Taxonomic Study of Corynebacterium Group ANF-1 Strains: Proposal of Corynebacterium afermentans sp. nov. Containing the Subspecies C. afermentans subsp. afermentans subsp. nov. and C. afermentans subsp. lipophilum subsp. nov.

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We have determined the cell wall composition, guanine-plus-cytosine (G+C) contents of the DNA, rRNA gene restriction patterns, and the levels of DNA-DNA relatedness of 11 strains identified biochemically as Centers for Disease Control (CDC) Corynebacterium group absolute nonfermenter 1 (Corynebacterium group ANF-1). For seven of these strains, growth is abundant on 5% sheep blood agar, which differentiates them from the other four strains, whose growth requires a lipid supplement such as Tween 80. Two of the lipid-requiring strains produced mucoid colonies on 1% Tween 80-supplemented sheep blood agar. All strains possess cell wall component type IV, short-chain mycolic acids, and G+C contents of DNA of 66 to 68 mol% as determined by reverse-phase high-performance liquid chromatography. DNA-relatedness experiments by an S1 nuclease procedure showed that nine of these strains, including two of the lipid-requiring strains, constitute a new genomic species less than 40% related to Corynebacterium species and other coryneform groups. The lipid-requiring strain T18502 exhibited 98% DNA relatedness with another lipid-requiring strain, T88593 (difference in thermal denaturation midpoint \( \Delta T_m \) range of from 2 to 5°C). Conversely, the DNA relatedness between strain LCDC 88199 and the six other nonlipophilic strains ranged from 86 to 100% (\( \Delta T_m \) range of from 1 to 3°C) and was only 73 and 76% with the lipophilic strains T18502 and T88593, respectively (\( \Delta T_m \) 3 and 4°C). These results indicated that these two cultural types of bacteria constitute two subspecies within the new genomic species. These subspecies can be identified within the genus Corynebacterium by their phenotypic characteristics and rRNA gene restriction patterns by 

PvuII and EcoRI endonuclease digestion. The two mucoid strains were not related to other Corynebacterium group ANF-1 strains or Corynebacterium species reference strains further studies should allow the determination of the taxonomic status of these mucoid strains. Therefore, we propose a new species, Corynebacterium afermentans sp. nov., which contains two subspecies: C. afermentans subsp. afermentans subsp. nov. (type strain, LCDC 88199 = CIP 103499) for nonlipophilic Corynebacterium group ANF-1 strains and C. afermentans subsp. lipophilum subsp. nov. (type strain, T18502 = CIP 103500) for two lipid-requiring Corynebacterium group ANF-1 strains.

Corynebacterium group absolute nonfermenter 1 (ANF-1) is a bacterial taxon first described in 1981 at the Centers for Disease Control (CDC) (Atlanta, Ga.) in a guide to the identification of medically important gram-positives organisms (12). This unnamed taxon consists of pleomorphic gram-positive rods which do not produce acid from any sugars and do not possess urease activity. Corynebacterium group ANF-1 differs from Corynebacterium group ANF-3 in its inability to reduce nitrate to nitrite.

Although their natural habitat is not well known, Corynebacterium group ANF-1 strains have been recovered from human clinical specimens, particularly from blood samples and ears (12), but no clinical infections were reported for this taxon in a review of the clinical aspects of coryneform bacteria (5).

The taxonomic status of Corynebacterium group ANF-1 is not clearly established. The presence of straight-chain or unsaturated cellular fatty acids (2) and the presence of short-chain mycolic acids in some strains (7) were reported to be common features for this and other Corynebacterium species, but no complete chemotaxonomic study and, to date, no genomic studies have been performed. Therefore, the purpose of this study was to characterize Corynebacterium group ANF-1 strains by cell wall composition, G+C contents of DNA, ribotyping, and DNA-DNA hybridization.

We studied 11 strains which have been identified according to the biochemical classification of the CDC as belonging to Corynebacterium group ANF-1. We compared biochemical properties and determined the genetic relationship of these Corynebacterium group ANF-1 strains with medically relevant Corynebacterium species and related organisms. On the basis of DNA-DNA hybridization and ribotyping results, we propose that two types of Corynebacterium group ANF-1 should be recognized as subspecies of a new species in the genus Corynebacterium for which we propose the name Corynebacterium afermentans.

MATERIALS AND METHODS

Bacterial strains. The 11 strains identified as Corynebacterium group ANF-1 and the reference strains or clinical isolates of Corynebacterium species and related organisms studied are listed in Table 1.

Corynebacterium group ANF-1 LCDC 88199 was a gift from K. A. Bernard, Laboratory Centre for Disease Control.

* Corresponding author.
TABLE 1. G+C contents of and levels of DNA relatedness between strains of *C. afermentans* and other *Corynebacterium* species or related organisms

<table>
<thead>
<tr>
<th>Source(s) of DNA</th>
<th>G+C content (mol%)*</th>
<th>% Relative binding at 65°C with the following labeled strain:</th>
<th>LCDC 88199&lt;sup&gt;T&lt;/sup&gt;</th>
<th>T18502&lt;sup&gt;T&lt;/sup&gt;</th>
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<td>100 (0.0)</td>
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<td>86 (2.0)</td>
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<td>T51462</td>
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<td>94 (3.0)</td>
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*G+C contents are data from this study and reference 16.*

<sup>a</sup> Values in parentheses are AT<sub>50</sub> values (in degrees Celsius).

<sup>b</sup> ND, not determined.

(Ottawa, Ontario, Canada). The other 10 *Corynebacterium* group ANF-1 clinical strains had been isolated from blood cultures (strains T53478, T94156, T18953, T8354, T51462, T18502, T88593, T30014, T88284, and T71705). These clinical isolates were from patients hospitalized in Strasbourg (France). The following strains of recognized *Corynebacterium* species were obtained from the Collection of the Institut Pasteur (CIP), the American Type Culture Collection (ATCC), the National Collection of Type Cultures (NCTC), and the Institute of Applied Microbiology (IAM): *C. bovis* NCTC 3324 (ATCC 7715, type strain), *C. cystitidis* CIP 69.40, *C. diphtheriae* CIP A102 (Park-Williams 8), *C. glutamicum* IAM 12435 (ATCC 13022, type strain), *C. jeikeium* CIP 82.51, *C. minutissimum* NCTC 10288 (ATCC 23348, type strain) and ATCC 23346, *C. mycetoides* CIP 55.51 (ATCC 21134, type strain), *C. pilosum* ATCC 29592 (type strain), *C. pseudodiphtheritcicum* CIP 102940, *C. pseudotuberculosis* bv. equi CIP 52.97, *C. renale* CIP 69.37, *C. striatum* CIP 82.51 (ATCC 6940, type strain), *C. urealyticum* ATCC 43042 (type strain), and *C. xerosis* ATCC 373 (type strain). The reference strains which are not the type strains of the named species were verified to possess the same biochemical characteristics as and G+C% contents of DNA similar to those of those reported for the type strains (4, 14). The strains of *Corynebacterium* groups F-1 (G5911), G-1 (F8156), G-2 (G795), and I-1 (F435) were a generous gift from R. E. Weaver, CDC. The *Corynebacterium* group ANF-3 strain B77159 was isolated in our laboratory from a human respiratory tract specimen and identified according to the method of Hollis and Weaver (12).

**Growth conditions and biochemical tests.** Bacteria were grown aerobically at 37°C on 5% sheep blood agar (Trypticase soy agar, Biomerieux, Marcy l’Etoile, France) and on 5% sheep blood agar supplemented with 1% (vol/vol) Tween 80 (polyoxyethylene sorbitan mono-oleate) (Merck, Darmstadt, Germany).

The procedures used for the determination of biochemical characteristics have been described elsewhere (16). The isolates were identified according to the method of Hollis and Weaver (12). Fermentation tests were verified with API Coryne systems (Biomerieux). Enzymatic activities were examined by the API Zym system according to the manufacturer’s instructions (Biomerieux). Assimilation tests were performed by the API 20 NE system. For acetate, propionate, and lactate assimilation tests, a mineral base (15) was supplemented with 2 g of these components per liter. Antimicrobial susceptibilities were determined by the Bauer diffusion method with 5% sheep blood agar and an inoculum of approximately 10<sup>5</sup> CFU/ml (1).

**Cell wall analysis.** Samples for amino acid and sugar determinations were prepared and separated by thin-layer chromatography, and the components were identified as described previously (20). Reverse-phase high-performance liquid chromatography was performed for the determination of mycic acids. Saponification, derivatization conditions, and comparison of mycic acid patterns were carried out by following a previously published procedure (7).

**DNA base composition.** Cultures were grown for 48 h on Mueller-Hinton agar (Oxoid, Basingstoke, England) supplemented with 1% (vol/vol) Tween 80. Cells were harvested and resuspended in 20 ml of 0.2 M sucrose–0.05 M Tris-HCl (pH 8.0). Incubation at 37°C for 2 h in the presence of 2.5 mg of lysozyme per ml was followed by the addition of 10 mg of sodium dodecyl sulfate (SDS) at 55°C for 2 h. DNA was then extracted and purified by the method of Brenner et al. (3). After being purified on cesium chloride gradients, the DNA for G+C content determination was hydrolyzed with DNase I and nuclease P1 (8) and the moles percent G+C was determined by reverse-phase high-performance liquid chromatography of the deoxynucleotides as described previously (16).

**DNA-DNA hybridization.** DNAs from *Corynebacterium*
group ANF-1 strains LCDC 88199T and T18502T were labeled by nick translation with [3H]dCTP (Amersham International, Amersham, United Kingdom) with a commercial kit (Boehringer GmbH, Mannheim, Germany).

DNA-DNA hybridization was carried out at 65°C for 16 h in 0.42 M NaCl by the S1 nuclease-trichloroacetic method (11). The denaturation temperature ($T_m$) at which 50% of the reassociated DNA becomes hydrolyzable by S1 nuclease was determined in 0.2 M NaCl by the method of Croca et al. (6). $T_m$ is the difference between the $T_m$ of a homoduplex (in a homologous reaction) and the $T_m$ of heteroduplexes (in heterologous reactions).

Ribotyping. Ribotyping was performed as described by Grimont and Grimont (9). DNA was digested for 4 h at 37°C with EcoRI or PstI restriction endonuclease. DNA fragments were separated overnight by agarose gel electrophoresis (2 V/cm) in 0.5× TEB (10× TEB is 0.89 M Tris, 0.89 M boric acid, and 25 mM EDTA Na$_2$ [pH 8.3]) and then transferred onto an Immobilon P membrane (Millipore) with 0.5 M NaOH by the method of Southern (19). Prehybridization was performed in 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA [pH 7.5]) (17), 5× Denhardt’s solution (17), and 0.1% (wt/vol) SDS at 50°C for 2 h. Escherichia coli 16S and 23S rRNAs, previously dephosphorylated with calf intestine alkaline phosphatase (Boehringer), were 5' labeled with [γ-32P]ATP by T4 polynucleotide kinase. Hybridization between Corynebacterium DNA and E. coli rRNA was performed overnight at 60°C in the prehybridization solution supplemented with 2.5 × 10⁶ dpm (50 ng) of 5'32P-labeled rRNA per 20 ml and per membrane. After hybridization, the filters were washed twice in 0.1× SSPE–0.1% (wt/vol) SDS at 37°C for 15 min. Hybridized bands were visualized by autoradiography at −70°C with intensifying screens for a maximum of 24 h.

RESULTS

Phenotypic characterization. The 11 strains were identified as belonging to Corynebacterium group ANF-1 by Hollis and Weaver’s identification scheme (12). These strains do not produce acid from any sugars or nitrite from nitrate, and they do not possess urease activity. Four of these strains (T18502T, T88593, T30014, and T71705) showed a lipid requirement, such as Tween 80, which has not been described for this taxon group. Tween 80 is the most commonly used component in studies characterizing lipophilic or lipid-dependent strains. The term “lipid requiring” is appropriate for the four strains of Corynebacterium group ANF-1 with which no visible growth occurs in brain heart infusion broth in 48 h and with which only very small colonies appear after several days on sheep blood agar alone. In contrast, on 1% Tween 80-supplemented sheep blood agar, large colonies are observed in 48 h. Furthermore, we observed that two of these nonfermenter lipid-requiring strains (T30014 and T71705) developed mucoid colonies on the Tween 80-supplemented medium. The nonlipophilic Corynebacterium group ANF-1 strains show widespread growth on either sheep blood agar or 1% Tween 80-supplemented sheep blood agar (Fig. 1).

By a high-performance liquid chromatography method which has been validated as a rapid and reliable method for the identification of coryneform bacteria (7), the esters of mycolic acids from the seven nonlipophilic Corynebacterium group ANF-1 strains and two of the lipid-requiring strains, T18502T and T88593, exhibited characteristic patterns which differentiated these strains from the mucoid Corynebacterium species group ANF-1 strains and the other Corynebacterium species.

Cell wall components and DNA base composition. The cell walls of the 11 strains identified as Corynebacterium group ANF-1 were found to contain cell wall component type IV (meso-diaminopimelic acid, arabinose, and galactose) and mycolic acids of short chain lengths (C₂₆ to C₃₀). These results support the assignment of these strains to the genus Corynebacterium (4). The G+C contents of all strains studied are listed in Table 1. The values for nonlipophilic and Tween-requiring Corynebacterium group ANF-1 strains show a tight cluster (66 to 68 mol%) and are similar to those for C. urealyticum and C. mycoloides. The other Corynebacterium species and related organisms, notably Corynebacterium group ANF-3, showed quite different values.

DNA-DNA hybridization. As shown in Table 1, DNA-DNA hybridization of DNAs from Corynebacterium group ANF-1 strains with labeled DNAs from strains LCDC 88199T and T18502T of the two proposed subspecies, respectively, revealed the division of group ANF-1 into two genomic groups. The DNA relatedness between strain LCDC 88199T and the other six nonlipophilic Corynebacterium group ANF-1 strains ranged from 86 to 100%, with a difference in melting temperature $ΔT_m$ range of from 1 to 3°C (mean = 1.7°C). The DNA relatedness between strain LCDC 88199T and two of the Tween-requiring strains, T18502T and T88593, was 73 and 76%, respectively, and the $ΔT_m$ was 3 and 4°C, respectively. Conversely, strain T18502T exhibited 98% DNA relatedness with another Tween-requiring strain, T88593 ($ΔT_m = 2°C$), and 71 to 77% similarity with typical
strains belonging to Corynebacterium group ANF-1 ($\Delta T_m$ range of from 2 to 5°C; mean = 3.6°C).

Corynebacterium species and mycolic acid-containing coryneform groups were 1 to 24% related to strain LCDC 88199T at 65°C, and Corynebacterium species with G+C contents near 66% were 12 to 40% related to Tween-requiring Corynebacterium group ANF-1 strain T18502T. The mucoid Corynebacterium group ANF-1 strains T30014 and T71705 exhibited 30 to 39% hybridization with DNA from either LCDC 88199T or T18502T. Further studies should allow the determination of the taxonomic status of these mucoid, lipid-requiring strains.

Ribotyping. In order to further characterize the Corynebacterium group ANF-1 strains, we determined the rRNA gene restriction patterns of these strains and those of some medically important Corynebacterium species. After PvuII cleavage of DNA, heterogeneous but closely related patterns were observed for the seven nonlipophilic strains and for the two lipid-requiring strains T18502T and T88593.

The DNA band profiles of these nine strains for this DNA restriction hydrolysis showed the presence of at least three common bands among the four bands of approximately 3.9, 4.9, 5.9, and 6.4 kb, which differentiate between the mucoid Corynebacterium group ANF-1 strain T30014 and the other Corynebacterium species (one or no bands) (Fig. 2). The profiles of the two Tween-requiring strains T18502T and T88593 showed an additional band of 4.6 kb that was not found in the other strains. The two mucoid Corynebacterium group ANF-1 strains T30014 and T71705 were clearly differentiated from the other Corynebacterium group ANF-1 strains by EcoRI endonuclease digestion of total DNAs (Fig. 3).

These results agree with DNA-DNA hybridization values and indicate that these two digestions with restriction enzymes may serve as a discriminatory genomic marker for studying rRNA genes of the Corynebacterium group ANF-1 strains within the genus Corynebacterium.

DISCUSSION

A species is considered to include strains with 70% or greater DNA-DNA relatedness and with 5°C or less $\Delta T_m$ (21). According to this generally accepted criterion defining a genomic species, the seven strains of nonlipophilic Corynebacterium group ANF-1 and the Tween-requiring strains T18502T and T88593 constitute a new genomic species in the genus Corynebacterium for which we propose the name C. afermentans.

Thus, this chemotaxonomic and genomic study has confirmed that Corynebacterium group ANF-1, as described by Hollis and Weaver (12), is a distinct taxon group in the genus Corynebacterium. Moreover, we have found that lipid-requiring strains should be included in this group. In the genus Corynebacterium, a variant intermedius is described for lipophilic strains of C. diphtheriae, and Smith considered some lipid-requiring diphtheroids to be identical to C. xerosis on the basis of biochemical tests (18). Here, we have found that a single genomic group can contain both lipophilic and nonlipophilic strains.

The reassociation values and $\Delta T_m$ values indicate significant sequence divergence in this genomic species between the typical strains and two of the Tween-requiring strains. On the basis of frequency of distribution of divergence values, Grimont found that a $\Delta T_m$ of approximately 3°C corresponds to divergence values occurring among different
subspecies within a species (10). Our results show a 3.6°C 
$\Delta T_m$ with levels of DNA-DNA relatedness of 70 to 80%
between nonlipophilic strains and the Tween-requiring
strains. Thus, we believe that these DNA-DNA hybridization
and $\Delta T_m$ values provide sufficient genomic evidence to
recognize these two closely related genomic groups at the
subspecies level in this new species.

*C. afermentans* can be distinguished from other Coryne-
*bacterium* species by its biochemical characteristics (Table
2). However, some difficulties in identifying coryneform
isolates at the genus level have been reported (5). Thus, cell
component analysis, including mycolic acid determination,
may provide the identification at the genus level for
coryneform strains which do not produce acid from sugars.

On the basis of culture, biochemical, and nucleic acid
characteristics, we propose a new species, *C. afermentans*
sp. nov., which contains two subspecies: *C. afermentans*
subsp. *afermentans* nov., formerly nonlipophilic
*Corynebacterium* group ANF-1 strains, and *C. afermentans*
subsp. *lipophilum* nov., formerly lipid-requiring
*Corynebacterium* group ANF-1 strains.

*C. afermentans* subsp. *afermentans* sp. nov. *C. afermentans*
afermentans (a.fer.men’tans. Gr. pref. a, not; L. part. adj. *fer*
ments: nonfermenting carbohy-
drates).

This description is based on observations of the authors
and descriptions of the literature of *Corynebacterium*
group ANF-1 strains (2, 4, 7, 12, 13).

The bacteria are gram-positive, irregular rods or coccoba-
cilli that sometimes contain metachromatic granules. All are
nonmotile and nonspore forming and are arranged in typical
V-shaped forms or palisades. They are not acid fast, and no
rod-coccus cycle is present. They are catalase positive and
oxidase negative. They exhibit good growth under aerobic
conditions and very slight growth under anaerobic condi-
tions. Nonhemolytic colonies (1 to 2 mm in diameter) are
greyish-white and smooth after 24 h at 37°C on fresh sheep
blood agar. On 1% Tween 80-supplemented medium, bright
colonies (2 mm in diameter) are beige, and Tween 80
esterase activity is detected. Acid is not produced from
D-glucose, glycerogen, lactose, sucrose, ribose, D-xylose, D-
mannose, D-galactose, trehalose, and D-mannitol or on

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<tr>
<td><em>C. diphtheriae</em></td>
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<tr>
<td><em>C. bovis</em></td>
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<td><em>C. jeikeium</em></td>
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<td><em>C. minutissimum</em></td>
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<tr>
<td><em>C. pseudodiphtheriticum</em></td>
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<tr>
<td><em>C. pseudotuberculosis</em></td>
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<td><em>C. renale</em></td>
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<td><em>C. striatum</em></td>
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<tr>
<td><em>C. urealyticum</em></td>
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<td><em>C. xerosis</em></td>
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* Data are from references 4 and 12 and our observations.

a, +, 90% or more positive strains; -, 10% or less positive strains; d, 11 to 89% of positive strains.
are large (2 mm in diameter) and without a mucoid consistency. Bacteria are pleomorphic gram-positive, nonspore-forming rods or coccobacilli that sometimes contain metachromatic granules. They are nonmotile and arranged in palisades and V shapes. They are not acid fast, and no rod-coccus cycle is present. They are catalase positive and oxidase negative. Acid is not produced from D-glucose, glycogen, lactose, sucrose, ribose, D-xylene, D-mannose, D-galactose, trehalose, and D-mannitol or on triple sugar iron. Tyrosine, gelatin, DNA, and urea are not degraded. Esculin is not hydrolyzed. The methyl red test is negative, and acetoin, indole, and H₂S (on triple sugar iron agar) are not produced. Hippurate is not hydrolyzed. Alkaline phosphatase, esterase, lipase, and acid phosphatase are produced. α-Galactosidase, β-galactosidase, α-glucuronidase, α-glucosidase, and β-glucosidase are not produced. Cells utilize acetate and lactate. Propionate, D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, maltose, gluconate, caprate, dodecanoate, citrate, and phenyl acetate are not utilized. The strains are susceptible to ampicillin, mezlocillin, piperacillin, ticarcillin, cephalothin, cefotaxime, gentamicin, netilmicin, amikacin, norfloxacin, fosfomycin, tetracycline, and fusidic acid, and some strains are susceptible to erythromycin. All strains are resistant to fosfomycin.

The cell walls contain meso-diaminopimelic acid, arabinose, galactose, and corynomycolic acids. Straight-chain saturated fatty acids are mainly palmitic and stearic acids. Branched saturated fatty acids are heptadecanoic acid. The DNA base composition is 68 mol% G+C. Bacteria were isolated from human blood cultures.

The type strain is strain T18502 (= CIP 103500). It was isolated at Strasbourg (France) from a human blood culture. Strain CIP 103500 has the characteristics described for the species.

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REFERENCES