Corynebacterium durum sp. nov., from Human Clinical Specimens

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A new *Corynebacterium* species, *Corynebacterium durum*, was isolated from respiratory tract specimens of five human patients. The strains of this species exhibited similar morphologic and biochemical features that differentiated them from all recognized species. Notably, all of these strains developed irregular and strongly adherent colonies under aerobic conditions and produced acid from mannitol and galactose. The cells are long pleomorphic rods with some filaments. This species has characteristics of the genus *Corynebacterium*, such as 55 mol% guanine plus cytosine in the DNA and the presence of corynomycolic acids, meso-diaminopimelic acid, arabinose, and galactose in the cell wall. These isolates formed a homogeneous group in which the DNA-DNA similarity values (as determined by an S1 nuclease procedure) compared with reference strain IBS G15036^T (T = type strain) ranged from 71 to 100%. The analysis of the nearly complete 16S rRNA gene sequence of IBS G15036^T indicated that this new species represents a distinct taxon within the genus *Corynebacterium*. This new species can be identified on the basis of its colony morphology, fermentation of sugars, and enzymatic activities. Strain IBS G15036 (= CCUG 37331) is the type strain of *C. durum*.

The genus *Corynebacterium* has recently been subjected to considerable taxonomic revisions, which have resulted in the proposal of several new species and subspecies, many of them representing previous Centers for Disease Control coryneform groups defined by Hollis and Weaver (10). The recent review of Funke et al. provided a very comprehensive overview of the taxonomic changes among coryneform bacteria (7). The genus *Corynebacterium* is currently defined on the basis of chemotaxonomic features (3). The salient chemotaxonomic features include cell wall component type IV (arabinose, galactose, and meso-diaminopimelic acid), short-chain-length mycolic acids, and a DNA base composition ranging from 52 to 65 mol%, although there are some exceptions to this general rule, with *C. amycolatum* lacking mycolic acids and *C. afermentans* and *C. auris* exhibiting guanine-plus-cytosine (G+C) contents of more than 65 mol% (7). Comparative analysis of sequences of genes coding for 16S rRNA (16S rDNA) confirmed that the members of the genus *Corynebacterium* constitute a single unit among the high-G+C-content gram-positive bacteria (11, 14). In contrast, species of the genus *Corynebacterium* exhibit significant phenotypic diversity in colony appearance (size, color, and morphology), requirements for growth factors (lipids), and biochemical activities. During the course of characterization of medically relevant coryneform rods, we isolated five strains that had the main characteristics of the genus *Corynebacterium* but that differed markedly from the established species because these strains grew slowly and exhibited irregular colonies strongly adherent to agar. Furthermore, all these strains were found to produce acid from mannitol, which is rarely observed in coryneform bacteria. In this paper we describe a new species, *Corynebacterium durum*, for these strains on the basis of biochemical, morphological, and cultural data, as well as our results of DNA-DNA hybridization experiments and 16S rDNA analyses.

MATERIALS AND METHODS

Strains, media, and growth conditions. The five isolates included in this study were isolated at the Institute of Bacteriology of Strasbourg, France, from respiratory tract specimens of different patients, mainly bronchiole washing specimens. As is usually done in our laboratory, these clinical materials were streaked onto 5% (vol/vol) sheep blood–Trypticase–soy agar (bioMérieux, Marcy-l'Etoile, France) and incubated in a 10% CO₂ atmosphere for 24 h. In addition, the samples were inoculated on both nonselective buffered charcoal-yeast extract (BCYE) agar and vancomycin-containing BCYE agar and incubated in a 2.5% CO₂ atmosphere for 3 weeks in order to recover *Legionella* species.

FIG. 1. Gram-stained smear of *C. durum* IBS G15036^T taken from a colony after 72 h of aerobic growth on blood agar. Bar = 10 µm.

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FIG. 2. Dendrogram based on an alignment of 1,423 nucleotides of the 16S rRNA genes of strain IBS G15036^T and representative *Corynebacterium* species. Strains studied are designated by their culture collection accession number, followed by the EMBL accession number. The tree was inferred from similarity values by the CLUSTAL method (12). The distance between two species is obtained by adding the lengths of the connecting horizontal branches using the scale at the bottom of the figure (percentage of sequence difference).

Biochemical tests. The API-Coryne system was used according to the manufacturer's instructions (API bioMérieux) except for the fermentation tests, for which the time of incubation was fixed at 48 h. Other tests were performed by conventional procedures as described previously (12). The end products of bacterial metabolism of glucose were analyzed by high-performance liquid chromatography (HPLC) according to a previously described procedure (13). Antimicrobial susceptibilities were determined by the disk diffusion method with Mueller-Hinton blood agar (Sanofi Diagnostic Pasteur, Marnes-La-Coquette, France) and an inoculum of approximately 10^5 CFU/ml.

Cell wall analysis and determination of DNA base composition. Samples for amino acid and sugar determinations were prepared and separated by thin-layer chromatography, and the components were identified as described previously (16). A reversed-phase HPLC method was used for the determination of mycolic acids (5). The guanine-plus-cytosine (G+C) content of DNA was determined by a capillary electrophoresis method as described previously (13).

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

Small-subunit rDNA sequencing. Chromosomal DNA was extracted as described previously (6). DNA amplification was performed with *Taq* DNA polymerase (Gibco BRL, Cergy-Pontoise, France), and the two 16S rDNA eubacterial universal primers were 5'-AGAGTTTGATCTGGCTCAG-3' (positions 8 to 28 in the *Escherichia coli* numbering system) and 5'-AAGGAGGTGATCCA GCCGA-3' (positions 1544 to 1525 in the *E. coli* numbering system). Thermal cycling consisted of denaturation at 93°C for 5 min, followed by 30 cycles of denaturation at 93°C for 30 s, primer annealing at 52°C for 1 min, and primer extension at 72°C for 1 min. After the last cycle, a final extension was performed at 72°C for 8 min and the mixture was cooled to 5°C. Thirty amplification cycles were performed in order to limit the number of sequence errors generated by the *Taq* DNA polymerase. The amplified DNA fragment was purified by phenol extraction and isopropanol precipitation (1). Sequencing was performed with a Thermo Sequenase fluorescence-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Life Science, Buckinghamshire, England) according to the manufacturer's instructions. Six different primers 5' labelled with fluorescein isothiocyanate were used for the sequencing reactions: P8 and Pc1544 (see above) and four internal primers, 5'-GTATTACCGCGGCTGCTGGCAC-3' (positions 535 to 514 in the *E. coli* numbering system), 5'-GTGCCAGCAGCCG CGGTAAKAC-3' (positions 514 to 535), 5'-GACTACCAGGGTATCTAAT CC-3' (positions 804 to 784), and 5'-GGATTAGATACCTGGGTAGTC-3'

(positions 784 to 804). The sequence was obtained with an Automated Laser Fluorescence (A.L.F.) DNA sequencer (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions.

Nucleotide sequence accession number. The amplified sequence was compared to other sequences of bacteria contained in the EMBL-GenBank database. The sequences were aligned by using the CLUSTAL method (9) and DNASTar software (DNASTar, Inc., Madison, Wis.). The nucleotide sequence of strain IBS G15036^T has been deposited in the EMBL sequence database under accession number Z97069.

RESULTS AND DISCUSSION

Isolation of strains and cultural, morphological, and biochemical properties. The new strains were isolated from sputum or bronchiole washing specimens of patients with a history of pneumonia. All but one of the five patients were immunocompromised (neoplasms, leukemia, and renal failure). All but one of the five coryneform isolates were recovered from non-selective BCYE agar after 2 to 3 days of incubation, the other being recovered from a blood plate. Lack of isolation on the vancomycin-containing BCYE agar is not surprising since these strains were fully susceptible to this antibiotic. Afterwards, these strains showed satisfactory growth on most of the usual media in an aerobic atmosphere, but their isolation may take longer than for the other common corynebacteria. After 48 h of incubation on blood agar plates in an aerobic atmosphere, cultures appeared as small, convoluted, raised beige colonies with irregular edges and no hemolytic activity was detected. Strong adherence to the medium occurred. Cells are long gram-positive pleomorphic rods (3 to 4 µm long) or occasionally filaments (15 to 30 µm long) with swollen ends (Fig. 1). Strains incubated in an aerobic atmosphere supplemented with 10% CO₂ exhibited larger colonies slightly adherent to agar, and under these conditions, microscopic observation re-

vealed no filaments. In liquid media, the organisms produced a sediment, leading to a granular appearance of the culture after the broth was shaken. The organisms were not partially acid fast.

Biochemical reactions obtained with the API-Coryne system or by conventional methods were remarkable for being similar among the five isolates. The strains were nitrate reductase and pyrazinamidase positive and alkaline phosphatase and β -glucuronidase negative. Slight degradation of urea occurred for four of the five strains with the API-Coryne system, urea broth medium, or Christensen's medium. The conventional media revealed two urease-positive strains, whereas urease was positive for three strains with the API-Coryne system. Some strains hydrolyzed esculin weakly (two strains were positive with tubes of esculin agar and one strain was positive with the API-Coryne system). The strains fermented glucose, sucrose, maltose, and fructose but not lactose, trehalose, or ribose, and it should be noted that all of the strains produced acid from mannitol, which is a phenomenon rarely observed for strains belonging to the genus *Corynebacterium*. The API-Coryne numerical code for all strains was 3000135, 3040135, or 3001135 for which there was no identification with the current database. The strains showed large inhibition zones around the following antimicrobial agents: ampicillin, amoxicillin-clavulanic acid, piperacillin, cefotaxime, gentamicin, ciprofloxacin, minocycline, erythromycin, rifampin, teicoplanin, and vancomycin.

Taxonomic analyses. The cell walls of the five isolates were found to contain cell wall component type IV (*meso*-diaminopimelic acid, arabinose, and galactose) and short-chain mycolic acids (C_{26} to C_{36}). The G+C contents of two strains studied were 55.4 (strain IBS G15036^T) and 55.0 mol%. These results support the assignment of these strains to the genus *Corynebacterium* (3). DNA-DNA hybridization experiments revealed that the five isolates form a single genomic group at the species level. The levels of relatedness between labeled DNA of strain IBS G15036^T (= CCUG 37331) and unlabeled DNAs of the four other isolates ranged from 71 to 100%.

PCR amplification of the 16S rDNA of strain IBS G15036^T with six primers produced a 1,500-base fragment from the 3' end, and we sequenced 1,423 bases from this fragment. Comparison of this sequence with sequences of other high-G+C gram-positive rods available from the EMBL database demonstrated that strain IBS G15036^T is related to the robust monophyletic unit grouping all available *Corynebacterium* sequences as one branch among the actinomycetes. The phylogenetic tree in Fig. 2 indicated that strain IBS G15036^T represents a distinct taxon among all the *Corynebacterium* species. The highest levels of similarity were the levels of similarity to *Corynebacterium matruchotii*, *Corynebacterium macginleyi*, and *Corynebacterium accolens* (93%), whereas the levels of similarity between strain IBS G15036^T and the other representatives of the genus *Corynebacterium* ranged from 90.2 to 92.8%. DNA-DNA hybridization experiments showed no significant similarity (7%) between strain IBS G15036^T and the type strain of *C. matruchotii*. Phylogenetic analysis based on rDNA sequences was demonstrated as one of the most powerful methods for estimating relationships of *Corynebacterium* strains since the results of such an analysis exhibited an excellent correlation with physiological characteristics (11, 14). It is remarkable that the relatively close position between the new species as represented by strain IBS G15036^T and *C. matruchotii* confirms their cultural similarity, e.g., irregular and adherent slowly growing colonies. Therefore, according to recent criteria defining a species (15), the chemotaxonomic data, the 16S rDNA sequence data, and the DNA-DNA hybridization data clearly showed that the five isolates form a new genomic

TABLE 1. Phenotypic characteristics^a differentiating *Corynebacterium durum* from some other actinomycetes

Characteristic	<i>C. durum</i>	<i>C. matruchotii</i>	<i>Rothia dentocariosa</i>	<i>Actinomyces</i> spp.	<i>Propionibacterium</i> spp.	<i>Nocardia</i> spp.
Cell morphology	Long pleomorphic rods with some filaments	Rods attached to a filament ("whip-handle")	Irregular rods with rudimentary branching	Rods, filaments some branching and cocci	Irregular rods, branched forms and cocci	Filaments with some coccoid forms
Partially acid-fast stain	Negative	Negative	Negative	Negative	Negative	Positive
Optimal atmospheric growth conditions	Aerobic	Aerobic	Aerobic	Anaerobic/aerobic	Anaerobic/aerobic	Aerobic
Mycolic acids (no. of carbons)	Presence (22-36)	Presence (22-36)	Absence	Absence	Absence	Presence (44-60)
Major product of glucose fermentation	Propionic acid	Propionic acid	Acetic acid	Succinic acid	Propionic acid	No fermentation
Acid production from:						
Galactose	+ ^b	-	v	v	v	v
Mannitol	+	-	-	v	v	v
Presence of:						
β -Galactosidase	-	-	-	v	+	+
α -Glucosidase	-	+	+	v	v	v

^a Data are from the publication of Funke et al. (7), *Bergey's Manual of Systematic Bacteriology* (3), and our observations.

^b +, 90% or more of strains positive; -, 10% or fewer of strains positive; v, 11 to 89% of strains positive.

TABLE 2. Characteristics^a used for differentiation of *Corynebacterium durum* from fermentative and nonlipophilic *Corynebacterium* species

Organism	Value for the indicated characteristic ^b							
	Urease	Nitrate reductase	β -Glucuronidase	Alkaline phosphatase	Acid from:			
					Galactose	Maltose	Mannitol	Sucrose
<i>C. durum</i>	(-) ^c	+	-	-	+	+	+	+
<i>C. amycolatum</i>	v	v	-	+	-	v	-	v
<i>C. argentoratense</i>	-	-	-	v	-	-	-	-
<i>C. callunae</i>	+	-	-	ND	-	+	-	+
<i>C. coyleae</i>	-	-	-	+	-	-	-	-
<i>C. cystitidis/C. renale</i>	+	-	+	-	-	+	-	-
<i>C. diphtheriae</i>	-	+	-	-	+	+	-	-
<i>C. flavescentis</i>	-	-	-	-	+	-	+	-
<i>C. glutamicum</i>	+	+	-	ND	-	+	-	+
<i>C. kutscheri</i>	+	+	-	-	-	+	-	+
<i>C. matruchotii</i>	-	+	-	-	-	+	-	+
<i>C. minutissimum</i>	-	-	-	+	ND	v	v	+
<i>C. pilosum</i>	+	-	+	-	-	v	-	-
<i>C. pseudotuberculosis</i>	+	v	-	v	-	+	-	-
<i>C. seminale/C. glucurono-lyticum</i>	v	v	+	-	ND	v	-	+
<i>C. striatum</i>	-	+	-	+	v	-	-	+
<i>C. xerosis</i>	-	+	-	+	ND	+	-	+

^a Data are from the publication of Funke et al. (7), *Bergey's Manual of Systematic Bacteriology* (3), and our observations.

^b +, 90% or more of strains positive; -, 10% or fewer of strains positive; v, 11 to 89% of strains positive; ND, not determined.

^c Some strains can exhibit a weak activity.

species within the genus *Corynebacterium*, for which we propose the name *C. durum*.

Differentiation from other actinomycetes. The present investigation provides evidence that differentiation of this new species from other actinomycetes can be accurately achieved by chemotaxonomic analyses or 16S rRNA sequence analyses, but these methods are not available to most clinical laboratories. Phenotypically, this new species can be confused with some other actinomycetes forming long rods, sometimes filamentous, or exhibiting irregular and slowly growing colonies. Characteristics which differentiate *C. durum* from these other actinomycetes are given in Table 1. Strains of *C. durum* may be differentiated easily from other morphologically related taxa as follows. (i) They are not partially acid fast, unlike *Nocardia* spp. (ii) They are catalase positive, while most of the *Actinomyces* species are catalase negative. (iii) They grow under anaerobic conditions only weakly, while *Actinomyces* spp. and *Propionibacterium* spp. are preferentially anaerobic. (iv) They produce acid from mannitol and do not exhibit α -glucosidase activity, unlike *C. matruchotii* and *Rothia dentocariosa*, and furthermore, acid is produced from galactose, which is not the case for *C. matruchotii*. Other partially acid-fast actinomycetes (*Gordonia* spp., *Rhodococcus* spp., and *Tsukamurella* spp.) exhibit, for the most part, pigmented colonies, unlike *C. durum*, and are rods or coccobacilli without filaments. More sophisticated methods such as determination of mycolic acids or products of glucose fermentation can be also used to characterize *C. durum*.

Differentiation from other *Corynebacterium* species. The strains of *C. durum* exhibit peculiar cell and colony morphologies (see above), which can be used for presumptive identification. The discriminating features of these nonlipophilic strains include the presence of nitrate reductase and pyrazinamidase and the production of acid from glucose, sucrose, maltose, and mannitol. These features readily distinguish *C. durum* from the other recognized *Corynebacterium* species (Table 2). Some phenotypic characteristics examined were identical to those of *C. matruchotii*, considering their peculiar cultural properties, but the "whip handle" forms of the cells are

not present in *C. durum*, unlike *C. matruchotii*. Also, the ability of *C. matruchotii* to hydrolyze esculin and produce acid from mannitol is debatable since hydrolysis of esculin is positive in the original description (2) and variable or negative in other publications (4, 7, 10). Similarly, production of acid from mannitol is considered either negative or variable (4, 7). Nevertheless, *C. durum* produces a strong acidification from galactose, unlike *C. matruchotii*, as it was given in the original description (2, 3) and determined by ourselves by the study of the type strain.

Description of *Corynebacterium durum* sp. nov. *Corynebacterium durum* (du'rum. L. adj. *durus*, hard, tough). The description of morphological and physiological characteristics below is based on the data for the five strains included in this study. The bacteria are gram-positive, nonmotile, non-spore-forming pleomorphic rods. After aerobic incubation at 37°C for 72 h, surface colonies are 0.5 to 1 mm in diameter; they are beige, convex, and rough, with convolutions and an irregular margin, and strongly adhere to the agar; cells are long rods, sometimes filamentous. Colonies incubated in an aerobic atmosphere supplemented with 10% CO₂ have a dense center and nearly regular margins; they adhere weakly to the agar. Formation of filaments under these conditions is rare. Growth under anaerobic conditions is relatively weak.

Nitrate is reduced to nitrite, and urea can be degraded weakly. Acid is produced from glucose, maltose, fructose, sucrose, galactose, and mannitol but not from ribose, lactose, trehalose, glycogen, or xylose. Gelatin and tyrosine are not degraded. A weak hydrolysis of esculin can be observed sometimes after at least 72 h of incubation. Pyrazinamidase is produced, but pyrrolidonyl arylamidase, alkaline phosphatase, β -glucuronidase, β -galactosidase, α -glucosidase, and *N*-acetyl- β -glucosaminidase are not. Propionic acid, but not succinic acid, is produced as the end product of anaerobic metabolism of glucose.

The cell wall contains *meso*-diaminopimelic acid, arabinose, and galactose. Mycolic acids with short chain lengths (C₂₆ to C₃₆) are present (determined by a HPLC procedure). The DNA base composition is 55 mol% G+C (determined by a

capillary electrophoresis procedure). *C. durum* strains were isolated from human respiratory tract specimens. The type strain is Institut de Bactériologie de Strasbourg (IBS) G15036 (= Culture Collection of the University of Göteborg [CCUG] 37331). It was isolated in Strasbourg, France, from the sputum of a patient. This strain has all of the above-described properties for the species.

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