

## Phylogenetic Placement and Characterization of a New Alpha-2 Proteobacterium Isolated from a Patient with Sepsis

GUNILLA BLOMQUIST,<sup>1</sup> LARS WESSLÉN,<sup>2</sup> CARL PÅHLSON,<sup>2\*</sup> EVA HJELM,<sup>2</sup> BERTIL PETTERSSON,<sup>3</sup>  
TAPIO NIKKILÄ,<sup>4</sup> ULRIKA ALLARD,<sup>1</sup> OLGA SVENSSON,<sup>2</sup> MATHIAS UHLÉN,<sup>3</sup> BROR MOREIN,<sup>1</sup>  
AND GÖRAN FRIMAN<sup>2</sup>

*Department of Infectious Diseases and Clinical Microbiology, Uppsala University Hospital,<sup>2</sup> Department of Virology, National Veterinary Institute,<sup>1</sup> and Department of Pathology, Swedish University of Agricultural Sciences,<sup>4</sup> Uppsala, and Department of Biochemistry and Biotechnology, Royal Institute of Technology, Stockholm,<sup>3</sup> Sweden*

Received 16 May 1997/Accepted 22 May 1997

**An alpha-2 proteobacterium, previously unknown as determined by its phylogenetic characteristics and the DNA sequence of its 16S rRNA gene, was isolated from a patient who presented an unusual clinical picture, including high remitting fever and multiorgan involvement. The bacterium was detected in multiple plasma samples, obtained during the acute phase of the disease, after cocultivation in cell culture media. Electron microscopy of the organism showed a three-layer laminar cell wall and electron-dense granules within the cytoplasm, as well as a polar flagellum. By means of PCR followed by sequencing of amplified 16S ribosomal DNA fragments, the bacterium was found to differ from all species for which ribosomal sequence information is available. It is here provisionally named the Rasbo bacterium. At a subsequent relapse, the bacterium was identified in pericardial fluid both by PCR/sequencing and by direct electron microscopy. At a second relapse, it was again cultured from plasma. After *in vitro* adaptation to solid media, the MICs of various antibiotics could be determined. A transient immunoglobulin M (IgM) but no IgG response to the bacterium was found by an indirect immunofluorescence test, as well as by an immobilization test during the acute phase of the disease.**

Few species of the alpha subdivision of the proteobacteria (30) have been confirmed to be pathogenic to humans. The most studied of those species are members of the genus *Rickettsia*, including species like *Rickettsia prowazekii* and *Rickettsia rickettsii*, the causative agents of louse-borne typhus and Rocky Mountain spotted fever, respectively. Other pathogens of the alpha subdivision of the proteobacteria belong to the genus *Bartonella*, which, until recently, was classified in the order *Rickettsiales*. *Bartonella* spp. are not obligate intracellular parasites, and when a classification based on molecular biology techniques is used, they are separated from the rickettsiae (4). *Bartonella bacilliformis* and *Bartonella quintana* (previously *Rochalimaea quintana*) are the causative agents of oroya fever and trench fever, respectively. These conditions have been known for almost a century (17, 18, 31). During the last decade, two more species have been discovered, namely, *Bartonella henselae* (25, 26), which is the agent most often causing cat scratch disease, and *Bartonella elizabethae* (7), which has been found in a patient with endocarditis. *Afipia felis* has also been found in patients with cat scratch disease (3, 11) but does not seem to be a common cause of this disease. *Caulobacter*, *Methylobacter*, and *Rhodomicrobium* are examples of other genetically related genera that have previously been considered commensals.

In this communication we report the isolation on cell cultures of an alpha-2 proteobacterium from a patient with high remitting fever and multiorgan involvement. Sequence comparison of rRNA genes of the ribosomal small subunit has been shown to be a powerful tool for phylogenetic analysis of

bacterial species (21, 37). Therefore, this bacterium was characterized phylogenetically by determination of the 16S rRNA sequence and subsequent comparison with relatives of the alpha subclass of the *Proteobacteria* (16, 30). The new alpha-2 isolate was found to differ from the other species of this group for which 16S rRNA nucleotide data have been deposited. This organism is here referred to as the Rasbo bacterium, a provisional name derived from the county in which the patient was born and where he may have acquired the bacterium.

### MATERIALS AND METHODS

**Case report.** A previously healthy 33-year-old man was admitted to the hospital 8 days after the onset of respiratory symptoms and severe myalgia followed by the development of fever. One of the most conspicuous clinical features was remitting fever continuing for 3 months and with extreme quotidian amplitude, with peak values reaching 42.3°C. Severe symptoms involving serous membranes and visceral organs gradually developed, i.e., profuse diarrhea and pericardial, pleural, and peritoneal effusions, as well as signs of myocardial, hepatic, and splenic involvement. Intensive care was required for several weeks. An extensive search for a microbial cause by conventional methods failed to demonstrate an infectious agent, and broad-range testing for systemic, endocrine, and malignant diseases resulted in negative findings. Presumptive treatment was given with a great number of antibacterial drugs, with no clear response to any specific drug. After 3 months, when antimicrobial chemotherapy had been discontinued due to suspected side effects, the fever and other symptoms gradually disappeared and the patient could leave the hospital. In the meantime, an infectious agent from the patient's plasma had adapted to *in vitro* growth on artificial media (see below). Two months after the patient was discharged from the hospital a relapse occurred, and the patient was readmitted with chest symptoms and a temperature above 40°C. Seven hundred milliliters of pericardial fluid was evacuated. The patient recovered within 2 weeks on doxycycline treatment that was continued until a second relapse occurred 7 months later. Compliance was checked by determination of the doxycycline concentration in serum on admission at the second relapse. At this time chest discomfort and fever had reappeared but were successfully treated with imipenem for 2 weeks. After 10 more symptom-free months, now without antibiotics, a third relapse with similar but milder symptoms occurred. After 10 days of continuous deterioration of the patient's condition, antibiotic treatment was begun. The patient again responded to a 2-week course of imipenem. During the first two relapses, the same organism as the one that was isolated from plasma during the initial phase of the disease was cultured

\* Corresponding author. Mailing address: Section of Clinical Microbiology, Department of Infectious Diseases and Clinical Microbiology, Uppsala University Hospital, S-751 85 Uppsala, Sweden. Phone: 46-18-663922. Fax: 46-18-559157.

from plasma or pericardial fluid. During the third relapse bacteria could be visualized directly in blood as rods stained by acridine orange, but no growing strain was isolated. The patient has been healthy since then (June 1997).

**Primary isolation.** Repeated heparinized or EDTA-treated blood samples were obtained from the patient over several weeks, beginning on day 13 after the onset of the disease. Moreover, during the first relapse pericardial fluid was obtained.

The blood samples and pericardial fluid were aliquoted into 2- to 3-ml volumes before further processing. Blood samples obtained on days 13 and 14 of the disease were prepared for cultivation of plasma and centrifuged blood cells. From subsequent samples only plasma was used for cultivation. Plasma was obtained as the supernatant after centrifugation of whole blood for 15 min at  $3,000 \times g$ . Buffy coat cells were separated by Ficoll centrifugation.

For cultivation, 0.5 ml of plasma supernatant or 1.0 ml of whole blood was added to a ventilated tissue culture flask containing 4 ml of RPMI 1640 medium (Gibco) with 5% fetal calf serum (FCS) plus gentamicin (50 mg/liter) and amphotericin B (2.5 mg/liter) and incubated at 37°C in a humidified atmosphere supplemented with 5% CO<sub>2</sub>. A negative isolation control encompassing all reagents, including FCS, the sole biological constituent, was run in parallel during the entire culture period. Plasma was also obtained from a patient requiring intensive care for multitrauma and from a healthy member of the hospital staff, who served as controls. After 2 days of incubation, 0.5 ml of the contents from each bottle was inoculated onto a monolayer of Vero cells in 25-cm<sup>2</sup> plastic cell culture flasks (Costar, Cambridge, Mass.). After an adsorption period of 1 h at 37°C, fresh full Dulbecco minimal essential medium (National Veterinary Institute, Uppsala, Sweden) supplemented with 5% FCS was added.

The cell cultures were incubated at 37°C and examined daily by phase-contrast microscopy. Isolations were also attempted on aerobic and anaerobic blood culture media (Bactalert; Organon Teknika, Durham, N.C.) incubated at 35°C for  $\geq 3$  weeks, Columbia agar (Difco Laboratories, Detroit, Mich.) with 5% sheep blood, hematin agar (same agar base), MacConkey agar (Oxoid, Unipath Ltd., Basingstoke, United Kingdom), and CLED agar (BBL, Becton Dickinson) incubated aerobically as well as in a 5% CO<sub>2</sub> atmosphere at 37°C for 3 weeks. Anaerobic incubation was performed on Fastidious Anaerobe agar (Lab M, Bury, United Kingdom) with 5% horse blood and on Rogosa agar (Difco). Regular culture for fungi was performed on Sabouraud agar. *Chlamydia trachomatis*, *Chlamydia psittaci*, and *Chlamydia pneumoniae* cultivation was carried out on McCoy cells (27) and HL cells (6). *Mycoplasma* was screened for by culturing the samples in SP4 broth (33).

**Adaptation procedure.** Isolates were kept in cell culture flasks. Once a week, supernatants were inoculated onto different cell-free media.

**Biochemical testing.** Enzyme activity was tested by an API-ZYM enzyme test kit (API System S.A., Montalieu-Versieu, France), and  $\beta$ -galactosidase activity was confirmed by a conventional aerobic *o*-nitrophenyl- $\beta$ -D-galactopyranoside test (19). The urease broth test and tests for oxidase and catalase activity were performed according to the *Manual of Clinical Microbiology* (19). The bacteria were also tested by API 20 NE (API System S.A.).

**Microscopic examination and staining procedures.** Gram, Giemsa, and acridine orange staining and staining specific for flagella (Gray flagellar stain), capsule endospores (Wirz-Conclon stain), and acid-fast-cell walls (Ziehl-Neelsen stain) were performed according to the *Manual of Clinical Microbiology* (19). The culture flasks were regularly examined with a phase-contrast microscope at a magnification of  $\times 400$ . All stained slides were studied with oil immersion at a magnification of  $\times 1,000$ .

**Bacterial strains.** *B. henselae* Houston 1 was obtained from the Centers for Disease Control and Prevention, Atlanta, Ga., and *A. felis* CCUG 30456 was obtained from the culture collection of the University of Gothenburg, Gothenburg, Sweden.

**Electron microscopy.** The bacteria were sedimented for 15 min at 6,000 rpm. The pellet was mixed with 1% glutaraldehyde sodium cacodylate buffer, pH 7.4, and fixed overnight. Post-fixation was performed in 1% OsO<sub>4</sub> in distilled H<sub>2</sub>O. The bacteria and cell material were then dehydrated with an ethanol gradient and embedded in Agar 100 resin (Agar Scientific Ltd., Stansted, Essex, United Kingdom). Ultrathin sections were cut and stained with uranyl acetate and lead citrate. The cells were inspected in a transmission electron microscope (Philips TEM 420) at 60 kV on 200-mesh hexagonal copper grids (Agar Scientific Ltd.).

For negative staining, samples of bacteria were applied to carbon-coated 400-mesh copper grids prior to contrasting by 2% ammonium molybdate. These specimens were examined in a Zeiss CEM 902 electron microscope with an 80-kV acceleration voltage.

**DNA isolation and amplification of the 16S rRNA gene.** Bacteria used for sequencing of 16S rRNA gene (rDNA) were propagated on Vero cells cultured with RPMI 1640 medium supplemented with 5% FCS. The bacteria were harvested from cell culture fluid by centrifugation. After being washed in sterile water, the bacteria were lysed with 200  $\mu$ g of proteinase K/ml and 0.5% sodium dodecyl sulfate at 55°C for 1 h. DNA was purified by centrifugation in Microcon 100 (Amicon Inc., Beverly, Mass.) concentrators according to the manufacturer's instructions, washed twice in distilled water, and finally resuspended in 50  $\mu$ l of PCR buffer (Boehringer Mannheim, Mannheim, Germany). Five-microliter samples were used for PCR amplification, which was performed with general primers for bacterial rRNA genes (24). The primary amplification was performed with primers Tp U1 (5'AGA GTT TGA TC[C/A] TGG CTC AG) and Rt U8 (5'AA

GAG GTG ATC CA[T/G] CC[G/A] CA), corresponding to the *Escherichia coli* 16S rDNA from positions 7 to 27 and 1522 to 1541, respectively. The PCR was carried out according to the instructions of the manufacturer of the *Taq* DNA polymerase kit (catalog no. 1435 094; Boehringer Mannheim). The PCR products were run on 1.5% agarose gels in  $0.5 \times$  Tris-borate-EDTA and examined after ethidium bromide staining (29). The gels were examined with UV light from a TM 20 transilluminator (wavelength, 302 nm; UVP Inc., Upland, Calif.). DNA amplification for detection of bacteria in clinical specimens was performed by a modified method previously described by Anderson (2). Briefly, a dilution of 150  $\mu$ l of blood or pericardial fluid was added to 150  $\mu$ l of 10 mM Tris-10 mM NaCl, pH 8.0. To this mixture were added 30  $\mu$ l of 10% sodium dodecyl sulfate and 50  $\mu$ g of proteinase K, and after 1 h of incubation at 60°C the lysates were extracted with Tris-EDTA-buffered saturated phenol-chloroform-isoamyl alcohol (25:24:1) three times (or until no debris was observed at the interface). The aqueous layer was purified and concentrated in Microcon 100 (Amicon Inc.) concentrators based on the manufacturer's instructions and used as a template in the PCR assays.

**Determination and analysis of 16S rDNA sequences.** Immobilization of the biotinylated PCR products, followed by strand separation and template preparation, was performed with superparamagnetic beads, Dynabeads M-280 streptavidin (DynaL AS, Oslo, Norway). The nucleotide sequences from both strands were determined by automated solid-phase DNA sequencing with the A.L.F. DNA Sequencer (Pharmacia Biotech, Uppsala, Sweden). All primers used for 16S rDNA sequencing, protocols for solid-phase DNA sequencing, and running conditions for the electrophoretic separation of the sequencing products have been described elsewhere (13, 14, 22-24, 34). The obtained sequence was tested against all known bacterial 16S rDNA sequences by a BLAST search, without any perfect fit. Furthermore, it was aligned with a selection of 16S rDNA sequences from relatives and clinically interesting species belonging to the alpha subdivision of the *Proteobacteria*, which were retrieved from the sequence collection of the Ribosomal Database Project (16). Ambiguously aligned positions and gaps introduced in the alignment procedure were removed prior to phylogenetic analysis, and the final data set consisted of 1,061 nucleotide positions. Similarity matrices were constructed by the method of Jukes and Cantor (15). Phylogenetic trees were constructed by using the neighbor-joining method of Saitou and Nei (28) included in the NEIGHBOR program in phylogenetic inference package PHYLIP 3.51c (12). Bootstrap analysis was performed with the SEQBOOT program by carrying out 500 resamplings of the data set.

**Antibiotic susceptibility testing.** Before the Rasbo bacterium could be grown on cell-free media, antibiotic susceptibility of bacteria cocultivated with Vero cells was tested. Approximately  $10^6$  bacteria in cell culture medium were added to the wells of a 96-well microtiter plate. A variety of antimicrobial substances was added to reach concentrations comparable with the maximal concentrations achievable in serum in vivo; final concentrations were as follows: quinine dihydrochloride, ciprofloxacin, clindamycin, and doxycycline, 10  $\mu$ g/ml each; trimethoprim, 70  $\mu$ g/ml; amphotericin B, 3  $\mu$ g/ml; chloramphenicol, 20  $\mu$ g/ml; metronidazole, 20  $\mu$ g/ml; tobramycin, 10  $\mu$ g/ml; pentamidine, 600  $\mu$ g/ml; and rifampin, 10  $\mu$ g/ml. The plate was examined daily for motility and changes in the number of bacteria as roughly estimated by the visual impressions obtained when, by phase-contrast microscopy, comparing wells containing different antibiotics and also including wells without any antibiotics. Three months later, when the strain had been adapted in vitro to grow on semisolid media, the screening for antimicrobial susceptibility was performed by disc diffusion test on hematin agar (Difco) incubated at 26°C for 2 days. The discs were bought from Oxoid, Unipath Ltd., and contained amikacin, ampicillin, aztreonam, cephalaxin, cefotaxime, ceftazidime, ceftriaxone, cefuroxime, chloramphenicol, ciprofloxacin, clindamycin, doxycycline, erythromycin, fusidic acid, gentamicin, imipenem, penicillin V, piperacillin, piperacillin-tazobactam, rifampin, tobramycin, trimethoprim-sulfamethoxazole, and vancomycin. MICs were determined by E-test (Bio-Disc, Stockholm, Sweden) for amikacin, azithromycin, cefuroxime, chloramphenicol, clindamycin, imipenem, gentamicin, piperacillin-tazobactam, rifampin, and tobramycin. Further, for cefotaxime, clindamycin, and doxycycline the MIC was determined by the Bioscreen analyzing system (Labsysteme, Helsinki, Finland) for continuous turbidimetric measurements at 540 nm. The MICs chosen as the limits for sensitivity or resistance were those recommended by the Swedish Reference Group for Antibiotics, Swedish Medical Society (32).

**Serological analyses. (i) Immobilization test.** The bacteria were grown for 2 days in brain heart infusion broth at 30°C, harvested, and washed three times in phosphate-buffered saline (PBS). One hundred microliters of the bacterial suspension (about  $10^6$  CFU/ml) was added into each well of a flat-bottom 96-well microtiter plate (Costar). Plasma samples were diluted twofold in PBS, and 50  $\mu$ l of each serum dilution was added to the wells. Pooled sera from healthy blood donors and PBS were used as negative controls. The microtiter plates were incubated at 35°C for 18 h before they were analyzed by inverted phase-contrast microscopy, and motility was registered (20). Activity of immunoglobulin M (IgM) was abolished by treatment with  $\beta$ -mercaptoethanol (5).

**(ii) Indirect immunofluorescence.** The antigen consisted of bacteria grown for 3 days in brain heart infusion broth at 30°C, washed three times in PBS, suspended in PBS to the desired concentration (about  $10^6$  CFU/ml), transferred to 10-well coated slides (10  $\mu$ l/well), and gently heat fixed (35, 36). The sera to be tested were diluted 10-fold in PBS and were allowed to react with the antigen for 1 h at 37°C. After being washed in PBS, the samples were incubated for 30 min

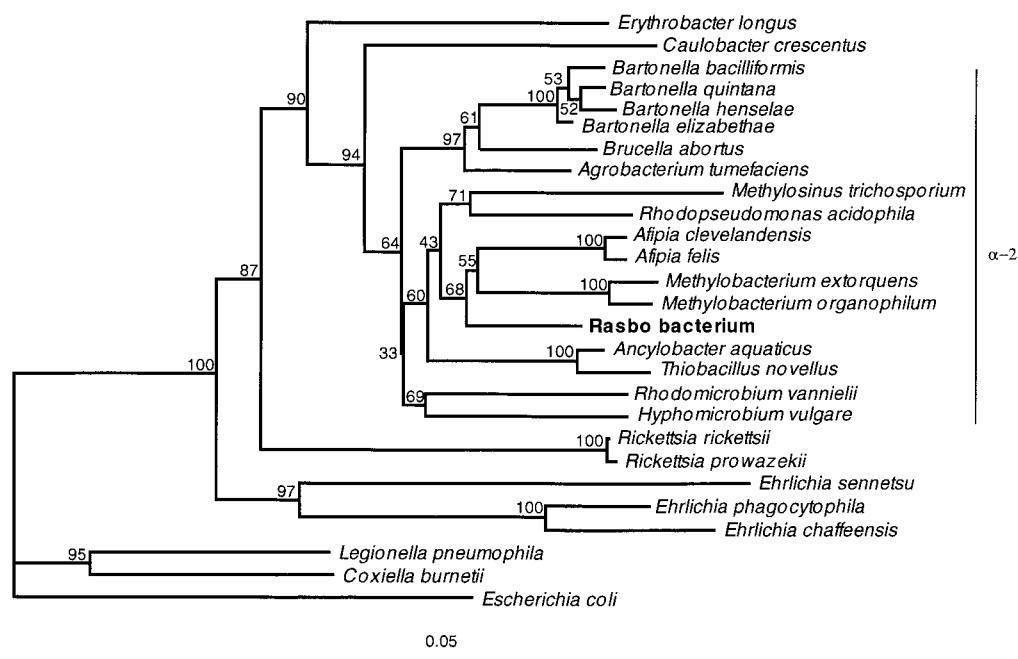


FIG. 1. Evolutionary distance tree of representatives of the alpha subclass of the *Proteobacteria* (30). The tree was based on 16S rDNA sequence comparison and derived from the similarity matrix corrected by the method of Jukes and Cantor (15) by using the neighbor-joining algorithm of Saitou and Nei (28). *E. coli* was used as an outgroup. Bootstrap values are given at the nodes and are the percentages of times among 500 replicates that a taxon to the right of the node occurred. The scale bar represents 0.05 substitution per nucleotide.

at 37°C with fluorescein isothiocyanate-conjugated anti-human IgG, IgA, or IgM (Dakopatts AB, Copenhagen, Denmark). The slides were washed in PBS and studied in a microscope equipped with incident UV illumination. Before being tested for IgM antibodies, the sera were absorbed with protein A-Sepharose (Pharmacia).

(iii) **Control sera.** Ninety-nine blood donors were used to obtain reference sera. The mean age of the donors was 40 years (range, 20 to 60 years). Sixty-four percent were males.

**Nucleotide sequence accession number.** The GenBank/EMBL accession number for the 16S rDNA sequence of the Rasbo bacterium is AF 007948.

## RESULTS

**Analysis of 16S rDNA.** Almost the complete 16S rDNA sequence of the new alpha-2 proteobacterium, the Rasbo bacterium, as obtained from our Vero cell cocultures, was determined and used for phylogenetic placement of the organism. Both strands were sequenced, resulting in an unambiguous final sequence consisting of 1,430 nucleotides. The 16S rDNA sequence of the Rasbo bacterium was used for a search against sequences deposited in different databases by using the BLASTN software (1) at the National Center for Biotechnology Information. This showed a <93% sequence similarity of the Rasbo bacterium to *Rhodomicrobium vannielii*, for which the Rasbo bacterium showed the highest probability score. The search was used as a guideline for subsequent phylogenetic placement of the Rasbo bacterium. The sequence was aligned and compared with 16S rDNA sequences of the members of the alpha subclass of the *Proteobacteria* obtained from the Ribosomal Database Project (16). The 16S rDNA sequence of the Rasbo bacterium was compared with those of 24 members of the alpha subclass of the *Proteobacteria* and 3 members of the gamma subclass of the *Proteobacteria*. A similarity matrix illustrating the results of this comparison is presented in Fig. 1. A phylogenetic tree was constructed by using the neighbor-joining algorithm and demonstrated that the Rasbo bacterium is a member of the alpha subclass of the *Proteobacteria*. The

Rasbo bacterium clusters with the species of the alpha-2 subgroup within the alpha subclass of the *Proteobacteria*. This study shows that the *Afipia* strains and the *Methylobacterium* strains are the closest relatives. The Rasbo bacterium was found to be 95.7 and 96.1% similar to *Afipia clevelandensis* and *Afipia felis* and 95.0 and 95.7% similar to *Methylobacterium extorquens* and *Methylobacterium organophilum*, respectively (Table 1).

**Morphological and biochemical features of the isolated bacterium.** (i) **Cultivation and light microscopy.** Isolations were made by cocultivation of plasma and pericardial fluid from the patient on Vero cells. Due to vivid motility, the Rasbo bacterium was observed for the first time after 24 h of culture of plasma samples obtained on day 13 of the disease.

In spite of the large number of bacteria observed by light microscopy, the medium was transparent, with no color change. The majority of bacteria were rod shaped, and a minor part (approximately 10%) were coccoid. The bacteria showed a shaking/twisting motility, and some bacteria occasionally moved rapidly and decisively across the view field. Specimens from the cell cultures were inoculated once a week onto solid media for bacterial growth. Following such inoculations for one of the specimens that had been kept for 3 months in cell culture (subcultivated twice on fresh cells) before inoculation onto semisolid media, growth on several different semisolid media occurred simultaneously following incubation for a couple of days. The bacterium grew well on hematin agar, MacConkey agar, and CLED agar but not on ordinary blood agar. After adaptation to artificial media and subsequent reinoculation onto Vero cells, cultures yielded (after 3 days of incubation) a monoculture of a coccoid variant. Parts of the 16S rRNA gene of the adapted variant was sequenced and found to be identical to the sequences obtained from the motile bacteria isolated by cocultivating the patient's plasma in cell cultures. Incubation for an additional 6 days resulted in the almost



exclusive prevalence of a rod-shaped variant. The colonies on hematin agar were small (1 to 2 mm in diameter) and greyish pink and had a slightly musty odor. After Gram staining, the coccoid variant of the bacteria was envisaged as coccobacilli, 1 to 3 by 0.7 to 1  $\mu\text{m}$ . The corresponding measurements of the rod-shaped variant were 4 to 7 by 1  $\mu\text{m}$ . Stainings from one colony on an agar plate always revealed a mixture of the two variants. If the incubation time was only 2 days, the coccoid variant predominated, but after 6 days of incubation, the rods predominated. It was also noted that, as the number of subcultures was increased, the adapted strain showed a tendency toward more rapid transformation to rod forms. The bacteria were gram negative and non-acid-fast, and neither endospores nor capsules could be detected by specific staining procedures. However, flagella could be visualized. The bacteria stained orange by acridine orange staining and blue by Giemsa staining.

Altogether, growth of the Rasbo bacterium was initially observed in three of eight plasma samples, in one of five specimens of centrifuged blood cells, and in one of two specimens of pericardial fluid. Adaptation to growth on artificial media was successful for two isolates, which were designated 3F (plasma from day 13) and 4B (plasma from day 27). Another isolate obtained during the second relapse was later lost during the laboratory procedures. Samples from one control patient with multitrauma admitted to the same intensive-care unit as the patient studied and from one member of the hospital staff were run in parallel, without any microbial findings.

(ii) **Electron microscopy.** Transmission electron microscopic examination revealed a three-layer laminar cell wall and electron-dense bodies within the cytoplasm of the cell (Fig. 2b). By negative staining a polar flagellum could be demonstrated (Fig. 2a). The size of the rod variant was 3 by 0.6  $\mu\text{m}$ .

(iii) **Biochemical testing.** The Rasbo bacterium was found to produce oxidase, urease, alkaline phosphatase, esterase, lipase, leucine arylamidase, trypsin, acid phosphatase, and naphthol phosphohydrolase. Furthermore, no fermentation of carbohydrates was observed, conversion of tryptophan to indole was not found, and no reduction of nitrate occurred. The biochemical activities of the Rasbo bacterium, in comparison with those of *B. henselae* and *A. felis*, are shown in Table 2.

**Susceptibility to antibiotics.** Before adaptation of the Rasbo bacterium to solid media, no decline in bacterial number or motility could be observed in the cell cultures subsequent to exposition for quinine dihydrochloride, ciprofloxacin, clindamycin, doxycycline, trimethoprim, amphotericin B, chloramphenicol, metronidazole, tobramycin, pentamidine, or rifampin in fluid media. The antibiotic concentrations were chosen to be comparable to those achieved in the serum during standard dose treatment. After the adaptation, the susceptibility of the Rasbo bacterium (cultures where the rod-shaped variant predominated) to several antibiotics was screened by disc diffusion, and those antibiotics yielding a detectable inhibition zone, and also gentamicin and rifampin, were tested to determine their MICs. The antibiotics to which the bacteria were susceptible included doxycycline (MIC, 0.25 mg/liter; MBC, 0.5 mg/liter), chloramphenicol (MIC, 2 mg/liter), and imipenem (MIC, 0.125 mg/liter). The bacteria showed intermediate susceptibility to azithromycin (MIC, 0.75 mg/liter) (Table 3).

**Serological host response.** As determined by indirect immunofluorescence, the Rasbo bacterium was reactive with IgM antibodies in the patient's serum on all occasions tested during the first month of the disease (Fig. 3). No antibodies of the IgG or IgA class were found on any occasion. In sera from 99 blood donors tested by the same method no IgG antibodies were

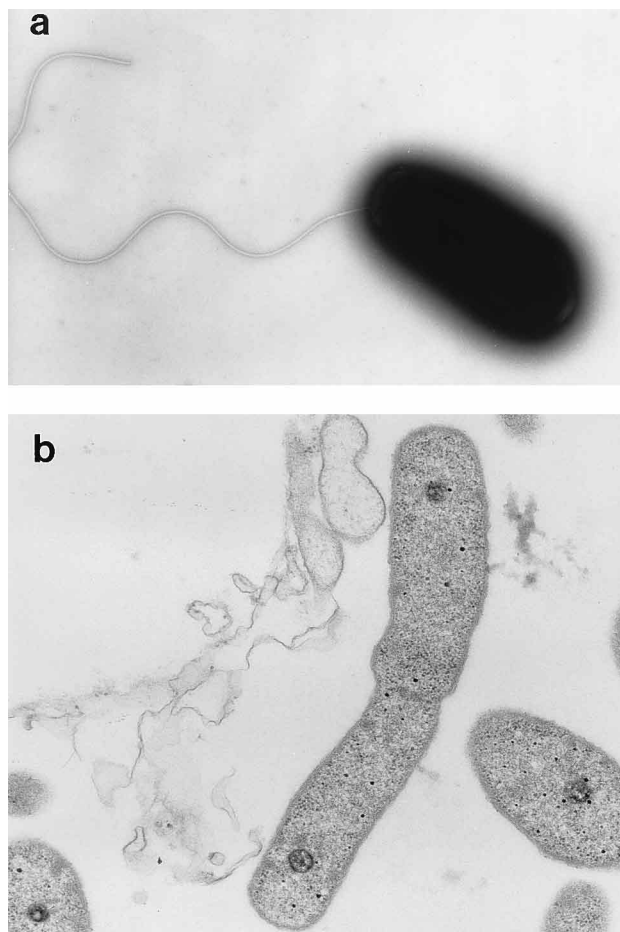


FIG. 2. Transmission electron microscopy of isolated bacteria showing one polar flagellum in negative staining (a) and electron-dense formations within the cells and a three-layer cell wall (b).

found, whereas IgM antibodies were found in two sera in the low reciprocal titer of 10.

Concomitantly elevated antibody titers were found by an immobilization assay (Fig. 3). This antibody response was totally abolished after treatment with  $\beta$ -mercaptoethanol, indicating an IgM response.

Thus, similar kinetics were observed in the two tests, with low titers recorded on day 8 of the disease (day of admission to the hospital), higher titers recorded during the following 2 weeks, and a decline in titer thereafter.

## DISCUSSION

A previously undescribed alpha-2 proteobacterium (Fig. 1), with the provisional name of the Rasbo bacterium, was repeatedly isolated from the blood, and the pericardial fluid, of a 33-year-old Swedish man with a clinical presentation of sepsis and multiorgan disease. The patient initially had influenza-like symptoms, after which his condition deteriorated and septicemia and perimyocarditis were suspected. During the subsequent course the patient ran a malaria-like fever with an extreme amplitude of spikes during the day. The fever persisted for 3 months, evidently uninfluenced by the large number of antibiotic combinations that were tried. Pleural effusion, ascites, diarrhea, and a 15-kg weight loss were other features. Evolving anemia required multiple blood transfusions during

TABLE 2. Comparison of the biochemical activities of the isolated organism and *B. henselae* and *A. felis*

Test or characteristic	Rasbo bacterium	<i>B. henselae</i> Houston 1	<i>A. felis</i> CCUG 30456
Gram reaction	—	—	—
Catalase	—	—	—
Oxidase	+	—	+
Nitrate reduction	—	—	+
Indole	—	—	—
Urease	+	—	+
Acid from carbohydrates <sup>a</sup>	—	—	—
Optimal temp (°C)	25–30	35–37	25–30
Flagella	+	—	+

<sup>a</sup> Arabinose, mannose, mannitol, *N*-acetylglucosamine, glucose, galactose, maltose, lactose, and fucose.

the first month. Further, thrombocytopenia and epistaxis occurred. The patient developed jaundice and later developed arthritis of the right elbow joint. After almost 3 months in the hospital, the fever slowly abated and the patient was discharged from the hospital without any medication.

After another 2 months, a relapse occurred, with fever and exudative pericarditis. Seven hundred milliliters of pericardial fluid was evacuated and doxycycline was initiated, resulting in the rapid disappearance of symptoms. Doxycycline was then continued for 8 months until a second, similar relapse occurred; this was successfully treated with imipenem in addition to doxycycline. The patient had a third but milder similar relapse 11 months later that also resolved during imipenem treatment. He has been well since then (June 1997).

The clinical picture of the disease suggests the involvement of an infectious agent, and there are strong indications that the Rasbo bacterium was involved in the disease in this case. Firstly, the bacterium was repeatedly isolated from blood and could be detected by microscopic examination of the cultures or by PCR, and adaptation to cell-free media was successful for two isolates. The sequences from the 16S rRNA gene from the isolates were equal to those of the strains that could be grown only by cocultivation with Vero cells. The bacterium was detected in pericardial fluid by means of PCR directly as well as after cell culture and by electron microscopy. Secondly, an IgM response to the organism occurred during the acute phase of the disease (Fig. 3). No specific IgG antibodies could, however, be detected, which might be attributable to antigenic differences between in vivo strains and the adapted, faster-growing, non-cell-dependent isolate that was used for antigen preparations. Thirdly, although presumptive treatment with a variety of antimicrobial drugs did not result in a clear response during the acute phase, recovery followed when the subsequent relapses were treated with antimicrobial drugs that had low MICs for the isolated organism; at the time of the relapses the organism had adapted to solid media, allowing conventional antimicrobial susceptibility testing. It is noteworthy, however, that the second relapse occurred during ongoing therapy with doxycycline, a drug to which the bacterium was sensitive. This suggests that the bacterium is harbored in tissues or compartments which doxycycline does not readily penetrate or that the bacterium can be harbored in certain forms that are insensitive to this agent, as none of the in vitro-tested antibiotics, including doxycycline, was able to affect the motility of the non-adapted forms in cell cultures.

It was noted that, for the original isolates, no decline in motility or bacterial density could be seen when nonadapted

bacteria were mixed with antibiotics in the presence of Vero cells. This is consistent with the clinical observation that there was no effect of doxycycline or imipenem treatment during the early course of the disease, at which time blood samples containing these isolates were drawn. However, when an inoculate of the faster-growing adapted strain of the Rasbo bacterium was used in disc diffusion tests, inhibition zones were seen, and E-tests or the Bioscreen procedure yielded results that were consistent, although somewhat conflicting, as noted for cefotaxime (Table 3).

The primary isolation was achieved with cell cultures that had been incubated with the patient's plasma specimens. The extreme motility of the Rasbo bacterium was a conspicuous feature in the absence of a cytopathic effect. Because the routine microbiological diagnostic procedures applied were not successful, a coculturing technique was employed, yielding growth of the Rasbo bacterium in the liquid medium overlaying a Vero cell monolayer; whether intracellular growth occurred was not determined. The remarkable observation that the Rasbo bacterium, despite growth in cell culture, was not able to be cultivated on ordinary bacteriological media, even with inoculation doses of  $10^6$  cells, is of evident clinical interest. Standard blood cultures are usually discarded after about 10 days if no bacteria are found. Here growth on conventional media was recorded only after several weeks of incubation, allowing for further studies, including antibiotic susceptibility

TABLE 3. Antibiotic susceptibility of the Rasbo bacterium as tested by disc diffusion, E-test, and Bioscreen

Antibiotic	Disc diffusion test		MIC (mg/liter) by:	
	Substance amt (µg/disc)	Zone diam <sup>a</sup> (mm)	E-test <sup>b</sup>	Bioscreen
Amikacin	30	32	16 (S ≥ 16)	
Ampicillin	10	0		
Azithromycin	— <sup>c</sup>		0.75 (S ≤ 0.5, R > 4)	
Aztreonam	30	0		
Cephalexin	30	0		
Cefotaxime	30	32		>4 (R ≥ 4)
Ceftazidime	30	40		
Ceftriaxone	30	0		
Cefuroxime	30	18	>256 (R ≥ 16)	
Chloramphenicol	30	33	2 (S ≤ 8)	
Ciprofloxacin	10	0		
Clindamycin	15	27	8 (R ≥ 4)	>4 (R ≥ 4)
Doxycycline	30	38		0.25 (S ≤ 1)
Erythromycin	15	0		
Fusidic acid	50	0		
Gentamicin	30	0	>256 (R ≥ 8)	
Imipenem	10	52	0.125 (S ≤ 4)	
Penicillin V	10	0		
Piperacillin	30	0		
Piperacillin-Tazobactam	36	33	96 (R ≥ 32)	
Rifampin	5	0	32 (R ≥ 2)	
Tobramycin	30	10	>256 (R ≥ 8)	
Trimethoprim-sulfamethoxazole	24/1 <sup>d</sup>	0		
Vancomycin	30	0		

<sup>a</sup> Means of two experiments.

<sup>b</sup> Values in parentheses are those at which the bacterium exhibited susceptibility (S) or resistance (R).

<sup>c</sup> —, Discs not available.

<sup>d</sup> The value on the left is for trimethoprim; the value on the right is for sulfamethoxazole.

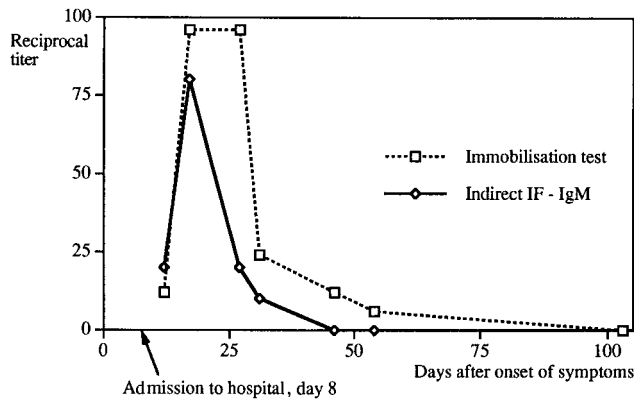


FIG. 3. Titers of anti-human IgM antibody in serum as measured by the immobilization test and the indirect immunofluorescence (IF) test.

testing. It is also noteworthy that the fluid culture media remained clear and transparent even in those samples where massive growth could be detected by acridine orange or Gram staining, implying a risk for such samples to be accidentally discarded. Prolonged cultivation of the coccoid motile variant of the bacterium resulted in transition to a rod-shaped and less motile variant. The conditions required for this transformation are not known, but the transformation might be induced by changed environmental conditions, or it might be a time-dependent constitutive change.

In serum samples obtained during the acute phase of the disease, a transient antibody response to the Rasbo bacterium was recorded by both an immunofluorescence test and an immobilization test. This antibody response was restricted to the IgM class. In a recent report of a culture-confirmed case of *B. quintana* infection, no serum antibody response either to the *B. quintana* type strain, as well as the patient's own isolate, or to *B. henselae* or *A. felis* was detected (9). Similarly, among patients showing characteristic histopathological features of cat scratch disease, serological confirmation was obtained in only 39.6% of the cases (10). The Rasbo bacterium, when adapted to fast in vitro culture on semisolid media, was used as the antigen in our serologies, and there is the possibility of a difference in antigenic properties from the in vivo form or the form that was cocultured with Vero cells as noted for *B. quintana* (8). This might be an explanation for the failure to detect antibodies of the IgG class.

Phylogenetic analysis based on 16S rDNA sequence comparison clearly placed the Rasbo bacterium within the alpha-2 subgroup of the alpha subclass of the *Proteobacteria*. Members of this group of bacteria are generally difficult to cultivate and thus to diagnose and are seldom identified by routine diagnostic procedures. Furthermore, the present study indicated that the Rasbo bacterium is most closely related to *Afipia* and *Methylobacterium*. The Rasbo bacterium formed a monophyletic subcluster with these genera. Moreover, the Rasbo bacterium branched off early, as shown in the tree in Fig. 1. Both the branch length and the values for similarity to the relatives of the alpha-2 subgroup suggest that the Rasbo bacterium is a new bacterial species phylogenetically located within this subgroup.

By transmission electron microscopy, the Rasbo bacterium was found to have a three-layer cell wall containing electron-dense rounded formations 0.1 to 0.3  $\mu\text{m}$  in diameter in the cytoplasm (Fig. 2). The function of these rounded formations is unknown.

In conclusion, the primary isolation of the Rasbo bacterium was achieved by not following established microbiological diagnostic procedures. When growth of bacteria occurs in cell cultures normally used for virus isolation, the cultivation material is usually discarded. In this case, however, because an infectious agent of an unusual nature was strongly suspected as the cause of disease in a severely ill patient, and because no bacteria could be observed in the control cell cultures, numerous specimens were taken and saved for further investigations. It is also notable that the presence of the Rasbo bacterium in tissue culture would probably have remained unnoticed, had the bacterium not been motile.

#### ACKNOWLEDGMENTS

We extend our thanks to Karl-Erik Johansson for sharing his knowledge regarding bacterial PCR technology and to Jan Fohlman for advice on serology.

This study was supported by funds from the Swedish Heart and Lung Foundation and from the Swedish Foundation for International Cooperation in Research and Higher Education (STINT).

#### REFERENCES

- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403-410.
- Anderson, B. E. 1993. PCR detection of *Rickettsia rickettsii* and *Ehrlichia chaffeensis*, p. 197-202. In D. H. Persing, T. F. Smith, F. C. Tenover, and T. J. White (ed.), *Diagnostic molecular microbiology: principles and applications*. American Society for Microbiology, Washington, D.C.
- Birkness, K. A., V. G. George, E. H. White, D. S. Stephens, and F. D. Quinn. 1992. Intracellular growth of *Afipia felis*, a putative etiologic agent of cat scratch disease. *Infect. Immun.* **60**:2281-2287.
- Brenner, D. J., S. P. O'Connor, H. H. Winkler, and A. G. Steigerwalt. 1993. Proposals to unify the genera *Bartonella* and *Rochalimaea*, with descriptions of *Bartonella quintana* comb. nov., *Bartonella vinsonii* comb. nov., *Bartonella henselae* comb. nov., and *Bartonella elizabethae* comb. nov., and to remove the family *Bartonellaceae* from the order *Rickettsiales*. *Int. J. Syst. Bacteriol.* **43**:777-786.
- Claffin, A. J., O. Smithies, and R. K. Meyer. 1966. Antibody responses in bursa-deficient chickens. *J. Immunol.* **97**:693-699.
- Cles, L. D., and W. E. Stamm. 1990. Use of HL cells for improved isolation and passage of *Chlamydia pneumoniae*. *J. Clin. Microbiol.* **28**:938-940.
- Daly, J. S., M. G. Worthington, D. J. Brenner, C. W. Moss, D. G. Hollis, R. S. Weyant, A. G. Steigerwalt, R. E. Weaver, M. I. Daneshvar, and S. P. O'Connor. 1993. *Rochalimaea elizabethae* sp. nov. isolated from a patient with endocarditis. *J. Clin. Microbiol.* **31**:872-881.
- Drancourt, M., J. L. Mainardi, P. Brouqui, F. Vandenesch, A. Carta, F. Lehnert, J. Etienne, F. Goldstein, J. Acar, and D. Raoult. 1995. *Bartonella (Rochalimaea) quintana* endocarditis in three homeless men. *N. Engl. J. Med.* **332**:419-423.
- Drancourt, M., V. Moal, P. Brunet, B. Dussol, Y. Berland, and D. Raoult. 1996. *Bartonella (Rochalimaea) quintana* infection in a seronegative hemodialyzed patient. *J. Clin. Microbiol.* **34**:1158-1160.
- Dupon, M., A. M. Savin De Larclause, P. Brouqui, A. De Mascarel, D. Raoult, J. Y. Lacut, and le Group d'étude. 1995. Contribution of serology testing in patients with characteristic histopathologic features of cat-scratch disease, abstr. K34, p. 293. In *Abstracts of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy*. American Society for Microbiology, Washington, D.C.
- English, C. K., D. J. Wear, A. M. Margileth, C. R. Lissner, and G. P. Walsh. 1988. Cat-scratch disease. Isolation and culture of the bacterial agent. *JAMA* **259**:1347-1352.
- Felsenstein, J. 1993. PHYLIP: phylogeny inference package (version 3.51c). University of Washington, Seattle.
- Hultman, T., S. Bergh, T. Moks, and M. Uhlen. 1991. Bidirectional solid phase sequencing of *in vitro*-amplified plasmid DNA. *BioTechniques* **10**:84-93.
- Hultman, T., S. Ståhl, E. Hornes, and M. Uhlen. 1989. Direct solid phase sequencing of genomic and plasmid DNA using magnetic beads as solid support. *Nucleic Acids Res.* **17**:4937-4946.
- Jukes, T. H., and C. R. Cantor. 1969. Evolution of protein molecules, p. 21-132. In H. N. Munro (ed.), *Mammalian protein metabolism*, vol. 3. Academic Press, Inc., New York, N.Y.
- Maidak, B., G. Olsen, N. Larsen, R. Overbeek, M. McCaughey, and C. Woese. 1996. The ribosomal database project (RDP). *Nucleic Acids Res.* **24**:82-85.
- McNee, J. W., A. Renshaw, and E. H. Brunt. 1916. Trench fever: a relapsing fever occurring with the British forces in France. *Br. Med. J.* **12**:225-234.

18. **Munk, R.-L.** 1917. Klink and Aetiologi des sogen "Wolhynischen Fiebers." Muench. Med. Wochenschr. **64**:1422–1426.
19. **Nash, P., and M. M. Krentz.** 1991. Culture media, p. 1226–1288. In A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.
20. **Nelson, R. A., and M. M. Mayer.** 1949. Immobilization of Treponema pallidum in vitro by an antibody produced in syphilitic infection. J. Exp. Med. **89**:369–393.
21. **Olsen, G. J., and C. R. Woese.** 1993. Ribosomal RNA: a key to phylogeny. FASEB J. **7**:113–123.
22. **Pettersson, B., K.-E. Johansson, and M. Uhlén.** 1994. Sequence analysis of 16S rRNA from mycoplasmas by direct solid-phase DNA sequencing. Appl. Environ. Microbiol. **60**:2456–2461.
23. **Pettersson, B., T. Leitner, M. Ronaghi, G. Bölske, M. Uhlén, and K.-E. Johansson.** 1996. Phylogeny of the *Mycoplasma mycoides* cluster as determined by sequence analysis of the 16S rRNA genes from the two rRNA operons. J. Bacteriol. **178**:4131–4142.
24. **Pettersson, B., M. Uhlén, and K.-E. Johansson.** 1996. Phylogeny of some mycoplasmas from ruminants based on 16S rRNA sequences and definition of a new cluster within the hominis group. Int. J. Syst. Bacteriol. **46**:1093–1098.
25. **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.
26. **Reiman, D. A., J. S. Loutit, T. M. Schmidt, S. Falkow, and L. S. Tomkins.** 1990. The agent of bacillary angiomatosis—an approach to the identification of uncultured pathogens. N. Engl. J. Med. **323**:1573–1580.
27. **Ripa, K. T., and P.-A. Mårdh.** 1977. Cultivation of *Chlamydia trachomatis* in cycloheximide-treated McCoy cells. J. Clin. Microbiol. **6**:328–331.
28. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**:406–425.
29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
30. **Stackebrandt, E., R. G. E. Murray, and H. G. Trüper.** 1988. *Proteobacteria* classis nov., a name for the phylogenetic taxon that includes the "purple bacteria and their relatives." Int. J. Syst. Bacteriol. **38**:321–325.
31. **Strong, R. P., E. Tyzzer, C. T. Brues, A. W. Selards, and J. C. Gastiaturú Veruga Peruviana.** 1913. Oroya fever and uta. JAMA **61**:1713–1716.
32. **The Swedish Reference Group for Antibiotics, Swedish Medical Society.** 1990. Referens—och metodbok för resistensbestämning av bakterier mot antibiotika, Swedish Medical Society, Stockholm, Sweden.
33. **Tully, J. G., R. F. Whitcomb, H. F. Clark, and D. L. Williamson.** 1977. Pathogenic Mycoplasmas: cultivation and vertebrate pathogenicity of a new Spiroplasma. Science **195**:892–894.
34. **Wahlberg, J., A. Holmberg, S. Bergh, T. Hultman, and M. Uhlén.** 1992. Automated magnetic preparation of DNA templates for solid phase sequencing. Electrophoresis **13**:547–551.
35. **Wang, S. P., and J. T. Grayston.** 1970. Immunologic relationship between genital TRIC, lymphogranuloma venereum and related organisms in a new microtiter indirect immunofluorescence test. Am. J. Ophthalmol. **70**:367–374.
36. **Wilkinson, H. W., B. J. Fikes, and D. D. Cruce.** 1979. Indirect immunofluorescence test for serodiagnosis of Legionnaires disease: evidence for serogroup diversity of Legionnaires disease bacterial antigens and for multiple specificity of human antibodies. J. Clin. Microbiol. **9**:379–383.
37. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51**:221–271.