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NOTE

Corynebacterium kroppenstedtii sp. nov., a novel corynebacterium that does not contain mycolic acids

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A strain of a hitherto undescribed coryneform bacterium from human clinical material was characterized by phenotypic and molecular taxonomic methods. Comparative 16S rRNA gene sequence analysis demonstrated the strain represents a novel and deep lineage within the genus *Corynebacterium sensu stricto*. Chemical analyses revealed the unidentified strain was unusual in that it lacked mycolic acids. Based on the phylogenetic and phenotypic distinctiveness of the unknown isolate, it is proposed that the bacterium be classified as a new *Corynebacterium* species, for which the name *Corynebacterium kroppenstedtii* sp. nov. is proposed. The type strain is CCUG 35717^T.

Keywords: *Corynebacterium kroppenstedtii* sp. nov., 16S rRNA, phylogeny, taxonomy

The genus *Corynebacterium* comprises a phenotypically heterogeneous group of Gram-positive non-acid-fast asporogenous rod-shaped organisms with a high DNA G+C content (Collins & Cummins, 1986). In recent years there has been a growing recognition of the importance of corynebacteria as opportunistic pathogens of man (Funke *et al*., 1997c). As a result of increased clinical interest, combined with intensive chemical and molecular taxonomic investigations of these organisms, many new species have been described in recent years from human sources [e.g. *Corynebacterium accolens* (Neubauer *et al*., 1991), *Corynebacterium afermentans* (Riegel *et al*., 1993a), *Corynebacterium argentoratense* (Riegel *et al*., 1995a), *Corynebacterium auris* (Funke *et al*., 1995b), *Corynebacterium coyleae* (Funke *et al*., 1997b), *Corynebacterium lipophiloflavum* (Funke *et al*., 1997a), *Corynebacterium macginleyi* (Riegel *et al*., 1995b), *Corynebacterium propinquum* (Riegel *et al*., 1993b), *Corynebacterium glucuronolyticum* (Funke *et al*., 1995a), *Corynebacterium urealyticum* (Pitcher *et al*., 1992)]. Despite the considerable metabolic diversity and wide DNA base composition range (approx. 47–70 mol% G+C) of the genus *Corynebacterium* (Collins & Cummins, 1986; Pitcher, 1983), 16S rRNA gene sequencing has revealed the genus to be monophyletic (Pascual *et al*., 1995). Members of the genus *Corynebacterium* are characterized by the presence of distinctive low-molecular-mass (approx. 22–36 carbon atoms) α-alkyl-β-hydroxy long-chain fatty acids (designated corynomycolic acids) (Collins & Cummins, 1986). Currently *Corynebacterium amycolatum*, a species found on human skin and encountered in clinical specimens, is the only recognized species of the genus that lacks corynomycolic acids (Collins *et al*., 1988). *Turicella otitidis*, an organism also from human sources which forms a deep subline within the genus *Corynebacterium*, also lacks these characteristic lipids (Funke *et al*., 1994). In this report, we describe the characteristics of a strain of a hitherto unknown *Corynebacterium* species, which lacks corynomycolic acids, isolated from human sputum. Based on phenotypic and molecular genetic findings we formally propose a new species, *Corynebacterium kroppenstedtii*, for the amycolate bacterium.

The human clinical isolate (CCUG 35717^T) was referred to the Culture Collection of the University of Göteborg, Sweden for identification. Strain CCUG 35717^T originated from sputum (82-year-old female with pulmonary disease) and was cultured on horse blood agar (Columbia base; Difco) at 37 °C. The biochemical profile of the strain was determined using the commercial API CORYNE system (bioMérieux). Enzyme reactions were read after 24 h incubation at

Abbreviation: TMS-MAME, trimethylsilylated mycolic acid methyl ester.

The EMBL under accession number for the 16S rRNA sequence of strain CCUG 35717^T is Y10077.

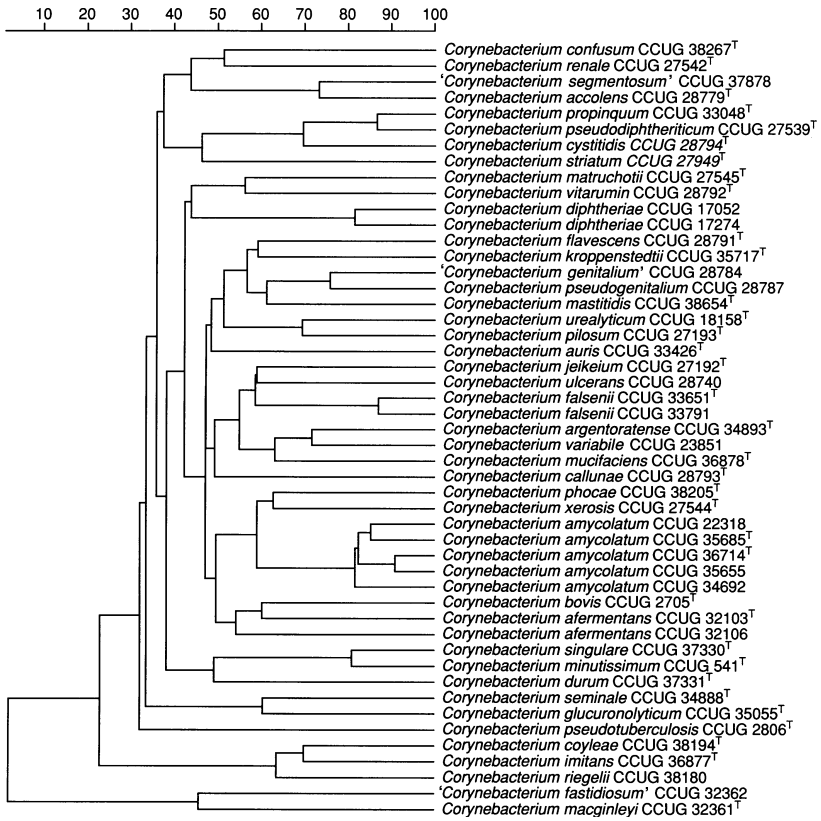

Fig. 1. Similarity dendrogram based on whole-cell protein patterns of *Corynebacterium kroppenstedtii* and related species. Levels of correlation are expressed as percentages of similarity for convenience.

37 °C whereas acid production from carbohydrates was observed after 48 h. Further enzymic reactions were studied by means of the API ZYM system (bioMérieux). PAGE of whole-cell proteins was performed as described previously (Pot *et al*., 1994). For densitometric analysis, normalization and interpretation of protein patterns, the GelCompar 3.0 software package (Applied Maths) was used. Cell wall murein was prepared by mechanical disruption of cells and complete acid hydrolysates analysed as described by Schleifer & Kandler (1972). Fatty acid methyl esters were prepared and analysed as described by Kämpfer & Kroppenstedt (1996). The presence of mycolic acids was determined by the TLC method of Minnikin *et al*. (1980). The presence of mycolic acids was also investigated by GLC analysis of trimethylsilylated mycolic acid methyl ester (TMS-MAME) derivatives (Klatte *et al*., 1994). Preparation of DNA and determination of mol% G+C content was as described previously (Farrow *et. al*., 1983). For phylogenetic studies a large fragment of the 16S rRNA gene was amplified from DNA by PCR using universal primers pA (positions 8–28, *Escherichia coli* numbering) and pH* (1542–1522). The amplified product was sequenced directly using primers to conserved regions of the rRNA gene. Sequencing was performed using a PRISM DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and reaction products were electrophoresed using an Applied Biosystems model 373A automatic DNA Sequencer according to the manufacturer's protocols. To establish the relatives of the unknown strain, preliminary searches in the EMBL database were performed with the program FASTA. Sequences of close relatives were retrieved from EMBL and GenBank databases and aligned with the newly determined sequence using the program PILEUP (Devereux *et al*., 1984). The rRNA alignment was corrected manually and approximately 100 bases at the 5′ end of the molecule were omitted from further analyses because of alignment uncertainties due to the highly variable region VI. Percentage sequence similarities were calculated and corrected for substitution rates by using Kimura's parameters. A phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987). The stability of relationships was assessed by using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE of the PHYLIP package (Felsenstein, 1989). Parsimony analysis was also performed (Felsenstein, 1989).

The human isolate consisted of Gram-positive, nonmotile, short asporogenous rod-shaped cells which exhibited a coryneform morphology. The bacterium was catalase-positive and facultatively anaerobic. The biochemical characteristics of the organisms were as follows: acid produced from glucose, maltose (weak) and sucrose but not from lactose, mannitol, glycogen, ribose or D-xylose. Positive for leucine arylamidase, esterase C4, ester lipase C8 and pyrazinamidase. Negative for alkaline and acid phosphatases, *N*-acetyl-β-glucosaminidase, cystine arylamidase, chymotrypsin, α-fucosidase, α-galactosidase, β-galactosidase,

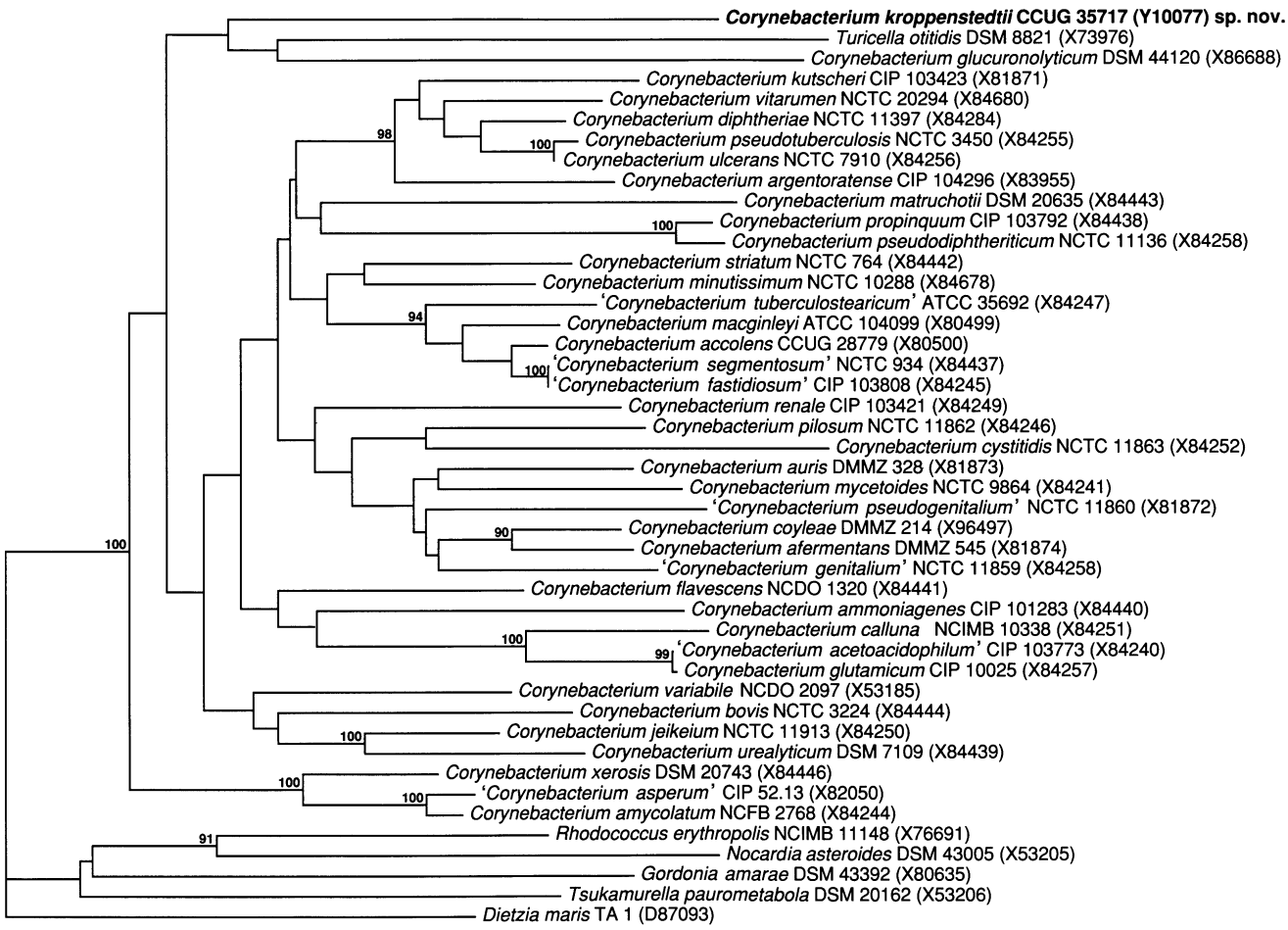

Fig. 2. Unrooted tree showing the phylogenetic relationships of *Corynebacterium kroppenstedtii* and members of the genus *Corynebacterium* and some other related taxa. The tree constructed using the neighbour-joining method was based on a comparison of approximately 1320 nucleotides. Statistically significant bootstrap values, expressed as a percentage of 500 replications, are given at the branching points.

α-glucosidase, β-glucosidase, β-glucuronidase, lipase C14, α-mannosidase, pyrrolydonyl arylamidase, trypsin, urease and valine arylamidase. Nitrate reduction negative. Aesculin hydrolysis positive. Gelatin hydrolysis negative. The organism was sensitive to penicillin (10 μg gives 30/36 mm zone) and vancomycin (23/25 mm zone).

Cell wall murein analysis revealed *meso*-diaminopimelic acid as the dibasic amino acid, which is consistent with the genera *Brevibacterium*, *Dermabacter*, *Turicella* and *Corynebacterium*. Analysis of whole-organism hydrolysates also showed the presence of arabinose and galactose consistent with a wall arabinogalacton polymer. The cellular fatty acids of the isolate also resembled those of corynebacteria and *Turicella*, with hexadecanoic ($C_{16:0}$) 37%, octadecanoic ($C_{18:0}$) 22%, and octadecenoic ($C_{18:1}$ ω9) 26% acids predominating; small amounts of tetradecanoic ($C_{14:0}$) 2% and tuberculostearic 13% acids were also present. By contrast, brevibacteria contain high levels of anteiso-methyl branched acids in addition to straight-chain saturated acids. TLC analysis of whole-cell methanolysates failed to detect any MAMEs. High-temperature GLC analysis of TMS-MAME derivatives confirmed the absence of mycolic acids in the strain. The results of PAGE analysis of whole-cell proteins are shown in Fig. 1. The unknown clinical isolate was found to be very distinct and did not display a close affinity with any of the reference *Corynebacterium* species examined, including the amycolate species *C. amycolatum* and *Turicella otitidis*. To determine the phylogenetic position of the unknown organism comparative 16S rRNA gene sequence analysis was performed. The determined sequence consisted of >1450 nucleotides. Sequence searches of EMBL and GenBank databases using the FASTA program revealed the newly determined sequence was most closely related to species of the genus *Corynebacterium* (data not shown). The newly determined sequence was subjected to pairwise analysis with those of *Corynebacterium* spp. and some close relatives, and derived

Table 1. Similarity values based on the 16S rRNA sequences of some *Corynebacterium* species, other related species and *Corynebacterium kroppenstedtii* strain CCUG 35717^T

Species	EMBL accession no.	CCUG 35717^T (%)
Corynebacterium accolens	X80500	91·7
'*Corynebacterium acetoacidophilum*'	X84240	91·8
Corynebacterium afermentans	X81874	93·0
Corynebacterium ammoniagenes	X84440	91·3
Corynebacterium amycolatum	X84244	93·6
Corynebacterium argentoratense	X83955	91·5
'*Corynebacterium asperum*'	X82050	93·4
Corynebacterium auris	X81873	93·6
Corynebacterium bovis	X84444	92·9
Corynebacterium callunae	X84251	91·1
Corynebacterium coyleae	X96497	92·8
Corynebacterium cystitidis	X84252	91·2
Corynebacterium diphtheriae	X84248	92·1
'*Corynebacterium fastidiosum*'	X84245	91·8
Corynebacterium flavescens	X84441	92·0
'*Corynebacterium genitalium*'	X84253	92·9
Corynebacterium glucuronolyticum	X86688	91·1
Corynebacterium glutamicum	X84257	91·5
Corynebacterium jeikeium	X84250	93·4
Corynebacterium kutscheri	X81871	92·0
Corynebacterium macginleyi	X80499	91·9
Corynebacterium matruchotii	X84443	92·7
Corynebacterium minutissimum	X84678	92·2
Corynebacterium mycetoides	X84241	92·6
Corynebacterium pilosum	X84246	92·2
Corynebacterium propinquum	X84438	91·1
Corynebacterium pseudodiphtheriticum	X84258	91·4
'*Corynebacterium pseudogenitalium*'	X81872	92·0
Corynebacterium pseudotuberculosis	X84255	92·6
Corynebacterium renale	X84249	92·5
'*Corynebacterium segmentosum*'	X84437	92·0
Corynebacterium striatum	X84442	91·8
'*Corynebacterium tuberculostearicum*'	X84247	92·2
Corynebacterium ulcerans	X84256	92·6
Corynebacterium urealyticum	X84439	92·4
Corynebacterium variabile	X53185	93·4
Corynebacterium vitarumen	X84680	92·1
Corynebacterium xerosis	X84446	93·4
Dietzia maris	X81920	91·2
Gordonia amarae	X80635	91·7
Nocardia asteroides	X57949	90·5
Rhodococcus erythropolis	X81929	91·2
Tsukamurella paurometabola	Z37151	90·2
Turicella otitidis	X73976	92·3

evolutionary distances were used to determine its phylogenetic relations. A tree, constructed using the neighbour-joining method, incorporating all described *Corynebacterium* species and five outgroups (viz. *Dietzia*, *Gordonia*, *Nocardia*, *Rhodococcus* and *Tsukamurella*) is shown in Fig. 2. The unknown bacterium formed a relatively long line, branching within the boundaries of the genus *Corynebacterium*, and did not display a close affinity with any currently recognized *Corynebacterium* species. Additional outgroups were added to the dataset but this did not alter the phylogenetic position of strain CCUG 35717^T. The phylogenetic placement of the unknown bacterium was also investigated by parsimony analysis. The unknown bacterium was again recovered proximal to the base, but clearly within the genus *Corynebacterium*

Table 2. Characteristics that differentiate *Corynebacterium kroppenstedtii* from related taxa

Species	*C. kroppenstedtii*	*C. amycolatum*	*T. otitidis*
Hydrolysis of:			
Aesculin	+	−	−
Urea	−	v	−
Acid from:			
Glucose	+	+	−
Ribose	−	+	−
Sucrose	+	v	−
Production of:			
Alkaline phosphatase	−	+	+
Reduction of:			
Nitrate	−	v	−
Tuberculostearic acid			
Present	+	−	+

v, Variable.

(data not shown). The treeing analyses and sequence divergence values of $>6\%$ with its closest relatives clearly demonstrates that the unknown bacterium represents a hitherto unknown subline within the genus *Corynebacterium sensu stricto* (Pascual *et al*., 1995).

The results of both the phylogenetic and phenotypic studies show that the unknown bacterium from human sputum constitutes a new species of the genus *Corynebacterium sensu stricto*. Phylogenetically the unknown bacterium forms a long isolated line and did not show a close affinity with any other species of this genus. The bacterium is phenotypically readily distinguished from most other *Corynebacterium* spp. in lacking corynomycolic acids. Although the bacterium resembles *C. amycolatum* and *Turicella otitidis* in failing to produce mycolic acids, it is biochemically very distinct from these species. Tests which serve to differentiate the unknown bacterium from the amycolate species, *C. amycolatum* and *Turicella otitidis*, are given in Table 2. For the designation of a new species, it is normally desirable to have more than one strain. However, we consider that the unusual chemical features (absence of mycolic acids and presence of tuberculostearic acid) of the described bacterium together with its isolated phylogenetic position justifies its recognition as a distinct species. Therefore, on the basis of the phenotypic and phylogenetic findings, a new species, *Corynebacterium kropppenstedtii*, is proposed for the bacterium from human sputum.

Description of *Corynebacterium kroppenstedtii* sp. nov.

Corynebacterium kroppenstedtii (krop.pen.sted.ti.i. N. L. gen. n. *kroppenstedtii* of Kroppenstedt, to honour Reiner M. Kroppenstedt, a contemporary German microbiologist, for his many contributions to the microbiology of actinomycetes).

Cells are Gram-positive, non-acid-fast, non-motile, non-spore-forming diphtheroids that occur as single cells or are arranged in V-shaped forms or palisades. Colonies on blood agar are non-pigmented, small, smooth and convex. Non-haemolytic. Facultatively anaerobic. Catalase is produced. Grows in 10% NaCl and at 42 °C. Acid is produced from glucose, maltose (weak reaction) and sucrose. Acid is not produced from lactose, ribose, D-xylose, mannitol and glycogen. Leucine arylamidase, esterase C4, ester lipase C8, and pyrazinamidase are produced. Alkaline and acid phosphatase, *N*-acetyl-β-glucosaminidase, cystine arylamidase, chymotrypsin, α-fucosidase, α-galactosidase, β-galactosidase, α-galactosidase, β-glucosidase β-glucuronidase, lipase C14, α-mannosidase, pyrrolydonyl arylamidase, trypsin, urease and valine arylamidase are not detected. Aesculin is hydrolysed. Gelatin is not hydrolysed. Nitrate is not reduced to nitrite. Cell wall murein contains *m*-diaminopimelic acid as the dibasic amino acid. Wall sugars arabinose and galactose are detected in hydrolysates. $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ $\omega 9$ are the major long-chain cellular fatty acids. Tuberculostearic acid present. Mycolic acids are not present. Sensitive to cefuroxim, erythromycin, klindamycin, penicillin G and vancomycin. The DNA base composition of CCUG 35717^T is 62 mol% G+C (T_m). Habitat unknown. Isolated from human sputum. The type strain is CCUG 35717^T.

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