Corynebacterium freneyi* sp. nov., α -glucosidase-positive strains related to *Corynebacterium xerosis

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Three coryneform strains from clinical specimens were studied. They belonged to the genus *Corynebacterium*, since they had type IV cell walls containing corynemycolic acids. They had phenotypic characteristics that included α -glucosidase, pyrazinamidase and alkaline phosphatase activities and fermentation of glucose, ribose, maltose and sucrose. These are the characteristics of *Corynebacterium xerosis*. Since this species is very rare in human pathology, the strains were studied in more detail by comparing the 16S–23S intergenic spacers, rDNA sequences and levels of DNA similarity of these three strains and those of the reference strains *C. xerosis* ATCC 373^T and *Corynebacterium amycolatum* CIP 103452^T. According to DNA–DNA hybridization data, the three novel strains are members of the same species (level of DNA similarity > 72%). Phylogenetic analysis revealed that these strains are closely related to *C. xerosis* and *C. amycolatum*, but DNA-relatedness experiments showed clearly that they constitute a distinct new species, with levels of DNA relatedness of less than 23% to *C. xerosis* ATCC 373^T and less than 5% to *C. amycolatum* CIP 103452^T. Two other α -glucosidase-positive strains presenting the same biochemical characteristics were included in the study and proved to be *C. amycolatum*. This new species can be differentiated from *C. xerosis* and *C. amycolatum* strains by carbon source utilization, intergenic spacer region length profiles and some biochemical characteristics such as glucose fermentation at 42 °C and growth at 20 °C. The name *Corynebacterium freneyi* sp. nov. is proposed with the type strain ISPB 6695110^T (= CIP 106767^T = DSM 44506^T).

Keywords: *Corynebacterium freneyi* sp. nov., new α -glucosidase-positive strain

INTRODUCTION

From five clinical samples, we have isolated five strains of coryneform bacilli (irregular Gram-positive bacilli) the biochemical characteristics of which, especially the presence of an α -glucosidase, pointed to the species *Corynebacterium xerosis*. Since this species is isolated very rarely (Funke *et al.*, 1996; Riegel *et al.*, 1996; Wauters *et al.*, 1998), we wanted to verify that these isolates did indeed belong to this species. We tested carbon substrate assimilation patterns, 16S–23S rDNA intergenic spacer PCR, the sequences of the

rRNA (rDNA) and DNA–DNA hybridization of these five strains and the type strains of *C. xerosis* and *Corynebacterium amycolatum*. The results showed that two of the strains belonged to the species *C. amycolatum*, whereas the other three strains were grouped within the same species, distinct from *C. amycolatum* and *C. xerosis*, for which we propose the name *Corynebacterium freneyi* sp. nov.

METHODS

Strains, media and growth conditions. Two of the five isolates of unidentified coryneform rods were isolated at the Institute of Bacteriology, Strasbourg; IBSG 95888 came from a wound in pure culture and IBSV 5533 came from the sperm of a hypofertile patient. The three other strains were

The GenBank accession numbers of the 16S rDNA sequences of strains ISPB 6695110^T and ISPB 20395347 are AJ292762 and AJ292763.

Table 1. Results of the identification tests for *C. freneyi*

Taxa are identified as: 1, *C. amycolatum*; 2, *C. xerosis*; 3, *C. minutissimum*; 4, *C. striatum*; 5, *C. freneyi* (three strains); 6, two α -glucosidase-positive *C. amycolatum* strains isolated in this study. Tests were carried out according to Wauters *et al.* (1998) and are scored as: +, > 80% positive; –, < 20% positive; v, between 20 and 80% positive.

Characteristic	1	2	3	4	5	6
Growth at 20 °C	–	+	–	+	+	v
Fermentation of glucose at 42 °C	+	–	+	+	+	+
Alkalinization of formate	–	–	+	+	–	–
Acidification of ethylene glycol	v	v	–	+	–	+

isolated at E. Herriot Hospital (Lyon, France); ISPB 6695110^T came from pus from a toe, ISPB 20395347 came from a varicose ulcer and ISPB 16799604 came from a sample of a subcutaneous abscess fistula. The only strain that was in pure culture within the sample was strain IBSG 95888. In addition, we tested the following reference strains: *C. amycolatum* CIP 103452^T, *C. xerosis* ATCC 373^T and *C. xerosis* IAM 12431 (= ATCC 7711). Bacteria were grown aerobically at 37 °C on trypticase soy agar supplemented with 5% (v/v) sheep blood (bioMérieux) in an atmosphere containing 10% CO₂.

Antibiotic sensitivity. Antibiotic sensitivity was determined by using the ATP Staph gallery (bioMérieux) or on Mueller–Hinton medium supplemented with 5% (v/v) sheep blood (bioMérieux) when the culture was insufficient on the API gallery.

Carbon substrate assimilation tests. The Biotype 100 system (bioMérieux) was cultured using the method modified by Renaud *et al.* (1998). The readings were taken after 6 d of incubation at 30 °C in a humid chamber. The results for the 99 wells were recorded using the RECOGNIZER software package (TAXOTRON package; Taxolab, P. A. D. Grimont, Institut Pasteur, Paris, France). The complement Jaccard coefficient was used to calculate the distances between the strains and the unweighted pair group method with averages (UPGMA) was used to form the clusters (TAXOTRON).

Cell wall analysis and determination of DNA base composition. Amino acid analysis, mycolic acid determinations and sugar determinations were carried out by performing TLC using the procedures described by Barreau *et al.* (1993). DNA was extracted as described previously (Riegel *et al.*, 1995). Experiments involving hybridization between labelled DNA and fragmented DNA preparations were carried out at 65 °C for 16 h in 0.42 M NaCl by the nuclease/trichloroacetic acid method (Grimont *et al.*, 1980).

ITS-PCR analysis. PCR analysis of the 16S–23S intergenic spacer region length (ITS-PCR) was performed according to the method of Mendoza *et al.* (1998) adapted to corynebacteria (Aubel *et al.*, 1997). The amplified products were analysed by electrophoresis at a constant 2 V cm^{–1} for 3 h at 10 °C. For digestion, the enzyme *Cfo*I was used.

rDNA sequencing. Strains were grown overnight at 37 °C on Mueller–Hinton agar (bioMérieux). Total cellular DNA was extracted by the Chelex technique (de Lamballerie *et al.*, 1992). Briefly, a single colony was picked from a plate and

resuspended in 2% (v/v) Chelex/0.05% (v/v) SDS. The suspension was heated at 98 °C for 20 min. DNA concentrations were estimated on agarose gels. PCR-mediated amplification of the 16S rRNA gene and sequence determination were performed by cycle sequencing with fluorescently labelled dideoxynucleotide terminators (Applied Biosystems). The sequencing reactions were analysed with a 377 automated DNA sequencer (Applied Biosystems) (Weisburg *et al.*, 1991).

Analysis of DNA sequences. Following initial alignment with CLUSTAL W version 1.60 (Higgins *et al.*, 1992), unweighted phylogenetic analyses were performed on the individual and combined datasets by using the heuristic search option in PAUP* 4.0b1 (Swofford, 1998) with 1000 stepwise random sequence additions.

RESULTS AND DISCUSSION

Macroscopic and microscopic morphology

The colonies were rough, whitish and 0.5–1 mm in diameter after 48 h of incubation at 37 °C. The edges were irregular. Gram staining revealed Gram-positive, non-spore-forming diphtheroids.

Biochemical characteristics

All the strains had the code API CORYNE (bioMérieux) 3110325 except strain ISPB 16799604, which was nitrate negative (code 2110325). This indicates that they had the following positive enzymic characteristics: pyrazinamidase, alkaline phosphatase and α -glucosidase. They had none of the following enzymes: pyrrolidonyl arylamidase, β -glucuronidase, β -galactosidase, *N*-acetyl β -glucosamidase, aesculinase (β -glucosidase) and urease. They did not hydrolyse gelatin. The only sugars from the API CORYNE gallery that were fermented were glucose, ribose, maltose and sucrose. This fermentation could appear slowly (after 48 h of incubation). Identification with the second database of the API CORYNE gallery (Funke *et al.*, 1997a) gave *Corynebacterium striatum*/*C. amycolatum* with an abnormal positive characteristic, α -glucosidase. This characteristic encouraged us to identify it as *C. xerosis* (Funke *et al.*, 1996). Moreover, identification tests were carried out according to Wauters *et al.* (1998). The *C. freneyi* strains (ISPB 6695110^T, ISPB 20395347 and ISPB 16799604) were able to grow at 20 °C and to ferment glucose at 42 °C, but they did not alkalinize formate and did not acidify ethylene glycol (Table 1).

The Biotype 100 results showed that strains IBSG 95888 and IBSV 5533 were grouped together with the reference strain of *C. amycolatum*, whereas strains ISPB 20395347, ISPB 6695110^T and ISPB 16799604 formed another homogeneous group. Neither of the two groups was linked to the classical reference strain of *C. xerosis*. Discriminatory substrates were: lactose (negative for *C. freneyi* and *C. amycolatum* and positive for *C. xerosis*), D-turanose (positive for *C. freneyi* and *C. xerosis* and negative for *C. amycolatum*), L-histidine (negative for *C. freneyi* and positive for *C. amycolatum* and *C. xerosis*), succinate (positive for *C. freneyi* and

Table 2. Characteristics that differentiate *C. freneyi* sp. nov. from α -glucosidase-positive *Corynebacterium* strains

Taxa are identified as: 1, *C. freneyi*; 2, *C. xerosis*; 3, *C. amycolatum*; 4, *C. diphtheriae*; 5, *C. kutscheri*; 6, *C. ulcerans*; 7, *C. sundsvallense*; 8, *C. pseudotuberculosis*. Data were taken from Funke *et al.* (1997b), Wauters *et al.* (1998) and Collins *et al.* (1999). See legend to Table 1 for scoring of characters other than susceptibility to O/129. ND, Not determined; s, susceptible; R, resistant.

Characteristic	1	2	3	4	5	6	7	8
Nitrate reduction	v	v	v	v	v	—	—	v
Urea hydrolysis	—	—	v	—	+	+	+	+
Aesculin hydrolysis	—	—	—	—	+	—	—	—
Pyrazinamidase	+	+	+	—	v	—	v	—
Alkaline phosphatase	+	+	+	—	—	+	v	v
Acid production from:								
Glucose	+	+	+	+	+	+	+	+
Maltose	+	+	v	+	+	+	+	+
Sucrose	+	+	v	—	+	—	+	v
Fermentation of glucose at 42 °C	+	—	+	—	ND	ND	ND	ND
Growth at 20 °C	+	+	—	+	ND	ND	ND	ND
Susceptibility to O/129	s	s	Mostly R	ND	ND	ND	ND	ND

C. amycolatum and negative for *C. xerosis*) and L-aspartate (negative for *C. freneyi* and *C. xerosis* and positive for *C. amycolatum*). This method did not differentiate strains IBSG 95888 and IBSV 5533 from *C. amycolatum*.

Differentiation from other previously described species

Only a few members of the genus *Corynebacterium* present α -glucosidase activity (Table 2). Of these, *C. xerosis* does not ferment glucose at 42 °C. *Corynebacterium diphtheriae* does not present any real problem of identification since pyrazinamidase and alkaline phosphatase are absent. *Corynebacterium kutscheri*, which is frequently isolated from rodents (Baker, 1998), hydrolyses urea and aesculin. *Corynebacterium ulcerans*, which is responsible for respiratory infections and mastitis in cows (some strains that produce a diphtheric toxin can provoke diphtheric syndromes; Gilbert & Stewart, 1926), is urease-positive. *Corynebacterium pseudotuberculosis*, which possesses α -glucosidase in one case out of two, is of veterinary origin and is distinguished from *C. freneyi* by the presence of urease and the absence of pyrazinamidase. *Corynebacterium sundsvallense*, a recently described α -glucosidase-positive strain, hydrolyses urea (Collins *et al.*, 1999).

Antimicrobial susceptibility testing

There were no significant differences in antibiotic sensitivity between the *C. freneyi* strains and α -glucosidase-positive *C. amycolatum* strains. They were all sensitive to penicillin, tobramycin, gentamicin, tetracycline, pristinamycin, fusidic acid, vancomycin and oxacillin. They were resistant to fosfomycin. They were also resistant to nitrofurantoin, with the exception of strain ISPB 20395347. Strain IBSG 95888 was the only one to present resistance to pefloxacin

Fig. 1. PCR profiles of the 16S–23S spacer regions of *Corynebacterium* species. Lanes: 1 and 10, Ready-Load 100 bp DNA ladder (Gibco-BRL); 2, *C. amycolatum* CIP 103452^T; 3, IBSG 95888; 4, IBSV 5533; 5, ISPB 6695110^T; 6, ISPB 20395347; 7, ISPB 16799604; 8, *C. xerosis* ATCC 7711; 9, *C. xerosis* ATCC 373^T.

and rifampicin. Behaviour towards erythromycin was varied; strains IBSG 95888 and ISPB 16799604 were resistant, strain IBSV 5533 was intermediate and strains ISPB 6695110^T and ISPB 20395347 were sensitive.

Chemotaxonomic analysis

Strains ISPB 6695110^T and ISPB 20395347 had type IV cell walls (containing *meso*-diaminopimelic acid, arabinose and galactose) that contained mycolic acids. These are characteristics of bacteria belonging to the genus *Corynebacterium*.

Fig. 2. Unrooted tree showing the relationships of *C. freneyi* strains CIP 106767^T (= ISPB 6995110^T) and CIP 106768 (= ISPB 20395347) to other members of the genus *Corynebacterium*. The tree was obtained by the neighbour-joining method using the 16S rDNA sequence of *Rhodococcus equi* as the outgroup. The tree was validated by a bootstrap analysis (1000 replications) and values greater than 85% are indicated as percentages above the branches. Sequences were retrieved from the EMBL database under the accession numbers indicated.

Table 3. DNA–DNA hybridization experiments between strains of *C. freneyi*, *C. amycolatum* and *C. xerosis*

Values are percentages. ND, Not determined.

Source of unlabelled DNA	<i>C. freneyi</i> ISPB 6695110 ^T	<i>C. amycolatum</i> CIP 103452 ^T	<i>C. xerosis</i> ATCC 373 ^T
<i>C. freneyi</i> ISPB 6695110 ^T	100	1	21
<i>C. freneyi</i> ISPB 20395347	79	2	13
<i>C. freneyi</i> ISPB 16799604	72	5	23
<i>C. amycolatum</i> IBSG 95888	5	87	ND
<i>C. amycolatum</i> IBSV5533	1	72	ND
<i>C. xerosis</i> ATCC 7711	18	2	64

Genotypic identification

Analysis of the 16S–23S intergenic spacer (Fig. 1) showed that strains ISPB 6695110^T, ISPB 20395347 and ISPB 16799604 presented the same profile. This group was distinct from that formed by strains IBSG 95888 and IBSV 5533 and the reference strain of *C. amycolatum*. The profiles of the reference strains *C. xerosis* ATCC 373^T and ATCC 7711 were identical and differed from the profiles of the other strains. This result allows three groups of strains to be differentiated genomically: one containing *C. xerosis* ATCC 373^T and ATCC 7711, another containing *C. amycolatum* strains, of which two are α -glucosidase-positive (IBSG 95888 and IBSV 5533), and the third containing strains ISPB 6695110^T, ISPB 20395347 and ISPB 16799604 (*C. freneyi*).

Phylogenetic analyses (Fig. 2) confirmed that strains ISPB 6695110^T and ISPB 20395347 belong to the robust monophyletic unit that groups all available *Corynebacterium* sequences. These strains were grouped together in a reliable fashion with *C. xerosis* and *C. amycolatum*. The percentage similarity between the rDNA sequences of these two strains and all known sequences of *C. xerosis* and *C. amycolatum* varied from 99 to 97%. From the data presented in Table 3, it is apparent that strains ISPB 6695110^T, ISPB 20395347 and ISPB 16799604 should be assigned to the same genomospecies, since they showed hybridization levels between 72 and 100%. These strains were genomically different from *C. amycolatum*, for which the percentage of hybridization with the type strain CIP 103452^T was less than 5%. They were also different from *C. xerosis*, for which the level of hybridization did not exceed 23%. The results obtained from ITS-PCR were confirmed, since strains IBSG 95888 and IBSV 5533 hybridized at over 72% with the reference strain of *C. amycolatum* and were indeed *C. amycolatum* strains possessing α -glucosidase.

It has been shown that the reference strains *C. xerosis* ATCC 373^T and ATCC 7711 have similar protein

profiles (Jackman, 1982) but their mycolic acids are slightly different (De Briel *et al.*, 1992) and their DNA–DNA hybridization is weak (Coyle *et al.*, 1993). Our experiment showed that their ITS profiles were identical, which could confirm that they belong to the same genomic group (Funke *et al.*, 1996). However, the relatively weak DNA–DNA hybridization (64%) could explain the results obtained by Coyle *et al.* (1993).

If differential diagnosis between *Corynebacterium minutissimum*, *C. striatum* and *C. amycolatum* in the routine clinical laboratory no longer presents a problem (Riegel *et al.*, 1996; Funke *et al.*, 1997b; Renaud *et al.*, 1998; Wauters *et al.*, 1998), identifying *C. xerosis* remains delicate, since this species has recently been taken out of the database of the API CORYNE gallery (Funke *et al.*, 1997a). Reference strains present problems of heterogeneity (Coyle *et al.*, 1993) and it seems that only strains ATCC 373^T and ATCC 7711 can be considered to belong to the species *C. xerosis* (Funke *et al.*, 1996). The presence of α -glucosidase was considered a major characteristic of differentiation between *C. xerosis* and *C. amycolatum* (Funke *et al.*, 1996). A few exceptions have appeared in publications on the subject: Wauters *et al.* (1996) showed that, among 60 strains of *C. amycolatum* (producers of propionic acid), two possessed α -glucosidase activity. In our study, two strains of *C. amycolatum* with α -glucosidase activity could not be differentiated from other *C. amycolatum* strains by either phenotypic or genotypic characteristics. In another publication (Wauters *et al.*, 1998), it was shown that, among 210 strains of *C. amycolatum* isolated, three exhibited α -glucosidase activity, whereas the only strain of *C. xerosis* isolated did not present any α -glucosidase activity. To summarize, if strains of *C. xerosis* can exist without α -glucosidase, strains of *C. amycolatum* with this characteristic can also be found. The presence of this enzyme can no longer separate the two species clearly. In this study, we have shown that, alongside these atypical strains, a new species exists, *C. freneyi*, the phenotypic properties of which are very similar to those of *C. xerosis* and *C. amycolatum*.

Description of *Corynebacterium freneyi* sp. nov.

Corynebacterium freneyi (fre'ney.i. N.L. gen. n. *freneyi* of Freney, to honour Jean Freney, a contemporary French microbiologist).

The description given below is based on the results of studies of three strains. Cells are Gram-positive, non-spore-forming and non-motile. They are typical club-shaped rods. Colonies are white, dry and rough, 0.5–1 mm in diameter after 48 h of incubation on a blood-enriched medium. The edges are irregular. Growth is not encouraged in a medium containing lipids. The organism is catalase-positive. Reduction of nitrates is variable. The strains express α -glucosidase, pyrazinamidase and alkaline phosphatase. They do not produce pyrrolidonyl arylamidase, β -glucuronidase, β -galactosidase or *N*-acetyl- β -glucosaminidase. They do not hydrolyse aesculin, gelatin or urea. They produce acid from glucose, maltose and sucrose. Ribose acidification is slow and weak. Xylose, mannitol, lactose and glycogen are not fermented. D-Turanose and succinate are used as carbon substrates but lactose, L-histidine and L-aspartate are not used. Glucose is fermented at 42 °C and growth occurs at 20 °C. The cell wall contains *meso*-diaminopimelic acid, arabinose, galactose and mycolic acids.

The type strain, ISP 6695110^T (= CIP 106767^T = DSM 44506^T), was isolated from pus from a toe.

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