Stenotrophomonas africana sp. nov., an Opportunistic Human Pathogen in Africa

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A gram-negative bacterium was isolated from a cerebrospinal fluid sample from an HIV-seropositive Rwandan refugee with primary meningoencephalitis. This Marseille-Goma sample B isolate, strain MGB^T (T = type strain), was found to exhibit evolutionary homology with *Stenotrophomonas maltophilia*, as determined by a 16S rRNA gene sequence analysis, and this finding was reflected by similar phenotypic traits. MGB^T could, however, be distinguished from the *S. maltophilia* type strain by using a number of biochemical and physiological tests, and a genotypic analysis of the two strains in which DNA homology was used revealed only 35% homology between them. Furthermore, the antibiotic susceptibility of MGB^T was restricted to netilmicin, ciprofloxacin, trimethoprim-sulfamethoxazole, and colimycin. On the basis of these results we propose that MGB^T is a representative of a new species in the genus *Stenotrophomonas*, *Stenotrophomonas africana*.

In August 1994, an outbreak of typhus was suspected among predominantly Rwandan refugees suffering from AIDS in Goma, Zaire, following the observation of cases of meningoencephalitis associated with bilateral conjunctivitis which led to death or were cured with chloramphenicol (1). Patients with the illness were found to be infested with body lice, and cerebrospinal fluid (CSF) samples were collected and were clear. However, a diagnosis of typhus was subsequently ruled out following examination of patients and serological testing. Furthermore, efforts to detect rickettsiae in body lice by molecular methods were unsuccessful. CSF samples were therefore studied further in order to identify the etiological agent of this outbreak. From one of these samples a highly resistant gram-negative bacterium was isolated. Polyphasic characterization methods demonstrated that this isolate was similar to, yet distinct from, *Stenotrophomonas (Xanthomonas) maltophilia*, a species known to cause hospital-acquired infections and recently implicated in opportunistic infections in HIV-infected patients (2).

MATERIALS AND METHODS

Bacterial strains. *Stenotrophomonas maltophilia* ATCC 13637^T (T = type strain) was obtained from the American Type Culture Collection, Rockville, Md. Isolate MGB^T (Marseille-Goma sample B) was obtained as described below.

Isolation, culturing, and phenotypic characterization of isolate MGB^T. CSF samples that were obtained by lumbar puncture of Rwandan refugees with febrile meningoencephalitis in Goma, Zaire, were studied. Gram-stained, cyto-centrifuged smears were microscopically observed at magnifications of $\times 100$ and $\times 1,000$, each smear was plated onto Trypticase soy agar (BioMérieux, Marcy-l'Etoile, France), chocolate agar (BioMérieux), and 5% sheep blood Colombia agar (BioMérieux), and the preparations were incubated at 37°C for up to 10 days.

The culture requirements of the strain referred to below as isolate MGB^T were further characterized by inoculating Trypticase soy agar, chocolate agar, 5% sheep blood agar, and MacConkey agar (BioMérieux) plates and incubating the preparations for 10 days at both 30 and 37°C. Phenotypic characterization of MGB^T was based on Gram staining, a motility test, a respiratory (the strain was incubated at room temperature in Schäedler broth containing 0.2% agar), the catalase test, and the oxidase test (Diagnostic Pasteur, Marnes, France). Further biochemical analysis was performed by inoculating API 20NE and API 50CHE strips (BioMérieux) according to the manufacturer's instructions. The strips were incubated at 25°C for 24 h. Carbon source utilization tests were performed by inoculating a Biotype-99 carbon source strip (BioMérieux) with Biotype 2 as-

simulation medium (BioMérieux) (4) and incubating the preparation at 30°C. Results were read on days 2 and 4. The vigor of the strain was defined as the number of positive characteristics observed on the strips (3). Specific tests for Tween 80 hydrolysis, lecithinase production, and requirements for methionine, nitrate, and disaccharides were performed as described previously (18). Antibiotic susceptibility tests were performed by the disk diffusion method on Mueller-Hinton medium after 24 h of incubation at 37°C under aerobic conditions (24). The MICs of the following antibiotics were determined: ticarcillin (Beecham-Sévigé, Paris, France), ticarcillin-clavulanic acid (Beecham-Sévigé), piperacillin (Lederle, Rungis, France), mezlocillin (Bayer-Pharma, Sens, France), imipenem (MSD-Chibert, Paris, France), cephalothin (Lilly, Saint-Cloud, France), moxalactam (Lilly), cefoperazone (Pfizer, Orsay, France), cefotaxime (Roussel, Paris, France), ceftazidime (Glaxo, Paris, France), gentamicin (Schering-Plough, Levallois Perret, France), tobramycin (Lilly), amikacin (Bristol, Paris, France), netilmicin (Unicet, Levallois Perret, France), trimethoprim-sulfamethoxazole (Roche, Neuilly sur Seine, France), ciprofloxacin (Bayer Pharma), rifampin (Merrell Dow, Neuilly sur Seine, France), fosfomycin (Clin Midy, Paris, France), and colimycin (Roger Bellon, Neuilly sur Seine, France).

Cellular fatty acids. Colonies of isolate MGB^T grown on Trypticase soy agar (Becton Dickinson Microbiology, Meylan, France) at 28°C for 24 h were saponified, and cell wall fatty acid methyl esters were extracted and analyzed by gas chromatography as described previously (14). The profile was analyzed with MIDI software based on the profiles determined for 22 *Stenotrophomonas maltophilia* strains.

DNA-DNA hybridization. DNA was extracted from Trypticase soy agar-grown MGB^T colonies and prototype strain colonies as described previously (5). Two micrograms of native DNA was labelled by nick translation with a nick translation kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) by using tritium-labelled nucleotides (Amersham International, Amersham, England). Hybridization experiments in which the S1 nuclease-trichloroacetic acid method was used were performed as described previously (8). Experiments were performed twice to verify reproducibility. Both DNAs were labelled and hybridized with unlabelled DNA to avoid errors due to a possible difference in genome sizes.

16S ribosomal DNA (rDNA) sequencing. The DNA extracted from isolate MGB^T was amplified by using PCR technology and universal primers fD1 and rp2 (23) (Eurogentec, Seraing, Belgium). PCR amplifications were performed in 50- μ l reaction mixtures by adding each deoxynucleoside triphosphate to a concentration of 100 mM, each primer to a concentration of 0.2 μ M, and 0.4 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). Amplifications were carried out with a Perkin-Elmer model 9600 thermal cycler by using 35 cycles consisting of denaturation at 90°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 60 s. The quality of the amplification was checked by ethidium bromide-stained 1% agarose gel electrophoresis. Each experiment included sterile water (no DNA) as a negative control and *Escherichia coli* DNA as a positive control. Amplified products were sequenced by using 5'-fluorescein-labelled primers fD1, 800f (5'-ATT AGA TAC CCT GGT AG-3'), 1050f (5'-TGT CGT CAG CTC GTG-3'), and RW1 (5'-TGA TGA CTT GAT GTC ATT CC-3') for direct sequencing, 5'-fluorescein-labelled primers rp2, 1050r, and 800r for reverse sequencing (primers 1050r and 800r were reverse complementary to primers 1050f and 800f, respectively), *Tth* DNA polymerase, and an AutoCycle sequencing kit (Pharmacia Biotech, Uppsala, Sweden) and were electrophoresed and analyzed with a model ALF DNA automatic sequencer (Pharmacia Biotech). To identify the 16S rRNA sequences most similar to the MGB^T sequence, searches of the EMBL and GenBank data libraries were performed by using the FASTA program (7). The sequence determined was then aligned with the se-

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FIG. 1. Negatively stained electron micrograph of strain MGB^T, showing two polar flagella (arrow). The micrograph was taken with a JEOL, model 1,200 electron microscope. Magnification, $\times 33,200$. Bar = 1 μm .

quences of *Stenotrophomonas maltophilia* (GenBank accession number M59158), *Pseudomonas aeruginosa* (GenBank accession number X06684), *Burkholderia cepacia* (GenBank accession number M22518), *Comamonas testosteroni* (GenBank accession number M11224), and *Cardiobacterium hominis* (GenBank accession number M35014) through Bisance (6) by using the Clustal package (9).

Serology. Agar-grown isolate MGB^T suspended in a 0.1% sodium azide solution was used as an antigen to test the CSF samples for the presence of immunoglobulin G (IgG), IgM, and IgA anti-MGB^T antibodies. A microimmunofluorescence test performed by the standard procedure (15) was used.

Nucleotide sequence accession number. The nucleotide sequence of the 16S rRNA gene of isolate MGB^T has been deposited in the GenBank database under accession number U62646.

RESULTS

Isolation and culture. Of the seven CSF samples studied, three were sterile, one yielded *Neisseria meningitidis*, one yielded *Streptococcus pneumoniae*, and one yielded *Bacillus cereus*. A gram-negative rod (isolate MGB^T) was observed microscopically in the last sample, which was found to contain anti-HIV antibodies. Isolate MGB^T formed faint fluorescent green colonies on Trypticase soy agar plates and grey colonies on sheep blood agar, chocolate agar, and MacConkey agar at both 30 and 37°C. Growth was not accompanied by the ammonia odor typical of *Stenotrophomonas maltophilia* and was strictly aerobic.

Phenotypic characterization and antibiotic susceptibility. Isolate MGB^T formed curved, *Vibrio*-like, gram-negative bacilli which were oxidase and indole negative and catalase positive. Motility was related to the presence of two polar flagella (Fig. 1 and Table 1). The isolate hydrolyzed Tween 80, gave a negative lecithinase reaction, and required methionine for growth. Growth was obtained when cellobiose, maltose, and lactose were used as carbon sources, but not when nitrate was

used as a nitrogen source. The isolate formed acid from glucose, fructose, mannose, sucrose, lactose, and maltose and hydrolyzed gelatin, esculin, and *ortho*-nitrophenyl- β -D-galactopyranoside. Lysine decarboxylase activity was present (Table 1). In the carbon source study, isolate MGB^T yielded the same auxanogram as the *Stenotrophomonas maltophilia* type strain except for the assimilation of *cis*-aconitate, which was positive for isolate MGB^T but not for the *Stenotrophomonas maltophilia* type strain. The cell wall fatty acid pattern of MGB^T was similar to that reported for *Stenotrophomonas maltophilia* (25) and identical to the identifying profile determined by an analysis of 22 strains by the MIDI software; i.e., C₁₅ fatty acids represented 45% of the total fatty acids and C₁₆ fatty acids represented 10% (C_{16:0} iso, 2.87%) of the total fatty acids. Isolate MGB^T was susceptible to netilmicin (MIC, 4 mg/liter), ciprofloxacin (MIC, <1 mg/liter), trimethoprim-sulfamethoxazole (MIC, 1 mg/liter), and colimycin (MIC, <2 mg/liter). It was resistant to all other antibiotics tested, including ticarcillin-clavulanic acid, cefoperazone, and imipenem (Table 2).

Genotypic identification. A total of 1,451 positions of the 16S rRNA gene of isolate MGB^T were sequenced. The *Stenotrophomonas maltophilia* 16S rDNA sequence, however, included 74 undetermined positions, and when these were removed from the analysis, 24 of the 1,377 paired positions of the isolate MGB^T 16S rDNA sequence and the *Stenotrophomonas maltophilia* 16S rDNA sequence differed, resulting in a level of similarity of 98.3%. The MGB^T sequence exhibited of 92.9% similarity with the *Stenotrophomonas (Xanthomonas) maltophilia* sequence, 83.3% similarity with the *Pseudomonas aerugi-*

TABLE 1. Characteristics of strain MGB^T

Characteristic	<i>Stenotrophomonas maltophilia</i> ATCC 13637 ^T	Strain MGB ^T	<i>Stenotrophomonas</i> sp. ^a
Gram stain reaction	Negative	Negative	Negative
Motility	+ ^b	+	+
No. of flagella	>1	2	NR
Oxidase	—	—	+
Catalase	+	+	NR
Growth requirements			
Methionine	—	—	NR
Nitrate	—	—	NR
Cellobiose	+	+	NR
Maltose	+	+	NR
Lactose	+	+	NR
Indole production	—	—	NR
Nitrate reduction	—	—	+
Acid produced from sucrose	+	+	—
Acid produced from lactose	+	+	—
Lactico dehydrogenase	+	+	—
Lecithinase production	—	—	NR
Gelatin hydrolysis	+	+	+
Tween 80 hydrolysis	+	+	NR
% 16S rRNA homology with ATCC 13637 ^T	100	98.7	94
% DNA hybridization with ATCC 13637 ^T	100	35	NR

^a New *Stenotrophomonas* species described by Bernard et al. (2).

^b +, positive; —, negative; NR, not reported.

TABLE 2. MICs of selected antibiotics against isolate MGB^T as determined by the disk diffusion method

Antibiotic	MIC (mg/liter)
Ticarcillin	256
Ticarcillin-clavulanate	256
Piperacillin	256
Mezlocillin	256
Imipenem	32
Cephalothin	128
Moxalactam	256
Cefoperazone	256
Cefotaxime	1,024
Ceftazidime	1,024
Gentamicin	32
Tobramycin	32
Amikacin	32
Netilmicin	4
Trimethoprim-sulfamethoxazole	1
Ciprofloxacin	<1
Rifampin	32
Fosfomycin	>32
Colimycin	<2

nosa sequence, 81.8% similarity with the *Burkholderia cepacia* sequence, and 81.5% similarity with the *Comamonas testoteroni* sequence. DNA-DNA hybridization revealed 35% similarity between isolate MGB^T and the *Stenotrophomonas maltophilia* type strain.

Serology. Five of the seven CSF samples tested were non-reactive, one sample exhibited a 1:10 IgG titer, and the sample which yielded isolate MGB^T exhibited titers of 1:10 for IgG, 1:8 for IgM, and 1:8 for IgA.

DISCUSSION

The role of isolate MGB^T in meningoencephalitis in a Rwandan refugee was determined by direct observation in and isolation from a CSF sample from one patient, by the vigor of the isolate determined after biochemical characterization, which indicated that the source of the isolate was clinical rather than environmental (3), and by the demonstration of specific antibodies in the CSF sample from which isolate MGB^T was recovered. Although the phenotypic characteristics of isolate MGB^T suggested that it was a *Stenotrophomonas* species, its isolation from the CSF of a patient suffering from primary meningoencephalitis and not nosocomial meningoencephalitis, its unusual vibrio-like morphology, and its broad-spectrum antibiotic resistance, which is unusual for a community-acquired strain, led us to question its relationship to the only previously recognized member of the genus *Stenotrophomonas*, *Stenotrophomonas maltophilia*, and to use a polyphasic approach for its identification. The 98.3% similarity of 16S rDNA sequences confirmed that there is a specific relatedness between isolate MGB^T and *Stenotrophomonas maltophilia*, but suggested that some divergence between the two organisms had occurred. DNA-DNA hybridization remains the ultimate criterion for determination of a bacterial species (22). At present, there is no consensus regarding the determination of bacterial taxa based on 16S rRNA gene sequencing, although it has been noted that strains sharing less than 97% similarity usually do not belong to the same species (17). A DNA hybridization value of 35% clearly indicates that strain MGB^T is distinct from *Stenotrophomonas maltophilia*. The genus *Stenotrophomonas* was recently created for *Stenotrophomonas mal-*

tophilia (16), a species initially assigned to the genus *Pseudomonas* (10, 11) and then to the genus *Xanthomonas* (19). The latter classification was considered controversial, especially because *Xanthomonas* and *Stenotrophomonas* spp. differ in basic properties that would require redefinition of the genus *Xanthomonas* to accommodate *Xanthomonas maltophilia* (20) and because the genus *Xanthomonas* previously included only plant pathogens (21).

Stenotrophomonas maltophilia has been shown to be responsible for nosocomial infections in patients exposed to large inocula or patients with impaired host defenses (12) and was implicated in catheter-related bacteremia and septicemia, urinary tract infections, respiratory tract infections, meningitis, serious wound infections, mastoiditis, epididymitis, conjunctivitis, and endocarditis (26). Recently, two groups of *Stenotrophomonas maltophilia*-related strains have been identified from environmental and clinical (immunocompromised patient) sources (2). The phenotypic characteristics of these isolates are different from those of our isolate (Table 1). Apart from primary pneumonia, *Stenotrophomonas maltophilia* has never been implicated in primary, nonnosocomial infections in humans. Isolate MGB^T is not nosocomial in origin, but was isolated from an HIV-infected patient. MGB^T is the most naturally resistant gram-negative bacterium isolated from a community-acquired infection. Antibiotics are not widely available in Central Africa, and the refugees in the area had received only chloramphenicol. Selection of a resistant strain is therefore very unlikely. *Stenotrophomonas maltophilia* is also highly resistant to antibiotics, but it is regularly susceptible to trimethoprim-sulfamethoxazole and some newer quinolones (13) and irregularly susceptible to ticarcillin-clavulanic acid, moxalactam, cefoperazone, and ciprofloxacin (12). Trimethoprim-sulfamethoxazole is the treatment of choice. Isolate MGB^T was even more antibiotic resistant than *Stenotrophomonas maltophilia*, since it was susceptible only to netilmicin, ciprofloxacin, trimethoprim-sulfamethoxazole, and colimycin. The recently reported *Stenotrophomonas maltophilia*-related isolates discussed above, however, were not multiresistant (2). A comprehensive polyphasic review of the characteristics of isolate MGB^T suggests that this strain belongs to a new *Stenotrophomonas* species. The name *Stenotrophomonas africana* is proposed for this new species.

Description of *Stenotrophomonas africana*. *Stenotrophomonas africana* (a. fri. ca' na. L. adj. *africana*, from Africa, the continent where the organism was first isolated) is a curved, gram-negative, nonsporulating bacterium whose cells are typically 0.5 µm wide and 1.5 µm long. It is motile by means of two polar flagella. Tests for oxidase and indole are negative, but catalase is present. It forms grey to green colonies lacking an ammonia odor. The biochemical pattern is identical to that of *Stenotrophomonas maltophilia*, except for the lack of assimilation of *cis*-aconitate. Antibiotic susceptibility is restricted to netilmicin, ciprofloxacin, trimethoprim-sulfamethoxazole and colimycin. A total of 24 of 1,377 16S rDNA base positions determined are different from those of *Stenotrophomonas maltophilia*, and the level of DNA hybridization between the species is 35%. *Stenotrophomonas africana* has been isolated once from the CSF of an African patient who tested positive for HIV antibodies and exhibited primary meningoencephalitis. The type strain of *Stenotrophomonas africana* is strain MGB, which has been deposited in the Collection de l'Institut Pasteur, Paris, France, as strain 10-48-54.

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