JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1998, p. 3698–3702 Vol. 36, No. 12
0095-1137/98/$04.00+0

Differentiation of *Corynebacterium amycolatum*, *C. minutissimum*, and *C. striatum* by Carbon Substrate Assimilation Tests

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Received 21 May 1998/Returned for modification 2 July 1998/Accepted 10 September 1998

We tested the carbon substrate assimilation patterns of 40 *Corynebacterium amycolatum* strains, 19 *C. minutissimum* strains, 50 *C. striatum* strains, and 1 *C. xerosis* strain with the Biotype 100 system (bioMérieux, Marcy-l'Étoile, France). Twelve carbon substrates of 99 allowed discrimination among the species tested. Additionally, assimilation of 3 of these 12 carbon substrates (maltose, *N*-acetyl-D-glucosamine, and phenylacetate) was tested with the API 20 NE identification system (bioMérieux). Since concordant results were observed with the two systems for these three carbon substrates, either identification system can be used as a supplementary tool to achieve phenotypic differential identification of *C. amycolatum*, *C. minutissimum*, and *C. striatum* in the clinical microbiology laboratory.

Recent progress in molecular taxonomy (DNA-DNA hybridization and 16S rRNA sequencing) and in chemotaxonomy has profoundly modified the classification of coryneform bacteria. Since 1987, 24 former CDC groups have been assigned a new genus and/or species name (8). *Corynebacterium amycolatum*, *C. minutissimum*, and *C. striatum* are frequently encountered in the routine clinical microbiology laboratory (11, 15). Their normal habitat is the human skin and mucous membranes, and they are therefore sometimes isolated as contaminants in clinical samples. However, they have also been reported to be responsible for various types of infection such as pneumonia, endocarditis, and septicemia, especially in immunocompromised patients (8, 11). Consequently, they should not always be considered contaminants and should be identified to the species level. In published case reports, *C. amycolatum* (13) and *C. striatum* (3, 10, 12, 14, 16, 20) were all found to be responsible for infection. However, differential identification of these three species by biochemical tests remains difficult, and several misidentifications have been reported previously (7, 8, 21, 23). Furthermore, interpretation of the clinical importance of these species is still difficult. These species have been easily differentiated by methods that cannot easily be used in the routine laboratory, such as chromatography of mycolic acids (2), determination of propionic acid and lactic acid production by gas-liquid chromatography (5, 21), amplified ribosomal DNA restriction analysis (18), and amplification of the 16S-23S gene spacer regions (1). Identification schemes which simplify correct identification in the routine laboratory have recently been reported (8, 15, 19). Additionally, four new tests which allow convenient differentiation of *C. amycolatum*, *C. minutissimum*, and *C. striatum* have recently been established by Wauters et al. (22).

We report here on a study of carbon substrate assimilation by 110 strains belonging to these three *Corynebacterium* species and conclude with a simple scheme allowing identification of *C. amycolatum*, *C. minutissimum*, and *C. striatum* in the routine microbiology laboratory.

Bacterial strains. We tested 110 *Corynebacterium* strains isolated from various clinical samples from nonduplicate patients. They were obtained from the bacterial collections of the Département d'Étude et de Recherche en Bactériologie Médicale (Lyon, France), IUT A Lyon 1 (Lyon, France), bioMérieux Laboratories (La Balme-les-Grottes, France), and the Microbiology Laboratory, Faculty of Medicine (Strasbourg, France). In preparation for this study, these strains were identified to the species level: *C. amycolatum* (40 strains), *C. minutissimum* (19 strains), *C. striatum* (50 strains), and *C. xerosis* (1 strain) by recently described methods (1, 8, 15, 19).

In brief, as a first step we inoculated API Coryne systems with these strains (bioMérieux, Marcy l'Étoile, France). The interpretations of the results were based on the second-generation database (9). In addition to the API Coryne system, we also determined the strains' capability for growth under anaerobic conditions and in the absence of lipids and tested them for the presence of a tyrosinase by previously described protocols (15). In cases of ambiguity, the identification was confirmed by PCR-based amplification of the 16S-23S gene spacer region recently described by Aubel et al. (1). Identification of *C. amycolatum* strains was confirmed by the absence of mycolic acids according to the method described by Barreau et al. (2). Additionally, we tested the following reference strains: *C. amycolatum* CIP 103452^T (Collection de l'Institut Pasteur, Paris, France), *C. minutissimum* ATCC 23348^T, *C. striatum* ATCC 6940^T, and *C. xerosis* ATCC 373^T (American Type Culture Collection, Manassas, Va.).

Culture conditions. *Corynebacterium* strains were grown at 37°C for 48 h on Columbia agar supplemented with 5% (vol/vol) sheep blood (bioMérieux) in an atmosphere containing 10% CO_2.

Carbon substrate assimilation tests. (i) Biotype 100 system (bioMérieux). The system is composed of 99 test wells, each one containing a single dehydrated carbohydrate, organic acid,

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or amino acid, plus one control well without carbon substrate. As a minimal growth medium, we used Biotype Medium 2 (bioMérieux), which contains 31 growth factors and is therefore adapted to fastidious microorganisms. A bacterial suspension was prepared in 5 ml of distilled water and adjusted to the density of a 6.0 McFarland standard. Two milliliters of this suspension was transferred in 60 ml of Biotype Medium 2, and this final suspension was used to inoculate the system's wells. The inoculated system was incubated at 30°C in a humid chamber. Growth was indicated by a higher turbidity observed in the test well than in the control well. In contrast to what is recommended for members of the family *Enterobacteriaceae* by the manufacturer, only growth (and not color development) was recorded in test wells 19 (esculin), 39 (hydroxyquinoline β-glucuronide), 59 (L-tryptophan), and 79 (L-histidine). Test results for the 99 wells were recorded with the Recognizer software package (P. A. D. Grimont, Taxolab, Institut Pasteur). The interstrain distances were calculated by using the complement of the Jaccard coefficient, which is not able to score double-negative characteristics. Clusters were formed with the unweighted pair group method with average (Taxotron package; Taxolab).

(ii) API 20 NE system (bioMérieux). This system is commercialized for the identification of gram-negative bacilli. To adjust it to coryneform bacteria, we modified the manufacturer's inoculation protocol by using a bacterial suspension adjusted to the density of a 6.0 McFarland standard. Ten drops of this suspension was then transferred in the AUX medium provided with the system, and this final suspension was used to inoculate the system's auxanogram wells. The inoculated system was incubated at 30°C, and growth in the maltose, *N*-acetyl-D-glucosamine, and phenylacetate wells was observed after 2 and 4 days.

Results and discussion. Use of the API Coryne system presents several problems, including difficulties in reading some enzymatic reactions, absence of *C. amycolatum* from the database, and often the need for supplementary tests to identify the two other species (6). The new API Coryne system database includes *C. amycolatum*, and *C. xerosis* is no longer included in this database (9). *C. amycolatum*, *C. minutissimum*, and *C. striatum* give the same code: (2-3)100(1-3)(0-2)(4-5). Certain *C. striatum* strains give a code such as 3100115; furthermore, a few *C. amycolatum* strains are urease positive (4).

The results obtained with the Biotype 100 system are reported in Table 1. Among the 99 carbon substrates tested, only 32 gave more than 20% positive results for at least one of the species tested (Table 1). *C. amycolatum*, *C. minutissimum*, and *C. striatum* used 13, 28, and 26 different substrates as sole carbon source, respectively. In general, we find that the metabolic activity of *C. amycolatum* is much lower than that of the two other species. The more discriminating carbon substrates were D-galactose, maltotriose, maltose, *N*-acetyl-D-glucosamine, phenylacetate, 4-aminobutyrate, 5-aminovalerate, L-glutamate, D-alanine, L-alanine, L-serine, and L-tyrosine.

In our study, the dendrogram performed on the results of 99 carbon substrate assimilation tests clearly showed three clusters representing the three species: *C. amycolatum*, *C. minutissimum*, and *C. striatum* (Fig. 1). However, the reference strain for *C. xerosis*, ATCC 373^T, was included in the *C. amycolatum* cluster, which we do not consider a major problem given the extremely rare occurrence of *C. xerosis* in clinical samples. *C. xerosis* has been found only once in 750 isolates as reported by Wauters et al. (22) and was completely absent in all of the 415 human isolates described by Riegel et al. (15). Table 1 clearly shows that we can distinguish all three *Coryne-*

TABLE 1. Percentages of positive carbon substrate assimilation reactions for *C. amycolatum*, *C. minutissimum*, and *C. striatum*[a]

Test	*C. amycolatum* (*n* = 40)	*C. minutissimum* (*n* = 19)	*C. striatum* (*n* = 50)
D-Glucose	96	100	98
D-Fructose	36	100	96
D-Galactose[c]	18	0	92
D-Trehalose	11	66	0
D-Mannose	25	100	98
Sucrose	50	89	92
Maltotriose[c]	78	95	0
Maltose[c]	66 (82)[b]	94 (89)	0 (0)
Gentiobiose	0	22	0
1-*O*-Methyl-β-D-glucoside	0	22	0
D-Ribose	10	55	64
Glycerol	73	100	98
L-Malate	58	100	100
N-Acetyl-D-glucosamine[c]	3 (0)	94 (89)	2 (0)
D-Gluconate	3	83	66
Phenylacetate[c]	3 (0)	78 (74)	90 (88)
Putrescine	3	45	66
4-Aminobutyrate[c]	0	100	100
DL-Lactate	83	100	100
L-Histidine	58	94	96
Succinate	95	100	100
Fumarate	78	100	100
5-Aminovalerate[c]	0	84	80
L-Aspartate	23	95	100
L-Glutamate[c]	10	100	94
L-Proline	0	95	46
D-Alanine[c]	0	6	90
L-Alanine[c]	0	6	84
L-Serine[c]	0	100	98
Propionate	6	77	84
L-Tyrosine[c]	0	100	92
2-Ketoglutarate	0	6	76

[a] Only for the carbon substrates that showed more than 20% positive reactions for at least one of the three species (Biotype 100).
[b] Values in parentheses are those for the API 20 NE test.
[c] Discriminatory substrate.

bacterium species by using the Biotype 100 system under the above-mentioned conditions.

To facilitate the use of carbon substrate assimilation tests in the routine clinical microbiology laboratory, we selected the most discriminating tests, i.e., maltose, *N*-acetyl-D-glucosamine, and phenylacetate, among those present in the API 20 NE system, a commonly used carbon substrate assimilation system. Since the system is commercialized for use with gram-negative bacilli, we modified the manufacturer's protocol to adapt the system to coryneform bacteria and used a much denser bacterial suspension as an inoculum. Under these conditions, we observed a good correlation between the assimilation results observed with the two systems, and the use of only these three tests allowed a simple and reliable differential identification of the three species (Table 1). However, there exists an ambiguity in the maltose assimilation of *C. amycolatum* (66% positive scores by the Biotype 100 system compared to 82% by the API 20 NE system). This difference could be explained by the relatively higher inoculum used for the API 20 NE system (0.5 ml of a 6.0-McFarland standard suspension in 7 ml of minimal medium) than for the Biotype 100 system (2 ml of the same suspension in 60 ml of minimal medium). However, the different inocula are expected to play only a minor role since no difference between the systems could be detected for the assimilation of other substrates. The difference in maltose assimilation may therefore reside rather in different substrate con-

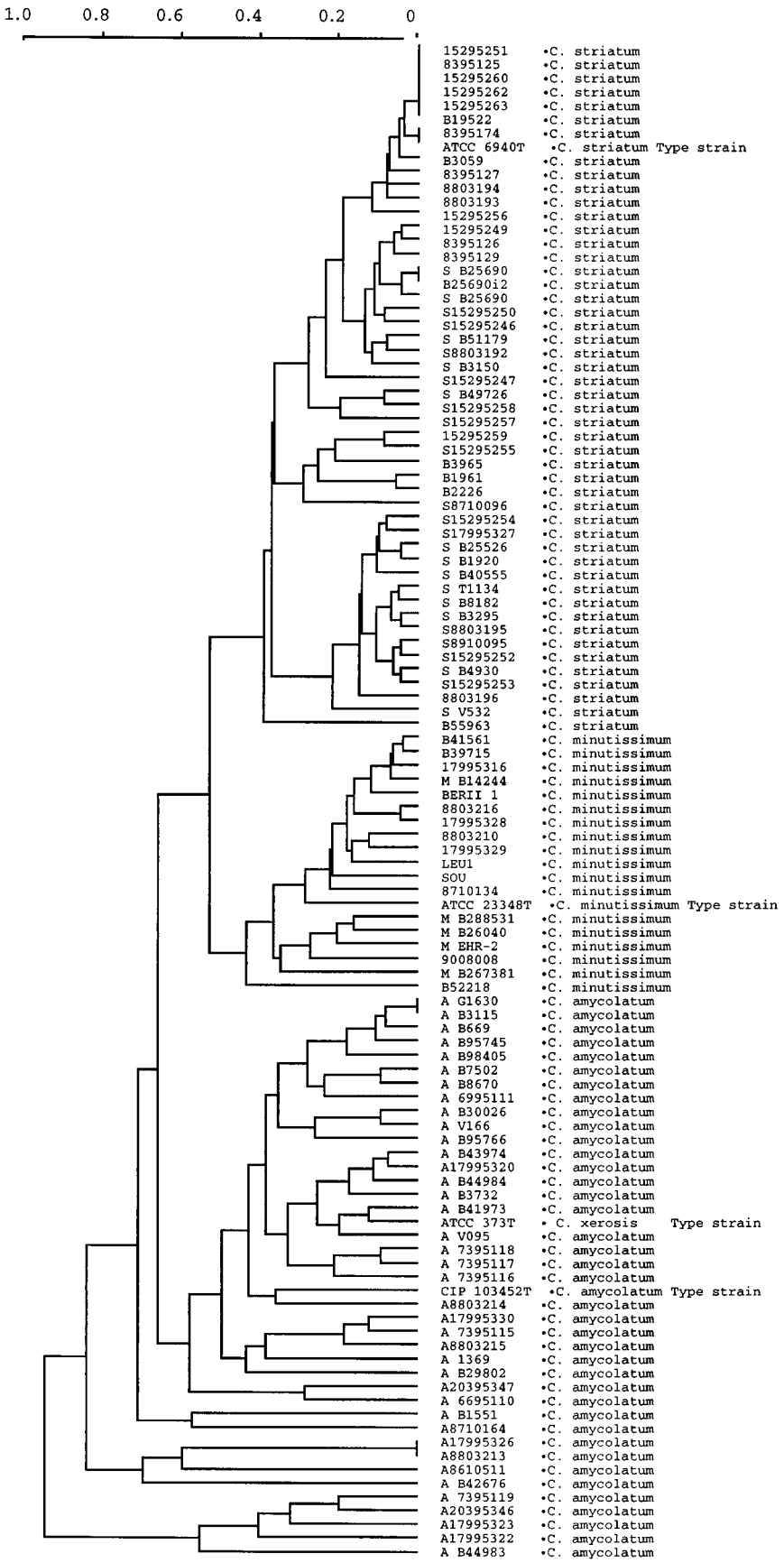

FIG. 1. Dendrogram of hierarchical aggregation clustering of 110 *Corynebacterium* strains belonging to the species *C. amycolatum*, *C. minutissimum*, *C. striatum*, and *C. xerosis* (99 substrates of Biotype 100).

TABLE 2. Differential phenotypic characteristics of *C. amycolatum*, *C. minutissimum*, and *C. striatum* (8, 11, 12, 15, 19)

Sp.	Value for characteristic[a]										
	Nitrate reductase	Acid from maltose	Acid from sucrose	Tyrosine hydrolysis	DNase	Ampicillin	MAL[b]	NAG[b]	PHE[b]	Colony phenotype	Other
C. amycolatum	V	V	V	−	−	R or S	82	0	0	Dry, irregular margin	O/129, [d]mostly R
C. minutissimum	−	+	V	+	+	S	89	89	74	Large, moist, convex	O/129, S
C. striatum	+	−	+	+	−	S[c]	0	0	88	Small at 24 h, large at 72 h, moist	

[a] −, negative; +, positive; V, variable; R, resistant; S, sensitive; MAL, maltose; NAG, *N*-acetyl-D-glucosamine; PHE, phenylacetate.
[b] Percent positive assimilation reactions by API 20 NE system.
[c] Seven percent of strains are resistant.
[d] Vibriocidal compound.

centrations found in the test chambers of the two diagnostic systems.

Since they are all irregularly shaped gram-positive coryneform bacteria, it is very difficult to differentiate these species by their microscopic characteristics. However, the aspect, shape, size, and color of the colonies provide the clinical microbiologist with useful identification characteristics. After 24 h of incubation, *C. amycolatum* produces characteristic dry colonies with an irregular margin and a diameter of 0.5 mm. The colonies have a diameter of 1 to 1.5 mm after 48 h of incubation and 2 mm after 72 h. *C. minutissimum* colonies are smooth, convex, and shiny, and their diameter varies from 1 to 1.5 mm after 24 h to 2.5 to 3 mm after 72 h of incubation. *C. striatum* colonies are round, regular, and smooth (somewhat like coagulase-negative staphylococci) after 24 h and measure between 2 and 3 mm after 72 h of incubation. The colony morphologies and sizes of all three species are identical when they are grown on blood-supplemented Trypticase soy agar or on Columbia agar, except for *C. amycolatum*, the colony size of which appears slightly smaller on Trypticase soy agar (about 0.5 mm).

Resistance to antibiotics, in particular ampicillin, could represent a further diagnostic feature: *C. amycolatum* is relatively resistant to antibiotics, with one strain of two being resistant to ampicillin (8, 15, 17). This represents a different characteristic from *C. striatum* and *C. minutissimum*, most strains of which are susceptible to this antibiotic.

In summary, routine identification of catalase-positive, nonlipophilic, coryneform gram-positive bacilli with the code (2-3)100(1-3)(0-2)(4-5) when the API Coryne system is used can be performed according to the following scheme. (i) If the colony is rather dry with an irregular margin, it is *C. amycolatum*. Confirmation will be obtained by resistance to ampicillin (one of two strains) and phenylacetate and *N*-acetyl-D-glucosamine assimilations as tested in the API 20 NE system, which will remain negative despite positive maltose assimilation. (ii) If the colony is moist, convex, and large after 72 h of incubation and is nitrate reductase negative and maltose positive, it is *C. minutissimum*. Confirmation is obtained by assimilation of the three substrates in the API 20 NE system and susceptibility to ampicillin. (iii) If the colony is moist, with a diameter not larger than 1 mm after 48 h of incubation but reaching 2.5 to 3 mm after 72 h, and is nitrate reductase positive and maltose negative, it is *C. striatum*. Confirmation will be obtained by assimilation of phenylacetate and absence of assimilation of maltose and *N*-acetyl-D-glucosamine in the API 20 NE system. Apart from a few exceptions, the strain is sensitive to ampicillin. The main characteristics allowing differentiation among these three species are summarized in Table 2.

We are grateful to D. Monnet and M. Fussenegger for translation of and critical comments on the manuscript.

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