Classification of Coryneform Bacteria Associated with Human Urinary Tract Infection (Group D2) as *Corynebacterium urealyticum* sp. nov.

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Urealytic strains of coryneform bacteria that are designated *Corynebacterium* group D2 and are isolated from human urine are a cause of urinary tract infections. Cell wall and lipid analyses confirmed that these organisms are members of the genus *Corynebacterium* but can be separated from other species in the genus on the basis of DNA base composition and DNA-DNA hybridization values. Biochemically, strains in this taxon can be distinguished from other *Corynebacterium* spp. by their failure to produce acid from carbohydrates, by their failure to reduce nitrates, and by their ability to hydrolyze urea. We regard these bacteria as a new species of the genus *Corynebacterium* and propose the name *Corynebacterium urealyticum*. The type strain is strain NCTC 12011 (= ATCC 43042).

The identification system for medically important coryneform bacteria devised by the Centers for Disease Control, Atlanta, Ga., designates one group of urealytic, nonfermentative bacteria as *Corynebacterium* group D2 and suggests that these organisms may be nitrate-negative variants of *Corynebacterium pseudodiphtheriticum* (9). This group has tentatively been referred to as *Corynebacterium urealyticum* (23), but this name has not been validly published previously. Strains identified as *C. urealyticum* have been associated with cases of encrusted cystitis and other urinary tract infections in hospital patients (20), where they have been highly resistant to antibiotics (22). The natural habitat of these strains is human skin (24), but they readily colonize urinary tracts (22); strains have also been isolated from blood (14, 17) and wounds (28). *C. urealyticum* is relatively inactive in conventional tests apart from its ability to hydrolyze urea rapidly. Colonially and microscopically, this species resembles *Corynebacterium jeikeium*, with which it shares a common habitat and resistance to antibiotics (4). However, the latter species is consistently nonurealytic and acidifies glucose and maltose. Evidence from lipid analysis (7, 21), from pyrolysis-mass spectrometry (8), and from rRNA gene restriction fragment polymorphism studies (26) indicates that there are clear differences between the two species. Chemotaxonomic evidence that group D2 is related to the genus *Corynebacterium* has been provided by Herrera-Alcaez et al. (7), who determined that the cell wall type is chemotype IV, with mycolic acids having chain lengths of 26 to 36 carbon atoms, and that the isoprenoid quinone MK-9(H₂) is present.

In this study, we estimated the DNA base compositions of *Corynebacterium* strains and levels of DNA-DNA relatedness between strains of *C. urealyticum* and other *Corynebacterium* species; we concluded that this taxon merits status as a new species in the genus *Corynebacterium*.

MATERIALS AND METHODS

Bacterial strains and culture. The strains used in this study are listed in Table 1. Strains identified as *C. urealyticum* were isolated in Madrid, Spain, from the urine (strains A516/89, A524/90, A532/90, A548/90, and NCTC 12011^T [T = type strain]) and skin (strain A545/90) of hospital patients. Cultures were maintained in the freeze-dried state, plated onto 5% horse blood nutrient agar, and incubated at 37°C for 48 h. To prepare DNA, strains were subcultured in 100 ml of brain heart infusion broth (Oxoid, London, United Kingdom) supplemented with 0.4% yeast extract and 0.2% (vol/vol) Tween 80 and incubated aerobically with shaking at 37°C for 48 h.

Preparation of DNA. Cells (0.5 to 1.0 g) were pelleted by centrifugation, washed once with TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0), and incubated in 0.5 ml of a lysozyme solution (50 mg · ml⁻¹ in TE buffer) at 37°C for 1 h. DNA was extracted as described previously (19), except that five times the stated volumes of reagents were used. The DNA was further purified by incubating it in RNase-SET (20 µg of RNase A per ml in 150 mM NaCl–15 mM EDTA–60 mM Tris-HCl [pH 8.3]) at 37°C for 1 h. Proteinase K was added to a final concentration of 50 µg · ml⁻¹, and incubation was continued for 1 h. The solution was mixed with an equal volume of TE buffer-saturated phenol-chloroform (1:1, vol/vol) and centrifuged at 20,000 × g for 30 min; this was followed by extractions with equal volumes of chloroform until all of the visible precipitate was removed.

Purified DNA was precipitated with 1 volume of 2-propanol, washed three times with 70% ethanol, and dried under a vacuum for 10 min.

DNA base composition. Purified DNA samples were dissolved in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate, pH 7.0), and the A₂₈₀, A₂₆₀, and A₂₃₀ were determined to estimate concentration and purity. The concentration was adjusted to approximately 50 µg · ml⁻¹ with 0.1× SSC, and the guanine-plus-cytosine (G+C) content was determined by using the thermal denaturation method (16). A DNA sample from *Escherichia coli* NCTC 9001 was used as a standard (52 mol% G+C).

DNA-DNA hybridizations. Nitrocellulose-bound DNA was

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TABLE 1. DNA base compositions and levels of DNA relatedness between strains of *C. urealyticum* and other *Corynebacterium* species

Source of unlabeled DNA		G+C content (mol%) ^a	Relative % of binding with ³ H-labeled DNA from:			
Species or subspecies	Strain		Strain NCTC 12011 ^T	Strain A516/89	Strain A545/90	Strain A548/90
<i>C. urealyticum</i>	NCTC 12011 ^T (= ATCC 43042 ^T)	62	100			
	A516/89	62	84	100		
	A545/90	62	88	76	100	
	A548/90	62	81	78	82	100
	A532/90	62	88	70	100	93
	A524/90	61	71	84	96	89
<i>C. bovis</i>	NCTC 3224 ^T (= ATCC 7715 ^T)	70	4	8	3	6
<i>C. cystiditis</i>	NCTC 11863 ^T (= ATCC 29593 ^T)	70	3	1	1	4
<i>C. diphtheriae</i>	NCTC 11397 ^T (= ATCC 27010 ^T)	54	2	2	1	3
<i>C. flavesces</i>	NCIMB 8707 ^T (= ATCC 10340 ^T)	59	2	2	1	5
<i>C. jeikeium</i>	NCTC 11913 ^T (= ATCC 43734 ^T)	60	22	8	8	9
<i>C. matruchotii</i>	NCTC 10254 ^T (= ATCC 14266 ^T)	56	3	2	1	5
<i>C. minutissimum</i>	NCTC 10288 ^T (= ATCC 23348 ^T)	59	3	3	2	6
<i>C. mycetoides</i>	NCTC 9864 ^T	65	5	4	4	5
<i>C. pilosum</i>	NCTC 11862 ^T (= ATCC 29592 ^T)	61	19	2	4	10
<i>C. pseudodiphtheriticum</i>	NCTC 11136 ^T (= ATCC 10700 ^T)	55	2	3	1	5
<i>C. pseudotuberculosis</i>	NCTC 3450 ^T (= ATCC 19410 ^T)	52	1	1	1	2
<i>C. renale</i>	NCTC 7448 ^T (= ATCC 19412 ^T)	58	19	3	4	8
<i>C. striatum</i>	NCTC 764 ^T (= ATCC 6940 ^T)	58	6	3	1	4
<i>C. variabilis</i>	NCIMB 9455 ^T (= ATCC 15753 ^T)	65	2	3	1	3
<i>C. xerosis</i>	NCTC 11861 ^T (= ATCC 373 ^T)	68	6	4	2	4
<i>Alcaligenes xylosoxidans</i> subsp. <i>denitrificans</i>	NCTC 10807 (= ATCC 27061)	69	1	1	0	4

^a Data from this study and reference 10.

prepared, and the direct membrane hybridization procedure was performed as described by Johnson (11). Radiolabeled DNA was prepared by random primer extension, using a kit (Boehringer Mannheim Ltd., Lewes, United Kingdom) that incorporated deoxy [1',2',5-³H]CTP (Amersham International PLC, Aylesbury, United Kingdom). According to the manufacturer's instructions, 25 ng of DNA was labeled for every 10 hybridization reactions. Labeled DNA was purified on a Sephadex G-50 spun column (15) and sonicated for 2 min, and volumes containing 20,000 cpm were added to each membrane filter (diameter, 6 mm). Hybridization was carried out in 5× SSC–50% formamide at the optimum reassociation temperature (45°C) in a shaking water bath for 20 h. Filters were washed three times in 2× SSC at the stringent temperature (84°C) with agitation. The filters were placed in scintillation vials containing 2 ml of Ready Protein+ scintillation cocktail (Beckman Instruments Ltd., High Wycombe, United Kingdom) and dissolved overnight, and radioactivity was measured with a Tri-Carb 4000 liquid scintillation counter (United Technologies, Packard, Pangbourne, United Kingdom). DNA from *Alcaligenes xylosoxidans* subsp. *denitrificans* NCTC 10807 was used as a nonhomologous control (G+C content, 69 mol%) (13).

RESULTS AND DISCUSSION

The DNA base compositions of the six strains of *C. urealyticum* which we used ranged from 61 to 62 mol% G+C. A comparison with the type strains of 15 *Corynebacterium* species (Table 1) showed that *Corynebacterium flavesces* NCIMB 8707, *C. jeikeium* NCTC 11913, *Corynebacterium minutissimum* NCTC 10288, *Corynebacterium pilosum* NCTC 11862, *Corynebacterium renale* NCTC 7448, *Corynebacterium striatum* NCTC 764, and *Corynebacterium variabilis* NCIMB 9455 all had DNA base composition

values within 5% of this range, which is consistent with the range for strains within a species (3, 12). However, the results of DNA pairing experiments (Table 1) showed that none of the type strains of the *Corynebacterium* species tested, including the type species, *Corynebacterium diphtheriae*, exhibited close DNA relatedness with *C. urealyticum*. The levels of interstrain homology among the six strains of this species (>60%) are consistent with the criterion for a homogeneous species (12), and the particularly low values obtained in the comparisons of *C. urealyticum* DNA with DNAs of other species (<23%) suggest that the proposed species has no close relatives among the other species which we examined. In addition, the low DNA base composition of *C. urealyticum*, together with the presence of mycolic acids (C₂₆ to C₃₆) that yield low-molecular-weight pyrolysis products (C₈ to C₁₂ methyl esters), excludes this organism from the genera *Rhodococcus* and *Gordona*.

Our data confirmed previous findings which indicated that the taxon designated *Corynebacterium* group D2 by the Centers for Disease Control (9) and referred to as *C. urealyticum* (23) could represent a single species on the basis of evidence from phenotypic tests (including API profiles) (5, 27), pyrolysis-mass spectrometry (8), fatty acid, isoprenoid quinone, and phospholipid analyses (7, 21), and rRNA gene profiling (26). The presence of a *meso*-diaminopimelic acid-based peptidoglycan, the presence of a cell wall arabinogalactan, and the presence of short-chain mycolic acids are characteristics which *C. urealyticum* shares with the type species of the genus *Corynebacterium*, *C. diphtheriae*, indicating that the new species should be placed in this genus (2).

C. urealyticum contains tuberculostearic acid (10-methyl-octadecanoic acid) (21), a lipid that is associated with the genera *Rhodococcus*, *Gordona*, *Nocardia*, *Mycobacterium*, and *Tsukamurella* ("aurantiaca" taxon) (6). However, the presence of tuberculostearic acid has been detected in other

TABLE 2. Some characteristics that are useful for differentiating *C. urealyticum* from other commensal *Corynebacterium* species found on human skin

Species	Aerobic acid production from ^a :			Nitrate ^a reduction	Urease ^a activity	Isoprenoid quinone(s) ^b
	Glucose	Maltose	Sucrose			
<i>C. urealyticum</i>	— ^c	—	—	—	+	MK-9(H ₂)
<i>C. jeikeium</i>	+	v	—	—	—	ND ^d
<i>C. bovis</i>	+	—	—	—	—	MK-9(H ₂)
<i>C. minutissimum</i>	+	+	+	—	—	MK-8(H ₂) + MK-9(H ₂)
<i>C. pseudodiphtheriticum</i>	—	—	—	+	+	MK-8(H ₂)
<i>C. striatum</i>	+	—	+	+	—	MK-8(H ₂)
<i>C. xerosis</i>	+	v	v	+	—	MK-8(H ₂)

^a Data from reference 9.^b Data from reference 3.^c +, positive; —, negative; v, variable.^d ND, not determined.

Corynebacterium species, notably *Corynebacterium bovis* (6) and *C. variabilis* (1). Although the presence of tuberculostearic acid in *C. minutissimum* has been reported by Collins (1), this compound was not detected by Herrera-Alcarez et al. (7). The latter authors also found that this fatty acid is present in *Corynebacterium cystitidis* and *C. pseudodiphtheriticum* in small amounts. Another lipid which has been suggested as a taxonomic marker is phosphatidylethanolamine. This compound was found by Minnikin et al. (18) to be absent in the genus *Corynebacterium* but present in the genera *Rhodococcus*, *Gordona*, *Nocardia*, and *Mycobacterium*, although sometimes it was present in trace amounts. However, Herrera-Alcarez et al. (7) have reported the presence of phosphatidylethanolamine in *C. bovis*, *C. cystitidis*, *Corynebacterium ammoniagenes*, and *C. pseudodiphtheriticum*. These findings suggest that the presence of tuberculostearic acid and the presence of phosphatidylethanolamine are unreliable as taxonomic markers and may depend on culture conditions.

The most significant phenotypic characteristic which *C. urealyticum* shares with some other *Corynebacterium* species is the possession of a potent urease activity. This property is considered to be important in the ability of the organism to infect human urinary tracts and to induce the formation of struvite (ammonium magnesium phosphate) stones (22, 23), a property that so far has not been reported for other *Corynebacterium* species. Other urealytic species (*C. renale*, *C. cystitidis*, and *C. pilosum*) which may be urinary tract pathogens in animals differ from *C. urealyticum* in their ability to produce acid from sugars. *C. pseudodiphtheriticum*, a urealytic, nonfermentative commensal of human skin, differs from *C. urealyticum* in being able to reduce nitrate. Most *C. urealyticum* isolates are resistant to antibiotics, but a few (<5%) harbor plasmids (25); strains of this species appear to colonize the skin of hospital patients and are similar in this respect to isolates of *C. jeikeium*, which they resemble morphologically and colonially. In addition, the growth of both species is considerably enhanced by including Tween 80 in the media, and these properties may make the two taxa difficult to distinguish on initial isolation. However, *C. jeikeium* is consistently nonurealytic and produces acid from glucose, and on this basis an identification can be made. Table 2 lists some of the characteristics that distinguish *C. urealyticum* from other commensal *Corynebacterium* species that are commonly encountered on human skin.

We believe that there is sufficient evidence to justify the creation of a new species in the genus *Corynebacterium* to

accommodate strains that were previously designated group D2 strains. The name *Corynebacterium urealyticum* is proposed for this taxon.

Description of *Corynebacterium urealyticum* sp. nov. *Corynebacterium urealyticum* (u.re.a.ly'ti.cum. M.L. fem. n. urea, urea; Gr. adj. lyticus, dissolving; M.L. neut. adj. urealyticum, urea dissolving). Gram-positive cells that are not acid fast and are nonsporing and after prolonged culture may be coccoid. Cells (diameter, 0.5 to 1 µm) are arranged in palisades and V shapes with no tendency to branch. The bacteria grow on blood agar as pinpoint colonies after 48 h of incubation at 25, 37, and 42°C. Colonies are whitish, opaque, smooth, convex, and nonhemolytic. Does not grow on blood agar incubated anaerobically for 48 h or on MacConkey agar. Nonmotile, catalase positive, and oxidase negative. Acid is not produced from glucose, sucrose, maltose, mannitol, xylose, ribose, L-arabinose, sorbitol, lactose, trehalose, inulin, raffinose, starch, and glycogen. Does not hydrolyze gelatin or esculin. Nitrate is not reduced. Does not degrade DNA. Possesses strong urease activity. Hydrolyzes Tween 80. Growth is stimulated by Tween 80. A few strains hydrolyze hippurate or give a positive Voges-Proskauer reaction. The peptidoglycan contains meso-diaminopimelic acid. The cell wall contains arabinose and galactose. The saturated and nonsaturated fatty acids (C₁₄ to C₁₈) consist of tetradecanoic, hexadecanoic, hexadecenoic, octadecanoic, octadecenoic, octadecadienoic, and 10-methyloctadecanoic (tuberculostearic) acids. Mycolic acids C₂₆ to C₃₆ are present. The major menaquinone is MK-9(H₂). Contains diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylinositol mannosides.

The DNA base composition is 61 to 62 mol% G+C.

Source: human urine, skin, and blood.

The type strain is strain NCTC 12011 (= ATCC 43042), which has a G+C content of 62 mol% (as determined by the thermal denaturation method).

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