Description of *Tropheryma whipplei* gen. nov., sp. nov., the Whipple's disease bacillus

¹ Unité des Rickettsies, Université de la Méditerranée, Faculté de Médecine, CNRS UPRESA 6020, 27 Boulevard Jean Moulin, 13385 Marseille, France

² Department of Medical Microbiology, University of Zürich, Gloriastrasse 30, 8028 Zürich, Switzerland

Bernard La Scola,¹ Florence Fenollar,¹ Pierre-Edouard Fournier,¹ Martin Altwegg,² Marie-Noëlle Mallet¹ and Didier Raoult¹

Author for correspondence: Didier Raoult. Tel: +33 4 91 32 43 75. Fax: +33 4 91 38 77 72. e-mail: Didier.Raoult@medecine.univ-mrs.fr

A detailed characterization was performed of the Whipple's disease bacillus, strain Twist-Marseille^T, isolated from the cardiac valve of a patient with Whipple's disease bacillus endocarditis. This strain was isolated and maintained on human embryonic lung fibroblast monolayers, but could not be cultivated in the absence of living eukaryotic cells. Two morphological forms were observed, with differing staining properties; an intracellular form with intact and degenerating bacteria within vacuoles of infected cells and an extracellular form with masses of bacteria embedded in an extracellular matrix. Determination of the DNA G+C content confirmed that it belongs to the high-G+C Gram-positive bacteria. Strain Twist-Marseille^T (= CNCM I-2202^T) is proposed as the type strain of a new species within a new genus, *Tropheryma whipplei* gen. nov., sp. nov., that was provisionally created solely on the basis of 16S rRNA gene sequence data.

Keywords: *Tropheryma whipplei*, Whipple's disease, '*Tropheryma whippelii*', cell culture, molecular characterization

INTRODUCTION

Whipple's disease is a systemic bacterial infection characterized by fever, weight loss, diarrhoea, lymphadenopathy and polyarthritis. Occasionally, it is also associated with cardiac manifestations such as myocarditis, pericarditis and endocarditis (Vital-Durand *et al.*, 1997; Ratliff *et al.*, 1984) or central nervous system involvement, including dementia, lethargy and neurological deficits (Maizel *et al.*, 1970). The disease was first described by Whipple (1907) and its bacterial aetiology was confirmed in 1961 by electron microscopy (Yardley & Hendrix, 1961). Diagnosis of infection is usually based on histopathological examination of a duodenal biopsy showing infiltration by large macrophages that contain periodic acid–Schiff (PAS)-positive material (Black-Schaffer, 1949) or bacteria with a typical trilamellar membrane using electron microscopy. Wilson *et al.* (1991) and then Relman

et al. (1992) used broad-range primers to amplify bacterial 16S rRNA genes directly from infected tissue and then determined and compared the sequence to demonstrate that this bacterium belongs to the class *Actinomycetes*. On this basis, the bacterium was tentatively proposed as '*Tropheryma whippelii*' (Relman *et al.*, 1992). Determination of the nucleotide sequence of the 16S rRNA gene of '*T. whippelii*' provided the basis for the development of species-specific diagnostic PCR systems (Ramzan *et al.*, 1997). These PCR-based methods have become the standard diagnostic method for Whipple's disease. Recent PCR studies have demonstrated that '*T. whippelii*' is probably a ubiquitous or commensal micro-organism (Street *et al.*, 1999; Ehrbar *et al.*, 1999; Dutly *et al.*, 2000). '*T. whippelii*' has remained uncultivable, despite much effort (Relman, 1997), and its characteristics have remained enigmatic. The report of the isolation of the Whipple's disease bacterium in 1997 using human macrophages inactivated with interleukin-4 (Schoedon *et al.*, 1997) stimulated renewed interest in culture of the micro-organism, but this isolate could unfortunately not be subcultured. Using a shell-vial cell culture system, we recently isolated the Whipple's disease bacterium from the cardiac valve of a patient with Whipple's disease-related endocarditis and subse-

Abbreviations: HEL, human embryonic lung; ITS, internal transcribed spacer; PAS, periodic acid–Schiff.

The GenBank accession numbers for the 16S rDNA, ITS and 23S rDNA domain III sequences of strain Twist-Marseille^T are AF251035, AF248312 and AF248313, respectively.

quently established this strain in stable culture (Raoult *et al.*, 2000a). The strain has been deposited at the Collection Nationale de Culture de Micro-organismes de l'Institut Pasteur, Paris, France, as strain Twist-Marseille^T (= CNCM I-2202^T). We now report the extensive characterization of this bacterium and formally propose to name this micro-organism *Tropheryma whippelii* gen. nov., sp. nov.

METHODS

Strain cultivation. Details of the isolation of '*T. whippelii*' strain Twist-Marseille^T have been described elsewhere (Raoult *et al.*, 2000a). The strain was routinely subcultured in 150 cm² cell culture flasks on human embryonic lung (HEL) cell monolayers (CCL-137; ATCC, Manassas, VA, USA) grown in 30 ml minimal essential medium (Gibco) with 10% foetal calf serum (Gibco) and 2 mM L-glutamine (Gibco) and incubated at 37 °C in a 5% CO₂ atmosphere. Every 3–4 weeks (according to the infected cell ratio), supernatant from each of the infected cell culture flasks was removed and frozen at –80 °C for further studies. Infected cells were harvested by trypsinization and inoculated into three 150 cm² cell culture flasks with fresh medium.

Subculture assays. Propagation was attempted in the mammalian cell lines CACO-2 (HTB-37; ATCC), HL-60 (CCL-240; ATCC), MDCK (CCL-34; ATCC) and MRC-5 (CCL-171; ATCC) and on solid or in liquid axenic media, chocolate agar (bioMérieux), Columbia sheep blood agar (bioMérieux), BCYE agar (bioMérieux), Coletsos (Sanofi Pasteur), Middlebrook and Cohn 7H10 (Becton Dickinson), Schaedler (bioMérieux) and brain-heart infusion broth (Sanofi Pasteur), incubated at 32 and 37 °C, under 5% CO₂, microaerophilic and anaerobic atmospheres. Culture was also attempted in the cell culture medium alone and cell culture medium with lysate of HEL cells incubated at 32 and 37 °C in a 5% CO₂ atmosphere.

Production of mouse polyclonal antibodies. Six- to eight-week-old immunocompetent BALB/c mice were inoculated subcutaneously with 0.5 ml of a suspension of 10⁶ bacterial cells ml⁻¹ obtained from the supernatant of infected cells and Freund's complete adjuvant. Inoculations were repeated on days 10, 20 and 30. In order to check whether the bacteria were immunogenic to mice, 500 µl blood was sampled by puncture of the retro-orbital sinus on day 19. After centrifugation, antibody titres were estimated with anti-mouse globulin–fluorescein conjugate (Immunotech). On day 40, the mice were sacrificed and blood was sampled by intracardiac puncture. Before use, sera were adsorbed with HEL cells in order to remove non-specific anti-cell antibodies. Sera were mixed with a pellet of washed HEL cells. Cells were suspended in the serum and the mixture was shaken overnight at room temperature. After centrifugation at 10000 g for 10 min, the supernatant was removed and frozen at –80 °C for further studies.

Staining for optical microscopy. Harvested infected cells and supernatant were cytocentrifuged separately for staining. Slides were stained by acridine orange, Gram, Ziehl, Gimenez, May–Grünwald Giemsa and PAS (Chapin, 1995; Gimenez, 1964; Woods & Walker, 1996).

Immunofluorescence staining. Cells and supernatant were cytocentrifuged separately and fixed for 10 min in methanol. Immunofluorescence detection was also carried out on

infected cells cultured in shell vials (Raoult *et al.*, 2000a). Four weeks after inoculation, monolayers in shell vials were fixed for 10 min in methanol and washed twice with PBS. One-hundred microlitres of the primary antibody (patient serum or mouse polyclonal antibody), diluted 1:200 in PBS with 3% (w/v) non-fat dried milk, was added to the slides and incubated in a moist chamber at 37 °C for 30 min. After three washes in PBS, the slides were incubated for 30 min at 37 °C with 100 µl fluorescein isothiocyanate-conjugated goat anti-human or anti-mouse Ig (bioMérieux) diluted 1:200 in PBS containing 0.2% Evans blue. After three washes with PBS, the cover slips were mounted (cells face down) in phosphate-buffered glycerol medium pH 8 (Sanofi Pasteur) and examined using an epifluorescence microscope at 400 × magnification.

Electron microscopy. Harvested infected cells and supernatant were suspended separately for fixation in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 0.1 M sucrose for 1 h at 4 °C. Fixed cells were washed overnight with the same buffer and then fixed for 1 h at room temperature with 1% osmium tetroxide in 0.1 M cacodylate buffer. Dehydration was performed through washes of increasing concentrations (25–100%) of acetone. The cells were then embedded in Araldite (Fluka). Thin sections were cut from embedded blocks using an Ultracut microtome (Reichert-Leica) and were post-stained with a saturated solution of methanol/uranyl acetate and lead nitrate with sodium citrate in water before examination using a JEOL 1220 electron microscope. The presence of flagella was assessed by depositing supernatant on copper grids coated with Formvar (Sigma) and staining with a 0.33% solution of uranyl acetate.

Purification of bacteria for SDS-PAGE and G+C content analysis. HEL cells infected with bacteria were harvested from forty 150 ml flasks into 40 ml PBS. Trypsin (Gibco) was added to the suspension to a final concentration of 5 mg ml⁻¹ and the suspension was incubated at 30 °C for 45 min. Next, the suspension was subjected to sonication (three periods of 1 min on ice), following which unlysed cells were removed by centrifugation at 100 g for 15 min. The supernatant was layered onto a 25% (w/v) sucrose solution in PBS. After centrifugation at 9000 g for 30 min at 4 °C, the pellet containing bacteria was resuspended in 2 ml PBS and layered carefully onto a 25:45% (w/v in PBS) renograffin step gradient. This gradient was subjected to centrifugation at 130000 g for 1 h at 5 °C. The bacteria were then harvested from the 25:45% interface and washed twice in PBS. For SDS-PAGE, bacteria were resuspended in sterile distilled water at a final concentration of 1 mg ml⁻¹. For DNA G+C content analysis, bacteria were resuspended in 2 ml PBS and proteinase K was added to a final concentration of 10 mg ml⁻¹. This suspension was incubated overnight at 37 °C and then submitted to a further centrifugation on a 25:45% renograffin step gradient. The bacteria were harvested and washed as described above and then resuspended in the smallest possible volume of sterile distilled water before use.

SDS-PAGE and Western blotting. In order to characterize the total protein profile of strain Twist-Marseille^T, samples were sonicated (three periods of 1 min on ice) and the proteins were then extracted in SDS-PAGE sample buffer [buffer 1 containing 200 mM Tris/HCl, pH 8.8, 5 mM EDTA, 1 M sucrose and 0.01% bromophenol blue; buffer 2 containing 18% SDS, 0.3 M dithio-1,4-erythritol and 5% (v/v) 2-mercaptoethanol; the sample buffer corresponds to 1 vol. buffer 2 to 4 vols buffer 1] and heated, for 15 min at 100 °C.

Fig. 1. Cultivated cells of *T. whipplei* observed using an inverted microscope on a 6-week-old culture. Bars, 30 µm. (a) Large, coarse, rounded structures observed on cell monolayers (arrow). (b) Long, rope-like structures due to aggregated extracellular bacteria.

SDS-PAGE was performed by the method of Laemmli (1970) on 9–16% polyacrylamide linear gradient gels (18 cm × 20 cm × 1.5 mm) at 60 mA constant current per gel and 10 °C, until the dye front reached the bottom of the gel. Proteins were visualized with silver stain according to the modified Blum procedure (Nesterenko *et al.*, 1994).

For Western blotting, proteins were extracted and heated as in SDS-PAGE prior to electrophoresis on 8% separating gels. A total of 10 µg total protein was loaded per lane. Following electrophoresis, the proteins were electroblotted

to nitrocellulose membranes at 100 V for 1 h at 10 °C. The membranes were blocked with 5% (w/v) non-fat dried milk in Tris buffer (10 mM Tris/HCl, pH 8.8, 150 mM NaCl, 0.1% Tween 20) overnight and then incubated with mouse antiserum against strain Twist-Marseille^T diluted 1:2000 in 3% (w/v) non-fat dried milk in PBS for 1 h at room temperature. After three 10 min washing steps in Tris buffer, the membranes were incubated with goat anti-mouse Ig G + M (Jackson ImmunoResearch) diluted to 1:3000 with PBS. After three washes in Tris buffer, immunodetection was performed by using the ECL chemiluminescence system (Amersham) followed by exposure to Boehringer Mannheim film for 15 s.

PCR amplification and sequencing of the 16S rRNA gene, internal transcribed spacer (ITS) region and domain III of the 23S rRNA gene. For the study of extracellular bacteria, 500 µl cell culture supernatant was centrifuged at 1000 *g* for 10 min in order to remove cells. The supernatant was then centrifuged at 12000 *g* for a further 5 min. The pellet was resuspended in 90 µl TE buffer. For the study of intracellular bacteria, cells from an infected cell culture flask were harvested in 500 µl culture medium. Cells were rinsed three times in PBS by centrifugation at 1000 *g* for 10 min. The resulting pellet was resuspended in 90 µl TE buffer. Suspensions of intracellular and extracellular bacteria were frozen separately in liquid nitrogen and thawed immediately at 100 °C in a heat block. This procedure was repeated three times. Ten µl of a stock solution of 10 g lysozyme l⁻¹ (Research Organics) was added and the tubes were incubated at 37 °C for 1 h. DNA was extracted using Qiagen columns (QIAamp tissue kit; Qiagen) as described by the manufacturer. Using the broad-range primer pair fD1 and rP2 (Weisburg *et al.*, 1991), the 16S rRNA gene was amplified by PCR. This fragment was sequenced as described previously (La Scola *et al.*, 1998). The 16S–23S rDNA ITS was amplified and sequenced by using the primers tws3/f (5'-CCGGTGACTTAACCTTTTGGAGA-3') and tws4/r (5'-TCCCCGAGGCTTATCGCAGATTG-3') as described previously (La Scola *et al.*, 1997; Hinrikson *et al.*, 1999). A fragment of domain III of the 23S rDNA was amplified and sequenced using the primers TW-23InsF (5'-GGTTGATA-TTCCCGTACCGGCAAAG-3') and TW-23InsR2 (5'-GCATAGGATCACCAATTTTCGCGCC-3') as described previously (Hinrikson *et al.*, 2000a). A commercially available sequencing kit was used for sequencing (dRhodamine Terminator cycle-sequencing ready reaction kit with AmpliTaq Polymerase FS; PE Applied Biosystems). Sequencing products were purified by ethanol/magnesium precipitation and then resolved on a 5% polyacrylamide gel (Long Ranger Singel packs, type 377-36cm WTR; Tebu) by electrophoresis on an ABI PRISM 377 DNA sequencer (Perkin Elmer).

Analysis of sequence data. Sequences of the 16S rRNA gene, ITS and domain III of 23S rRNA gene of strain Twist-Marseille^T were compared with DNA sequence databases using the program BLAST 2.0 (NCBI).

DNA G + C content analysis. DNA was extracted as described previously (Ausubel *et al.*, 1995). Nucleosides residue analysis was performed by HPLC using a model L6200A system pump and a UV detector L4250 (Merck Clevenot). An aliquot of 5 µl of the sample was applied onto a Nucleosil 100-SC18 Lichrocart column (4 × 250 mm) (Merck). Elution was carried out at room temperature using a mixture of 0.2 M NH₄H₂PO₄ (pH 4.5) and acetonitrile (96:4, v/v). A flow rate of 1 ml min⁻¹ was used and absorbance was

monitored at 270 nm. The calibration curve was obtained from a mixture of four standard nucleosides (Sigma) at a concentration of 0.1 mg ml^{-1} each in distilled water. After chromatography, the relative concentration of each nucleotide was calculated on the basis of peak areas in the HPLC dilution profile and corrected by coefficients of relative molar absorption, as described previously (Tamaoka & Komagata, 1984).

RESULTS

Strain cultivation

After inoculation of fresh cell culture medium, HEL cells multiplied for approximately 10 d, as observed by using an inverted microscope. Small, coarse and dark inclusions appeared within cells (Fig. 1a). As incubation continued, large, coarse and rounded structures appeared on the cell monolayer and long rope-like

Fig. 2. Staining of extracellular *T. whipplei* by Gram staining (a) and PAS staining (b). Bars, 15 μm .

Fig. 3. Immunofluorescence staining of the Whipple's disease bacterium isolate in an infected HEL cell monolayer in a shell vial, revealed by patient serum. Bar, 6 μm .

structures appeared in the supernatant (Fig. 1b). As these structures increased in number, the cell population began to decline. Good bacterial growth was obtained on subculture with MRC-5 cells. In HL-60 cells, bacteria were observed mostly as extracellular rods, but these disappeared after three subcultures. Despite incubation for up to 12 weeks, no growth was obtained on any of the axenic media used. The time required to split and passage a cell culture into three subcultures and then to obtain the same degree of infection in the subcultures as observed in the initial culture prior to splitting was about 4 weeks, a period in accordance with the 18 d doubling time determined previously (Raoult *et al.*, 2000a). To date, we have achieved 29 successful passages of the Whipple's disease bacterium.

Morphological studies

Stains for optical microscopy. Similar structures were observed with all staining methods. Small, slender bacilli within large vacuoles were observed after cytocentrifugation of cells. Aggregates of bacilli, arranged regularly as long rope-like structures, were observed after cytocentrifugation of supernatants. On Gimenez staining, intracellular rods were poorly stained and appeared as pale-pink structures. Vacuoles appeared bright pink within blue cytoplasm. Aggregates of extracellular bacilli appeared pale blue. On Gram staining, bacterial structures were all poorly stained and always appeared as Gram-negative (Fig. 2a). Bacteria were not visible by Ziehl-Neelsen staining. On May-Grünwald Giemsa, both intracellular bacteria and aggregates were visualized, but intra-

Fig. 4. Electron microscopy of *T. whipplei* cultured in HEL cell monolayers. (a) Transmission electron microscopy of *T. whipplei* (arrowheads) in HEL cells within vacuoles. Bar, 2 μ m. (b) Transmission electron microscopy of two extracellular *T. whipplei* cells, of which one is dividing. Bar, 100 nm. (c) Transmission electron microscopy and negative staining of individual extracellular *T. whipplei* cells. Bar, 500 nm. (d) Scanning electron microscopy of aggregated extracellular *T. whipplei* cells embedded in an extracellular matrix. Bar, 2 μ m.

cellular bacteria were clustered. Using acridine orange staining, bacteria were visible within cells but were difficult to distinguish from cell components. Aggre-

gates of extracellular bacteria appeared bright orange. Better results were obtained with PAS staining, as PAS-positive bacilli appeared more numerous than

with other stains. Intracellular bacteria were clustered into vacuoles that were clearly visible within the cytoplasm of infected cells. Individual bacteria were also seen within the cytoplasm of infected cells. Thus, cells appeared filled with coarse, PAS-positive conglomerates and short, slender, PAS-positive rods. Most long, slender bacilli were observed extracellularly, usually as aggregates (Fig. 2b).

Immunofluorescence staining. Visualization of bacteria varied according to the source of bacteria used and the antibody used for detection. Patient serum and cyto-centrifuged cells revealed few intracellular bacteria on immunofluorescence staining. The same serum on cell monolayer shell-vial cultures allowed good visualization of intracellular bacteria, which appeared brightly fluorescent. The morphology observed with immunofluorescence staining was the same as that observed with PAS staining, with several individual bacteria within vacuoles and large fluorescent vacuoles with no individual bacteria (Fig. 3). Most extracellular bacteria, especially the long rope-like structures, could not be stained. The use of mouse polyclonal antibodies yielded the same results, but allowed detection of bacteria on cyto-centrifuged cells. Aggregates in long rope-like structures were again not stained. The success of immunofluorescence staining of cultures was dependent on the age of culture; the presence of large fluorescent vacuoles and non-fluorescent extracellular rods was noted with increasing frequency, the older a culture became.

Electron microscopy. Transmission electron microscopy confirmed that PAS-positive inclusions and immunopositive material corresponded to intact and degenerating bacteria within vacuoles (Fig. 4a). Dividing cells were observed within vacuoles. The cell wall had a specific structure consistent with the previous description of the Whipple's disease bacterium (Silva *et al.*, 1985; Raoult *et al.*, 2000a); the plasma membrane was surrounded by a thin, homogeneous wall and a plasma membrane-like structure, resulting in the typical trilamellar appearance. Long, rope-like structures were aggregates of extracellular bacteria, all orientated in the same direction (Fig. 4b). Dividing cells were observed among the extracellular bacteria. These extracellular bacteria adhered to each other through an extracellular matrix. On negative staining, there was a tendency to aggregate and no flagella were observed (Fig. 4c). On scanning electron microscopy, the bacteria appeared aggregated, orientated in the same direction and embedded in an extracellular matrix (Fig. 4d).

SDS-PAGE analysis and Western blot immunoassay

SDS-PAGE showed the presence of proteins from 213 to 10 kDa, with major protein bands of 213, 110 and 85 kDa (Fig. 5). Polyclonal mouse serum recognized several antigens with dominant bands of approximately 40, 110 and 213 kDa and several less intense bands (Fig. 5).

Fig. 5. Silver-stained SDS-PAGE of whole-cell protein preparations of *T. whipplei* (left) and Western blot of *T. whipplei* with mouse polyclonal antiserum (right).

PCR amplification and sequencing of the 16S rRNA gene, ITS region and domain III of 23S rRNA gene

After amplification and sequencing of the 16S rRNA gene of the isolate, a 1484 bp sequence was compared with DNA sequences accessible in public databases using the program BLAST 2.0. The sequence showed 100% similarity to two 16S rRNA gene sequences of '*T. whipplei*' available in GenBank (X99636.1 and M874841). The 472 bp sequence derived from the ITS of the isolate showed 100% similarity to the ITS sequence of '*T. whipplei*' (GenBank accession no. AF100950) designated by Hinrikson *et al.* (1999) as genotype 2. The 225 bp sequence derived from domain III of the 23S rDNA sequence from the isolate showed 100% similarity to the sequence available in GenBank (accession no. AF148136) designated by Hinrikson *et al.* (2000a) as genotype A. Thus, '*T. whipplei*' strain Twist-Marseille^T (CNCN I-2202^T) corresponds to genotype 2A, as defined by Hinrikson *et al.* (2000b). The same data were obtained when analysis was performed separately on intracellular or extracellular bacteria.

DNA G + C content

The DNA G + C content was determined to be 59.4 mol%.

DISCUSSION

Whipple's disease has long been recognized as a bacterial disease, but the causative agent was not established successfully *in vitro* until 1999 (Raoult *et al.*, 2000a). The first non-controversial identification of the organism that causes Whipple's disease was provided by Wilson *et al.* (1991) and was based on partial 16S rDNA gene amplification and sequencing from a single patient. This work has been confirmed and extended with specimens from several patients and controls (Relman *et al.*, 1992). Although no type strain has been available, the provisional name '*T. whippelii*' is now widely accepted (Relman *et al.*, 1992; Murray & Schleifer, 1994). A single report of the propagation of '*T. whippelii*' from cardiac valves was published and two passages of the organism were achieved using human macrophages (Schoedon *et al.*, 1997). However, this isolate no longer exists and was not deposited in a culture collection. We propagated '*T. whippelii*' using a human fibroblast cell line with no specific culture conditions except for a prolonged incubation period, probably necessary because of the long doubling time (18 d) (Raoult *et al.*, 2000a). Cell lines with short lives, such as human macrophages, cannot therefore be used effectively to propagate the bacterium. Moreover, cells that lack contact inhibition or cannot grow at a slow rate (such as mouse macrophages) are probably also inappropriate.

In cell cultures, *Tropheryma whipplei* appeared in two forms, an intracellular form located within vacuoles of infected cells and an extracellular form that appeared as massive aggregates of bacteria embedded in an extracellular matrix. The two forms of the bacterium did not have the same staining characteristics. All bacteria were readily visualized by acridine orange or PAS staining. The bacteria always appeared as Gram-negative, despite phylogenetic studies that place *T. whipplei* in the group of Gram-positive bacteria (Maiwald *et al.*, 1996). Intracellular bacteria were stained poorly by Gimenez stain. Immunofluorescence staining of infected cells in shell-vial culture using either mouse polyclonal antibody or serum from a patient with Whipple's endocarditis clearly visualized intracellular bacteria. Cyto-centrifuged cells were immunopositive only with mouse polyclonal anti-serum. This peculiarity led us to develop an immunofluorescence assay that used labTek slides (Raoult *et al.*, 2000a) rather than our routine assay (La Scola & Raoult, 1996). Extracellular bacteria were not stained by Gimenez and could not be observed by immunofluorescence. We hypothesize that these differences in staining are due to the extracellular matrix observed around bacteria, which may mask reactive antigens that are exposed when bacteria are degraded by lysosomal enzymes. When harvested separately and inoculated separately on non-infected cells, the two forms of the bacterium (intracellular and extracellular) are able to grow intracellularly, producing long, rope-like structures (data not shown). As no growth was obtained on axenic media in this study or in culture

media with lysed eukaryotic cells (Raoult *et al.*, 2000a), the presence of intact cells appears to be necessary for bacterial growth. Although numerous bacteria are observed within cells, the presence of large aggregates of bacteria including dividing cells in the culture supernatant indicates that *T. whipplei* may not be a strictly intracellular bacterium.

The advent of molecular tools such as 16S rDNA amplification and sequencing has led to the identification of bacteria without prior cultivation. Nevertheless, knowledge of a 16S rDNA gene sequence alone is not considered a sufficient basis for the formal description of a bacterium. As outlined by several authors (Murray & Schleifer, 1994; Stackebrandt & Goebel, 1994), although 16S rDNA sequences may be helpful in separating species, defining new species genetically may require DNA–DNA hybridization. It is well established that different species (as in the genus *Bacillus*) may have identical 16S rDNA gene sequences (Fox *et al.*, 1992; Ash *et al.*, 1991) and there may also be variation of these sequences within a well-defined species, such as *Bartonella henselae* (Drancourt *et al.*, 1996). When the 16S rRNA genes of two bacteria are identical, the analysis of alternative, less well-conserved genes may help in assessing the taxonomic juxtaposition of the two strains and therefore provide an indication as to whether or not they belong to the same species. The remarkable diversity in domain III of the 23S rRNA gene found between various bacterial species but not among strains of the same species has led to this DNA fragment being proposed as a suitable alternative to 16S rRNA. Indeed, the usefulness of analysis of this domain has previously been demonstrated in defining *Actinobacterium* taxonomy (Roller *et al.*, 1992). The identity of the 23S rRNA gene sequences of the strain Twist-Marseille^T and those of bacteria detected in other cases of Whipple's disease is therefore a good indicator that all these strains form a single species. Combined analysis of the ITS and the 23S rRNA gene indicates that strain Twist-Marseille^T possesses the 2A genotype, as proposed by Hinrikson *et al.* (2000a, b). However, as this classification is based only on data derived from DNA sequences amplified directly from infected tissues of Whipple's disease patients, determination of its correlation with other, specifically pangenomic typing schemes has not been possible. Clearly, such an undertaking can only be performed when a number of representative strains have been isolated.

That the strain Twist-Marseille^T is indeed a member of the bacterial species implicated in Whipple's disease is further witnessed by the observation that polyclonal antibodies that recognize this strain specifically are also reactive in immunohistochemical visualization of organisms infecting duodenal biopsies taken from patients with typical Whipple's disease (Raoult *et al.*, 2000b). To assist the recording of new putative taxa of prokaryotes based on limited data such as 16S rDNA gene sequences, it was proposed that such microorganisms should be given '*Candidatus*' status until

sufficient data become available to give an official name (Murray & Schleifer, 1994). Following these recommendations, the organism described by Relman *et al.* (1992) as '*T. whippelii*' has never been validated. After successful cultivation, including stable subcultures (Raoult *et al.*, 2000a), we provide additional phenotypic and genotypic data that now allow formal description and naming of the new species. To quote Murray & Schleifer (1994), 'it must be hoped that the candidate names used should be validated, but this cannot be guaranteed, and other names might then be more appropriate'. In order to avoid any confusion that could result from the introduction of a new name, especially for clinicians with patients suffering from Whipple's disease, we propose to retain the name *Tropheryma whippelii*. We have corrected '*whippelii*' to '*whipplei*', as the name Whipple must be correctly Latinized to *whippleus* and thus the genitive is *whipplei*.

Description of *Tropheryma* gen. nov.

Tropheryma (Tro.phe.ry'ma. Gr. n. *trophe* nourishment; Gr. n. *eryma* barrier; N.L. fem. n. *Tropheryma* barrier to nourishment, so named because it causes malabsorption).

The type species is *Tropheryma whipplei*. The genus and species descriptions are combined below until further species are described.

Description of *Tropheryma whipplei* sp. nov.

Tropheryma whipplei (whipp'le.i. N.L. gen. n. *whipplei* of Whipple, after George Whipple, who described the first patient with the clinical syndrome that is today known as Whipple's disease).

Formerly named '*Tropheryma whippelii*'. Short rods, 0.25–0.3 µm in diameter and 0.8–1.7 µm long, sometimes longer when cell division is impaired. Non-motile. Flagella do not occur. The plasma membrane is surrounded by a thin homogeneous wall and by a plasma membrane-like structure, resulting in a trilamellar appearance of the cell wall under electron microscopy. Appear Gram-negative. Ziehl-negative. Retain basic fuchsin when stained by the method of Gimenez when intracellular. Well stained by PAS staining. Although we have shown that the bacterium can be observed extracellularly, it has not been cultivated in the absence of host cells. Grows well in or associated with HEL and MRC-5 cells in minimal essential medium with 10% foetal calf serum and 2 mM L-glutamine when incubated at 37 °C in a 5% CO₂ atmosphere. May be observed in an intracellular or extracellular location. When extracellular, has a tendency to aggregate in masses of bacteria embedded in an extracellular matrix, with the appearance of long, rope-like structures. Doubling time has been estimated to be 18 d. Well preserved by rapid freezing and storage at –80 °C. On SDS-PAGE, proteins of 213 to 10 kDa are detected, with major bands of 213 and 85 kDa. Sequence of the 16S rDNA gene places it in the class *Actinomycetes* and sequences of the 16S–23S

rDNA ITS and domain III of the 23S rDNA identify the strain as genotype 2A. The DNA G + C content of strain Twist-Marseille^T is 59.4 mol%. Opportunistic pathogen for humans. Responsible for Whipple's disease, a systemic infection with symptoms of fever, weight loss, diarrhoea, polyadenopathy and polyarthritis. Occasionally responsible for cardiac manifestations such as myocarditis, pericarditis and endocarditis or central nervous system involvement that may include dementia, lethargy and neurological deficits or ocular diseases such as uveitis.

The type strain is Twist-Marseille^T (= CNCM I-2202^T), isolated from the aortic valve of a patient with endocarditis. This strain has been deposited in the Collection Nationale de Cultures de Micro-organismes (CNCM, Institut Pasteur, Paris, France) and the Collection de l'Unité des Rickettsies (WHO Collaborative Center for Rickettsial Reference and Research, Marseilles, France), and is available upon request from the authors or from the Institut National de la Propriété Industrielle (Paris, France).

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