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Identification of *Corynebacterium amycolatum* and Other Nonlipophilic Fermentative Corynebacteria of Human Origin

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Four identification tests, proposed in addition to conventional methods, were evaluated with 320 fermentative nonlipophilic *Corynebacterium* strains: growth at 20°C, glucose fermentation at 42°C, alkalinization of sodium formate, and acid production from ethylene glycol. These tests were highly discriminant. *Corynebacterium amycolatum* displayed a unique profile, allowing it to be distinguished from similar species, such as *C. xerosis*, *C. striatum*, and *C. minutissimum*.

Nondiphtheric corynebacteria are of growing importance as opportunistic pathogens, especially in nosocomial settings (6). *Corynebacterium amycolatum* is one of the diphtheroids most often isolated from clinical samples (3, 11, 13). Many conventional tests have variable results with this species, leading to confusion with other fermentative corynebacteria, such as *C. striatum* or *C. minutissimum* and, more particularly, *C. xerosis* (3, 13). Moreover, *C. amycolatum* was not included in some commercial identification systems, resulting in no identification or misidentification of the strains (1, 13), although it now appears in the updated API Coryne database 2.0 (5). Recently, several new nonlipophilic fermentative corynebacteria, isolated from humans, have been described: *C. argentoratense* (12), *C. coyleae* (4), and *C. imitans* (2). This may also increase the need to improve the existing identification schemes by additional discriminant characteristics.

In this study, we have evaluated some cultural and biochemical properties not yet applied to the identification of corynebacteria. These tests are easy to perform in the routine laboratory and may contribute to the differentiation of nonlipophilic fermentative corynebacteria.

Bacterial strains. The following 16 reference strains were used: *C. amycolatum* CIP 103452^T; *C. minutissimum* NCTC 10288^T and NCTC 10284; *C. striatum* ATCC 6940^T; *C. xerosis* ATCC 373^T, ATCC 7711, and DSM 43607; *C. glucuronolyticum* DSM 44120^T, DMMZ (Department of Medical Microbiology, Zürich) 845, and DMMZ 987; *C. argentoratense* CIP 104296^T; *C. coyleae* DSM 44184^T and DSM 44185; *C. imitans* DSM 44264^T; and *C. diphtheriae* DSM 44123^T and DSM 43988.

Three hundred four isolates of nonlipophilic fermentative corynebacteria of human origin were included in the study. They were collected from various clinical samples by several laboratories. Strains were identified according to existing identification schemes by the methods described by other authors (6, 7, 12, 13). Production of propionic acid from glucose was detected in glucose broth by gas-liquid chromatography in all strains as previously described (13).

Identification tests proposed in this study. (i) Growth at 20°C. A suspension (±2 McFarland standard) was made from a 24-h blood agar culture. One loopful was evenly streaked onto the surface of half of a blood agar plate and incubated at 20 ± 1°C. Obvious macroscopic growth was recorded after 1, 2, and 3 days.

(ii) Glucose fermentation at 42°C. The same suspension described for growth at 20°C was used for glucose fermentation at 42°C. Two drops were inoculated into 4 ml of phenol red broth base (Difco, Detroit, Mich.) supplemented with 1% (wt/vol) glucose and 0.1% (vol/vol) Tween 80. Tubes were incubated in a water bath at 42°C (±0.1°C) for 3 days. A yellow color shift was recorded as a positive fermentation test.

(iii) Alkalinization of a sodium formate solution. Strains were cultured for 24 h on brain heart agar, heart infusion agar, or brucella agar. No tryptic soy agar or blood-supplemented media should be used. A heavy suspension (4 to 6 McFarland standard) was made in 0.5 ml of distilled water distributed in small tubes, and 0.5 ml of the following solution was added: sodium formate, 5 g; 0.5% cresol red solution, 1 ml; 0.001 M phosphate buffer (pH 6), 100 ml. After overnight incubation at 37°C, positive reactions were characterized by a purple color shift. Negative reactions were yellow. Additional readings were made after 2 and 3 days.

(iv) Acid production from ethylene glycol in a low-peptone-content medium. Acid production was measured with a low-peptone-medium consisting of casitone (Difco) (1 g), ethylene glycol (20 ml), NaCl (5 g), agar (17 g), phenol red (0.025 g), and distilled water (1 liter). No buffer was added, and the pH was adjusted to ±7.2. After melting, the medium was distributed in 5-ml amounts in tubes and distributed to slants after sterilization. The slants were heavily inoculated with bacteria from a 24-h blood agar plate and incubated for 2 days. The medium turned yellow, usually within 24 h, when acid was produced from ethylene glycol.

Genetic studies. Strain K132 was submitted to S. Pradella, Deutsche Sammlung von Mikroorganismen und Zelkulturen (DSMZ), Braunschweig, Germany, for genomic analysis. Approximately 95% of the 16S rRNA gene sequences of this strain were determined by direct sequencing of PCR-amplified 16S ribosomal DNA (rDNA). Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA, and purification of the PCR products were carried out as described previously (9). Purified PCR products were sequenced with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Weiterstadt, Germany) as directed in the manufacturer's protocol. Sequence reaction mixtures were electrophoresed with the Applied Biosystems 373A DNA Se-

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TABLE 1. Results of the identification tests described in this study for 320 reference strains and clinical isolates of nonlipophilic fermentative corynebacteria

Corynebacterium species (*n*)	Positive result for parameter (%)			
	Growth at 20°C (3 days)	Glucose fermentation at 42°C (3 days)	Formate alkalinization (24 h)	Ethylene glycol acidification (48 h)
C. amycolatum (211)	0	100	0	68
C. xerosis (4)	100	0	0	75
C. minutissimum (36)	0	94	100	0
C. striatum (30)	100	97	97	100
C. glucuronolyticum (17)	0	0	100	0
C. diphtheriae (17)	100	0	0	100
C. argentoratense (1)	100	100	0	100
C. coyleae (2)	0	0	0	100
C. imitans (2)	0	100	100	50

quencer. The resulting sequence data from strain K132 were put into the alignment editor ae2 (8), aligned manually, and compared with the 16S rDNA sequence of *C. xerosis* ATCC 373^T (AC X81914).

Results and discussion. Identification by conventional methods and detection of propionic acid production allowed us to assign the 304 clinical strains to the following species: 210 were *C. amycolatum*, 34 were *C. minutissimum*, 29 were *C. striatum*, 15 were *C. diphtheriae*, 14 were *C. glucuronolyticum*, 1 was *C. xerosis*, and 1 was *C. imitans.*

As expected, *C. amycolatum* strains were variable in fermentation tests of sucrose, maltose, and galactose and in nitrate reduction or urease. Three strains exhibited α-glucosidase activity. All of the strains formed dry colonies and produced propionic acid from glucose.

Only one *C. xerosis* strain (K132) was isolated during the study. This species is very rarely encountered in clinical samples, since we have identified only a single strain out of more than 750 corynebacterial isolates collected in our laboratory over several years (unpublished data). Riegel et al. did not find any *C. xerosis* strains among 415 human isolates (11). Therefore, the identification of K132 was confirmed by 16S rRNA gene sequence analysis. Comparison of 1,300 nucleotides gave a 16S rDNA similarity value of 100% and indicated identity between the sequences of strain K132 and *C. xerosis* ATCC 373^T. It should be noted that the K132 strain, unlike the three reference strains of *C. xerosis*, did not exhibit α-glucosidase activity, which can no longer be considered as a distinctive characteristic of this species.

The results obtained by the identification tests proposed in this study are reported in Table 1. As can be seen, most results yielded a 100 or 0% positivity rate, providing a high level of discrimination between species.

Tests based on growth temperature were already included in the *Guide for Identification of Gram Positive Rods* by Hollis and Weaver, but the temperature and the methodology used were different (7). This may explain why Hollis and Weaver more often recorded variable results than those achieved in our study. Organic salts are currently used in assimilation tests with or without alkalinization on a minimal mineral base. Alkalinization of a slightly buffered sodium formate solution by some *Corynebacterium* species was not correlated with assimilation of this substrate on minimal media. Glycols have been used in assimilation or degradation tests, but rarely for acid production, except for propylene glycol in the medium described by Rambach for the detection of *Salmonella* (10). Differentiation between *C. amycolatum* and *C. xerosis* can be achieved by chemotaxonomic procedures, such as mycolic acid analysis and detection of propionic acid production from glucose, but routine identification is difficult and the colonial appearances are very similar. Temperature-dependence tests distinguished, without exception, the four *C. xerosis* strains from all of the *C. amycolatum* strains. *C. amycolatum* is a moderate grower but a strong fermenter, whereas *C. xerosis* is a strong grower but a fairly poor fermenter. Therefore, growth on solid media at the lower temperature of 20°C and fermentation of glucose at the higher temperature of 42°C gave the most discriminant results.

Alkalinization of formate was strongly positive in *C. minutissimum* and in the two strains of *C. imitans*. The reaction was also positive, although sometimes weaker, in *C. striatum* and *C. glucuronolyticum. C. amycolatum* mainly differs from *C. minutissimum* and *C. striatum* by the absence of tyrosine decomposition and Tween esterase, but alkalinization of formate also clearly separates the two latter species from *C. amycolatum.*

Ethylene glycol acidification was variable and therefore of limited value in *C. amycolatum*. However, the results were uniformly positive and negative in *C. striatum* and *C. minutissimum*, respectively. Both ethylene glycol acidification and galactose fermentation are as reliable as maltose fermentation for differentiating the two species (13).

The new API Coryne database allows identification of *C. amycolatum*, but extra tests are required. Moreover, *C. xerosis* was removed from the database because of its rarity (5).

Considering the tests proposed in this study, *C. amycolatum* has a unique and highly discriminant profile not found in any other species included in this study. The versatility of *C. amycolatum* and the multiplicity of fermentative *Corynebacterium* species require a revision of the current routine identification schemes. In this respect, the tests proposed in this study may complete existing schemes, or they may be used when the results obtained are questionable.

Nucleotide sequence accession number. The nucleotide sequence of strain K132 has been assigned GenBank accession no. AF022653.

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