Taxonomic characterization of nine strains isolated from clinical and environmental specimens, and proposal of Corynebacterium tuberculostearicum sp. nov.

Carole Feurer, Dominique Clermont, François Bimet, Adina Candréa, Mary Jackson, Philippe Glaser, Chantal Bizet and Catherine Dauga

INTRODUCTION

Corynebacterium species have been detected in various habitats, including dairy products, soil and vegetables (Brennan et al., 2001; Fudou et al., 2002), and in a large variety of hosts such as tortoises, cats (Collins et al., 2001b), dogs (Collins et al., 1999b), seals (Pascual et al., 1998), goats, cows (Fernandez-Garayzabal et al., 1997) and sheep (Collins et al., 2001a). Several species have been implicated in human disease. Corynebacterium diphtheriae is the aetiological agent of diphtheria. Other species are recognized as opportunistic pathogens, and have been isolated from diverse clinical specimens, including the genito-urinary tract (Collins et al., 1999a; Funke et al., 1995; Furness et al., 1979), the respiratory tract (Riegel et al., 1995b, 1997b; Zimmermann et al., 1998), blood samples (Riegel et al., 1997a; Funke et al., 1997c) and surgical infections (Esteban et al., 1999; Yassin et al., 2002b). In the 2 years before this paper was written, nine novel species had been described, namely Corynebacterium capitosi (Collins et al., 2001a), Corynebacterium testudinoris and Corynebacterium felinum (Collins et al., 2001b), Corynebacterium frenesi (Renaud et al., 2001), Corynebacterium aurimucosum (Yassin et al., 2002a), Corynebacterium mooreparkense (Brennan et al., 2001), Corynebacterium appendicis (Yassin et al., 2002b), Corynebacterium efficiens (Fudou et al., 2002) and Corynebacterium sphenisorum (Goyache et al., 2003).

Some species of the genus Corynebacterium belong to the normal flora of the skin (Funke et al., 1997a; Collins et al., 1988), while others were isolated from specific cutaneous lesions. In leprosy, Corynebacterium spp., referred to as leprosy-derived corynebacteria (LDC) (Beaman et al., 1974), were initially reported as a homogeneous and unique group of organisms within the genus Corynebacterium, and were later included in the ‘corynebacteria pathogenic for humans’ (Danhaive et al., 1982). A novel species, ‘Corynebacterium tuberculostearicum’, was described as a lipid-requiring LDC, differing from other corynebacteria by the production of tuberculostearic (10-methyl-octadecanoic) acid (Brown et al., 1984), although this name was never validly published. Since this description, other...
members of the genus Corynebacterium have been shown to produce tuberculostearic acid (Funke et al., 1998; Yassin et al., 2002a, b).

Identification of lipophilic corynebacteria appears to be problematic with respect to classic bacteriological methods (Riegel et al., 1995a; Watts et al., 2000). The US Centers for Disease Control and Prevention (CDC) separated lipophilic corynebacteria into groups ANF-1, G-1 and G-2 in addition to groups JK (Corynebacterium jeikeium) and D-2 (Corynebacterium urealyticum) using biochemical tests (Hollis & Weaver, 1981; Riegel et al., 1992, 1993). According to their ability to ferment fructose, bacteria isolated from urethritis samples were separated into 'Corynebacterium genitalium' pathogenic biotypes and 'Corynebacterium pseudogenitalium' saprophytic biotypes (Furness et al., 1979). In disagreement with these data, DNA-DNA hybridizations distinguished five genomic groups of lipid-requiring corynebacteria (Riegel et al., 1995a). Genomospecies I included 'C. tuberculostearicum' LDCC8, strain CDC G 5840 (G-2 group), strain CDC F 8156 (G-1 group) and three different 'C. pseudogenitalium' biotypes. Numerous descriptions of novel lipophilic species of the genus Corynebacterium required the use of molecular-based approaches (Riegel et al., 1995a; Neubauer et al., 1991; Funke et al., 1997b; Fernandez-Garayzabal et al., 1997; Yassin et al., 2002b). The implementation of 16S rRNA gene sequencing combined with improved phenotypic approaches (in particular, biochemical characterization using bioMérieux API 50 CH, API ZYM or API Coryne) has provided an accurate system for the identification of novel corynebacteria. Recently, this strategy has led to the incrimination of 'C. tuberculostearicum' as a causative agent in mastitis (Paviour et al., 2002).

During the course of the characterization of strains deposited in the Collection of the Institut Pasteur (CIP), we identified nine isolates from various clinical and food samples as lipophilic Corynebacterium spp. On the basis of phylogenetic investigations, it is suggested that they be classified as 'C. tuberculostearicum'. Phenotypic comparison of the nine isolates, 'C. tuberculostearicum' strains CIP 107291T and CIP 107067 and 'C. pseudogenitalium' CIP 106714 led us to an emended description of 'C. tuberculostearicum' and the formal proposal of Corynebacterium tuberculostearicum sp. nov.

**METHODS**

**Bacterial strains and culture conditions.** The nine unidentified strains were isolated from different sources (Table 1). 'C. tuberculostearicum' strain CIP 107291T (=CCUG 45418T=ATCC 35529T), also named Medalle X5 or LDC-205 (Brown et al., 1984; Danhaive et al., 1982), and 'C. tuberculostearicum' strain CIP 107067 (=CCUG 41662) were originally obtained from the Culture Collection of the University of Göteborg. 'C. pseudogenitalium' (C-1 type) strain CIP 106714 (=CECT 763=ATCC 33035) (Furness et al., 1979) was obtained from the Spanish Type Culture Collection (CECT). The 12 strains were grown on trypticase soy agar (Bio-Rad) supplemented with 1% (v/v) Tween 80 (Riegel et al., 1995a; Watts et al., 2000).

**Biochemical characterization.** Four commercially available galleries, API Coryne, API ZYM, API 50 CH and Biotype-100 strips, were used according to the instructions of the manufacturer (bioMérieux) to determine biochemical characteristics, i.e. fermentation profiles, enzymic reactions and assimilation tests. They were inoculated with a bacterial suspension enriched with 1% (v/v) Tween 80.

**PAGE analysis of whole-cell protein.** Cellular protein extracts for PAGE analysis were prepared as described previously (Barreau & Hansen, 2000). Densitometric analysis, normalization and comparison of protein patterns were performed using GelCompar version 2.5 (Applied Maths).

**Fatty acid analysis and tuberculostearic acid detection.** Fatty acid methyl esters and mycolic acid methyl esters were extracted as described previously (Phetsukrit et al., 1999). Detection of mycolic acids was performed with TLC (Minnikin et al., 1980). Fatty acid methyl esters were analysed by GC on a Shimadzu GC-14A chromatograph using a methyl silicone 5% phenyl column operating at a temperature of 175 °C for 2 min followed by a programmed increase of 8 °C min⁻¹ to 300 °C. The eluted peak of tuberculostearic

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### Table 1. 'C. tuberculostearicum' reference strains and unnamed lipophilic corynebacteria used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Date</th>
<th>Country (town) of origin</th>
<th>Clinical or environmental source (pathology)</th>
</tr>
</thead>
<tbody>
<tr>
<td>'C. tuberculostearicum' CIP 107291T</td>
<td>1901</td>
<td>Philippines</td>
<td>Bone marrow (leprosy)</td>
</tr>
<tr>
<td>'C. tuberculostearicum' CIP 107067</td>
<td>1999</td>
<td>Sweden</td>
<td>Contaminant, industrial laboratory</td>
</tr>
<tr>
<td>CIP 101775</td>
<td>1985</td>
<td>France (Lille)</td>
<td>Inguinal node (soft chancre)</td>
</tr>
<tr>
<td>CIP 102076</td>
<td>1985</td>
<td>France</td>
<td>Unknown</td>
</tr>
<tr>
<td>CIP 102124</td>
<td>1986</td>
<td>France (Bordeaux)</td>
<td>Tinned tuna</td>
</tr>
<tr>
<td>CIP 102211</td>
<td>1986</td>
<td>France (Royan)</td>
<td>Lymph node</td>
</tr>
<tr>
<td>CIP 102346</td>
<td>1986</td>
<td>France (Le Rancy)</td>
<td>Blood</td>
</tr>
<tr>
<td>CIP 102590</td>
<td>1986</td>
<td>France (Bordeaux)</td>
<td>Urethra</td>
</tr>
<tr>
<td>CIP 102622</td>
<td>1986</td>
<td>France (Paris)</td>
<td>Skin (no leprosy lesion)</td>
</tr>
<tr>
<td>CIP 102645</td>
<td>1986</td>
<td>France (Sens)</td>
<td>Peritoneum (peritonitis)</td>
</tr>
<tr>
<td>CIP 102857</td>
<td>1987</td>
<td>France (Paris)</td>
<td>Urine</td>
</tr>
<tr>
<td>'C. pseudogenitalium' CIP 106714</td>
<td>1979</td>
<td>Unknown</td>
<td>Healthy urogenital tract</td>
</tr>
</tbody>
</table>
acid was identified by comparing its retention time with the retention time of the tuberculostearic acid standard of *Mycobacterium tuberculosis*.

**Genomic DNA extraction.** Bacteria were scraped from the agar and resuspended in 200 μl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7.4). After the addition of 50 μl lysozyme (5 mg ml⁻¹, pH 7) and 30 μl RNase A from bovine pancreas (10 mg ml⁻¹), the samples were incubated for 30 min at 37°C. Each lysate was transferred to a 2 ml tube (Sarstedt) containing 20 μl 10 % SDS, 0.2 g acid-washed glass beads (212–300 μm; Sigma) and 200 μl phenol/chloroform/isooamyl alcohol (25:24:1 by vol.). The tubes were shaken for two periods of 40 s at a speed of 6 m s⁻¹ in a Savant FastPrep apparatus and then centrifuged at 14,000 r.p.m. for 20 min. The upper phase containing DNA was precipitated with absolute ethanol at −20°C. After centrifugation, the pellets were washed with 70 % ethanol, dried and dissolved in 200 μl TE buffer.

**16S rRNA gene amplification.** 16S rRNA genes were amplified by PCR using the universal primers A (5′-AGAGTTTGATCCTGGCTCAG-3′, positions 8–27 according to the *Escherichia coli* numbering system; Brosius et al., 1978) and H (5′-AAGGAGTTGATCCTGGCTCAGC-3′, positions 1541–1522) (Böttger, 1989). Each reaction was performed in a 1 μl volume containing 20 pmol each primer, 2.5 U Taq DNA polymerase (Roche Applied Science), 10 mM reaction buffer containing MgCl₂, 0.3 mM each dNTP (Pharmacia Biotech) and 1 μl template DNA. PCR amplification was carried out in a GeneAmp thermal cycler (Applied Biosystems), using the following thermal profile: 94°C for 4 min, followed by 25 cycles of 94°C for 1 min, 57°C for 1 min and 72°C for 2 min, with a final extension step at 72°C for 5 min. PCR products were examined using 1 % (w/v) agarose gel electrophoresis and ethidium bromide staining. SmartLadder (Eurogentec) was used as a molecular size marker.

**16S rRNA gene sequencing.** Each PCR product was treated using the ExoSAP-IT exonuclease I and shrimp alkaline phosphatase protocol (USB). PCR products were then sequenced using the Taq DyeDeoxy terminator cycle sequencing kit and an ABI PRISM 3700 DNA sequencer according to the instructions of the manufacturer (PE Applied Biosystems). Primers A and H and sequencing primers 5′-CTCTACGGGGAGCCAGCAGT-3′ (positions 339–358 according to the *E. coli* numbering system), 5′-ACTGGTCGCTCCCCGAG-3′ (positions 358–339), 5′-GGTGGCAGCAGCCGGGTAAAT-3′ (positions 514–534), 5′-GATGTGTTATTTAATTCGA-3′ (positions 947–964), 5′-TGGATTAAACACATGCG-3′ (positions 964–947) and 5′-AGGTTGGGCCCTGTTCCGG-3′ (positions 1115–1097) were used. The GenBank/EMBL/DDJB accession numbers for the 16S rRNA gene sequences determined in this study are shown in Fig. 2.

**Computational analysis.** The 16S rRNA sequences were compared with known sequences in the GenBank/EMBL/DDJB databases by using the BLASTN 2.2.4 algorithm (Altschul et al., 1997). Sequences were then aligned using the CLUSTAL V algorithm (Higgins, 1994) with default gap penalties. The initial alignment was further refined by eye, introducing gaps to improve the overall alignment, using the alignment editor of the PAUP* version 4.0 software package (Swofford, 1998). Sequence-distance matrices were established in pairwise comparisons by applying the algorithm of Tamura & Nei (1993). Phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987) using the PHYLIP version 3.5 software package (Felsenstein, 1993). A maximum-likelihood analysis was performed using the same model and an estimated rate gamma shape of 0.7484 with PAUP* 4.0 version (Felsenstein, 1981). Maximum-parsimony trees were obtained using 100 random-addition heuristic search replicates and the tree bisection–reconnection branch-swapping option. Statistical significance was evaluated by bootstrap analysis (Felsenstein, 1985), with 100 iterations of bootstrap samplings.

**RESULTS AND DISCUSSION**

**Phenotypic characterization of strains**

The nine unidentified strains were non-motile, non-spore-forming, pleomorphic, Gram-positive rods, frequently swollen at one or both ends, and aerobic to facultatively anaerobic. On trypto casein soy agar supplemented with 1 % (v/v) Tween 80, colonies were whitish and glistening with entire edges (1 mm diameter after 24–48 h at 37°C). All isolates were oxidase-negative, catalase-positive and urease-negative. Most had enzymic activities similar to those of *C. tuberculostearicum* CIP 107291¹, CIP 107067 and ‘*C. pseudogenitalium*’ CIP 106714 in the API ZYM system. Reactions for esterase (C4) and naphthol-AS-BI-phosphohydrolase were positive for all strains. No activity was detected for α-galactosidase, β-galactosidase, α-fucosidase, lipase (C14), trypsin or α-chymotrypsin. Results for nitrate reductase, esterase lipase (C8), leucine arylamidase and alkaline phosphatase production are shown in Table 2. With API 50 CH, acid was produced from galactose, glucose, glycerol, fructose, mannose, ribose and 5-ketogluconate but not from mannitol, glycogen, starch, lactose or xylose. Sucrose and maltose were not fermented by any of the strains, even after 3 days incubation (Table 2). Biotype-100 strips showed that the substrates D-glucose, sucrose, D-ribose, glycerol, L-malate, 2-ketogluconate, succinate, fumarate, L-aspartate, L-glutamate, L-lysine and D-fructose were utilized.

### Table 2. Differential characteristics of *C. tuberculostearicum* and its nearest phylogenetic relatives

<table>
<thead>
<tr>
<th>Species/strains</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tr>
<td><strong>Characteristic</strong></td>
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<td>Production of:</td>
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<tr>
<td>Nitrate reductase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>27</td>
<td>-</td>
</tr>
<tr>
<td>Pyrazinamidase</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>100</td>
<td>+</td>
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<tr>
<td>Alkaline phosphatase</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>91</td>
<td>+</td>
</tr>
<tr>
<td>Leucine arylamidase</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>91</td>
<td>+</td>
</tr>
<tr>
<td>α-Glucosidase</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Esterase lipase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>91</td>
<td>+</td>
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<td>Fermentation of:</td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>Sucreose</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>82</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>91</td>
<td>–</td>
</tr>
<tr>
<td>Glycerol</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>100</td>
<td>+</td>
</tr>
<tr>
<td>Galactose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>100</td>
<td>+</td>
</tr>
</tbody>
</table>

Species/strains: 1, *C. accolens* CIP 104783¹; 2, ‘*C. fastidiosum*’ CIP 103808; 3, *C. magnum* CIP 104999⁡; 4, ‘*C. segemontorum*’ CIP 107068; 5, *C. tuberculostearicum* (n=11); 6, ‘*C. pseudogenitalium*’ CIP 106714. Values for *C. tuberculostearicum* are percentages of strains giving positive results.
L-glutamate, L-proline and L-serine were always used as sole carbon sources. L-Alanine was assimilated by all strains except 'C. tuberculostearicum' CIP 107067. 2-Ketogluconate was assimilated by all strains except 'C. pseudogenitalium' CIP 106714. The API Coryne system generated the numerical code 6100324 for 'C. tuberculostearicum' strains CIP 107291\textsuperscript{T} and CIP 102590, 6100325 for strains CIP 102211, CIP 102346, CIP 101775, 'C. tuberculostearicum' CIP 107067 and 'C. pseudogenitalium' CIP 106714, 7100325 for strains CIP 102124, CIP 102857 and CIP 102076, 6100325 for strain CIP 102622 and 2100325 for strain CIP 102645. These codes corresponded to Corynebacterium CDC group G in the API Coryne profile index. GC analyses of non-hydroxylated fatty acid methyl esters showed that C\textsubscript{18:0}, C\textsubscript{18:1}\text{cis\textsubscript{9}} and C\textsubscript{18:2} were the predominant cellular fatty acids in the nine strains, 'C. tuberculostearicum' CIP 107291\textsuperscript{T} and CIP 107067, in agreement with a previous study (Bernard et al., 1991). Small amounts of tuberculostearic acid (0\textsuperscript{0}83–4\textsuperscript{0}42 %) and mycolic acids were also detected in the strains. All these strains clustered together in SDS-PAGE analysis and formed a distinct branch with a correlation level of more than 72\textsuperscript{\%} (Fig. 1). These chemotaxonomic findings demonstrated that the nine strains, 'C. tuberculostearicum' strains CIP 107291\textsuperscript{T} and CIP 107067 and 'C. pseudogenitalium' CIP 106714 represented a homogeneous group.

**Phylogenetic analysis**

To ascertain the phylogenetic positions of the isolates, complete 16S rRNA gene sequences (1512 nucleotides) of the nine unidentified strains, 'C. tuberculostearicum' CIP 107291\textsuperscript{T} and CIP 107067 and 'C. pseudogenitalium' CIP 106714 were determined and subjected to a comparative analysis. Sequence searches of the GenBank/EMBL/DDBJ databases revealed that the nine unidentified strains were closely related to species of the genus Corynebacterium. The 16S rRNA gene sequences of the nine strains displayed high levels of similarity (89\textsuperscript{\%}–98\textsuperscript{\%}) to those of previously described members of the genus Corynebacterium. A preliminary phylogenetic analysis based on Corynebacterium 16S rRNA gene sequences deposited in international databases showed that the nine sequences clustered with 'C. tuberculostearicum' ATCC 35692\textsuperscript{T} (accession no. X84247) and 'C. pseudogenitalium' ATCC 33035 (U87822), confirming that the isolates were members of the genus Corynebacterium (data not shown). A neighbour-joining phylogenetic tree depicting the relationships between the strains and the nearest Corynebacterium species is shown in Fig. 2. The 16S rRNA gene sequences of the isolates grouped with those of 'C. pseudogenitalium' CIP 106714 and 'C. tuberculostearicum' strains CIP 107291\textsuperscript{T} and CIP 107067. Comparison of sequences from this monophyletic group revealed 0–8 nucleotide differences (corresponding to 100–99\textsuperscript{\%} similarity). The high levels of 16S rRNA gene sequence similarity suggested that the nine strains, 'C. tuberculostearicum' strains CIP 107291\textsuperscript{T} and CIP 107067 and 'C. pseudogenitalium' CIP 106714 belonged to the same species (Drancourt et al., 2000).

**Differential properties**

The monophyletic group of 'C. tuberculostearicum' and related strains formed a distinct lineage within a small
Corynebacterium tuberculostearicum sp. nov.

Description of Corynebacterium tuberculosis sp. nov.

Corynebacterium tuberculosis (tu.ber.cu.lo.stea’ri.cum. N.L. neut. adj. tuberculosis of tuberculostearic acid, which is contained in the cells).

Cells are pleomorphic, Gram-positive rods, non-motile and non-spore-forming, developing coccoid forms in stationary cultures. Colonies on trypto casein soy agar supplemented with Tween 80 are circular, convex, glistening and 1 mm in diameter. Aerobic to facultatively anaerobic. Lipophilic. Oxidase-negative and catalase-positive. Corynebacterium mycolic acids are present and the fatty acid profiles contain tuberculostearic acid. Acid is produced from galactose, glucose, glycerol, fructose, mannose, ribose and 5-ketogluconate but not from mannitol, glycerogen, starch, sorbitol, lactose, inulin or xylose. Production of acid from trehalose, maltose, gluconate, sucrose and N-acetylglucosamine is variable. Urea, aesculin and gelatin are not hydrolysed. Presence of nitrate reductase is variable. DNase is absent. Esterase (C4) and naphthol-AS-BI-phosphohydrolase activities are detected. Presence of esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase and alkaline phosphatase is variable. No activity is detected for β-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, α-fucosidase, lipase (C14), trypsin or ξ-chymotrypsin. The following substrates are utilized in 2 or 4 days: D-glucose, sucrose, D-ribose, glycerol, L-malate, 2-ketogluconate, succinate, fumarate, L-aspartate, L-glutamate, L-proline and L-serine. The following substrates are not utilized in 6 days: caprate, citrate and DL-glycerate.

The type strain, Medalle XT (=LDC-20T=CIP 107291T=CCUG 45418T=ATCC 35529T), was isolated from a case of lepromatous leprosy in the Philippines (Brown et al., 1984). The type strain has all the properties given for the species. In addition, it assimilates D-mannose.

ACKNOWLEDGEMENTS

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