Marinomonas brasilensis sp. nov., isolated from the coral *Mussismilia hispida*, and reclassification of *Marinomonas basaltis* as a later heterotypic synonym of *Marinomonas communis*

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A Gram-negative, aerobic bacterium, designated strain R-40503^T, was isolated from mucus of the reef-builder coral *Mussismilia hispida*, located in the São Sebastião Channel, São Paulo, Brazil. Phylogenetic analyses revealed that strain R-40503^T belongs to the genus *Marinomonas*. The 16S rRNA gene sequence similarity of R-40503^T was above 97 % with the type strains of *Marinomonas vaga*, *M. basaltis*, *M. communis* and *M. pontica*, and below 97 % with type strains of the other *Marinomonas* species. Strain R-40503^T showed less than 35 % DNA–DNA hybridization (DDH) with the type strains of the phylogenetically closest *Marinomonas* species, demonstrating that it should be classified into a novel species. Amplified fragment length polymorphism (AFLP), chemotaxonomic and phenotypic analyses provided further evidence for the proposal of a novel species. Concurrently, a close genomic relationship between *M. basaltis* and *M. communis* was observed. The type strains of these two species showed 78 % DDH and 63 % AFLP pattern similarity. Their phenotypic features were very similar, and their DNA G+C contents were identical (46.3 mol%). Collectively, these data demonstrate unambiguously that *Marinomonas basaltis* is a later heterotypic synonym of *Marinomonas communis*. Several phenotypic features can be used to discriminate between *Marinomonas* species. The novel strain R-40503^T is clearly distinguishable from its neighbours. For instance, it shows oxidase and urease activity, utilizes L-asparagine and has the fatty acid $C_{12:1}$ 3-OH but lacks $C_{10:0}$ and $C_{12:0}$. The name *Marinomonas brasilensis* sp. nov. is proposed, with the type strain R-40503^T (=R-278^T =LMG 25434^T =CAIM 1459^T). The DNA G+C content of strain R-40503^T is 46.5 mol%.

Mussismilia hispida is one of the major reef-builder corals along the north-eastern Brazilian coast, and it also has the widest geographical distribution among its genus (from Maranhão to Santa Catarina state, approx. 5000 km) (Leão & Kikuchi, 2005). The ability of *Mussismilia* corals to survive in different regions indicates their adaptation to wide environmental gradients, such as temperature, water turbidity and pollution. However, recent studies have revealed that *Mussismilia hispida* and *Mussismilia braziliensis* are threatened by extinction (de Castro *et al.*, 2010; Francini-Filho *et al.*, 2008). Micro-organisms appear to play a key role in coral health. Micro-organisms and the coral make up the holobiont (Rosenberg *et al.*, 2007). The holobiont microbiota appears to protect its host by providing nourishment and antibiotics (Raina *et al.*, 2009; Shnit-Orland & Kushmaro, 2009). It is also recognized that the holobiont harbours a wide microbial diversity. In the last 10 years, a growing number of studies have focused on

Abbreviations: AFLP, amplified fragment length polymorphism; DDH, DNA–DNA hybridization.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain R-40503^T and *M. basaltis* LMG 25279^T determined in this study are GU929940 and GU929941.

Two supplementary figures and a supplementary table are available with the online version of this paper.

the diversity and ecology of the coral microbiota (Alves *et al.*, 2010; Dinsdale *et al.*, 2008; Rohwer *et al.*, 2001).

The genus *Marinomonas* was created in 1983 to accommodate *Alteromonas communis* and *Alteromonas vaga* (Baumann *et al.*, 1972), which were distinct from the other species of *Alteromonas* (van Landschoot & De Ley, 1983). At the time of writing, the genus *Marinomonas* comprised 15 species, mainly originating from seawater from different geographical locations. *Marinomonas communis* and *Marinomonas vaga* (Baumann *et al.*, 1972; van Landschoot & De Ley, 1983) were isolated from the Pacific Ocean, *Marinomonas pontica* (Ivanova *et al.*, 2005) from the Black Sea, *Marinomonas dokdonensis* (Yoon *et al.*, 2005) from the East Sea of Korea and *Marinomonas mediterranea* (Solano & Sanchez-Amat, 1999) and *Marinomonas aquimarina* (Macián *et al.*, 2005) from the Mediterranean Sea. *Marinomonas polaris* (Gupta *et al.*, 2006) and *Marinomonas ushuaiensis* (Prabagaran *et al.*, 2005) were isolated from subantarctic regions, while *Marinomonas primoryensis* (Romanenko *et al.*, 2003) and *Marinomonas arctica* (Zhang *et al.*, 2008) were isolated from sea-ice. *Marinomonas ostreistagni* (Lau *et al.*, 2006) and some *M. aquimarina* strains (Macián *et al.*, 2005) were isolated from oysters. *Marinomonas basaltis* (Chang *et al.*, 2008) and *Marinomonas arenicola* (Romanenko *et al.*, 2009) were isolated from marine sediment, while *Marinomonas balearica* and *Marinomonas pollencensis* (Espinosa *et al.*, 2010) were isolated from seagrass *Posidonia oceanica*.

In the present study, isolate R-40503^T, obtained from mucus of an apparently healthy *Mussismilia hispida* coral located on the rocky shore of Grande beach (23° 50′ 25″ S 045° 24′ 59″ W) in São Sebastião Channel, São Paulo, Brazil, in the summer of 2005, during a survey of the heterotrophic bacterial diversity associated with cnidarians in São Paulo (Brazil) (Chimetto *et al.*, 2008, 2009), was investigated using a polyphasic taxonomic approach. The strain was isolated using nitrogen-free (NFb) selective medium supplemented with 3 % NaCl after 4 days of incubation at 28 °C.

Five strains (R-236, R-237, R-249, R-256 and R-278^T) isolated at the time of collection as described in Chimetto *et al.* (2008) clustered together in a new taxon by partial 16S rRNA gene sequence comparison, but only strain R-278^T (=R-40503^T) maintained viability. The almost-complete 16S rRNA gene sequence of R-40503^T (1425 nt) was obtained as described previously (Chimetto *et al.*, 2008, 2009). The raw sequence data were transferred to the ChromasPro version 1.34 software (Technelysium Pty. Ltd), with which a consensus sequence was determined. The sequence was aligned with sequences from EMBL using the CLUSTAL W software (Chenna *et al.*, 2003). Pairwise similarities were calculated with the BioNumerics 4.61 software (Applied Maths), using an open gap penalty of 100 % and a unit gap penalty of 0 %. Similarity matrices and phylogenetic trees were constructed using MEGA version 4.0 (Tamura *et al.*, 2007) and the BioNumerics 4.61 software. Trees were drawn using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Eck & Dayhoff, 1966) methods. The robustness of the tree topologies was checked by bootstrap replications (Felsenstein, 1985). The gene sequence data obtained in this study are also available through our website TAXVIBRIO (http://www.taxvibrio.lncc.br/).

The novel strain R-40503^T was closely related to the type strain of *M. vaga*, with 97.9 % 16S rRNA gene sequence similarity. R-40503^T had 97.2 % 16S rRNA gene sequence similarity towards the type strains of *M. basaltis*, *M. communis* and *M. aquimarina* (Fig. 1 and Supplementary Fig. S1, available in IJSEM Online). DNA–DNA hybridizations (DDH) were performed between strain R-40503^T and the type strains of its closest phylogenetic neighbours, *M. vaga*, *M. basaltis*, *M. communis* and *M. aquimarina* (Table 1), using the microplate method described by Ezaki *et al.* (1989) with minor modifications (Willems *et al.*, 2001). Hybridizations were performed at 40.7 °C in the presence of 50 % formamide. Reciprocal reactions were performed for every DNA pair and the variation was within the limits of this method (Goris *et al.*, 1998). DDH between R-40503^T and the tested type strains was below 70 % (Table 1), demonstrating that the novel strain represents a novel species in the genus *Marinomonas* (Wayne *et al.*, 1987; Stackebrandt & Ebers, 2006). DDH between *M. basaltis* LMG 25279^T and *M. communis* LMG 2864^T was above 70 % (78 %), which suggests that these species are synonymous. Chang *et al.* (2008) obtained 56.2 % DDH between the same pair of type strains, but additional data

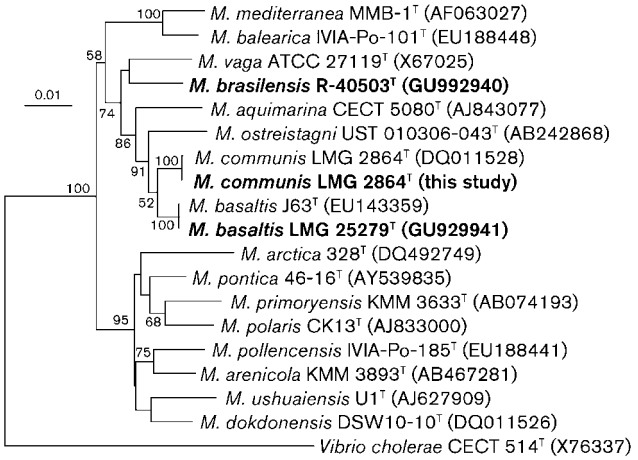

Fig. 1. Neighbour-joining phylogenetic tree showing the phylogenetic position of strain R-40503^T based on 16S rRNA gene sequences. The sequence of *M. communis* LMG 2864^T determined in this study was identical to the sequence deposited as DQ011528 for the same strain and was not deposited in GenBank. Evolutionary distances were computed by BioNumerics 4.61 software (Applied Maths). Bootstrap values (>50 %) based on 1000 repetitions are shown. *Vibrio cholerae* CECT 514^T was used as an outgroup. Bar, 1 % estimated sequence divergence.

Table 1. DDH and 16S rRNA gene sequence similarity between strain R-40503^T and the type strains of phylogenetically related *Marinomonas* species

Strain	DNA G+C content (mol%)	16S rRNA gene sequence similarity to strain R-40503^T (%)	DNA–DNA relatedness (%) to:				
			1	2	3	4	5
1. *M. brasilensis* sp. nov. R-40503^T	46.5	(100)	100	42	23	22	17
2. *M. vaga* LMG 2845^T	47.5	97.9	27	100	16	15	21
3. *M. basaltis* LMG 25279^T	46.3	97.2	18	19	100	84	13
4. *M. communis* LMG 2864^T	46.3	97.2	16	21	73	100	12
5. *M. aquimarina* LMG 25236^T	49.0	96.7	5	3	12	11	100

from the present study (see below) support our value of 78 %. The authenticity of *M. basaltis* LMG 25279^T and *M. communis* LMG 2864^T used in this study was verified by 16S rRNA gene sequencing. The sequences obtained in this study for the two type strains (1501 nt for LMG 25279^T and 1499 nt for LMG 2864^T) showed 100 % similarity with sequences deposited in GenBank for *M. basaltis* J63^T (GenBank accession no. EU143359) and *M. communis* LMG 2864^T (DQ011528), respectively (Fig. 1). The 16S rRNA gene sequence similarity between *M. basaltis* LMG 25279^T and *M. communis* LMG 2864^T was 98.7 %. As further support for the proposed synonymy, *M. basaltis* LMG 25279^T and *M. communis* LMG 2864^T had identical DNA G+C contents (46.3 mol%) and related amplified fragment length polymorphism (AFLP) patterns. DNA G+C contents were determined for R-40503^T, *M. basaltis* LMG 25279^T and *M. communis* LMG 2864^T by HPLC as described previously (Mesbah *et al.*, 1989). The DNA G+C content of strain R-40503^T was 46.5 mol% (Table 1).

AFLP analysis was performed for strain R-40503^T, *M. basaltis* LMG 25279^T, *M. communis* LMG 2864^T, *M. vaga* LMG 2845^T and three *M. aquimarina* strains (Supplementary Fig. S2), as reported by Beaz Hidalgo *et al.* (2008) and Thompson *et al.* (2001). Briefly, 1 μg DNA was digested with *Taq*I (cut site 5′-TCGA-3′) and *Hin*dIII (cut site 5′-AAGCTT-3′) (Amersham Pharmacia Biotech) and subsequently ligated with double-stranded adaptors complementary to the ends of the restriction fragments, using T4 ligase (Amersham Pharmacia Biotech), to generate template DNA for PCR amplification. A selective PCR was then performed with primers H01-6FAM (5′-GACTGCGTACCAGCTTA-3′, labelled at the 5′ end with the fluorescent dye 6-FAM) and T13 (5′-GTTTCTTATGA-GTCCTGACCGAG-3′), using the conditions described by Thompson *et al.* (2001), in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems). Separation of selective PCR products was performed using a capillary ABI Prism 3130XL DNA sequencer (Applied Biosystems). The level of reproducibility was controlled by generating the AFLP pattern of strain R-40305^T three times, starting from different subcultures. Normalization of the resulting electrophoretic patterns was performed using the Gene Mapper 4.0 software (Applera Co.). For subsequent analysis, patterns of fragments of 20–600 bp were transferred into the BioNumerics 4.61 software (Applied Maths). For numerical analysis, the zone from 40 to 580 bp was used. Similarity values were calculated using the Dice coefficient (tolerance value of 0.15 %), and a dendrogram was constructed using the UPGMA algorithm. The similarity between the patterns of R-40503^T ranged from 93.0 to 94.4 %. The similarity level chosen to delineate AFLP clusters was 63 %, as proposed previously by Beaz Hidalgo *et al.* (2008). Strains with AFLP profiles showing more than 63 % similarity can be considered as members of the same species. The AFLP data supported the DDH data obtained in this study. R-40503^T showed at most 46 % pairwise band pattern similarity with its closest phylogenetic neighbours, below the cut-off similarity level of 63 %, while the type strains of *M. basaltis* and *M. communis* constituted a distinguishable cluster with 69 % mutual AFLP pattern similarity (Supplementary Fig. S2). AFLP has been reported as a widely applicable technique with high discriminatory power and reproducibility (Janssen *et al.*, 1996; Savelkoul *et al.*, 1999). It has been shown to be useful for discrimination at the species and intraspecies levels for *Aeromonas*, *Acinetobacter*, *Campylobacter*, *Xanthomonas* (Savelkoul *et al.*, 1999), *Vibrionaceae* (Thompson *et al.*, 2001), *Bradyrhizobium* (Willems *et al.*, 2001), *Arcobacter* (On *et al.*, 2003) and *Pantoea* (Brady *et al.*, 2007). The present study provides enough evidence to consider *Marinomonas basaltis* Chang *et al.* 2008 a later heterotypic synonym of *Marinomonas communis* (Baumann *et al.* 1972) van Landschoot and De Ley 1984.

Phenotypic characteristics were determined in order to demonstrate that strain R-40503^T belongs to a novel species. Phenotypic analysis was carried out of the novel strain and the type strains of the phylogenetically closest *Marinomonas* species, *M. vaga*, *M. basaltis*, *M. communis* and *M. aquimarina*. Analysis of fatty acid methyl esters was carried out as described by Huys *et al.* (1994). Cells for fatty acid analysis were grown on MA (Difco) for 24 h at 28 °C under aerobic conditions. Phenotypic characterization was performed using the API ZYM, API 20E and API 20NE kits (bioMérieux) and Biolog GN2 microwell plates (Biolog Inc.) according to the manufacturers' instructions with minor modifications. Cell suspensions for inoculation of the API tests were prepared in 3 % (w/v) NaCl and those for the Biolog GN2 microwell plates showed turbidity

(transmission) of 20 %. Cells for the suspensions were grown on Biolog medium for 24 h at 28 °C under aerobic conditions. The results of the tests were recorded after 24–48 h of incubation at 28 °C. Growth at 4–42 °C was determined by incubation on TSA (Difco) for 72 h. Growth at 0–14 % NaCl was determined by incubation on TSA (Difco) at 28 °C for 72 h. Catalase activity was determined by adding young cells to a drop of a 3 % H_2O_2 solution and observation of O_2 production. Oxidase activity was tested using 1 % *N*,*N*,*N*′,*N*′-tetramethyl *p*-phenylenediamine (Kovács, 1956).

The novel strain R-40503^T could be differentiated from its closest phylogenetic neighbours by several phenotypic features (Table 2). It grew in medium containing 13 % NaCl and used Tween 80, sucrose and L-asparagine but not α-ketoglutaric acid, L-aspartic acid, L-serine, L-ornithine or bromosuccinic acid. It had oxidase activity, and was not able to grow at 40 °C (Table 2). The novel strain could also be differentiated from its neighbours on the basis of the presence of the fatty acid $C_{12:1}$ 3-OH and the absence of $C_{10:0}$ and $C_{12:0}$. The major cellular fatty acids of R-40503^T were $C_{18:1}\omega7c$ (48.8 %), summed feature 3 (iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$; 19 %), $C_{16:0}$ (10.5 %) and $C_{10:0}$ 3-OH (8 %) (Supplementary Table S1). The phenotypic features of *M. basaltis* LMG 25279^T and *M. communis* LMG 2864^T were very similar, except for some features, namely that *M. communis* LMG 2864^T utilized sucrose, D-fructose, succinamic acid, urocanic acid and putrescine and had urease activity, whereas *M. basaltis* LMG 25279^T did not. Some phenotypic results for *M. basaltis* LMG 25279^T obtained in this study conflict with those reported by Chang *et al.* (2008). They reported no growth in less than 1 % or more than 7 % NaCl and no esterase (C4), esterase lipase (C8) or naphthol-AS-BI-phosphohydrolase activities, but activities for trypsin and *N*-acetyl-β-glucosaminidase were present, and assimilation of L-arabinose, L-aspartic acid and glycerol. However, in this study, growth was observed at 0.5–11 % NaCl, and activities of esterase (C4), esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase were detected. Trypsin and *N*-acetyl-β-glucosaminidase activities and assimilation of L-arabinose, L-aspartic acid and glycerol were not observed. In our hands, no significant phenotypic or genotypic differences were found between *M. communis* LMG 2864^T and *M. basaltis* LMG 25279^T.

Table 2. Phenotypic differences between strain R-40503^T and its phylogenetically closest neighbours

Strains: 1, *M. brasilensis* sp. nov. R-40503^T; 2, *M. vaga* LMG 2845^T; 3, *M. basaltis* LMG 25279^T; 4, *M. communis* LMG 2864^T; 5, *M. aquimarina* LMG 25236^T. Data were obtained in this study under the same laboratory conditions unless indicated. +, Positive; −, negative; W, weakly positive; NA, no data available.

Characteristic	1	2	3	4	5
Growth with:					
12 % (w/v) NaCl	+	+	−	−	+
13 % (w/v) NaCl	W	+	−	−	W
Growth at 40 °C	−	W	+	+	+
Activity of:					
Oxidase	+	−	+	+	+
Urease	+	+	−	+	+
Utilization of:					
Tween 80	+	W	−	−	−*
Sucrose	+	+	−	−	−*
α-D-Glucose	+	W	+	+	NA
Alaninamide	+	+	−	−	NA
L-Asparagine	+	−	+	+	NA
L-Arabinose	W	−	−	−	−*
Cellobiose	W	−	W	W	−*
Glycerol	W	−	−	−	−*
Turanose	W	+	−	−	NA
α-Hydroxybutyric acid	W	+	+	+	NA
α-Ketobutyric acid	W	−	+	+	NA
Methylpyruvate	−	−	W	+	+*
α-Ketoglutaric acid	−	+	−	−	+*
L-Aspartic acid	−	+	−	−	+*
L-Serine	−	+	+	+	+*
L-Ornithine	−	+	−	−	+*
Putrescine	−	W	−	+	−*
Bromosuccinic acid	−	+	−	−	NA
Glycyl L-aspartic acid	−	W	−	−	NA

*Data from Macián *et al.* (2005).

Based on the phylogenetic, genomic and phenotypic data, the novel species *Marinomonas brasilensis* sp. nov. is proposed to encompass strain R-40503^T.

Description of *Marinomonas brasilensis* sp. nov.

Marinomonas brasilensis (bra.si.len′sis. N.L. fem. adj. *brasilensis* of or belonging to Brazil).

Cells are Gram-negative, aerobic, halophilic, motile, straight rods, approx. 1 μm wide and 1.5–3 μm long. Catalase- and oxidase-positive. Colonies on MA are circular, undulate, convex, smooth, beige in colour and 1 mm in diameter after 1 day of incubation at 28 °C. Prolific growth occurs at 20–35 °C and in the presence of 1–11 % (w/v) NaCl. No growth is observed in the absence of NaCl or in ⩾14 % NaCl or at ⩽7 °C or ⩾40 °C. The type strain exhibits alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, urease and tryptophan deaminase enzyme activities, but not lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase or gelatinase activities. It produces acetoin (Voges–Proskauer reaction), but not H_2S or indole. It does not ferment glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin or arabinose. It is negative for reduction of nitrate to nitrite or N_2 gas. It is able to assimilate citrate, Tweens 40 and 80, D-fructose, α-D-glucose,

D-mannose, sucrose, monomethyl succinate, DL-lactic acid, D-saccharic acid, succinic acid, alaninamide, L-asparagine, L-glutamic acid, L-proline, inosine and uridine. Positive for hydrolysis of aesculin. Weakly positive reactions for assimilation of α-cyclodextrin, L-arabinose, cellobiose, turanose, α-hydroxybutyric acid, α-ketobutyric acid, urocanic acid and glycerol. Negative for assimilation of dextrin, glycogen, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, D-arabitol, i-erythritol, L-fucose, D-galactose, gentiobiose, *myo*-inositol, lactose, lactose lactulose, maltose, D-mannitol, melibiose, methyl β-D-glucoside, psicose, raffinose, L-rhamnose, D-sorbitol, trehalose, xylitol, methyl pyruvate, acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketoglutaric acid, α-ketovaleric acid, malonic acid, propionic acid, quinic acid, sebacic acid, bromosuccinic acid, succinamic acid, glucuronamide, D- and L-alanine, L-alanyl glycine, L-aspartic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-pyroglutamic acid, D- and L-serine, L-threonine, DL-carnitine, γ-aminobutyric acid, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, DL-α-glycerol phosphate, glucose 1-phosphate, glucose 6-phosphate, potassium gluconate, capric acid, adipic acid, malate and trisodium citrate. The main cellular fatty acids are $C_{18:1}\omega7c$, summed feature 3 (iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$), $C_{16:0}$ and $C_{10:0}$ 3-OH, corresponding to 86 % of the total profile. The following fatty acids are present in small amounts (<5 %): unknown fatty acid ECL 11.799, $C_{12:1}$ 3-OH, $C_{18:0}$ and $C_{14:0}$ (Supplementary Table S1). The phenotypic profile is at present based on one strain. The DNA G+C content of the type strain is 46.5 mol%.

The type strain, R-40503^T (=R-278^T =LMG 25434^T= CAIM 1459^T), was isolated from mucus of the endemic coral *Mussismilia hispida* located in the São Sebastião channel, São Paulo, Brazil.

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