Corynebacterium crudilactis sp. nov., isolated from raw cow's milk

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A Gram-stain-positive, rod-shaped bacterium (strain JZ16T) was isolated from raw cow’s milk from the bulk tank of a dairy farm in Germany. The 16S rRNA gene sequence of the isolate showed a similarity of 98.3 % to the nearest related type strain Corynebacterium glutamicum ATCC 13032T, a similarity of 97.6 % to Corynebacterium deserti GIMN1.010T and a similarity of 97.4 % to Corynebacterium callunae DSM 20147T. Determination of chemotaxonomic characteristics revealed oleic acid (18 : 1 cis 9) as the predominant fatty acid, major amounts of hexadecanoic acid (16 : 0) and minor amounts of heptadecanoic acid (17 : 0). The isolate showed an acetyl type of peptidoglycan and corynemycolic acids. The menaquinones MK-8(H4) and MK-9(H4) and the phospholipids diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannoside were detected, which was in agreement with the description of the genus Corynebacterium. Strain JZ16T was positive for reduction of nitrate to nitrite, pyrazinamidase, β-glucuronidase, β-glucosidase and urease activities. Acid was produced from d-glucose, d-ribose and d-mannitol, but not from d-xylose, maltose, lactose, sucrose and glycogen. The results of phylogenetic, phenotypic and chemotaxonomic analyses enabled the differentiation of the isolated strain from other closely related species of the genus Corynebacterium. Therefore, strain JZ16T represents a novel species of the genus Corynebacterium, for which the name Corynebacterium crudilactis sp. nov. is proposed. The type strain is JZ16T (=DSM 100882T =CCUG 69192T =LMG 29813T).

The genus Corynebacterium contains 119 validly published species at the time of writing (June 2016; Euzéby, 1997), which were recovered from various ecological niches. Corynebacterium diphtheriae, a well-known serious human pathogen, was the first described species of the genus (Lehmann & Neumann, 1896). Members of the genus Corynebacterium were frequently detected in raw milk and raw milk products (Watts et al., 2000; Weber et al., 2014). Some of the species were pathogenic, e.g. Corynebacterium bovis, Corynebacterium amycolatum, Corynebacterium minutissimum and Corynebacterium ulcerans, and were associated with subclinical or clinical mastitis in cows or sheep (Hommez et al., 1999; Watts et al., 2000). Corynebacterium ammoniagenes, Corynebacterium xerosis and Corynebacterium diphtheriae were found in the mammary glands of cows with mastitis (Watts et al., 2000) and C. xerosis were also detected in bulk tank raw cow’s milk (Weber et al., 2014). However, there were also some beneficial species of the genus Corynebacterium detected in raw milk, which can be used in food processing. For example, Corynebacterium casei, Corynebacterium variabile and Corynebacterium glutamicum were detected in raw milk (Fricker et al., 2011; Weber et al., 2014) and were also part of the surface microbiota of smear-ripened cheese (Brennan et al., 2001; Dolci et al., 2009).

During an investigation of the microbiota in raw cow’s milk, several species of the genus Corynebacterium were
isolated from bulk tank milk of different dairy farms in Germany. Some of the isolated strains were identified as *Corynebacterium glutamicum* based on 16S rRNA gene sequence analyses. Strain JZ16<sup>T</sup> shared a 16S rRNA gene sequence similarity of 98.3% to the type strain of this species. According to Kim et al. (2014), a 16S rRNA gene sequence similarity of 98.7% is appropriate as the threshold for species delineation, so strain JZ16<sup>T</sup> might represent a novel species of the genus *Corynebacterium*. Strain JZ16<sup>T</sup> was isolated on brain-heart infusion agar (Oxoid) supplemented with 0.25 g l<sup>–1</sup> potassium tellurite (Merck) and 1.0% Tween 80 (Merck) at 30 °C under aerobic conditions and was subcultivated on trypticase soy agar (TSA; Merck).

The oxidase activity of the isolated strain was tested with Bactident oxidase test strips (Merck). Catalase activity and Gram-staining behaviour were determined according to Gerhardt (1981). KOH lysis and aminopeptidase activity were tested with amino-peptidase test strips (Merck). Cell morphology, motility, the presence of spores and cell dimensions were determined with a Zeiss Axio Observer phase-contrast microscope at ×1000 magnification. Average cell size was calculated based on measuring of at least 100 cells by Zen 2012 software. Strain JZ16<sup>T</sup> was catalase-positive, oxidase-negative and Gram-stain-positive. The strain was aminopeptidase-negative and KOH-lysis-negative, which confirmed the positive Gram-staining behaviour. The cells showed a rod-shaped morphology and cell dimensions of 0.9–1.8×0.6–1.3 μm, and neither motility nor spore formation was detected (Fig. 1). The determination of biochemical characteristics was achieved with the API Coryne, API ZYM and API 50CH (with API 50CHB medium) test systems (bioMérieux) according to the manufacturer’s specifications. Reaction profiles of the isolate and reference strains are listed in Tables S1–S3 (available in the online Supplementary Material).

Fatty acid patterns, presence and length of mycolic acids, the acyl type of peptidoglycan, quinones and polar lipid patterns of strain JZ16<sup>T</sup> and closely related type strains were determined as described by Wiertz et al. (2013). Strain JZ16<sup>T</sup> showed straight-chain fatty acids, as described for species of the genus *Corynebacterium* (Bernard & Funke, 2012), with octadecenoic acid (18:1 cis 9) as the main compound, major amounts of hexadecanoic acid (16:0) and minor amounts of heptadecanoic acid (17:0). Additionally, traces of pentadecanoic acid (15:0), octadecanoic acid (18:0) and two unknown compounds were present (Table 1). The unknown compounds of the fatty acid pattern were identified as saturated and unsaturated aldehydes according to their mass spectra. For strain JZ16<sup>T</sup>, mycolic acids were detected that showed a chromatographic mobility compared to the mycolic acids of *C. glutamicum* DSM 20300<sup>T</sup>, *C. callunae* DSM 20147<sup>T</sup> and *C. deserti* GIMN 1.010<sup>T</sup> (Fig. 2), and strain JZ16<sup>T</sup> had an acetyl type of peptidoglycan. Strain JZ16<sup>T</sup> and the *Corynebacterium* reference strains contained MK-9(H<sub>2</sub>) as main menaquinone and MK-8(H<sub>2</sub>) as minor compound. Diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannoside were detected as the main polar lipids of strain JZ16<sup>T</sup> (Fig. 3).

Susceptibility patterns to antimicrobial agents were obtained by the agar disc diffusion method on Mueller Hinton agar (Oxoid) with 16 different antimicrobial agents: penicillin G (6 μg; Oxoid), oxacillin (1 μg; Oxoid), ampicillin (10 μg; Oxoid), tetracycline (30 μg; bestbion), gentamicin (10 μg; bestbion), erythromycin (15 μg; bestbion), trimethoprim/sulfonamide (1.25/23.75 μg; bestbion), cefotaxime (30 μg; bestbion), cefazolin (30 μg; bestbion), cephalothin (30 μg; bestbion), pirlimycin (2 μg; bestbion), amoxicillin/clavulanic acid (20/10 μg; bestbion), kanamycin (30 μg; bestbion), streptomycin (10 μg; bestbion), tobramycin (30 μg; bestbion) and amikacin (30 μg; bestbion). The strains were considered resistant, intermediate or susceptible according to zone diameters of the CLSI document.
M100-S23 (M02-A11) (CLSI, 2013). Strain JZ16<T> showed resistance against oxacillin, ampicillin, trimethoprim/sulfonamide, kanamycin and streptomycin, whereas C. glutamicum DSM 20300<T> showed resistance only against oxacillin.

Extraction of genomic DNA, amplification and sequencing of 16S rRNA genes was performed as described previously (Wiertz et al., 2013). The rpoB gene sequence of strain JZ16<T> was determined as an additional marker for identification according to Khamis et al. (2004). Amplification of the partial rpoB gene was performed with the Corynebacterium-specific primers C2700F and C3130R (Khamis et al., 2004). These primers were also used as sequencing primers. The obtained sequences were manually edited with Chromas Lite 2.1.1. (Technelysium) and assembled with BioEdit 7.2.5. (Hall, 1999) to obtain either almost-complete 16S rRNA gene sequences (1400–1500 bp) or partial rpoB gene sequences (300–400 bp). The 16S rRNA gene sequences were compared to the sequences of type strains by the EzTaxon server (Kim et al., 2012) and rpoB gene sequences were compared to the reference sequences on the Basic local alignment search tool (BLAST; Altschul et al., 1990). Strain JZ16<T> showed a 16S rRNA gene sequence similarity of 98.3 % and a rpoB gene sequence similarity of 87.0 % to C. glutamicum ATCC 13032<T>. Kim et al. (2014) suggested that a 16S rRNA gene sequence similarity of 98.7 % is appropriate as the threshold for species delineation and Khamis et al. (2005) proposed a cut-off value of 95.0 % for the definition of species based on the rpoB gene sequence. Both criteria indicated that strain JZ16<T> represents a novel species of the genus Corynebacterium. This was confirmed by comparison of whole genome sequence of strain JZ16<T> with those of the three next related type strains. For isolate JZ16<T>, genomic DNA was extracted and sequenced in a combined approach using a whole-genome shotgun library and a mate pair library. The whole-genome shotgun library was constructed with the TruSeq DNA PCR-free library preparation kit (Illumina) and was sequenced in a paired-end run using the MiSeq reagent kit v3 (600 cycles) on the MiSeq desktop sequencer (Illumina). This shotgun sequencing resulted in 1905056 paired reads and 455067044 detected bases. The paired reads were assembled with the Roche GS De Novo Assembler software (Newbler; release 2.8), yielding 25 scaffolds with 35 scaffolded contigs. A 7-kb mate pair library was prepared with the Nextera mate pair sample preparation kit according to the gel-plus protocol. The mate pair library was sequenced with the MiSeq reagent kit v3 (600 cycles) and 117222 mate pair reads were added to the initial Newbler assembly. The gap closure step was facilitated by the Consed software (version 26; Gordon & Green, 2013). The genome sequence revealed a circular chromosome of 3047 kb length accompanied by two plasmids with sizes of 25 and 145 kb, respectively. The 145-kb plasmid represents the largest so far detected plasmid within the genus Corynebacterium. This plasmid codes for an aminoglycoside acetyltransferase and...
Corynebacterium crudilactis sp. nov.

Fig. 4. Phylogenetic position of the novel isolate within the genus Corynebacterium, obtained by maximum-likelihood analysis from 16S rRNA gene sequences. Bootstrap values >70% based on 1000 replicates are indicated at each node. Bar, 0.01 substitutions per nucleotide position.

streptomycin and hygromycin kinases, which is in accord with the resistances detected by disc diffusion method. The average nucleotide identities (ANIs) were calculated as a replacement for DNA–DNA hybridization according to Goris et al. (2007). Strain JZ16T showed an ANI of 81.85% (reverse 81.74%) with C. glutamicum ATCC 13032T. This was clearly below the threshold of 95–96% proposed by Kim et al. (2014) for the differentiation of bacterial species. ANI values calculated for strain JZ16T and several closely related species are given in Table S4. The G+C content for strain JZ16T was calculated from the genome sequence and is 51.59 mol%, 2.25 mol% less than that of C. glutamicum ATCC 13032T.

In order to determine the phylogenetic position of the isolate, the 16S rRNA gene and rpoB sequences were aligned with sequences of other type strains using CLUSTALW (Thompson et al., 1994). The phylogenetic position of the isolate JZ16T was analysed by the maximum-likelihood, maximum-parsimony and neighbour-joining algorithms with MEGA 6.06. software (Tamura et al., 2011). For maximum-likelihood analyses, model parameters were estimated using the ‘find best DNA/Protein option’ and models were selected according to the lowest BIC and AIC values (Tamura et al., 2011). As best-fit substitution model for 16S rRNA gene and rpoB sequences, the Tamura–Nei model was chosen with a discrete gamma distribution, and for 16S rRNA gene sequences additionally coupled with an allowance for the presence of invariant sites. The nearest-neighbour-interchange search method was used to reconstruct the maximum-likelihood tree. For neighbour-joining trees, the maximum composite likelihood model was used, and for maximum-parsimony trees, the subtree-pruning-regrafting search method was chosen. All trees were reconstructed using bootstrap test with 1000 replications to test phylogeny and the partial deletion option to treat with gaps. Trees reconstructed with the maximum-likelihood procedure are given in Figs 4 and 5. All three algorithms revealed a similar tree topology, in which strain JZ16T clustered with the next related type strains of C. glutamicum, C. deserti, C. callunae and C. efficiens.

Isolate JZ16T could be differentiated from the closest relatives C. glutamicum DSM 20300T, C. callunae DSM 20147T...
Therefore, it is concluded that strain JZ16 and closely related species of the genus Corynebacterium, for which the name Corynebacterium crudilactis sp. nov. is proposed.

**Description of Corynebacterium crudilactis sp. nov.**

*Corynebacterium crudilactis* (cru.di.lac’tis. L. adj. crudus raw; L. n. lac, lactis milk; N.L. gen. n. crudilactis of raw milk).

Colonies appear on TSA after two days of incubation under aerobic conditions at 30 °C. Colonies are 3 mm in diameter, beige, shiny and entire-edged. Good growth also occurs on brain-heart infusion agar supplemented with 0.25 g l⁻¹ potassium tellurite and 1.0 % Tween 80. Cells are non-motile and non-spore-forming rods, with cell dimensions of 0.9-1.8 x 0.6-1.3 µm. Cells are Gram-stain-positive, catalase-positive, oxidase-negative, KOH-lysis-negative and aminopeptidase-negative. The numerical code obtained by the API Coryne test system (bioMérieux) is 3241304. Positive for reduction of nitrate to nitrite, pyrazinamidase, β-

Combined phylogenetic, phenotypic and chemotaxonomic data enabled the differentiation of the isolated strain from other closely related species of the genus *Corynebacterium*. Therefore, it is concluded that strain JZ16 represents a novel species of the genus *Corynebacterium*, for which the name *Corynebacterium crudilactis* sp. nov. is proposed.

**Table 2. Differentiating physiological characteristics of strain JZ16 and closely related type strains of species of the genus Corynebacterium**

Strains: 1, JZ16; 2, *C. glutamicum* DSM 20300; 3, *C. callunae* DSM 20147; 4, *C. deserti* GIMN1.010. All data from this study.

<table>
<thead>
<tr>
<th>Characteristic</th>
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<th>2</th>
<th>3</th>
<th>4</th>
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<tr>
<td>Biochemical test (API Coryne and API ZYM):</td>
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<tr>
<td>β-Glucuronidase</td>
<td>+</td>
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<td>Nitrate reduction</td>
<td>+</td>
<td>-</td>
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<tr>
<td>β-Glucosidase (aesculin)</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Urease</td>
<td>+</td>
<td>+</td>
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<td>Esterase (C4)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>Esterase Lipase (C8)</td>
<td>+</td>
<td>+</td>
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<td>Trypsin</td>
<td>-</td>
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<td>Acid phosphatase</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<td>Fermentation of (API 50 CHB):</td>
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<tr>
<td>Maltose</td>
<td>-</td>
<td>+</td>
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<td>Sucrose</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Mannitol</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Presence of heptadecanoic acid</td>
<td>+</td>
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*Opposite result from the original description (Zhou et al., 2012).*
glucuronidase, β-glucosidase and urease and negative for pyrrolidonyl arylamidase, alkaline phosphatase, β-galactosidase, α-glucosidase, N-acetyl-β-glucosaminidase and hydrolysis of gelatin. Acid is produced from D-glucose, D-ribose and D-mannitol, but not from D-xylene, maltose, lactose, sucrose and glycerogen. Contains the menaquinones MK-9 (H2) and MK-8(H2) and corynemycolic acids. The peptidoglycan is of the acetyl type and major polar lipids are phosphatidylglycerol, diposphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannoside. The fatty acid profile contains 18 : 1 cis 9 and 16 : 0 as main compounds.

The type strain is JZ16T (=DSM 100882T =CCUG 69192T =LMG 29813T), isolated from bulk tank raw cow’s milk produced in Germany. The DNA G+C content of the type strain is 51.59 mol%.

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References


