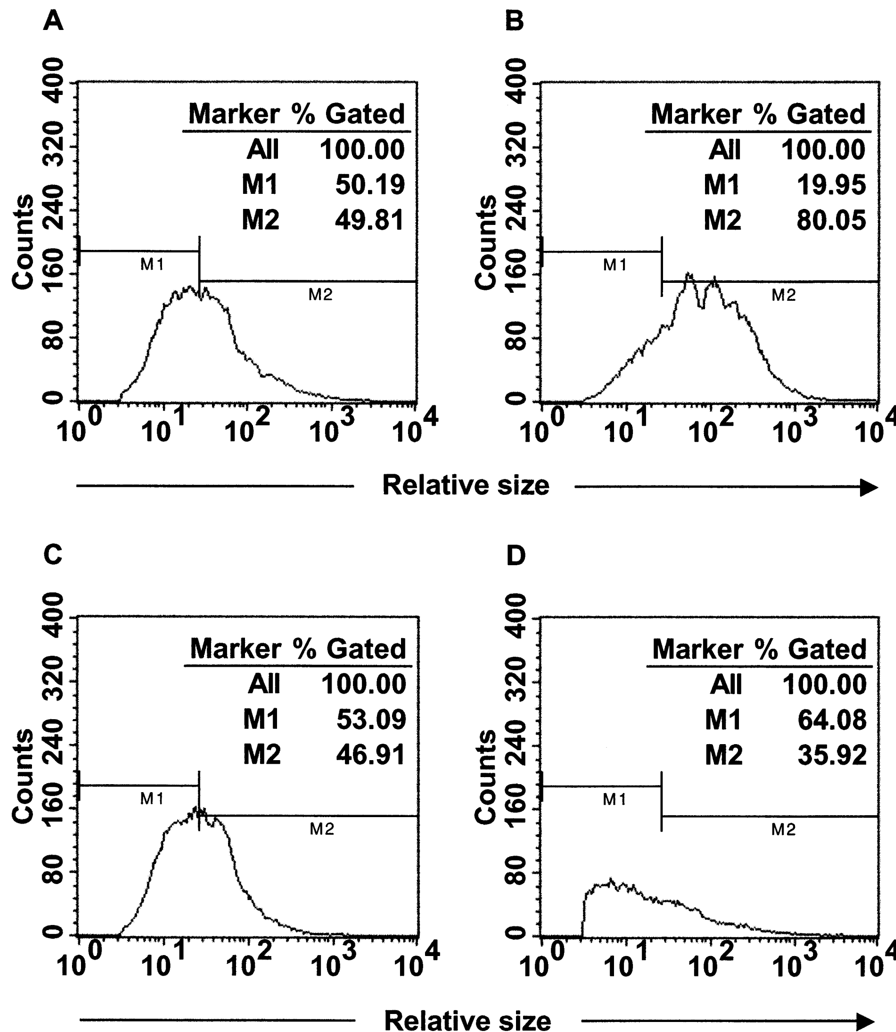

Fig. 3. Substrate responsiveness in the presence of nalidixic acid analyzed by flow cytometry. Upper row: *F. tularensis* cells starved for 140 days before (A) and after (B) incubation for 1 week in broth containing nalidixic acid. Lower row: Formalin-killed *F. tularensis* cells before (C) and after (D) incubation for 1 week in broth containing nalidixic acid. Markers 1 and 2 (M1 and M2) both mark the median of the cell size distribution before incubation in rich medium. An increase in M2 indicates an increase in size and an increase in M1 a decrease.

days in cold water increased in size (Fig. 3), while none of the formalin-killed cells increased in size. Taken together, the results showed that at least 30% of the 140-day starved cells could be defined as VBNC.

3.3. VBNC F. tularensis cells are not virulent for mice

Approximately 10^5 VBNC *F. tularensis* cells starved for 140 days were injected intraperitoneally into mice. No signs or symptoms were detected during a 3-week observation period. The lack of onset of tularemia in mice was further disclosed by subsequent assay of the lymphocyte proliferative response. No lymphocyte response could be estimated with *F. tularensis* as recall antigen (Fig. 4). However, 3 weeks post injection, the spleen from each mouse was homogenized and aliquots were spread on agar plates. No colonies were observed 7 days after incubation at 37°C in 5% CO_2.

4. Discussion

Cultivation of bacteria on standard artificial media under laboratory conditions is fundamentally different from the growth conditions in nature. In contrast to laboratory conditions, bacteria in nature spend most of their lifetime in a state of starvation or nutrient limitation. To survive extended periods of starvation or other forms of environmental stress, many bacteria have developed strategies for metamorphosis into more or less sophisticated survival forms, where spores may constitute an extreme in terms of survival in adverse conditions. Similarly, the formation of VBNC cells by non-differentiating bacteria as a response to adverse conditions has been proposed as a survival strategy [25].

In the present study it is shown that *F. tularensis* can enter a state of VBNC after starvation in cold water for an extended period of time. Other bacteria have also been

Downloaded from https://academic.oup.com/femsec/article/31/3/217/461898 by guest on 24 April 2024

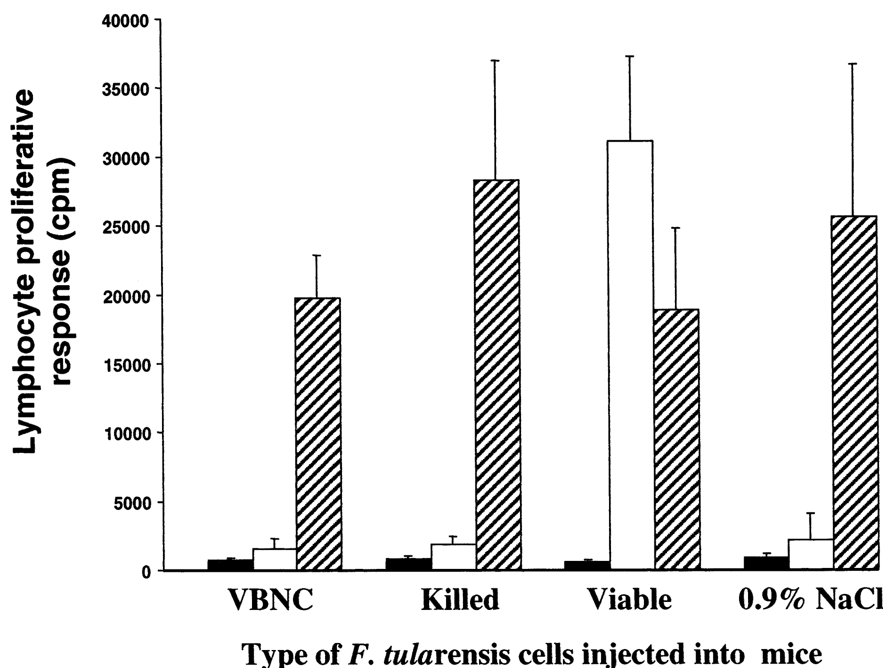

Fig. 4. Lymphocyte proliferative response (cpm) in lymphocytes prepared from mice injected intraperitoneally with *F. tularensis* cells starved for 140 days (VBNC), formalin-killed (Killed), grown for 2 days (Viable), and without bacteria (0.9% NaCl), respectively. The open bars indicate the proliferative responses of the lymphocytes prepared from the spleens from the injected mice, using heat-killed *F. tularensis* as recall antigen, the filled bars indicate the negative control for the lymphocyte proliferative assay (RPMI medium), and the hatched bars indicate the positive control for the lymphocyte proliferative assay (concanavalin A). Error bars indicate standard deviation ($n=5$).

shown to have this capability after encountering environmental stress [26]. However, factors that stimulate resuscitation are complex and unique for different bacteria. *Vibrio cholerae* and *Campylobacter jejuni* need passage through animals to regain their capacity to multiply in vitro [27,28]. *Legionella pneumophila* can be reactivated by passage in its ameba host [29]. VBNC cells of *Vibrio vulnificus* can be resuscitated by incubation for 1 or 2 days in artificial seawater at room temperature without nutrients [30]. It has also been reported that *V. cholerae* can leave the VBNC state after oral ingestion by humans [31]. Mukamolova et al. [32] have shown that starved or extensively washed cells of several Gram-positive strains reversibly lose culturability, which can be restored by the addition of an autocrine, proteinaceous growth factor. However, despite extensive attempts presented here, including preincubation at room temperature without nutrients, preincubation in spent medium, extended incubation in different media, passage through mice, all prior to cultivation on plates, it was not possible to resuscitate any colony of VBNC *F. tularensis* cells.

The maintenance of virulence in the VBNC state varies for different species of bacteria, and seems to be dependent on the environmental conditions. *C. jejuni* and *V. vulnificus* maintain virulence in the VBNC state [33,34]. In contrast, VBNC cells of *F. tularensis* did not seem to induce tularemia in mice and thus were not virulent under the conditions used in the present study.

The FC_{TOT} method of analysis showed a higher yield than the CC_{TOT} method. It is likely that CC analysis of *F. tularensis* cells is an underestimate since the size of *F. tularensis* is at the lower limit of the resolving capacity of the Coulter counter. The ratio of smaller cells increases when the culture is starved and CC cannot resolve bacteria smaller than 0.6 μm [18,19]. In the FC_{TOT} method the total number of *F. tularensis* cells was determined by specific targeting with monoclonal antibodies which is not particularly sensitive to cell size alterations. The relative estimation of cell number during starvation over time with quantitative PCR targeting the 16S rDNA of *F. tularensis* showed an increase in variability after 25 days and the signal was lost after 45 days. Loss of PCR-amplifiable products from bacterial cells subjected to starvation has also been reported for *V. vulnificus* [35]. These authors hypothesized that either DNA binding proteins or chromosomal supercoiling, or a combination of both, prevents PCR amplification in starved cells [35].

It is well documented that viable bacteria accumulate rhodamine 123, whereas non-viable ones do not [19,36]. Under appropriate conditions the extent to which individual bacteria take up rhodamine 123 quantitatively reflects the extent of their viability [37]. *F. tularensis* cells starved for 140 days showed a discrepancy between the ratio of metabolically active cells measured by rhodamine 123 accumulation and the ratio of cells able to elongate in the presence of nalidixic acid. Approximately 30% of the population increased in size after incubation in rich medium in the presence of nalidixic acid. In contrast, approximately

Downloaded from https://academic.oup.com/femsec/article/31/3/217/461898 by guest on 24 April 2024

65% showed metabolic activity as measured by rhodamine 123 accumulation. As previously suggested [38], it is possible that this discrepancy could be explained by the rapid production of superoxide and free radicals upon incubation in rich medium to which the bacterial cells are not phenotypically adapted. Nevertheless, if only 30% of the population were viable, this would have been more than sufficient to elicit tularemia in mice [39]. Typical signs and symptoms of tularemia in mice [1] manifest virulence of *F. tularensis*, which normally leads to death of the mice. No such clinical picture was observed during the experimental period. Thus, no virulence of VBNC *F. tularensis* could be detected in the animal model.

As a complement to the virulence test, the ability of the bacterium to trigger the host lymphocyte response was estimated. In this test it would be possible to find out whether or not passage through mice resuscitates the bacterium, since only viable bacteria elicit a cell-mediated immune response [39]. Hence, the fact that no lymphocyte proliferative response was found in mice after injection of VBNC *F. tularensis* cells indicates that no reactivation occurred in mice. This finding is also supported by the lack of viable count from homogenized spleens removed from mice 3 weeks after injection of 10^5 VBNC *F. tularensis* cells. However, it cannot be excluded that resuscitation of VBNC *F. tularensis* cells can occur if appropriate conditions to support culturability are provided. In conclusion, the data showed that VBNC *F. tularensis* cells were not virulent and did not resuscitate upon one passage through mice.

F. tularensis is an intracellular parasite in mammals. Many free-living soil and water protozoa mimic professional phagocytes in their ability to ingest and destroy large numbers of bacteria. The spectrum of pathogens able to survive and multiply to various degrees within amebas includes *F. tularensis*, [40] and are potential reservoirs and vectors for *F. tularensis*; they are ubiquitous in the environment, they have resistant cyst stages, and can grow and multiply in water supplies. It is possible that symbiosis with amebas is the phenotypic adaptation needed for further spread of *F. tularensis*, perhaps through other vectors such as arthropods, to man.

References

[1] Mörner, T. and Sandström, G. (1997) Tularemia. In: Manual of Standards for Diagnostic Tests and Vaccines, 3rd edn., Chapter 3.7.2. Office International des Epizooties, Paris.

[2] Olsufiev, N.G., Emelyanova, O.S. and Dunaeva, T.N. (1959) Comparative studies of strains of *B. tularense* in the Old and New World and their taxonomy. J. Hyg. Epidemiol. Microbiol. Immunol. (Prague) 3, 138–149.

[3] Jantzen, E., Berdal, B.P. and Omland, T. (1979) Cellular fatty acid composition of *Francisella tularensis*. J. Clin. Microbiol. 10, 928–930.

[4] Forsman, M., Sandstrom, G. and Jaurin, B. (1990) Identification of *Francisella* species and discrimination of type A and type B strains of *F. tularensis* by 16S rRNA analysis. Appl. Environ. Microbiol. 56, 949–955.

[5] Jellison, W.L. (1974) Tularemia in North America 1930–1974. University of Montana, Missoula, MT.

[6] Tärnvik, A. (1989) Nature of protective immunity to *Francisella tularensis*. Rev. Infect. Dis. 11, 440–451.

[7] Mitscherlich, E. and Marth, E.H. (1984) Microbial Survival in the Environment, pp. 741–742. Springer-Verlag, Berlin.

[8] Parker, R.R., Steinhaus, E.A., Kohls, G.M. and Jellison, W. (1951) Contamination of natural waters and mud with *Pasteurella tularensis* and tularemia in beavers and muskrats in the north-western United states. National Institute of Health Bulletin, No. 193. Rocky Montain Laboratory, Hamilton, MT.

[9] Gibby, I.W., Nicholes, P.S., Tamura, J.T. and Foshay, L. (1948) The effects of an extract of blood cells upon the cultivation of *Bacterium tularense* in liquid media. J. Bacteriol. 55, 855–863.

[10] Dahlstrand, S., Ringertz, O. and Zetterberg, B. (1971) Airborne tularemia in Sweden. Scand. J. Infect. Dis. 3, 7–16.

[11] Cristenson, B. (1984) An outbreak of tularemia in the northern part of central Sweden. Scand. J. Infect. Dis. 16, 285–290.

[12] Syrjälä, H., Kujala, P., Myllyla, V. and Salminen, A. (1985) Airborne transmission of tularemia in farmers. Scand. J. Infect. Dis. 17, 371–375.

[13] Luotonen, J., Syrjala, H., Jokinen, K., Sutinen, S. and Salminen, A. (1986) Tularemia in otolaryngologic practice. An analysis of 127 cases. Arch. Otolaryngol. Head Neck Surg. 112, 77–80.

[14] Wills, P.I., Gedosh, E.A., Nichols, D.R. and Smith, F. (1982) Head and neck manifestations of tularemia. Laryngoscope 92, 770–773.

[15] Pollitzer, R. (1967) History and incidence of tularemia in the Soviet Union. A review. The Institute of Contemporary Russian Studies, Fordham University, New York.

[16] Scharer, J.M., Klein, F. and Lincoln, R.E. (1968) Growth and metabolism of live vaccine strain of *Pasturella tularensis*. Appl. Microbiol. 16, 855–861.

[17] Chamberlain, R.E. (1965) Evaluation of live tularemia vaccine prepared in a chemically defined medium. Appl. Microbiol. 13, 232–235.

[18] Henningsson, E., Lundquist, M., Larsson, E., Sandström, G. and Forsman, M. (1997) A comparative study of different methods to determine the total number and the survival of bacteria in aerobiological samples. J. Aerosol Sci. 28, 459–469.

[19] Henningson, W.E., Krocova, Z., Sandström, G. and Forsman, M. (1998) Flow cytometric assessment of the survival ratio of *Francisella tularensis* in aerobiological samples. FEMS Microbiol. Ecol. 25, 241–249.

[20] Bartilson, M. and Shingler, V. (1989) Nucleotide sequences and expression of the catechol catechol 2,3-dioxygenase-encoding gene of phenol-catabolizing *Pseudomonas* CF600. Gene 85, 233–238.

[21] Kogure, K., Simidu, U. and Taga, N. (1979) A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. 25, 415–420.

[22] Böyum, A. (1968) Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Lab. Invest. 21 (Suppl.), 27–50.

[23] Sandström, G., Löfgren, S. and Tärnvik, A. (1988) A capsule deficient mutant of *Francisella tularensis* LVS exhibits enhanced sensitivity to killing by serum but diminished sensitivity to killing by polymorphonuclear leukocytes. Infect. Immun. 56, 1194–1202.

[24] Sjöstedt, A., Sandström, G. and Tärnvik, A. (1990) Several membrane polypeptides of the live vaccine strain *Francisella tularensis* LVS stimulate T cells from naturally infected individuals. J. Clin. Microbiol. 28, 43–48.

[25] Effendi, I. and Austin, B. (1995) Dormant/unculturable cells of fish pathogen *Aeromonas salmonicida*. Microb. Ecol. 30, 183–192.

[26] Oliver, J.D. (1995) The viable but non-culurable state in the human pathogen *Vibrio vulnificus*. FEMS Microbiol. Lett. 133, 203–208.

[27] Colwell, R.R., Rayton, P.R., Grimes, D.J., Roszak, D.B., Hug, S.A. and Palmer, L.M. (1985) Viable but not culturable *Vibrio cholerae*

Downloaded from https://academic.oup.com/femsec/article/31/3/217/461898 by guest on 24 April 2024

and related pathogen in the environment: implications for release of genetically engineered microorganisms. BioTechniques 3, 817–820.

[28] Jones, M., Sutcliffe, E.M. and Curry, A. (1991) Recovery of viable but non-culturable *Campylobacter jejuni*. J. Gen. Microbiol. 127, 2477–2482.

[29] Steinert, M., Emody, L., Amann, R. and Hacker, J. (1997) Resuscitation of viable but nonculturable *Legionella pneumphila* Philadelphia JR32 by *Acanthamoeba castellanii*. Appl. Environ. Microbiol. 63, 2047–2053.

[30] Whitesides, M.D. and Oliver, J.D. (1997) Resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. Appl. Environ. Microbiol. 63, 1002–1005.

[31] Colwell, R.R., Brayton, P., Herrington, D., Tall, B., Huq, A. and Levine, M.M. (1996) Viable but non-culturable *Vibrio cholerae* O1 revert to a cultivable state in the human intestine. World J. Microbiol. Biotechnol. 12, 28–31.

[32] Mukamolova, G.V., Kaprelyants, S.A., Young, D.L., Young, M. and Kell, D.B. (1998) A bacterial cytokine. Proc. Natl. Acad. Sci. USA 95, 8916–8921.

[33] Oliver, J.D. and Bockian, R. (1995) In vivo resuscitation, and virulence towards mice, of viable but nonculturable cells of *Vibrio vulnificus*. Appl. Environ. Microbiol. 61, 2620–2623.

[34] Rollins, D.M. and Colwell, R.R. (1986) Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the aquatic environment. Appl. Environ. Microbiol. 52, 531–558.

[35] Warner, J.M. and Oliver, J.D. (1998) Randomly amplified polymorphic DNA analysis of starved and viable but nonculturable *Vibrio vulnificus* cells. Appl. Environ. Microbiol. 64, 3025–3028.

[36] Diaper, J.P., Tither, K. and Edwards, C. (1992) Rapid assessment of bacterial viability by flow cytometry. Appl. Microbiol. Biotechnol. 38, 268–272.

[37] Kaprelyants, A.S. and Kell, D.B. (1992) Rapid assessment of bacterial viability and vitality by rhodamine 123 and flow cytometry. J. Appl. Bacteriol. 72, 410–422.

[38] Bloomfield, F.S., Stewart, G.S.A.B., Dodd, C.E.R., Booth, I.R. and Power, E.G.M. (1998) The viable but non-culturable phenomenon explained? Microbiology 144, 1–3.

[39] Eigelsbach, H.T. and Downs, C.M. (1961) Prophylactic effectiveness of live and killed tularemia vaccines. I. Production of vaccine and evaluation in the white mouse and guinea pig. J. Immunol. 87, 413–425.

[40] Berdal, B.P., Mehl, R., Meidell, N.K., Lorentzen-Styr, A.M. and Scheel, O. (1996) Field investigations of tularemia in Norway. FEMS Immunol. Med. Microbiol. 13, 191–195.

Downloaded from https://academic.oup.com/femsec/article/31/3/217/461898 by guest on 24 April 2024