Massilia norwichensis sp. nov., isolated from an air sample

Ivana Orthová,[1] Peter Kämpfer,[2] Stefanie P. Glaeser,[2] René Kaden[3] and Hans-Jürgen Busse[1]

Correspondence
Hans-Jürgen Busse
hans-juergen.busse@vetmeduni.ac.at

[1]Institut für Bakteriologie, Mykologie und Hygiene, Veterinärmedizinische Universität, A-1210 Wien, Austria

[2]Institut für Angewandte Mikrobiologie, Justus-Liebig-Universität Giessen, D-35392 Giessen, Germany

[3]Department of Medical Sciences, Clinical Bacteriology, University of Uppsala, SE-75185 Uppsala, Sweden

A Gram-negative, rod-shaped and motile bacterial isolate, designated strain NS9^T, isolated from air of the Sainsbury Centre for Visual Arts in Norwich, UK, was subjected to a polyphasic taxonomic study including phylogenetic analyses based on partial 16S rRNA, *gyrB* and *lepA* gene sequences and phenotypic characterization. The 16S rRNA gene sequence of NS9^T identified *Massilia haematophila* CCUG 38318^T, *M. niastensis* 5516S-1^T (both 97.7 % similarity), *M. aerilata* 5516S-11^T (97.4 %) and *M. tieshanensis* TS3^T (97.4 %) as the next closest relatives. In partial *gyrB* and *lepA* sequences, NS9^T shared the highest similarities with *M. haematophila* CCUG 38318^T (94.5 %) and *M. aerilata* 5516-11^T (94.3 %), respectively. These sequence data demonstrate the affiliation of NS9^T to the genus *Massilia*. The detection of the predominant ubiquinone Q-8, a polar lipid profile consisting of the major compounds diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol and a polyamine pattern containing 2-hydroxyputrescine and putrescine were in agreement with the assignment of strain NS9^T to the genus *Massilia*. Major fatty acids were summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH), $C_{16:0}$, $C_{18:1}\omega7c$ and $C_{10:0}$ 3-OH. Dissimilarities in partial *lepA* and *gyrB* gene sequences as well as results from DNA–DNA hybridizations demonstrate that strain NS9^T is a representative of an as-yet undescribed species of the genus *Massilia* that is also distinguished from its close relatives based on physiological and biochemical traits. Hence, we describe a novel species, for which we propose the name *Massilia norwichensis* sp. nov., with the type strain NS9^T (=CCUG 65457^T=LMG 28164^T).

The genus *Massilia* La Scola *et al.* 2000 belongs to the betaproteobacterial family *Oxalobacteraceae*, together with the closely related genera *Telluria*, *Pseudoduganella*, *Duganella* and *Janthinobacterium* (Kämpfer *et al.*, 2011; Wang *et al.*, 2012; Kong *et al.*, 2013). The genus was described on the basis of a single clinical isolate from the blood of a 25-year-old immunocompromised patient with meningoencephalitis, which was assigned to the type species *Massilia timonae* (La Scola *et al.*, 1998). Since then, 22 additional species have been described: *Massilia aerilata* (Weon *et al.*, 2008), *M. albidiflava* (Zhang *et al.*, 2006), *M. alkalitolerans* (Kämpfer *et al.*, 2011), *M. aurea* (Gallego *et al.*, 2006), *M. brevitalea* (Zul *et al.*, 2008), *M. consociata* (Kämpfer *et al.*, 2011), *M. dura* (Zhang *et al.*, 2006), *M. flava* (Wang *et al.*, 2012), *M. haematophila* (Kämpfer *et al.*, 2011), *M. jejuensis* (Weon *et al.*, 2010), *M. lurida* (Luo *et al.*, 2013), *M. lutea* (Zhang *et al.*, 2006), *M. namucuonensis* (Kong *et al.*, 2013), *M. niabensis* (Weon *et al.*, 2009), *M. niastensis* (Weon *et al.*, 2009), *M. oculi* (Kämpfer *et al.*, 2012), *M. plicata* (Zhang *et al.*, 2006), *M. suwonensis* (Kämpfer *et al.*, 2011), *M. tieshanensis* (Du *et al.*, 2012), *M. umbonata* (Rodríguez-Díaz *et al.*, 2014), *M. varians* (Kämpfer *et al.*, 2011) and *M. yuzhufengensis* (Shen *et al.*, 2013). These species were isolated from clinical and environmental specimens eye, blood, soil, air, water and ice. According to the genus description (La Scola *et al.*,

Abbreviation: DDH, DNA–DNA hybridization.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of NS9^T is HG798294. Sequences of *gyrB* and *lepA* of NS9^T and reference strains are deposited under accession numbers HG798295–HG798311, KF780159, KF780161, KF780163 and KF780165 (*gyrB*) and HG798312–HG798328, KF780160, KF780162, KF780164 and KF780166 (*lepA*).

Two supplementary figures are available with the online Supplementary Material.

1998; Kämpfer *et al.*, 2011), members of the genus *Massilia* are characterized as Gram-negative, aerobic, non-spore-forming, motile rods. The major isoprenoid quinone is Q-8. The polar lipid profile contains predominantly diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol. The DNA G+C content ranges from 62.4 to 68.9 mol%.

Strain NS9^T was isolated in summer 1997 from the air of the Sainsbury Centre for Visual Arts in Norwich, UK. The present investigation was designed to establish the taxonomic position of this isolate. Strain NS9^T was routinely grown on PYE agar (0.3 % yeast extract, 0.3 % peptone from casein, 1.5 % agar, pH 7.2) or the corresponding broth and 1/10 PYE agar at 28 °C. Colony morphology of strain NS9^T was studied using cells grown on PYE agar for 3 days at 28 °C. Gram-staining was carried out with cells grown for 2 and 7 days at 28 °C on PYE agar according to Gerhardt *et al.* (1994) and was observed under a Leitz Dialux 20 microscope at ×1000 magnification. In all samples, the cells stained Gram-negative and were rod-shaped. Growth was tested on MacConkey II agar (BD Biosciences), Columbia III agar supplemented with 5 % sheep blood and PYE agar supplemented with 4 % NaCl (w/v) at 28 °C. NS9^T did not grow on MacConkey agar or on PYE agar supplemented with 4 % NaCl, but grew well on blood agar without haemolysis. After growth on SIM soft agar (Merck) for 8 days, no H_2S or indole production was detected and there were no signs of motility. In order to avoid any influence of the medium, testing for motility was repeated in 1/10 PYE and PYE soft agar tubes (0.3 %; w/v) according to the test conditions applied in the SIM test. After 5 days of cultivation in 1/10 PYE soft agar, strain NS9^T showed diffuse growth surrounding the stab channel in a narrow area approximately 1–5 mm below the surface, indicating motility under microaerobic conditions. In the corresponding test in PYE soft agar tubes, strain NS9^T grew only on the surface. A colony of strain NS9^T that had been grown on PYE agar for 1 week at 28 °C did not show oxidase activity in the BBL DrySlide oxidase test (BD Biosciences). This trait is in line with the emended description of the genus, which lists this trait as variable between species of the genus (Kämpfer *et al.*, 2011). The test for the presence of catalase activity was positive, as demonstrated by the production of bubbles when cells were suspended in 3 % (v/v) H_2O_2.

Detailed physiological characterization of strain NS9^T in comparison with the type strains of the closest related species of *Massilia* was performed using the 96-well physiological and biochemical test panel described by Kämpfer (1990) and Kämpfer *et al.* (1991). The detailed carbon substrate assimilation pattern of strain NS9^T is listed in the species description. Differentiating characteristics from type strains of other species of the genus *Massilia* are given in Table 1.

The phylogenetic position of strain NS9^T was studied by analyses of partial 16S rRNA, *lepA* and *gyrB* gene sequences. Genomic DNA was extracted from a loop of biomass using the ULTRAClean Microbial DNA Isolation kit according to the manufacturer's protocol (MO BIO Laboratories). The 16S rRNA gene of strain NS9^T was amplified by a PCR containing 0.65 µl of each primer solution (50 pmol µl^{-1}) (27f and 1492r; Lane, 1991), 30 µl REDTaq ReadyMix PCR mixture with $MgCl_2$ (Sigma-Aldrich), 26.2 µl sterile water and 2.5 µl 1 : 5-diluted genomic DNA. The amplification was carried out in a MultiGene Gradient PCR thermal cycler (Labnet International) and consisted of an initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 90 s, annealing at 55 °C for 90 s and elongation at 72 °C for 5 min and a final extension at 72 °C for 10 min. PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions and were sequenced at LGC Genomics (Berlin, Germany). The 16S rRNA gene sequence of NS9^T was composed of 1404 bases and was compared to entries in databases using the EzTaxon-e server (eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012). Highest sequence similarities were found with *M. haematophila* CCUG 38318^T and *M. niastensis* 5516S-1^T (both 97.7 %), *M. aerilata* 5516S-11^T and *M. tieshanensis* TS3^T (both 97.4 %). For phylogenetic analyses, the 16S rRNA gene sequences of NS9^T and type strains of established species of *Massilia* were aligned using CLUSTAL W (Thompson *et al.*, 1994) and edited manually for gaps and ambiguous nucleotides using BioEdit (Hall, 1999). Comparison of the edited 16S rRNA gene sequences (1327 bp) resulted in slightly higher similarity values, but *M. aerilata* 5516S-11^T, *M. niastensis* 5516S-1^T (both 97.9 %) and *M. haematophila* CCUG 38318^T (97.8 %) were still identified as the closest relatives of strain NS9^T.

Phylogenetic calculations based on 16S rRNA gene sequences were carried out applying the maximum-likelihood algorithm using the Tamura–Nei model (Tamura & Nei, 1993) with 1000 bootstrap replications included in MEGA 5.2.2 (Tamura *et al.*, 2011). In this tree, strain NS9^T branched next to *M. aerilata* and was slightly more distant from *M. niastensis* and *M. tieshanensis*, whereas *M. haematophila* was found in a separate clade (Fig. 1). However, none of the branching nodes were supported by significant bootstrap values.

The phylogenetic position of NS9^T within the genus *Massilia* was further studied by employing partial sequences of the housekeeping genes *gyrB* and *lepA* (encoding DNA gyrase subunit B and GTP-binding protein, respectively) of *M. aerilata* DSM 19289^T, *M. albidiflava* CIP 109189^T, *M. aurea* AP13^T, *M. consociata* CCUG 58010^T, *M. dura* CCUG 52213^T, *M. haematophila* CCUG 38318^T, *M. lutea* CIP 109190^T, *M. niabensis* KACC 12632^T, *M. niastensis* KACC 12599^T, *M. plicata* DSM 17505^T, *M. timonae* CCUG 45783^T, *M. varians* CCUG 35299^T, *Telluria chitinolytica* CIP 104069^T and *Telluria mixta* CCUG 35206^T. Primers for amplification of partial *gyrB* and *lepA* sequences were designed based on corresponding sequences in closely related strains. For PCR amplification of partial housekeeping genes, the following primers were constructed: gyrB_f (5′-TCSTTCCTSAAYA-AYGGCG-3′)/gyrB_r (5′-TCGGTCATGATGATGATRCG-G-3′) and lepA_f (5′-ACSATCAAGGCCCAGACCGC-3′)/

Table 1. Physiological properties of strain NS9^T that distinguish it from type strains of related species of the genus *Massilia*

Strains: 1, NS9^T; 2, *M. niastensis* KACC 12599^T; 3, *M. aerilata* DSM 19289^T; 4, *M. tieshanensis* KACC 14940^T; 5, *M. haematophila* KACC 13771^T (unless indicated, data from Weon *et al.*, 2010); 6, *M. varians* KACC 13770^T (Weon *et al.*, 2010); 7, *M. aurea* DSM 18055^T (Gallego *et al.*, 2006); 8, *M. niabensis* KACC 12632^T (Weon *et al.*, 2009); 9, *M. suwonensis* 5414S-25^T (Weon *et al.*, 2010); 10, *M. consociata* CCUG 58010^T (Kämpfer *et al.*, 2011); 11, *M. alkalitolerans* YIM 31775^T (Xu *et al.*, 2005); 12, *M. brevitalea* DSM 18925^T (Zul *et al.*, 2008; Weon *et al.*, 2009); 13, *M. jejuensis* 5317J-18^T (Weon *et al.*, 2010); 14, *M. timonae* DSM 16850^T (Weon *et al.*, 2008). +, Positive; (+), weakly positive; −, negative; ND, not determined/no data available. Data are from this study unless indicated. For *M. alkalitolerans* KACC 12188^T (formerly *Naxibacter alkalitolerans* KACC 12188^T), the data for this strain published by Weon *et al.* (2010) were not considered because the Korean Agricultural Culture Collection does not list this species or the corresponding collection number in their catalogue.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Oxidase	−	+*	+*	+*	+[a]†	+[a]	(+)[b]	+	+	−	−	−[c]	+	+[e]
Hydrolysis of aesculin	+	+*	(+)*	+*	−	+	+	−	−	+	+	+[d]	+	+
Assimilation of:														
D-Glucose	(+)	(+)*	(+)*	+*	−	−	+	−	(+)	+	+	+[d]	−	+
L-Arabinose	+	(+)*	+*	+*	−	+	−	−	−	+	ND	−[d]	−	+
Gluconate	−	−	−*	(+)*	−	−	−	−	−	−	ND	−[d]	−	+
Adipate	−	−*	−*	−*	+	−	+	−	+	ND	ND	+[d]	−	−
Citrate	−	−*	−*	−*	−	−	+	−	−	+	−	−[d]	−	+
L-Rhamnose	−	−	−*	−*	−	+	+	−	−	−	ND	−	−	+
Suberate	−	−*	−*	−	+	−	+	−	+	−	ND	+	−	−
Lactate	(+)	−*	−*	+*	−	−	−	−	+	−	ND	−	−	+
L-Alanine	−	−	−*	−*	−	+	+	−	−	−	ND	−	−	+
3-Hydroxybenzoate	−	−	−*	−*	+	−	−	−	−	−	ND	−	−	−
L-Serine	+	−*	−	−*	−	+	+	−	+	−	ND	−	−	+
Propionate	−	+	−	−	+	+	(+)	−	+	+	ND	+	−	−
L-Histidine	−	−*	−	−*	−	−	−	−	+	−	+	−	−	+
3-Hydroxybutyrate	−	−	−	−*	+	+	+	−	+	+	ND	+	−	+
4-Hydroxybenzoate	−	−	−*	−*	+	−	−	−	−	ND	ND	−	−	−
L-Proline	−	−	−	−	−	+	+	−	+	+	+	+	−	+

*Result agrees with previously published data.

†Data from: *a*, Kämpfer *et al.* (2008); *b*, Gallego *et al.* (2006); *c*, Zul *et al.* (2008); *d*, Weon *et al.* (2009); *e*, Lindquist *et al.* (2003).

lepA_r (5′-ATCAGTTCGCGCATCTTGGC-3′). PCRs were performed in 50 µl volumes using the REDTaq ReadyMix PCR mixture with $MgCl_2$ (Sigma-Aldrich) each containing 21 µl distilled water, 25 µl RedTaq mixture, 1 µl of each primer (gyrB_f and gyrB_r or lepA_f and lepA_r) (10 pmol µl^{-1}) and 2 µl 1 : 5-diluted genomic DNA as a template. Conditions for *gyrB* amplification were initial denaturation at 94 °C for 5 min followed by 25 cycles of denaturation at 94 °C for 1 min, primer hybridization at 58.9 °C for 45 s and elongation at 72 °C for 1 min and a final elongation at 72 °C for 5 min. PCR products were separated electrophoretically on a 2.5 % LM agarose gel (D-1 LE agarose; Fisher Molecular Biology) and stained with ethidium bromide and bands of the expected size of approximately 910 bp were excised and extracted from the gel using the Wizard SV Gel and PCR Clean-Up System (Promega). Under these PCR conditions, only small amounts of the corresponding PCR product were obtained from *M. plicata* DSM 17505^T and *M. dura* CCUG 52213^T. Therefore, the excised bands were reamplified and treated as described above. Conditions for *lepA* gene amplification were an initial denaturation step at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 1 min, primer annealing for 30 s with stepwise decreasing temperature (0.2 °C steps) from 64.4 to 62.4 °C and 20 cycles at 59 °C and elongation at 72 °C for 90 s and final extension at 72 °C for 5 min. In almost all strains, specific PCR products of the expected size of approximately 1400 bp were detected by electrophoretic separation on a 2 % agarose gel (agarose NEEO; ROTH) and ethidium bromide staining. In *M. lutea* CIP 109190^T and *M. dura* CCUG 52213^T, specific PCR products were obtained by lowering the primer annealing temperature to 59 °C. An appropriate PCR product from *M. plicata* DSM 17505^T could only be obtained by using another primer pair, lepA_f_2 (5′-ARCGYGGCATYACSATYAARGC-3′)/lepA_r_2 (5′-ACRTCRWACATYTGRCGCGG-3′), and an annealing temperature of 55 °C. Purified PCR products were sequenced by LGC Genomics (Berlin, Germany). Resulting sequences of *lepA* and *gyrB* and corresponding sequences retrieved from the GenBank/EMBL/DDBJ databases were aligned using CLUSTAL W provided in BioEdit (Thompson *et al.*, 1994; Hall, 1999). Sequences were edited manually by removal of 5′ and 3′ ends to obtain sequences matching in length (*gyrB*, 810 bp; *lepA*, 1299 bp) and removal of gaps. The partial *gyrB*

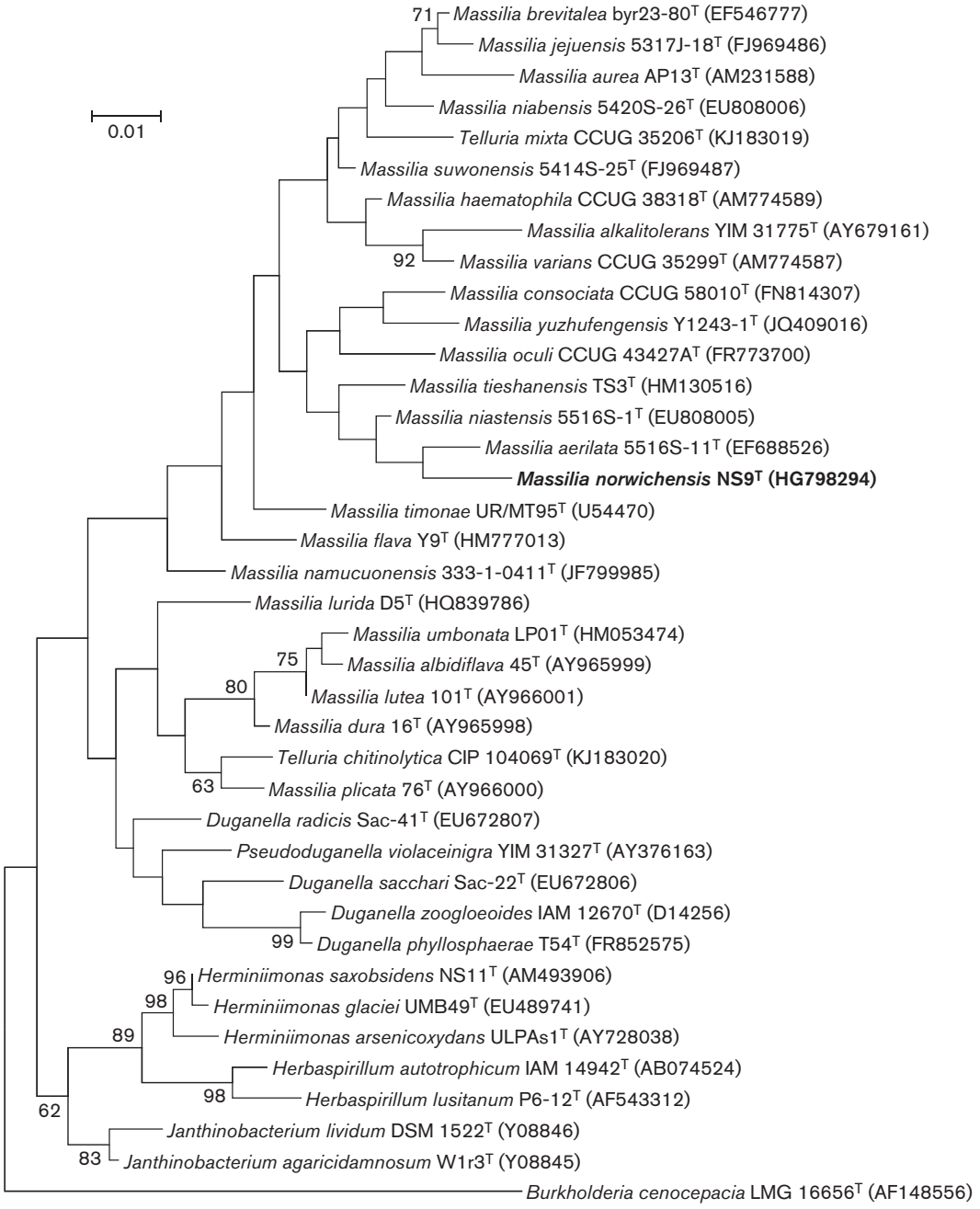

Fig. 1. Maximum-likelihood tree based on 16S rRNA gene sequences (1327 nt) showing the phylogenetic position of strain NS9^T. Confidence of branching nodes was investigated by 1000 bootstrap replications; only bootstrap values >60 % are shown. The tree was calculated applying the software package MEGA 5.2.2 (Tamura *et al.*, 2011) using the Tamura–Nei evolutionary model (Tamura & Nei, 1993). *Burkholderia cenocepacia* LMG 16656^T was used as an outgroup. Bar, 0.01 accumulated changes per nucleotide.

and *lepA* gene sequence data were used to infer phylogenetic trees employing MEGA 5.2.2 (Tamura *et al.*, 2011) (Figs S1 and S2, respectively, available in the online Supplementary Material) applying the Tamura–Nei evolutionary model (Tamura & Nei, 1993) and the maximum-likelihood algorithm. The neighbour-joining algorithm and Kimura's two-parameter model (Kimura, 1980) were employed for analysis of the phylogeny based on amino acid sequences translated from corresponding nucleotide sequences (results not shown). *Burkholderia cenocepacia* J2315^T was used as an outgroup. Among representative species of the genus *Massilia*, including the two species of *Telluria*, *gyrB* sequence

similarities were in the range 89.1–97.6 % and *lepA* gene sequence similarities were between 83.2 and 98.8 %. These data suggest that species of the genus *Massilia* are separated from each other in the *gyrB* gene sequence at values below 98 % similarity and in the *lepA* gene at values below 99 % similarity. In the *gyrB* sequence, strain NS9^T shared the highest similarity with *M. haematophila* CCUG 38318^T (94.5 %), and similarity to the other strains included in the comparison was between 88.5 % (*M. lutea* CIP 109190^T) and 93 % (*M. aerilata* DSM 19289^T). The partial *lepA* gene sequence of NS9^T shared the highest similarity with *M. aerilata* DSM 19289^T (94.3 %), and similarity to other strains was between 84.9 % (*M. lutea* CIP 109190^T) and 93.6 % (*M. varians* CCUG 35299^T). For both housekeeping gene sequences, the highest similarity was clearly below the threshold value concluded from similarity values between established species. In amino acid sequences obtained after translation of the corresponding gene sequences, members of the *Massilia*/*Telluria* group showed GyrB similarities of 94.4–99.6 % and LepA similarities of 84.0–100 % over 270 and 433 amino acids, respectively. Strain NS9^T showed the highest sequence similarity in the GyrB sequence to *M. haematophila* CCUG 38318^T and in the LepA sequence to *M. aerilata* DSM 19289^T (99.6 and 98.3 %, respectively). Very high similarities (>99 %) between members of different species indicate that the partial amino acid sequences of the proteins GyrB and LepA are not useful for identification of novel strains at the species level. It is also worth mentioning that, among the set of strains included in our comparison of *gyrB* and *lepA* gene sequences and also of the corresponding protein sequences, *M. alkalitolerans* DSM 17462^T and *M. varians* CCUG 35299^T exhibited the highest similarity values for the two genes and proteins (*gyrB*, 97.6 %; *lepA*, 98.8 % GyrB, 99.6 %; LepA, 100 %), which reflects their close relatedness at the 16S rRNA gene sequence level (Figs 1, 2, S1 and S2; Kämpfer *et al.*, 2011; Kong *et al.*, 2013; Luo *et al.*, 2013; Shen *et al.*, 2013). In agreement with the observation of the highest sequence similarities in *gyrB* and the corresponding protein sequences, *M. haematophila* CCUG 38318^T was identified as the closest phylogenetic relative of strain NS9^T (Fig. S1). As in the 16S rRNA gene phylogeny (Fig. 1), the trees based on *lepA* and also the amino acid sequence of the corresponding protein identified *M. aerilata* DSM 19289^T as the closest relative of NS9^T (Fig. S2), and this was also indicated in trees reconstructed from concatenated sequences (Fig. 2).

DNA–DNA hybridization (DDH) experiments were performed between NS9^T and the type strains of closely related species of the genus *Massilia* according to the method of Ziemke *et al.* (1998) with a modification in nick translation; 2 µg DNA was labelled for 3 h of incubation at 15 °C. DDH values obtained after hybridization of NS9^T with *M. niastensis* KACC 12599^T, *M. aerilata* DSM 19289^T, *M. haematophila* CCUG 38318^T, *M. brevitalea* DSM 18925^T, *M. jejuensis* KACC 12634^T, *M. aurea* AP13^T, *M. niabensis* KACC 12632^T, *M. consociata* CCUG 58010^T and *M. yuzhufengensis* CGMCC 1.12041^T were respectively 37.1, 43.5, 45.1, 59.9, 7.3, 11.8, 40.9, 21.6 and 55.0 %. These results demonstrate that strain NS9^T can be considered to be a representative of a novel species. Furthermore, in comparison with 16S rRNA gene sequence similarities, the DDH values support the view of Kim *et al.* (2014) that the threshold for species demarcation can be raised from 97 % to approximately 98.5 % 16S rRNA gene sequence similarity.

For production of biomass for quinone and polar lipid analyses, NS9^T and the reference strains were grown in PYE broth at 28 °C and harvested when cells had reached the stationary growth phase. Quinone and polar lipid analyses were carried out using lyophilized biomass as described previously (Tindall, 1990a, b; Altenburger *et al.*, 1996). Biomass subjected to polyamine analyses was harvested in the late exponential growth phase as recommended by Busse & Auling (1988) and analysed as described by Busse *et al.* (1997). For HPLC analysis, the instrumentation described by Stolz *et al.* (2007) was used. The predominant respiratory ubiquinone was Q-8 (96.4 %), followed by minor amounts of Q-7 (2.4 %) and Q-9 (1.2 %). The polar lipid profile of NS9^T (Fig. 3) and all reference strains (results not shown) showed diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine in major amounts, in accordance with the emended description of the genus *Massilia* (Kämpfer *et al.*, 2011). Additionally, NS9^T possessed an unidentified polar lipid (L1) in moderate amounts that was also detected in trace to moderate amounts in *M. albidiflava* CIP 109189^T, *M. aurea* AP13^T, *M. consociata* CCUG 58010^T, *M. haematophila* CCUG 38318^T, *M. dura* CCUG 52213^T, *M. lutea* CIP 109190^T, *M. niastensis* KACC 12599^T, *M. plicata* DSM 17505^T, *M. timonae* CCUG 45783^T, *M. varians* CCUG 35299^T, *M. aerilata* DSM 19289^T and *T. chitinolytica* CIP 104069^T but not in *T. mixta* CCUG 35206^T. Moreover, strain NS9^T showed minor amounts of an unidentified aminophospholipid (APL) that was detected as a major compound in *M. aurea* AP13^T and in moderate to minor amounts in *M. plicata* DSM 17505^T, *M. timonae* CCUG 45783^T and *M. dura* CCUG 52213^T. *M. albidiflava* CIP 109189^T, *M. consociata* CCUG 58010^T, *M. haematophila* CCUG 38318^T, *M. lutea* CIP 109190^T, *M. niastensis* KACC 12599^T, *M. varians* CCUG 35299^T and *T. chitinolytica* CIP 104069^T showed the presence of minor to trace amounts of an unidentified aminolipid (AL) with chromatographic motility corresponding to that of the aminophospholipid (APL) of NS9^T (Fig. 3). However, the absence of a positive reaction in staining for the presence of phosphate might be due to the low abundance of this aminolipid in these strains. Neither APL nor AL was detected in *M. aerilata* DSM 19289^T and *T. mixta* CCUG 35206^T. Another unidentified polar lipid (L2) was detected only in NS9^T and *T. mixta* CCUG 35206^T. NS9^T showed a polyamine pattern characteristic of the majority of members of the class *Betaproteobacteria*, with the major compounds putrescine [74.4 µmol (g dry weight)$^{-1}$] and 2-hydroxyputrescine [13.2 µmol (g dry weight)$^{-1}$]. Spermidine and spermine were detected in smaller amounts [3.5 and 1.6 µmol (g dry

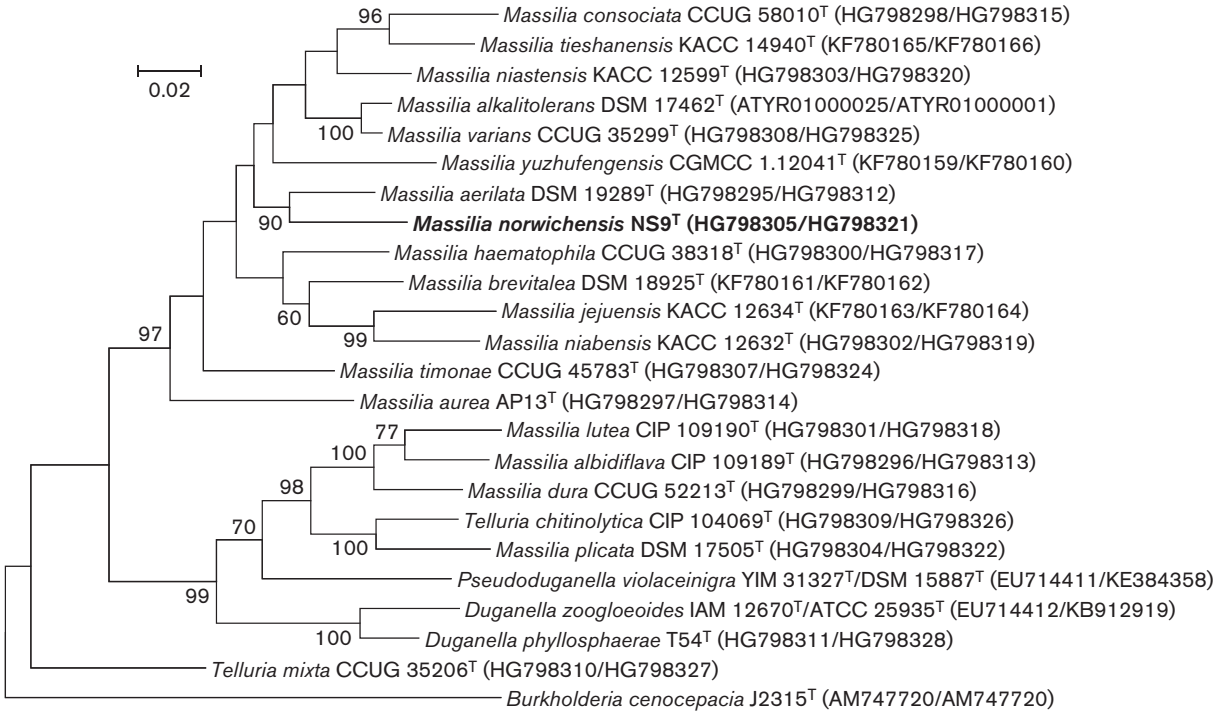

Fig. 2. Phylogenetic tree based on concatenated partial *gyrB* and *lepA* gene sequences applying the maximum-likelihood algorithm. The tree was reconstructed using the software package MEGA 5.2.2 (Tamura *et al.*, 2011) and confidence of branching nodes was analysed by 1000 bootstrap replications; only bootstrap values $\geqslant$60 % are indicated. *Burkholderia cenocepacia* J2315^T was used as an outgroup. Bar, 0.02 accumulated changes per nucleotide. Strain names and accession numbers for *gyrB*/*lepA* sequences are shown.

weight)$^{-1}$, respectively], and only traces of 1,3-diaminopropane [0.1 μmol (g dry weight)$^{-1}$] were detected. Similar polyamine patterns containing the major diamines 2-hydroxyputrescine and putrescine and significantly smaller amounts of spermidine, spermine and 1,3-diaminopropane were also detected in all reference strains analysed in this study, including *M. aerilata* DSM 19289^T, *M. albidiflava* CIP 109189^T, *M. aurea* AP13^T, *M. consociata* CCUG 58010^T, *M. dura* CCUG 52213^T, *M. lutea* CIP 109190^T, *M. niastensis* KACC 12599^T, *M. plicata* DSM 17505^T, *T. chitinolytica* CIP 104069^T and *T. mixta* CCUG 35206^T (results not shown). Hence, the polyamine patterns of species of the genus *Massilia* are in good agreement with those of the majority of species of the class *Betaproteobacteria* (Busse, 2011).

Biomass of strain NS9^T and reference strains for fatty acid analysis was grown on TS agar at 28 °C for 48 h. Fatty acid extraction and analysis was performed as described by Kämpfer & Kroppenstedt (1996) using an HP 6890 gas chromatograph with the Sherlock MIDI software version 2.11 and the TSBA peak-naming table version 4.1. The major fatty acids of strain NS9^T were summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH; 41.5 %), $C_{16:0}$ (26.6 %), $C_{18:1}\omega7c$ (8.9 %) and $C_{10:0}$ 3-OH (7.4 %); minor amounts (<5.0 %) of $C_{17:0}$ cyclo, $C_{12:0}$, $C_{14:0}$ and $C_{14:0}$ 2-OH were also present. The fatty acid profile of strain NS9^T is typical of species of the genus *Massilia*, with minor differences from other type strains of the genus in the relative abundance of fatty acids (Table 2).

Results from sequence analysis of partial 16S rRNA, *gyrB* and *lepA* genes clearly demonstrate that strain NS9^T is a member of the genus *Massilia*, and its polar lipid profile, fatty acid profile, quinone system and polyamine pattern are in agreement with this classification. Furthermore, *gyrB* and *lepA* gene sequences and DDH results suggest that NS9^T is a representative of a novel species, which is supported by its physiological characteristics. Hence, we here propose a novel species, *Massilia norwichensis* sp. nov., to accommodate strain NS9^T.

Description of *Massilia norwichensis* sp. nov.

Massilia norwichensis (nor.wi.chen′sis. N.L. fem. adj. *norwichensis* of or belonging to Norwich, the city where the type strain was isolated from air of the Sainsbury Centre for the Visual Arts).

Cells are rod-shaped and stain Gram-negative. Motility is only observed under microaerobic conditions in 1/10 PYE soft agar (0.3 %). Colonies are approximately 1–2 mm in diameter, circular, bright, smooth, semi-translucent, whitish pale to ochre–yellow with lightly coloured entire

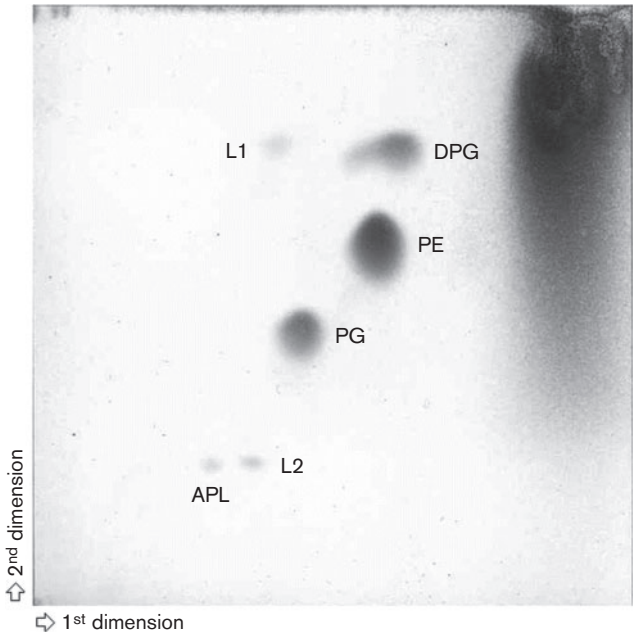

Fig. 3. Two-dimensional TLC displaying the polar lipid profile of strain NS9^T after detection with molybdatophosphoric acid. DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; L1–2, unidentified polar lipids; APL, unidentified aminophospholipid.

margins on PYE agar after 3 days of cultivation at 28 °C. Catalase-positive and oxidase-negative. Grows well on PYE, 1/10 PYE and Columbia agar supplemented with 5 % defibrinated sheep blood, but haemolysis is not observed. No growth on MacConkey agar or on PYE agar supplemented with 4 % (w/v) NaCl. Sulphide, indole production and motility on SIM agar are negative. Positive for assimilation of L-arabinose, maltose, D-ribose, D-xylose, acetate, fumarate, glutarate, L-malate, 2-oxoglutarate, pyruvate, L-aspartate and L-serine, weakly positive for assimilation of cellobiose, D-glucose, salicin and DL-lactate and negative for assimilation of *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, *p*-arbutin, D-fructose, D-galactose, gluconate, D-mannose, melibiose, L-rhamnose, sucrose, trehalose, adonitol, *myo*-inositol, maltitol, D-mannitol, D-sorbitol, putrescine, propionate, *cis*- and *trans*-aconitate, adipate, 4-aminobutyrate, azelate, citrate, DL-3-hydroxybutyrate, itaconate, mesaconate, suberate, L-alanine, *β*-alanine, L-histidine, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-tryptophan, 3-hydroxybenzoate, 4-hydroxybenzoate and (DL-3-) phenylacetate. The respiratory quinone system consists of the major compound ubiquinone Q-8 and small amounts of Q-7 and Q-9. Major compounds of the polar lipid profile are diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol. Furthermore, moderate amounts of two unidentified lipids, L1 and L2, and minor amounts of an unidentified aminophospholipid are present (Fig. 3). The polyamine

Table 2. Cellular fatty acid compositions of strain NS9^T and type strains of closely related species of the genus *Massilia*, including the type species *M. timonae*

Strains: 1, NS9^T; 2, *M. niastensis* KACC 12599^T (data in parentheses from Weon *et al.*, 2009); 3, *M. aerilata* DSM 19289^T (data in parentheses from Weon *et al.*, 2008); 4, *M. tieshanensis* KACC 14940^T; 5, *M. haematophila* KACC 13771^T (data from Kämpfer *et al.*, 2011); 6, *M. varians* KACC 13770^T (Kämpfer *et al.*, 2011); 7, *M. aurea* DSM 18055^T (Gallego *et al.*, 2006); 8, *M. niabensis* KACC 12632^T (Weon *et al.*, 2009); 9, *M. suwonensis* 5414S-25^T (Weon *et al.*, 2010); 10, *M. consociata* CCUG 58010^T (Kämpfer *et al.*, 2011); 11, *M. alkalitolerans* KACC 12188^T (Weon *et al.*, 2010); 12, *M. brevitalea* DSM 18925^T (Zul *et al.*, 2008; Weon *et al.*, 2009); 13, *M. jejuensis* 5317J-18^T (Weon *et al.*, 2010); 14, *M. timonae* DSM 16850^T (Weon *et al.*, 2008). −, <1 % or not detected. Data were obtained in this study unless indicated.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12	13	14
$C_{10:0}$ 3-OH	7.4	9.3 (5.5)	6.6 (4.7)	14.7	3.0	3.3	4.8	6.6	5.4	4.3	3.2	4.9	6.1	4.6
$C_{12:0}$	4.5	7.9 (4.7)	4.5 (3.4)	10.9	3.3	3.7	4.6	8.9	4.4	3.4	3.3	5.0	5.9	3.3
$C_{12:0}$ 2-OH	−	3.4 (2.4)	−	6.3	1.5	2.0	1.9	−	2.4	1.8	2.2	2.0	1.7	2.2
$C_{14:0}$	3.9	−	2.1 (1.9)	1.8	−	−	0.6	−	1.2	−	−	−	−	−
$C_{14:0}$ 2-OH	2.2	−	2.8 (2.5)	−	−	−	−	−	−	−	−	−	−	−
anteiso-$C_{15:0}$	−	−	−	−	1.5	−	−	−	−	−	−	−	−	−
$C_{16:0}$	26.6	18.6 (26.9)	26.5 (30.6)	16.3	26.2	28.5	36.8	23.6	28.8	21.5	26.6	23.0	22.5	30.5
iso-$C_{16:0}$	−	−	−	−	2.1	−	−	−	−	2.2	−	−	−	−
$C_{17:0}$ cyclo	4.8	3.4	1.7 (6.1)	−	2.7	−	−	−	2.9	−	−	−	1.0	3.7
iso-$C_{17:0}$	−	−	−	−	1.6	−	−	−	−	−	−	−	−	−
iso-$C_{17:1}\omega 9c$	−	−	−	−	1.4	−	−	−	−	−	−	−	−	−
$C_{18:1}\omega 7c$	8.9	16.5 (12.3)	8.7 (11.7)	8.1	8.7	8.1	2.5	7.8	7.8	12.8	6.6	9.0	12.1	7.9
$C_{20:0}$	−	2.6	−	−	−	−	−	−	−	−	−	−	−	−
Summed feature 3*	41.5	38.1 (38.1)	43.8 (35.2)	40.7	44.7	51.7	48.3	46.0	45.8	54.0	55.2	54.2	49.0	47.0

*Summed feature 3 includes $C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$ 2-OH. Since branched fatty acids were not detected (with the exception of *M. haematophila* KACC 13771^T), it appears most likely that summed feature 3 indicates the presence of $C_{16:1}\omega 7c$.

pattern is composed of the major compounds putrescine and 2-hydroxyputrescine, small amounts of spermidine and spermine and traces of 1,3-diaminopropane. Major fatty acids are summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH), $C_{16:0}$, $C_{18:1}\omega7c$ and $C_{10:0}$ 3-OH; minor amounts of $C_{17:0}$ cyclo, $C_{12:0}$, $C_{14:0}$ and $C_{14:0}$ 2-OH are also present.

The type strain is strain NS9^T (=CCUG 65457^T=LMG 28164^T), isolated from air of the Sainsbury Centre for Visual Arts in Norwich, UK.

References

Altenburger, P., Kämpfer, P., Makristathis, A., Lubitz, W. & Busse, H.-J. (1996). Classification of bacteria isolated from a medieval wall painting. *J Biotechnol* **47**, 39–52.

Busse, H.-J. (2011). Polyamines. *Methods Microbiol* **38**, 239–259.

Busse, H.-J. & Auling, G. (1988). Polyamine pattern as a chemotaxonomic marker within the *Proteobacteria*. *Syst Appl Microbiol* **11**, 1–8.

Busse, H.-J., Bunka, S., Hensel, A. & Lubitz, W. (1997). Discrimination of members of the family *Pasteurellaceae* based on polyamine patterns. *Int J Syst Bacteriol* **47**, 698–708.

Du, Y., Yu, X. & Wang, G. (2012). *Massilia tieshanensis* sp. nov., isolated from mining soil. *Int J Syst Evol Microbiol* **62**, 2356–2362.

Gallego, V., Sánchez-Porro, C., García, M. T. & Ventosa, A. (2006). *Massilia aurea* sp. nov., isolated from drinking water. *Int J Syst Evol Microbiol* **56**, 2449–2453.

Gerhardt, P., Murray, R. G. E., Wood, W. A. & Krieg, N. R. (editors) (1994). *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**, 95–98.

Kämpfer, P. (1990). Evaluation of the Titertek-Enterobac-Automated System (TTE-AS) for identification of members of the family *Enterobacteriaceae*. *Zentralbl Bakteriol* **273**, 164–172.

Kämpfer, P. & Kroppenstedt, R. M. (1996). Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. *Can J Microbiol* **42**, 989–1005.

Kämpfer, P., Steiof, M. & Dott, W. (1991). Microbiological characterization of a fuel-oil contaminated site including numerical identification of heterotrophic water and soil bacteria. *Microb Ecol* **21**, 227–251.

Kämpfer, P., Falsen, E. & Busse, H.-J. (2008). *Naxibacter varians* sp. nov. and *Naxibacter haematophilus* sp. nov., and emended description of the genus *Naxibacter*. *Int J Syst Evol Microbiol* **58**, 1680–1684.

Kämpfer, P., Lodders, N., Martin, K. & Falsen, E. (2011). Revision of the genus *Massilia* La Scola *et al.* 2000, with an emended description of the genus and inclusion of all species of the genus *Naxibacter* as new combinations, and proposal of *Massilia consociata* sp. nov. *Int J Syst Evol Microbiol* **61**, 1528–1533.

Kämpfer, P., Lodders, N., Martin, K. & Falsen, E. (2012). *Massilia oculi* sp. nov., isolated from a human clinical specimen. *Int J Syst Evol Microbiol* **62**, 364–369.

Kim, O.-S., Cho, Y.-J., Lee, K., Yoon, S.-H., Kim, M., Na, H., Park, S.-C., Jeon, Y. S., Lee, J.-H. & other authors (2012). Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol* **62**, 716–721.

Kim, M., Oh, H.-S., Park, S.-C. & Chun, J. (2014). Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. *Int J Syst Evol Microbiol* **64**, 346–351.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Kong, B.-H., Li, Y.-H., Liu, M., Liu, Y., Li, C.-L., Liu, L., Yang, Z.-W. & Yu, R. (2013). *Massilia namucuonensis* sp. nov., isolated from a soil sample. *Int J Syst Evol Microbiol* **63**, 352–357.

La Scola, B., Birtles, R. J., Mallet, M. N. & Raoult, D. (1998). *Massilia timonae* gen. nov., sp. nov., isolated from blood of an immunocompromised patient with cerebellar lesions. *J Clin Microbiol* **36**, 2847–2852.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.

Lindquist, D., Murrill, D., Burran, W. P., Winans, G., Janda, J. M. & Probert, W. (2003). Characteristics of *Massilia timonae* and *Massilia timonae*-like isolates from human patients, with an emended description of the species. *J Clin Microbiol* **41**, 192–196.

Luo, X., Xie, Q., Wang, J., Pang, H., Fan, J. & Zhang, J. (2013). *Massilia lurida* sp. nov., isolated from soil. *Int J Syst Evol Microbiol* **63**, 2118–2123.

Rodríguez-Díaz, M., Cerrone, F., Sánchez-Peinado, M., SantaCruz-Calvo, L., Pozo, C. & López, J. G. (2014). *Massilia umbonata* sp. nov., able to accumulate poly-β-hydroxybutyrate, isolated from a sewage sludge compost-soil microcosm. *Int J Syst Evol Microbiol* **64**, 131–137.

Shen, L., Liu, Y., Wang, N., Yao, T., Jiao, N., Liu, H., Zhou, Y., Xu, B. & Liu, X. (2013). *Massilia yuzhufengensis* sp. nov., isolated from an ice core. *Int J Syst Evol Microbiol* **63**, 1285–1290.

Stolz, A., Busse, H.-J. & Kämpfer, P. (2007). *Pseudomonas knackmussii* sp. nov. *Int J Syst Evol Microbiol* **57**, 572–576.

Tamura, K. & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol* **10**, 512–526.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**, 2731–2739.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Tindall, B. J. (1990a). Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol Lett* **66**, 199–202.

Tindall, B. J. (1990b). A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. *Syst Appl Microbiol* **13**, 128–130.

Wang, J.-W., Zhang, J.-L., Pang, H., Zhang, Y.-B., Li, Y.-Y. & Fan, J.-P. (2012). *Massilia flava* sp. nov., isolated from soil. *Int J Syst Evol Microbiol* **62**, 580–585.

Weon, H.-Y., Kim, B.-Y., Son, J.-A., Jang, H. B., Hong, S. K., Go, S.-J. & Kwon, S.-W. (2008). *Massilia aerilata* sp. nov., isolated from an air sample. *Int J Syst Evol Microbiol* **58**, 1422–1425.

Weon, H.-Y., Kim, B.-Y., Hong, S.-B., Jeon, Y.-A., Koo, B.-S., Kwon, S.-W. & Stackebrandt, E. (2009). *Massilia niabensis* sp. nov. and *Massilia niastensis* sp. nov., isolated from air samples. *Int J Syst Evol Microbiol* **59**, 1656–1660.

Weon, H.-Y., Yoo, S.-H., Kim, S.-J., Kim, Y.-S., Anandham, R. & Kwon, S.-W. (2010). *Massilia jejuensis* sp. nov. and *Naxibacter suwonensis* sp. nov., isolated from air samples. *Int J Syst Evol Microbiol* **60**, 1938–1943.

Xu, P., Li, W.-J., Tang, S.-K., Zhang, Y.-Q., Chen, G.-Z., Chen, H.-H., Xu, L.-H. & Jiang, C.-L. (2005). *Naxibacter alkalitolerans* gen. nov., sp.

nov., a novel member of the family '*Oxalobacteraceae*' isolated from China. *Int J Syst Evol Microbiol* **55**, 1149–1153.

Zhang, Y. Q., Li, W.-J., Zhang, K.-Y., Tian, X.-P., Jiang, Y., Xu, L.-H., Jiang, C.-L. & Lai, R. (2006). *Massilia dura* sp. nov., *Massilia albidiflava* sp. nov., *Massilia plicata* sp. nov. and *Massilia lutea* sp. nov., isolated from soils in China. *Int J Syst Evol Microbiol* **56**, 459–463.

Ziemke, F., Höfle, M. G., Lalucat, J. & Rosselló-Mora, R. (1998). Reclassification of *Shewanella putrefaciens* Owen's genomic group II as *Shewanella baltica* sp. nov. *Int J Syst Bacteriol* **48**, 179–186.

Zul, D., Wanner, G. & Overmann, J. (2008). *Massilia brevitalea* sp. nov., a novel betaproteobacterium isolated from lysimeter soil. *Int J Syst Evol Microbiol* **58**, 1245–1251.