Revisiting Corynebacterium glyciniphilum (ex Kubota et al., 1972) sp. nov., nom. rev., isolated from putrefied banana

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A strain of a species of the genus Corynebacterium, designated AJ 3170T, was isolated during the 1980s from putrefied bananas. Since then, there have been no further updates on the description of the strain or its phylogenetic classification. However, phylogenetic analysis of this strain using 16S rRNA and in silico DNA–DNA hybridization has confirmed that it is a member of the genus Corynebacterium and that strain AJ 3170T clusters with Corynebacterium variabile DSM 44702T, Corynebacterium terpenotabidum Y-11T and Corynebacterium nuruki S6-4T in one subgroup. Furthermore, a combination of enzymatic, chemical, and morphological characterization techniques was applied in order to describe strain AJ 3170T further. The strain grew well at pH values of 6–10 and at temperatures of 30–41°C. The major fatty acids were C16:0 (42.15 %), C18:1ω9c (41.6 %) and C18:0 10-methyl (TBSA) (8.56 %). The whole-cell sugars were determined to comprise galactose, arabinose and ribose. On the basis of this phenotypic, chemotaxonomic and phylogenetic characterization, it is proposed that strain AJ 3170T represents a novel species, for which the name Corynebacterium glyciniphilum sp. nov. is proposed; the type strain is AJ 3170T (=DSM 45795T =ATCC 21341T).

The genus Corynebacterium belongs to the order Actinomycetales, which in turn belongs to the phylum Actinobacteria (Bernard & Funke, 2012). This genus was first described as a pathogenic species with morphological similarities to the diphtheria-causing bacterium, Corynebacterium diphtheriae (Barksdale, 1970). Today, the genus Corynebacterium consists of Gram-positive bacteria including pathogenic and non-pathogenic bacteria isolated from a wide range of habitats, which are host-associated as well as found living free in the environment (Ventura et al., 2007).

The strain, AJ 3170T, was first isolated from putrefied bananas during a search for bacteria with the potential to produce amino acids and was associated with the family Corynebacteriaceae (Kubota et al., 1972). It was found that strain AJ 3170T is capable of producing large amounts of L-serine (up to 13.9 g l−1) in cultivation media containing glycine as a precursor for L-serine synthesis (Kubota & Yokozeki, 1989; Kubota, 1985; Kubota et al., 1972).

Although the characteristics of this strain have been associated with the genus Corynebacterium, the species name ‘Corynebacterium glycinophilum’ has not yet been validly published. In this paper the taxonomic characterization of the proposed type strain, ‘Corynebacterium glycinophilum’, is addressed together with the proposal of a new species.

Strain AJ 3170T was obtained from the American type Culture Collection (ATCC) as ATCC 21341T. For morphological descriptions, the strain was cultivated in an aerobic shaking flask culture in complex rich medium (CASO, casein and soybean peptone medium, ROTH), washed and visualized by scanning electron microscopy (Hitachi S-450; Hitachi High-Technologies Europe). In addition to the Gram-stain positive character, which was shown by Kubota et al. (1972), cells of strain AJ 3170T exhibited the shape of species of the genus Corynebacterium (Fig. 1), being club-shaped rods; they were also non-motile.
and non-spore-forming (Lehmann & Neumann, 1896). The cells were approximately 0.3–0.4 μm in width and 1 μm in length and showed the typical v-type arrangement (snapting division) of cell pairs after cell division. Determination of the optimal growth temperature was carried out on CASO agar complex medium, for temperatures ranging from 5°C to 45°C. Strain AJ 3170T displayed optimum growth at 37°C, with significant growth observed between 25°C and 41°C. After being cultivated on CASO agar plates for two days, cell colonies were circular, up to 1–2 mm in diameter, with a smooth surface, yellowish to yellow and seemed to have a sticky consistency. To determine the pH range for growth, MME medium (Vogel & Bonner, 1956) was buffered with 25 mM MES (pH adjusted to 6) or 25 mM Tris (pH adjusted to 9) and 25 mM CAPSO (pH adjusted to 10) in addition to varying concentrations of NaH2PO4 and K2HPO4. Growth occurred between pH 6 and pH 10, with optimal growth at pH 8. Strain AJ 3170T showed a time for doubling growth of approximately 2 h under optimal growth conditions of pH 8 and at 37°C in CASO medium in shaking flasks at 180 r.p.m., with a maximal OD600 of 25.

The metabolic and phenotypic properties of strain AJ 3170T were obtained using two kinds of analytical methods. The API ZYM and API Coryne test stripes from bioMérieux (Nürtingen) were used for testing enzyme activities of the strain according to the manufacturer’s instructions. The second type of phenotypic characterization was determined by using Phenotype MicroArrays (PM1, PM2 and PM3) from Biolog. Based on the API ZYM and API Coryne test stripes, strain AJ 3170T was positive for β-glucosidase, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine and cystine arylamidase, trypsin, acid phosphatase and naphthol-AS-Bl-phosphohydrolase, but not valine arylamidase, α-chymotrypsin, α- or β-galactosidases, β-glucuronidases, α-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, or α-fucosidase. Furthermore, it had positive pyrazinamidase, urease and catalase activities. Strain AJ 3170T was able to ferment the sugar sources: D-glucose, D-ribose and D-xylose. The combination of positive reactions for urease and pyrazinamidase activities and negative reactions for maltose fermentation reveals the non-toxigenic character of strain AJ 3170T (Efstratiou & George, 1999). According to Phenotype MicroArrays (Biolog), strain AJ 3170T was shown to metabolize the following compounds as sole carbon sources: N-acetyl-D-galactosamine, D-trehalose, D-mannose, D-gluconic acid, D-ribose, Tween 20, Tween 40, Tween 80, D-fructose, acetic acid, α-D-glucose, β-methyl-D-glucoside, L-malic acid, L-lyxose, pyruvic acid, D-arabitol, arbutin, salicin, D-glucosamine, quinic acid, and dihydroxy acetone. Based on phylogenetic analysis (see below) its closest relatives are Corynebacterium variabile DSM 44702T (Schröder et al., 2011), Corynebacterium nuruki S6-4T (Shin et al., 2011a), Corynebacterium terpenotabidum Y-11T (Takeuchi et al., 1999). In comparison to those relatives, the following carbon sources could only be metabolized by AJ 3170T: succinic acid, L-aspartic acid, L-proline, L-glutamic acid, D,L-malic acid, L-glutamine, citric acid, fumaric acid, butyric acid, caproic acid, and putrescine (complete list in Table S1, available in the online Supplementary Material). Inter alia, D-sorbitol, D-galucuronic acid, D-mannitol, L-rhamnose, maltose, α-D-lactose, sucrose, myo-inositol, and dextrin could not be fermented by strain AJ 3170T. In addition to analysis of the profile of carbon-utilization, the nitrogen-source utilization profile was obtained by using the Phenotype MicroArray PM3 (Biolog). Strain AJ 3170T was shown to metabolize the following compounds as a sole nitrogen source (complete list in Table S1): ammonia, L-aspartic acid, L-glutamic acid, glycine, L-proline, N-aminomaleine, N-acetyl-D-glucosamine, N-butyramine, putrescine, agmatine, histamine, β-phenylethylamine, tyramine, acetamide, guanosine, xanthosine, allantoin, and, less efficiently, L-methionine, L-threonine, D-alanine, D-serine, L-homoserine, ethylamine, ethanolamine, alloxa, adenosine and α-amino-N-valeric acid. Furthermore, like its closest relatives C. variabile DSM 44702T (Schröder et al., 2011), C. nuruki S6-4T (Shin et al., 2011a) and C. terpenotabidum Y-11T (Takeuchi et al., 1999), strain AJ 3170T is also able to metabolize ammonia, L-asparagine, L-glutamic acid, L-cysteine, L-serine, guanine and γ-amino-N-butyric acid as sole nitrogen sources. Strain AJ 3170T is not able to metabolize nitrite, nitrate or L-citulline as the nitrogen source (Table S1).

The sensitivity of strain AJ 3170T to 17 antibiotics was examined by inhibition zone assays based on the agar disc-diffusion method (Table 1). For this purpose, 100 μl of a culture with OD600 20 were streaked on CASO agar plates.
with commercial antibiotic assay discs (Oxoid) and cultivated overnight at 37°C. Interestingly, ‘C. glycinophilum’ showed, with the one exception of trimethoprim (5 μg), an identical resistance pattern for the antibiotics tested to that of C. variabile DSM 4470T. Conversely, there are clear differences between the resistance patterns of ‘C. glycinophilum’ as compared to those of the other tested strains of species of the genus Corynebacterium, C. nuruki S6-4T and C. terpenotabidum Y-11T.

Analyses of polar lipids, respiratory quinones, mycolic acids, cell-wall composition, and whole-cell sugars were carried out by the Identification Service, Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. Polar lipids were determined by two-dimensional TLC (Tindall et al., 2007). The polar lipid content of strain AJ 3170T consists of phosphatidylglycerol, diphosphatidylglycerol, two glycolipids, and phosphoglycolipids (Fig. S1). In addition, further lipids and phospholipids of unknown structure were present. The analysis of mycic acids (as trimethylsilylated derivatives) was carried out using gas chromatography coupled with mass spectrometry (GS-MS) (Klatte et al., 2007). The polar lipid content of strain AJ 3170T was hydrolysed completely with 4M HCl at 100°C for 16 h, and tested by the Identification Service of the DSMZ, Braunschweig, Germany. Whole cells were hydrolysed and analysed by TLC to analyse the 2,6-diaminopimelic acid content (Rhuband et al., 1955) and the whole-cell sugars (Staneck & Roberts, 1974). The cell-wall diamino acid of strain AJ 3170T was meso-diaminopimelic acid (meso-DAP). The presence of meso-DAP in the cell wall is responsible for accommodating strain AJ 3170T within the genus Corynebacterium (Schleifer & Kandler, 1972; Schumann, 2011). The whole-cell hydrolysates of strain AJ 3170T comprised galactose, arabinose and ribose. Corynebacterium efficiens shows a similar cell-wall and whole-cell sugar composition (Fudou et al., 2002).

Strain AJ 3170T was classified by Kubota et al. (1972) in the genus Corynebacterium. Since 1972, no further updates on the phylogenetic classification of this species have been reported. To obtain the 16S rRNA gene sequence and for later analyses, e.g. in silico DNA–DNA hybridization, genomic DNA was isolated and a whole genome sequencing approach was applied. The complete 16S rRNA gene sequence, which is present in four identical copies, was subsequently compared to other corynebacterial 16S rRNA gene sequences using the Ribosomal Database (RDP) (Cole et al., 2014). RDP uses the weighted neighbour-joining tree-building algorithm (Bruno et al., 2000). The phylogenetic tree (Fig. 2) shows that strain AJ 3170T clustered within the genus Corynebacterium. The 16S rRNA sequence of strain AJ 3170T shows no sequence identities of more than 98.3% with any type strain of a species of the genus Corynebacterium. The phylogenetic tree indicated that the nearest relatives of strain AJ 3170T were C. variabile DSM 4470T (Schröder et al., 2011) and C. terpenotabidum Y-11T (Takeuchi et al., 1999) with sequence similarities of 98.3% and 97.6%, respectively (Fig. 2). C. variabile DSM 4470T was isolated from the surface of smear-ripened cheese and C. terpenotabidum Y-11T is a squalene-degrading bacterium. Furthermore, C. nuruki S6-4T, which was isolated from an alcohol fermentation starter (Shin et al., 2011a) clusters in the same subgroup with a sequence similarity of 96.4% to the 16S rRNA of AJ 3170T (Fig. 2). These three most closely related species were shown to be non-pathogenic bacteria, similarly to strain AJ 3170T. As 98.3% and 97.6% identities, respectively, of the 16S rRNA gene sequences of C. variabile DSM 4470T and C. terpenotabidum Y-11T to those of strain AJ 3170T indicate that strain AJ 3170T might be a member of one of the two species, the complete genome sequence of strain AJ 3170T was used to perform an in silico DNA–DNA hybridization (DDH) using GGDC 2.0 (Meier-Kolthoff et al., 2013). The GGDC server calculates the genomic distance between one reference genome and up to ten other genomes, analogous to the classical DDH methods. We calculated all combinations of the genomes of the three other closely related species (Rückert et al., 2014; Schröder et al., 2011; Shin et al., 2011a, b) and of strain AJ 3170T. Even without complex bioinformatics, the high divergence in the G+C content already provided strong circumstantial evidence that strain

**Table 1. Results of antibiotic resistance/sensitivity tests for strain AJ 3170T** and its closest relatives

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin (25 μg)</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Trimethoprim (5 μg)</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sulfonamides (300 μg)</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Norfloxacin (5 μg)</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Streptomycin (25 μg)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</tbody>
</table>
AJ 3170T is indeed a species distinct from the three others: strain AJ 3170T displays a G+C content of 64.82%, while the related species have G+C contents above 67% (Table 2). This hypothesis is backed by the results of the in silico DDH, with no related strain displaying more than 21% similarity (Table 2).

Therefore, on the basis of phenotypic, chemotaxonomic and phylogenetic data, strain AJ 3170T should be placed in the genus Corynebacterium as the type strain of a novel species for which we propose the name Corynebacterium glyciniphilum (ex Kubota, et al., 1972) sp. nov., nom. rev.

Table 2. G+C contents of and DDH similarities between strain AJ 3170T and its closest relatives

<table>
<thead>
<tr>
<th>Strain</th>
<th>G+C content (mol%)</th>
<th>DDH* (%) with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
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<tr>
<td>1</td>
<td>64.8</td>
<td>–</td>
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<tr>
<td>2</td>
<td>67.2</td>
<td>20.7 ± 2.32</td>
</tr>
<tr>
<td>3</td>
<td>67.0</td>
<td>20.8 ± 2.33</td>
</tr>
<tr>
<td>4</td>
<td>69.5</td>
<td>20.8 ± 2.33</td>
</tr>
</tbody>
</table>

*DDH similarities were calculated in silico using whole genome sequences with GGDC server version 2.0 using formula 2 (Meier-Kolthoff et al., 2013).

**Description of Corynebacterium glyciniphilum (ex Kubota et al., 1972) sp. nov., nom. rev.**

Corynebacterium glyciniphilum (ex Kubota et al., 1972) sp. nov., nom. rev. corrig. (gly.ci.ni’phi.lum. N.L. glycine; N.L. adj. philus -a -um (from Gr. adj. philos -e-on) friend, loving; N.L. neut. adj. glyciniphilum glycine-loving).

Cells are Gram-stain-positive (ex Kubota et al., 1972), non-motile, non-spore-forming club-shaped rods, which are approximately 0.3–0.4 μm in width and 1 μm in length. Cell pairs are formed by snapping division. Cells are aerobic (ex Kubota et al., 1972; negative for nitrate...
reductase) and catalase-positive. They grow on CASO agar at 30–41 °C and at pH 6.0–10.0. Yellowish to yellow, circular, convex, very smooth and opaque colonies form within 48 h, with a diameter of approximately 1.5–2 mm. *Inter alia,* the following carbon compounds are metabolized as the sole energy source: N-acetyl-D-glucosamine, succinic acid, D-trehalose, D-mannose, L-aspartic acid, L-proline, L-glutamic acid, DL-malic acid, D-glucose acid, D-ribose, Tween 20, Tween 40, Tween 80, D-fructose, acetic acid, α-D-glucose, β-methyl-D-gluco side, L-malic acid, L-lyxose, pyruvic acid, D-arabitol, arbutin, salicin, D-glucosamine, quinic acid, dihydroxy acetone, L-glutamine, citric acid, fumaric acid, butyric acid, caproic acid, and putrescine. Additionally, able to metabolize more nitrogenous compounds as sole sources, including: ammonia, L-aspartic acid, L-glutamic acid, glycine, L-methionine, L-proline, L-serine, L-threonine, N-amy lamine, N-butylamine, agmatine, histamine, β-phenylethylamine, xanthine and allantoin. No activity is observed for the carbon sources maltose, sucrose, uridine, maltotriose, inositol, N-acetyl-neuraminic acid and L-proglutamic acid and for the nitrogen sources nitrite, nitrate, L-proglutamyl acid and D-mannosamine. According to the API ZYM gallery produces β-glucosidase, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine and cystine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase. Urea is hydrolyzed, but gelatin is not. Pyrazinamidase and alkaline phosphatase activities are phosphohydrolase. Urea is hydrolysed, but gelatin is not.

The type strain, AJ 3170T (DSM 44721T), was isolated from putrefied banana from an unknown location. The genomic DNA G+C content is 64.82 mol%.

References


