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Colony Formation and Morphology in *Borrelia burgdorferi*†

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Two strains of *Borrelia burgdorferi*, B31 and 297, formed colonies when plated onto Barbour-Stoenner-Kelly medium solidified with agarose (1.3%) and incubated in a candle jar at 34°C. Colonies differing in morphology were observed in both strains after 2 to 3 weeks of incubation. Strain B31 colonies were either compact, round (mean diameter, 0.43 mm), and restricted to the surface of the agarose medium or diffuse (mean diameter, 1.80 mm) and penetrating into the solid medium. Strain 297 colonies (mean diameter, 1.43 mm) either showed a raised center surrounded by a diffuse ring of spirochetes or consisted of numerous small spirochetal aggregates. Both colony types expanded into the agarose medium. Scanning electron and light microscopy confirmed that the colonies were formed by spirochetes. Twisted tangles of intertwined spirochetes were visible on the surface, with numerous spherical bodies among them, especially in the central regions. At the periphery, the borreliae were more loosely packed, and individual coils were discernible.

Borrelia burgdorferi, the etiological agent of Lyme disease, infects a number of vertebrates, including humans and domestic as well as feral animals (1, 2). The spirochetes, transmitted primarily by ticks of the genus *Ixodes* (7, 9, 10), were first isolated from the midgut of a deer tick, *Ixodes dammini*, in modified Kelly medium (8). Variations of this liquid Barbour-Stoenner-Kelly (BSK) medium are routinely used to culture the spirochetes in vitro (3, 4). When supplemented with certain antibiotics, it permits the selective isolation of spirochetes from tick tissues (14). Strains that have been extensively passaged in vitro can be grown in liquid BSK medium from single spirochetes, i.e., cloned (4), but the growth of spirochetes as isolated colonies on a solid medium has not been achieved. Barbour (3) described the growth of *B. burgdorferi* as a "lawn" on BSK medium solidified with agarose, but colony formation was not reported. For the selection of variants, spontaneous mutants, and recombinant clones carrying specific DNA inserts, an agar-cloning procedure is a necessity. In this communication we present a culture system that permits colonial growth of *B. burgdorferi*. The growth of the colonies and their light and electron microscopic appearance are described.

MATERIALS AND METHODS

Spirochetes. Two strains of *B. burgdorferi* were used: strain B31, isolated from *I. dammini* (8) and previously cloned by limiting dilution (4), and the uncloned 297 strain, isolated from human spinal fluid (18).

Culture media. Spirochetes were routinely maintained in liquid BSK medium prepared as described by Barbour (3). Cultures were incubated at 34°C and transferred at intervals of 7 to 10 days with a 1% (vol/vol) inoculum.

The solid medium was prepared as follows. Fraction 1: to 900 ml of water (18 MΩ resistivity Milli-Q water; Millipore Corp., Bedford, Mass.) were added 50 g of bovine serum albumin (fraction V; Armour Pharmaceuticals, Kankakee, Ill.), 5 g of Neopeptone (Difco Laboratories, Detroit, Mich.), 6 g of HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (Sigma Chemical Co., St. Louis, Mo.), 0.7 g of sodium citrate, 5 g of glucose, 0.8 g of sodium pyruvate, 0.4 g of *N*-acetylglucosamine (Sigma), 2.2 g of sodium bicarbonate, and 2.53 g of TC Yeastolate (Difco). This was filtered through 0.22-μm-pore-size membranes and stored at 4°C in 90-ml portions until needed. Fraction 2 (made fresh every time): 2.8 g of gelatin (Difco) was dissolved in 20 ml of water (60°C; Millipore), and then 1.7 g of agarose (SeaKem LE, low electroendosmosis; Polysciences, Inc., Warrington, Pa.) was added. The mixture was solubilized and sterilized by autoclaving (15 min at 121°C) and then kept warm in a 60°C water bath. Fraction 1 and heat-inactivated rabbit serum (Pel-Freez Biologicals, Rogers, Ariz.) were also warmed to 60°C in the water bath. All solutions were then transferred to a heating plate inside a laminar flow hood, where the final medium was made by mixing 90 ml of fraction 1, 6.4 ml of rabbit serum, and 20 ml of the gelatin-agarose solution. Thorough mixing of the warm medium components was necessary to ensure their complete blending. Finally, 10 ml of prewarmed CMRL (Connaught Medical Research Laboratories) 1066 without glutamine (GIBCO Laboratories, Grand Island, N.Y.) and 5.3 ml of a 5% aqueous $NaHCO_3$ solution were added. This medium was dispensed into petri dishes (Nunc, Roskilde, Denmark; 35-mm diameter; 2 to 3 ml per plate), and the plates were allowed to cool and dry for 15 min in a laminar flow hood.

Inoculation and incubation of plates. Spirochetes from liquid cultures were counted in a Petroff-Hausser bacteria counting chamber. Cultures containing 10^7 to 10^8 spirochetes per ml were serially diluted 10-fold to give a range of dilutions from about 1,000 to less than 1 spirochete per 25 μl. For each dilution, three replicate plates were inoculated by placing 25 μl of spirochete suspension onto the surface of the agarose medium. The dishes were tilted to spread the fluid evenly onto the surface and then placed in a glass desiccator jar with a stopcock on the lid. Each jar contained 50 to 100 ml of distilled water on the bottom and a white paraffin candle on the porcelain plate. The candle was lit and the jar was covered, leaving the stopcock open. Immediately after the flame subsided, the stopcock was closed and the jar was placed in a 34°C incubator. After 2 to 4 weeks of incubation, the plates were examined for the presence of colonies by a dissecting microscope with transmitted light. The colonies

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were counted, and their numbers were compared with those obtained by direct enumeration in the Petroff-Hausser chamber.

Stability of colony morphology. To obtain an indication of the phenotypic stability of colony morphology, well-isolated colonies from each strain were selected by morphological characteristics. They were picked with a Pasteur pipette and transferred to liquid BSK. After 1 week of incubation, serial 10-fold dilutions of spirochetes were made from each clone, and 25-μl portions of each dilution were inoculated onto fresh plates of agarose-solidified BSK and incubated in a candle jar as outlined above.

SEM. Agarose blocks containing individual colonies were excised and fixed in 2.5% glutaraldehyde in 0.05 M phosphate buffer (pH 7) at 4°C for 1 to 3 days. Samples were postfixed with the OTO (osmium-thiocarbohydrazide-osmium) double osmium tetroxide application method (15) and dehydrated in a graded ethanol series of 20, 40, 60, 80, and 99% ethanol, followed by three changes of absolute ethanol for 10 min each. Blocks were dried out of liquid CO_2 in a Ladd critical-point dryer. They were mounted on aluminum pin stubs with colloidal graphite paste prior to coating in a Kinney vacuum evaporator with approximately 200 nm of 60% gold–40% palladium metal. Samples were viewed in a Philips 500X scanning electron microscope (SEM) at 12 kV acceleration voltage, and the image was recorded on Polaroid type 55 film.

RESULTS

Previous studies (3) in which *B. burgdorferi* failed to produce discrete colonies on a medium solidified with 0.8% agarose suggested that Lyme disease spirochetes might need a firmer medium to restrict their movements. Furthermore, they were known to require microaerophilic conditions for growth (5). To meet these criteria, we doubled the concentration of gelatin and increased the agarose concentration to 1.3%. The candle jar was chosen because it is a simple device that can provide a humidified atmosphere of reduced O_2 and increased CO_2 (13).

Colonies were visible after 2 weeks of incubation, but 3 to 4 weeks were needed for accurate enumeration and evaluation of morphology. With the unaided eye, the colonies generally appeared as small, white disks, but when examined with a dissecting microscope, differences in morphology became apparent.

On plates inoculated with B31 spirochetes, two types of colonies developed: small, compact, round colonies with an average diameter of 0.43 ± 0.03 (standard deviation [SD]) mm which were restricted to the surface (Fig. 1A), and larger diffuse colonies (1.80 ± 0.57 [SD] mm) which penetrated into the agarose (Fig. 1B) to an average depth of 0.60 ± 0.24 (SD) mm. The small surface colonies were composed of tangles of coiled spirochetes at the periphery and numerous spherical cells, along with very densely packed coiled spirochetes in the center (Fig. 2). The edges of such colonies were sharp and well defined, with no isolated free spirochetes present on the surrounding agar surface. In contrast, diffuse colonies contained fewer spherical bodies and were less tightly packed, and groups of spirochetes were seen in the process of migrating away from the edges (not shown).

Colonies formed by strain 297 were more variable in morphology and intermediate forms were seen. Two main types were discernible: diffuse colonies with or without a conspicuous raised center surrounded by a diffuse ring of spirochetes (Fig. 3A), and colonies comprising numerous

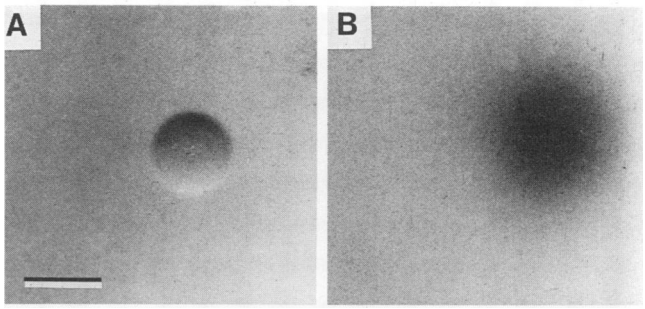

FIG. 1. Two types of *B. burgdorferi* B31 colonies as seen under a dissecting microscope with transmitted light after 3 weeks of incubation at 34°C. Bar, 0.33 mm. (A) Smaller, compact colony with distinct borders. (B) Larger colony with diffuse borders.

spirochete aggregates (granular type) (Fig. 3B). The raised-colony centers comprised tightly packed and intertwined spirochetes (Fig. 4) surrounded by a flat layer of spirochetes. After 3 weeks of incubation, the average colony size was 1.43 ± 0.71 (SD) mm. All colonies penetrated into the agarose to an average depth of 0.57 ± 0.28 (SD) mm.

At high spirochete concentrations (>500 per 25 μl), the number of colonies could not be accurately determined, as they tended to overlap extensively and form lawns. At lower concentrations of spirochetes (>100 per 25 μl), the number of colonies observed on a plate correlated approximately with the calculated number of spirochetes inoculated. The average number of colonies observed for each spirochete counted in the inoculum in the Petroff-Hausser chamber was 1.12 for strain 297 (12 trials; range of plating efficiency, 5 to 272%) and 2.06 for strain B31 (7 trials; range of plating efficiency, 56 to 636%).

Well-isolated colonies subcultured into liquid medium and replated onto solid medium gave rise to colonies similar in morphology to the parental type that was picked. The small, compact, round colonies of strain B31 again formed small colonies, most of which were of the compact, round type, and others were more diffuse, whereas a strain B31 colony of the larger diffuse type gave rise again to large, diffuse colonies. Strain 297 colonies of either type formed colonies of both morphological types after replating.

DISCUSSION

The passage of borreliae through vertebrate hosts, arthropod vectors, and culture systems is postulated to cause changes in their physiology, antigenicity, viability, virulence, and infectivity (5). Prolonged in vitro culture may lead to loss of infectivity for vertebrates, while continued passage from vertebrate to vertebrate host via needle inoculation of infected blood reduces the infectivity for the arthropod vector. Antigenic changes in the relapsing fever spirochete *Borrelia hermsii* within vertebrates occur at a rate higher than can be accounted for by mutations and are achieved via transposable genes (6, 16, 17). Whether such genetic mechanisms are also responsible for phenotypic variability in other borreliae remains to be determined. To study and characterize the changes induced by environmental conditions and to separate any variants from a heterogeneous mixture, the agar-cloning method has been widely used. It permits one to link morphological traits, such as colony shape, with physiological ones, such as virulence, and to isolate clones that exhibit the desired characteristic.

As far as we are aware, this is the first report describing colonial growth of *B. burgdorferi*. Barbour (3) cultured strain

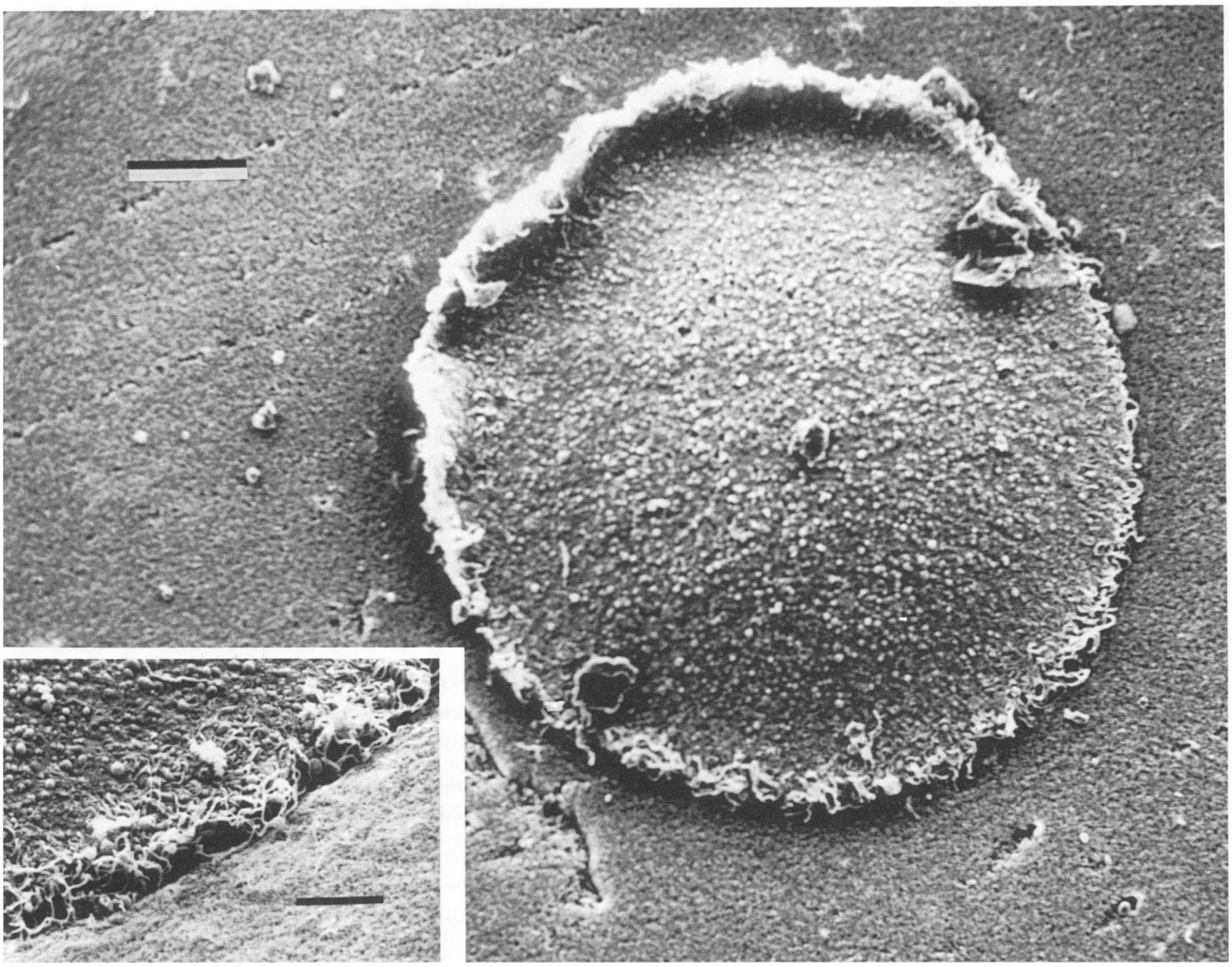

FIG. 2. SEM of 3-week-old *B. burgdorferi* B31 colony, small, compact type, on agarose. Bar, 30 μm. Inset: Note the sharp, distinct border (bar, 10 μm).

B31 on BSK medium solidified with 0.8% agarose. Cultures incubated in a candle jar grew to form a lawn of spirochetes. We used a higher concentration of agarose (1.3%) in BSK medium with twice the amount of gelatin, but the composition of the atmosphere was the same. These conditions were suitable for the formation of discrete colonies by the microaerophilic borreliae. This candle jar system, which provides a gaseous environment consisting of 3% CO_2, 17% O_2, and 80% N_2 (13), is similar to that used for the cultivation of malarial parasites in vitro. We also tested other concentrations of agarose and confirmed that 0.8% is unsatisfactory because of extensive migration of the spirochetes through the medium. While 1.5% agarose yielded satisfactory colony growth, it was difficult to evenly dissolve the agarose in the BSK medium.

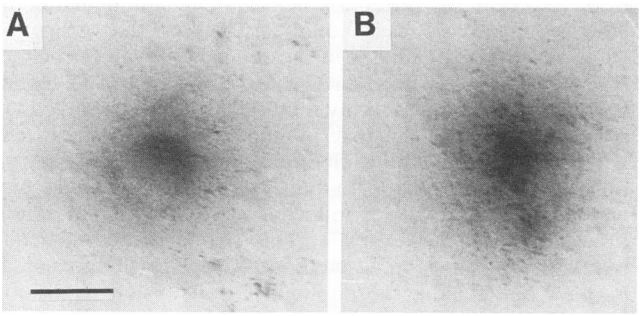

FIG. 3. *B. burgdorferi* 297 colonies as seen under a dissecting microscope with transmitted light after 3 weeks of incubation at 34°C. (A) Colony with raised center and diffuse borders. (B) Colony comprising numerous aggregates of spirochetes. Bar, 0.33 mm.

SEM observation of whole colonies and light microscopic observation of subcultured spirochetes in fluid medium confirmed that the colonies were formed by spirochetes. Also, the number of colonies observed on plates inoculated with dilute spirochete suspensions approximated the number of spirochetes calculated to be present from direct count data obtained with the Petroff-Hausser chamber. Various methods have been used to estimate the number of borreliae in a suspension (19). However, they do not evaluate viability, nor can they be used with low numbers of spirochetes. The seemingly large variation in plating efficiency might be explained by the fact that we used young, logarithmically growing cultures with relatively low concentrations of spi-

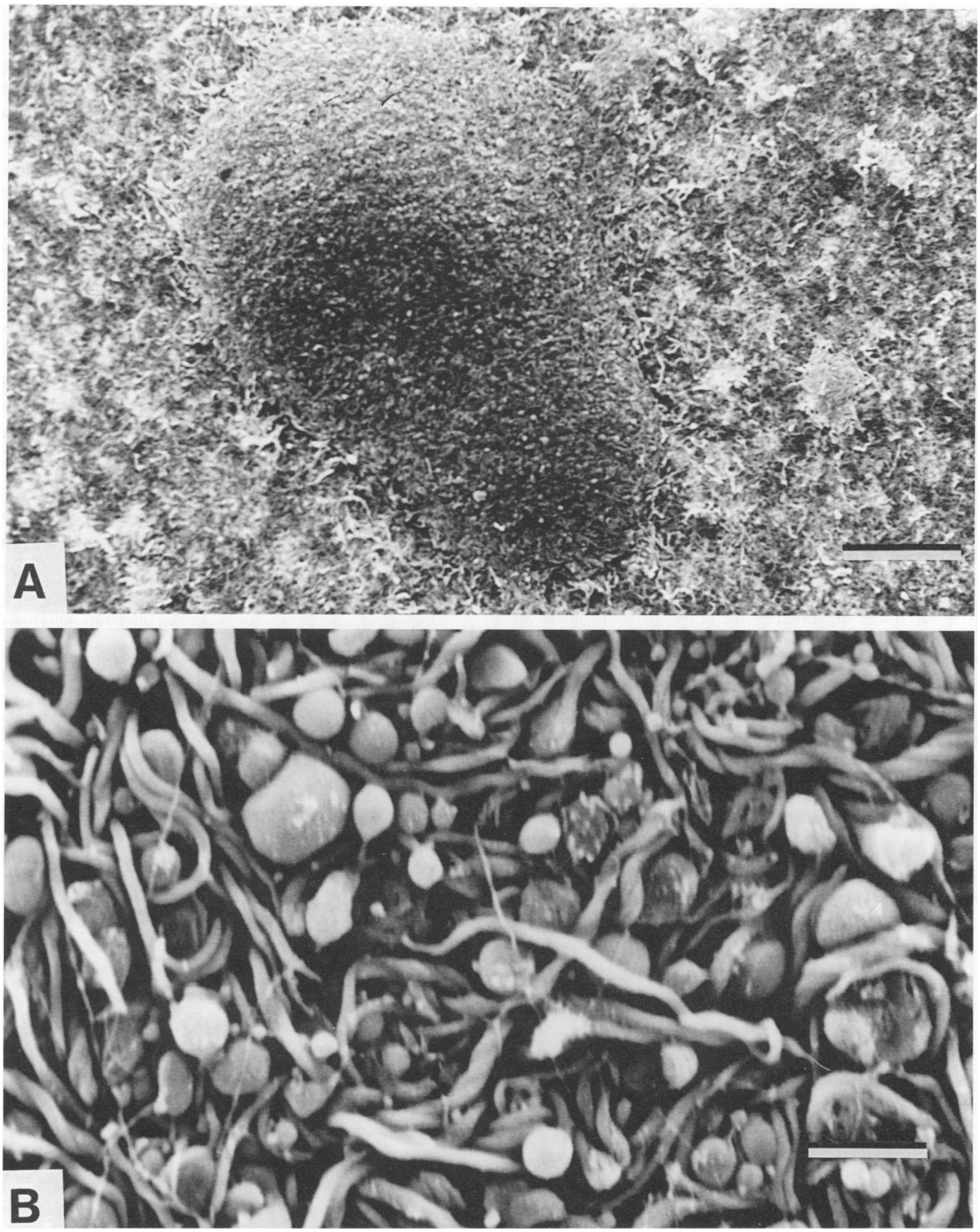

FIG. 4. (A) SEM of *B. burgdorferi* 297 colony with raised center and surrounding flat spirochete layer. (B) Masses of intertwined spirochetes and spherical bodies seen in the center of the colony. Bars: 30 μm (A); 2 μm (B).

rochetes. Under such circumstances, counts obtained by use of the Petroff-Hausser chamber tend to be less accurate. The method described here should overcome the difficulty of enumerating viable spirochetes and be applicable to genetic studies as well as antibiotic susceptibility assays.

We noted morphological variations in the colonies formed between and within strains B31 and 297. The SEM observations of selected colonies indicated that the spirochetes constituting the various colonies differed in their ability to spread over and migrate in the solid medium and to grow as aggregates. These characteristics may reflect important differences in spirochete mobility or adhesiveness that can, in turn, have a bearing on their in vivo behavior, i.e., their tendency to penetrate or adhere to the tissues of the vertebrate host or vector tick. Spirochetes subcultured from the round, compact strain B31 colonies were indistinguishable in liquid BSK medium from those transferred from a diffuse, spreading colony. Whether such differences in colony morphology can be correlated with other biological features of *B. burgdorferi* (e.g., virulence, infectivity, antigenicity) remains to be determined. Among leptospires, virulent and avirulent organisms are distinguishable on the basis of colony morphology (11, 12). Diffuse colonies of *Leptospira icterohaemorrhagiae* were found to contain leptospires virulent for rodents, but small compact colonies did not (12). Finally, by using clones derived from single agarose colonies, we can be certain that variability in a strain is not due to a mixed population, but rather to an inherent genetic or phenotypic variation.

ACKNOWLEDGMENTS

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