

Analysis of a Novel Insect Cell Culture Medium-Based Growth Medium for *Bartonella* Species[∇]

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Human- and animal-pathogenic *Bartonella* species are fastidious and slow-growing bacteria difficult to isolate and cultivate. We describe a novel, easy-to-prepare liquid medium for the fast and reliable growth of several *Bartonella* spp. that does not affect bacterial protein expression patterns or interactions with host cells.

Bartonella spp. are important pathogens in human and veterinary medicine, of which *B. henselae* and *B. quintana* are considered to represent the most-relevant human-pathogenic *Bartonella* species (8). New members of the genus with unclear epidemiology (e.g., “*B. rochalimae*”) were recently described (10). *Bartonella* spp. are present in a broad spectrum of mammals (4, 14), which either suffer from these infections (11) or serve as asymptomatic reservoir hosts (8).

Usually, *Bartonella* spp. are cultivated using highly supplemented blood agars, which still results in long incubation periods. To date, the slow growth of *Bartonella* spp. on solid media has limited diagnostic (e.g., primary isolation) and experimental (e.g., analyses of *B. henselae* pathogenicity) approaches. In light of these problems, a laboratory diagnosis of a *Bartonella* infection is generally made by serology (5) or molecular approaches (7).

Here, we describe the discovery, characterization, and optimization of a liquid growth medium for *Bartonella* spp. This inexpensive and easy-to-prepare medium allows for relatively fast planktonic growth of the fastidious bacteria. It facilitates approaches for analyzing, e.g., *B. henselae* pathogenicity and might also be evaluated for its use in the primary cultivation of *Bartonella* spp. from human or veterinary samples in the future.

Development of a liquid growth medium for *Bartonella henselae*. While analyzing the interaction of *B. henselae* Marseille (9) with Schneider cells from *Drosophila melanogaster* (19), we noticed the growth of *B. henselae* in the insect cell culture medium (Schneider’s drosophila powder medium, revised [Serva, Heidelberg, Germany], supplemented with 10% fetal calf serum [FCS] and 2 mM glutamine [data not shown]). As no easy-to-prepare liquid growth medium for *Bartonella* spp. has been described before, we further evaluated Schneider’s medium for its use as a growth medium. For all of the following experiments, *Bartonella* spp. were grown either in liquid media in cell culture flasks with constant, slow shaking

(60 rpm/min) or on Columbia blood agar (CBA) in a humidified atmosphere at 37°C with 5% CO₂.

First, Schneider’s medium supplemented with 10% FCS was inoculated with *B. henselae* (1.0 × 10⁶ CFU/ml) and incubated for 9 days. Bacterial growth was determined by measuring the optical densities at 600 nm (OD₆₀₀) at 24-h intervals, revealing a typical growth curve with a lag phase, a logarithmic growth phase, and a stationary phase (Fig. 1A). At the same time points, viable bacteria were quantified by plating serial dilutions on CBA (Fig. 1B). As described previously (6, 13), the number of viable bacteria decreased rapidly after the exponential growth phase (death phase), with no detectable stationary phase. We speculate that this early death phase might be caused by a lack of nutrients in the culture medium and not by lytic phage induction as described earlier (6), as the OD does not decrease during the death phase. Our observation has direct consequences when, e.g., infection experiments are performed with such liquid-grown *Bartonella* spp.; here, for each bacterial strain, the time point of entering the death phase needs to be evaluated to avoid an excess of dead bacteria.

Interestingly, no growth of *B. henselae* was observed in Schneider’s medium without FCS or in Luria-Bertani broth, in brain heart medium, or in endothelial cell growth medium, all supplemented with 10% FCS (data not shown). Cultivation of *B. henselae* in *Bartonella* Alphaproteobacteria growth medium (BAPGM [13]; provided by Ricardo Maggi, Chapel Hill, NC) supplemented with 10% FCS (instead of 5% sheep blood) revealed growth curves similar to those of the cultures in Schneider’s medium with 10% FCS. Remarkably, the maximum bacterial numbers were higher in Schneider’s medium (1.9 × 10⁸ CFU/ml) than in BAPGM (1.2 × 10⁸ CFU/ml). In contrast to BAPGM, our medium is not hampered by turbidity due to the addition of blood, thus allowing the detection of bacterial growth by visual inspection or by measurement of OD. Furthermore, it does not contain hemin (a necessary component of other liquid *Bartonella* media [17, 20, 21]), of which various concentrations were shown to be essential for the growth of different *Bartonella* species (20).

While evaluating different methods for the genetic manipulation of *B. henselae*, we realized that the addition of 5% (wt/vol) sucrose to Schneider’s medium with 10% FCS resulted in even faster bacterial growth. Schneider’s medium with 10%

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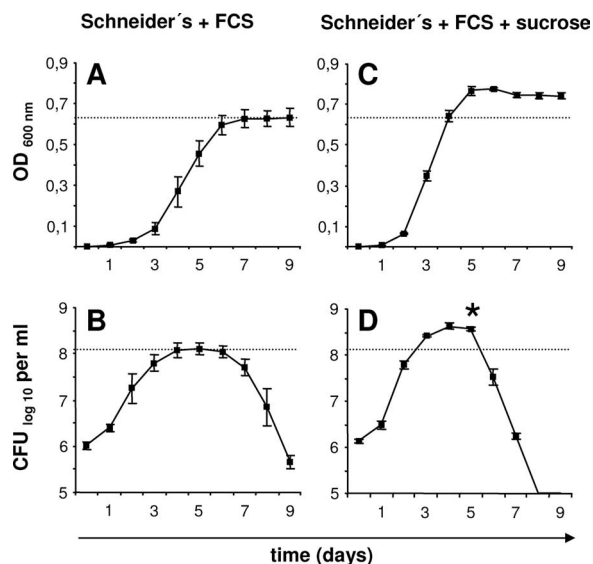


FIG. 1. Growth curves of *B. henselae* in Schneider's medium containing 10% FCS depending on the addition of 5% sucrose. Media were inoculated with *B. henselae* Marseille (1.0×10^6 CFU/ml), and bacterial growth was determined in triplicates by measuring the OD₆₀₀ (A, C) and by quantifying the number of viable bacteria (CFU/ml) at 24-h intervals (B, D). Note that the growth rates and the maximum number of viable bacteria are higher in the presence of sucrose. *, significant difference from the control value ($P < 0.01$).

FCS and 5% sucrose was inoculated with *B. henselae* (1.0×10^6 CFU/ml), and the OD and numbers of CFU/ml were determined at 24-h intervals. In accordance with the OD, the maximum bacterial number was ~ 3.3 -fold higher when sucrose was added, and again, we observed an early death phase after the exponential growth phase (Fig. 1C and D). The maximum growth rate between days 1 and 2 allowed us to calculate a generation time of ~ 5.6 h [to calculate the generation time, we used the equation $1 \times \lg 2(t - t_0) / \lg N - \lg N_0$, where t is time, t_0 is time zero, N is the bacterial number, and N_0 is the bacterial number at time zero].

To analyze the influence of further carbohydrates on the growth of *B. henselae*, Schneider's medium containing 10% FCS was supplemented with 5% (wt/vol) fructose, glucose,

mannose, or galactose. While the addition of fructose showed no significant effect, the addition of glucose lowered bacterial growth rates, and remarkably, the addition of mannose or galactose suppressed the growth of *B. henselae* completely (data not shown). Right now, it is unclear why sucrose improves the growth of *B. henselae* but other carbohydrates do not influence, reduce, or even inhibit bacterial growth. Several ABC transporters (including a sugar ABC transporter) are present in the genome of *B. henselae* (1) and might facilitate the use of sucrose as a carbon source, whereas mannose and galactose could be toxic for *B. henselae*. For example, mannose might be taken up in an energy-consuming process by a phosphoenolpyruvate-dependent sugar phosphotransferase system (2), causing phosphoenolpyruvate starvation and killing of *B. henselae*.

Growth of different *Bartonella* spp. in Schneider's medium containing 10% FCS. Next, the growth of medically and veterinarily important *Bartonella* spp. (*B. henselae* Marseille, *B. henselae* Houston-1 [ATCC 49882], *B. quintana* Toulouse [Collection de l'Institut Pasteur, Paris, France], *B. quintana* JK31 [22], and *B. vinsonii* subsp. *berkhoffii* [ATCC 51672]) was analyzed (inoculum, 1.0×10^5 bacteria/ml; OD determination at 24-h intervals [Fig. 2]). All five strains grew, but the amount of time needed to reach the respective exponential phase and the maximum OD differed from strain to strain. The best-growing strain was *B. quintana* JK31 (OD₆₀₀, 1.37 at day 7), and the slowest-growing strains were *B. henselae* Marseille (OD₆₀₀, 0.76 at day 9) and *B. vinsonii* (OD₆₀₀, 0.95 at day 10), demonstrating that even within a certain species, generation times and maximum growth rates deviate significantly (e.g., for *B. henselae* strains Houston-1 and Marseille).

Functional analysis of *B. henselae* grown in Schneider's medium-based *Bartonella* growth medium. Expression of *Bartonella* adhesin A (BadA) depends on the cultivation procedures used (3, 16). Therefore, we analyzed *B. henselae* Marseille grown in our liquid medium for BadA expression. Immunofluorescence using specific antibodies and electron microscopy (done as described previously [15]) demonstrated a strong surface expression of BadA (Fig. 3A). BadA was also present in trichloroacetic acid-precipitated culture supernatants (inoculum, *B. henselae* [1.0×10^7 CFU/ml]; incubation

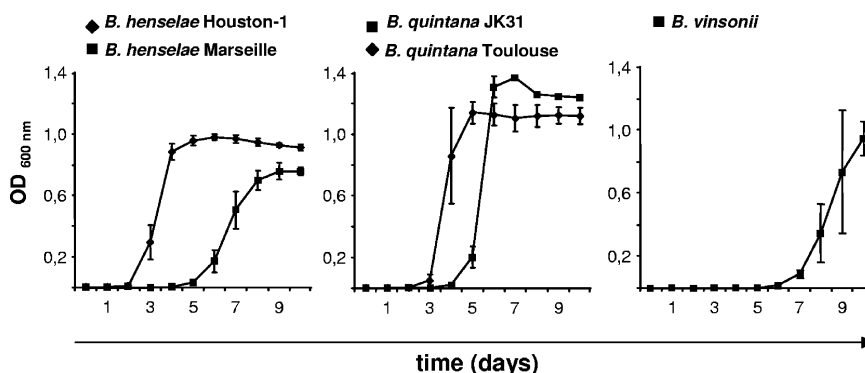


FIG. 2. Growth curves of different *Bartonella* strains in Schneider's medium. Schneider's medium with 10% FCS was inoculated with *B. henselae* Marseille, *B. henselae* Houston-1, *B. quintana* Toulouse, *B. quintana* JK31, and *B. vinsonii* (1.0×10^5 bacteria/ml), and bacterial growth was determined in triplicates by measuring the OD₆₀₀ at 24-h intervals. Note that all strains exhibited growth in this medium, although the intervals before the start of the logarithmic growth phase (lag phase) and the maximum OD differ.

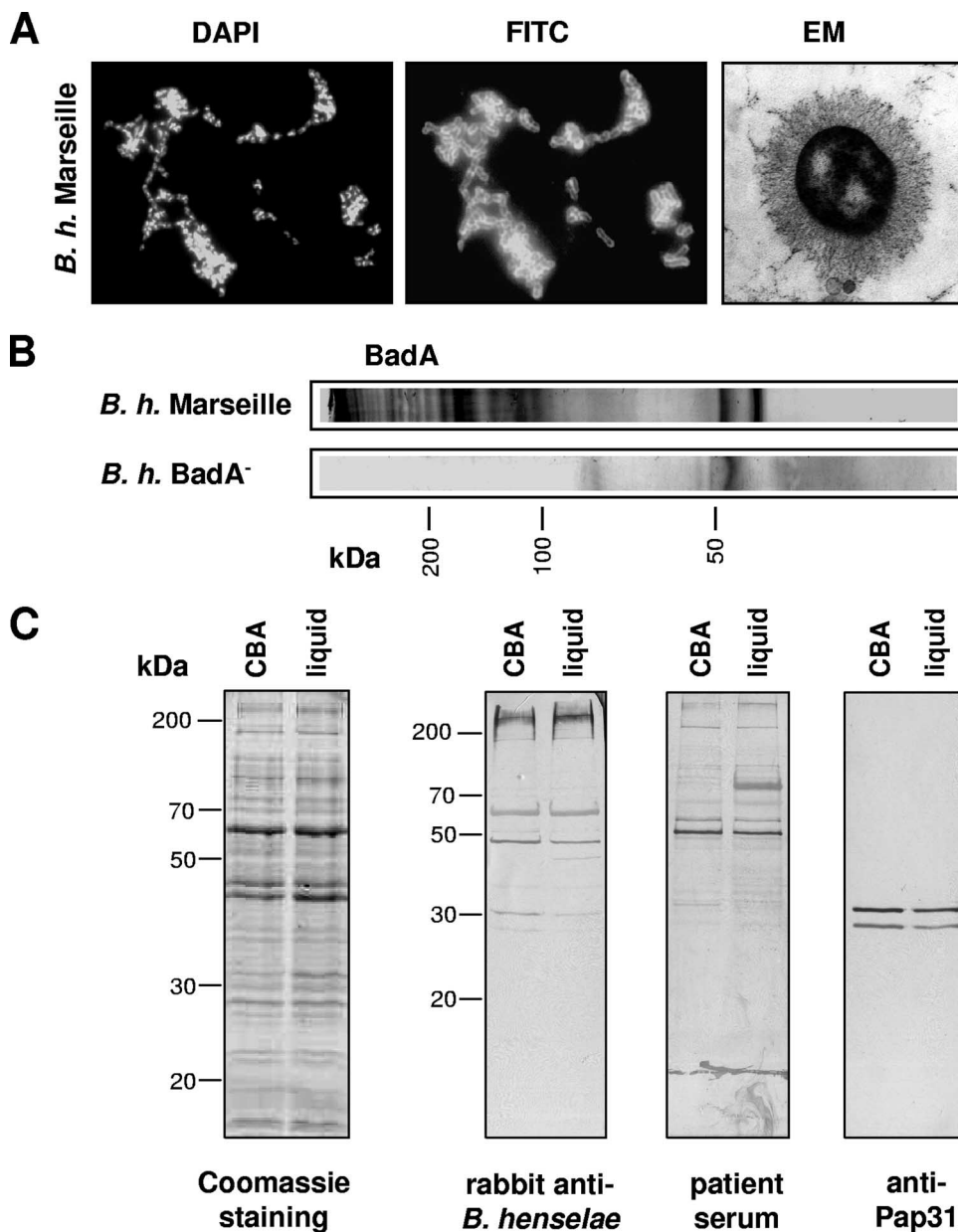


FIG. 3. Protein expression pattern and antigenic profile of *B. henselae* (*B. h.*) grown on CBA and in liquid medium. (A) BadA expression by *B. henselae* Marseille grown in Schneider's medium containing 10% FCS and 5% (wt/vol) sucrose is shown by BadA immunostaining (fluorescein isothiocyanate [FITC] represents specific fluorescence, and DAPI [4',6-diamidino-2-phenylindole] is used as an internal control) and electron microscopy (EM) (long, "hairy" structures). (B) Detection of BadA in liquid culture supernatants of *B. henselae* Marseille by BadA immunoblotting. A *B. henselae* BadA⁻ strain (not expressing BadA [15]) was used as a negative control. (C) Protein composition of *B. henselae* grown on CBA or in liquid medium (Coomassie blue-stained sodium dodecyl sulfate gel) and immunoblots incubated with sera from a *B. henselae*-infected rabbit and from a patient suspected of having a *B. henselae* infection (indirect immunofluorescence assay titer, 1:400). The expression of Pap31 was detected with a specific monoclonal antibody (VK29).

period, 10 days) and detectable by the appearance of the typical ladder-like pattern of BadA bands (15, 16) in immunoblotting (Fig. 3B). No eminent differences were detected in the total protein compositions and immunoreactivities of *B. henselae* Marseille grown on CBA and in liquid medium (exponential growth phase), compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting using the sera of a rabbit infected with *B. henselae* Marseille (15) and of a patient suspected of having a *B. henselae* infection (indirect immunofluorescence assay titer, 1:400). Finally, no differ-

ence was observed in the levels of expression of Pap31 (detected by immunoblotting using the monoclonal antibody VK29 [23]) (Fig. 3C).

B. henselae adheres to and invades endothelial cells (12). We next analyzed the interaction of liquid-grown *B. henselae* with human umbilical vein endothelial cells (HUVEC) which were infected with *B. henselae* Marseille grown either on CBA or in Schneider's medium containing 10% FCS and 5% sucrose for 6 days. Adhesion and invasion were analyzed 30 min, 2 h, and 24 h after infection by confocal laser scanning microscopy as

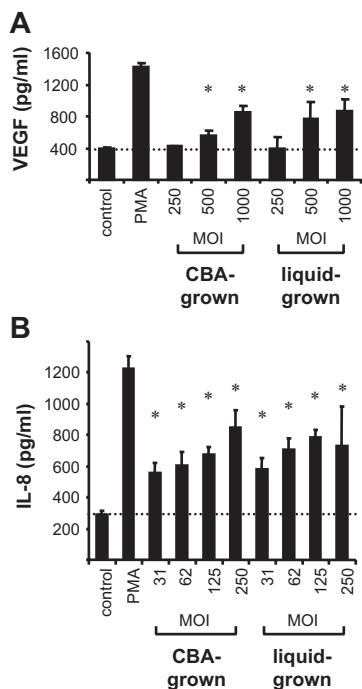


FIG. 4. Induction of VEGF and IL-8 secretion by *B. henselae* grown in Schneider's medium. Bacteria were grown either on CBA or in Schneider's medium containing 10% FCS and 5% (wt/vol) sucrose. (A) HeLa 229 cells (VEGF secretion) were infected with *B. henselae* Marseille (multiplicity of infection [MOI], 250, 500, and 1,000). (B) HUVEC (IL-8 secretion) were infected with *B. henselae* ATCC 49882 (MOI, 31, 62, 125, and 250). Phorbol myristate acetate (PMA; 25 ng/ml) was used as a positive control. Cytokines were quantified by enzyme-linked immunosorbent assay from cell culture supernatants 24 h after infection. Note that no significant differences in their abilities to trigger VEGF or IL-8 secretion were observed between bacteria grown on agar and bacteria grown in liquid culture. Uninfected cells were used as a control. *, significant difference from the control value (VEGF, $P < 0.05$; IL-8, $P < 0.01$).

described previously (12), revealing no differences in the amounts of adherent and intracellular *B. henselae* (data not shown). Moreover, experiments investigating the secretion of vascular endothelial growth factor (VEGF) from HeLa cells (15) or of interleukin-8 (IL-8) from HUVEC (18) demonstrated that using our liquid medium to grow *B. henselae* (exponential growth phase) does not affect bacterium-triggered cytokine secretion (Fig. 4).

Taken together, we describe the development of an inexpensive and easy-to-prepare liquid medium for the cultivation of *Bartonella* spp. that uses only three widely used ingredients (Schneider's medium, FCS, and sucrose). This medium allows relatively fast planktonic growth and does not affect host-cell interaction in vitro. The use of Schneider's medium-based growth medium for microbiological diagnostics of *Bartonella* infections in veterinary or human medicine and for the cultivation of other fastidious bacteria will be evaluated in the future.

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