***Corynebacterium confusum* sp. nov., isolated from human clinical specimens**

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Three strains of a previously unknown coryneform bacterium were isolated from two patients with foot infections and from a blood culture of a third patient. The three non-lipophilic strains exhibited very slow fermentative acid production from glucose but not from maltose or sucrose, nitrate reductase activity, no tyrosinase activity and the presence of small amounts of tuberculostearic acid as the most significant phenotypic features. Differentiation of these strains from all other presently defined coryneform bacteria was readily achieved. Chemotaxonomic investigations revealed that the three strains unambiguously belonged to the genus *Corynebacterium*. Comparative 16S rRNA gene sequence analysis demonstrated that the isolates were almost identical and represented a new subline within the genus *Corynebacterium*, for which the designation *Corynebacterium confusum* sp. nov. is proposed. The type strain of *Corynebacterium confusum* is CCUG 38267^T.

Keywords: *Corynebacterium*, *Corynebacterium confusum* sp. nov., phenotypical delineation, 16S rRNA gene, phylogeny

INTRODUCTION

Clinical microbiologists have only in recent years started to realize the enormous diversity of coryneform bacteria encountered in human clinical specimens. Within the group of coryneform bacteria (i.e. aerobically growing, irregular, asporogenous, Gram-positive rods) the genus *Corynebacterium* is the one comprising the largest number of validated species (more than 40 as of January 1998) (Collins & Cummins, 1986; Fernandez-Garayzabal *et al.*, 1997; Funke *et al.*, 1997a–e; Riegel *et al.*, 1997a, b; Sjöden *et al.*, 1998; Zimmermann *et al.*, 1998). It is important to note that 16 of these species had been defined within the past 5 years and that all of them, with the exception of *Corynebacterium mastitidis*, were recovered from human clinical materials. This was mainly because of the development of more sophisticated phenotypical identification systems, the more consistent application of chemotaxonomic methods, as well as the extensive use of molecular genetic methods such as 16S rRNA

gene analysis. This report is on yet another novel *Corynebacterium* isolated from humans. Using a polyphasic approach to taxonomy, we describe three strains of a previously unknown member of the genus *Corynebacterium*. Based on the results of this study a new species, *Corynebacterium confusum*, is proposed.

METHODS

Strains used and culture conditions. The three strains studied were DMMZ (Department of Medical Microbiology, University of Zurich, Switzerland) 2439^T, DMMZ 3259 and IBS (Institut de Bactériologie, Strasbourg, France) T88848. The first two strains had primarily been isolated from Columbia agar plates (Difco) supplemented with 5% sheep blood agar (SBA) and incubated for 24 h at 37 °C in a 5% CO₂-enriched atmosphere. The same conditions were used for subculturing of all three strains. Strain IBS T88848 was initially grown from a Bactec aerobic NR6A blood culture system (Becton Dickinson).

Biochemical profiles. The methods used for determination of the biochemical profiles have been described previously (Funke *et al.*, 1993). The API CORYNE strips (bioMérieux) were read after 24 h incubation, except for acid production from carbohydrates, in which case they were read after 72 h. For the API 50CH system applying the 50 CHE medium

The EMBL accession number for the 16S rRNA of strain DMMZ 2439^T (CCUG 38267^T) is Y15886.

(both from bioMérieux), the end reading was performed after 240 h incubation at 37 °C in ambient air. Hydrolysis of tyrosine was observed on a medium described previously (Nash & Krenz, 1991); a positive reaction was defined as clearing of the medium after 240 h incubation at 37 °C in ambient air.

Antimicrobial susceptibility patterns. The minimal inhibitory concentrations (MICs) of 19 agents were determined with the Merlin Micronaut system (Merlin Diagnostics, Bornheim-Hersel, Germany) and interpreted according to breakpoints established by the National Committee for Clinical Laboratory Standards as described previously (Funke *et al.*, 1997f).

Chemotaxonomic investigations. Cellular fatty acid patterns were determined with the Sherlock system (Microbial ID) as outlined previously (von Graevenitz *et al.*, 1991). Techniques used for analyses of whole-cell hydrolysates for the presence of *meso*-diaminopimelic acid and of mycolic acids were also as described previously (Funke *et al.*, 1993).

Molecular genetic investigations. A large fragment (around 1500 bases) of the 16S rRNA gene of strains DMMZ 2439^T and 3259 was amplified by PCR by using universal primers pA and pH* as described previously by Hutson *et al.* (1993). The PCR products were purified by using a Prep-A-Gene kit (Bio-Rad) and were sequenced by using a *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) and a model 373A automatic sequencer (Applied Biosystems). The sequences determined were aligned with those of phylogenetically close reference species (i.e. other high-G+C-content actinomycetes) obtained from the EMBL database by using the program PILEUP (Devereux *et al.*, 1984), and the alignment was corrected manually. An unrooted phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987). Stability of relationships was assessed using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE of the PHYLIP package (Felsenstein, 1989).

RESULTS AND DISCUSSION

Strain DMMZ 2439^T was isolated from a 36-year-old man with a plantar abscess and strain DMMZ 3259 grew from bone material of a 40-year-old man with osteomyelitis of the calcaneus. Neither of the patients had diabetes. Strain IBS T88848 was isolated only once: from the blood of a 20-year-old, non-immuno-compromised man with transient fever after thoracic surgery. When cultured on aerobic SBA plates, the three strains grew as whitish, glistening, convex, creamy colonies of up to 1.5 mm in diameter after 48 h incubation. The strains were non-lipophilic and grew only weakly in a strict anaerobic atmosphere to colonies of 0.5 mm diameter after 48 h incubation. Gram stains showed often relatively short but typically club-shaped coryneform bacteria (seen in genuine *Corynebacterium* species only: Collins & Cummins, 1986; Funke *et al.*, 1997e) of 1–3 µm in length which were arranged as single cells, in pairs or in small clusters. The three strains were not partially acid-fast.

When initially applying the phenotypic identification system for coryneform bacteria outlined recently (von Graevenitz & Funke, 1996), we observed the following

reactions: catalase-positive; non-motile rods; nitrate reduction-positive; no hydrolysis of urea or aesculin; no acid production from glucose, maltose, sucrose, mannitol or xylose in cystine–trypticase agar (CTA) medium after 10 d incubation, although the alkalization reaction of glucose was judged as weaker than for the other four carbohydrates. However, when acidification of glucose was determined in the independent API CORYNE and API 50CH systems, acid formation from glucose was positive although delayed, i.e. the reaction became positive after 48–72 h only. Obviously, the stronger buffering capacity of the CTA medium prevented the indication of an acid production from glucose. For all three strains, we observed the numerical code 3100304 (after 72 h) when applying the API CORYNE system. This corresponded to a very good identification of the strains on the genus level as *Corynebacterium* and on the species level as either *Corynebacterium striatum*/*Corynebacterium amycolatum* or *Corynebacterium jeikeium* according to the system's database (API CORYNE Analytical Profile Index 2.0; 1997). If the strains had been incubated for only 24 h, as recommended by the manufacturer, a numerical code of 3100004 would have been generated, which corresponds to an identification of the isolates as *Corynebacterium propinquum*. The same result was obtained when using the identification scheme of von Graevenitz & Funke (1996). However, as mentioned above, the three unknown coryneform bacteria produced acid from glucose in the above API systems, which is not observed in *C. propinquum* isolates (Riegel *et al.*, 1993). In addition, unlike *C. propinquum* (Riegel *et al.*, 1993), all three strains did not hydrolyse tyrosine. The same test served to distinguish the isolates from *C. striatum* strains, which also invariably hydrolyse tyrosine (Table 1). The three strains were initially also considered to represent *Corynebacterium coyleae* strains, but the latter species does not reduce nitrate and exhibits a strong CAMP reaction (Table 1). Strains DMMZ 2439^T, DMMZ 3259 and IBS T88848 phenotypically also resemble *Corynebacterium argentoratense* (Table 1), but strains belonging to this taxon are always nitrate reductase-negative and exhibit chymotrypsin activity, so that an assignment of the three unknown strains to this taxon could also be ruled out. Finally, *Corynebacterium amycolatum* strains may have a biochemical profile identical to the one of the three unknown coryneform bacteria DMMZ 2439^T, DMMZ 3259 and IBS T88848, but *C. amycolatum* exhibits dry and rough colonies, which is incompatible with the morphology of the unknown coryneforms described in this report (see above).

Further evidence for the distinctiveness of the three clinical isolates came from the chromatographic analysis of mycolic acids. All three strains were found to contain short-chain mycolic acids, whereas *C. amycolatum* strains always lack these lipids. *Meso*-diaminopimelic acid was found to be the diamino acid of the peptidoglycan. The major cellular fatty acids were

Table 1. Characteristics that differentiate *Corynebacterium confusum* from other fermenting, non-lipophilic *Corynebacterium* spp. encountered in human clinical specimens

Data according to Collins & Cummins (1986), Funke *et al.* (1997a, c–e), Riegel *et al.* (1997a, b), Sjöden *et al.* (1998), Zimmermann *et al.* (1998). (+), Delayed reaction; v, variable reaction; w, weak reaction; REV, reverse CAMP reaction.

Species	Nitrate reduction	Urea hydrolysis	Aesculin hydrolysis	Pyrazinamidase	Alkaline phosphatase	Acid produced from:			CAMP reaction	Other traits
						Glucose	Maltose	Sucrose		
<i>C. confusum</i>	+	–	–	+	+	(+)	–	–	–	Tyrosine hydrolysis –
<i>C. amycolatum</i>	v	v	–	+	+	+	v	v	–	Mycolic acids –
<i>C. argenteovirescens</i>	–	–	–	+	v	+	–	–	–	Chymotrypsin +
<i>C. coyleae</i>	–	–	–	+	+	(+)	–	–	+	
<i>C. diphtheriae</i>	v	–	–	–	–	+	+	–	–	Cystinase +
<i>C. durum</i>	+	(v)	(v)	+	–	+	+	+	–	Adherence to agar
<i>C. falsenii</i>	–	(+)	–	w	+	(+)	v	–	–	Yellowish
<i>C. glucuronolyticum</i>	v	v	v	+	v	+	v	+	+	β -Glucuronidase +
<i>C. imitans</i>	–	–	–	w	+	+	+	w	+	Tyrosine hydrolysis –
<i>C. minutissimum</i>	–	–	–	+	+	+	+	v	–	Tyrosine hydrolysis +
<i>C. pseudotuberculosis</i>	–	+	–	–	v	+	+	v	REV	Glycogen –
<i>C. rieglia</i>	–	+	–	v	v	–	(+)	–	–	
<i>C. singulare</i>	–	+	–	+	+	+	+	+	–	Tyrosine hydrolysis +
<i>C. striatum</i>	+	–	–	+	+	+	–	v	v	Tyrosine hydrolysis +
<i>C. thomassenii</i>	–	+	–	+	+	+	+	+	–	N-Acetylglucosaminidase +
<i>C. ulcerans</i>	–	+	–	–	+	+	+	–	REV	Glycogen +
<i>C. xerosis</i>	v	–	–	+	+	+	+	+	–	Yellowish

C_{16:0} (27–29% of all cellular fatty acids), C_{18:0} (34–35%) and C_{18:1} (7–9%). Significant amounts of tuberculostearic acid (2–3%) were also detected. As a result of the chemotaxonomic investigations, the three unknown coryneform bacteria could be unambiguously identified as *Corynebacterium* species (Collins & Cummins, 1986). Tuberculostearic acid is relatively rarely detected in non-lipophilic fermentative corynebacteria. In fact, *Corynebacterium minutissimum* and *Corynebacterium xerosis* are the only two non-lipophilic, fermentative corynebacteria in which tuberculostearic acid is detected (Bernard *et al.*, 1991; von Graevenitz *et al.*, 1991).

Determination of MICs (in $\mu\text{g ml}^{-1}$) revealed that the strains were susceptible to penicillins (e.g. penicillin G 0.0625–0.25, ticarcillin 0.5–2), most cephalosporins (e.g. cephalothin $\leq$ 0.125–0.25, ceftriaxone 0.5–1), aminoglycosides (e.g. amikacin 0.25–1, tobramycin 0.125–0.25), tetracyclines (e.g. doxycycline 0.25–1, minocycline 0.25–1), quinolones (e.g. ciprofloxacin 0.0625–0.25, ofloxacin 0.5–1), chloramphenicol (2–4), fusidic acid (0.0625–0.5) and glycopeptides (e.g. teicoplanin 0.5, vancomycin 1). Strain DMMZ 3259 was resistant to rifampicin (MIC > 32) whereas strains DMMZ 2439^T and IBS T 88848 were susceptible (MIC $\leq$ 0.02). Strain IBS T 88848 was resistant to macrolides (e.g. erythromycin and clarithromycin), whereas the other two strains were susceptible. All strains were resistant to aztreonam (> 64) and fosfomycin (> 256). Overall, the antimicrobial susceptibility pattern of the three unknown coryneform bacteria corresponded to that of many other non-lipophilic corynebacteria (Funke *et al.*, 1997f).

To determine the phylogenetic relatedness of strains DMMZ 2439^T and DMMZ 3259, their 16S rRNA

genes were amplified by PCR and subjected to sequence analysis. The almost complete 16S rRNA gene sequences (> 1400 nucleotides) of both strains were determined and comparative sequence analysis revealed only four nucleotide differences between the isolates (approx. 99.7% sequence similarity), thereby demonstrating their genealogical homogeneity. Sequence searches of EMBL/GenBank databases using the FASTA program revealed that the newly determined sequences were most closely related to species of the genus *Corynebacterium* (16S rRNA sequence similarities > 92%). Significantly lower levels of relatedness were shown with other actinomycete taxa (data not shown). A tree depicting the phylogenetic relationships of the unidentified bacterium within the genus *Corynebacterium* is shown in Fig. 1. *C. propinquum* and *Corynebacterium pseudodiphtheriticum* were found to be the closest phylogenetic relatives of the new bacterium. However, a sequence divergence value of > 4% unambiguously demonstrates that the unknown *Corynebacterium* represents a new species (Stackebrandt & Goebel, 1994). Riegel *et al.* (1993) had already demonstrated that strain IBS T 88848 exhibits only a 5–10% DNA–DNA homology with the type strain of *Corynebacterium propinquum* and only 1–2% DNA–DNA homology with *C. pseudodiphtheriticum*. In addition, strain IBS T 88848 did not show high DNA–DNA relatedness with a variety of other *Corynebacterium* species examined (Riegel *et al.*, 1993). These data are consistent with the findings of the comparative 16S rRNA gene sequence analysis and confirm that the three unknown strains represent a new species.

It is apparent that the new *Corynebacterium* species is only rarely encountered in the clinical environment, but it seems most likely that other clinical micro-

Fig. 1. Unrooted tree showing the phylogenetic position of *Corynebacterium confusum* within the genus *Corynebacterium*. The tree, constructed using the neighbour-joining method, was based on a comparison of approximately 1330 nucleotides. Bootstrap values, expressed as a percentage of 200 replications, are given at the branching points. Strains used are given and their corresponding 16S rRNA sequence accession numbers are given in parentheses.

biologists will also recognize it once it has been described in the literature. As many clinical laboratories use the API CORYNE strip for identification of coryneform bacteria, the weak acid formation from glucose exhibited by the new *Corynebacterium* may be recognized more easily, leading to the correct identification and avoiding the false-negative result observed in CTA media. Weak acidification of CTA media is also observed in the recently described *C. coyleae* (Funke *et al.*, 1997d), *Corynebacterium mucifaciens* (Funke *et al.*, 1997c) and *Corynebacterium riegelii* (Funke *et al.*, 1998), all of which might be misidentified if acidification reactions are not read after an extended time period, i.e. 48 or 72 h instead of 24 h.

This report once more demonstrates the importance of identifying coryneform bacteria from clinical specimens to the species level, as in this way new bacterial species might be discovered. Based on the results of the phenotypic and molecular genetic findings, we therefore propose that the above-described coryneform bacterium should be classified as a new species of the genus *Corynebacterium*, for which the name *Corynebacterium confusum* sp. nov. is proposed.

Description of *Corynebacterium confusum* sp. nov.

Corynebacterium confusum (con.fu'sum. L. past part. *confusum* confusing, to indicate that this bacterium

might be phenotypically confused with many other *Corynebacterium* species).

The description given below is based on the results of the studies of three strains. Cells are Gram-positive, non-spore-forming and non-motile. They are typically club-shaped rods that occur as single cells, in pairs or in small clusters. Colonies are whitish, glistening, convex, creamy and up to 1.5 mm in diameter after 48 h incubation. Weak anaerobic growth. Catalase-positive. Acid is produced from D-glucose, ribose, D-fructose, tagatose and 5-ketogluconate, but acid is not produced from maltose, sucrose, mannitol, D-xylose, glycerol, erythritol, arabinose, adonitol, β -methyl-xyloside, galactose, L-sorbose, rhamnose, dulcitol, inositol, sorbitol, methyl α -D-mannoside, methyl α -D-glucoside, N-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, lactose, melibiose, trehalose, inulin, melezitose, D-raffinose, glycogen, xylitol, D-turanose, D-lyxose, fucose, arabitol, gluconate or 2-ketogluconate. Acid production from β -gentiobiose is variable. Nitrate is reduced. Urea, aesculin and tyrosine are not hydrolysed. The CAMP reaction is negative. Activities of pyrazinamidase, alkaline phosphatase, esterase (C_4) and esterase lipase (C_8) are detected, whereas pyrrolidonyl arylamidase, valine arylamidase, trypsin, chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase are not detected. Activities of leucine arylamidase and phosphoamidase are variable. The cell wall contains meso-diaminopimelic acid. Mycolic acids are present. The main straight-chain saturated fatty acids are palmitic and stearic acids; oleic acid is the predominant unsaturated fatty acid. Minor amounts of tuberculostearic acid are present. Isolated from human clinical specimens. The type strain DMMZ 2439^T has been deposited in the Culture Collection of the University of Gothenburg, Sweden, as strain CCUC 38267^T. It has the features described above except that acid production from β -gentiobiose is positive and activities of leucine arylamidase and phosphoamidase are detected.

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