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Growth Characteristics of *Bartonella henselae* in a Novel Liquid Medium: Primary Isolation, Growth-Phase-Dependent Phage Induction, and Metabolic Studies

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Bartonella henselae **is a zoonotic pathogen that usually causes a self-limiting infection in immunocompetent individuals but often causes potentially life-threatening infections, such as bacillary angiomatosis, in immunocompromised patients. Both diagnosis of infection and research into the molecular mechanisms of pathogenesis have been hindered by the absence of a suitable liquid growth medium. It has been difficult to isolate** ***B. henselae*** **directly from the blood of infected humans or animals or to grow the bacteria in liquid culture media under laboratory conditions. Therefore, we have developed a liquid growth medium that supports reproducible in vitro growth (3-h doubling time and a growth yield of approximately 5×10^8 CFU/ml) and permits the isolation of** ***B. henselae*** **from the blood of infected cats. During the development of this medium, we observed that** ***B. henselae*** **did not derive carbon and energy from the catabolism of glucose, which is consistent with genome nucleotide sequence data suggesting an incomplete glycolytic pathway. Of interest,** ***B. henselae*** **depleted amino acids from the culture medium and accumulated ammonia in the medium, an indicator of amino acid catabolism. Analysis of the culture medium throughout the growth cycle revealed that oxygen was consumed and carbon dioxide was generated, suggesting that amino acids were catabolized in a tricarboxylic acid (TCA) cycle-dependent mechanism. Additionally, phage particles were detected in the culture supernatants of stationary-phase** ***B. henselae*****, but not in mid-logarithmic-phase culture supernatants. Enzymatic assays of whole-cell lysates revealed that** ***B. henselae*** **has a complete TCA cycle. Taken together, these data suggest** ***B. henselae*** **may catabolize amino acids but not glucose to derive carbon and energy from its host. Furthermore, the newly developed culture medium should improve isolation of** ***B. henselae*** **and basic research into the pathogenesis of the bacterium.**

Bartonella henselae is a medically important gram-negative, zoonotic pathogen. Cats are the reservoir, and infections are generally asymptomatic. *B. henselae* causes a diverse and emerging disease spectrum in humans, from self-limiting lymphadenopathy to life-threatening conditions such as bacillary angiomatosis. Cat scratch disease (CSD), a generally benign infection characterized by regional lymphadenopathy and persistent fever, is the most-common manifestation of *B. henselae* infection in humans. CSD occurs in healthy individuals and affects an estimated 24,000 persons a year in the United States (11). In immunocompromised patients, *B. henselae* causes more-serious infections, including bacillary angiomatosis and bacillary peliosis, which can be fatal when misdiagnosed and improperly treated. A major contributing factor to misdiagnosis is the inherent difficulty in culturing the bacterium. *B. henselae* takes an average of 21 days to form colonies during primary isolation on blood agar plates, and no reliable liquid growth medium exists (8). In clinical settings, the protracted growth phase often leads to false-negative culture results, making the isolation of new strains very problematic. The lack of a suitable liquid medium has also severely hindered basic studies of *B. henselae* biology and pathogenesis.

Little is known of the metabolic requirements of *B. henselae.* When the bacterium was first isolated from the blood of patients with human immunodeficiency virus, preliminary characterization of the pathogen did not detect any carbohydrate utilization (19, 24). Similarly, the closely related pathogen *Bartonella quintana* does not metabolize glucose (10, 23). This is unusual for a pathogenic organism, since glucose is an abundant, readily catabolizable carbon source in host tissue and blood. Recently, it was reported that the unpublished *B. henselae* genome contains most of the genes necessary for the Embden-Meyerhoff-Parnas and Entner-Doudoroff pathways but lacks hexokinase and phosphofructokinase (4). Since these pathways are the most widely conserved mechanisms of glucose catabolism in eukaryotic and prokaryotic cells, the authors suggest that *B. henselae* may utilize a chimeric Embden-Meyerhoff-Parnas–Entner-Doudoroff pathway to generate energy from glucose. This speculation would seem to contradict previous data that *B. henselae* does not utilize carbohydrates.

To facilitate isolation of *B. henselae* and answer fundamental questions about its life cycle and pathogenesis, we developed a liquid culture medium that supports consistent *B. henselae* growth, yielding good cell densities and a 3-h doubling time.

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TABLE 1. Components of liquid media developed for cultivation of *B. henselae*

Medium	Base medium (g/liter)	Fe source (mg/liter)	Buffer (g/liter)	Supplement(s)	Final pH	Reference
BBH-H	*Brucella* broth (28)	Histidine-hematin (100)	HEPES (23.8)	Histidine (0.4%, wt/vol)	7.2	This study
Semidefined	*Brucella* broth (28)	Hemin (250)	None	Fildes solution (5%, vol/vol)	NR[a]	20
Isolation	RPMI 1640 (10.4)	Hemin (15)	HEPES (10)	L-Glutamine, Na pyruvate, nonessential amino acids (1% [wt/vol] each)	7.0	26

[a] NR, not reported in reference.

This novel medium has been used successfully for isolation of the bacterium from the blood of experimentally infected cats and has allowed us to elucidate the unusual central metabolism of the pathogen.

MATERIALS AND METHODS

Bacterial strains, growth conditions and chemicals. *B. henselae* strains Houston-1, passage 4 (P4), LSU16 (P4), and ATCC 49793 (HP), and *B. quintana* OK 90-268 (P2) were grown in *Brucella* broth histidine-hematin (BBH-H) (described below and summarized in Table 1), as well as in two previously published liquid media (20, 26). *B. henselae* Houston-1 was used for all metabolic studies. Liquid cultures were incubated at 37°C with shaking (180 rpm) under different gas mixtures. Aerobic cultures were grown under normal atmospheric oxygen (21% O_2); microaerobic cultures were grown under an atmosphere of 7% O_2, 5% CO_2, and 88% N_2; and anaerobic cultures were grown under 4% H_2, 5% CO_2, and 91% N_2. Microaerobic cultures were prepared by three 10-min cycles alternating between degassing by vacuum and replacing dissolved gas with premixed gas to achieve the desired gas concentrations. Anaerobic medium was prepared by degassing medium overnight in an anaerobic chamber containing the gas mix detailed above. Microaerobic and anaerobic media were prepared in sealed, sidearm flasks, and samples were withdrawn by syringe through a rubber septum to maintain the appropriate atmosphere within the flask. Unless otherwise noted, cultures were grown under aerobic conditions. To determine the cell density and viability of *B. henselae* in liquid culture, aliquots were serially diluted and plated on BBH-H agar (BBH-HA) (described below) to determine the number of CFU per milliliter and plot growth curves. Plates were incubated at 34°C under atmospheric oxygen with 3% CO_2. Cell viability was also determined using a Molecular Probes (Eugene, Oreg.) BacLight fluorescent staining kit with a Nikon Eclipse E800 fluorescent microscope according to the manufacturer's instructions. All chemicals and reagents were purchased from Sigma Chemical, St. Louis, Mo., unless stated otherwise.

Growth medium. A previously described medium (20) consisting of *Brucella* broth, Fildes solution, and hemin was modified to create an efficient growth medium. BBH-H was formulated as described below. First, 28 g of brucella broth (Fisher Scientific, Pittsburgh, Pa.) and 23.8 g of HEPES (free acid) were dissolved in 850 ml of distilled water, and the pH was adjusted to 7.0 with 10 N NaOH. Next, hematin was conjugated to histidine (14) as follows: 100 mg of hematin was dissolved in 100 ml of a 4% histidine solution, the pH was adjusted to 8.0 with 10 N NaOH, and the reaction mixture was stirred overnight at room temperature. One hundred milliliters of the hematin-histidine conjugate was added to 900 ml of *Brucella* broth base, and the resulting medium was adjusted to pH 7.2 with 10 N NaOH and filter sterilized, yielding BBH-H. Additional supplements were added to broth medium for some experiments as described in the text. Solid medium (BBH-HA) was made by adding filter-sterilized 2× BBH-H medium to an equal volume of molten (45°C) Bacto Agar (Fisher Scientific) and dispensing into petri plates.

Metabolite and amino acid analysis. The concentrations of common metabolic substrates and end products in uninoculated and spent media were determined as described below. Glucose, glutamate, ammonia, succinate, acetate, lactate, formate, malate, citrate, and ethanol levels were determined with Boehringer-Mannheim metabolite kits (R-Biopharm, Inc., South Marshall, Mich.). Carbon dioxide levels were measured with a CO_2 assay kit. Except for CO_2 and ethanol assays, all assays were performed using filtered culture supernatant according to the manufacturers' instructions. CO_2 and ethanol assays were performed without filtering to avoid loss of volatile products. Oxygen levels were determined using an oxygen electrode (model 733; Diamond General Development Corp., Ann Arbor, Mich.). To determine which amino acids *B. henselae* depleted from the growth medium, filtered culture supernatants were analyzed. Free amino acid analyses of uninoculated and spent media were performed on a Beckman Instruments model 6300 amino acid analyzer by the Scientific Research Consortium (St. Paul, Minn.) (www.aminoacids.com).

Enzyme assays. *B. henselae* cell lysate was assayed for tricarboxylic acid (TCA) cycle and amino acid catabolic enzymes. Cells were grown to a density of 2×10^8 CFU/ml in BBH-H, harvested by centrifugation (5,000 × *g*, 15 min, 4°C), suspended in 20 mM HEPES-5% sucrose (pH 7.6), and lysed by two passes in a French pressure cell (14,000 lb/in^2, 4°C). After lysis, cell debris was removed by centrifugation (15,000×*g*, 15 min, 4°C). Enzymatic assays for citrate synthase (21), aconitase (5), isocitrate dehydrogenase (25), α-ketoglutarate dehydrogenase (7), fumarase (9), glutamate dehydrogenase (6), and histidase (18) were performed as previously described. Succinate dehydrogenase was assayed as previously described (12) with the addition of fresh flavin adenine dinucleotide (0.10 mM, final concentration) to the reaction mixture. Malate dehydrogenase assays (13) were done in 50 mM potassium phosphate buffer, pH 7.3, containing NAD (final concentration, 10 mg/ml). All assays were performed at 37°C using a Beckman Coulter DU 640 spectrophotometer (Beckman Instruments, Palo Alto, Calif.).

Phage isolation. Growth-dependent phage induction in *B. henselae* was examined by isolating phage particles and extracting phage DNA from BBH-H culture supernatant at time points throughout the growth curve. Bacteria were removed from the medium by centrifugation (5,000 × *g*, 15 min, 4°C), and the supernatant was filtered with a 0.22-μm-pore-size filter (Millipore, Bedford, Mass.). Phage particles were harvested from the filtered supernatant (38 ml) by centrifugation (141,000 × *g*, 4 h, 10°C) in an SW-28 rotor (Beckman Instruments), suspended in phage buffer (5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.15 M NaCl in 10 mM Tris-HCl, pH 7.5) overnight at 4°C (15) and examined by electron microscopy as described previously (27). For isolation of phage DNA, 0.5 ml of a phage suspension was treated with 3 μl of Benzonase (Novagen, Madison, Wisc.) and DNase (Roche Applied Science, Indianapolis, Ind.) (1 h, 37°C) to remove any contaminating chromosomal DNA from the suspension. The phage coat proteins were then digested with proteinase K (6.67 mg/ml) in 3.3% sodium dodecyl sulfate–0.167 M EDTA (1 h, 37°C). Phage DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1), reextracted with chloroform-isoamyl alcohol, and precipitated with ethanol at −80°C. Purified phage DNA was electrophoresed in 0.5% agarose gels and visualized by ethidium bromide staining.

Isolation from experimentally infected cats. Cats were experimentally infected by intradermal injection with 6.1×10^8 CFU of *B. henselae* Houston-1 and monitored for bacteremia as described previously (17). Blood from bacteremic cats was collected in Vacutainer tubes (Becton Dickinson, Franklin Lakes, N.J.) supplemented with either 0.1 ml of erythrocyte lysis solution (4% saponin, 0.96% polyamethodesulfonic acid, and 2.5% polyethylene glycol in 0.9% saline) or 0.1 ml of heparin solution (500 U/ml in 0.9% saline), diluted 1:100 in BBH-H, and incubated as described above. Cultures were examined by microscopy every 24 h for growth, and cells were transferred to plates with BBH-HA when bacterial growth was evident. PCR was used to confirm that colonies from these plates were *B. henselae* (16). All federal and institutional guidelines were followed in the treatment and handling of animals.

RESULTS

Development of a liquid growth medium for *B. henselae* culture. The difficulty in isolating and studying the biology and

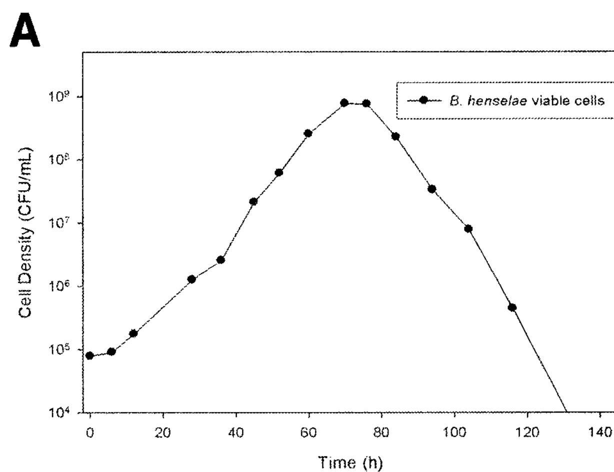
pathogenesis of *B. henselae* is due in part to the lack of a reliable growth medium. Two liquid media have been described for the growth of *B. henselae* (Table 1) (20, 26), but they are seldom used because they do not support consistent bacterial growth or yields. Our attempts to culture *B. henselae* in either medium gave very inconsistent results. However, the medium described by Schwartzman et al. (20) was a useful starting point for the development of a reliable medium. Initial growth experiments which finally resulted in the development of BBH-H demonstrated that three factors were critical for optimal growth. The form and quantity of heme were the first modifications. Hematin was substituted for hemin and was conjugated to histidine to improve solubility, and the concentration was reduced from 250 to 100 mg/liter (Table 1). When using this form of BBH-H in static culture, the cells grew very well at the medium-atmosphere interface, suggesting that the amount of dissolved oxygen was affecting growth. Therefore, the gas headspace was increased to 2:1 (vol/vol) in proportion to the culture medium and cultures were shaken at 180 rpm during growth. These modifications significantly improved growth rate and yield.

B. henselae growth was unexpectedly sensitive to the pH of the medium, and optimal growth occurred over a very narrow pH range (pH 6.8 to 7.2). The doubling time in BBH-H at pH 7.2 was approximately 3 h (Fig. 1A), which represents a threefold reduction compared to previously reported growth rates (20). Growth rates under acidic conditions began to decrease at pH 6.6. The growth rate of *B. henselae* was >6 h at pH 7.4, and no growth was observed at pH 7.6 or more (data not shown). Significantly, cell densities reached 5×10^8 to 1×10^9 CFU/ml in BBH-H (Fig. 1A). Growth of cultured *B. henselae* on BBH-HA was similar to that on blood agar plates, with colonies forming in 5 to 7 days.

BBH-H was tested for its ability to support growth of *B. henselae* strains LSU16 and ATCC 49793 as well. Both strains exhibited growth levels similar to the Houston-1 strain, with approximately the same doubling time. The closely related pathogen *B. quintana* was cultured in BBH-H under aerobic and microaerobic conditions. Interestingly, *B. quintana* did not grow in 21% O_2 but did grow in 7% O_2. These data show that BBH-H supports rapid growth of multiple *B. henselae* strains, and it may also be used for the cultivation of another pathogenic *Bartonella* species, *B. quintana*. The ability of the new medium BBH-H to support rapid and reliable growth of multiple *Bartonella* strains and species represents a significant advance in *Bartonella* research.

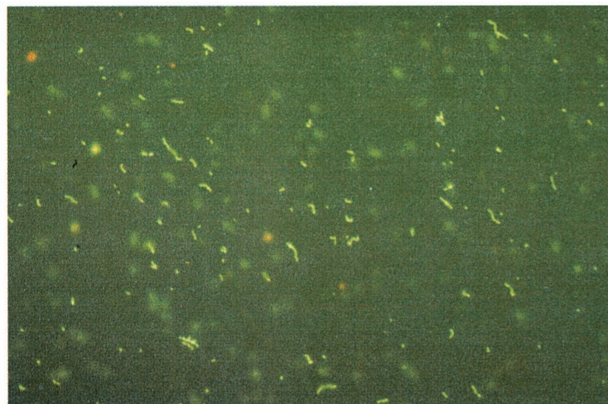
Substrate utilization and metabolite production by *B. henselae*. Little is known about the metabolic properties of *B. henselae*, and conflicting reports have obscured identification of its primary carbon and energy sources. Therefore, we examined culture supernatants for the depletion of carbon sources and correlated these with the appearance of secondary metabolites and end products. Metabolite concentrations in BBH-H were measured before and after *B. henselae* growth in the medium. Glucose levels were unchanged during growth, indicating that *B. henselae* does not utilize this carbohydrate under the conditions tested (Table 2). To determine which metabolic pathways *B. henselae* was utilizing, the concentrations of a number of fermentation products were assayed in conditioned BBH-H. Acetate, ethanol, lactate, and formate

A



B

Exponential phase



Death phase

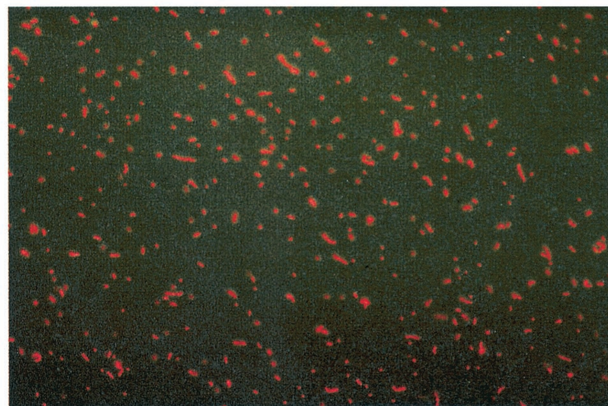


FIG. 1. Growth of *B. henselae* in BBH-H. (A) Growth curve of viable cell density in BBH-H. Viable cell counts were determined by plating from a culture grown aerobically as described in the text. (B) Microscopic photographs (magnification, ×400) of viability-stained *B. henselae* cells in BBH-H of exponential-phase growth (top) and death-phase growth (bottom), illustrating the drastic decline in viable cell density.

TABLE 2. Metabolite concentrations in BBH-H before and after *B. henselae* growth

Metabolite	Mean concn ± SD[a] (mM)	
	T_0[b]	T_F[c]
Glucose	4.42 ± 0.21	4.45 ± 0.19
Acetate	2.16 ± 0.08	2.08 ± 0.15
Ethanol	0.09 ± 0.01	0.10 ± 0.02
Formate	1.09 ± 0.08	1.12 ± 0.11
Citrate	0.04 ± 0.01	0.03 ± 0.01
Lactate	2.54 ± 0.09	1.96 ± 0.09
Succinate	0.49 ± 0.02	0.26 ± 0.02
Oxygen	26.0 ± 0.1	ND[d]
Glutamate	2.75 ± 0.19	1.27 ± 0.11
Carbon dioxide	ND	8.74 ± 0.64
Malate	0.02 ± 0.00	0.06 ± 0.00
Ammonia	4.70 ± 0.46	11.6 ± 0.35

[a] All values are from triplicate assays on two independent cultures. Values were determined by enzymatic assay except for oxygen levels, which were measured with an oxygen electrode.
[b] T_0 values are from uninoculated medium.
[c] T_F values are from conditioned medium from death-phase cultures.
[d] ND, none detected.

are common end products of fermentation pathways. No changes in the levels of acetate, ethanol, and formate were detected; however, the concentration of lactate decreased, suggesting that *B. henselae* could metabolize lactate (Table 2). The absence of secondary metabolites suggested that carbon sources utilized by *B. henselae* were completely catabolized to CO_2. To determine if CO_2 was produced, *B. henselae* cultures were grown in controlled atmospheres and the CO_2 concentrations were assayed before and after growth. Initial levels of CO_2 in BBH-H were undetectable but increased to 8.75 mM as *B. henselae* reached a postexponential phase of growth (Table 2). Since the production of CO_2 is accompanied by O_2 consumption, the levels of dissolved O_2 in the culture medium were also determined. As *B. henselae* grew in BBH-H, dissolved oxygen dropped from 26 mM to undetectable levels (<20 ppm) (Table 2).

The absence of glucose catabolism indicated that *B. henselae* was utilizing alternative carbon sources. A very closely related bacterium, *B. quintana*, has been reported to utilize succinate and glutamate (10, 23), leading us to speculate that *B. henselae* could utilize these compounds as well. As expected, the concentrations of succinate and glutamate in BBH-H decreased significantly during the growth of *B. henselae* (Table 2). The catabolism of amino acids usually produces NH_3 when the amine group is removed. To determine indirectly if glutamate was catabolized, we measured the concentration of NH_3 in the culture supernatants. Ammonia was produced at a level greater than the amount of glutamate depleted from the medium (Table 2), suggesting that additional amino acids were being catabolized. To test this hypothesis, we measured the concentrations of free amino acids present in BBH-H before and after growth. The results confirmed that, in addition to consuming glutamate, *B. henselae* consumed histidine, asparagine, glycine, and serine from the growth medium (Table 3). Tryptophan and cysteine were also consumed but at significantly lower levels than the other amino acids (Table 3). Taken together, these data suggest that *B. henselae* was catabolizing amino acids rather than glucose as a carbon source.

Enzymatic activities in *B. henselae* cell lysate. Based upon amino acid depletion and NH_3 generation, we hypothesized that *B. henselae* catabolized amino acids. To test this hypothesis, *B. henselae* lysate was assayed for amino acid catabolism enzymes. Since glutamate was the predominant amino acid depleted from BBH-H, the enzyme responsible for the degradation of this amino acid was assayed. NADH-dependent glutamate dehydrogenase catalyzes the reaction that deaminates glutamate to α-ketoglutarate, a TCA cycle intermediate. Activity was detected for this enzyme in *B. henselae* lysate, but not for NADPH-dependent glutamate dehydrogenase, a biosynthetic enzyme that produces glutamate from α-ketoglutarate and NH_3 (Table 4). The presence of a glutamate-catabolizing enzyme suggested that histidine, the other amino acid depleted from the medium in large quantities, was also entering a catabolic pathway. To test this, *B. henselae* lysate was assayed for histidase activity, the first step in the degradation of histidine to glutamate; histidase activity was detected in *B. henselae* cell lysate (Table 4). Since histidase deaminates histidine, the high levels of this enzyme and NADH-dependent glutamate dehydrogenase in *B. henselae* could account for the elevated NH_3 measured during growth. The presence of these key enzymes indicated that *B. henselae* was able to catabolize amino acids to TCA cycle intermediates.

Utilization of the TCA cycle produces CO_2 and consumes O_2, and during *B. henselae* growth, we observed O_2 consumption and CO_2 production. This led us to hypothesize that *B. henselae* harbors the enzymes for a complete TCA cycle. Enzymatic activities for all of the TCA cycle enzymes or enzyme complexes were detected in cell lysate, indicating that *B. henselae* does possess the enzymatic components of a full TCA cycle (Table 4). To generate energy from the reducing potential produced by the TCA cycle, organisms typically utilize the electron transport chain (ETC). Under aerobic conditions, oxygen is consumed as the terminal electron acceptor of the ETC. During growth, *B. henselae* consumed all of the oxygen present in the medium (Table 2). However, oxidative decarboxylation reactions of the TCA cycle cannot account for all of the O_2 depleted, based on the amount of CO_2 produced (Table 2). These data, coupled with the presence of a complete TCA cycle, suggest that *B. henselae* uses the ETC to generate ATP

TABLE 3. Depletion of free amino acids from BBH-H by *B. henselae*

Amino acid	Mean concn ± SD[a] (mM)		Change
	T_0[b]	T_F[c]	
Asn	0.67 ± 0.02	0.01 ± 0.00	0.66
Cys	0.18 ± 0.00	0.07 ± 0.00	0.12
Glu	4.19 ± 0.05	1.72 ± 0.01	2.47
Gly	3.75 ± 0.03	3.43 ± 0.07	0.32
His	33.0 ± 0.30	30.7 ± 0.67	2.30
Ser	0.89 ± 0.01	0.45 ± 0.02	0.44
Trp	0.46 ± 0.00	0.40 ± 0.00	0.05

[a] Values were determined by mass spectrometry and are from two independent experiments assayed in triplicate.
[b] T_0 values are from uninoculated medium.
[c] T_F values are from conditioned medium from death-phase cultures.

TABLE 4. Metabolic enzyme activities present in *B. henselae* lysate

TCA enzyme or amino acid degradation	Activity[a]
TCA enzyme	
Citrate synthase	+
Aconitase	+
Isocitrate dehydrogenase	+
α-Ketoglutarate dehydrogenase	+
Succinate dehydrogenase	+
Fumarase	+
Malate dehydrogenase	+
Amino acid degradation	
Glutamate dehydrogenase	
NADH dependent	+
NADPH dependent	−
Histidase	+

[a] Symbols: +, presence of enzymatic activity; −, no activity detected.

from the NADH and flavin adenine dinucleotide-H_2 produced by the TCA cycle. Other bacteria are able to utilize alternate electron acceptors, such as NO_3 and NO_2, when grown anaerobically. A number of terminal electron acceptors (NO_3, fumarate, dimethyl sulfoxide, NO_2, NO_2-formate, or $Na_2S_2O_3$) were used at concentrations of 20 mM to supplement anaerobic BBH-H; however, none supported *B. henselae* growth (data not shown). These data suggest that during in vitro growth, *B. henselae* utilizes the TCA cycle to generate reducing potential, which then enters the ETC to generate energy with oxygen as the terminal electron acceptor and that alternate electron acceptors are not utilized by *B. henselae*.

Postexponential growth phase of *B. henselae* in BBH-H. When *B. henselae* cultures grown in BBH-H reached the postexponential phase, there was a rapid decline in the number of viable cells in the medium as determined by plating on BBH-HA (Fig. 1A). This precipitous drop in CFU over a 24-h period was defined as the "death phase." These results were confirmed using the BacLight live/dead stain, a cell viability assay based upon membrane integrity. All of the cells visible at peak density (5×10^8 CFU/ml) fluoresced green, indicating intact cell membranes, whereas 24 h later, >99% of the bacterial cells fluoresced red, indicating that they were not viable (Fig. 1B). The death phase of *B. henselae* was examined in more detail to determine the potential cause of the rapid decrease in cell viability.

Substrate depletion or toxic metabolite (i.e., NH_3) production were possible explanations for postexponential death. To ascertain if this was the case, spent BBH-H from a death-phase culture was filtered and reinoculated with *B. henselae*. This "spent" medium supported growth, with doubling times and cell densities very similar to those observed in fresh medium, suggesting that loss of viability was not due to the depletion of an essential nutrient or the production of a toxic metabolite (data not shown). *B. henselae* does not grow in BBH-H under anaerobic conditions, but does deplete O_2 from the medium as it grows. To determine if the bacteria were dying because of O_2 depletion, cells were grown under atmospheric (21%) and microaerobic (7%) oxygen levels. We hypothesized that if O_2 deprivation was the cause of the death phase, then the decline in viable cells would occur at a lower cell density in microaerobic cultures due to the O_2 being depleted more rapidly. However, both conditions produced the death phase phenotype at similar cell densities, suggesting that O_2 deprivation is not the cause (data not shown). Taken together, these data suggest that substrate depletion, accumulation of toxic metabolites, and production of anaerobic conditions were not responsible for the death phase.

Phage induction in *B. henselae*. Since *B. henselae* and other *Bartonella* species harbor defective phages (1, 3, 22), phage induction was examined as a possible cause of the death phase. When phage are induced, bacterial membrane integrity becomes compromised, resulting in host cell death. To investigate whether this was the cause of the *B. henselae* death phase, culture supernatants taken from bacterial cultures were examined for the presence of intact phage by electron microscopy (Fig. 2A). Phage particles with multiple head sizes were detected, although it was not clear if the variation in particle size was an artifact of the isolation protocol, as ultracentrifugation can alter the appearance of phage particles. Consistent with the observations of Anderson and Barbian (1, 3), no phage tails were evident in the phage preparations.

To verify that particles in the culture supernatants were phage, samples were treated with nucleases to remove any free DNA, as phage DNA packaged in capsid proteins is DNase resistant. The putative phage DNA was extracted and analyzed by agarose gel electrophoresis. A 14-kb DNA segment was recovered from death-phase *B. henselae* culture supernatants that was not evident in exponential-phase culture supernatants (Fig. 2B). The size of the DNA fragment is consistent with the size of the *Bartonella* phage DNA described by other researchers (1, 3). The appearance of this DNase-protected, extrachromosomal DNA correlates with the onset of death phase, suggesting that the rapid decline in *B. henselae* cell density is the result of phage induction and host lysis.

Isolation from experimentally infected cats. Current methods for isolating *B. henselae* utilize blood agar plates; however, colonies typically take 2 to 6 weeks to form when the bacteria are first isolated from the mammalian host. To determine if BBH-H would be effective for the isolation of *B. henselae* from experimentally infected cats, blood samples from bacteremic cats (3×10^2 to 5×10^4 CFU/ml blood) and from noninfected cats were diluted 1:100 in 10 ml BBH-H and incubated at 37°C with aeration. No bacterial growth was detected in BBH-H inoculated with uninfected cat blood. Within 4 days, cultures inoculated from samples with 5×10^2 CFU reached maximum cell density (5×10^8 CFU/ml), while those inoculated with <50 CFU required an additional 24 h to reach the same cell density. Interestingly, treatment of the blood sample during collection had a dramatic effect on isolation time. BHH-H inoculated with samples collected in Vacutainer-saponin resulted in the shortest isolation times (above) while cultures inoculated from Vacutainer-heparin blood took 11 days to reach similar cell densities. These data suggested that lysing the red blood cells during sample collection improved isolation efficiency. When these same samples were used to inoculate BBH-HA, colonies formed within 5 days. PCR screening confirmed that the bacteria isolated from these blood samples were *B. henselae* (data not shown). The reduction in incubation time from 2 to 6 weeks using blood agar plates to 5 days in BBH-H medium or BBH-HA represented a significant improvement and sug-

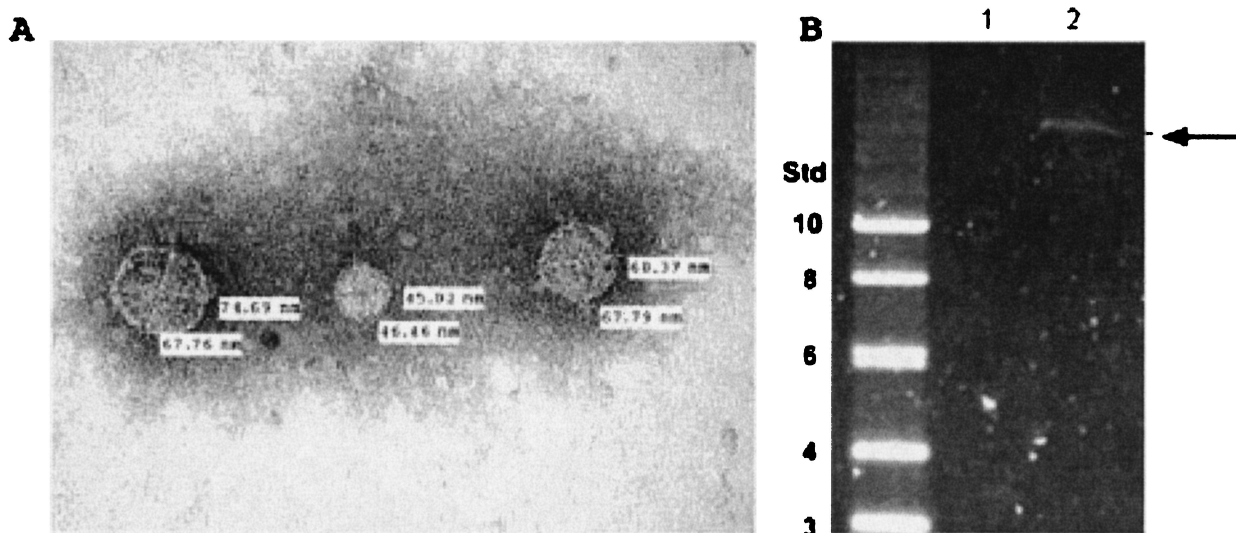


FIG. 2. Growth-dependent phage induction in *B. henselae* grown in BBH-H. (A) Electron micrograph (magnification, ×200,000) of phage from death-phase culture supernatant revealing different particle sizes present in the medium. The numbers present on the image represent the diameter in nanometers. (B) Extracted phage DNA separated in 0.5% agarose. Lane 1, exponential-phase culture; lane 2, early-death-phase culture. The arrow indicates a 14-kb DNA band isolated from death-phase culture supernatant. Standards (in kilobase pairs) are indicated to the left.

gested that BBH-H could be used for more rapid and reliable isolation of *B. henselae*.

DISCUSSION

Bartonella research has been hampered by the lack of a reliable growth medium. By modifying a previously published formula, we have developed a reliable *B. henselae* liquid growth medium. Several factors ultimately were key for more rapid, reproducible growth. First, was the form of heme. When hematin was substituted for hemin and conjugated to histidine to improve solubility, growth yields and rates improved. Second, unreacted histidine in the conjugation reaction mixture was utilized by *B. henselae* as a carbon source. Third, aerating the cultures provided sufficient dissolved oxygen to support maximum growth. *B. henselae* would not grow under anaerobic conditions and consumed all available dissolved O_2 in the growth medium. And finally, although seemingly trivial, the addition of 100 mM HEPES to maintain the pH within tolerable limits was critical. *B. henselae* growth was sensitive to the pH of the medium, with optimal growth occurring over a very narrow pH range (pH 6.6 to 7.2). The growth rate of *B. henselae* in BBH-H was 3 h, and cell densities reached 5×10^8 to 1×10^9 CFU/ml.

The development of BBH-H has allowed us to investigate the metabolism of *B. henselae*, which appeared to be unusual when compared to other blood-borne bacterial pathogens. Almost all bacterial pathogens that thrive and grow in blood preferentially utilize glucose as a carbon and energy source. Atypically, *B. henselae* did not use glucose under the growth conditions tested. These results were contradictory to a recently published genomic study which concluded that *B. henselae* had the metabolic potential to catabolize glucose (4). However, the same study noted that *B. henselae* lacked the gene encoding phosphofructokinase, a key regulatory enzyme of glycolysis. Since glucose was not depleted from the culture medium during growth, *B. henselae* must be deriving carbon and energy from alternative sources. The accumulation of NH_3 and the coordinate depletion of five amino acids (Asn, Glu, Gly, His, and Ser) from the culture medium during growth, suggested a possible mechanism for *B. henselae* metabolism. Amino acids can be used for biosynthesis or they can be deaminated and used as carbon and/or energy sources resulting in the production of NH_3. Therefore, biochemical analysis of culture supernatants before and after growth suggested that *B. henselae* catabolized amino acids to provide carbon and energy.

One way to utilize amino acids efficiently is to convert them to TCA cycle intermediates. In the obligate intracellular pathogen *Rickettsia prowazekii*, glutamate is catabolized to TCA intermediates while glucose is not utilized (2). If amino acids were catabolized by *B. henselae* via the TCA cycle, the pathogen must (i) possess the enzymes for a complete TCA cycle, (ii) demonstrate TCA cycle activity (i.e., CO_2 production from the oxidative decarboxylation reactions and O_2 consumption), (iii) contain the enzymes necessary to convert amino acids into TCA cycle intermediates, and (iv) have a source of acetyl coenzyme A and either a C_4 or C_5 intermediate to maximize the reducing potential and ATP production from the TCA cycle. Our results clearly demonstrated that *B. henselae* had enzymatic activities for all the TCA cycle enzymes or enzyme complexes, produced CO_2, and consumed O_2 during growth (Table 2). Additionally, we detected enzymes which could convert histidine or glutamine to glutamate, and glutamate to α-ketoglutarate, a C_5 intermediate in the TCA cycle. *B. henselae* also removed lactate, serine, and glycine from the medium during growth. These compounds are readily converted to pyruvate, a potential source of acetyl coenzyme A. Taken together, these data strongly suggest that *B. henselae* derives carbon and energy from the catabolism of amino acids rather than glucose. Based upon the pattern of substrate uti-

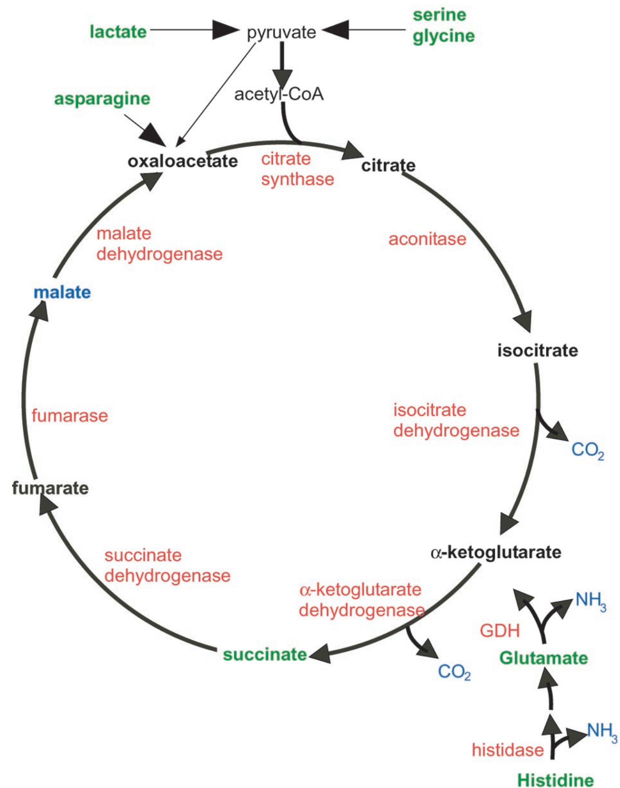


FIG. 3. Proposed model of *B. henselae* metabolism. Substrates utilized are shown in green, and secondary metabolites produced are shown in blue. Enzyme complexes present in *B. henselae* lysate are shown in red. Arrows without labels are reactions inferred from the substrates utilized and pathways present.

lization in BBH-H, we have developed a model for central metabolism in *B. henselae* (Fig. 3). While other pathogenic bacteria can derive energy using the same metabolic pathways, *B. henselae* is unusual in its preference for this catabolic strategy.

A curious aspect of *B. henselae* growth was the rapid decline in cell density after the culture reached stationary phase. Several *Bartonella* species, including *B. henselae*, harbor defective phages. Conditions for induction have been investigated by several groups; however, mitomycin C and UV radiation treatments did not lead to phage induction (1). We have found that phage induction was dependent on *B. henselae*'s growth phase and correlated with the death phase of the bacterium. At this point, we cannot determine if phage induction was the cause or consequence of the death phase.

We have developed a liquid medium for rapid growth of *B. henselae* and used it to identify the unusual metabolic strategy of this pathogen. Importantly, the medium was also used to isolate *B. henselae* from the blood of infected cats. The growth rates of *B. henselae* in BBH-H or BBH-HA inoculated with infected cat blood samples represented a significant decrease in isolation time over previously described media, including blood agar. However, it should be noted that *B. henselae* strains LSU-16 and Houston-1 can be successfully isolated in 5 to 7 days from experimentally infected cats using chocolate agar. Unfortunately, chocolate agar does not permit the reproducible isolation of *Bartonella* species from naturally infected cats or humans (8). Studies are currently under way to determine if BBH-H and BBH-HA will support primary isolation of *B. henselae* from the blood of naturally infected cats. Overall, the development of BBH-H medium represents a major step forward for the study of *B. henselae* pathogenesis and may ultimately prove efficacious for the isolation of *B. henselae* and *B. quintana*.

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REFERENCES

1. **Anderson, B., C. Goldsmith, A. Johnson, I. Padmalayam, and B. Baumstark.** 1994. Bacteriophage-like particle of *Rochalimaea henselae.* Mol. Microbiol. **13:**67–73.
2. **Andersson, S. G.** 1998. Bioenergetics of the obligate intracellular parasite *Rickettsia prowazekii.* Biochim. Biophys. Acta **1365:**105–111.
3. **Barbian, K. D., and M. F. Minnick.** 2000. A bacteriophage-like particle from *Bartonella bacilliformis.* Microbiology **146:**599–609.
4. **Canback, B., S. G. Andersson, and C. G. Kurland.** 2002. The global phylogeny of glycolytic enzymes. Proc. Natl. Acad. Sci. USA **99:**6097–6102.
5. **Craig, J. E., M. J. Ford, D. C. Blaydon, and A. L. Sonenshein.** 1997. A null mutation in the *Bacillus subtilis* aconitase gene causes a block in Spo0A-phosphate-dependent gene expression. J. Bacteriol. **179:**7351–7359.
6. **Doherty, D.** 1971. Glutamate dehydrogenase. Methods Enzymol. **17:**850–856.
7. **Fisher, S. H., and B. Magasanik.** 1984. Synthesis of oxaloacetate in *Bacillus subtilis* mutants lacking the 2-ketoglutarate dehydrogenase enzymatic complex. J. Bacteriol. **158:**55–62.
8. **Fournier, P. E., J. Robson, Z. Zeaiter, R. McDougall, S. Byrne, and D. Raoult.** 2002. Improved culture from lymph nodes of patients with cat scratch disease and genotypic characterization of *Bartonella henselae* isolates in Australia. J. Clin. Microbiol. **40:**3620–3624.
9. **Hill, R. L., and R. A. Bradshaw.** 1969. Fumarase. Methods Enzymol. **13:**91–99.
10. **Huang, K. Y.** 1967. Metabolic activity of the trench fever rickettsia, *Rickettsia quintana.* J. Bacteriol. **93:**853–859.
11. **Jerris, R. C., and R. L. Regnery.** 1996. Will the real agent of cat-scratch disease please stand up? Annu. Rev. Microbiol. **50:**707–725.
12. **Kasahara, M., and Y. Anraku.** 1974. Succinate dehydrogenase of *Escherichia coli* membrane vesicles. Activation and properties of the enzyme. J. Biochem. (Tokyo) **76:**959–966.
13. **Kitto, G. B.** 1969. Intra- and extramitochondrial malate dehydrogenases from chicken and tuna heart. Methods Enzymol. **13:**106–116.
14. **Kotarski, S. F., and A. A. Salyers.** 1984. Isolation and characterization of outer membranes of *Bacteroides thetaiotaomicron* grown on different carbohydrates. J. Bacteriol. **158:**102–109.
15. **Nida, S. K., and J. J. Ferretti.** 1982. Phage influence on the synthesis of extracellular toxins in group A streptococci. Infect. Immun. **36:**745–750.
16. **Norman, A. F., R. Regnery, P. Jameson, C. Greene, and D. C. Krause.** 1995. Differentiation of *Bartonella*-like isolates at the species level by PCR-restriction fragment length polymorphism in the citrate synthase gene. J. Clin. Microbiol. **33:**1797–1803.
17. **O'Reilly, K. L., R. W. Bauer, R. L. Freeland, L. D. Foil, K. J. Hughes, K. R. Rohde, A. F. Roy, R. W. Stout, and P. C. Triche.** 1999. Acute clinical disease in cats following infection with a pathogenic strain of *Bartonella henselae* (LSU16). Infect. Immun. **67:**3066–3072.
18. **Rechler, M. M., and H. Tabor.** 1970. Histidine ammonia-lyase (*Pseudomonas*). Methods Enzymol. **17:**63–69.
19. **Regnery, R. L., B. E. Anderson, J. E. Clarridge III, M. C. Rodriguez-Barradas, D. C. Jones, and J. H. Carr.** 1992. Characterization of a novel *Rochalimaea* species, *R. henselae* sp. nov., isolated from blood of a febrile, human immunodeficiency virus-positive patient. J. Clin. Microbiol. **30:**265–274.
20. **Schwartzman, W. A., C. A. Nesbit, and E. J. Baron.** 1993. Development and evaluation of a blood-free medium for determining growth curves and optimizing growth of *Rochalimaea henselae.* J. Clin. Microbiol. **31:**1882–1885.
21. **Srere, P. A.** 1969. Citrate synthase. Methods Enzymol. **13:**3–11.
22. **Umemori, E., Y. Sasaki, K. Amano, and Y. Amano.** 1992. A phage in *Bartonella bacilliformis.* Microbiol. Immunol. **36:**731–736.
23. **Weiss, E., G. A. Dasch, D. R. Woodman, and J. C. Williams.** 1978. Vole agent

identified as a strain of the trench fever *Rickettsia*, *Rochalimaea quintana.* Infect. Immun. **19:**1013–1020.
24. **Welch, D. F., D. A. Pickett, L. N. Slater, A. G. Steigerwalt, and D. J. Brenner.** 1992. *Rochalimaea henselae* sp. nov., a cause of septicemia, bacillary angiomatosis, and parenchymal bacillary peliosis. J. Clin. Microbiol. **30:**275–280.
25. **Williamson, J. R., and B. E. Corkey.** 1969. Assays of intermediates of the citric acid cycle and related compounds by flourometric enzyme methods. Methods Enzymol. **13:**434–513.
26. **Wong, M. T., D. C. Thornton, R. C. Kennedy, and M. J. Dolan.** 1995. A chemically defined liquid medium that supports primary isolation of *Rochalimaea* (*Bartonella*) *henselae* from blood and tissue specimens. J. Clin. Microbiol. **33:**742–744.
27. **Xiong, L. W., L. D. Raymond, S. F. Hayes, G. J. Raymond, and B. Caughey.** 2001. Conformational change, aggregation and fibril formation induced by detergent treatments of cellular prion protein. J. Neurochem. **79:**669–678.