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 nonacarolytic. 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 RNA 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 2026T (SICGH 158T, JCM 12819T, CCUG 50093T).

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, acarolytic, 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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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* DSM 44609 and *Corynebacterium jeikeium* DSM 6817 (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 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 were 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 C18:1ω9c (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. urealyticum*

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**TABLE 1. MICs of 12 antimicrobial agents against the five strains**

| GTC strain no. | MIC (μg/ml) |
|---------------|-------------|
| PEN | CFZ | CTM | CMZ | FEP | IPM | AMK | CLI | CIP | MIN | TEC | VAN |
| 2023 | >64 | >64 | >64 | >64 | >64 | >32 | >32 | >16 | >32 | 16 | ≤0.5 |
| 2024 | >64 | >64 | >64 | >64 | >64 | >0.13 | >32 | >16 | >32 | 0.25 | ≤0.5 |
| 2025 | >64 | >64 | >64 | >64 | >64 | >32 | >32 | >16 | >32 | 16 | ≤0.5 |
| 2026 | >64 | >64 | >64 | >64 | >64 | >32 | >32 | >16 | >32 | 16 | ≤0.5 |
| 2027 | >64 | >64 | >64 | >64 | >64 | >32 | >32 | >16 | >32 | 16 | ≤0.5 |

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**Notes:**

- **PEN:** penicillin; **CFZ:** cefazolin; **CTM:** cefotiam; **CMZ:** cefmetazole; **FEP:** cefepime; **IPM:** imipenem; **AMK:** amikacin; **CLI:** clindamycin; **CIP:** ciprofloxacin; **MIN:** minocycline; **TEC:** teicoplanin; **VAN:** vancomycin.
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 *Corynebacterium 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 shown for nitrate reduction, pyrazinamidase, esculin, urease, gelatin, glucose, ribose, xylose, mannitol, 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 glyceral, erythritol, D-arabinose, L-arabinose, D-xyllose, L-xyllose, adonitol, β-methylhexoside, 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, lactose, melibiose, sucrose, inulin, melezitose, D-raffinose, starch, glycerol, xylitol, β-gentiobiose, D-turanose, D-lyxose, D-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%).

**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 non-lipophilic 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.

**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 non-lipophilic 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
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 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-sporulating, 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-sorbos were positive.

All strains were negative for glycerol, erythritol, d-arabinose, l-arabinose, d-xyllose, L-xylose, adonitol, β-methyl-D-xylloside, galactose, d-fructose, d-mannose, rhamnose, dulcitol, inositol, sorbitol, α-methyl-d-mannoside, α-methyl-D-glucoside, N-acetylglicosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, inulin, melizitose, d-rafarnose, starch, glycogen, xyitol, β-gentiobiose, d-turanose, d-lyxose, d-fucose, L-fucose, l-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 ν9 (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 5093 T).

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