**Brevibacterium massiliense** (Roux and Raoult 2009) is a later heterotypic synonym of **Brevibacterium ravenspurgense** (Mages, Frodl, Bernard and Funke 2009), using whole-genome sequence analysis as a comparative tool

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A patient strain derived from urine was found by 16S rRNA gene sequencing to be closely related (99.6 % identity) to sequences derived from both **Brevibacterium ravenspurgense** CCUG 56047T and **Brevibacterium massiliense** CCUG 53855T. Those species had been described during the same 11 month period in 2008–2009. Further characterization revealed that those isolates could not be readily distinguished from each other biochemically, by cellular fatty acids, antimicrobial susceptibility, MALDI-TOF MS, 16S rRNA gene sequencing or by whole-genome sequence (WGS) analyses. By WGS comparison, these isolates had an aerage nucleotide identity using BLASTn (ANIb) scores of 95.7 % or higher to each other, DNA G+C content in the range of 62.3 mol%–62.4 mol%, with genome sizes ranging from 2.28 × 10⁶ to 2.41 × 10⁶ bases. Based on these data, we propose that the name **B. massiliense** is a later heterotypic synonym of **B. ravenspurgense** and provide an emended description of **B. ravenspurgense**.

In 2014, a Gram-stain-positive bacterium strain NML 140868 received as a diphtheroid derived from midstream urine of a 91-year-old male with pyuria, was referred to the National Microbiology Laboratory (NML) for definitive identification. Further clinical information was not available. Preliminary assessment of strain NML 140868 by 16S rRNA gene sequencing indicated that this isolate had 99.6 % identity with two species with validly published names, **Brevibacterium ravenspurgense** and **Brevibacterium massiliense**. **B. ravenspurgense** had been effectively described in 2008 for isolates derived from a wound (CCUG 56047T) and a human blood culture (CCUG 46391) (Mages et al., 2008); subsequently, this epithet was validly published in Validation List 125 in January 2009 (Euzéby, 2009). The name **B. massiliense** was validly published for a Gram-stain-positive isolate recovered from discharge of a human ankle wound (CCUG 53855T) in August 2009 (Roux & Raoult, 2009). This observation raised the possibility that these names may be heterotypic synonyms which had been coincidently described during an 11 month period in 2008–2009. To investigate this, **B. ravenspurgense** CCUG 56047T and **B. massiliense** CCUG 53855T were acquired from the culture collection of the University of Göteborg (CCUG) Sweden and tested in comparison to strain NML 140868 biochemically, by cellular fatty acid (CFA) composition, MALDI-TOF, antimicrobial susceptibility testing (AST) and by whole-genome sequencing (WGS) analyses.
The two type strains and the clinical isolate were observed after 48 h incubation to have 4+ growth [scoring described previously (Bernard et al., 2002a)] on sheep blood agar (SBA) in air at 35 °C or in air supplemented with 5% CO₂ at 35 °C, with 0.5–1 mm, rounded, entire, convex and grey-whitish or beige colonies. At the NML, we found B. ravenspurgeense CCUG 56047ᵀ and the patient isolate to be only slightly sticky and neither grew to 2 mm in diameter after 24 h incubation, as described previously (Mages et al., 2008). None of these strains grew under strictly anaerobic conditions. All grew poorly (1+) on SBA at 25 °C, grew 4+ at 42 °C, and were catalase-positive but oxidase-negative. All three isolates were asporogenic, Gram-stain-positive, short, irregular rods.

Species of the genus Brevibacterium have been observed to be non-reactive with commonly-used biochemical test panels, making timely but definitive identification difficult without the use of molecular methods (Trujillo & Goodfellow, 2012). Some species of the genus Brevibacterium, but not B. ravenspurgeense, had been found to be reactive to various substrates using the API 50CH assimilation panel (Funke & Carlotti, 1994; Mages et al., 2008). To examine biochemical results, the three strains were characterized using several phenotypic test platforms. Biochemically, all three strains were found here to be non-reactive towards a variety of substrates (Bernard et al., 2002a), including dextrose, xylene, mannitol, lactose, sucrose, maltose, fructose, galactose, glycerol, glyco- gen, mannose, ribose, salicin and trehalose. The three strains did not reduce nitrate to nitrite or nitrogen, alkalize citrate or hydrolyze gelatin or aesculin and motility was not observed. Triple sugar iron slants were neutral/neutral without hydrogen sulphide being detected. Christie–Atkins–Munch–Petersen assay (CAMP) or reverse CAMP reactions were not observed. These organisms grew well in tryptic soy, brain–heart infusion and nutrient broths, the latter in the presence or absence of 1% (v/v) Tween 80 or 6% NaCl. These three strains did not grow in the API 50CH panel (BioMérieux). All three bacteria were reactive with all substrates found in the Biolog Gen III panel using the C₁ test protocol as described by the manufacturer at 33 °C (Biolog) and so could not be interpreted. In contrast, reactivity has been observed previously for some substrates found in Biolog G2 (Roux & Raoult, 2009); those reactions were outlined in the emended species description. The code obtained using the API Coryne panel (BioMérieux) was 2000004 for B. ravenspurgeense CCUG 56047ᵀ and B. massiliense CCUG 53855ᵀ; the code observed for NML 140868 was 2001004 (urea positive) and urease activity was confirmed using Christiansen’s urea tube test for this strain.

In our hands, all three strains had identical results according to the API ZYM system, that is, all were reactive or weakly reactive only for esterase (C4), esterase lipase (C8), leucine arylamidase and pyrazinamidase (API Coryne). No reactivity was observed for other enzymes in the API ZYM or API Coryne panels as outlined below in the emended species description, except that Mages et al. (2008) found B. ravenspurgeense CCUG 56047ᵀ to also be reactive with naphthol-AS-BI-phosphohydrolase. Enzyme activity was not detected using the API 32A panel (Biomerieux) for B. massiliense (Roux & Raoult, 2009). After review, phenotypic test reactions found here were deemed to be consistent with those obtained previously for B. ravenspurgeense and B. massiliense (Mages et al., 2008; Roux & Raoult, 2009).

Each strain was extracted then analyzed using the Sherlock system (MIDI) for cellular fatty acid (CFA) composition as described previously (Bernard et al., 1991), except that version 4.5 of the MIDI software was used. These three strains could not be distinguished by this approach, as all had profiles which were typical for CDC group A-4, CDC group A-5 (Bernard et al., 1991) or species of the genus Brevibacterium, with CFAs anteiso-C₁₅:₀, anteiso-C₁₇:₀, iso-C₁₅:₀ and iso-C₁₆:₀ predominating, as described previously (Mages et al., 2008; Roux & Raoult, 2009) (Table S1, available in the online Supplementary Material). Mages et al. (2008) had also reported meso-diaminopimelic acid as the diamino acid type in the peptidoglycan for B. ravenspurgeense.

MALDI-TOF MS analysis was performed for six replicates for each strain after growth for 24 h on SBA at 35 °C in 5% CO₂. Each isolate was extracted as described by Bruker Daltronics (2011) and analyzed using software version 3.1. Spectra were compared to the Bruker Biotyper library (5627 version) and evaluated using standard scoring, that is, log (score) values between 0.00 and 3.00 were calculated, with scores ≥2.0 considered to infer excellent identification at species level. Biotyper library was found to contain mass spectral profiles (MSPs) for three strains of B. ravenspurgeense (DSM 21258ᵀ, 2RLT and 3RLT) but no MSPs for B. massiliense. As outlined in Table S2, six replicates of B. ravenspurgeense CCUG 56047ᵀ, B. massiliense CCUG 53855ᵀ and strain NML 140868 gave rise to ranges of scores of 2.16–2.36, 2.10–2.18 and 2.12–2.23 respectively towards the Biotyper MSP for B. ravenspurgeense DSM 21258ᵀ as first choice. Subsequently, in-house MSPs were created for both B. ravenspurgeense CCUG 56047ᵀ and B. massiliense CCUG 53855ᵀ (Daltronics, 2014) and then individually retested along with strain NML 140868, with respect to each other. All three strains gave rise to scores ranging from 2.44 to 2.85 towards MSPs created for either B. ravenspurgeense CCUG 56047ᵀ or B. massiliense CCUG 53855ᵀ, indicating that those two species could not be unambiguously separated by MALDI-TOF MS analysis.

Antimicrobial susceptibility testing (AST) was done as outlined by CLSI methods for species of the genus Corynebacterium, using Table 5 from guideline M45-2A, for broth microdilution methods, breakpoints and interpretation (Clinical Laboratory Standards Institute, 2010). Trek Sensititre panels GN3F and STP6F were used for each strain. For antibiotics where breakpoints have been described, B. ravenspurgeense CCUG 56047ᵀ, B. massiliense CCUG 53855ᵀ and strain NML 140868 were found to be susceptible to penicillin, cephalosporins, cefotaxime, ceftriaxone, meropenem, vancomycin, daptomycin, rifampin, quinupristin/dalfopristin and linezolid. All three isolates were intermediate or...
resistant to ciprofloxacin, erythromycin and clindamycin. Results for gentamicin (GEN), tetracycline (TET) and trimethoprim-sulfamethoxazole (T/S) varied, with *B. ravenspurgen*us CCUG 56047\(^\text{T}\) being susceptible to GEN, TET and T/S, *B. massiliense* CCUG 53855\(^\text{T}\) being resistant to GEN and T/S but susceptible to TET and NML 1400868 being susceptible to GEN but resistant to TET and T/S. AST data were not provided previously for *B. massiliense*.

16S rRNA gene sequencing was performed on all three strains as previously outlined (Bernard *et al.*, 2002b) generating >1450 bps per strain; all were found to be highly similar to each other, with 99.5 % (or higher) identity. This close relationship was illustrated using the neighbour-joining algorithm found in MEGA 6.06 (Tamura *et al.*, 2013) after alignment using CLUSTAL W (MEGA) using curated sequences obtained from Genbank for species in the genus *Brevibacterium*. Sequences from the type strains of *Spelaeicoccus albus* and *Cellulomonas hominis* were used as unrelated outgroups (Fig. 1).

Comparative analyses were then done on these three strains using whole-genome sequencing (WGS), to ascertain if these taxa could be differentiated at that level as well as to enable comparison with the draft genome described for *B. massiliense* 5401308\(^\text{T}\) (Roux *et al.*, 2012). Whole-genome shotgun paired end and mate pair libraries were prepared, sequenced and assembled as previously described (Bernard *et al.*, 2016).
Genomes were annotated with Prokka (Seemann, 2014) and assembly and annotation data are presented in Table 1. Assembled whole genomes were compared using iSpeciesWS to calculate the average nucleotide identity using BLASTN (ANlb) values (Richter et al., 2016). Using this approach, these genomes were observed to have ANlb scores exceeding ~95% identity to each other, a value suggested to encompass members of the same species (Richter & Rosselló-Móra, 2009) the strains also demonstrated essentially identical DNA G+C contents at 62.3–62.4%, numbers of coding regions (CDS) and genome sizes (Table 1). Therefore, the type strains of B. ravenspurgense and B. massiliense and NML 140868 were deemed to be members of the same taxon group.

Based on evidence provided here and in accordance with Rule 24b (2) ascribed for nomenclature of prokaryotes (Parker et al., 2015), the name B. massiliense Roux & Raoult (2009) was deemed to be a later heterotypic synonym of B. ravenspurgense Mages et al. 2009 and the latter epithet took priority. NML 140868 was classified as representing a member of this species. As a result of this investigation, the following sequences have been deposited in Genbank: 16S rRNA gene sequence, KU976967 for NML 140868; whole genome sequences: Brevibacterium ravenspurgense CCGU 56047T, LQQC00000000; Brevibacterium massiliense CCUG 53855T, LPXX00000000 and NML 140868, LPXW00000000.

**Emended Description of Brevibacterium ravenspurgense** (Mages, Frodl, Bernard and Funke, 2009).

*Brevibacterium ravenspurgense* (ra.vens.pur.gen’se. N.L. adj. from Ravenspurgum, Latin name of the town of Ravensburg, Germany, where the type strain of this species was isolated).

| Table 1. Comparison of whole genome sequence assembly and annotation results for study isolates |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| NML 140868     | B. ravenspurgense CCG 56047T | B. massiliense CCUG 53855T | B. massiliense* 5401308T |
| NCBI accession no. | LPXW0000000000 | LQQC0000000000 | LPXX0000000000 | CAJD01000000 |
| Bases           | 2 417 684        | 2 297 397        | 2 365 554        | 2 349 262       |
| ANlb values† towards B. ravenspurgense CCGU 56047T LQQC0000000000 | 96.6        | –     | 95.7        | 95.88          |
| CDS             | 2167             | 1964             | 2123             | 2038            |
| tRNA            | 42               | 45               | 43               | 42              |
| CRISPR          | 0                | 0                | 2                | 2               |
| Contigs         | 38               | 14               | 22               | 26              |
| Scaffolds       | 13               | 5                | 5                | 26              |
| Contig N50      | 192 755          | 252 487          | 355 659          | 266 120         |
| Coverage        | 120              | 77               | 118              | –               |
| DNA G+C content (%) | 62.4         | 62.4             | 62.3             | 62.3            |

*As described by Roux & Raoult (2009).
†ANlb scores ranged from 69.11 to 70 when compared with species of the genus Brevibacterium for which WGS are available in Genbank - species identifier (accession number): B. album DSM 18621T (AUFF0000000001.1), B. casei S18 (AMSP01000001.1), B. epidermidis NBR1 14811T (BCS10000001.1), B. linens BL2 (AAGP01000001.1), B. meibomi ATCC49030T (ADNU0000000001.1) and B. senegalense JC43T (CAHK00000001.1).
methyl α-D-galactoside, methyl β-D-galactoside, 3-methyl glucose, methyl α-D-glucoside, methyl β-D-glucoside, methyl α-D-mannoside, palatinose, D-psicose, raffinose, L-rhamnose, salicin, sedoheptulose, D-sorbitol and stachyose. Enzymes detected include esterase (C4), esterase lipase (C8), leucine arylamidase (API ZYM) and pyrazinamidase (API Coryne) and is variable for detection of naphthol-AS-BI-phosphohydrolase; arginine arylamidase, proline arylamidase, leucine glycine arylamidase, phenylalnine arylamidase, leucine arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, serine arylamidase and histidine arylamidase, which were weakly detected using API 32A. The following enzymes are not detected: alkaline phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, α-glucosidase, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase (API ZYM); pyrolydonylarylamidase (PYRα), β-glucuronidase, β-galactosidase, α-galactosidase, N-acetyl-β-glucosaminidase (API Coryne); urease, arginine dihydrolase, α-galactosidase, α-glucosidase, β-galactosidase, 6-phospho-β-galactosidase, β-glucosidase, α-arabinosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, glutamic acid decarboxylase, α-fucosidase, alkaline phosphatase, pyrog glutamic acid arylamidase and glutamy glutamic acid arylamidase. Meso-diaminopimelic acid and anteiso-C_{15:0}, anteiso-C_{17:0} iso-C_{15:0} and iso-C_{16:0} CFAs are detected.

The type strain of *B. ravenspurgense* is CCUG 56047^T (=DSM 21258^T). The DNA G+C content of the type strain is 62.4 % (by WGS) with a genome size of 2 285 636 bps. The name *B. massiliense* (Roux & Raoult, 2009, 5401308 = CCUG 53855 = CIP 109422 =CSUR P26 = JCM 18108) is a later heterotypic synonym.

**Acknowledgements**

We gratefully acknowledge the NML’s Proteomic Core Facility under Dr G. Westacott for MALDI-TOF MS studies, the DNA Core Facility under Dr M. Graham and S. Tyler for 16S and WGS, and B. Balc ewich of the Special Bacteriology Unit for technical assistance.

**References**


Published online 13 March 2012.