Description of *Kribbella italic* sp. nov., isolated from a Roman catacomb

Gareth J. Everest,¹ Sarah M. Curtis,¹ Filomena De Leo,² Clara Urzi² and Paul R. Meyers¹

¹Department of Molecular and Cell Biology, University of Cape Town, Private Bag X3, Rondebosch, 7701 Cape Town, South Africa
²Department of Biological and Environmental Sciences, University of Messina, Viale F. Stagno d’Alcontres 31, 98166 Messina, Italy

A novel actinobacterium, strain BC637ᵀ, was isolated from a biodeteriorative biofilm sample collected in 2009 in the Saint Callixtus Roman catacomb. The strain was found to belong to the genus *Kribbella* by analysis of the 16S rRNA gene. Phylogenetic analysis using the 16S rRNA gene and the gyrB, rpoB, relA, recA and atpD concatenated gene sequences showed that strain BC637ᵀ was most closely related to the type strains of *Kribbella lupini* and *Kribbella endophytica*. DNA–DNA hybridization experiments confirmed that strain BC637ᵀ is a genomic species that is distinct from its closest phylogenetic relatives, *K. endophytica* DSM 23719ᵀ (63% DNA relatedness) and *K. lupini* LU14ᵀ (63% DNA relatedness). Physiological comparisons showed that strain BC637ᵀ is phenotypically distinct from the type strains of *K. endophytica* and *K. lupini*. Thus, strain BC637ᵀ represents the type strain of a novel species, for which the name *Kribbella italic* sp. nov. is proposed (≡DSM 28967ᵀ≡NRRL B-59155ᵀ).

Proposed in 1999 (Park *et al.*, 1999), the genus *Kribbella* contains nocardioform actinobacteria with L-DAP in the cell wall peptidoglycan. The genus description was recently emended by Everest *et al.* (2013). Another recent development associated with the genus was the publication of a multilocus sequence analysis (MLSA) method, which can be used to assess whether an isolate belongs to a novel species without the need for DNA–DNA hybridization (DDH) experiments (Curtis & Meyers, 2012). The MLSA study extended previous phylogenetic analysis of strains of the genus *Kribbella* using the gyrB gene alone (Kirby *et al.*, 2010). With the recent descriptions of *Kribbella albertannoniae* (Everest *et al.*, 2013), *Kribbella endophytica* (Kaewkla & Franco, 2013) and *Kribbella shirazensis* (Mohammadipanah *et al.*, 2013), at the time of writing there are 20 species of the genus *Kribbella* with validly published names (Parte, 2014). Here we describe the characterization of a novel member of the genus, isolated from the same site as the previously described species *K. albertannoniae*, *Kribbella catacumbeae* and *Kribbella sancticalisti* in the Saint Callixtus catacomb in Rome (Everest *et al.*, 2013; Urzi *et al.*, 2008).

Ocean cubicle (CSC13) is located inside the Saint Callixtus catacomb in Rome (Italy) and was extensively studied under the project financed by the European Community called CATS (Cyanobacteria AtTack RockS). During this project, an interdisciplinary group of researchers investigated the damage caused by the growth of microbial communities, mainly cyanobacteria-containing biofilms, as well as white/greyish patinas, as described by Albertano *et al.* (2003). At the end of the project, the Ocean cubicle was closed to the public and blue light was used for illumination in order to control the growth of photosynthetic micro-organisms. Strain BC637ᵀ was isolated from a white biofilm on a frescoed surface during a periodic monitoring campaign in 2009.

Site CSC13 harboured different species of the genus *Kribbella*, among which were *K. sancticalisti* BC633ᵀ, *K. catacumbeae* BC631ᵀ (both isolated before the microclimatic changes induced by the blue light) and *K. albertannoniae* BC640ᵀ, which was isolated in 2009, but from a different sampling site than strain BC637ᵀ. **Fig. S1** (available in the online Supplementary Material) shows the sampling locations in the CSC13 cubicle from which the novel species of the genus *Kribbella* were isolated (Urzi *et al.*, 2008; Everest *et al.*, 2013; present study). Three of the

**Abbreviations:** DAP, diaminopimelic acid; MLSA, multilocus sequence analysis.

The GenBank accession numbers for the 16S rRNA, atpD, gyrB, recA, relA and rpoB gene sequences of strain BC637ᵀ are KJ875927, KJ875925, KJ875917, KJ875921, KJ875923 and KJ875919, respectively.

Three figures and two tables are available with the online Supplementary Material.
novel species were associated with white/greyish biofilms, while K. albertanoniae BC640T was isolated from a dark green biofilm.

Sampling was carried out using the non-destructive adhesive tape technique (Fungi Tape, DID; Urzi & De Leo, 2001). Growth of colonies (quantified as the number of c.f.u. per cm² of adhesive tape) was carried out on R2A medium (Reasoner & Geldreich, 1985) at 28°C for 15 days. Ten to 20 colonies were randomly selected, and preliminarily characterized after transferring to tryptic soy agar (TSA, BBL); these were subsequently maintained on yeast extract-malt extract agar [International Streptomyces Project (ISP) medium 2; Shirling & Gottlieb, 1966].

Genomic DNA was extracted as described by Everest & Meyers (2008). The 16S rRNA gene was amplified as described by Cook & Meyers (2003), the gyrB gene as described by Kirby et al. (2010) and the atpD, recA, relA and rpoB genes as described by Curtis & Meyers (2012). Approximately 500 ng of template DNA was used in the PCR amplification of the 16S rRNA and gyrB genes, with 1 μg of DNA being used for the amplification of the atpD, recA, relA and rpoB genes. The PCR products were purified using an MSB Spin PCRapace kit (STRATEC Molecular) and sequenced (Macrogen, Seoul, South Korea). Sequence analysis was performed using DNAMAN version 5.2.9 (Lynnon BioSoft). Phylogenetic analyses were conducted, and the gyrB and concatenated-gene genetic distances calculated using Kimura’s two-parameter model (Kimura, 1980), using MEGA version 5.05 (Tamura et al., 2011). Phylogenetic trees were reconstructed using the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Takahashi & Nei, 2000) and neighbour-joining (Saitou & Nei, 1987) methods. Accession numbers for the genes used in generating the concatenated-sequence tree are listed in Table S1.

All ISP media were prepared according to Shirling & Gottlieb (1966). Morphological properties were determined on ISP 2 and inorganic salts-starch agar (ISP 4). Physiological tests were carried out as described by Williams et al. (1989). All plates were incubated at 30°C for the recommended time periods, unless stated otherwise. Carbon source utilization was tested as in the methods of Shirling & Gottlieb (1966), with all carbon sources being filter-sterilized and tested at a final concentration of 1% (w/v), with the exception of the sodium salts, which were tested at 0.1% (w/v). Nitrogen source utilization was performed as described by Williams et al. (1989). All nitrogen sources were filter-sterilized and tested at a final concentration of 0.1% (w/v). NaCl tolerance was determined on ISP 2 agar incubated for 14 days. Growth at pH 4.3, 7 and 10 and growth at different temperatures (20, 30 and 37°C) was determined on Bennett’s agar (Atlas, 2004) incubated for 14 days.

Analysis of the isomer of DAP, the whole cell sugar pattern and the phospholipid pattern were carried out as described by Everest et al. (2013). Analysis of the respiratory quinones was carried out by the identification service at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). All chemotaxonomic analyses were performed on freeze-dried cells of a culture of strain BC637T grown in ISP 2 broth, with moderate shaking at 30°C for 3 days. Fatty acid analysis was performed as a service by the BCCM/LMG Culture Collection (Ghent, Belgium), according to the recommendations of the commercial identification system MIDI (Microbial Identification System; MIDI Sherlock version 3.10; database: TSBA 50 rev 5.0) on cells grown at 28°C for 3 days on TSA (11768, BBL).

DDH analysis was performed as a service by the BCCM/LMG Culture Collection as described by Everest et al. (2013). Data are displayed as mean DNA–DNA hybridization values with the differences between the means of the reciprocal values given in parentheses.

A query against the EzTaxon-e server (Kim et al., 2012) with 1467 bp of 16S rRNA gene sequence indicated that strain BC637T belonged to the genus Kribbella and was most closely related to Kribbella flavida DSM 17836T (99.45% sequence similarity), K. cattacumbae DSM 19601T (99.32%), K. albertanoniae BC640T (99.32%) and Kribbella alba YIM 31075T (99.31%). However, the reconstruction of a 16S rRNA gene maximum-likelihood phylogenetic tree showed that strain BC637T grouped with Kribbella lupini LU14T (99.17% sequence similarity), with low bootstrap support (60%) (Fig. 1). Phylogenetic analyses based on the gyrB-rpoB-recA-relA-atpD concatenated gene sequences showed an association of strain BC637T with the type strains of K. endophytica (99.09% 16S rRNA gene sequence similarity) and K. lupini, which was strongly supported (bootstrap value of 100% in the maximum-likelihood tree; Fig. 2).

Genetic distance values were calculated between strain BC637T and all type strains of species of the genus Kribbella to assess the likelihood of this strain representing a novel genomic species. The gyrB-based genetic distance values ranged from 0.016 to 0.096, with those between strain BC637T and the type strains of K. lupini, K. endophytica, K. flavida, K. cattacumbae, K. albertanoniae and K. alba being 0.026, 0.016, 0.051, 0.078, 0.078 and 0.094, respectively. The values for K. lupini (0.026) and K. endophytica (0.016) were below the 0.04 gyrB genetic distance threshold proposed to represent novel species of the genus (Kirby et al., 2010). The concatenated five-gene genetic distance values ranged from 0.025 to 0.114. The values between strain BC637T and the type strains of K. lupini, K. endophytica, K. flavida, K. cattacumbae, K. albertanoniae and K. alba were 0.025, 0.025, 0.046, 0.059, 0.065 and 0.070, respectively. The proposed threshold for this concatenated-gene sequence, above which strains can be assumed to belong to distinct genomic species, is 0.04 (Curtis & Meyers, 2012). As the concatenated-gene genetic distances between strain BC637T and K. endophytica and between strain BC637T and K. lupini were <0.04, DDH
analysis was required to determine whether strain BC637<sup>T</sup> belongs to a different genomic species.

DDH revealed that strain BC637<sup>T</sup> shared 63 (7) % DNA relatedness with *K. endophytica* DSM 23718<sup>T</sup> and 63 (0) % DNA relatedness with *K. lupini* LU14<sup>T</sup>. Strain BC637<sup>T</sup> was thus found to represent a separate genomic species when the threshold value of 70 % DNA relatedness by DDH was used to delineate bacterial species (Wayne et al., 1987). Although the DDH values are close to the 70 % cut off, the MLSA results strongly suggest that BC637<sup>T</sup> represents a distinct genomic species: in all cases where there are multiple strains of a species of the genus *Kribbella*, the gyrB and concatenated-gene genetic distances between the type strain and all non-type strains of the same species are 0.000 (i.e. the gyrB, rpoB, relA, recA and atpD gene sequences for all strains of a species are identical). Strain BC637<sup>T</sup> did not have any genetic distance values lower than 0.016 and 0.025 for the gyrB and concatenated-gene sequences, respectively, indicating sequence differences between strain BC637<sup>T</sup> and all type strains and non-type strains of species of the genus *Kribbella*. Based on this evidence, strain BC637<sup>T</sup> is not a strain of any of the species of the genus *Kribbella* with validly published names. The identical sequences between type strains and non-type strains are shown clearly in Fig. 2 for the multi-strain species, *K. catacumbae*, *K. sancticallisti*, *Kribbella solani* and *Kribbella aluminosa*. The grouping of strain BC637<sup>T</sup> with *K. endophytica* DSM 23718<sup>T</sup> and *K. lupini* LU14<sup>T</sup> indicates a strong association of the strains, but the tree topology in Fig. 2 and the genetic distance values suggest that the strains belong to distinct species. This pattern is also seen with the grouping of the closely related *Kribbella ginsengisoli* DSM 17941<sup>T</sup> and *Kribbella koreensis*.

![16S rRNA gene phylogenetic tree](image-url)
CIP 108301T (concatenated-gene genetic distance value 0.015) and K. solani CIP 108508T and Kribbella hippodromi S1.4T (concatenated-gene genetic distance value 0.017) in Fig. 2. DDH analysis between strain BC637T and the type strains of K. flavida, K. catacumbae, K. albertanoniae and K. alba was not performed, as the genetic distances between strain BC637T and these strains were above the thresholds for distinguishing genomic species using both the gyrB and concatenated-gene sequences.

The cell wall peptidoglycan was found to contain LL-DAP (Fig S2) and glycine (chemotype I); Lechevalier & Lechevalier, 1970). The polar lipid profile included diphostatidylglycerol, phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannoside, six unidentified aminolipids, four unidentified glycolipids, five unidentified phospholipids, an unidentified aminophospholipid and seven unidentified phosphoglycolipids (Fig S3). The full fatty acid profile of the type strain is shown in Table S2.

Physiological characterization of strain BC637T showed that it was phenotypically distinct from the type strains of K. endophytica and K. lupini. The results of the phenotypic characterization of strain BC637T are presented in Table 1.

**Fig. 2.** gyrB-rpoB-recA-relA-atpD phylogenetic tree showing the position of strain BC637T within the genus Kribbella. The tree was reconstructed using the maximum-likelihood method based on 4099 bp of sequence. Values at each node are the percentage bootstrap values of 1000 replications (only values 70 % are shown), with asterisks (*) indicating the branches that were conserved in the maximum-likelihood, neighbour-joining and maximum-parsimony trees. Microlunatus phosphovorus NM-1T was used as an outgroup. Accession numbers of all the gene sequences used are listed in Table S1. Bar, 2 nt substitutions per 100 nt.
and in the species description. Chemotaxonomic analysis of strain BC637<sup>T</sup> showed that it had chemotaxonomic characteristics consistent with it being a member of the genus *Kribbella*. LL-DAP as the diagnostic diamino acid in the peptidoglycan; a phospholipid pattern characterized by the presence of phosphatidylcholine (and the absence of phosphatidylethanolamine); MK-9(H<sub>4</sub>) as the major menaquinone and ai-C<sub>15:0</sub> as the predominant fatty acid.

**Description of Kribbella italica sp. nov.**

*Kribbella italica* (i.ta’li.ca. N.L. fem. adj. *italica* from Italy). Gram-stain-positive, catalase- and oxidase-positive, non-motile actinobacterium. Colonies appear convoluted with irregular edges on most media. Vegetative mycelium appears cream to white. Hyphae are highly branched and irregular edges on most media. Aerial mycelium is fragmented in both liquid and agar cultures. Aerial mycelium appears white on ISP 4. No diffusible pigment is produced on ISP 5. Melanin is not produced on peptone-yeast-extract-iron agar (ISP 6) or tyrosine agar (ISP 7).

### Table 1. Phenotypic characteristics that allow the differentiation of strain BC637<sup>T</sup> from closely related species of the genus *Kribbella*

Strains: 1, BC637<sup>T</sup>; 2, *K. endophytica* DSM 23718<sup>T</sup>; 3, *K. lupini* LU14<sup>T</sup>. All data are from this study. Conflicting data are indicated in parentheses. + +, strongly positive; +, positive; +w, weakly positive; −, negative.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>+w</td>
<td>+</td>
</tr>
<tr>
<td>Degradation of:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenine</td>
<td>+w</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Gelatin</td>
<td>+</td>
<td>+</td>
<td>(−)*</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>+</td>
<td>+</td>
<td>(−)*</td>
</tr>
<tr>
<td>Urea</td>
<td>−</td>
<td>−</td>
<td>(+)*</td>
</tr>
<tr>
<td>Xanthine</td>
<td>−</td>
<td>+w</td>
<td>(−)*</td>
</tr>
<tr>
<td>Xylan</td>
<td>−</td>
<td>−</td>
<td>+w</td>
</tr>
<tr>
<td>Hydrolysis of starch</td>
<td>+</td>
<td>(−)*</td>
<td>−</td>
</tr>
<tr>
<td>Growth at/in:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37 °C</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>pH 4.3</td>
<td>−</td>
<td>+</td>
<td>(+)*</td>
</tr>
<tr>
<td>5% (w/v) NaCl</td>
<td>+w</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Utilization as the sole carbon source</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inulin</td>
<td>+</td>
<td>+</td>
<td>w</td>
</tr>
<tr>
<td>Raffinose</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Salicin</td>
<td>+w</td>
<td>+w</td>
<td>+</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>+w</td>
<td>+w</td>
<td>−</td>
</tr>
<tr>
<td>Utilization as the sole nitrogen source</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>l-Cysteine</td>
<td>+</td>
<td>+w</td>
<td>+</td>
</tr>
<tr>
<td>l-Methionine</td>
<td>+</td>
<td>+w</td>
<td>+w</td>
</tr>
</tbody>
</table>

*Data from Kaewkla & Franco, 2013.
†Data from Trujillo et al. 2006.

Nitrile is reduced to nitrite. Produces H<sub>2</sub>S. Utilizes adonitol, (+)-l-arabinose, (+)-cellobiose, (−)-d-fructose, (+)-d-glucose, *myo*-inositol, inulin, α-lactose, (+)-D-mannose, (−)-d-mannitol, melibiose, raffinose, rhamnose, sucrose and (+)-d-xylene as sole carbon sources, with weak growth on salicin and sodium acetate. Unable to utilize sodium citrate as a sole carbon source. Utilizes DL-α-amino-n-butyric acid, l-arginine, l-asparagine, l-cysteine, l-histidine, 4-hydroxy-l-proline, l-methionine, potassium nitrate, l-serine, l-threonine and l-valine as sole nitrogen sources, with weak growth on l-phenylalanine. Grows at 20 °C and 30 °C, but not at 37 °C. Grows optimally at pH 7, weakly at pH 10, but not at pH 4.3. Starch is hydrolysed. Casein, gelatin, hypoxanthine, Tween 80 and l-tyrosine are degraded. Adenine is weakly degraded. Allantoin, urea, xanthine and xylan are not degraded. Grows weakly in the presence of 5% (w/v) NaCl. The cell wall peptidoglycan contains LL-DAP and glycine (chymotype 1). Glucose, ribose and an unidentified pentose are present in the whole-cell sugar hydrolysate. The polar lipid profile includes diphosphatidylglycerol, phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannoside, six unidentified aminolipids, four unidentified glycolipids, five unidentified phospholipids, an unidentified aminophospholipid and seven unidentified phosphoglycolipids. The major menaquinone of the type strain is MK-9(H<sub>4</sub>) (75%), with minor amounts of MK-9 (4%) and MK-9(H<sub>2</sub>) (18%). The major fatty acids (present as >10% of total fatty acids) are ai-C<sub>15:0</sub> and C<sub>17:1ω9c</sub>.

The type strain BC637<sup>T</sup> (=DSM 28967<sup>T</sup> = NRRL B-59155<sup>T</sup>) was isolated from site CSC13 of the Saint Callixtus catacomb in Rome.

**Acknowledgements**

The authors are grateful to Dr Laura Bruno of Tor Vergata University in Rome and Dr R. Giuliani of the Pontificia Commissione di Archaeologia sacra (C.d.V.) for their kind collaboration. G. J. E. held a Claude Leon Foundation Postdoctoral Fellowship. S. M. C. holds a National Research Foundation (NRF) Innovation Doctoral Scholarship. The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at are those of the authors and are not necessarily to be attributed to the NRF. This work was supported by research grants to P. R. M. from the National Research Foundation NRF (grant number: 85476) and the University Research Committee (University of Cape Town).

**References**


