Corynebacterium singulare sp. nov., a New Species for Urease-Positive Strains Related to *Corynebacterium minutissimum*

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We studied two coryneform strains from clinical specimens. These strains had type IV and corynemycolic acids in their cell walls and also had phenotypic characteristics, such as urease activity and fermentation of glucose and sucrose but not trehalose, which did not permit assignment to any previously recognized taxon. According to DNA-DNA hybridization data, these two strains are members of the same species (level of DNA similarity, 86%). Phylogenetic analysis based on comparisons of almost complete small-subunit ribosomal DNA sequences revealed that these strains are closely related to *Corynebacterium minutissimum*, but DNA relatedness experiments clearly showed that they constitute a distinct new species with a level of DNA relatedness to the *C. minutissimum* type strain of less than 40%. This new species can be differentiated from *C. minutissimum* strains by its enzymatic activities and carbon source utilization, and the name *Corynebacterium singulare* is proposed for it. The type strain is strain IBS B52218 (= CCUG 37330), which was isolated from a semen specimen.

During an attempt to identify coryneform rods encountered in clinical sources, we isolated two strains which exhibited similar phenotypic characteristics but differed from previously recognized coryneform taxa. The presence of type IV and corynemycolic acids in the cell walls of the organisms supported their assignment to the genus *Corynebacterium*, and these two strains were found to possess a urease but not a nitrate reductase and to produce acid from glucose, sucrose, and maltose. In fact, except for the presence of a urease, these two strains resembled *Corynebacterium minutissimum* based on the morphology of colonies or commonly used biochemical tests (4). In order to determine the taxonomic position of these two unidentified isolates, we studied them and strains of *Corynebacterium* species by performing a biochemical analysis, a DNA-DNA hybridization analysis, and a small-subunit ribosomal DNA (rDNA) sequences analysis. On the basis of our results and previous phylogenetic studies of the genus *Corynebacterium* (11, 18), we propose a new species, *Corynebacterium singulare*, for these two urease-positive strains related to *C. minutissimum*.

MATERIALS AND METHODS

Strains, media, and growth conditions. The two strains of unidentified coryneform rods were isolated at the Institute of Bacteriology of Strasbourg from a semen specimen (strain IBS B52218¹) and a blood specimen (strain IBS T22913) from two patients. Type strain NCTC 10288 (= ATCC 23348) of *C. minutissimum* was obtained from the National Collection of Type Cultures. In addition, 18 *C. minutissimum* wild strains were obtained from the Institute of Bacteriology of Strasbourg or from the personal collection of F. Renaud, University Institute of Technology, Villeurbanne, France. These strains were identified as *C. minutissimum* strains by using recently published schemes (13, 22, 23). Bacteria were grown aerobically at 37°C on Trypticase soy agar (bioMérieux, Marcy-l'Etoile, France) and on Trypticase soy agar supplemented with 5% (vol/vol) sheep blood.

Biochemical tests. For the most part, the API Coryne system and a biotype 100 gallery were used with all strains according to the instructions of the manufac-

turer (API bioMérieux, La Balme-Les-Grottes, France); the only exceptions were the fermentation tests of the API Coryne system, for which the time of incubation was changed to 48 h. The biotype 100 gallery was inoculated by using a bacterial suspension in biotype medium 2 (bioMérieux) with a turbidity equivalent to 6 on the McFarland standard scale. Readings were made after 2, 4, and 6 days. Cupules 19 (esculin), 39 (hydroxyquinoline β -glucuronide), 59 (L-tryptophan), and 79 (L-histidine) were read taking into account the turbidity. Other tests (hydrolysis of tyrosine, gelatin, and esculin; fermentation of trehalose) were performed by conventional procedures, as described previously (12, 13). Leucine aminopeptidase activity was tested by using commercial tablets (Rosco, Eurobio, Les Ulis, France) according to the manufacturer's recommendations.

Analysis of fatty acids. The end products of bacterial metabolism of glucose were analyzed by high-performance liquid chromatography by using a previously described procedure (14). Cellular fatty acids (CFAs) were analyzed by gas-liquid chromatography as described previously (6).

Cell wall analysis and determination of DNA base composition. Amino acid and sugar determinations were carried out by performing thin-layer chromatography by using the procedure of Stanek and Roberts (20). A reverse-phase high-performance liquid chromatography method was used for the mycolic acid determination (5). The guanine-plus-cytosine (G+C) content of DNA was determined by using a capillary electrophoresis method as described previously (14).

DNA-DNA hybridization. DNA was extracted as described previously (12). Hybridization between labeled DNA and a fragmented DNA preparation was carried out at 60°C for 16 h in 0.42 M NaCl by using the S1 nuclease-trichloroacetic acid method (8, 12).

Small-subunit rDNA sequencing. One hundred nanograms of DNA was used in a PCR to amplify the small-subunit rDNAs. A pair of primers corresponding to positions 8 to 28 and 1384 to 1400 of the *Escherichia coli* small-subunit rDNA sequence was used. The procedures used for amplification and direct sequencing of PCR products have been described previously (17).

Phylogenetic analysis. The phylogenetic data described below were obtained (i) by using successive alignments and phylogeny procedures and (ii) by reinvestigating deep branching patterns after close relationships were determined (17). A neighbor-joining method was used for a preliminary analysis; the resulting topologies were further investigated by performing maximum-likelihood and maximum-parsimony analyses. For the maximum-likelihood analyses we used the fdnaml program rewritten by G. J. Olsen (University of Illinois, Urbana) and compiled on a Hewlett-Packard model 700 workstation, while the maximum-parsimony analyses were performed with the PAUP program for Macintosh computers (21). The robustness of each topology was evaluated with the neighbor-joining method through 1,000 bootstrap replications. Trees were plotted by using the njplot program for Macintosh computers, as developed by M. Gouy (Unité de Recherche Associée 243, Centre National de la Recherche Scientifique, Université Claude Bernard, Lyon, France), which permits transformation of a formal tree representation (Newick's format) into McDraw drawings. Only topologies that were found to be similar by all three methods were retained as

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FIG. 1. Unrooted tree showing the phylogenetic position of *C. singulare* IBS B52218^T within the genus *Corynebacterium* and related taxa. The tree was obtained by a neighbor-joining method. The values above the lines (only values greater than 90% are shown) indicate how branches were supported by a bootstrap analysis (1,000 replications). The branches that were also found to be significantly positive at $P > 0.01$ by the maximum-likelihood analysis are indicated by double asterisks. The plus signs indicate the branches also found in the most-parsimonious tree (maximum-parsimony analysis). Scale bar = 0.0062 accumulated change per nucleotide. Sequences were retrieved from the EMBL database under the accession numbers indicated.

true trees. Recent theoretical works have demonstrated that convergence of the results of all three methods is a strong indication that the correct phylogeny has been determined (10).

Nucleotide sequence accession number. The DNA sequence of strain IBS B52218^T has been deposited in the EMBL sequence database under accession no. Y10999.

RESULTS AND DISCUSSION

Taxonomic analyses. The cell walls of the two strains were found to be type IV cell walls (containing *meso*-diaminopimelic acid, arabinose, and galactose) and to contain mycolic acids with short chain lengths (C_{26} to C_{36}), and the G+C contents of the two strains were 62 mol%. These are typical features of

the genus *Corynebacterium* (3). Phylogenetic analyses confirmed that strain IBS B52218^T belonged to the robust monophyletic unit grouping all available *Corynebacterium* sequences as one branch of the *Corynebacterium-Mycobacterium-Nocardia* group. The comparison of nearly complete rDNA sequences revealed that the closest relatives of strain IBS B52218^T were *C. minutissimum* and *Corynebacterium striatum* (Fig. 1). The sequence of this strain is similar to all available sequences of the *C. minutissimum* type strain (similarity range, 99.1 to 99.6%) and differed from the sequence of the *C. striatum* type strain by 48 nucleotides (96.5% sequence similarity). The DNA similarity results for each pair given below are the means of the

TABLE 1. Biochemical characteristics of *C. singulare* and strains of *C. minutissimum*

Characteristic	<i>C. singulare</i> (n = 2) ^a	<i>C. minutissimum</i> (n = 19)
Urease	+ ^b	—
Nitrate reductase	—	—
Acid produced from:		
Sucrose	+	v
Maltose	+	v
Utilization of:		
D-Ribose	—	v
D-Turanose	+	—
N-Acetyl-D-glucosamine	—	+
D-Gluconate	—	v
3-Hydroxybutyrate	+	—
D-Alanine	+	v

^a n is the number of strains examined.^b +, 90% or more of the strains are positive; —, 10% or fewer of the strains are positive; v, 11 to 89% of the strains are positive.^c Data obtained by using the API biotype 100 system.

values obtained in two separate DNA hybridization experiments. We found a level of DNA binding of 86% between isolate IBS B52218^T and the other urease-positive isolate, strain IBS T22913, indicating that these two strains should be assigned to the same genomospecies. DNA-DNA hybridization experiments between labeled DNA of strain IBS B52218^T and unlabeled DNA of the type strain of *C. minutissimum* confirmed the relationship with *C. minutissimum* but revealed significant genomic divergence at the species level, with only 28% similarity. Conversely, the levels of DNA relatedness between labeled DNA of the *C. minutissimum* type strain and unlabeled DNAs of strains IBS B52218^T and IBS T22913 were 38 and 30%, respectively. The level of DNA similarity between strain IBS B52218^T and the type strain of *C. striatum* was 16%. Therefore, on the basis of both the phylogenetic analysis and DNA similarities, we concluded, in agreement with recent criteria for defining species (19), that the two previously unrecognized strains belong to a new genomic species in the genus *Corynebacterium*.

Cultural, morphological, and biochemical properties.

Strains IBS B52218^T and IBS T22913 were gram-positive pleomorphic rods occurring in typical V-shaped forms or palisades. They produced similar widespread growth on either Trypticase soy agar or sheep blood agar supplemented with Tween 80 or not supplemented with Tween 80. On sheep blood agar, they formed grayish glistening colonies with regular margins. The API Coryne numerical code for both strains was 6101125, for which there was no identification. These strains were urease positive and nitrate reductase negative, characteristics which were confirmed by using traditional methods (Christensen's medium and urea test broth for urease activity and a nitrate reduction broth for nitrate reductase activity). The CFA analysis of strain IBS B52218^T revealed that hexadecanoic acid (36% of the total CFAs), oleic acid (49%), and octadecanoic acid (4%) were the predominant CFAs. These results are nearly identical to those previously found for *C. minutissimum* strains (1).

Differentiation from *C. minutissimum*. It is worth noting that the two new strains differ phenotypically from *C. minutissimum* in an important number of characteristics. The discriminating features include the presence of a urease and a distinctive carbon source utilization pattern (Table 1). As determined with the API biotype 100 gallery, the two previously unrecognized strains utilized D-turanose and 3-hydroxybutyrate but not

N-acetyl-D-glucosamine, whereas none of 19 *C. minutissimum* strains exhibited this pattern. In two recent studies of the taxonomy of the genus *Corynebacterium*, workers reinvestigated the status and identification of *C. minutissimum* in more detail by using biochemical characteristics and DNA-DNA hybridization since it was demonstrated that there could be confusion with strains of *Corynebacterium amycolatum* (22, 23). As a result, accurate identification of *C. minutissimum* should be based on a lack of urease, nitrate reductase, α -glucosidase, and β -glucuronidase and the presence of leucine aminopeptidase and tyrosine clearing. Therefore, on the basis of our data and previous reports on *C. minutissimum* identification, we concluded that the two urease-positive strains studied here belong to a taxon clearly distinct from all strains identified as *C. minutissimum*.

Differentiation from other previously described species. Using the characteristics that differentiate the species of the genus *Corynebacterium* described in *Bergey's Manual of Systematic Bacteriology* (3) and recent descriptions of new species (13), we found 16 taxa belonging to the genus *Corynebacterium* which contain urease-positive strains. Comparisons of the rDNA sequences revealed that strain IBS B52218^T is not a member of any of these urease-positive taxa (Fig. 1). Phenotypically, the two previously unrecognized strains differ from these taxa as follows: (i) they ferment glucose and sucrose, whereas *Corynebacterium urealyticum* and *Corynebacterium pseudodiphtheriticum* do not produce acids from any sugar; (ii) they do not reduce nitrate to nitrite, while *Corynebacterium pilosum*, *Corynebacterium vitrumen*, *Corynebacterium glutamicum*, *Corynebacterium kutscheri*, and *Corynebacterium matruchotii* reduce nitrate invariably; (iii) they do not possess a β -glucuronidase activity, unlike *C. pilosum*, *Corynebacterium renale*, *Corynebacterium cystitidis*, *Corynebacterium glucuronolyticum*, and *Corynebacterium seminale* (7, 14); (iv) a pyrazinamidase is present, while *Corynebacterium pseudotuberculosis* and *Corynebacterium ulcerans* do not possess this enzyme (16); and (v) acid is not formed from trehalose, unlike *Corynebacterium calumae* and *C. glutamicum* (Table 2). The results of biochemical screening reactions (urea hydrolysis positive, nitrate reduction negative, and production of acids from glucose and sucrose) could be consistent with assignment of the new strains to Centers for Disease Control and Prevention (CDC) coryneform group F-1 and *C. amycolatum* (2, 9, 13, 15). However, the two previously undescribed strains do not require lipid and are alkaline phosphatase positive, unlike the CDC group F-1 strains previously studied (15). Nevertheless, the CDC group F-1 strains were not referred to by the CDC as lipophilic, and therefore, it is not unlikely that similar strains were included in this group as delineated initially (9). Our two previously undescribed strains possess mycolic acids, unlike *C. amycolatum*, and using the findings of Wauters et al. (22), we found that they differ from *C. amycolatum* by not producing propionic acid. In addition, we found the following differences in the biochemical and morphological properties of the previously undescribed strains and *C. amycolatum*: the two previously undescribed strains are positive for leucine aminopeptidase activity and degradation of tyrosine and produce colonies with a glistening and creamy appearance, whereas *C. amycolatum* is negative for leucine aminopeptidase activity and degradation of tyrosine and produces dry colonies (13, 22, 23). Finally, the results of this study indicate that the two previously undescribed strains should belong to a new species distinct from *C. minutissimum* and other *Corynebacterium* species, particularly because of their urease activity, which is one of the key reactions in the identification of corynebacteria. On the basis of phenotypic and genotypic differences, we propose that these

TABLE 2. Characteristics used for differentiation of *C. singulare* from urease-positive *Corynebacterium* species and related organisms^a

Organism(s)	Lipid requirement	Nitrate reductase	Pyrazinamidase	Alkaline phosphatase	β -Glucuronidase	Acid produced from:			
						Glucose	Sucrose	Maltose	Trehalose
<i>C. singulare</i>	— ^b	—	+	+	—	+	+	+	—
<i>C. amycolatum</i> ^c	—	v	+	+	—	+	v	v	v
<i>C. callunae</i>	—	—	ND	ND	ND	+	+	+	+
<i>C. cystitidis</i> and <i>C. renale</i>	—	—	+	—	+	+	—	+	+
<i>C. glutamicum</i>	—	+	ND	ND	ND	+	+	+	+
<i>C. kutscheri</i>	—	+	+	—	—	+	+	+	v
<i>C. matruchotii</i>	—	+	+	—	—	+	+	+	—
<i>C. pseudodiphtheriticum</i>	—	+	+	v	—	—	—	—	—
<i>C. pseudotuberculosis</i>	—	v	—	v	—	+	v	+	—
<i>C. pilosum</i>	—	+	+	—	+	+	—	v	v
<i>C. seminale</i>	—	v	+	v	+	+	+	v	—
<i>C. ulcerans</i>	—	—	—	+	—	+	—	+	—
<i>C. urealyticum</i>	+	—	+	v	—	—	—	—	—
<i>C. vitrumen</i>	—	+	+	—	ND	+	+	+	+
CDC group F-1	+	v	+	—	—	+	+	+	—

^a Data from references 2, 3, 8, 14 through 16, 22, and 23.^b —, negative; +, positive; v, variable; ND, not determined.^c Differentiated from *C. singulare* by a lack of mycolic acids and tyrosine hydrolysis.

two strains belong to a new species in the genus *Corynebacterium*, *Corynebacterium singulare*.

Description of *Corynebacterium singulare* sp. nov. *Corynebacterium singulare* (sin.gu.la're. L. adj. *singulare*, single, unique). The description below is based on the data obtained for the two strains included in this study. The bacteria are gram-positive, irregular rods. All cells are nonmotile, do not form spores, and are arranged in typical V-shaped forms or palisades. They are not acid fast. They are catalase positive, oxidase negative, and facultatively anaerobic. Nonhemolytic colonies (1 to 2 mm in diameter) are grayish and smooth after 24 h at 37°C on sheep blood agar. The colonies are circular and slightly convex with entire margins. Nitrate is not reduced to nitrite. Urea and tyrosine are degraded, but gelatin and esculin are not hydrolyzed. Acid is produced from glucose, maltose, and sucrose but not from lactose, glycogen, ribose, trehalose, mannitol, and D-xylose. Alkaline phosphatase, pyrrolidonyl-arylamidase, and pyrazinamidase are produced but α -glucosidase, β -glucuronidase, β -galactosidase, and *N*-acetyl- β -glucosaminidase are not produced. Cells utilize D-glucose, D-fructose, D-trehalose, D-mannose, sucrose, maltose, glycerol, D-turanose, L-malate, phenylacetate, putrescine, DL-lactate, caprylate, L-histidine, succinate, fumarate, 3-hydroxybutyrate, L-aspartate, L-glutamate, D-alanine, L-serine, propionate, and L-tyrosine as sole carbon sources. D-Ribose, *N*-acetyl-D-glucosamine, and D-gluconate are not utilized. Propionic acid is not produced by anaerobic metabolism of glucose. The cell wall contains *meso*-diaminopimelic acid, arabinose, galactose, and short-chain mycolic acids (C₂₆ to C₃₆). The main fatty acids are oleic, hexadecanoic, and octadecanoic acids. The G+C content of the DNA is 62 mol%. The type strain is strain IBS B52218 (= CCUG 37330); it was isolated from a human semen specimen.

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