Corynebacterium frankenforstense sp. nov. and Corynebacterium lactis sp. nov., isolated from raw cow milk

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Two groups of Gram-stain positive, aerobic bacterial strains were isolated from raw cow’s milk, from a milking machine and from bulk tank milk. Based on their 16S rRNA gene sequences these isolates formed two distinct groups within the genus Corynebacterium. The sequence similarities of the isolates to the type strains of species of the genus Corynebacterium were below 98.4%. The presence of menaquinones MK-8(H2) and MK-9(H2), the predominant fatty acid 18:1 cis 9 and a polar lipid pattern with several phospholipids but without aminolipids was in accord with the characteristics of this genus. The results of DNA–DNA hybridization, biochemical tests and chemotaxonomic properties allowed genotypic and phenotypic differentiation of the strains from all known species of the genus Corynebacterium. Therefore, the isolates were assigned to two novel species of this genus for which the names Corynebacterium frankenforstense sp. nov. (type strain ST18T=DSM 45800T=CCUG 63371T), and Corynebacterium lactis sp. nov. (type strain RW2-5T=DSM 45799T=CCUG 63372T) are proposed, respectively.

The genus Corynebacterium represents one of the earliest described bacterial genera and was originally proposed for the causative organism of diphtheria, Corynebacterium diphtheriae (Lehmann & Neumann, 1896) which was defined as the type species of this genus. Actually, the genus comprises a broad range of additional important human pathogens like Corynebacterium ulcerans and Corynebacterium xerosis (Lipsky et al., 1982). Species of the genus Corynebacterium are also known to be associated with mastitis for several mammals. Corynebacterium bovis and Corynebacterium amycolatum are frequently isolated from bovine mammary glands (Watts et al., 2000). Four species, C. amycolatum, Corynebacterium pseudotuberculosis, C. ulcerans and Corynebacterium minutissimum, are known to be associated with mastitis of dairy cows (Hommez et al., 1999). Corynebacterium camporealesis and Corynebacterium mastitidis are two species described for isolates from sheep with subclinical mastitis (Fernández-Garayzabal et al., 1997, 1998). Corynebacterium kroppenstedtii, C. amycolatum and Corynebacterium tuberculosis are the most common species isolated from women with inflammatory breast disease (Paviour et al., 2002).

Together with the genera Staphylococcus, Escherichia and Enterococcus, members of the genus Corynebacterium represent one of the most relevant mastitis-associated pathogens in dairy farming (Reyher et al., 2011). However, corynebacteria from raw milk of dairy cows are often difficult to identify, mainly due to inappropriate routine test systems. In a re-examination of 212 isolates identified as C. bovis by mastitis bacteriology laboratories, only 50 isolates were correctly re-identified as C. bovis by an expanded test set including 16S rRNA gene sequencing (Watts et al., 2000). This unsatisfying situation is also caused by dairy system associated corynebacteria that are not yet assigned to existing species of this genus and, therefore, are not covered by existing identification schemes. Actually, there is a continuous increase in species number for this genus. At time of writing this manuscript, there are 92 species of the genus Corynebacterium with three supplementary tables and two supplementary figures are available with the online version of this paper.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains ST18, RW2-45.1, RW2-5 and RW3-42 are HE983827, HE983828, HE983829 and HE983830, respectively.

Three supplementary tables and two supplementary figures are available with the online version of this paper.

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validly published names, 42 of which were described between 2000 and 2012 (Euzéby, 2013). The lack of reliable taxonomic data about corynebacteria associated with bovine mastitis results in frequent identification of those isolates as *Corynebacterium* spp., but not at the species level. Without detailed taxonomic information about dairy associated strains, the assessment of species of the genus *Corynebacterium* as relevant pathogens for dairy farming and potential milk spoiling organisms remains imprecise.

During an in-depth analysis of the microbiota of a milking parlour, we regularly isolated strains of the genus *Corynebacterium*. Foremilk samples (raw milk) were taken directly from udder quarters after discarding the first milk, from surfaces of the milking equipment and from bulk tank milk. The 16S rRNA gene sequences of two groups of these strains differed clearly from type strains of known species of the genus *Corynebacterium*. This prompted us to characterize these groups in more detail. The first group is represented by the isolates ST18<sup>T</sup> and RW2-45.1 and the second group by the isolates RW2-5<sup>T</sup> and RW3-42. Strain ST18<sup>T</sup> was isolated from a milk sample from a bulk milk storage tank on MRS agar under anaerobic incubation conditions. Isolate RW2-45.1 was isolated from foremilk samples of a mastitic cow. Isolate RW2-5<sup>T</sup> was isolated from milk of a cow without pathological findings and isolate RW3-42 from milk of a cow with unspecific mastitis. Strains RW2-45.1, RW2-5<sup>T</sup> and RW3-42 were isolated on Columbia blood agar (Oxoid) at 36 °C under aerobic incubation conditions. After isolation, all four strains were transferred to trypticase soy agar (TSA; Merck) and subcultivated aerobically at 30 °C.

On Columbia blood agar the isolates showed no haemolytic activity. Gram-staining and catalase activity was performed as described by Gerhardt *et al.* (1981). KOH lysis was tested according to Buck (1982). Oxidase activity was tested with Bactident oxidase test strips (Merck). Cell morphology, motility and presence of spores were analysed with an Olympus BH2 phase-contrast microscope at ×1000 magnification. Cell dimensions were measured by a Zeiss Axio Observer phase-contrast microscope equipped with Zen 2012 software at ×1000 magnification. All isolates were Gram-stain positive, KOH-lysis negative, catalase-positive and oxidase-negative. The cells showed a rod-shaped morphology. Motility or sporulation was not detected. Biochemical characterization was performed by the API Coryne system, the API ZYM and the API 50 CH (with API 50 CHB medium) test systems (bioMérieux) according to the instructions of the manufacturer. The reaction profiles of the isolates and the reference strains are listed in Tables S1–S3, available in IJSEM Online. Growth at 20 and 42 °C was tested on TSA. Fermentation of glucose at 42 °C was tested according to Wauters *et al.* (1998). All four isolates and the reference strain *C. amycolatum* DSM 6922<sup>T</sup> grew at 42 °C and showed fermentation of glucose at this temperature but did not grow at 20 °C.

Biomass for chemotaxonomic analyses was cultured in trypticase soy broth (Merck) at 30 °C. Cells were washed with 0.9 % NaCl, centrifuged and cell pellets were lyophilized. Isoprenoid quinones and polar lipids were extracted and analysed using the small-scale integrated procedure of Minnikin *et al.* (1984). Menaquinones (MK) were analysed by a Hewlett Packard series 1050 HPLC equipped with an ODS Hypersil column and a diode-array detector. Methanol and isopropyl ether (9 : 2, v/v) was used as mobile phase with a flow rate of 1.0 ml min<sup>−1</sup> and a column temperature of 30 °C (Hu *et al.*, 1999). For isolates ST18<sup>T</sup> and RW2-45.1, MK-8(H<sub>2</sub>) was detected as the dominating quinone type with more than 90 % and MK-7(H<sub>2</sub>) as a minor compound with less than 10 %. Isolates RW2-5<sup>T</sup> and *C. amycolatum* DSM 6922<sup>T</sup> showed MK-9(H<sub>2</sub>) as major quinone with more than 85 % and MK-8(H<sub>2</sub>) with less than 15 % as minor compound. The polar lipid pattern revealed diphosphatidylglycerol (DPG), phosphatidylglycerol (PG) and phosphatidylinositol (PI) for isolates ST18<sup>T</sup> and RW2-5<sup>T</sup> and for the reference strain *C. amycolatum* DSM 6922<sup>T</sup>. Phosphatidylinositol mannosides and ninhydrin-positive aminolipids were not detected for the strains analysed (Fig. 1).

Mycolic acids were extracted from lyophilized biomass and characterized by TLC according to Minnikin *et al.* (1975). *C. amycolatum* DSM 6922<sup>T</sup>, *C. atypicum* DSM 44849<sup>T</sup>, *Corynebacterium glutamicum* DSM 20300<sup>T</sup>, *Rhodococcus rhodochrous* DSM 43241<sup>T</sup> and *Gordonia terrae* DSM 43249<sup>T</sup> were used as reference strains. Except for *C. amycolatum* DSM 6922<sup>T</sup>, mycolic acids were detected for all reference strains. This is in accord with published data (Bendinger *et al.*, 1992; Collins *et al.*, 1988), except for *C. atypicum*, which was reported to contain no mycolic acids (Hall *et al.*, 2003). For isolates ST18<sup>T</sup> and RW2-45.1, mycolic acids were detected, whereas no mycolic acids were detected for isolates RW2-5<sup>T</sup> and RW3-42 (Fig. 2).

The acyl type of the peptidoglycan was determined for the isolates and the two reference strains *C. amycolatum* DSM 6922<sup>T</sup> and *C. atypicum* DSM 44849<sup>T</sup> by the colorimetric method of Uchida & Aida (1977). The strains *C. glutamicum* DSM 20300<sup>T</sup> and *G. terrae* DSM 43249<sup>T</sup> were used as references for the acetyl and the glycolyl type of peptidoglycan. All four isolates and the three reference strains of the genus *Corynebacterium* showed the acetyl type peptidoglycan, whereas *G. terrae* DSM 43249<sup>T</sup> showed the glycolyl type.

Fatty acid profiles were analysed for the isolates and the reference strains using cells grown on TSA for 48 h at 30 °C. Saponification with 15 % NaOH in 50 % methanol, acid methylation with 6 M HCl in 50 % methanol, and extraction of fatty acid methyl esters (FAMEs) were performed as described by Sasser (1990). The FAME extracts were analysed by GC-MS with an Agilent model 7890A gas chromatograph equipped with a 5 % phenylmethyl silicone capillary column and a model 5975C mass selective detector. The chromatographic conditions were as described previously (Lipski & Altendorf, 1997). The positions of double bonds were given from the carboxyl
group of the fatty acid molecule according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (CBN) (1977). All isolates showed a straight-chain fatty acid pattern with oleic acid (18:1 cis 9) as the dominating compound. In addition, the isolates ST18T and RW2-45.1 showed hexadecanoic acid (16:0), octadecanoic acid (18:0) and an unknown compound with an equivalent chain-length (ECL) of 16.695. In contrast, for the isolates RW2-5T and RW3-42, 16:0, 18:0, cis-9-heptadecenoic (17:1 cis 9) and heptadecanoic acid (17:0) were detected (Table 1).

For 16S rRNA gene sequencing, genomic DNA was isolated with the DNeasy Blood and Tissue kit according to the instructions of the manufacturer (Qiagen). The 16S rRNA gene of the purified chromosomal DNA was amplified using the universal bacterial primers GM3F and GM4R (Muyzer et al., 1995). The PCR products were purified by a QIAquick PCR purification kit (Qiagen) and sequenced by Seqlab (Göttingen, Germany). Sequencing primers used were 518F, 787R and 1099F (Buchholz-Cleven et al., 1997). Sequences were checked manually by the Chromas program (Technelysium) and assembled by MEGA 5 (Tamura et al., 2011) to obtain almost complete 16S rRNA gene sequences. These sequences were compared to reference sequences of type strains by the EzTaxon server (Kim et al., 2012). The most closely related type strain to isolates ST18T and RW2-45.1 was \textit{C. atypicum} CCUG 45804T with a sequence similarity of 95.0 \% and 94.8 \%, respectively. For the isolates RW2-5T and RW3-42, the type strain with most similar 16S rRNA gene sequence was \textit{C. amycolatum} CIP 103452T with 98.3 \% and 97.9 \% similarity, respectively. The phylogenetic position of the isolates was analysed by maximum-likelihood, maximum-parsimony and neighbour-joining algorithms by MEGA 5. All methods revealed similar phylogenetic positions of the isolates within the genus \textit{Corynebacterium}. A tree reconstructed with the neighbour-joining procedure is presented in Fig. 3. Trees reconstructed with maximum-likelihood and maximum-parsimony algorithms are provided as Figs. S1 and S2.

The phylogenetic reconstruction shows that the novel isolates represent two distinct groups within the genus \textit{Corynebacterium}. Phenotypic characteristics were also in accord with the genus description (Bernard & Funke, 2012): positive for Gram-reaction and catalase activity, acetyl type of the peptidoglycan, straight-chain saturated and monounsaturated fatty acids and MK-8(H2) and/or...
MK-9(H2) as major quinones. Mycolic acids were detected only for one of the two groups (for isolates ST18T and RW2-45.1) but, in contrast to most species of the genus, absence of mycolic acids was also reported for five other species described since 1988 (Collins et al., 1988, 1998, 2004; Hall et al., 2003; Fernández-Garayzábal et al., 2004).

The sequence similarities between the novel isolates and the type strains of the genus *Corynebacterium* were below 98.4 % and indicated that these strains may represent two novel species. A statistical analysis of Keswani & Whitman (2001) predicted a DNA–DNA hybridization value of less than 70 % from a sequence similarity of 98.6 % with a high level of confidence (99 %). A DNA–DNA hybridization value of 70 % is still accepted as the level for species delineation (Wayne et al., 1987). To prove this prediction, hybridization experiments were performed with genomic DNA from the isolates RW2-5T and RW3-42 and DNA from the most closely related type strain *C. amycolatum* DSM 6922T. The method described by Ziemke et al. (1998) was used except that for nick-translation 2 μg DNA was labelled during a 3 h incubation at 15 °C. The extent of DNA–DNA hybridization between RW2-5T and the reference strain *C. amycolatum* DSM 6922T was 49 % (reciprocal hybridization 43 %) and between RW3-42 and this reference strain 55 % (reciprocal hybridization 32 %). These values were below 70 %, which confirms the differences of these strains to the most closely related species, *C. amycolatum*. The grouping of strains RW2-5T and RW3-42 in a single species was also confirmed by DNA–DNA hybridization experiments, which resulted in hybridization of 92 % (reciprocal hybridization 100 %). The same was true for isolates ST18T and RW2-45.1, with hybridization of 100 % (reciprocal hybridization 100 %). We abstained from hybridization experiments between the latter isolates and the most closely related type strains because sequence similarities are clearly below 97 %.

The isolates ST18T and RW2-45.1 could be differentiated from the most closely related species *C. atypicum* by their good growth on TSA under aerobic conditions. In contrast, the reference strain *C. atypicum* DSM 44849T showed growth on TSA only with a supplement of 1 % Tween 80 or on Columbia blood agar under O2-reduced and CO2-enriched atmosphere with pinpoint colonies. Biochemically, the isolates showed pyrazinamidase activity and acid production from glucose, arabinose, arabitol, ribose, fructose and mannose while the reference strain did not (Table 2).

Isolates RW2-5T and RW3-42 could be differentiated from the most closely related species *C. amycolatum* by their fatty acid profiles, which showed a significantly lower content of octadecanoic acid (18:0) but a higher content of 17:0 (Table 1). Biochemically, the isolates were negative for fermentation of glycerol and maltose, which is in contrast to the species *C. amycolatum* (Table 2).

### Table 1. Fatty acid profiles of isolates and reference strains of the genus *Corynebacterium*

<table>
<thead>
<tr>
<th>Fatty acid (%)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>12:0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.9</td>
</tr>
<tr>
<td>14:0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>16:0</td>
<td>48.0</td>
<td>38.8</td>
<td>12.3</td>
<td>11.7</td>
<td>36.3</td>
<td>5.8</td>
</tr>
<tr>
<td>ECL 16.695</td>
<td>2.1</td>
<td>3.4</td>
<td></td>
<td></td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>17:1 cis 9</td>
<td></td>
<td></td>
<td>6.4</td>
<td>4.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17:0</td>
<td></td>
<td></td>
<td>16.2</td>
<td>14.4</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>18:1 cis 9</td>
<td>49.1</td>
<td>56.4</td>
<td>50.9</td>
<td>51.6</td>
<td>34.7</td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>0.8</td>
<td>1.5</td>
<td>14.2</td>
<td>18.1</td>
<td></td>
<td>28.4</td>
</tr>
</tbody>
</table>

**Description of *Corynebacterium frankenforstense* sp. nov.**

*Corynebacterium frankenforstense* (fran.ken.forst.en’se N.L. neut. adj. *frankenforstense* of or belonging to Frankenforst, the name of the experimental farm, from where the type strain was isolated).

Cells are Gram-stain positive, non-spore-forming and non-motile short rods. Cell dimensions are 1.0–1.5 x 0.6–0.9 μm. Colonies on TSA after 3 days of incubation under aerobic conditions at 30 °C are about 2 mm in diameter, white, entire-edged and shiny. On Columbia blood agar after 3 days of incubation under aerobic conditions at 30 °C colonies are about 2 mm in diameter, white, entire-edged, shiny and non-haemolytic. Oxidase-negative and catalase-positive. Positive for fermentation of glucose at 42 °C. Growth at 42 °C but not at 20 °C. Gelatin and starch are not hydrolysed. Positive for pyrazinamidase, β-glucuronidase, esterase lipase (C8) and leucine arylamidase activity but negative for pyrrolidonyl arylamidase, alkaline phosphatase, acid phosphatase, β-galactosidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, urease, lipase (C14), valine arylamidase, cysteine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, N-galactosidase, β-galactosidase, z-mannosidase and α-fucosidase. Acid is produced from L-arabinose, D-ribose, D-glucose, D-fructose, D-mannose, sucrose, trehalose and...
Fig. 3. Phylogenetic positions of the novel isolates within the genus Corynebacterium based on neighbour-joining analysis of 16S rRNA gene sequences. Bootstrap values greater than 70% based on 1000 replicates are indicated at nodes. Bar, 0.01 substitutions per nucleotide position.
**Table 2. Differentiating characteristics between species of the genus Corynebacterium**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic growth on TSA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Presence of mycolic acids</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+*</td>
</tr>
<tr>
<td>Presence of heptadecanoic acid (&gt;10%)</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Major menaquinone</td>
<td>MK-8(H$_2$)</td>
<td>MK-9(H$_2$)</td>
<td>MK-9(H$_2$)</td>
<td>ND</td>
</tr>
<tr>
<td>Pyrazinamidase†</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Alkaline phosphatase†</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>β-Glucuronidase§</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Acid from (API 50 CH):</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol, maltose</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>l-Arabinose, D-arabitol</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>D-Ribose, D-fructose, D-mannose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

*Reported as mycolic acid-negative by Hall et al. (2003).
†Based on API Coryne system.
§Species is described as positive for pyrazinamidase activity by Bernard & Funke (2012).
||Some strains are negative for acid production from maltose (Collins et al., 1988).

D-arabitol, but not from glycerol, D-galactose, maltose, erythritol, D-arabinose, D-xylene, L-xylene, D-adonitol, methyl 8,9-D-xylopyranoside, L-sorbitose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl 8,9-D-mannopyranoside, methyl 8,9-D-glucoxyranoside, amygdalin, cellobiose, lactose, melibiose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol, potassium gluconate, potassium-2-ketogluconate or potassium-5-ketogluconate. Predominating fatty acids are hexadecanoic acid (16:0) and oleic acid (18:1 cis 9). Tuberculostearic acid is not present. Mycolic acids are present. Peptidoglycan is of the acetyl type. Major quinone is menaquinone-8(H$_2$).

The type strain is ST18$^{+}$ (=DSM 45800$^{T}$=CCUG 63371$^{T}$) and was isolated from a bulk milk tank of a dairy farm in Germany.

**Description of Corynebacterium lactis sp. nov.**

*Corynebacterium lactis* (lac’tis. L. gen. n. lactis from milk, according to the isolation source of the type strain).

Cells are Gram-stain positive, non-spore-forming and non-motile short rods. Cell dimensions are 1.0–1.6 × 0.6–0.8 μm. Colonies on TSA after 3 days of incubation under aerobic conditions at 30 °C are about 2 mm in diameter, white, with uneven edge and dry. On Columbia blood agar after 3 days of incubation under aerobic conditions at 30 °C colonies are about 4 mm in diameter, white, with uneven edge, dry and non-haemolytic. Oxidase-negative and catalase-positive. Positive for fermentation of glucose at 42 °C. Growth at 42 °C but not at 20 °C. Gelatin and starch are not hydrolysed. Positive for pyrazinamidase, alkaline phosphatase and leucine arylamidase activity but negative for pyrrolidonyl arylamidase, β-glucuronidase, β-galactosidase, α-glucosidase, N-acetyl-β-glucosaminidase, urease, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, α-galactosidase, α-mannosidase and α-fucosidase. Acid is produced from D-ribose, D-glucose, D-fructose and D-mannose, but not from L-arabinose, glycerol, N-acetylglucosamine, arbutin, salicin, maltose, D-arabitol, erythritol, D-arabinose, D-xylene, L-xylene, D-adonitol, methyl β-D-xylopyranoside, L-sorbitose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl β-D-mannopyranoside, methyl 8,9-D-glucoxyranoside, amygdalin, cellobiose, lactose, melibiose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol, potassium gluconate, potassium-2-ketogluconate or potassium-5-ketogluconate. Predominating fatty acids are hexadecanoic acid (16:0), heptadecanoic acid (17:0), octadecanoic acid (18:0) and oleic acid (18:1 cis 9). Tuberculostearic acid is not present. Mycolic acids are absent. Peptidoglycan is of the acetyl type. Major quinone is menaquinone-9(H$_2$).

The type strain is strain RW2-5$^{+}$ (=DSM 45799$^{T}$=CCUG 63372$^{T}$) and was isolated from raw cow’s milk produced in Germany.

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