TECHNICAL NOTE

Growth temperature ranges of *Borrelia burgdorferi sensu lato* strains

Z. HUBÁLEK, J. HALOUZKA and M. HEROLDOVÁ*

*Institute of Landscape Ecology, Academy of Sciences, Kvetná 8, CZ-60365 Brno and *Department of Microbiology, Faculty of Science, Masaryk University, Brno, Czech Republic*

Three strains of *Borrelia burgdorferi sensu lato*, representing three human pathogenic genomospecies (B31, *B. burgdorferi sensu stricto*; BR14, *B. garinii*; BR75, *B. afzelii*) were grown in BSK-H medium at different temperatures and the spirochaetal cells were counted by dark-field microscopy after 0, 4, 8, 16 and 48 days. Approximate optimum (minimum–maximum) temperatures for the in-vitro growth were found to be 33°C (22–39°C) in strain B31, 35°C (20–40°C) in strain BR75 and 37°C (20–41°C) in strain BR14. Maximum, optimum and minimum growth temperatures seem to be important characteristics of *B. burgdorferi* s.l. strains, with relevance for the symptomatology, epidemiology and epizootology of Lyme borreliosis.

Introduction

Borrelia burgdorferi sensu lato (s.l.), the causative agent of Lyme borreliosis, is a tick-borne spirochaete ‘shuttling’ principally between the vector tick and a homeotherm vertebrate host. This life cycle requires the bacterium to be able to replicate (or at least survive for a long time) in three or four very different environments and temperatures: (i) unfed tick (at ambient temperature); (ii) blood-sucking tick (c. 30–35°C) and (iii) homeotherm vertebrate (mammalian or avian) tissue, either on the skin (32–37°C) or in internal organs (37–41°C). However, information on temperature requirements of *B. burgdorferi* s.l. is limited. The optimum temperature for its growth *in vitro* has been given widely as 30–37°C [1]. The spirochaete is usually grown in BSK-II (or BSK-H, Sigma) medium at 32–34°C (most often at 33°C), but sometimes at 30–31°C or 35°C. Nevertheless, no exact studies on the optimum temperature for the growth of *B. burgdorferi* s.l. strains have been published. As yet, even less is known about the range of temperatures for the growth of *B. burgdorferi* s.l. The maximum temperature for multiplication of *B. burgdorferi sensu stricto* (s.s.) and *B. afzelii* (strain PKo) was reported to be 39°C, while no growth was observed at temperatures of $\geq 40^\circ\text{C}$ [1, 2]. These observations contradict some reported isolations of *B. burgdorferi* s.s. from birds that have normal body temperature of c. 41°C, e.g., from the liver of a veery (*Catharus fuscescens*) [3], the

blood of a song sparrow (*Melospiza media*) [4] and the blood and internal organs of experimentally inoculated mallard ducks (*Anas platyrhynchos*) [5] and canary finches (*Serinus canaria*) [6]. The isolate from the veery liver (VL) was also recovered from chicks inoculated experimentally [3]. Moreover, *B. garinii* was re-isolated from the skin and several organs of Japanese quails (*Coturnix japonica*) between 7 and 56 days after inoculation [7]. Only one strain of *B. burgdorferi* (ATCC 35211, isolated from *Ixodes ricinus* in Switzerland) was found to survive at 41°C *in vitro* for up to 4 days [2]. In contrast, there are no reports about the growth parameters of *B. burgdorferi* s.l. at ecologically meaningful temperatures $<30^\circ\text{C}$ which prevail in vector ticks during their non-parasitic stage of life cycle; only one recent paper has mentioned a slow in-vitro growth of *B. burgdorferi* at 23°C [8]. The present study investigated the maximum, minimum and optimum growth temperatures for selected strains of *B. burgdorferi*, *B. garinii* and *B. afzelii*.

Materials and methods

Three strains of *B. burgdorferi* s.l., representing the three known human pathogenic genomic groups (‘genomospecies’ or ‘genospecies’), were tested: (1) type strain B31 (ATCC 35210) of *B. burgdorferi* s.s., isolated from *Ixodes scapularis* collected on Shelter Island, NY and passaged >20 times *in vitro*, including eight passages in this laboratory at 33°C; (2) strain BR14 (*B. garinii*), isolated from nymphal *Ixodes ricinus* collected in South Moravia, Czech Republic and passaged 11 times *in vitro* at 33°C [9]; and (3)

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Corresponding author: Dr Z. Hubálek.

strain BR75 (*B. afzelii*), isolated from *Ctenophthalmus agyrtus* fleas collected on *Clethrionomys glareolus* in South Moravia and passaged nine times *in vitro* at 33°C [9]. The spirochaetes were grown at 33°C in BSK-H medium (Sigma) supplemented with rabbit serum (Sigma) 5%, phosphomycin 100 µg/ml and rifampicin 50 µg/ml. About 1×10^5 cells from the logarithmic phase of growth (4–5-day-old cultures, with a cell motility of 90–99% and a cell density of $(5–10) \times 10^7$ /ml) were inoculated into 4-ml glass tubes containing 3.5 ml of BSK-H medium with rabbit serum 5% and antibiotics, tightly stoppered and incubated for up to 48 days at various temperatures as follows (their ecological relevance is given in parentheses): 20°C (mean daily air temperature in July, Central Europe); 28°C (maximum daily air temperature in summer, Central Europe); 33°C (approximate skin temperature of mammals); 37°C (average body temperature of mammals and approximate skin temperature of birds) and 41°C (average body temperature of birds). The temperature of incubators was checked continuously by thermometers placed at the level of culture racks; the temperature fluctuation did not exceed $\pm 0.2^\circ\text{C}$ in any of the incubators used. For each of the three strains, tubes were incubated for 4, 8, 16 and 48 days at each of the five temperatures (60 tubes in total). The number of organisms present was determined by dark-field microscopy of 10-µl volumes, after appropriate dilution and in triplicate by two observers, at time 0, and after each incubation period at each temperature. The cell density was calculated as the mean number of spirochaetes (motile plus non-motile) per ml. Motility rate (percentage of total number of spirochaetes) was evaluated separately in undiluted samples by inspecting at least 100 spirochaetes and taken as an indication of viability. Statistical analyses (ANOVA, *t* test) were performed with the SOLO package (BMDP Statistical Software, Los Angeles, CA, USA).

Results

The log numbers and motility rates of borreliae at different temperatures and intervals are shown in Fig. 1. All three strains grew at 28–37°C, and the number of spirochaetes increased by 2–3 log₁₀ during the cultivation period. Only strain BR14 multiplied at 41°C: 63% of the spirochaetes were still motile after 8 days, and 1% remained motile until the 16th day. Strains BR14 and BR75 both multiplied well, although slowly, at 20°C. The optimum temperature, i.e., that giving the highest growth rate, was found to be 33°C for strain B31, 33–37°C for BR75 and 37°C for BR14. Maximum cell density values were reached in all three strains at 33°C. After attaining the maximum cell density of $c. (1–10) \times 10^7$ /ml, the motility rate of the spirochaetes generally decreased quite rapidly at all permissive temperatures. Strains BR14 and BR75 remained motile for >48 days at the lowest temperatures of 20–28°C and 20°C, respectively.

Statistical analysis demonstrated significant effects ($p < 0.0001$) of the strain, temperature and time period on the number of borrelial cells. The spirochaetal numbers among all 63 cultures (i.e., three strains $\times$ five temperatures $\times$ four time periods plus all three strains at time 0) were compared by the *t* test. Of the 2775 pair-wise comparisons, the differences were significant ($p < 0.05$) in 2333 (84.1%). In general, all histogram differences > 0.3 log were revealed as significant ($p < 0.05$) (Fig. 1). For instance, at 33°C, the numbers of cells in the strain B31 culture decreased significantly between days 8 and 16, and 16 and 48, while those in strain BR75 decreased significantly only between days 16 and 48, but not between days 8 and 16.

In separate tests, no growth or motility of any strain was observed after 4 days at 42.0°C. Spirochaetes of strains BR14 and BR75 (but not B31) multiplied 40–50 times in 8 days at 40.2°C; the motility rate after 8 days was 2% in strain BR75 and 6% in strain BR14. Strain B31 multiplied at 39°C, 25°C (well) and 22°C. One culture of each strain was tested after prolonged incubation for 81 days at 20°C; the mean cell numbers/ml (and motility rates) in strains B31, BR14 and BR75 were 0 (0%), 6.2878×10^7 (98%) and 3.7333×10^7 (74%), respectively.

Discussion

Different temperature requirements for the growth of three strains of *B. burgdorferi* s.l., representing the three known human pathogenic genomic groups, were detected. Approximate optimum (and minimum to maximum) temperatures were 33°C (22–39°C) for strain B31 (*B. burgdorferi* s.s.), 35°C (20–40°C) for strain BR75 (*B. afzelii*) and 37°C (20–41°C) for strain BR14 (*B. garinii*). Although only one strain of each genomic group was used, and strain variability in temperature requirements within each group might exist, the present data indicate that *B. garinii* is the most thermotolerant and *B. burgdorferi* the least thermotolerant genomic group among human pathogenic *B. burgdorferi* s.l.. The 1–2°C difference in optimum and maximum temperatures between *B. afzelii* and *B. garinii* could explain in part the observed difference in clinical symptomatology caused by these genospecies: whereas *B. afzelii* usually predominates in skin manifestations (erythema migrans, acrodermatitis chronica atrophicans), *B. garinii* is relatively more frequently associated with internal forms like neuroborreliosis (e.g., meningoradiculoneuritis) [10].

The normal rectal temperature of birds is *c.* 41°C, slightly varying among species (e.g., 40.1–42.1°C in chicken, 40.9°C in turkey, 41.1°C in duck, 40.6°C in goose, 40.6–41.9°C in pigeon), but reaching up to 44°C during intense activity [11]. Borrelial strains which do not grow at 41°C *in vitro* obviously cannot

Fig. 1. The growth rate of strains B31 (*B. burgdorferi sensu stricto*, **a**), BR75 (*B. afzelii*, **b**) and BR14 (*B. garinii*, **c**) at various temperatures: ▨, 20°C; ▤, 28°C; ■, 33°C; □, 37°C; ▩, 41°C. The figures above histograms show the motility rate (%) of the spirochaetes.

replicate in avian bodies. Birds are therefore probably incompetent as long-term hosts for *B. burgdorferi*. However, some avian species might be able to amplify *B. burgdorferi* s.l. because the spirochaetes have been isolated from them under natural [3, 4] or experi-

mental [5–7] conditions. In addition, a higher prevalence (21.4%) of the spirochaetes was detected in larval *Ixodes scapularis* ticks parasitising veeries (*Catharus fuscescens*) than other avian species (usually <10%) in north-eastern USA [12]. This indicates

that some of the veeries carried the spirochaetes in their skin tissue or blood. Isolations of *B. burgdorferi* s.s. from birds probably reflect strain variability in maximum temperature for growth within this genospecies; there must be some strains that tolerate a temperature of 41°C. *B. garinii*, the genospecies with the highest maximum growth temperature as found in this study, has been detected in, or isolated from, ixodid ticks parasitising Eurasian birds significantly more often than the other *Borrelia* genospecies [13–16]. In contrast, *B. afzelii* has been isolated more frequently from small mammals than from birds [9, 15]. In addition, the results of the present study also show the potential of *B. burgdorferi* s.l. spirochaetes to multiply in unfed vector ticks during summer temperatures of the north temperate (mid-latitude) zone. The maximum and minimum temperatures for growth thus seem to be phenotypic characters important in the ecology of *B. burgdorferi* s.l. strains, with relevance for the symptomatology, epidemiology and epizootiology of Lyme borreliosis. Moreover, the temperature shift from ambient temperature in the vector tick to the higher temperature of an endothermic vertebrate host (and *vice versa*) has significant implications for differential expression of outer surface [8] or heat-shock [17] proteins by *B. burgdorferi* s.l. This antigenic α protein 'dimorphism' of the agent could greatly affect the immune response of the vertebrate host as well as the pathogenesis of Lyme borreliosis.

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