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Corynebacterium resistens sp. nov., a New Multidrug-Resistant Coryneform Bacterium Isolated from Human Infections

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Five strains of an unknown, multidrug-resistant coryneform, gram-positive rod were isolated from blood, bronchial aspirate, and abscess specimens. Four of the five strains isolated were highly resistant to antimicrobial agents, including β-lactams, aminoglycosides, macrolides, quinolones, and tetracyclines, except for glycopeptides. In immunocompromised patients, bacteremia associated with this organism was rapidly fatal. This coryneform bacterium was nonmotile, lipophilic, and nonsaccharolytic. Lack of pyrazinamidase activity differentiated this organism from other lipophilic corynebacteria. Chemotaxonomic studies indicated that this multidrug-resistant coryneform bacterium belongs to the genus *Corynebacterium*. Comparative 16S rRNA gene sequencing and DNA-DNA hybridization analyses revealed that the five isolates were genetically identical and that they represent a new subline within the genus *Corynebacterium*, for which we propose the designation *Corynebacterium resistens* sp. nov. The type strain of *Corynebacterium resistens* is GTC 2026^T (SICGH 158^T, JCM 12819^T, CCUG 50093^T).

With the exception of *Corynebacterium diphtheriae*, the pathogenicity of corynebacteria has been underestimated and often underappreciated, despite an increasing number of reports associating corynebacteria with human disease (2, 7). Not only is the increase in case reports of corynebacteria involved in infections consistent with improved recognition of these bacteria, it also reflects the growing number of immunocompromised patients who are at risk for opportunistic infections. With respect to opportunistic infections associated with corynebacteria, lipophilic *Corynebacterium jeikeium* and *Corynebacterium urealyticum* are multidrug-resistant species frequently associated with bacteremia in patients with underlying hematological dyscrasia (6, 7, 13, 18). When lipophilic multidrug-resistant corynebacteria are isolated from blood cultures, those that oxidize only glucose and are negative for urease are tentatively identified as *C. jeikeium*.

During the management of two patients, one with acute myelocytic leukemia and one with myelodysplastic syndrome, we recovered two multidrug-resistant, lipophilic, asaccharolytic, urease-negative isolates from blood cultures. In addition, three other clinically significant, lipophilic, multidrug-resistant corynebacteria were recovered, bringing the total to five isolates resistant to antimicrobial agents at a level not previously observed. Because the isolates could not be assigned to any of the established taxa of coryneform bacteria, we studied these five strains further using a polyphasic taxonomic approach that included both phenotypic and molecular genetic methods. On the basis of the results of this investigation, we propose that our isolates represent a new *Corynebacterium* species, *Corynebacterium resistens* sp. nov.

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MATERIALS AND METHODS

Strains and culture conditions. Strains SICGH 158 (Social Insurance Central General Hospital 158 = GTC 2026) and SICGH 279 (GTC 2027) included in this study were recovered from positive blood cultures BD BACTEC Plus Aerobic/F bottles (Becton Dickinson, Maryland) of samples taken from patients with acute myelocytic leukemia and myelodysplastic syndrome, respectively. SICGH 102 (GTC 2023) and SICGH 108 (GTC 2024) were isolated from bronchial aspirates of one patient with malignant lymphoma and one patient with subarachnoid hemorrhage. SICGH 221 (GTC 2025) was recovered from the right thigh of a patient with cellulitis. Following isolation, all strains were subcultured on Trypticase soy agar (TSA) plates supplemented with 5% sheep blood (Becton Dickinson, Tokyo, Japan) for 24 h at 37°C in ambient air. *Staphylococcus aureus* ATCC 25923 was used as a control in assessing the Christie-Atkins-Munch-Peterson (CAMP) reaction of the strains in this study.

Antimicrobial agent susceptibility test. MICs for penicillin G, cefazolin, cefotiam, cefmetazole, cefepime, imipenem, amikacin, clindamycin, ciprofloxacin, minocycline, teicoplanin, and vancomycin were determined with a CLSI (formerly NCCLS) microtiter broth dilution method citation supplemented with 3% lysed horse blood (Eiken Chemical Co., Ltd., Tokyo, Japan) (12). On the basis of a consultation with an infectious disease, we set the clinically relevant drug concentration range at 0.13 to 64 μg/ml, with the exception of clindamycin and glycopeptides, which were set at 0.06 to 16 μg/ml and 0.5 to 16 μg/ml, respectively. Because no CLSI interpretive standard for corynebacteria exists, MICs were analyzed for comparative purposes. Microdilution trays were incubated aerobically at 35°C for 48 h.

Chemotaxonomic investigations. Cellular fatty-acid compositions were measured by means of the Sherlock Microbial Identification System (MIDI, Inc., Newark, DE) and the method described by Kosako et al. (9).

16S rRNA gene sequence and analysis. The 16S rRNA genes of the isolates in this study were amplified by PCR as described previously (5). The nucleotide sequence was determined with an automatic sequencer (model 3100, Applied Biosystems, Calif., USA) and a dye-terminator reaction kit (Applied Biosystems). The sequences of other *Corynebacterium* species used for alignment and for calculating homology levels were obtained from the DNA Data Bank of Japan (DDBJ), GenBank, and the European Molecular Biology Laboratory (EMBL) databases. CLUSTAL W software, originally described by Thompson et al. (17), was used to align the sequences, and the phylogenetic distance was calculated with the neighbor-joining method. The phylogenetic tree was drawn with TREEVIEW software (14).

DNA-DNA hybridization. DNA from each strain was prepared by the standard procedure of Marmur (10). Quantitative microplate DNA-DNA hybridization was carried out as described previously (3). Hybridization experiments were

TABLE 1. MICs of 12 antimicrobial agents against the five strains[a]

GTC strain no.	SICGH strain no.	MIC (μg/ml)											
		PEN	CFZ	CTM	CMZ	FEP	IPM	AMK	CLI	CIP	MIN	TEC	VAN
2023	102	>64	>64	>64	>64	>64	>32	>32	>16	>32	16	≤0.5	2
2024	108	>64	>64	>64	>64	>64	>32	32	>16	>32	8	≤0.5	2
2025	221	>64	>64	>64	>64	>64	≤0.13	>32	>16	>32	0.25	≤0.5	2
2026	158	>64	>64	>64	>64	>64	>32	32	>16	>32	16	≤0.5	2
2027	279	>64	>64	>64	>64	>64	>32	32	>16	>32	8	≤0.5	2

[a] PEN, penicillin; CFZ, cefazolin; CTM, cefotiam; CMZ, cefmetazole; FEP, cefepime; IPM, imipenem; AMK, amikacin; CLI, clindamycin; CIP, ciprofloxacin; MIN, minocycline; TEC, teicoplanin; VAN, vancomycin.

carried out at 37°C (optimal conditions) and 47°C (stringent conditions) with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 50% formamide. The optimal temperature was 30°C lower than the denaturation temperature because the formamide lowered the hybridization temperature (11). The type strains used for DNA-DNA hybridization were *Corynebacterium auriscanis* GTC 1995^T (= DSM 44609) and *Corynebacterium jeikeium* GTC 681^T (= IFO 15298). Because *C. auriscanis* showed the highest 16S rRNA gene sequence homology (98.5%), and *C. jeikeium*, which also phylogenetically related species, was the multidrug resistant along with our isolates, we selected these two species to clarify whole genome DNA-DNA relationships with our isolates.

Biochemical profiles. The strains were characterized biochemically with the API Coryne, API ZYM, and API 50CH systems (all from bioMérieux, Tokyo, Japan). API Coryne reactions were read after 24 h of incubation at 37°C, and API ZYM reactions were read after 4 h of incubation at 37°C, whereas acid production from carbohydrates was observed after 48 h. API 50CH reactions performed with 50 CHE medium were read after seven days of incubation at 37°C in ambient air.

G+C content. The G+C content of DNA from the isolates was determined by high-pressure liquid chromatography (HPLC) as described previously (4). Briefly, 10 μl of purified DNA (1 mg/ml) was heat denatured, after the DNA solution was cooled, and 10 μl nuclease P1 solution (2 U/ml) was added and incubated at 50°C for 1 h. Then 10 μl alkaline phosphatase solution (2 · 4 U/ml) was added, and the mixture was incubated at 37°C for 30 min. The digested DNA solution was analyzed by HPLC with a packed column (Wakosil 5C18, Wako Co., Ltd., Osaka, Japan). The mol% G+C content was calculated by using that of the *Escherichia coli* K-12 strain DNA as a standard (51.12 mol% G+C).

Nucleotide sequence accession number. The nucleotide sequence of the 16S rRNA of strain GTC 2026 has been deposited in DDBJ under accession number AB128981.

RESULTS

Clinical significance. Of the five strains of coryneform bacteria analyzed in the present study, two strains were recovered from blood cultures of patients with leukemia. Of these two strains, one was recovered from two different blood cultures, and the other was recovered from three different blood cultures. The third and fourth strains were recovered from bronchial aspirates of one patient with malignant lymphoma and one patient with subarachnoid hemorrhage. Gram-stained smears of the bronchial aspirates demonstrated the presence of increased polymorphonucleocytes and phagocytized coryneform bacteria. The fifth isolate was recovered together with *Staphylococcus epidermidis* from an aspirate collected via syringe from the right thigh of a 32-year-old male with cellulitis. These five strains are isolated among from June 1998 to October 2001. On the basis of an assessment by an infectious disease physician, all five isolates were judged to be clinically significant isolates.

Colony morphology. The five strains of coryneform bacteria grew as grayish-white, glistening, pearly colonies of up to 1.0 mm in diameter after 48 h of incubation on TSA with 5% sheep blood. All strains were lipophilic. When Tween 80 was added to a concentration of 1%, colony growth was enhanced, resulting in a colony diameter of 2 to 3 mm.

Susceptibility to antimicrobial agents. The MICs of various antimicrobial agents are given in Table 1. With respect to penicillin, cephalosporins, amikacin, clindamycin, and ciprofloxacin, all five isolates showed MICs beyond the clinically relevant drug concentration range utilized in this study. Four isolates exhibited MICs beyond the clinically relevant drug concentration range established for imipenem and minocycline. Only isolate GTC 2025, which was initially recovered from an outpatient, showed low MICs for imipenem and minocycline. All five isolates exhibited low MICs for the glycopeptides tested in the study.

Chemotaxonomic investigations. The predominant fatty acids were C18:1 ωc (37.34%), C16:0 (22.17%), and C18:0 (16.84%), which was consistent with values for other members of the genus *Corynebacterium*.

16S rRNA analysis. To determine the phylogenetic relatedness of the unknown coryneform isolates, the almost complete 16S rRNA gene sequence (1,418 bases) of a representative strain (GTC 2026) was determined. As shown in Fig. 1, sequence searches of the DDBJ, GenBank, and EMBL databases revealed that the 16S rRNA sequence was highly related to sequences of species within the genus *Corynebacterium*, with *C. auriscanis*, *C. falsenii*, *C. jeikeium*, and *C. urealyticum* displaying the highest levels of sequence relatedness (98.5, 96.7, 96.2, and 95.9% sequence similarity, respectively, with the unknown isolate). The unidentified bacterium formed a distinct subline that was close to, albeit distinct from, *C. auriscanis*, *C. falsenii*, *C. jeikeium*, and *C. urealyticum*. We also determined the partial sequence of the 16S rRNA gene (about 800 bp from

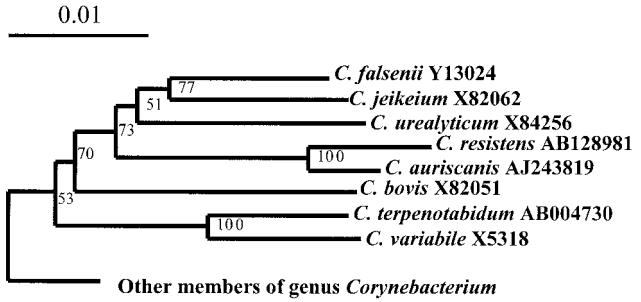

FIG. 1. Dendrogram showing phylogenetic relations of the 16S rRNA gene sequences of *Corynebacterium resistens* sp. nov. Distances were calculated by the neighbor-joining method. The numbers at the branch points are bootstrap values.

TABLE 2. DNA-DNA hybridization similarity values

Species	Strain	Temp (°C)					
		C. resistens		*C. auriscanis*		*C. jeikeium*	
		Optimal	Stringent	Optimal	Stringent	Optimal	Stringent
Corynebacterium resistens	GTC 2026^T (SICGH 158^T)	100.0	100.0	25.8	12.2	24.5	11.6
Corynebacterium auriscanis	GTC 1995^T (DSM 44609^T)	23.1	13.9	100.0	100.0	17.7	13.8
Corynebacterium jeikeium	GTC 681^T (IFO 15298^T)	18.0	13.2	14.4	9.9	100.0	100.0

the 5′ end) from another four strains (GTC 2023, GTC 2024, GTC 2025, and GTC 2027). The four strains and GTC 2026 shared almost identical sequences within this 800bp region only one or two different bases were observed).

DNA-DNA hybridization. DNA-DNA hybridization results under optimal and stringent conditions are shown in Table 2. GTC 2026 showed less than 23.1% DNA similarity to *C. auriscanis*, which was phylogenetically related. The data indicating that the unknown bacterium was a new *Corynebacterium* species. GTC 2023, GTC 2024, GTC 2025, and GTC 2027 showed 94.6 ± 4.52% (mean ± standard deviation) relatedness to GTC 2026.

Biochemical profiles. The five strains were facultative anaerobic, catalase positive, nonmotile, and CAMP reaction negative. Biochemical characterization with the API Coryne system yielded in positive reactions for only pyrrolidonyl arylamidase and alkaline phosphatase, in contrast, negative reactions were showed for nitrate reduction, pyrazinamidase, esculin, urease, gelatin, glucose, ribose, xylose, mannitol, maltose, lactose, sucrose, and glycogen, which corresponds to a profile number of 4100004.

With API ZYM, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase were clearly positive, whereas lipase (C14), cystine, and arylamidase were weakly positive. Reactions for valine arylamidase, trypsin, chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase were negative.

With API 50CH, no oxidation was noted even after 7 days reaction. With the overlay of sterile mineral oil, D-tagatose and 5-ketogluconate were positive within 24 h whereas ribose and D-glucose were weakly positive at 24 h and clearly positive when reactions were extended to 72 h. With respect to trehalose, only GTC 2025 was negative among the five strains tested. For L-sorbose, only GTC 2026 was negative. All strains were negative for glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, β-methylxyloside, galactose, D-fructose, D-mannose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, *N*-acetylglucosamine, amygdaline, arbutin, esculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, inulin, melezitose, D-raffinose, starch, glycogen, xylitol, β-gentiobiose, D-turanose, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, and 2-ketogluconate.

G+C content. The G+C content for strain GTC 2026 was 54.643 mol% (standard deviation, 0.03%).

DISCUSSION

Phylogenetic relatedness based on 16S rRNA sequence revealed that the present unknown coryneform isolate is most closely related to *C. auriscanis*; however, *C. auriscanis* is nonlipophilic and has not been reported to be resistant to antimicrobial agents (1). The lipophilic *C. jeikeium* is also related phylogenetically. The results of our DNA-DNA hybridization studies with type strains of *C. auriscanis* and *C. jeikeium* revealed that the unknown coryneform is not related specifically to any other species. Comparison of the biochemical characteristics of lipophilic *Corynebacterium* with those of our isolates (Table 3) shows that only *C. afermentans* subsp. *lipophilum* and *C. urealyticum* are unable to oxidize glucose, maltose, sucrose, mannitol, or xylose. *C. urealyticum* and *C. afermentans* subsp. *lipophilum* can be differentiated from our isolates because they are urease and pyrazimidase positive, respectively.

With the API Coryne system a profile code of 4100004 is considered a doubtful profile, which makes our isolates readily recognizable in clinical laboratories. Our newly identified corynebacterium is similar to *C. jeikeium* and *C. urealyticum* in that it is resistant to a number of antimicrobial agents, which makes this strain clinically relevant. *C. jeikeium* and *C. urealyticum* are susceptible to teicoplanin and vancomycin as well as to the tetracyclines (16), whereas our isolates are resistant to tetracyclines. Among corynebacteria, our isolates appear to have the greatest degree of resistance to antimicrobial therapies, which complicates patient management. While vancomycin is considered the first choice for eradication of multidrug-resistant corynebacteria, the use of vancomycin is restricted to methicillin-resistant *Staphylococcus aureus* in Japan because of potential nephrotoxicity and to prevent its overuse. Therefore, minocycline is considered the first choice, both for safety and for economic considerations.

In the present study, GTC 2027 was isolated on three occasions over 2 days during an episode of sepsis from a 68-year-old male with myelodysplastic syndrome. On the basis of the clinical findings and the recovery of corynebacteria from blood cultures, we considered *C. jeikeium* the most likely etiologic agent, and minocycline (200 mg/day) was administered. Because the MIC of 8 μg/ml was considered high, we believe that lack of effective therapy contributed to the subsequent death of the patient from sepsis.

Our isolates present a clinical challenge because glycopeptides appear to be the only antimicrobial agents with low MICs. Of the five strains in the present study, only GTC 2025, which was recovered from a patient in an outpatient setting, showed low MICs for imipenem and minocycline. It is thought that

TABLE 3. Biochemical characteristics of lipophilic *Corynebacterium* spp.[a]

Organism	Fermentation (F)/oxidation (O)	Nitrate reduction	Urea hydrolysis	Esculin hydrolysis	Pyrazina-midase activity	Alkaline phosphatase activity	Acid production from:					CAMP reaction	Other characteristics
							Glucose	Maltose	Sucrose	Mannitol	Xylose		
C. resistens	F	−	−	−	−	+	+	−	−	−	−	−	Slow growing in anaerobic conditions
C. accolens	F	+	−	−	V	−	+	−	V	V	−	−	
C. afermentans subsp. *lipophilum*	O	−	−	−	+	+	−	−	−	−	−	V	
C. bovis	O	−	−	−	V	+	+	−	−	−	−	−	β-Galactosidase positive
C. diphtheriae biotype *intermedius*	F	+	−	−	−	−	+	+	−	−	−	−	
C. jeikeium	O	−	−	−	+	+	+	V	−	−	−	−	Fructose negative, no growth anaerobically
C. kroppenstedtii	F	−	−	+	+	−	+	V	−	−	−	−	
C. lipophiloflavum	O	−	−	−	+	+	−	−	−	−	−	−	Yellow
C. macginleyi	F	+	−	−	−	+	+	−	+	V	−	−	
C. urealyticum	O	−	+	−	+	V	−	−	−	−	−	−	
CDC group F-1	F	V	+	−	+	−	+	+	+	−	−	−	
CDC group G	F	V	−	−	+	+	+	V	V	−	−	−	Fructose positive, growth anaerobically

[a] Modified from reference 7 with permission of the publisher. Symbols −, negative reaction; +, positive reaction; V, variable reaction.

exposure to antimicrobial agents in an inpatient setting contributes to increased resistance. Furthermore, as with *C. jeikeium*, the potential for nosocomial spread increases the clinical significance of our isolates (8, 15). On the basis of our phenotypic and molecular genetic findings, we propose that the unknown multidrug-resistant corynebacteria described above be classified as a new species within the genus *Corynebacterium* and that the name *Corynebacterium resistens* sp. nov. be used.

Description of *Corynebacterium resistens* sp. nov. *Corynebacterium resistens* (L. adj. *resistens*, resistant). The descriptive characteristics given below are based on the results of the studies of the five strains. Cells are gram positive, non-spore-forming, and nonmotile. They are typically club-shaped rods, coryneform bacteria (indicative of true *Corynebacterium* spp.) 1 to 3 μm in length, and arranged as single cells, in pairs, or in small clusters. Growth on TSA with 5% sheep blood demonstrated nonpigmented, grayish-white, glistening, pearly colonies up to 1.0 mm in diameter. Colonies were catalase positive, oxidase negative, nonhemolytic, and very slow growing under anaerobic conditions. Tween 80 enhanced growth, resulting in colonies 2 to 4 mm in diameter; CAMP negative, lipophilic, and nitrate was not reduced. There was no oxidizing resolution of any carbohydrates. However, the fermenting resolution was as follows: D-tagatose, 5-ketogluconate, ribose, and D-glucose were positive. For four of the five strains, trehalose and L-sorbose were positive.

All strains were negative for glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, β-methylxyloside, galactose, D-fructose, D-mannose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, *N*-acetylglucosamine, amygdaline, arbutin, esculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, inulin, melezitose, D-raffinose, starch, glycogen, xylitol, β-gentiobiose, D-turanose, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, and 2-ketogluconate. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase were clearly positive, whereas lipase (C14), cysteine, and arylamidase were weakly positive. Reactions for valine arylamidase, trypsin, chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase were negative. Fatty acids were C18:1 ωc (37.34%), C16:0 (22.17%), and C18:0 (16.84%). The G+C content of the DNA was 54.643 mol% (standard deviation = 0.03%) by HPLC. The type strain is GTC 2026^T (SICGH 158^T, JCM 12819^T, CCUG 50093^T)

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