Corynebacterium simulans* sp. nov., a non-lipophilic, fermentative *Corynebacterium

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Three coryneform strains isolated from clinical samples were analysed. These strains fitted the biochemical profile of *Corynebacterium striatum* by conventional methods. However, according to recently described identification tests for fermenting corynebacteria, the strains behaved rather like *Corynebacterium minutissimum*. The three isolates could be distinguished from *C. minutissimum* by a positive nitrate and nitrite reductase test and by not fermenting maltose; from *C. striatum* by their inability to acidify ethylene glycol and to grow at 20 °C. Genetic studies based on 16S rRNA showed that the three strains were in fact different from *C. minutissimum* and *C. striatum* (96.9 and 98% similarity, respectively) and from other corynebacteria. They represent a new species for which the name *Corynebacterium simulans* sp. nov. is proposed. The type strain is DSM 44415^T (= UCL 553^T = Co 553^T).

Keywords: *Corynebacterium simulans* sp. nov., fermentative non-lipophilic *Corynebacterium*, 16S rRNA analysis

INTRODUCTION

Bacteria of the genus *Corynebacterium* are frequently isolated from clinical samples. Among non-diphtheric corynebacteria, *Corynebacterium striatum* and *Corynebacterium minutissimum* are part of the commensal flora of humans but they are occasionally isolated as opportunistic pathogens in patients with immunosuppressive disease or other risk factors. Thanks to both phenotypic and molecular characterization methods, about 20 new species of *Corynebacterium* have been described during the last decade. Most of them have been revised by Funke *et al.* (1997a). More recently, additional species have been described (Fernandez-Garayzabal *et al.*, 1997, 1998; Funke *et al.*, 1997b, c, d, e, 1998a, b; Collins *et al.*, 1998, 1999; Pascual *et al.*, 1998; Riegel *et al.*, 1997a, b; Sjöden *et al.*, 1998; Takeuchi *et al.*, 1999; Zimmermann *et al.*, 1998). Using recently developed identification tests (Wauters *et al.*, 1998), three strains were isolated from human clinical samples displaying identical but atypical phenotypic and metabolic profiles. Based on chemotaxonomic properties, these bacteria clearly

belonged to the genus *Corynebacterium*. An accurate characterization of the three strains including 16S rRNA sequencing revealed that they all belonged to a yet unidentified *Corynebacterium* species for which the name *Corynebacterium simulans* sp. nov. is proposed.

METHODS

Bacterial strains. The three strains Co 301 (= UCL 301 = DSM 44392), Co 553^T (= UCL 553^T = DSM 44415^T) and Co 557 (= UCL 557 = DSM 44416) were primarily isolated on blood agar plates. Subcultures were made on Columbia agar with 5% (v/v) sheep blood (Becton Dickinson) incubated at 37 °C in air.

Biochemical properties. Biochemical profiles were determined as described elsewhere (Wauters *et al.*, 1998; Funke *et al.*, 1997a). Nitrite reduction was investigated in peptone water supplemented with either 0.01 or 0.001% (w/v) NaNO₂. The medium was heavily inoculated and, after overnight incubation, disappearance of nitrite was detected by adding Griess' reagents. Pyrazinamidase was detected in a medium containing 1% (w/v) peptone and 0.25% (w/v) pyrazinamide (Sigma). After overnight incubation, a few drops of a 1% (w/v) ferrous sulfate solution were added. API coryne strips were read after 24 h, API ZYM strips after 4 h and API 50 CH after 7 d using the API coryne medium (bioMérieux).

Antimicrobial susceptibility. The MIC of antimicrobial agents were determined by E-test strips and interpreted

Abbreviation: SSU, small ribosomal subunit.

The EMBL accession numbers for the 16S rDNA sequences of *Corynebacterium* sp. Co 301, Co 553^T and Co 557 are AJ012836, AJ012837 and AJ012838, respectively.

according to criteria established for staphylococci by the National Committee for Clinical Laboratory Standards (1997).

Chemotaxonomic studies. Cellular fatty acid composition was determined by GLC using a Delsi DI 200 chromatograph (Intersmat) as described elsewhere (Wauters *et al.*, 1996). Mycolic acids were detected by TLC (Minnikin *et al.*, 1980). Peptidoglycan analysis was performed by N. Weiss at DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), using a TLC method (Schleifer & Kandler, 1972). Cell wall sugars were determined as alditol acetates by GC (Saddler *et al.*, 1991) by P. Schumann at the DSMZ.

DNA preparation, restriction and sequencing. Total DNA from strains Co 301, Co 553^T and Co 557 was prepared according to the method of Marmur (1961) except that cells were disrupted by sonification rather than by lysozyme treatment. A 1.5 kb PCR fragment corresponding to the 16S rDNA was generated with the Dynazyme thermostable polymerase (Finnzymes) using the 16S rDNA PCR primers '27F' and '1522R' (Johnson, 1994) according to the following PCR protocol: 95 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s (30 cycles). Sequencing reactions were obtained directly on ethanol-precipitated PCR products with the Thermosequenase kit (Amersham) using ³³P-labelled oligonucleotides as sequencing primers. The universal forward and reverse 16S rDNA sequencing primers 321, 530, 685, 1100, 1242 and 1392, numbered relative to the *Escherichia coli* 16S rRNA numbering, were used in this study (Johnson, 1994). The sequencing reactions were analysed by electrophoresis on 6% (w/v) acrylamide, 8 M urea sequencing gels on a Macrophor unit (Pharmacia). Restriction analysis was assayed by incubating approximately 0.1 µg ethanol-precipitated, PCR-amplified DNA with the relevant restriction enzyme in a buffer supplied by the manufacturer (Pharmacia). The restricted DNA was separated on a 1.5% (w/v) agarose gel by electrophoresis at constant voltage in TBE buffer (Tris 0.5 M, boric acid 0.5 M and EDTA 10 mM, pH 8.3) together with a molecular weight standard (Life Technologies) and stained with ethidium bromide.

Nucleotide sequence comparison and phylogenetic analysis. The FASTA software was used for DNA homology searches (Pearson & Lipman, 1988). When available, pre-aligned 16S rDNA sequences from other *Corynebacterium* species were retrieved from the small ribosomal subunit (SSU) RNA database (Van de Peer *et al.*, 1998) and aligned with the newly determined sequences. 16S rDNA sequences not found in the SSU RNA database were retrieved from the EMBL and Genbank databases and aligned manually with the pre-aligned sequences. Multiple sequence alignments were generated manually and approximately 100 bases at the 5' end of the molecule were removed to avoid alignment uncertainties due to hypervariable domain V1. The multiple alignment encompassed nucleotides 122–1361 relative to the *E. coli* 16S rRNA numbering. Phylogenetic tree data were calculated using the PAUPSEARCH software (Swofford, 1990) run on a SUN Enterprise 450 workstation. The neighbour-joining, maximum-likelihood and parsimony methods of tree construction were applied on the aligned sequences. The Kimura two-parameter distance correction method was used for calculating the neighbour-joining tree. Branches that were found by all the three methods were considered relevant and validated by a bootstrap analysis (1500 replications) using PAUPSEARCH. The RETREE and DRAWGRAM programs from the PHYLIP software package version 3.5 (Felsenstein,

1989) were used to manipulate and draw the phylogenetic trees generated by PAUPSEARCH.

RESULTS AND DISCUSSION

Strains Co 301, Co 553^T and Co 557 were isolated from a foot abscess, a biopsy of an axillar lymph node and a boil, respectively. Although the first isolate grew in pure culture from deep-sited pus, disease association was not proved and a possible pathogenic role of such strains remains to be established.

On Columbia blood agar, colonies were about 0.5–1.0 mm in diameter after 24 h of incubation in air at 37 °C and 1–2 mm after 48 h. They were greyish-white, opaque and glistening, similar to colonies of *C. striatum* and *C. minutissimum*. The strains were not lipophilic. Growth was facultatively anaerobic but was enhanced in air. Gram stain showed Gram-positive rods with a typical diphtheroid arrangement.

Biochemical characterization by conventional methods yielded a profile close to that of *C. striatum* (Wauters *et al.*, 1998; Funke *et al.*, 1997a). The strains were catalase-positive, non-motile and fermentative. Nitrate reduction was positive, urease was negative. Pyrazinamidase was positive in two strains and negative in one. Glucose and sucrose were fermented but not maltose and mannitol. Tyrosinase and Tween esterase were positive. The CAMP (Christie–Atkins–Munch–Petersen) reaction was negative. The three strains displayed strong nitrite reduction in nitrite broths with both low and high concentrations. Nitrite-reducing strains have been described in organisms resembling *C. striatum* (Markowitz & Coudron, 1990). In our experience, most strains of *C. striatum* reduce nitrite at the lower concentration of 0.001% but not at 0.01%. A few strains (including the type strain) do not reduce nitrite at either concentration and a few others are strong reducers even at 0.01% (unpublished data).

The numerical code obtained with the API CORYNE system (V2-0) was 2100305 for strain Co 553^T. According to the manufacturer, this code corresponds to *Corynebacterium* 'CDC group G' and *C. striatum*/*Corynebacterium amycolatum* with a confidence level of 87.0% and 10.7%, respectively. For strain Co 557, the code was 0100305 which corresponds to *Corynebacterium macginleyi*, 'CDC group G' and *C. striatum*/*C. amycolatum* with confidence levels of 84.4%, 11% and 2.7%, respectively. For strain Co 301, the code was 2100105 corresponding to *C. striatum*/*C. amycolatum* with a confidence level of 88.7%. However, upon addition of zinc dust after reagents for nitrate reduction, as recommended for the API 20NE strips, it appeared that the nitrate reduction was falsely negative because of the strong nitrite reduction. After correction of the nitrate result, the numerical codes of the three strains became 3100305 (*Corynebacterium* 'CDC group G' 55.0%, *C. striatum*/*C. amycolatum* 43.0%), 1100305 (*C. macginleyi* 99.4%) and 3100105 (*C. striatum*/*C. amycolatum* 98.5%), respectively.

Table 1. Distinctive characters of *C. simulans* and related nonlipophilic fermentative corynebacteria

NO₃, nitrate reduction; NO₂, nitrite reduction; Ur, urease; Pyz, pyrazinamidase; Suc, sucrose fermentation; Mal, maltose fermentation; Gal, galactose fermentation; CAMP, CAMP test; E Gl, acid production from ethylene glycol; 20 °C, growth at 20 °C; 42 °C, glucose fermentation at 42 °C; Form, formate alkalization; v, variable.

	NO ₃	NO ₂	Ur	Pyz	Suc	Mal	Gal	CAMP	E Gl	20 °C	42 °C	Form
<i>C. simulans</i>	+	+	—	v	+	—	v	—	—	—	v	+
<i>C. striatum</i>	+	v	—	+	v	—	+	v	+	+	+	+
<i>C. minutissimum</i>	—	—	—	+	v	+	—	—	—	—	v	+
<i>C. singulare</i>	—	—	+	+	+	+	—	—	—	—	+	+
<i>C. amycolatum</i>	v	—	v	+	v	v	v	—	v	—	+	—
<i>C. xerosis</i>	v	—	—	+	+	+	v	—	v	+	—	—

The enzymic profile obtained after 4 h incubation with the API ZYM strips was as follows: alkaline phosphatase, esterase, esterase-lipase, leucine arylamidase, trypsin and naphthol-AS-BI-phosphohydrolase were positive. Lipase, valine arylamidase, cystine arylamidase, chymotrypsin, acid phosphatase, α - and β -galactosidase, β -glucuronidase, α - and β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase were negative. By testing sugar fermentations with 50 CH strips, the three strains were positive for glucose, fructose, mannose and sucrose within 24 h. Ribose and *N*-acetyl-glucosamine were positive in two strains. Using 50 CH strips, a positive reaction for galactose fermentation was observed, but the reaction was sometimes delayed; by conventional methods, galactose fermentation was sometimes delayed or the reaction was negative.

With respect to the tests recently described for the identification of fermenting corynebacteria (Wauters *et al.*, 1998), the three strains showed strong alkalization of formate, like *C. striatum* and *C. minutissimum*. Glucose fermentation at 42 °C was variable. Ethylene glycol acidification and growth at 20 °C within 3 d were negative. The three isolates could be distinguished from *C. minutissimum* by a positive nitrate and nitrite reductase and by not fermenting maltose; from *C. striatum* by their inability to acidify ethylene glycol and to grow at 20 °C (Table 1).

The strains were susceptible to penicillin, ampicillin, amoxicillin/clavulanic acid, cephalotin, ciprofloxacin, erythromycin, fusidic acid, gentamicin and rifampicin.

Chemotaxonomic studies showed that arabinose and galactose were the cell-wall sugars, although galactose was found in smaller amounts than in other *Corynebacterium* species. The peptidoglycan diamino acid was *meso*-diaminopimelic acid. Cellular fatty acids were those found in other species of the genus *Corynebacterium*, predominantly C18:1*cis*9 (range 65.7–82.7%), C16:0 (range 7.7–21.5%), C16:1*cis*9 (range 3.5–7.0%) and C18:0 (range 2.3–7.0%). Mycolic acids were of the short-chain type (C22–C36).

These characteristics are consistent with the assignment of the strains to the genus *Corynebacterium* (Collins & Cummins, 1986).

A large part of the 16S rRNA sequence from strains Co 301, Co 553^T and Co 557 was determined (1468 residues). Strain Co 553^T and Co 557 had exactly the same 16S rRNA sequence whereas only two nucleotides at position 200 and 1444 relative to the *E. coli* numbering were found to differ when compared to strain Co 301. All together, the three strains thus shared more than 99.86% 16S rRNA similarity. Database comparisons using FASTA revealed that these strains were members of the actinomycete high-G + C content subphylum of Gram-positive bacteria and showed the highest similarity with the genus *Corynebacterium*. However, no similarity greater than 98.0% could be found with any *Corynebacterium* 16S rDNA sequence in the EMBL or GenBank databases (see Table 2). These results suggest that strains Co 301, Co 553^T and Co 557 belong to a genetically distinct *Corynebacterium* species showing close (approx. 2% 16S rRNA divergence) and significant relatedness with *Corynebacterium striatum*, whereas *C. minutissimum* and *Corynebacterium singulare* were the next nearest relatives (Table 2). Taken individually, this similarity value of 98% with the 16S rRNA of *C. striatum* is too high to allow the definition of a new species since values below 97% and/or genomic DNA reassociation values below 70% are considered relevant for the establishment of new bacterial species (Stackebrandt & Goebel, 1994). However, the genus *Corynebacterium* contains a number of species for which numerous distinctive characters have been described that fully justify their classification in separate species but that exhibit only limited 16S rRNA divergence. For instance, the 16S rRNA of *Corynebacterium mucifaciens* is 98.5% similar to that of *Corynebacterium afermentans*, though the distinction between the two species has been convincingly illustrated by different characters and did not require the determination of DNA hybridization values (Funke *et al.*, 1997e). Similarly, the three species *Corynebacterium ulcerans*,

Table 2. Levels of 16S rRNA sequence similarity between *C. simulans* sp. nov. and other *Corynebacterium* species and subspecies

16S rRNA similarity values were determined with the FASTA program. Accession numbers labelled with an asterisk correspond to pre-aligned 16S rRNA sequences retrieved from the SSU RNA database (Van de Peer *et al.*, 1998) and were used to construct the phylogenetic tree shown in Fig. 1. Unlabelled accession numbers correspond to sequences retrieved from the EMBL or GenBank databases, aligned manually and used for tree construction as well.

Genus	Species or subspecies	Accession number	Strain	% 16S rRNA sequence similarity with <i>C. simulans</i> sp. nov.
<i>Corynebacterium</i>	<i>simulans</i>	AJ012837	DSM 44415 ^T	100
<i>Corynebacterium</i>	<i>simulans</i>	AJ012838	DSM 44416	100
<i>Corynebacterium</i>	<i>simulans</i>	AJ012836	DSM 44392	99.9
<i>Corynebacterium</i>	<i>striatum</i>	X81910*	ATCC 6940 ^T	98.0
<i>Corynebacterium</i>	<i>singulare</i>	Y10999	DSM 44357 ^T	97.0
<i>Corynebacterium</i>	<i>minutissimum</i>	X84678*	DSM 20651 ^T	96.9
<i>Corynebacterium</i>	<i>argenteratense</i>	X83955*	DSM 44202 ^T	96.1
<i>Corynebacterium</i>	<i>accolens</i>	X80500*	ATCC 49725 ^T	95.9
<i>Corynebacterium</i>	<i>macginleyi</i>	X80499*	ATCC 51787 ^T	95.9
<i>Corynebacterium</i>	<i>vitaeruminis</i>	X84680*	DSM 20294 ^T	95.6
<i>Corynebacterium</i>	' <i>tuberculoostearicum</i> '	X84247*	ATCC 35692 ^T	95.5
<i>Corynebacterium</i>	' <i>segmentosum</i> '	X84437*	NCTC 934	95.2
<i>Corynebacterium</i>	' <i>fastidiosum</i> '	X84245*	CIP 103808	95.0
<i>Corynebacterium</i>	<i>flavescens</i>	X82060*	ATCC 10340 ^T	95.2
<i>Corynebacterium</i>	<i>diphtheriae</i>	X82059	ATCC 27010 ^T	95.0
<i>Corynebacterium</i>	<i>jeikeium</i>	X84250*	ATCC 43734 ^T	94.7
<i>Corynebacterium</i>	<i>ulcerans</i>	X81911	DSM 46325 ^T	94.6
<i>Corynebacterium</i>	<i>imitans</i>	Y09044	DSM 44264 ^T	94.3
<i>Corynebacterium</i>	<i>mucifaciens</i>	Y11200	DSM 44265 ^T	94.3
<i>Corynebacterium</i>	<i>mycetoides</i>	X84241*	DSM 20632 ^T	94.1
<i>Corynebacterium</i>	<i>pseudodiphtheriticum</i>	X84258*	DSM 44287 ^T	94.2
<i>Corynebacterium</i>	' <i>pseudogenitalium</i> '	X81872*	NCTC 11860	94.1
<i>Corynebacterium</i>	<i>urealyticum</i>	X81913*	ATCC 43042 ^T	93.9
<i>Corynebacterium</i>	<i>propinquum</i>	X81917*	DSM 44285 ^T	93.9
<i>Corynebacterium</i>	<i>pseudotuberculosis</i>	D38576*	OR1	93.7
<i>Corynebacterium</i>	<i>callunae</i>	X84251*	ATCC 15991 ^T	93.6
<i>Corynebacterium</i>	<i>coyleae</i>	X96497*	DSM 44184 ^T	93.5
<i>Corynebacterium</i>	' <i>genitalium</i> '	X84253*	NCTC 11859	93.6
<i>Corynebacterium</i>	<i>kutscheri</i>	D37802*	ATCC 15677 ^T	93.6
<i>Corynebacterium</i>	<i>afermentans</i> subsp.	X82054	DSM 44280 ^T	93.4
	<i>afermentans</i>			
<i>Corynebacterium</i>	<i>glutamicum</i>	Z46753*	DSM 20300 ^T	93.4
<i>Corynebacterium</i>	<i>xerosis</i>	M59058*	ATCC 373 ^T	93.5
<i>Corynebacterium</i>	<i>auris</i>	X82493	DSM 44122 ^T	93.2
<i>Corynebacterium</i>	<i>amycolatum</i>	X82057*	DSM 6922 ^T	93.0
<i>Corynebacterium</i>	<i>variabile</i>	X53185*	ATCC 15763 ^T	93.1
<i>Corynebacterium</i>	' <i>acetoacidophilum</i> '	X84240*	ATCC 13870	92.9
<i>Corynebacterium</i>	<i>ammoniaenes</i>	X82056*	ATCC 6871 ^T	92.8
<i>Corynebacterium</i>	<i>renale</i>	M29553*	ATCC 19412 ^T	92.8
<i>Corynebacterium</i>	<i>bovis</i>	D38575*	ATCC 7715 ^T	92.7
<i>Corynebacterium</i>	<i>cystitidis</i>	D37914*	ATCC 29593 ^T	92.0
<i>Corynebacterium</i>	<i>pilosum</i>	D37915*	ATCC 29592 ^T	92.0
<i>Corynebacterium</i>	<i>matruchotii</i>	X82065*	DSM 20635 ^T	91.7
<i>Corynebacterium</i>	<i>seminale</i>	X84375*	DSM 44288 ^T	91.5
<i>Corynebacterium</i>	<i>glucuronolyticum</i>	X86688*	DSM 44120 ^T	91.2
<i>Turicella</i>	<i>otitidis</i>	X73976*	DSM 8821 ^T	90.6

Fig. 1. *HpaI* and *PstI* restriction analysis of PCR-amplified 16S rRNA from strains *C. simulans* Co 301 (lane 1), *C. simulans* Co 553^T (lane 2), *C. simulans* Co 557 (lane 3), *C. striatum* ATCC 6940^T (lane 4), *C. minutissimum* DSM 20651^T (lane 5) and *C. singulare* DSM 44357^T (lane 6). The size and position of molecular mass standards are shown.

Corynebacterium pseudotuberculosis and *Corynebacterium diphtheriae* share more than 98% 16S rRNA similarity (Pascual *et al.*, 1995). More dramatic examples are *C. singulare* and *C. minutissimum*, the 16S rRNA of which are 99.1% similar, whereas the total labelled genomic DNA of *C. singulare* exhibited only 28% similarity with *C. minutissimum* (Riegel *et al.*, 1997a). A similar situation is found for *Corynebacterium propinquum* and *Corynebacterium pseudodiphtheriticum* (>99% 16S rRNA similarity) (Pascual *et al.*, 1995; Ruimy *et al.*, 1995). The 97% limit is thus not always fulfilled in the genus *Corynebacterium* and additional distinctive characters must be determined to allow the definition of a new species. Given the 98% similarity value between the 16S rRNA of strains Co 301, Co 553^T and Co 557 and their closest relative *C. striatum* on the one hand and the biochemical tests presented in this paper (Table 1) to discriminate these strains from *C. striatum* on the other hand, we feel that it is reasonable to define a new species for which the name *Corynebacterium simulans* sp. nov. is proposed. According to the 16S rRNA sequences of strains Co 301, Co 553^T and Co 557, it might be possible to readily differentiate the proposed new species *C. simulans* sp. nov. from other *Corynebacterium* species by comparing the restriction profiles of DNA fragments corresponding to the 16S rRNA gene. To test this hypothesis, we analysed PCR-amplified DNA corresponding to the 16S rRNA sequence of *C. simulans* sp. nov., *C. striatum*, *C. minutissimum* and *C. singulare* after either *HpaI* or *PstI* digestion. Fig. 1 shows that the *HpaI* restriction pattern of the 16S rRNA of *C. simulans* sp. nov. is clearly different from that of *C. minutissimum* and *C. singulare*, whereas it is identical to that of *C. striatum*. In contrast, the profile observed after *PstI* restriction unambiguously differentiates *C. simulans* sp. nov. from *C. striatum*. A

multiple alignment made with the 16S rRNA sequences listed in Table 2 was used to compute a phylogenetic tree by the neighbour-joining method (Fig. 2) and validated by a bootstrap analysis (1500 replications). The tree computation data confirmed the close relatedness between *C. simulans* sp. nov. and *C. striatum*. A distinct phylogenetic sub-branching was predicted for the two strains by three different tree construction methods (neighbour-joining, maximum-likelihood and parsimony). The relevance of this separate branching was further supported by a significant bootstrap value (96.9). The three methods also predicted a monophyletic subunit in the *Corynebacterium* cluster including the four species *C. simulans* sp. nov., *C. striatum*, *C. minutissimum* and *C. singulare* with a fairly significant bootstrap value (86.7). It is noteworthy that, except for a few critical tests, the metabolic and phenotypic profiles of these species are very similar and are consistent with this common phylogenetic grouping.

Description of *Corynebacterium simulans* sp. nov.

Corynebacterium simulans (si'mu.lans. L. v. *simulare* to simulate, because it resembles *Corynebacterium striatum*).

Cells are Gram-positive, non-motile, non-spore forming, showing a diphtheroid arrangement. Colonies are greyish-white, glistening and 1–2 mm in diameter on blood agar after 48 h incubation at 37 °C. Growth is facultatively anaerobic and does not occur or is very weak at 20 °C within 3 d. The metabolism is fermentative. Catalase is positive. Acid is produced from: glucose, sucrose, fructose and mannose. Acid production from *N*-acetyl-glucosamine, galactose and ribose is variable. Acid is not produced from: mannitol, maltose, D- and L-xylose, glycerol, erythritol, D- and L-arabinose, adonitol, methyl β -D-xyloside, sorbose, rhamnose, dulcitol, inositol, sorbitol, methyl α -D-mannoside, methyl α -D-glucoside, amygdalin, arbutin, aesculin, salicin, cellobiose, lactose, melibiose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D- and L-fucose, D- and L-arabitol, gluconate, 2-keto-gluconate and 5-keto-gluconate. Urea, aesculin and gelatin are not hydrolysed. Nitrate and nitrite reduction is positive. Alkalization of buffered formate is positive. No acid is produced from ethylene glycol. Tween esterase and tyrosine clearing are positive. Pyrazinamidase is variable. The CAMP reaction is negative.

Using the API ZYM system, alkaline phosphatase, esterase, esterase-lipase, leucine arylamidase, trypsin and naphthol-AS-BI-phosphohydrolase are positive. Lipase, valine arylamidase, cystine arylamidase, chymotrypsin, acid phosphatase, α - and β -galactosidase, β -glucuronidase, α - and β -glucosidase, *N*-

Fig. 2. Unrooted tree showing the phylogenetic relationships of *C. simulans* strain Co 553^T with other members of the genus *Corynebacterium*. The tree was obtained by the neighbour-joining method and is drawn using the 16S rRNA sequences of *Dietzia maris* and *Tsukamurella paurometabola* as the outgroup. Relevant branches also found to be significant by the maximum-likelihood and the maximum-parsimony analysis methods are indicated by '+'. The tree was validated by a bootstrap analysis (1500 replications) and values greater than 85% are indicated above the branches. Scale bar, 0.01 accumulated change per nucleotide.

acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase are negative. Arabinose and galactose are present in the cell wall and the peptidoglycan diamino acid is *meso*-diaminopimelic acid. The main straight-chain saturated fatty acid is palmitic acid and oleic acid is the main unsaturated acid. Short-chain mycolic acids (C22–C36) are present. The three strains have been deposited in the DSMZ with the following accession numbers: Co 301 = DSM 44392; Co 553^T = DSM 44415^T; and Co 557 = DSM 44416. The type strain of *Corynebacterium simulans* is Co 553^T which is positive for ribose, *N*-acetyl-glucosamine, galactose and pyrazinamidase.

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