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

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A novel actinobacterium, strain BC637ᵀ, was isolated from a biodeteriogenic 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 23718ᵀ (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 italica sp. nov. is proposed (=DSM 28967ᵀ=NRRL B-59155ᵀ).

Proposed in 1999 (Park et al., 1999), the genus Kribbella contains nocardioform actinobacteria with lL-diaminopimelic acid (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 albertanoniae (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. albertanoniae, K. catacumbae and K. sancticallisti 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 ATack 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. sancticallisti BC633ᵀ, K. catacumbae BC631ᵀ (both isolated before the microclimatic changes induced by the blue light) and K. albertanoniae 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
novel species were associated with white/greyish biofilms, while \textit{K. albertanoniae} BC640\textsuperscript{T} 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 cm\textsuperscript{2} 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 \textit{gyrB} gene as described by Kirby et al. (2010) and the \textit{atpD}, \textit{recA}, \textit{relA} and \textit{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 \textit{gyrB} genes, with 1 μg of DNA being used for the amplification of the \textit{atpD}, \textit{recA}, \textit{relA} and \textit{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 \textit{DNAMAN} version 5.2.9 (Lynnnon BioSoft). Phylogenetic analyses were conducted, and the \textit{gyrB} and concatenated-gene genetic distances calculated using Kimura’s two-parameter model (Kimura, 1980), using \textit{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 BC637\textsuperscript{T} 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 BC637\textsuperscript{T} belonged to the genus \textit{Kribbella} and was most closely related to \textit{Kribbella flavida} DSM 17836\textsuperscript{T} (99.45% sequence similarity), \textit{K. catacumbae} DSM 19601\textsuperscript{T} (99.32%), \textit{K. albertanoniae} BC640\textsuperscript{T} (99.32%) and \textit{Kribbella alba} YIM 31075\textsuperscript{T} (99.31%). However, the reconstruction of a 16S rRNA gene maximum-likelihood phylogenetic tree showed that strain BC637\textsuperscript{T} grouped with \textit{Kribbella lupini} LU14\textsuperscript{T} (99.17% sequence similarity), with low bootstrap support (60%) (Fig. 1). Phylogenetic analyses based on the \textit{gyrB}-\textit{rpoB}-\textit{recA}-\textit{relA}-\textit{atpD} concatenated gene sequences showed an association of strain BC637\textsuperscript{T} with the type strains of \textit{K. endophytica} (99.09% 16S rRNA gene sequence similarity) and \textit{K. lupini}, which was strongly supported (bootstrap value of 100% in the maximum-likelihood tree; Fig. 2).

Genetic distance values were calculated between strain BC637\textsuperscript{T} and all type strains of species of the genus \textit{Kribbella} to assess the likelihood of this strain representing a novel genomic species. The \textit{gyrB}-based genetic distance values ranged from 0.016 to 0.096, with those between strain BC637\textsuperscript{T} and the type strains of \textit{K. lupini}, \textit{K. endophytica}, \textit{K. flavida}, \textit{K. catacumbae}, \textit{K. albertanoniae} and \textit{K. alba} being 0.026, 0.016, 0.051, 0.078 and 0.094, respectively. The values for \textit{K. lupini} (0.026) and \textit{K. endophytica} (0.016) were below the 0.04 \textit{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 BC637\textsuperscript{T} and the type strains of \textit{K. lupini}, \textit{K. endophytica}, \textit{K. flavida}, \textit{K. catacumbae}, \textit{K. albertanoniae} and \textit{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 BC637\textsuperscript{T} and \textit{K. endophytica} and between strain BC637\textsuperscript{T} and \textit{K. lupini} were <0.04, DDH
analysis was required to determine whether strain BC637 T belongs to a different genomic species.

DDH revealed that strain BC637 T shared 63 (7) % DNA relatedness with K. endophytica DSM 23718 T and 63 (0) % DNA relatedness with K. lupini LU 14 T. Strain BC637 T 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 T 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 T 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 T and all type strains and non-type strains of species of the genus Kribbella. Based on this evidence, strain BC637 T 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 T with K. endophytica DSM 23718 T and K. lupini LU14 T 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 T and Kribbella koreensis.

Fig. 1. 16S rRNA gene phylogenetic tree showing the position of strain BC637 T within the genus Kribbella. The tree was reconstructed using the maximum-likelihood method based on 1347 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. Accession numbers are indicated in parentheses after the strain numbers. Microlunatus phosphovorus NM-1 T (AP012204) was used as an outgroup. Bar, 1 nt substitution per 100 nt.
CIP 108301<sup>T</sup> (concatenated-gene genetic distance value 0.015) and <i>K. solani</i> CIP 108508<sup>T</sup> and <i>Kribbella hippodromi</i> S1.4<sup>T</sup> (concatenated-gene genetic distance value 0.017) in Fig. 2. DDH analysis between strain BC637<sup>T</sup> and the type strains of <i>K. flavida</i>, <i>K. catacumbae</i>, <i>K. albertanoniae</i> and <i>K. alba</i> was not performed, as the genetic distances between strain BC637<sup>T</sup> and these strains were above the thresholds for distinguishing genomic species using both the <i>gyrB</i> 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 diphasphatidylglycerol, 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 BC637<sup>T</sup> showed that it was phenotypically distinct from the type strains of <i>K. endophytica</i> and <i>K. lupini</i>. The results of the phenotypic characterization of strain BC637<sup>T</sup> are presented in Table 1.

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**Fig. 2.** <i>gyrB-rpoB-recA-relA-atpD</i> phylogenetic tree showing the position of strain BC637<sup>T</sup> within the genus <i>Kribbella</i>. 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. <i>Microlunatus phosphovorus</i> NM-1<sup>T</sup> 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 BC637T 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 phosphatidycholine (and the absence of phosphatidylethanolamine); MK-9(H4) as the major menaquinone and ai-C15:0 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 white to cream and are 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). Nitrate is reduced to nitrite. Produces H2S. Utilizes adonitol, (+)-L-arabinose, (+)-cellobiose, (-)-d-fructose, (+)-d-glucose, myo-inositol, inulin, r-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 1-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 (chomotype 1). Glucose, ribose and an unidentified pentose are present in the whole-cell sugar hydrolysate. The polar lipid profile includes diphasphatidylglycerol, phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol and 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(H4) (75 %), with minor amounts of MK-9 (4 %) and MK-9(H2) (18 %). The major fatty acids (present as >10 % of total fatty acids) are ai-C15:0 and C17:1ω9c.

The type strain BC637T (=DSM 28967T = NRRL B-59155T) was isolated from site CSC13 of the Saint Callixtus catacomb in Rome.

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**References**


Table 1. Phenotypic characteristics that allow the differentiation of strain BC637T from closely related species of the genus *Kribbella*

Strains: 1, BC637T; 2, *K. endophytica* DSM 23718T; 3, *K. lupini* LU14T. All data are from this study. Conflicting data are indicated in parentheses. +++, Strongly positive; +, positive; +w, weakly positive; −, negative.

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


