Corynebacterium coyleae sp. nov., Isolated from Human Clinical Specimens

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Over a 5-year period, six isolates of a previously unknown nonlipophilic coryneform bacterium were isolated from human clinical specimens. The most characteristic phenotypic reactions of these isolates included slow fermentative acid production from glucose but no acid production from maltose and sucrose and a strongly positive CAMP reaction. Chemotaxonomic investigations revealed that *meso*-diaminopimelic acid and mycolic acids were present, that palmitic, oleic, and stearic acids were the predominant cellular fatty acids, and that the G+C content was 62 to 64 mol%, characteristics which are consistent with assignment to the genus *Corynebacterium*. Phenotypically, the unknown coryneform bacterium could be readily differentiated from all other *Corynebacterium* species. A 16S rRNA gene sequence analysis and quantitative DNA-DNA hybridization experiments demonstrated unambiguously that the unknown coryneform bacterium is a member of the genus *Corynebacterium* and is genotypically distinct from all other members of this genus. Based on phenotypic and genotypic findings, a new species, *Corynebacterium coyleae* sp. nov., is proposed. The type strain of *C. coyleae* is strain DSM 44184 (= CCUG 35014).

In *Bergey's Manual of Systematic Bacteriology* 17 *Corynebacterium* species are listed (2). Since *Bergey's Manual of Systematic Bacteriology* was published, 11 new species that belong to the genus *Corynebacterium* and are encountered in human clinical specimens have been defined. The reasons for the large number of *Corynebacterium* species described in the last few years include greater interest in corynebacteria as serious opportunistic human pathogens (3) and the recognition and description of many Centers for Disease Control and Prevention (CDC) coryneform groups defined by Hollis and Weaver (9) as true *Corynebacterium* species. However, some of the newly defined *Corynebacterium* species, including *Corynebacterium argentoratense* (15) and *Corynebacterium glucuronolyticum* (5), were not included as CDC taxa on the charts of Hollis and Weaver. We describe here the recovery from human clinical specimens of a nonlipophilic coryneform bacterium which has not been represented by any of the previously defined CDC taxa. Based on a comprehensive study in which phenotypic and molecular genetic methods were used, we propose a new *Corynebacterium* species, *Corynebacterium coyleae*, for this previously unknown coryneform bacterium.

MATERIALS AND METHODS

Strains. The six strains examined in this study were recovered from SeptiChek (Hoffmann-La Roche, Basle, Switzerland) or BacT/Alert (Organon Teknika, Durham, N.C.) blood culture bottles after 0.1 ml of broth was plated on 5% sheep blood-containing Columbia agar plates (Difco Laboratories, Detroit, Mich.) and the plates were incubated for 24 h at 37°C in a 5% CO₂ atmosphere. *Staphylococcus aureus* ATCC 25923 was used for the CAMP reaction analysis.

Biochemical profiles. The methods used to determine biochemical profiles were the methods described previously (6), except that the API Coryne system results were read after 48 h of incubation. The API 50CH system for determination of carbohydrate fermentations was used according to the instructions provided by the manufacturer, and reactions were read after 120 h of incubation at 37°C. To determine the volatile and nonvolatile fatty acids produced from the fermentation of glucose (6), cells were grown for 72 h in brain heart infusion broth (Becton Dickinson, Cockeysville, Md.) supplemented with 1% glucose.

Antimicrobial agent susceptibility patterns. The MICs of 17 antimicrobial agents known to exhibit activity against coryneform bacteria were determined by the agar dilution method according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) (12). Cells were incubated for 24 h at 35°C in ambient air on Mueller-Hinton agar plates supplemented with 5% sheep blood. MICs were interpreted according to the NCCLS guidelines (13). Since the NCCLS did not provide specific criteria for coryneform bacteria, the categories for staphylococci were used.

Chemotaxonomic investigations. Cellular fatty acid (CFA) patterns were determined with the Sherlock system (Microbial ID, Inc., Newark, Del.) as outlined previously (18). Analyses of whole-cell hydrolysates for the presence of *meso*-diaminopimelic acid and of mycolic acids were performed as described by Schaal (17). The method used to determine the G+C contents of the strains has been described previously (7).

Molecular genetic investigations. A large fragment (ca. 1,500 bases) of the 16S rRNA gene of strains DMMZ 214^T (T = type strain) and DMMZ 1075 was amplified by PCR by using universal primers pA and pH* and was sequenced as previously described (10). Smaller fragments (ca. 500 bases) of the strain DMMZ 1415, DMMZ 1812, and DMMZ 1874 sequences were also determined. The sequences determined were aligned with the sequences of reference organisms obtained from the EMBL Data Library by using the program PILEUP (4), and a phylogenetic tree was constructed by using the neighbor-joining method (4). A bootstrap analysis was performed by using 200 replicates (4).

The DNA used for DNA-DNA relatedness studies was isolated and purified by the method of Marmur (11). DNAs were labelled with ³H-labelled nucleotides by using a Megaprime kit (Amersham International, Amersham, United Kingdom). DNA-DNA hybridization experiments were performed by using an S1 nuclease-trichloroacetic acid procedure (8), and the reaction mixtures were incubated at 70°C for 16 h.

Nucleotide sequence accession number. The nucleotide sequence of the 16S rRNA of strain DMMZ 214^T has been deposited in the EMBL Data Library under accession number X96497.

RESULTS AND DISCUSSION

The six strains included in this study were isolated in pure culture from different patients between 1992 and 1996 (Table 1). The patients from whom the blood cultures were taken had fevers of unknown origin. The patient from whom strain DMMZ 214^T was isolated was infected with the human immunodeficiency virus, whereas the other five patients had previous surgical intervention as the underlying condition.

The isolates grew as whitish, slightly glistening colonies with entire edges that were 1 mm in diameter after 24 h of incubation on Columbia agar containing 5% sheep blood at 37°C in a 5% CO₂ atmosphere. The consistency of the colonies was either creamy or sticky. Gram staining revealed gram-positive,

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TABLE 1. Strains included in the present study

Strain ^a	Year of isolation	Sex of patient ^b	Age of patient (yr)	Source
DMMZ 214 ^T (= DSM 44184 ^T = CCUG 35014 ^T)	1992	M	30	Blood culture
DMMZ 1075 (= DSM 44185 = CCUG 35015)	1994	F	81	Blood culture
DMMZ 1415	1995	F	74	Pleural fluid
DMMZ 1812	1995	F	22	Blood culture
DMMZ 1874	1996	M	23	Blood culture
DMMZ 1879	1996	M	78	Pleural fluid

^a DMMZ, Department of Medical Microbiology, University of Zürich; DSM, German Collection of Microorganisms and Cell Cultures; CCUG, Culture Collection, University of Göteborg.

^b M, male; F, female.

non-spore-forming diphtheroids. The organisms were not partially acid fast. Initial biochemical screening reactions revealed the following characteristics: absence of nitrate reductase; no hydrolysis of urea or esculin; and no acid production from maltose, sucrose, mannitol, and xylose. In contrast, we observed slow fermentative acid production from glucose in cysteine Trypticase agar, as well as in the commercial API Coryne and API 50CH systems. The API Coryne system generated the numerical code 2100304 for three strains and the code 6100304 for the three other strains; these results corresponded to identification of the isolates as *Corynebacterium jeikeium* (98.4% identification and *T* value of 1.00 and 81.0% identification and *T* value of 0.75, respectively) (1). However, all six strains were nonlipophilic, which clearly separated them from the lipophilic organism *C. jeikeium*. Moreover, we observed that the CAMP reaction was strongly positive for all six strains, which is never the case with *C. jeikeium* strains. The CAMP reaction was clearly visible after only 16 h of incubation. Further biochemical profiling with the API Zym system revealed the presence of the following enzymes: esterase (*C*₄), esterase-lipase (*C*₈), leucine arylamidase, cystine arylamidase, acid phosphatase, and phosphoamidase. Enzymatic activities of valine arylamidase, chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase were not detected. Acid was produced from ribose, D-fructose, D-mannose, and 5-keto-gluconate but not from glycerol, erythritol, D-arabinose, adonitol, β-methylxyloside, galactose, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, lactose, melibiose, trehalose, inulin, melezitose, D-raffinose, amidon, glycogen, xylitol, β-gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, gluconate, and 2-keto-gluconate. Succinic and acetic acids were the major end products of glucose metabolism.

The strains were susceptible to the following antimicrobial agents: amoxicillin-clavulanic acid, ampicillin, ceftriaxone, cefuroxime, cephalothin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, imipenem, oxacillin, penicillin G, rifampin, teicoplanin, tetracycline, and vancomycin. The MICs of clindamycin were intermediate for two strains.

The CFA analysis revealed that palmitic acid (45 to 49% of the total CFA), oleic acid (34 to 38%), and stearic acid (10 to 15%) were the predominant CFAs. *meso*-Diaminopimelic acid was the diamino acid of the peptidoglycan, and mycolic acids were also present in all six strains. The G+C contents of the

TABLE 2. Levels of 16S rRNA sequence similarity between *C. coyleae* sp. nov. and other *Corynebacterium* species

Species (EMBL no.) ^a	% 16S rRNA sequence similarity with <i>C. coyleae</i> sp. nov.
<i>C. accolens</i> (X80500).....	94.9
" <i>C. acetacidophilum</i> " (X84240)	94.1
<i>C. afermentans</i> subsp. <i>afermentans</i> (X81874).....	98.2
<i>C. afermentans</i> subsp. <i>lipophilum</i> (X82055)	97.9
<i>C. ammoniagenes</i> (X84440)	93.9
<i>C. amycolatum</i> (X84244)	94.0
<i>C. argenteorotense</i> (X83955).....	94.5
<i>C. auris</i> (X82493).....	95.8
<i>C. bovis</i> (X84444)	93.3
<i>C. callunae</i> (X84251).....	93.9
<i>C. cystitidis</i> (X84252).....	93.3
<i>C. diphtheriae</i> (X84248)	95.8
" <i>C. fastidiosum</i> " (X84245).....	94.5
<i>C. flavesces</i> (X84441)	94.5
<i>C. glucuronolyticum</i> (X86688)	92.1
" <i>C. genitalium</i> " (X84253).....	96.9
<i>C. glutamicum</i> (X84257).....	93.8
<i>C. jeikeium</i> (X84250).....	94.1
<i>C. kutscheri</i> (X81871).....	95.3
<i>C. macginleyi</i> (X80499)	94.9
<i>C. matruchotii</i> (X84443)	93.8
<i>C. minutissimum</i> (X84678).....	94.4
<i>C. mycetoides</i> (X84241).....	96.3
<i>C. pilosum</i> (X84246)	94.7
<i>C. propinquum</i> (X84438)	94.1
<i>C. pseudodiphtheriticum</i> (X84258).....	93.9
" <i>C. pseudogenitalium</i> " (X81874).....	96.4
<i>C. pseudotuberculosis</i> (X84255).....	95.6
<i>C. renale</i> (X84249).....	95.3
" <i>C. segmentosum</i> " (X84437).....	94.5
<i>C. striatum</i> (X84442).....	94.7
" <i>C. tuberculostearicum</i> " (X84247).....	94.6
<i>C. ulcerans</i> (X84256).....	95.7
<i>C. urealyticum</i> (X84439)	93.5
<i>C. vitrumen</i> (X84680).....	95.0
<i>C. xerosis</i> (X84446).....	93.7
<i>C. variabilis</i> (X53185).....	93.7

^a The numbers in parentheses are EMBL 16S rRNA nucleotide sequence accession numbers.

strains ranged from 62 to 64 mol%. These chemotaxonomic features, as well as the phenotypic characteristics, were compatible with assignment of the six unknown coryneform strains to the genus *Corynebacterium* (2).

In order to confirm the phenotypic data by molecular genetic methods, 16S rRNA gene sequence analyses of two representative strains (DMMZ 214^T and DMMZ 1075) were performed. Strains DMMZ 214^T and DMMZ 1075 had identical 16S rRNA gene sequences. The partial 16S rRNA gene sequences (ca. 500 bases, including variable regions V1 to V3) of three other clinical isolates (DMMZ 1415, DMMZ 1812, and DMMZ 1874) were also examined and were found to be identical to the strain DMMZ 214^T and DMMZ 1075 sequences, which confirmed the genotypic similarity of these organisms. A comparison of the sequence of strain DMMZ 214^T with the sequences of other high-G+C-content actinomycetes (obtained from the EMBL Data Library) revealed that the highest levels of sequence similarity (ca. 92 to 98%) were the levels of sequence similarity with species of the genus *Corynebacterium* (Table 2). Significantly lower levels of sequence similarity were found with members of the related genera *Mycobacterium*, *Nocardia*, *Gordona*, *Rhodococcus*, *Tsukamurella*, and *Dietzia*.

FIG. 1. Unrooted tree showing the phylogenetic position of *C. coyleae* within the mainly mycolic acid-containing high-G+C-content gram-positive bacteria. Significant bootstrap values ($\geq 80\%$) are indicated at the branch points.

An unrooted tree constructed from a matrix of sequence similarities is shown in Fig. 1, and this tree clearly demonstrates that the unknown coryneform bacterium is a member of the genus *Corynebacterium*. It was evident from both sequence divergence values and the treeing analysis that the closest phylogenetic relative of the unknown coryneform bacterium is *Corynebacterium afermentans* subsp. *afermentans* (level of sequence similarity, ca. 98.2%). The invalid lipophilic species "*Corynebacterium genitalium*" and "*Corynebacterium pseudogenitalium*," as well as some unnamed strains belonging to *Corynebacterium* CDC group F-1 (strains G4330 and G5911) also exhibited significant, albeit lower, levels of sequence similarity (ca. 96 to 97%) with the unknown coryneform bacterium (Table 2). Although it is not possible to derive precise 16S rRNA sequence divergence values for delineating species, the ca. 2% 16S rRNA divergence between *C. afermentans* (both subspecies) and the unknown coryneform bacterium strongly supports phenotypic data which indicate that the latter organism represents a new species. It is pertinent to note that within the genus *Corynebacterium* many genomically distinct species, including *Corynebacterium pseudodiphtheriticum* and *Corynebacterium propinquum* (>99% 16S rRNA similarity) and

Corynebacterium diphtheriae, *Corynebacterium ulcerans*, and *Corynebacterium pseudotuberculosis* (>98% 16S rRNA similarity), as well as CDC coryneform group G bacteria and *Corynebacterium accolens* (>98% 16S rRNA similarity) (14, 16), exhibit comparable or even higher levels of 16S rRNA relatedness. In order to check the genotypic separateness of the unknown coryneform bacterium and *C. afermentans* subsp. *afermentans*, chromosomal DNA-DNA hybridization experiments were performed with the type strain of *C. afermentans* subsp. *afermentans* (CIP 103499) and isolate DMMZ 214^T. When strain CIP 103499^T was used as the labelled strain, DMMZ 214^T exhibited 10.8% DNA similarity, whereas when DNA from strain DMMZ 214^T was labelled, a reciprocal similarity value of 11.2% was obtained with CIP 103499^T. These data confirm that DMMZ 214^T and *C. afermentans* subsp. *afermentans* are not related at the species level. Therefore, based on phenotypic and genealogical findings, it is evident that the unknown coryneform bacterium represents a new *Corynebacterium* species, for which we propose the name *C. coyleae*.

A synopsis of characteristics which differentiate *C. coyleae* from other nonlipophilic *Corynebacterium* species is given in Table 3. The fermentative organism *C. coyleae* is readily distinguished from the nonfermenting organism *C. afermentans*. *C. coyleae* may be confused with *C. argenteorotense*, *Corynebacterium flavesces*, or *Corynebacterium mycetoides*, all of which exhibit a negative CAMP reaction. The last two species also have a yellow pigment, whereas *C. coyleae* colonies are whitish. *C. argenteorotense* rapidly (within 24 h) produces acid from the fermentation of glucose and exhibits chymotrypsin activity (15), whereas *C. coyleae* slowly ferments glucose and does not exhibit chymotrypsin activity. Fermentation of ribose is always positive in *C. coyleae* strains, whereas *C. argenteorotense* strains are variable for this reaction. In addition, colonies of *C. argenteorotense* are slightly dry, whereas colonies of *C. coyleae* are more glistening.

The clinical significance of *C. coyleae* is not known, although so far strains have been isolated only from blood cultures and pleural fluids of immunocompromised patients.

***Corynebacterium coyleae* sp. nov.** *Corynebacterium coyleae* (coy'le.æ. N.L. gen. n. *coyleae*, of Coyle, to honor Marie B. Coyle, a contemporary American microbiologist, for her contributions to the clinical microbiology of coryneform bacteria). The description of morphological and physiological characteristics below is based on the results of studies of six strains.

Cells are gram-positive, nonmotile, non-spore-forming diphtheroids. Colonies are circular, convex, and slightly glistening; some colonies have a creamy consistency, and others have a sticky consistency. The colony diameter is about 1 mm after 24 h of incubation on sheep blood agar at 37°C. Very weak anaerobic growth. Catalase positive. Acid is produced from glucose, ribose, D-fructose, D-mannose, and 5-keto-gluconate but not from maltose, sucrose, mannitol, xylose, glycerol, erythritol, D-arabinose, L-arabinose, adonitol, β-methylxyloside, galactose, L-sorbose, rhamnose, dulcitol, inositol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, N-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, lactose, melibiose, trehalose, inulin, melezitose, D-raffinose, starch, glycogen, xylitol, β-gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, and 2-keto-gluconate. Nitrate is not reduced. Urea, esculin, casein, tyrosine, and xanthine are not hydrolyzed. The CAMP reaction is strongly positive. Pyrazinamidase, alkaline phosphatase, esterase (C₄), esterase-lipase (C₈), leucine arylamidase, cystine arylamidase, acid phosphatase, and phosphoamidase activities are detected, but valine arylamidase, chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glu-

TABLE 3. Characteristics that differentiate *C. coyleae* from other nonlipophilic *Corynebacterium* spp.^a

Organism	Nitrate reduction	Urea hydrolysis	Esculin hydrolysis	Pyrazinamidase	Alkaline phosphatase	Acid produced from:			CAMP reaction	Other traits
						Glucose	Maltose	Sucrose		
<i>C. coyleae</i>	— ^b	—	—	+	+	+	—	—	+	
<i>C. afermentans</i> subsp. <i>afermentans</i>	—	—	—	+	+	—	—	—	V	
<i>C. ammoniagenes</i>	+	+	—	+	+	+	—	—	—	Tyrosine positive, TBSC ^c positive, cleaved mycolic acids
<i>C. amycolatum</i>	V	V	—	+	+	+	V	V	—	Mycolic acids negative
<i>C. argentoratense</i>	—	—	—	+	V	+	—	—	—	Chymotrypsin positive
<i>C. auris</i>	—	—	—	+	+	—	—	—	+	Cleaved mycolic acids
<i>C. callunae</i>	—	+	—	ND	ND	+	+	+	—	
<i>C. cystitidis</i>	—	+	—	+	+	+	—	—	—	β-Glucuronidase positive
<i>C. diphtheriae</i>	V	—	—	—	—	+	+	—	—	Cystinase positive
<i>C. flavescentis</i>	—	—	—	—	—	+	—	—	—	Yellow pigment
<i>C. glucuronolyticum</i>	V	V	V	+	V	+	V	+	+	β-Glucuronidase positive
<i>C. glutamicum</i>	+	+	—	+	+	+	+	+	—	
<i>C. kutscheri</i>	V	+	+	V	—	+	+	+	—	
<i>C. matruchotii</i>	+	V	+	+	—	+	+	+	—	“Whip handle” (Gram)
<i>C. minutissimum</i>	—	—	—	+	+	+	+	V	—	
<i>C. mycetoides</i>	—	—	—	+	+	+	—	—	—	Yellow pigment
<i>C. pilosum</i>	+	+	—	+	+	+	—	—	—	Yellow pigment, β-glucuronidase positive
<i>C. propinquum</i>	+	—	—	V	V	—	—	—	—	
<i>C. pseudodiphtheriticum</i>	+	+	—	+	V	—	—	—	—	
<i>C. pseudotuberculosis</i>	—	+	—	—	V	+	+	V	REV	
<i>C. renale</i>	—	+	—	+	—	+	—	—	+	β-Glucuronidase positive
<i>C. striatum</i>	+	—	—	+	+	+	—	V	V	Tyrosine positive
<i>C. ulcerans</i>	—	+	—	—	+	+	+	—	REV	Glycogen positive
<i>C. variabilis</i>	V	+	+	—	—	—	—	—	—	
<i>C. vitarumen</i>	+	+	+	+	+	+	+	+	—	Yellow
<i>C. xerosis</i>	V	—	—	+	+	+	+	+	—	Yellowish

^a Data from references 1 and 2 and our observations.^b —, negative; +, positive; V, variable; ND, no data; REV, reverse.^c TBSC, tuberculostearic acid.

cosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase activities are not detected. Pyrrolidonyl arylamidase activity is 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. The DNA base composition is 62 to 64 mol% G+C. Isolated from human clinical specimens. Type strain DMMZ 214 has been deposited in the German Collection of Microorganisms and cell cultures, Braunschweig, Germany, as strain DSM 44184 and in the Culture Collection of the University of Göteborg, Göteborg, Sweden, as strain CCUG 35014. Strain DSM44184^T has the characteristics described above for the species except that it has a negative pyrrolidonyl arylamidase reaction and a G+C content of 62 mol%.

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