***Corynebacterium sundsvallense* sp. nov., from human clinical specimens**

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Three strains of a previously undescribed catalase-positive non-lipophilic coryneform bacterium isolated from human clinical specimens were characterized by phenotypic and molecular taxonomic methods. Morphologically the unknown bacterium consisted of pleomorphic rods, some of which displayed bulges/knobs at their ends. All three strains were similar in that they produced acid from fructose, glucose, maltose and sucrose and were urease-positive. Chemotaxonomic investigations revealed the presence of meso-diaminopimelic acid and short-chain mycolic acids consistent with the genus *Corynebacterium sensu stricto*. Comparative 16S rRNA gene sequencing showed that the three strains are genealogically highly related and constitute a new subline within the genus *Corynebacterium*, displaying >3% sequence divergence with recognized species. The unknown bacterium was distinguished from currently validly published *Corynebacterium* species by phenotypic tests, including electrophoretic analysis of whole-cell proteins. Based on phylogenetic and phenotypic evidence, it is proposed that the unknown bacterium from clinical specimens be classified as *Corynebacterium sundsvallense* sp. nov. The type strain is CCUG 36622^T.

Keywords: *Corynebacterium sundsvallense* sp. nov., taxonomy, phylogeny, rRNA

INTRODUCTION

During the past few years a plethora of new coryneform-like bacteria causing and/or associated with human disease have been described (Funke *et al.*, 1997d). The vast majority of these previously unrecognized coryneforms have been shown to be members of the genus *Corynebacterium*. The recognition of such a diversity of new coryneform-like organisms from humans probably stems from a growing awareness by clinical microbiologists and physicians of the pathogenic potential of some coryneforms, due to the implementation of improved diagnostic methods (e.g. miniaturized kits and databases) and the use of molecular-based approaches, in particular 16S rRNA sequencing, for investigating phylogenetic diversity. Indeed, the application of improved taxonomic methodologies has resulted in the description of over 20 *Corynebacterium* species from human sources since 1990, e.g. *Corynebacterium argen-*

toratense (Riegel *et al.*, 1995a), *Corynebacterium afermentans* (Riegel *et al.*, 1993a), *Corynebacterium auris* (Funke *et al.*, 1995b), *Corynebacterium coyleae* (Funke *et al.*, 1997c), *Corynebacterium durum* (Riegel *et al.*, 1997a), *Corynebacterium glucuronolyticum* (Funke *et al.*, 1995a), *Corynebacterium lipophiloflavum* (Funke *et al.*, 1997a), *Corynebacterium macginleyi* (Riegel *et al.*, 1995b), *Corynebacterium mucifaciens* (Funke *et al.*, 1997b), *Corynebacterium propinquum* (Riegel *et al.*, 1993b), *Corynebacterium singulare* (Riegel *et al.*, 1997b) and *Corynebacterium riegelii* (Funke *et al.*, 1998).

In this article we report the polyphasic taxonomic characterization of three coryneform-like isolates from human clinical specimens. Based on the results of this study, we propose a new species, *Corynebacterium sundsvallense* sp. nov.

METHODS

Strains. Two of the bacterial isolates (CCUG 36622^T and CCUG 37940) originating from human clinical specimens were referred to the Culture Collection of the University of Göteborg, Göteborg, Sweden, for identification. Strain

The GenBank accession number for the 16S rRNA gene sequence of strain CCUG 36622^T is Y09655.

CCUG 36622^T was isolated from an intrauterine device in a 46-year-old woman with suspected endometritis or pyosalpingitis. Strain CCUG 37940 was isolated in a haemoculture from a 67-year-old man with health problems, in particular with respect to dental status. The third strain, LCDC 93-0639, was recovered from sinus drainage from the left groin of a male patient (Kingston, Ontario, Canada) which had persisted for over 10 years.

Biochemical tests. All strains were cultured on Columbia agar (Difco) supplemented with 5% horse or sheep blood at 37 °C in air plus 5% CO₂. The strains were biochemically characterized by using the API CORYNE system (bioMérieux). Enzyme reactions were read after 24 h incubation at 37 °C, whereas acid production from carbohydrates was observed after 48 h. Further enzyme reactions were studied by means of the API ZYM system (bioMérieux). Other biochemical tests were performed as described previously (Bernard *et al.*, 1991).

For determination of end products of glucose fermentation, strain LCDC 93-0639 was grown for 4 d in CMC-PRAS medium obtained from Carr-Scarborough (Immunocor). Fermentation products were analysed by GC as described previously (Bernard *et al.*, 1997).

PAGE analysis of whole-cell proteins. PAGE analysis of whole-cell proteins was performed as described by Pot *et al.* (1994). For densitometric analysis, normalization and interpretation of protein patterns, the GelCompar GCW 3.0 software package (Applied Maths) was used.

Chemotaxonomic studies. Cell wall murein was prepared by mechanical disruption of cells using a Braun homogenizer and complete acid hydrolysates (4 M HCl) analysed as described by Stanek & Roberts (1974). The presence of mycolic acids was determined by the TLC method of Minnikin *et al.* (1980). DNA base composition was determined by thermal denaturation as described by Garvie (1978).

16S rRNA gene sequence analysis. A large fragment of the 16S rRNA gene (corresponding to positions 30–1521 of the *Escherichia coli* 16S rRNA gene) was amplified by PCR using conserved primers close to the 3' and 5' ends of the gene. The PCR products were purified using a Prep-A-Gene kit (Bio-Rad), according to the manufacturer's instructions, and directly sequenced using a *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and an automatic DNA sequencer (model 373A; Applied Biosystems). The closest known relatives of the new isolates were determined by performing database searches. These sequences and those of other known related strains were retrieved from the GenBank or Ribosomal Database Project (RDP) libraries and aligned with the newly determined sequences using the program PILEUP (Devereux *et al.*, 1984). The resulting multiple sequence alignment was corrected manually and a distance matrix was calculated using the programs PRETTY and DNADIST (using the Kimura 2-correction parameter) (Felsenstein, 1989). A phylogenetic tree was constructed according to the neighbour-joining method with the program NEIGHBOR and the stability of the groupings was estimated by bootstrap analysis (500 replications) using the programs DNABOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989).

RESULTS AND DISCUSSION

The three strains stained Gram-positive and consisted of pleomorphic coryneform rods. Bulges/knobs were observed on the end of some rods. The strains were

non-motile, non-spore-forming and not partially acid-fast. Colonies were buff or slightly yellowish in colour, opaque, shiny, heaped and very adherent to the medium after 2–3 d. They were non-haemolytic and all strains grew in 6% NaCl. The strains were catalase-positive and produced acid slowly from fructose, glucose, maltose and sucrose, hydrolysed hippurate and were urease-positive. Analysis of the end products of glucose fermentation of a single strain (LCDC 93-0639) revealed major amounts of lactate and minor amounts of succinate. Propionate was not detected. The isolates displayed α -glucosidase and phosphoramidase activities (weak reaction). The production of alkaline phosphatase, pyrazinamidase, leucine arylamidase, ester lipase C8 and esterase C-4 was variable. None of the isolates produced acid from amygdalin, *N*-acetylglucosamine, galactose, glycogen, lactose, mannitol, raffinose, salicin, trehalose, ribose or D-xylose and they were negative for acid phosphatase, cystine arylamidase, chymotrypsin, α -fucosidase, α -galactosidase, β -galactosidase, β -glucosidase, β -glucuronidase, lipase C14, α -mannosidase, pyrrolidonyl arylamidase, trypsin and valine arylamidase. The strains were CAMP (Christie-Atkins-Munch-Petersen)-negative and none were lipophilic or reduced nitrate. Although the above characteristics were consistent with an assignment to the genus *Corynebacterium*, the isolates did not conform exactly to any currently validly published species of this genus. Chemotaxonomic investigations revealed that C_{16:0} (38–43% of total acids) and C_{18:1 ω 9} (39–46%) were the predominant cellular fatty acids which was compatible with the assignment of the strains to the genus *Corynebacterium*. Tuberculostearic acid was not present. Additionally, TLC analysis demonstrated *meso*-diaminopimelic acid as the cell wall diamino acid and the presence of short-chain mycolic acids, thereby confirming the identity of the isolates as members of the genus *Corynebacterium*. The whole-cell protein profiles of two of the isolates (CCUG 36622^T, CCUG 37940) were examined by SDS-PAGE. A dendrogram derived from a numerical analysis of the protein patterns is shown in Fig. 1 and confirmed that the isolates are phenotypically highly related and distinct from all other corynebacteria examined.

To determine the phylogenetic relatedness of the strains, their 16S rRNA genes were amplified by PCR and subjected to sequence analysis. The almost complete 16S rRNA gene sequence (>1400 nt) of the three strains was determined. Comparative sequence analysis revealed 0–1 nucleotide differences between the strains (100–99.9% sequence similarity), thereby demonstrating their high genealogical relatedness. Sequence searches of EMBL/GenBank databases using the FASTA program revealed the newly determined sequences were most closely related to species of the genus *Corynebacterium* (16S rRNA sequence similarities >92%; Table 1). Significantly lower levels of relatedness were shown with other actinomycete taxa (data not shown). A tree depicting the phylogenetic

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

relationships of the unidentified bacterium, as exemplified by strain CCUG 36622^T, within the genus *Corynebacterium* is shown in Fig. 2. The new bacterium showed a close phylogenetic affinity to the subcluster of species embracing *Corynebacterium afermentans*, *Corynebacterium auris*, *Corynebacterium coyleae*, '*Corynebacterium genitalium*', *Corynebacterium imitans*, *Corynebacterium mucifaciens*, *Corynebacterium mycetoides*, *Corynebacterium lipophiloflavum*, *Corynebacterium riegelii* and '*Corynebacterium pseudogenitalium*'. It is evident from both sequence diver-

gence values and the tree analysis that the unknown bacterium is not specifically related to any other species and the >3% 16S rRNA sequence divergence unequivocally demonstrates the bacterium represents a new *Corynebacterium* species.

These three isolates from clinical specimens clearly constitute a previously unrecognized and highly related group of organisms which represent a new subline within the genus *Corynebacterium*. Phylogenetically, the recently described *Corynebacterium riegelii* is the

Table 1. Percentage 16S rRNA sequence similarities between *Corynebacterium sundsvallense* sp. nov. (Y09655) and some closely related species

Species	Similarity (%)	Species	Similarity (%)
<i>Corynebacterium accolens</i> (X80500)	95.0	<i>Corynebacterium macginleyi</i> (X80499)	95.0
<i>Corynebacterium afermentans</i> (X80255)	95.7	<i>Corynebacterium matruchotii</i> (X84443)	92.9
<i>Corynebacterium ammoniagenes</i> (X84440)	94.0	<i>Corynebacterium minutissimum</i> (X84678)	95.0
<i>Corynebacterium amycolatum</i> (X84244)	93.8	<i>Corynebacterium mucifaciens</i> (Y11200)	95.7
<i>Corynebacterium auris</i> (X82493)	94.0	<i>Corynebacterium mycetoides</i> (X84241)	94.5
<i>Corynebacterium bovis</i> (X84444)	92.8	<i>Corynebacterium pilosum</i> (X84246)	94.4
<i>Corynebacterium callunae</i> (X84251)	92.4	<i>Corynebacterium propinquum</i> (X84438)	93.4
<i>Corynebacterium coyleae</i> (X96497)	95.6	<i>Corynebacterium pseudodiphtheriticum</i> (X84258)	93.8
<i>Corynebacterium cystitidis</i> (X84252)	93.5	<i>Corynebacterium pseudogenitalium</i> (X81872)	95.6
<i>Corynebacterium diphtheriae</i> (X84248)	94.6	<i>Corynebacterium pseudotuberculosis</i> (X84255)	93.8
<i>Corynebacterium fastidiosum</i> (X84245)	94.3	<i>Corynebacterium renale</i> (X84249)	93.2
<i>Corynebacterium flavesens</i> (X84441)	93.3	<i>Corynebacterium riegelii</i> (Y14651)	96.5
<i>Corynebacterium genitalium</i> (X84253)	95.5	<i>Corynebacterium segmentosum</i> (X84437)	94.5
<i>Corynebacterium glucuronolyticum</i> (X86688)	91.7	<i>Corynebacterium striatum</i> (X84442)	93.4
<i>Corynebacterium glutamicum</i> (X84257)	92.9	<i>Corynebacterium tuberculostrictum</i> (X84247)	94.2
<i>Corynebacterium imitans</i> (Y09044)	95.8	<i>Corynebacterium ulcerans</i> (X84256)	94.1
<i>Corynebacterium jeikeium</i> (X84250)	94.1	<i>Corynebacterium urealyticum</i> (X84439)	93.4
<i>Corynebacterium kutscheri</i> (X81871)	93.5	<i>Corynebacterium variabilis</i> (X53185)	93.8
<i>Corynebacterium lipophiloflavum</i> (Y09045)	95.1	<i>Corynebacterium vitaeruminis</i> (X84680)	92.4
		<i>Corynebacterium xerosis</i> (X84446)	93.5

nearest relative of the unknown bacterium, displaying 3.5% 16S rRNA sequence divergence. Phenotypically, the new bacterium can be readily distinguished from *Corynebacterium riegelii* and other validly published *Corynebacterium* species. For example, it differs from *Corynebacterium riegelii* by producing acid from glucose and sucrose but failing to produce acid from ribose and trehalose. Tests which are useful in distinguishing the bacterium from its closest phylogenetic relatives and fermentative urease-positive *Corynebacterium* species encountered in clinical specimens are summarized in Table 2. In some respects (e.g. adherence to agar, α -glucosidase activity) the unknown bacterium superficially resembles *Rothia dentocariosa*. However, *Rothia dentocariosa* can be biochemically readily distinguished from the unknown species in not producing acid from sucrose and by reducing nitrate. *Rothia dentocariosa* also differs from the unknown *Corynebacterium* in wall murein composition, by the presence of predominately methyl branched cellular fatty acids and the absence of mycolic acids.

Based on the phylogenetic findings in conjunction with the phenotypic distinctiveness of the unidentified isolates, we propose they be assigned to a new species, *Corynebacterium sundsvallense* sp. nov.

Description of *Corynebacterium sundsvallense* sp. nov.

Corynebacterium sundsvallense (sunds.vall.en'se. N.L. gen. n. *sundsvallense* from Sundsvall, Sweden, named

after the city from where the bacterium was first isolated).

Cells are Gram-positive, non-spore-forming pleomorphic coryneform rods; some branching and bulges/knobs at the ends of some cells may be observed. Colonies are buff or yellowish, opaque, shiny, heaped and adherent to medium. Non-haemolytic. Non-lipophilic and CAMP-negative. Catalase-positive and oxidase-negative. Growth possible in 6% NaCl but not in 10% NaCl. Acid produced from fructose, glucose, maltose and sucrose but not from amygdalin, *N*-acetylglucosamine, galactose, glycogen, lactose, mannitol, ribose, raffinose, salicin, trehalose or D-xylose. Lactate and succinate are the major products of glucose fermentation. Hippurate is hydrolysed but not aesculin, gelatin or starch. Nitrate not reduced. α -Glucosidase- and urease-positive. Weak phosphoramidase activity detected. Some strains are positive for alkaline phosphatase, leucine arylamidase, pyrazinamidase, ester lipase C8 and esterase C-4. Acid phosphatase, cystine arylamidase, chymotrypsin, α -fucosidase, α -galactosidase, β -galactosidase, β -glucosidase, β -glucuronidase, lipase C14, α -mannosidase, pyrrolidonyl arylamidase, trypsin and valine arylamidase are not detected. Sensitive to penicillin (10 U) and vancomycin (30 μ g). The cell wall contains meso-diaminopimelic acid. Mycolic acids are present. The main cellular long-chain fatty acids are hexadecanoic acid and octadecenoic acid. Tuberculostrictic acid is not present. Isolated from human clinical specimens. Type strain is CCUG 36622^T. G + C con-

Fig. 2. Unrooted tree showing the phylogenetic relationships of *Corynebacterium sundsvallense* sp. nov. and members of the genus *Corynebacterium*. The tree constructed using the neighbour-joining method was based on a comparison of approximately 1320 nt. Bootstrap values, expressed as a percentage of 500 replications, are given at the branching points.

Table 2. Characteristics differentiating *Corynebacterium sundsvallense* from its nearest phylogenetic neighbours and some other fermentative urease-positive *Corynebacterium* species encountered in clinical specimens

–, Negative reaction; +, positive reaction; +^w, weakly positive reaction; v, variable reaction; REV, reverse CAMP reaction.

Organism	Urea hydrolysis	Pyrazinamidase	Acid production from:			Nitrate reduction	CAMP reaction	Other trait(s)
			Glucose	Maltose	Sucrose			
<i>Corynebacterium afermentans</i> subsp. <i>afermentans</i>	–	+	–	–	–	–	v	
<i>Corynebacterium afermentans</i> subsp. <i>lipophilum</i>	–	+	–	–	–	–	–	Lipophilic
<i>Corynebacterium amycolatum</i>	v	+	+	v	v	v	–	No mycolic acids
<i>Corynebacterium auris</i>	–	+	–	–	–	–	+	
<i>Corynebacterium coyleae</i>	–	+	+	–	–	–	+	
<i>Corynebacterium durum</i>	v	+	+	v	+	+	–	Adherent to agar; mannitol-positive
<i>Corynebacterium imitans</i>	–	+ ^w	+	+	+ ^w	–	+	
<i>Corynebacterium glucuronolyticum</i>	v	+	+	v	+	v	+	β-Glucuronidase-positive
<i>Corynebacterium lipophiloflavum</i>	+	+	–	–	–	–	–	Yellow pigment; lipophilic
<i>Corynebacterium matruchotii</i>	v	+	+	+	+	+	–	Whip-handle
<i>Corynebacterium mucifaciens</i>	–	+	+	–	v	–	–	Yellow mucoid colonies
<i>Corynebacterium mycetoides</i>	–	+	+	+	+	–	–	Yellow pigment
<i>Corynebacterium pseudotuberculosis</i>	+	–	+	+	v	–	REV	
<i>Corynebacterium sundsvallense</i> sp. nov.	+	+	+	+	+	–	–	Slightly yellowish colonies; adherent to agar
<i>Corynebacterium riegelii</i>	+	v	–	+	–	–	–	
<i>Corynebacterium ulcerans</i>	+	–	+	+	–	–	REV	Glycogen-positive

tent of the type strain is 64 mol %. Type strain has the features described above except weak reactions for pyrazinamidase and alkaline phosphatase are observed but leucine arylamidase, ester lipase C8 and esterase C-4 activities are not detected.

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