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Detection of a Previously Unsuspected Bacteremia in the Blood of  
Healthy Individuals

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# ABSTRACT

A doctrine of current medicine is that the bloodstream is devoid of actively metabolizing microorganisms in the absence of disease. This statement is based on the inability to culture microorganisms from blood of healthy individuals. However, modern technology has revealed microorganisms in many ecological niches where they were previously not detectable. In this study, we challenged the hypothesis that the human bloodstream is truly sterile.

Bacteria were cultured from 800 blood samples using a newly developed medium. Antibiotic susceptibilities were tested *in vitro*. Morphology was analyzed by transmission electron microscopy (TEM). Nucleic acids were extracted and bacteria identified by PCR amplification of 16S rRNA and subsequent sequence analyses. *In situ* hybridizations were conducted using labeled specific and universal 16S rRNA probes.

We obtained positive cultures from approximately 800 samples over a five-year period. These isolates were resistant to all known groups of antibiotics. TEM analyses revealed Gram negative rod shaped bacteria. 16S rRNA analyses from 36 isolates identified the  $\alpha$ -proteobacterium. *In situ* hybridization confirmed the presence of metabolically active bacteria in both human sera and cultures, however PCR products were obtained only from 95% of the samples tested.

We developed a system that allows us specifically to detect continuous bacteremia in the blood of healthy individuals. Our data demonstrate the startling finding that, in general, blood is not sterile but contains a multidrug resistant organism. These results imply that bacteremia is a natural property of blood, possibly another bacterial ecosystem.

# INTRODUCTION

A fundamental principle presented in many standard medical microbiology textbooks maintains that, as a rule, in the absence of disease, microorganisms are not found in the blood or healthy tissue<sup>1</sup>. This concept was based on the inability to culture microorganisms from the blood of healthy individuals. For this reason, bacteremia is always considered potentially serious regardless of symptoms present.

Recent advances in bacterial identification using molecular techniques have divulged a universe of uncultured species<sup>2</sup>. Furthermore, improvements in culturing techniques have allowed the identification of previously undetectable organisms in different ecological niches<sup>3</sup>. Throughout the past 10 years, multiple discoveries of unexpected microorganisms in the human body have been documented, for example *Helicobacter pylori* in the stomach and streptococci in the heart<sup>4,5</sup>. These advances suggest that a plethora of unidentified microbes are yet to be revealed.

# METHODS

**Strains and Culture Conditions.** *Methylobacterium organophilum* ATCC 27886, *M. fujisawaense* ATCC 43884, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923 were used as reference strains. LSM<sup>TM</sup> contains CaCl<sub>2</sub>, 0.15 g; MgCl<sub>2</sub>, 0.05 g; KCl<sub>2</sub>, 0.2 g; NaCl, 7 g; NaH<sub>2</sub>PO<sub>4</sub>, 0.17 g; glucose, 4.0 g; lactoalbumin hydrolysate, 6.5 g; yeast extract, 5.0 g; fructose, 4.0 g; sucrose, 4.0 g; MnCl<sub>2</sub>, 1.0 mg per liter of H<sub>2</sub>O (BRL). LSM<sup>TM</sup> was inoculated with 10% human serum in 25 cm<sup>2</sup> flasks and incubated at 30 °C or 37 °C. Growth on solid media was tested by spreading 100 µL of serum on plates. Media used: LSM<sup>TM</sup>, Luria Broth, Nutrient Broth, Nutrient Agar (Difco) with 5% methanol; BCYE agar, TSAII 5% SB, Modified Thayer Martin (MTMII), Seven H11, Sabouraud Dextrose, MacConkey (BBL); *Methylobacterium* Minimal Medium, (ATCC #784). All cultures were set in parallel with a media control.

**TEM.** Pellets from cultures were fixed in phosphate-buffered 4.0% paraformaldehyde-1.0% glutaraldehyde, pH 7.4 and post-fixed in 1% osmium tetroxide in PBS for 1 h. Thin sections were stained with uranyl acetate followed by Reynolds lead citrate<sup>12</sup> and viewed on a Hitachi H-7000 TEM at 75 kv.

**Nucleic acid manipulations.** DNA was isolated from 40 mL of culture or 5 mL of serum using a QIAamp Blood Kit (Qiagen) and the gram-positive bacterial protocol with a few modifications. Prior to lysozyme treatment, samples were resuspended in 180 µL of buffer (20mM Tris, pH 8, 2 mM EDTA and 1.2 % Triton (v/v) and heated to 80 °C for 20 min to inactivate DNases. Pronase E (Sigma) was added to the AL buffer at a concentration of 1.2 µg/µL in AL buffer and incubated at 50 °C for 2 h before Proteinase K treatment. Approximately 0.05 µg DNA was eluted in a final volume of 100 µL of AE buffer. Universal primers<sup>6</sup> for 16S rRNA were PA-1 5'-AGAGTTTG-ATCCTGGCTCAG-3'; PH-4 5'-AAGGAGGTGATCCAGCCGCA-3'. PCR was performed in 100 µL reactions containing 5-10 µL of template DNA using TAQ PCR Core Kit (Qiagen) and 12 nM of each primer. H<sub>2</sub>O used for PCR reactions and controls was sterile tissue culture H<sub>2</sub>O (BRL) that was boiled for 20 min then UV-irradiated for 20 min on a UVA shortwave transilluminator. PCR reactions were performed in an MJ Research thermocycler using 3 min at 96 °C; continued by 40 cycles of 94 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min and a final 7 min extension at 72 °C. To exclude false positive signals from PCR reactions, individual components of LSM<sup>TM</sup> were tested for bacterial DNA contamination and the following precautions adopted to remove it: H<sub>2</sub>O,

uninoculated medium and DNA prepared from uninoculated medium were used as negative controls; TAQ was UV-irradiated for 5 min; lactoalbumin (20% w/v), was UV-irradiated for 20 min rearrange and complete K2, 5'-GTGTTACCCAGAGAGATTGG-3' and K4, 5'-AGTCGCTGACCCTAC-*α-Proteobacterium* 16S rRNA, were designed from the sequence of previous PCR products and produce a product of approximately 480 bp. The PCR reactions contained 5 μL of Gene Releaser™ beads (BioVenture). Cycling conditions were: 96 °C for 3 min; 10 cycles of 94 °C, 1 min; 60 °C to 55 °C over 1 min; 72 °C, 1 min; followed by 10 cycles of 94 °C, 1 min; 55 °C to 50 °C over 1 min; 72 °C, 1 min; followed by 30 cycles 94 °C, 1 min; 50 °C, 1 min; 72 °C, 1 min and a final 7 min extension at 72 °C. PCR products were purified by preparative agarose gel electrophoresis and ligated into either pT7-Blue-3 (Novagen) or pPCR-ScriptAmpSK<sup>+</sup> (Stratagene) vectors. Sequencing reactions were completed with T3 or T7 primers in both directions by University of Florida Molecular Biology Core or by SeqWright, Houston, TX using a 373A DNA sequencer (PE Biosystems). Database searches were performed by BLAST<sup>11,12</sup>. Sequence analysis was performed using SeqMan II (Lasergene) computer software. The cladogram in Figure 3 was generated by Clustal analysis (using the residue weight table).

**Fluorescent *in situ* hybridization (FISH).** Bacterial cells were fixed in 4% paraformaldehyde, pH 7.2. FISH was performed following the method of Jurtschuk *et al.* with 30% formamide<sup>13</sup>. For solution hybridizations, fixed samples were dried in eppendorf tubes, 15 ul of hybridization cocktail was added and tubes were incubated at 42 °C for 16 h. Samples were centrifuged, washed two times in wash solution at 42 °C, resuspended in 10 ul PBS and spotted on slides. Slides were fixed over dry paraformaldehyde for 30 min at 60 °C and viewed as below. Nucleic acid probes for *Methylobacterium*, 5'-TCGCAGTTCCACCAAC-3' and 5'-CTGTGGTTGAGCCACA-3', were designed from sequence data and labeled with tetramethylrhodamine (TAMRA) at the 5' end. Conversely the universal probes, PA-1 and another 16S oligonucleotide from Jurtschuk *et al.*, were labeled with Oregon Green (Molecular Probes, Inc.) and used as non-specific probes to confirm the presence of bacterial cells. Vectashield mounting medium (Vector Laboratories, Inc.) containing 4', 6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) at 1 μg/ml was used as an antifade solution and nucleic acid counterstain. Slides were visualized using Nikon epifluorescence microscope with a TRITC/FITC filter, XF52 (Omega Optical, Inc.).

Figure 1. TEM of *Methylobacterium* species from cultures inoculated with human serum



Cultures of serum samples from human subjects in LSM™ media appeared visually to contain living microorganisms. These slow growing microorganisms appeared pleomorphic under light microscopy, ranging from short rods to cocci, often including complex budding structures. They varied markedly in size and demonstrated a tumbling motility. Analyses of thin sections of cell pellets using TEM established the presence of Gram negative rod-shaped cells with electron dense bodies. Large electron lucent vacuoles within the cytoplasm and a three-layered laminar cell

wall can also be observed. The arrow indicates the appearance of a pilus-like structure.

## **ANTIBIOTIC SENSITIVITY TESTING**

Antibiotic sensitivity was tested in liquid cultures. The sensitivity patterns of human isolates were different than that seen in the reference strains. Human isolates exhibited partial sensitivity to penicillins, ciprofloxacin, tetracyclines and isoniazid. Growth stimulation was observed with cefixime and clarithromycin. There was relatively no effect with most other antibiotics, including most cephalosporins other than cefixime.

**Table 1. Sequencing results from cultures of 6 individuals**  
**and media components**

Samples	1	2	3	4	5	6	7	LA	H <sub>2</sub> O
Methylobacterium	1	3	2	1			3		
Herbaspirillum						2			4
Sphingomonas				1					
Pseudomonas					1				
Stenotrophomonas								1	
Bacillus								1	
Leptothrix									1

The next step was to identify these microorganisms. DNA was prepared using a modification of the QIAamp bacterial DNA extraction protocol. DNA was isolated from cultures, PCR amplifications were done using universal primers originating from highly conserved regions of bacterial 16S rRNA sequences. PCR products of approximately 1500 bp were subcloned and sequenced.

# *Methylobacterium*

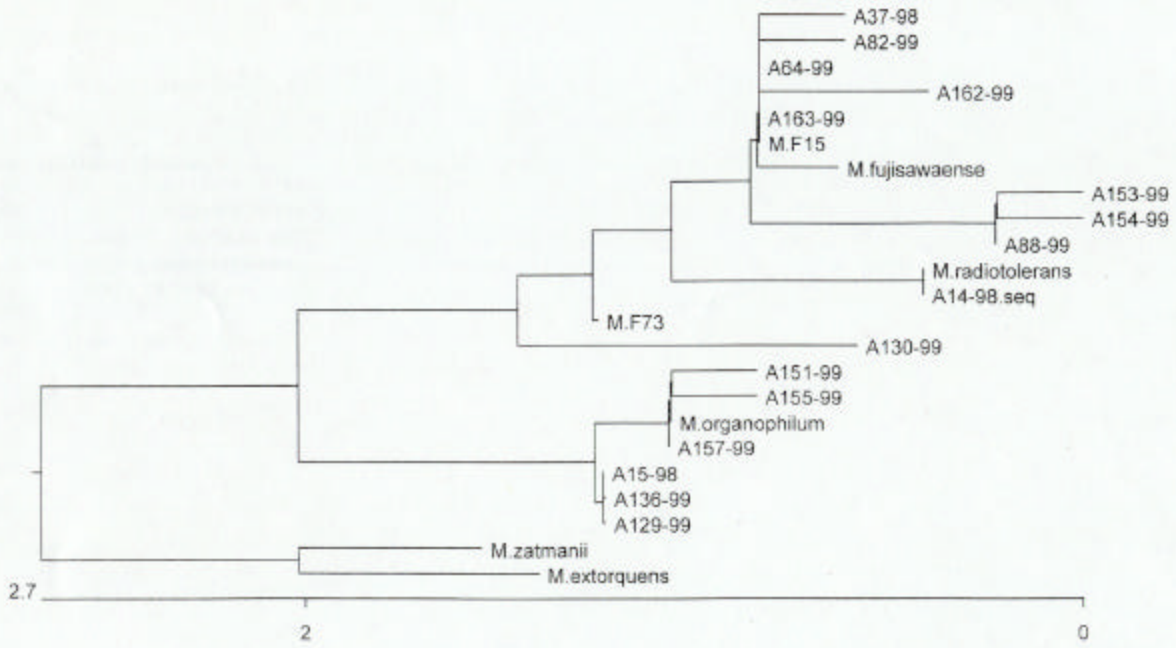
*Methylobacterium* spp. are characterized as pink-pigmented, facultatively methylotrophic, gram-negative pleomorphic bacteria. Cells appear as large vacuolated rods, which vary in size from 0.8-1.0 um X 1.0-8.0 um. Isolates are known to grow slowly on ordinary media producing 1 mm diameter colonies in 4 to 5 days. The genus is defined, in part, on the ability of its members to grow on simple media utilizing methyl-containing compounds. The genus is ubiquitous in nature being widely distributed in a variety of natural and man-made environments<sup>7</sup>. *Methylobacterium* have also been reported to be rare opportunistic pathogens in immunocompromised individuals<sup>8,9</sup>.

## **Figure 2. Alignment of sequence data of 500 bp 16S rRNA fragments**

Based on data in Table 1., a primer set specific for  $\alpha$ -*Proteobacterium* 16S rRNA was designed that excluded the contaminating sequences from media components. Multiple PCR products from cultures and/or serum of 27 individuals were then each subcloned. Subsequent analyses of a 500 bp region from 91 of these subclones, resulted in 97 to 99+ % identity to a number of *Methylobacterium* species, including *M. organophilum*, *M. fujisawaense*, *M. radiotolerans* and *M. sp. F73*.

Sets of DNA sequences from 16S rRNA of human isolates are aligned with their most closely related reference strain. Sequences beginning with 'A' are derived from human isolates and those beginning with 'M' are *Methylobacterium* from GenBank. The differences in nucleotide sequences were found to be statistically greater than would be expected from errors in the fidelity rate of DNA polymerase used in PCR<sup>6</sup>.

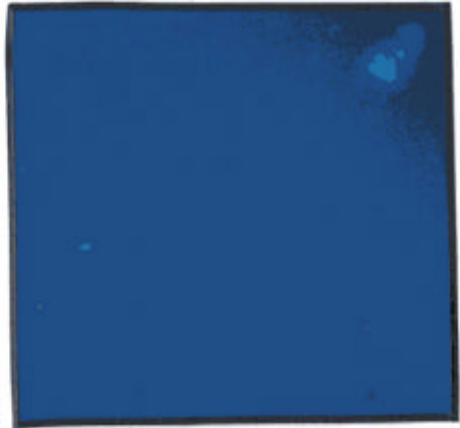
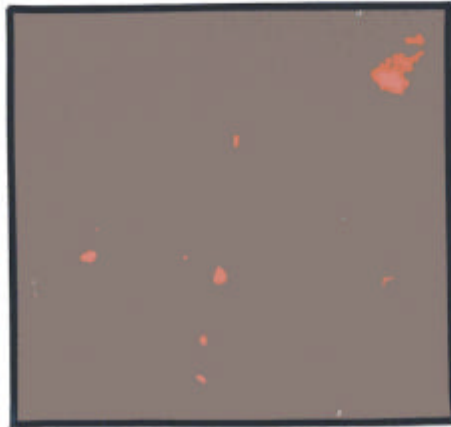
**Figure 3. Cladogram depicting putative phylogenetic relationships of sequences from Figure 2**



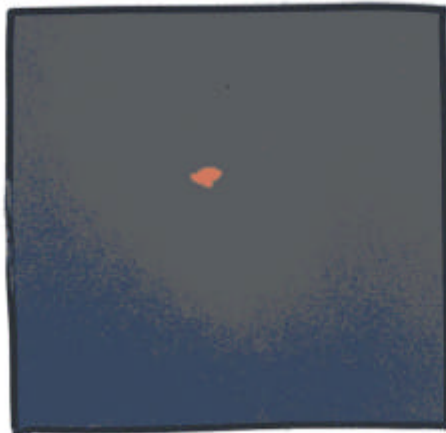
**FISH**

**DAPI**

**Culture**

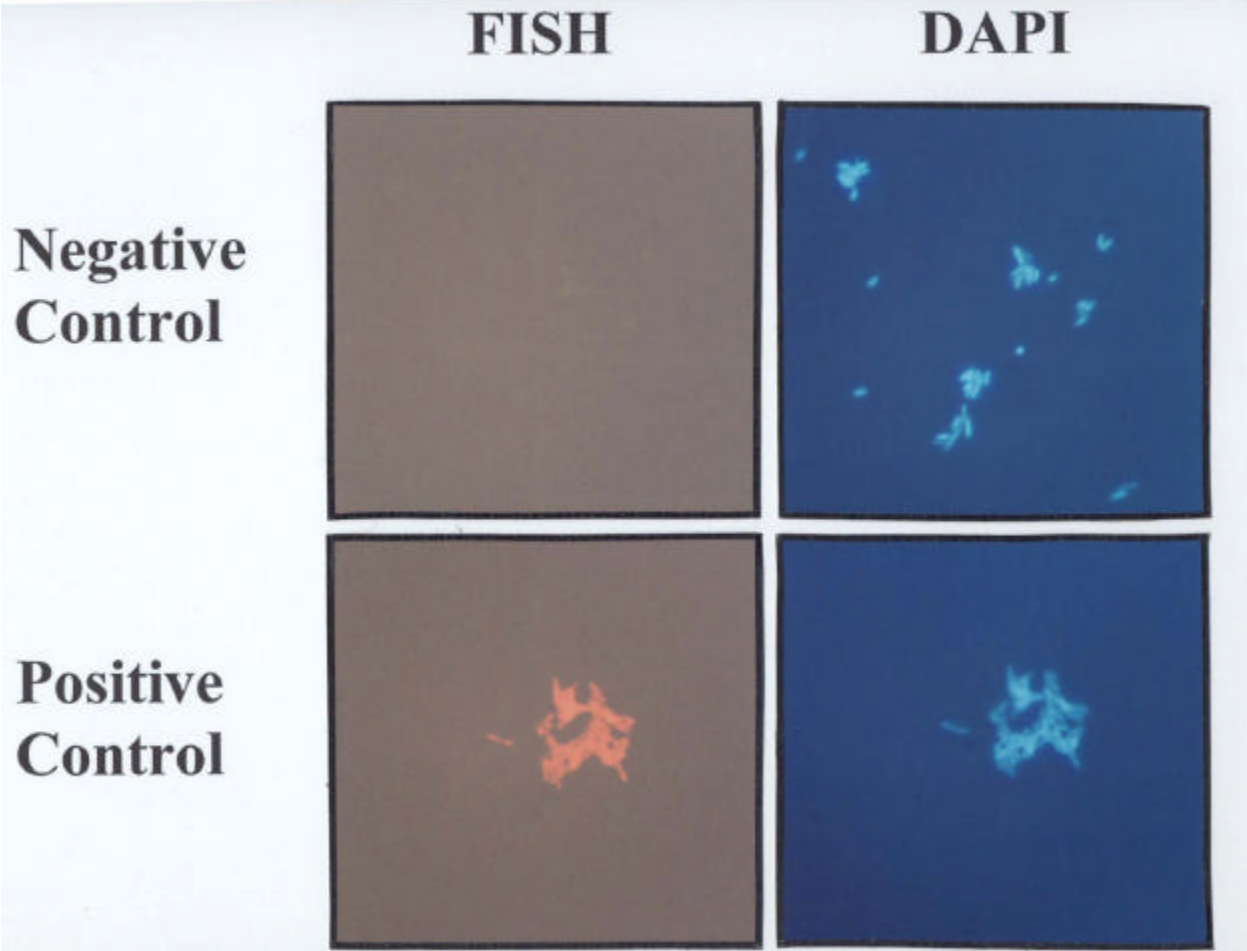


**Serum**



**Figure 4. Fluorescent *in situ* hybridization of fixed cells**

To confirm that the bacteria observed in serum were viable and identical to the bacteria sequenced, FISH assays were utilized. Cells stained with rhodamine-labeled oligodeoxynucleotides complementary to 16S RNA sequences of *Methylobacterium* spp. (left) or with DAPI (right) are shown. Culture, samples from culture of human isolate; Serum, samples from human serum; Negative Control, *E. coli* ; Positive Control, *M. organophilum*.



# Conclusions

- ***Methylobacterium* spp. regularly occurs in the human bloodstream.**
- **We developed a system that allows us to detect continuous bacteremia in human blood.**
- **The microorganism isolated from human serum differs from the reference strains in growth rate and characteristics, size and antibiotic sensitivity patterns.**
- **FISH data demonstrated the presence of extracellular, actively metabolizing *Methylobacterium* spp. in blood and serum.**
- **This data demonstrates that blood is not sterile.**

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<i>Product</i>	<i>Quantity</i>	<i>Cat No.</i>	<i>Size</i>	<i>Unit Price</i>
<b>Lindner Culture Media</b>	<b>1</b>	CM-015	500 mls	\$150.00
<b>Lindner Modified Media</b>	<b>1</b>	CM-016	500 mls	\$195.00
<b>Lindner Culture Media</b>	<b>2 – 4</b>	CM-015	500 mls	\$125.00
<b>Lindner Modified Media</b>	<b>2 – 4</b>	CM-016	500 mls	\$150.00
<b>Lindner Culture Media</b>	<b>≥5</b>	CM-015	500 mls	\$ 90.00
<b>Lindner Modified Media</b>	<b>≥5</b>	CM-016	500 mls	\$100.00

Lindner Culture Media or Modified Media is well suited for supporting the growth of the microorganism isolated from human blood, known as human blood bacteria (HBB).

**Recommended usage:**

Culture in sterile T25 flask containing 9 mls of Lindner culture media or modified media and 1 ml of human serum. Incubate flask at 30°C to 32°C in light (place 18 inches from light source containing 50 W, 120 V bulb, non-blue, grow light).

**Performance and quality testing:**

Filter sterilized and microbial tested. Performance tested to recognized reference standards

**Recommended Storage:** 2°C to 8°C

Several molecular products are available including PCR and FISH.