Corynebacterium argentoratense sp. nov., from the Human Throat

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A new *Corynebacterium* species, *Corynebacterium argentoratense* was isolated from the throats of four human patients. It is characterized by the presence of chemotype IV, a cell wall, corynomycolic acids, and a G+C content ranging from 60 to 61 mol%. Strains belonging to this species exhibit high levels of DNA relatedness as determined by DNA-DNA hybridization experiments (S1 nuclease procedure) but no close DNA relatedness with related *Corynebacterium* species. Phylogenies based on comparative analyses of nearly complete small-subunit rDNA sequences confirmed the inclusion of this new species within the genus *Corynebacterium* and grouped it in a cluster with *C. diphtheriae*, *C. ulcerans*, *C. pseudotuberculosis*, and *C. kutscheri*. PCR experiments revealed an absence of the gene coding for diphtheria toxin. This new species can be identified by its mycolic acid pattern, fermentation of sugars, and enzymatic activities. Strain IBS B10697 (CIP 104296) is the type strain of *C. argentoratense*.

The genus *Corynebacterium* contains irregular, gram-positive rods which have a type IV cell wall (arabinose, galactose, and meso-diaminopimelic acid) and short-chain mycolic acids (1). In a previous study that used high-performance liquid chromatography (HPLC) of mycolic acids as a tool for the identification of coryneform organisms, de Briel et al. observed that some strains exhibited a singular pattern which differed from that recognized for other *Corynebacterium* species (2). However, the estimated lengths of mycolic acids of these strains were in the range corresponding to *Corynebacterium* species. The bacteria had biochemical features, such as lack of nitrate reductase and urease activities and presence of acidification from glucose alone, which are characteristic of *Corynebacterium flavescentis* and *C. mycetoides*, but pending further data they were referred to as *Corynebacterium* sp. In the present study, we determined the DNA base composition, the levels of DNA relatedness between four of these isolates and several *Corynebacterium* species, and their phylogenetic position by using comparisons of small-subunit rDNA sequences. On the basis of genomic data and phenotypic differences, we propose a new species for these strains, for which the name *Corynebacterium argentoratense* is proposed.

MATERIALS AND METHODS

Strains, media, and growth conditions. The four strains of *Corynebacterium* sp. (IBS B6369, IBS B10697, IBS B63473, and IBS B22070) were isolated at the Institute of Bacteriology of Strasbourg from patients hospitalized at the University Hospitals of Strasbourg, France. Each of these four strains exhibited a distinct ribotyping pattern with the *PvuII* restriction endonuclease. All these isolates were from throat specimens of patients suffering from tonsillitis. The type strains of established *Corynebacterium* species (*C. amycolatum*, *C. cystitidis*, *C. diphtheriae*, *C. jeikeium*, *C. propinquum*, *C. renale*, and *C. striatum*) were obtained from the Collection of the Pasteur Institute and the National Collection of Type Cultures for *C. minutissimum* NCTC 10288^T. Bacteria were grown aerobically at 37°C on Trypticase soy agar (bioMérieux, Marcy-l'Etoile, France) supplemented with 5% (vol/vol) sheep blood agar.

Biochemical tests. The API-Coryne system was used according to the manufacturer's instructions (bioMérieux) except for the time of incubation, which was fixed at 48 h. Acid production from sugars was also tested by using a basal heart infusion (bioMérieux) at 25 g per liter, adjusted to pH 7.8 and containing 1% (wt/vol) sugar, 0.1% Tween 80 (Merck, Darmstadt, Germany), and red phenol as

FIG. 1. Phylogenetic position of *C. argentoratense* within the genus *Corynebacterium*. The unrooted tree summarizes the results of phylogenetic analyses using three different methods based on the comparisons of small-subunit rDNA sequences of *Corynebacterium* species (12) and of their closest outgroup, represented by *D. maris* and *T. paurometabolum*. The topology shown was obtained by a neighbor-joining method. Asterisks, branches that were also found by maximum likelihood ($P < 0.01$). The percentages of bootstrap replications are indicated (heuristic search; only values greater than 50% are shown); these are branches found in the most parsimonious tree. Scale bar, accumulated changes per nucleotide. Domains used for these analyses correspond to positions 126 to 138, 141 to 180, 232 to 417, 422 to 440, 492 to 600, 618 to 836, 881 to 928, 932 to 998, 1040 to 1131, 1137 to 1250, 1265 to 1283, and 1286 to 1355 (*E. coli* numbering). Organism names are followed by EMBL accession numbers.

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TABLE 1. Similarity matrix based on small-subunit rDNA sequences

Strain	% Similarity																										
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1. <i>C. argentoratense</i>	100																										
2. <i>C. accolens</i>	94.4																										
3. <i>C. afermentans</i> subsp. <i>afermentans</i>	93.7	94.5																									
4. <i>C. amoniagenes</i>	93.4	93.1	92.7																								
5. <i>C. amycolatum</i>	93.8	92.4	93.0	93.1																							
6. <i>C. bovis</i>	93.8	93.1	92.8	93.0	93.8																						
7. <i>C. callunae</i>	92.7	92.5	91.7	93.5	92.0	92.5																					
8. <i>C. cystitidis</i>	91.5	94.1	93.4	91.5	91.6	91.7	91.0																				
9. <i>C. diphtheriae</i>	96.1	94.7	94.3	94.3	93.5	95.1	93.1	92.2																			
10. <i>C. flavescens</i>	94.8	94.8	92.9	94.9	93.5	93.8	93.3	92.9	94.9																		
11. <i>C. glutamicum</i>	92.8	92.9	92.9	93.6	92.3	93.5	96.3	92.0	93.5	93.6																	
12. <i>C. jeikeium</i>	93.2	94.5	92.9	93.1	93.6	95.1	91.9	91.6	93.9	93.9	92.8																
13. <i>C. kutscheri</i>	94.8	93.9	94.2	93.4	92.9	93.2	91.5	91.6	96.3	93.9	92.5	92.6															
14. <i>C. macginleyi</i>	94.2	98.5	94.6	92.8	92.8	93.7	91.7	94.5	94.7	94.5	92.5	94.8	93.8														
15. <i>C. matruchotii</i>	91.6	91.6	91.3	90.3	90.2	90.9	88.9	89.7	91.9	91.8	89.6	91.5	91.4	91.8													
16. <i>C. minutissimum</i>	95.9	97.3	94.3	94.0	92.9	94.0	93.3	93.3	94.8	95.4	93.4	94.8	93.3	96.5	92.3												
17. <i>C. mycetoides</i>	93.4	93.8	95.7	92.0	92.0	92.0	91.6	93.0	93.2	92.7	92.3	91.8	92.1	93.4	90.1	94.6											
18. <i>C. pilosum</i>	93.6	93.7	93.2	94.1	92.4	92.5	92.2	94.0	94.0	94.2	93.1	92.4	93.0	93.5	90.9	94.4	93.5										
19. <i>C. propinquum</i>	93.1	94.4	93.4	92.2	92.2	92.6	90.9	91.4	93.2	92.9	91.7	93.5	92.0	94.4	90.8	94.6	92.0	92.5									
20. <i>C. pseudodiphtheriticum</i>	93.4	94.2	93.1	92.4	92.7	93.0	90.9	91.6	93.6	93.0	91.4	93.6	92.1	94.1	90.8	94.4	92.1	92.4	99.0								
21. <i>C. pseudotuberculosis</i>	95.5	94.4	93.9	93.2	93.0	94.8	92.5	91.4	97.7	94.2	93.1	93.4	95.5	94.6	91.7	95.0	92.6	92.9	93.6	93.6							
22. <i>C. renale</i>	94.1	94.6	94.2	93.1	93.4	93.2	92.1	92.7	95.0	93.7	92.3	92.9	94.7	94.6	91.3	94.6	93.8	94.1	92.8	92.8	94.6						
23. <i>C. striatum</i>	94.9	94.9	93.5	94.2	92.3	93.8	93.2	92.5	95.0	95.4	93.2	92.8	93.6	94.9	92.3	97.3	92.9	93.4	92.9	92.7	94.9	93.4					
24. <i>C. ulcerans</i>	96.0	94.5	94.2	93.5	93.2	95.0	92.5	91.7	98.1	94.4	93.2	93.4	95.7	94.9	91.7	95.5	93.1	93.4	93.8	93.7	99.3	94.9	95.3				
25. <i>C. urealyticum</i>	94.3	94.7	92.7	92.8	93.5	94.9	92.0	91.5	94.0	93.6	92.5	96.3	93.3	94.4	90.7	94.7	92.1	92.6	93.1	93.1	93.7	94.3	92.7	94.0			
26. <i>C. variabilis</i>	94.2	94.1	93.1	93.4	94.1	95.1	93.8	91.3	94.7	94.3	93.8	94.9	92.8	93.8	90.7	94.7	92.1	92.7	92.8	92.9	94.3	94.1	94.4	94.4	94.7		
27. <i>C. xerosis</i>	93.5	92.8	92.2	93.9	97.0	94.7	92.8	91.2	93.8	94.0	93.3	93.5	92.5	93.4	89.7	93.5	91.5	92.9	92.4	92.4	93.6	93.2	93.2	93.7	93.5	94.8	100

^a For 1,314 nucleotides.

TABLE 2. Characteristics used for differentiation of *C. argentoratense* from *Corynebacterium* species and related organisms^a

Organism	Lipid requirement	Urease	Nitrate reductase	Pyrazinamidase	Alkaline phosphatase	Acid from:		
						Glucose	Sucrose	Maltose
<i>C. argentoratense</i>	—	—	—	+	√ ^b	+	—	—
<i>C. accolens</i>	+	—	+	v	—	+	v	—
<i>C. afermentans</i> subsp. <i>afermentans</i>	—	—	—	+	+	—	—	—
<i>C. afermentans</i> subsp. <i>lipophilum</i>	+	—	—	+	+	—	—	—
<i>C. amycolatum</i> ^c	—	v	v	+	+	+	v	v
<i>C. bovis</i> ^d	+	—	—	v	+	+	—	—
<i>C. diphtheriae</i>	—	—	v	—	—	+	—	+
<i>C. flavesces</i>	—	—	—	—	—	+	—	—
<i>C. jeikeium</i> ^e	+	—	—	+	+	+	—	v
<i>C. macginleyi</i>	+	—	+	—	+	+	+	—
<i>C. minutissimum</i>	—	—	—	+	+	+	v	+
<i>C. mycetoides</i>	—	—	—	—	+	+	—	—
<i>C. propinquum</i>	—	—	+	v	v	—	—	—
<i>C. pseudodiphtheriticum</i>	—	+	+	+	v	—	—	—
<i>C. pseudotuberculosis</i>	—	+	v	—	v	+	v	+
<i>C. renale</i>	—	+	—	+	—	+	—	v
<i>C. striatum</i>	—	—	+	+	+	+	+	—
<i>C. urealyticum</i>	+	+	—	+	v	—	—	—
<i>C. xerosis</i>	—	—	+	+	+	+	+	+
CDC group G	+	—	v	+	+	+	v	v
CDC group F-1	+	+	v	+	—	+	+	+

^a The data are from the API-Coryne system database and our observations.^b v, variable.^c Lacks mycolic acids and includes CDC group I-2 and CDC group F-2 strains.^d Differentiated from *C. jeikeium* and CDC coryneform group G by the presence of β-galactosidase activity.^e Differentiated from strains of CDC coryneform group G by lack of growth under anaerobic conditions.

an indicator. Enzymatic activities were determined by the API ZYM system (bioMérieux) after 24 h of incubation. DNase was tested on plates containing agar supplemented with 1% (vol/vol) Tween 80 after incubation for 5 days at 37°C. Tests of degradation of gelatin, tyrosine, and starch were performed in nutrient plate culture. Hydrolysis of hippurate was detected after incubation for 2 h and with ninhydrin as reagent.

Cell wall analysis. Amino acid and sugar determinations were carried out by thin-layer chromatography using the procedure of Stanek and Roberts (15). A reverse-phase HPLC was performed for the determination of mycolic acids (2).

DNA base composition determination. After extraction and purification on cesium chloride gradients, DNA was hydrolyzed with DNase I and nuclease P1 and the G+C content in moles percent was determined by chromatography (HPLC) of the deoxynucleotides as described elsewhere (9).

DNA-DNA hybridization. DNA was extracted as described previously (9) except that 10 µg of proteinase K (Sigma) and 20 mg of sodium dodecyl sulfate were added per ml of lysozyme-treated bacteria. Hybridization between labeled DNA and fragmented DNA preparations was carried out at 60°C for 16 h in 0.42 M NaCl by the S1 nuclease-trichloroacetic acid method (3, 8).

Small-subunit rDNA gene sequencing. One hundred nanograms of DNA was used in a PCR to amplify the small-subunit rDNA genes. A pair of primers, corresponding to positions 8 to 28 and 1384 to 1400 of *Escherichia coli* small-subunit rDNA sequence was used. The procedures for amplification and direct sequencing of PCR products were as described previously (11).

Phylogenetic methods. The phylogenetic data described below were obtained (i) by using successive alignment and phylogeny procedures and (ii) by reinvestigating deep branching patterns after close relationships were determined (11). A neighbor-joining method (13) was used for a preliminary analysis; resulting topologies were further investigated by maximum likelihood and maximum parsimony methods. Maximum likelihood analyses used the fdnaml program rewritten by G. J. Olsen (University of Illinois, Urbana) and compiled on a Hewlett-Packard model 700 workstation, while maximum parsimony analyses were performed by using the PAUP program for the Macintosh (16). The robustness of each topology was evaluated under maximum parsimony through 100 bootstrap replications (heuristic search). Trees were plotted by using the njplot program for the Macintosh developed by M. Gouy (Université Claude Bernard, Lyon, France), which allows the transformation of a formal tree representation (Newick's format) into MacDraw drawings. Only topologies that were found to be similar by all three methods were retained as "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 (6).

PCR detection of the diphtheria toxin gene. DNA was prepared and PCR experiments were performed by a previously described procedure (5).

Nucleotide sequence accession numbers. The sequence of strain IBS B10697

has been deposited in the EMBL sequence database under accession number X83955.

RESULTS AND DISCUSSION

DNA similarities. The DNA base composition of the four isolates was similar and ranged from 60 to 61 mol% G+C. These data agree with the inclusion of these strains in the genus *Corynebacterium*, which has been previously suggested on the basis of their mycolic acid composition (2). DNA-DNA hybridization experiments revealed that the four isolates form a single genomic group at the species level. The levels of DNA relatedness between strain IBS B10697 and the three other *Corynebacterium* sp. isolates were 65, 73, and 93% for IBS B63473, IBS B22070, and IBS B6369 respectively. Less than 5% DNA relatedness was found with the type strain of *C. diphtheriae* and with type strains of *Corynebacterium* species exhibiting a similar G+C content, i.e., *C. amycolatum*, *C. cystitidis*, *C. jeikeium*, *C. minutissimum*, *C. propinquum*, *C. renale*, and *C. striatum* (7).

Phylogenetic analyses. General phylogenetic analyses confirmed that strain IBS B10697 belonged to the robust monophyletic unit grouping all available *Corynebacterium* sequences (12) as one branch of the CMN taxon (10) (data not shown). More detailed analyses including only *Corynebacterium* sequences and their closest outgroup, represented by *Dietzia maris* and *Tsukamurella paurometabolum* (Fig. 1), were then performed. All three phylogenetic methods placed the new bacterium in a monophyletic unit that included *C. kutscheri*, *C. diphtheriae*, *C. pseudotuberculosis*, and *C. ulcerans*. However, this cluster was supported by only moderate bootstrap values (69%), but the inclusion of the new species in the genus *Corynebacterium* is supported by high bootstrap values. The relatedness to *C. diphtheriae*, *C. pseudotuberculosis*, and *C. ulcerans* led us to investigate the presence of the *tox* gene

FIG. 2. HPLC patterns of *p*-bromophenacyl esters of mycolic acids from *C. argenteoratense* IBS B10697 (A), *C. flavescentis* CIP 69.5T (B), *C. minutissimum* NCTC 10288^T (C), and *C. mycetoides* CIP 55.51T (D). The peaks differentiating *C. argenteoratense* from the three other species are numbered.

coding for the diphtheria toxin since these species were found to possess phage β with the *tox* gene (4). A PCR procedure which had been demonstrated to be useful for the detection of this gene (5) did not demonstrate the presence of the gene in any of the four isolates. Because of the low genomic relatedness and since there was less than 96% similarity in rDNA sequences with any other *Corynebacterium* species (Table 1), we can conclude, in agreement with the recent criteria for defining a species (14), that strain IBS B10697 is a new species within the genus *Corynebacterium*. DNA similarities confirm that the four isolates constitute a single distinct genomic species.

Phenotypic characterization. The four isolates are typical coryneform gram-positive rods; they formed creamy colonies (2 mm in diameter after 48 h of incubation at 37°C) with an absence of hemolysis on sheep blood agar. They grow well under anaerobic conditions, and a lipid supplement is not required for optimal growth. Biochemical patterns as obtained with the API-Coryne system are similar for the four isolates. These strains are urease and nitrate reductase negative and do not produce acid from lactose, sucrose, maltose, or mannitol.

They ferment glucose, but strain IBS B6369 exhibits a weak acidification from this sugar with the API-Coryne system. These acidifications were confirmed by using a basal heart infusion as described in Materials and Methods, giving a higher sensitivity. Moreover, this strain possesses an alkaline phosphatase activity, whereas the three other strains are negative for this reaction. Differentiation from other *Corynebacterium* species can be achieved by using the scheme shown in Table 2. The isolates studied can be differentiated from the two species *C. flavescentis* and *C. mycetoides*, which also ferment glucose alone, by a positive pyrazinamidase test and absence of yellow pigmentation. It should be noted that, in the case of weak glucose fermentation, these strains can be distinguished from *C. afermentans* strains by using data provided by an API ZYM gallery such as α -chymotrypsin activity, which is negative for *C. afermentans* and positive for the four *Corynebacterium* sp. isolates. Identification of these organisms can also be performed by comparison of patterns of mycolic acids obtained by an HPLC procedure. The four isolates exhibit similar patterns containing 10 peaks which easily distinguish them from the

biochemically related species *C. flavescentis*, *C. minutissimum*, and *C. mycetoides* (Fig. 2). In conclusion, genomic divergence, difference in mycolic acid composition, and biochemical reactions allow the recognition of these four *Corynebacterium* sp. strains as a new species within the genus *Corynebacterium* for which the name *C. argentoratense* is proposed. This species was isolated from the throats of patients suffering from tonsillitis, but its clinical relevance remains to be determined since no pathogenic factors were detected and coryneforms are frequently isolated from this site.

Description of *Corynebacterium argentoratense* sp. nov. *Corynebacterium argentoratense* (ar.gen.to.ra.ten'se. N.L. adj. from Argentoratum, Latin name of the city of Strasbourg, where the organism was isolated). The description is based on the data of the four strains included in this study.

Bacteria are nonmotile pleomorphic rods that are gram positive but not partially acid-fast and nonsporing. Cells are arranged in palisades or V-shaped forms. They are catalase positive, oxidase negative, and facultatively anaerobic. The bacteria grow on blood agar as circular acuminate creamy colonies (diameter, 2 mm) after 48 h of incubation at 37°C which are not hemolytic. They are not lipid requiring. Nitrate is not reduced, and tyrosine, gelatin, DNA, and urea are not degraded or hydrolyzed. Esculin is not hydrolyzed. Hydrolyses of starch and hippurate occur variably. Pyrazinamidase, esterase lipase, cystine arylamidase, and α -chymotrypsin are present. Production of esterase, leucine arylamidase, and acid phosphatase is variable, and alkaline phosphatase is usually not produced. Pyrrolidonylamidase, β -glucuronidase, β -galactosidase, α -glucosidase, and *N*-acetyl- β -glucosaminidase are not produced. Acid is produced from D-glucose and fructose but not from sucrose, maltose, lactose, galactose, D-xylose, trehalose, glycogen, or D-mannitol. Acidification from ribose occurs variably. The cell wall contains *meso*-diaminopimelic acid, arabinose, and galactose. Mycolic acids of short chain lengths (C_{26} to C_{36}) are present. DNA base composition is 60 to 61 mol% G+C (HPLC procedure). *C. argentoratense* strains were isolated from human throat specimens. The type strain is strain IBS B10697 (CIP 104296). It was isolated in Strasbourg. This strain has all the above properties for the species. Starch, but not hippurate, is hydrolyzed. Acid is produced from ribose. Esterase and acid phosphatase, but not alkaline phosphatase, are present.

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