Corynebacterium rouxii sp. nov., a novel member of the diphtheriae species complex

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Abstract

A group of six clinical isolates previously identified as *Corynebacterium diphtheriae* biovar Belfanti, isolated from human cutaneous or peritoneum infections and from one dog, were characterized by genomic sequencing, biochemical analysis and MALDI-TOF mass spectrometry. The six isolates were negative for the diphtheria toxin gene. Phylogenetic analyses showed that the six isolates (including FRC0190^T) are clearly demarcated from *C. diphtheriae*, *C. belfantii*, *C. ulcerans* and *C. pseudotuberculosis*. The average nucleotide identity of FRC0190^T with *C. diphtheriae* NCTC11397^T was 92.6%, and was 91.8% with *C. belfantii* FRC0043^T. *C. diphtheriae* subsp. *lausannense* strain CHUV2995^T appeared to be a later heterotypic synonym of *C. belfantii* (ANI, 99.3%). Phenotyping data revealed an atypical negative or heterogeneous intermediate maltose fermentation reaction for the six isolates. MALDI-TOF mass spectrometry differentiated the new group from the other *Corynebacterium* taxa by the presence of specific spectral peaks. *rpoB* sequences showed identity to atypical, maltose-negative *C. diphtheriae* biovar Belfanti isolates previously described from two cats in the USA. We propose the name *Corynebacterium rouxii* sp. nov. for the novel group, with FRC0190^T (= CIP 111752^T = DSM 110354^T) as type strain.

Introduction

The genus *Corynebacterium* currently includes approximately 111 species [1–3]. The most important human pathogen of the genus is *Corynebacterium diphtheriae*, which causes diphtheria [2,4]. *C. diphtheriae* is genetically heterogeneous [5–8] and four biovars were defined: Gravis, Mitis, Belfanti and Intermedius [9–11], the latter being almost never reported in recent literature. In 2010, maltose-non fermenting strains of *C. diphtheriae* biovar Belfanti were reported from two cats in the USA, and were shown to have a divergent *rpoB* sequence [12]. In 2018, some biovar Belfanti isolates were classified as a novel species, *C. belfantii* [3], with 94.85% average nucleotide identity (ANI) with *C. diphtheriae*. Almost simultaneously, *C. diphtheriae* subsp. *lausannense* was also proposed for strains of biovar Belfantii [13]. The *tox* gene, which codes for diphtheria toxin, is carried on a corynephage that can lysogenize strains of *C. diphtheriae*. However, the *tox* gene was rarely reported in isolates of biovar Belfanti [5,14,15] and no strain of *C. belfantii* or *C. diphtheriae* subsp. *lausannense* was described as *tox*-positive [3][12]. The *tox* gene can also be harboured by strains of *C. ulcerans* and *C. pseudotuberculosis*, two species that are phylogenetically close to *C. diphtheriae* and *C. belfantii* [16]. Together, the above-mentioned species constitute a single phylogenetic clade nested within the *Corynebacterium* genus. We refer to this clade as the *C. diphtheriae* complex.

Here, we define the taxonomic status of six isolates initially identified as *C. diphtheriae* biovar Belfanti, isolated from five human infections and one dog in France.

Material and Methods

We compared the six atypical clinical isolates, among which is strain FRC0190^T, with 13 *C. diphtheriae* strains of biovars Gravis or Mitis (including *C. diphtheriae* type strain NCTC 11397^T) and 8 strains previously [3] identified as *C. belfantii* (including the type strain FRC0043^T; **Table 1; Table S1**). Type strains of *C. ulcerans* (CIP 106504^T = NCTC 7910^T) and of *C. pseudotuberculosis* (CIP 102968^T = ATCC 19410^T) were also included for comparison.

Clinical samples or isolates were received at the French National Reference Centre for Corynebacteria of the *diphtheriae* complex for isolation and/or characterization, respectively. Oxoid's Tinsdale agar with supplement medium (Thermo Fisher Diagnostics, Dardilly, France) was used to isolate *C. diphtheriae* from clinical samples. Isolates were frozen in Brain-Heart-Infusion (BHI) medium containing 30% of glycerol and stored at -80°C prior to this study. After thawing, isolates were grown at 37°C on tryptose-casein soy agar plates during 24 hours. DNA was extracted from a few colonies with the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). The six isolates were identified as *C. diphtheriae* by multiplex polymerase chain reaction (PCR) combining a *dtxR* gene fragment specific for *C. diphtheriae* [15] and a multiplex PCR [17,18] that targets a fragment of the *pld* gene specific for *C. pseudotuberculosis,* the gene *rpoB* (amplified in all species of the *C. diphtheriae* complex) and a fragment of 16S rRNA gene specific for *C. pseudotuberculosis* and *C. ulcerans*. The *tox* gene was also detected by PCR [19]. These PCR results were confirmed using a more recent four-plex qPCR [20].

For biochemical identification, standard methods were used [21][14,22]. More specifically, strains were characterized for pyrazinamidase, urease, nitrate reductase and for utilization of maltose and trehalose using API Coryne strips (BioMérieux, Marcy l'Etoile, France) and the Rosco Diagnostica reagents (Eurobio, Les Ulis, France) following provider's recommendations. The Hiss serum water test was used for glycogen fermentation. Briefly, this test was performed as follows. Solution A was obtained by dissolving 500 mg of bacteriological peptone (Oxoid, Hampshire, UK; ref. LP0037) in 100 mL of distilled water, adding 100 mg of Na2HPO4 (Sigma-Aldrich, Saint-Louis, Missouri, USA; ref: S7907), and homogenizing and heating the mixture until boiling. After cooling to room temperature, 18 ml of sterile horse serum were added and mixed. In parallel, solution B was prepared by adding 430 mg of acid fuchsin (Sigma-Aldrich, Saint-Louis, Missouri, USA, ref: F8129) into 86 mL of distilled water, after which 14 mL of 30% NaOH (ThermoFisher Scientific, Waltham, Massachusetts; ref. S/4950/PB15) were added. Solution B was stored up to 15 days in the dark. To prepare the complete Hiss serum water sugar medium, 780 µL of solution B were added to the total volume of solution A. The pH was adjusted to 7.7 using HCl 5N (Sigma-Aldrich, Saint-Louis,

Missouri, USA; ref. H1758). 100 mg of glycogen (Acros Organics, Geel, Belgium, ref: 422950050) were then added. The solution was mixed and distributed in 3.5 mL aliquots in 5 mL glass tubes, and sterilized at 108°C during 30 minutes. This medium was conserved up to 6 months at 5°C + 3°C. To perform the glycogen test, a loopful (10 µL) of a bacterial culture from Columbia blood agar or Tryptose-Casein-Soy agar was introduced into a tube containing 3.5 mL of sterile Hiss serum water sugar medium. Results were read manually after homogenization of the suspension and incubation at 37°C ± 2°C during 24 h. Strains NCTC 12077 and NCTC 764 were used as positive and negative controls, respectively (expected results: dark pink and light pink, respectively).

The biovar of isolates was determined based on the combination of nitrate reductase (positive in Mitis and Gravis, negative in Belfanti) and glycogen fermentation (positive in Gravis only).

Antimicrobial susceptibility was characterized by the disk diffusion method using impregnated paper disks (Bio-Rad, Marnes-la-Coquette, France) and minimum inhibitory concentrations were determined using ETEST strips (BioMérieux, Marcy l'Etoile, France). The sensitivity was interpreted using CA-SFM/EUCAST V.1.0 (Jan 2019) criteria for *Corynebacterium* (https://www.sfm-microbiologie.org/wp-content/uploads/2019/02/CASFM2019_V1.0.pdf). Susceptibility was tested for the following antimicrobial agents: fosfomycin, vancomycin, kanamycin, gentamycin, penicillin G, oxacillin, amoxicillin, imipenem, cefotaxime, clindamycin, azithromycin, spiramycin, clarithromycin, erythromycin, clindamycin, ciprofloxacin, trimethoprim-sulfamethoxazole, trimethoprim, sulfonamide, pristinamycin, rifampicin and tetracycline.

MALDI-TOF mass spectrometry was used for identification confirmation. For this purpose, an overnight culture on Trypto-Casein-Soy Agar (TSA) (37°C) was used to prepare the samples accordingly to the ethanol/formic acid extraction procedure proposed by in the manufacturer recommendations (Bruker Daltonics, Bremen, Germany). The cell extracts were then spotted onto an MBT Biotarget 96 target plate, air dried and overlaid with 1 µL of a saturated α-cyano-4-hydroxycinnamic acid (HCCA). 24 mass spectra per strain were acquired on a Microflex LT mass spectrometer (Bruker Daltonics, Bremen, Germany). Re-analysis of the spectra was performed for the purpose of this work. Spectra were first preprocessed by applying smoothing and baseline subtraction with FlexAnalysis software using default parameters, exported as text files from the Brucker system and then imported and analyzed in a dedicated BioNumerics v7.6.3 (Applied-Maths, Belgium) database following the protocol described by Rodrigues *et al.* [23]. To allocate proteins to the specific peaks detected, we extracted all the molecular weights from the genomes of the type strains (NTCT11397^T, FRC0043^T and FRC0190^T) using a Biopython script

(https://biopython.org/DIST/docs/api/Bio.SeqUtils-module.html) and performed sequence alignments with ClustalW for the candidate proteins.

Genomic sequencing was performed from Nextera XT libraries using a NextSeq-500 instrument (Illumina, San Diego, USA) with a 2 x 150 nt paired-end protocol. Contig sequences were assembled using SPAdes v3.12.0 [24] (**Table S1**). JSpeciesWS [25] was used to calculate the BLAST-based average nucleotide identity (ANIb). BLASTN was used to extract 16S rRNA and *rpoB* sequences from genome assemblies and to determine the presence or absence of the *narIJHGK* nitrate reduction gene cluster using as query the cluster of strain NCTC 13129 (RefSeq accession number: DIP_RS13820 to DIP_RS13845) [26]. *rpoB* and 16S rRNA gene sequences of atypical *C. belfantii* strains from cats [12] were included for comparison. For genome-based phylogenetic analysis, the pairwise *p*-distance (*i.e.*, proportion of aligned nucleotide differences) between each pair of genomes was estimated based on Mash [27] using a multiple hit correction [28] with JolyTree (https://gitlab.pasteur.fr/GIPhy/JolyTree). For 16S rRNA and rpoB gene sequences, sequences were aligned with MAFFT v7.407 [29] and the resulting alignment was used for phylogenetic tree inference with IQ-TREE v1.6.7.2 [30] using the GTR+I+G4 model. Branch support was obtained after 1000 bootstrap replicates.

Results and discussion

Six isolates were isolated from five cutaneous lesions and one ascitic fluid sample (**Table 1**). Strikingly, human cutaneous lesions were all ulcerations due to underlying chronic arteritis. Ascitic fluid was sampled on a patient with a suspicion of spontaneous peritonitis. The dog was investigated in the context of purulent orbital cellulitis.

The six isolates were *tox* negative (**Table S1**); more specifically, they were negative for amplification of the expected 910-bp PCR product encompassing fragments A and B of the toxin gene [19] and also negative for the amplification of a 117-bp region of diphtheria toxin fragment A [31] by multiplex qPCR [20]. We also confirmed by BLASTN that the *tox* gene sequence (query: *tox* gene sequence from strain NCTC 13129, RefSeq accession number: DIP_RS12515) was absent from the genomic assemblies. After species identification by multiplex PCR, the isolates were positive for *dtxR* and *rpoB* and negative for *C. ulcerans/C. pseudotuberculosis* 16S rDNA and *pld*, leading to initial identification as *C. diphtheriae*. Concordant with this identification, the six isolates were pyrazinamidase, urease and trehalose negative. Upon biotyping, the isolates were nitrate and glycogen negative, a pattern that corresponds to biovar Belfanti. Consistently, the *narKGHIJ* nitrate reduction gene cluster was not detected from the genomic assemblies of these isolates and those of *C. belfantii* (**Table S2**). The phenotypic aspect of colonies on Tinsdale or blood agar medium was undistinctive from *C. diphtheriae* Mitis and Gravis and *C. belfantii*. However, we noted that similar to the Gravis isolates, the colonies of the six atypical isolates looked dry and were friable on TCS medium. Distinctively, the maltose test was negative for the six isolates using API Coryne (**Table S1**). The same test was atypical using the Rosco Diagnostic method: results showed heterogeneous coloration that was neither as yellow as the typically positive strains, nor as purple as the negative strains (**Figure S3**). This atypical maltose result was not observed using API Coryne strips, with which the maltose test was clearly negative for the six isolates. We noted that the four genes of the maltose utilization pathway [32] are present and undisrupted in the six isolates, as in other members of the *C. diphtheriae* complex. Further work is required to elucidate the mechanisms of maltose utilization and its regulation, and why the two tests give different results.

Regarding their antimicrobial susceptibility (**Table S3**), the six isolates were resistant to fosfomycin, as is typical of *Corynebacteria* [33], and were susceptible to all other tested antimicrobial agents with the following exceptions: FRC0284 and FRC0527 were resistant to penicillin (minimum inhibitory concentration: 0.19 mg/L), and FRC0412 was resistant to penicillin and cefotaxime (0.19 mg/L and 1.0 mg/L, respectively).

Genomic sequencing results showed that the six isolates had a genome size of 2.4 Mb on average (**Table S1**), similar to *C. diphtheriae* biovars Mitis and Gravis isolates (average size: 2.45 Mb), but smaller than *C. belfantii* (average size: 2.7 Mb). A genome sequence-based phylogenetic tree (**Figure 1**) revealed three main clades. The first one contained all *C. diphtheriae* Mitis and Gravis isolates, whereas the second comprised all *C. belfantii* isolates, and the third comprised the six maltose-atypical isolates. The mean ANIb value of atypical isolates was 92.4% with the *C. diphtheriae* clade and was 91.4% with *C. belfantii* (**Table 2**). These data indicate that the six isolates forming the atypical clade correspond to a distinct genomic cluster, separated by a level of nucleotide divergence that is well above the currently accepted genomic species threshold of ~94-96% [34,35]. The atypical clade was genetically homogeneous, with ANIb values among the six isolates ranging from 99.21% to 99.94% (**Table 2**). Phylogenetic analysis of *rpoB* and 16S rRNA coding sequences was consistent with the distinction of the atypical isolates from *C. diphtheriae* and *C. belfantii* (**Figures S1 and S2**). However, the 16S rRNA gene sequence alignment showed only 3 insertions and 4 nucleotide substitutions shared among the six atypical isolates as compared to *C. diphtheriae*, resulting in low resolution of phylogenetic relationships (**Figure S1**). We noted that *rpoB* and 16S rRNA sequences of previously reported atypical biovar Belfanti isolates from cats in the USA [12] were indistinguishable from those of the atypical isolates from France, suggesting that the cat isolates from the USA belong to the same novel group. Supporting this observation, the USA cat isolates were also reported as maltose negative [12].

Recently, it was proposed that the *C. diphtheriae* taxon should be subdivided into two subspecies, *C. diphtheriae* subsp. *diphtheriae* and *C. diphtheriae* subsp. *lausannense* [13]. Here, we observed that the ANI value between the type strains of *C. diphtheriae* subsp. *lausannense* and *C. belfantii* was 99.3%. Besides, the former was positioned within the phylogenetic branch of *C. belfantii* (**Figure 1, S1 and S2**), and the descriptions of both taxa are very similar [3,13]. Given that *C. belfantii* was validly published in October 2018, a few months before the taxonomic proposal *C. diphtheriae* subsp. *lausannense* was validated (https://www.microbiologyresearch.org/content/journal/ijsem/10.1099/ijsem.0.003174; January 2019), the latter subspecies appears to be a later heterotypic synonym of *C. belfantii*.

Based on the MALDI Biotyper Compass database version 4.1.90 (Bruker Daltonics, Bremen, Germany), the six isolates were identified as *C. diphtheriae*. However, detailed analysis of their spectra led to the identification of six pairs of biomarkers (12 peaks corresponding to the same proteins, with either single and double-charged ion forms) corresponding to three different proteins within the range 3255–9495 *m/z*, which were associated either with the group of six isolates or with *C.*

diphtheriae and *C. belfantii* (**Figure S4, Table S4**). We presumptively identified the specific biomarkers as two ribosomal proteins, L30 and S20, and one putative stress response protein (CsbD). Consistently, their amino-acid sequences differed between the *C. rouxii* on the one hand, and *C. diphtheriae*/*C. belfantii* on the other hand (**Figure S5**). Based on the current dataset, the specificity and sensitivity of peak distribution among the three species ranged between 95–100% and 76–100%, respectively (**Table S4**). MALDI-TOF MS thus allows the discrimination between *C. rouxii* and *C. diphtheriae*/*C. belfantii*. These results warrant future updates of reference MALDI-TOF databases to incorporate the novel taxon.

Based on the above results, the isolates of the novel clade represent a novel species, which we propose to name *Corynebacterium rouxii.*

Description of *Corynebacterium rouxii* sp. nov. (rou'.xi.i. N.L. gen. n. *rouxii*, of Roux, a French scientist and former director of Institut Pasteur who made critical contributions to diphtheria toxin discovery and antitoxin treatment).

C. rouxii conforms biochemically to the description of *C. diphtheriae* strains belonging to biovar Belfanti [2,21], except that strains are negative for maltose fermentation (API Coryne), being nearly negative or weakly positive with the Rosco Diagnostica maltose test. Key characteristics that distinguish *C. rouxii* from other members of the *C. diphtheriae* complex are specific MALDI-TOF MS biomarkers as described herein. The G+C content of *C. rouxii* genomes ranges from 53.2% to 53.3%, with a value of 53.3% for the type strain. So far, strains were isolated from 5 humans and a dog in France, as well as from two related cats in the USA.

The type strain is FRC0190^T (= CIP 111752^T = DSM 110354^T), isolated in 2013 from a foot ulceration reported in Cahors, France. The genome accession number of strain FRC0190^T is ERS3795540.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Conceived the study: SB. Performed the experiments: EB, CR, VP, MD, ACL. Analyzed the data: MH, CR, LP, VB, SB. Curated data: VB, MD, EB, JT, SB. Wrote the initial draft of the manuscript: SB, CR, JT. Commented on working versions of the manuscript and agreed on the final version of the manuscript: all.

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Abbreviations

ANI: average nucleotide identity; MALDI-TOF: matrix-assisted laser desorption/ionisation time-of-flight

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Data Availability

Sequence data generated in this study were deposited in the European Nucleotide Archive database and are accessible under project number PRJEB22103. The EMBL (GenBank/DDBJ) accession numbers of the genomic sequences released in this study are ERS3795539 to ERS3795544. The annotated genomic sequence of strain FRC0190^{T} was deposited in the European Nucleotide Archive and is available under accession number ERZ1195831. *rpoB* and 16S rRNA gene sequences were also submitted individually under accession numbers MN542347 to MN542352 and MN535982 to MN535987, respectively.

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[33] Soriano F, Zapardiel J, Nieto E. Antimicrobial susceptibilities of Corynebacterium species and other non-spore-forming gram-positive bacilli to 18 antimicrobial agents. Antimicrob Agents Chemother 1995;39:208–14. https://doi.org/10.1128/aac.39.1.208.

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Figure 1. Phylogenetic relationships derived from the analysis of genomic sequences. The phylogenetic tree and branch supports were inferred using JolyTree [28] (https://gitlab.pasteur.fr/GIPhy/JolyTree). Strains *C. ulcerans* NCTC 7910^{T} and *C. pseudotuberculosis* ATCC 19410^{T} were used as outgroup as they are the closest phylogenetic neighbors to *C. diphtheriae*, *C. belfantii* and *C. rouxii*. Branch support is indicated using grey circles (see key; only values >50 are shown). Each taxonomic type strain is shown in bold; note that *C. diphtheriae* subsp. *lausannense* type strain falls within *C. belfantii*. The scale bar corresponds to an estimated evolutionary distance of 0.01.

Figure S1. Phylogenetic analysis of 16S rRNA gene sequences.
16S rRNA gene sequences of *C. rouxii* isolates have accession numbers MN535982 to MN535987 (**Table S1**). *C. diphtheriae* strains isolated from domestic cats in the USA [12] were added for comparison (strain codes CD443-CD450; GenBank accession numbers: FJ409572 to FJ409575). Each taxonomic type strain is shown in bold. The scale bar indicates the number of substitutions per site. The sizes of grey circles correspond to bootstrap support of branches (-b 1000 option in IQ-TREE) as indicated by the key.

Figure S2. Phylogenetic analysis of *rpoB* coding sequences.
rpoB gene sequences of *C. rouxii* isolates have accession numbers MN542347 to MN542352 (**Table S1**). *C. diphtheriae* strains isolated from domestic cats in the USA [12] were added for comparison (strain codes CD443 and CD450; GenBank accession numbers: FJ415317 and FJ415318). Each taxonomic type strain is shown in bold. The scale bar indicates the number of substitutions per site. The sizes of grey circles correspond to bootstrap support of branches (-b 1000 option in IQ-TREE) as indicated by the key.

Figure S3. Maltose fermentation results.
The maltose test was performed using the Rosco Diagnostica reagents. Tube 1: NCTC 10648, *C. diphtheriae* biovar Gravis; Tubes 2 to 7: *C. rouxii* isolates FRC0071, FRC0297, FRC0284, FRC0527, FRC0190, FRC0412; Tube 8: NCTC 10356, *C. belfantii*; Tube 9: NCTC 12077 (*C. ulcerans*, positive control); Tube 10: NCTC 764 (*C. striatum*, negative control).

Figure S4. Peak positions (*m/z*) observed for strains of C. *diphtheriae, C. belfantii* and *C. rouxii*. Stars denote those peaks that are useful for species identification, as detailed in the corresponding supplementary Table.

Figure S5. Amino acid sequence alignments and the respective molecular weight of the proteins presumptively associated with specific MALDI-TOF MS peaks detected in *Corynebacterium diphtheriae, C. belfantii* and *C. rouxii*

Figure 1: Phylogenetic relationships derived from the analysis of genomic sequences

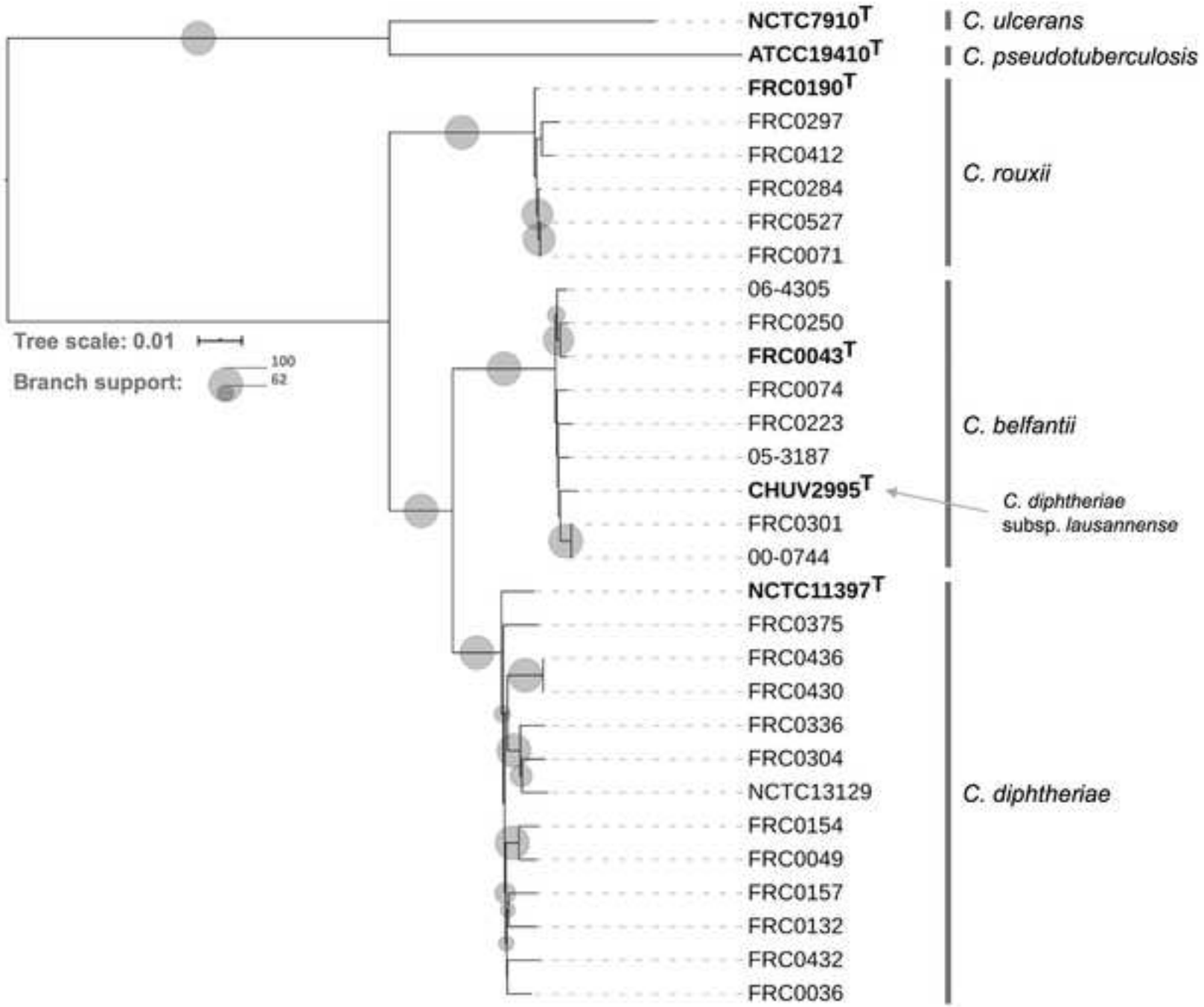

Table 1 : Strains used in this study and their characteristics

Isolate &	Species	biovar #	Isolation year	Country	Geographic origin @	*tox* gene	Isolation source	Disease	Reference
FRC0190^T	*C. rouxii*	Belfanti	2013	France	Lot, Cahors	Negative	Cutaneous	Foot ulceration, chronic arteritis	This study
FRC0071	*C. rouxii*	Belfanti	2011	France	Haute-Garonne, Toulouse	Negative	Cutaneous	Leg ulceration on chronic arteritis - diabetes	This study
FRC0284	*C. rouxii*	Belfanti	2015	France	Rhone, Lyon	Negative	Cutaneous	Limb amputation - vasculitis	This study
FRC0297	*C. rouxii*	Belfanti	2015	France	Herault, Beziers	Negative	Ascitic fluid	Spontaneous peritonitis	This study
FRC0412	*C. rouxii*	Belfanti	2016	France	Lot, Cahors	Negative	Cutaneous	Purulent orbital cellulitis (dog)	This study
FRC0527	*C. rouxii*	Belfanti	2017	France	Savoie, Chambery	Negative	Cutaneous	Foot ulceration on chronic arteritis	This study
FRC0043^T	*C. belfantii*	Belfanti	2009	France	Corrèze, Brives	Negative	Pharyngeal membrane	Laryngitis	Dazas *et al.* 2018 IJSEM
06-4305	*C. belfantii*	Belfanti	2006	France	Rhone, Lyon	Negative	Expectoration	Bronchopathy	Dazas *et al.* 2018 IJSEM
00-0744	*C. belfantii*	Belfanti	2000	France	Calvados, Caen	Negative	Expectoration	Cystic fibrosis	Dazas *et al.* 2018 IJSEM
FRC0074	*C. belfantii*	Belfanti	2011	France	Cote d'Or, Dijon	Negative	Expectoration	Cystic fibrosis	Dazas *et al.* 2018 IJSEM
FRC0223	*C. belfantii*	Belfanti	2014	France	Pas-de-Calais, Coquelles	Negative	Sinusal swab	Sinusitis	Dazas *et al.* 2018 IJSEM
05-3187	*C. belfantii*	Belfanti	2005	France	Seine-Maritime, Rouen	Negative	Nasal swab	Rhinitis	Dazas *et al.* 2018 IJSEM
FRC0250	*C. belfantii*	Belfanti	2014	France	Bas-Rhin, Strasbourg	Negative	Bronchoalveolar wash	Pneumonia	Dazas *et al.* 2018 IJSEM
FRC0301	*C. belfantii*	Belfanti	2015	France	Calvados, Lisieux	Negative	Expectoration	n.a.	Dazas *et al.* 2018 IJSEM
NCTC 11397^T	*C. diphtheriae*	Gravis	1969	USA	New York, USA	Negative	n.a.	n.a.	Dazas *et al.* 2018 IJSEM
NCTC 13129	*C. diphtheriae*	Gravis	1997	United Kingdom	Unknown	Positive	Pharyngeal membrane	Diphtheria	Dazas *et al.* 2018 IJSEM
FRC0336	*C. diphtheriae*	Gravis	2015	France	Ille-et-Vilaine, Rennes	Positive	Cutaneous	Leishmaniasis	Dazas *et al.* 2018 IJSEM
FRC0304	*C. diphtheriae*	Gravis	2015	France	La Reunion, St Denis	Negative	Cutaneous	Bullous skin lesion	Dazas *et al.* 2018 IJSEM
FRC0375	*C. diphtheriae*	Mitis	2015	France	Oise, Creil	Positive	Cutaneous	Ankle ulceration	Dazas *et al.* 2018 IJSEM
FRC0432	*C. diphtheriae*	Mitis	2016	France	Seine-et-Marne, Vaires sur Marne	Negative	Cutaneous	Purulent scalp skin injury	Dazas *et al.* 2018 IJSEM
FRC0157	*C. diphtheriae*	Mitis	2013	France	Paris	Negative	Cutaneous	Left ankle wound	Dazas *et al.* 2018 IJSEM
FRC0132	*C. diphtheriae*	Mitis	2012	France	Yvelines, Le Chesnay (return from Mali)	Negative	Cutaneous	Necrotic lesions	Dazas *et al.* 2018 IJSEM
FRC0036	*C. diphtheriae*	Mitis	2009	France	Mayotte, Mamoudzou	Negative	Cutaneous	Burn wound	Dazas *et al.* 2018 IJSEM
FRC0154	*C. diphtheriae*	Mitis	2012	France	Haut-Rhin, Colmar	Positive	Cutaneous	Cutaneous infection	Dazas *et al.* 2018 IJSEM
FRC0049	*C. diphtheriae*	Mitis	2009	France	Mayotte, Mamoudzou	Positive	Cutaneous	Genital lesion	Dazas *et al.* 2018 IJSEM
FRC0430	*C. diphtheriae*	Mitis	2016	France	Rhone, Bron	Positive	Cutaneous	Leg ulcerations	Dazas *et al.* 2018 IJSEM
FRC0436	*C. diphtheriae*	Mitis	2016	France	Ille-et-Vilaine, Rennes	Positive	Cutaneous	Cutaneous infection	Dazas *et al.* 2018 IJSEM
ATCC 19410^T	*C. pseudotuberculosis*	not applicable	1931	n.a.	South America	Negative	Infected gland (sheep)	n.a.	PMID: 13882624 (Cummins, 1962)
NCTC 7910^T	*C. ulcerans*	not applicable	1948	United Kingdom	n.a.	Negative	Throat	n.a.	PMID: 7729671 (Riegel et al., 1995)

Biovar of *C. diphtheriae* as classically defined

& FRC: collection of the French National Reference Center for the Corynebacteria of the C. diphtheriae complex; NCTC: National Collection of Type Cultures (Public Health England); ATCC: American Type Culture Collection

@ Geographic origin for French isolates is given as "French Department, city"

n.a.: not available

Table 2. Average nucleotide identity values.

***Corynebacterium* species**	**Strain identifier (1)**	**FRC0071**	**FRC0190T**	**FRC0284**	**FRC0297**	**FRC0412**	**FRC0527**	**NCTC11397T**	**FRC0043T**	**NCTC7910T**	**ATCC19410T**
C. rouxii sp. nov.	FRC0071	100	99.60	99.89	99.21	99.37	99.94	92.30	91.22	71.34	70.94
C. rouxii sp. nov.	FRC0190	99.68	100	99.75	99.24	99.34	99.68	92.41	91.36	71.40	70.93
C. rouxii sp. nov.	FRC0284	99.94	99.71	100	99.22	99.35	99.92	92.30	91.28	71.26	70.85
C. rouxii sp. nov.	FRC0297	99.26	99.28	99.27	100	99.29	99.26	92.44	91.43	71.16	70.67
C. rouxii sp. nov.	FRC0412	99.45	99.26	99.40	99.23	100	99.44	92.39	91.37	71.04	70.79
C. rouxii sp. nov.	FRC0527	99.95	99.55	99.85	99.21	99.40	100	92.30	91.27	71.27	70.99
C. diphtheriae	NCTC11397T	92.33	92.45	92.30	92.32	92.34	92.32	100	95.07	71.29	70.86
C. belfantii	FRC0043T	90.92	91.06	90.93	90.99	90.99	90.92	94.77	100	71.12	70.76
C. ulcerans	NCTC7910T	71.42	71.51	71.43	71.32	71.30	71.42	71.40	71.31	100	84.33
C. pseudotuberculosis	ATCC19410T	71.31	71.30	71.31	71.25	71.25	71.31	71.19	71.06	84.29	100

(1) A trailing T after the strain identifier denotes that the strain is the type strain of its corresponding taxon

Figure S1: Phylogenetic analysis of 16S rRNA gene sequences

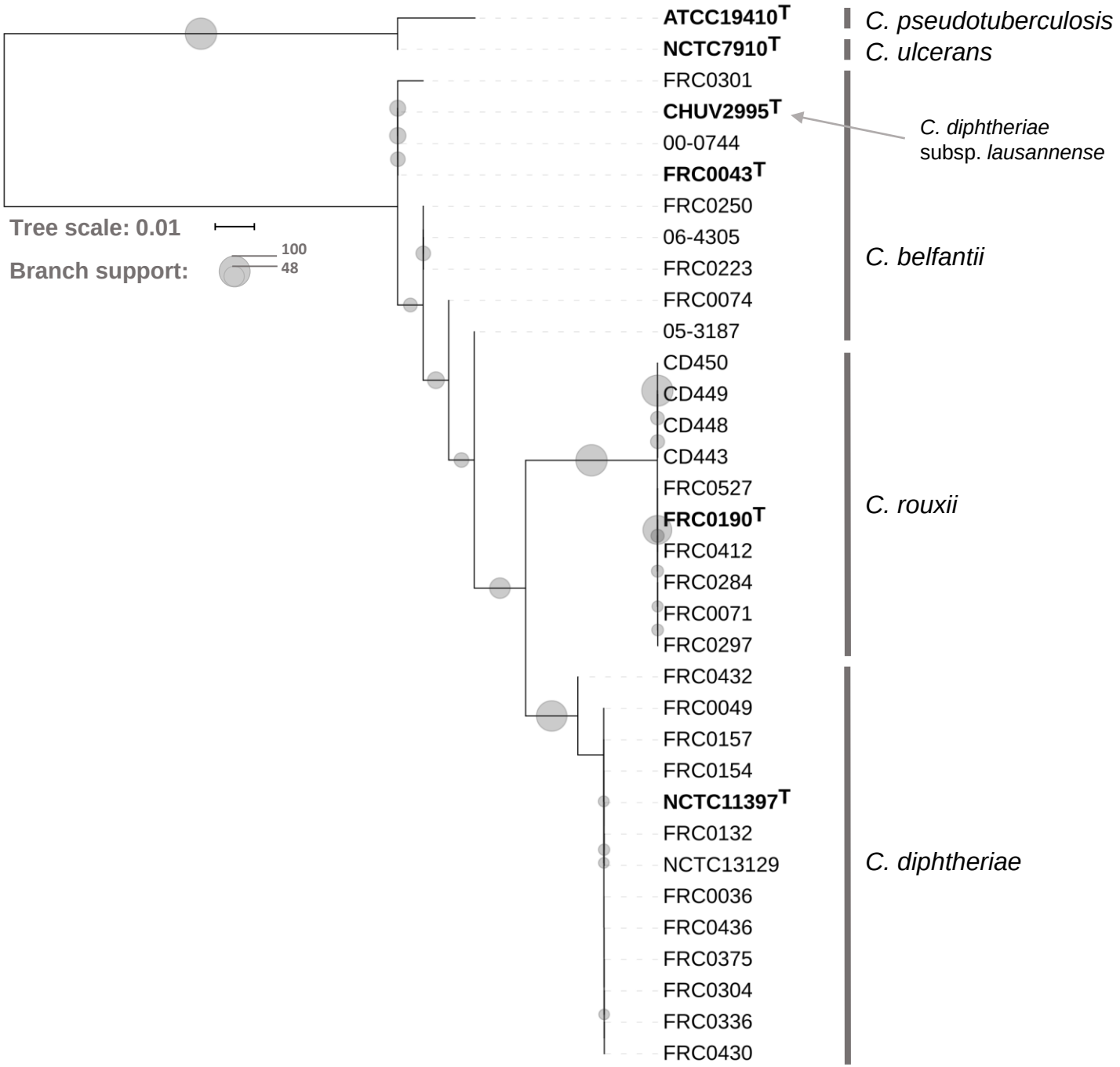

Figure S2: Phylogenetic analysis of *rpoB* coding sequences

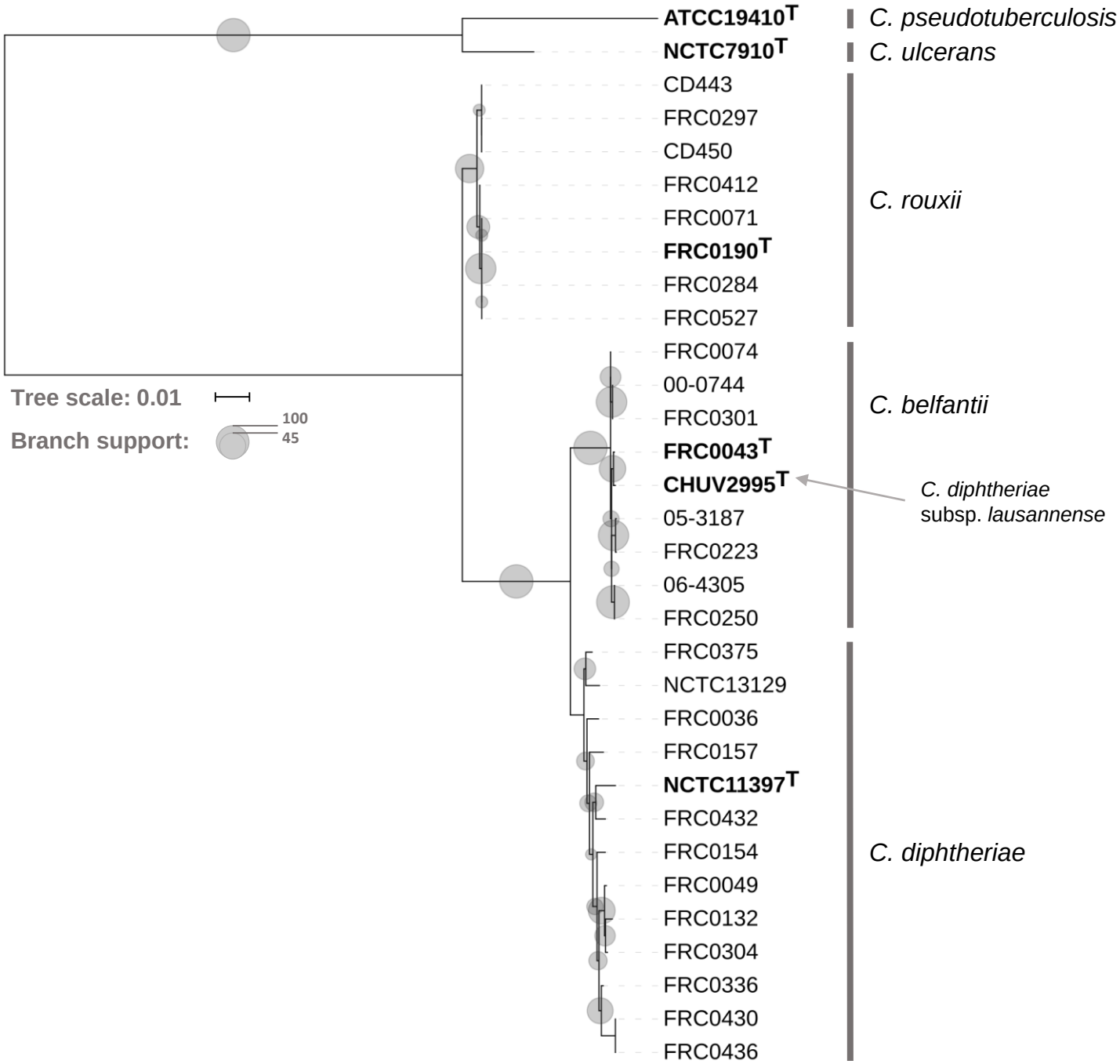

Figure S3. Maltose fermentation results (Rosco Diagnostica method)

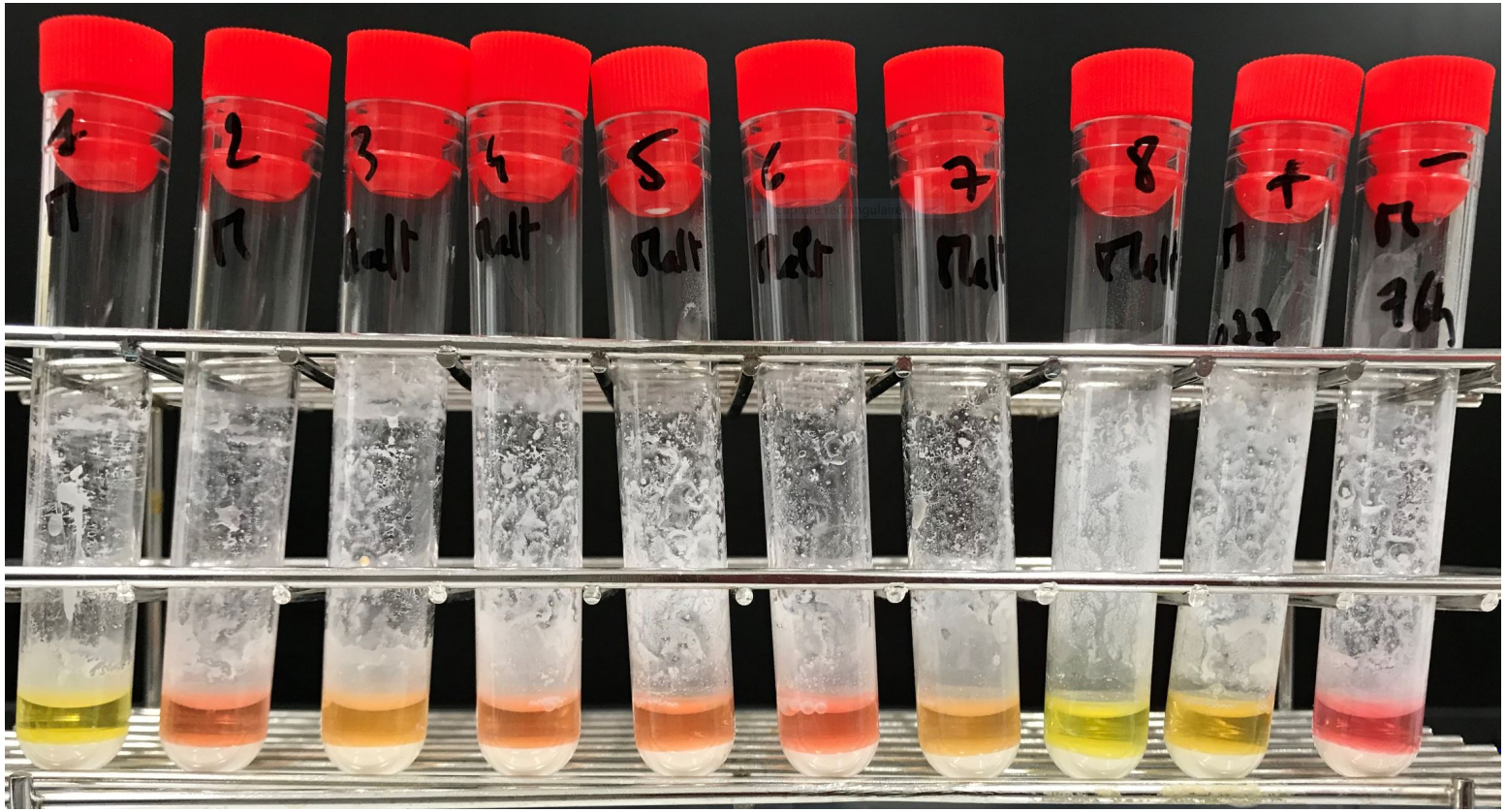

Figure S4. Peak positions (m/z) observed for strains of C. *diphtheriae, C. belfantii* and *C. rouxii*.

Stars denote those peaks that are useful for species identification, as detailed in the corresponding supplementary Table.

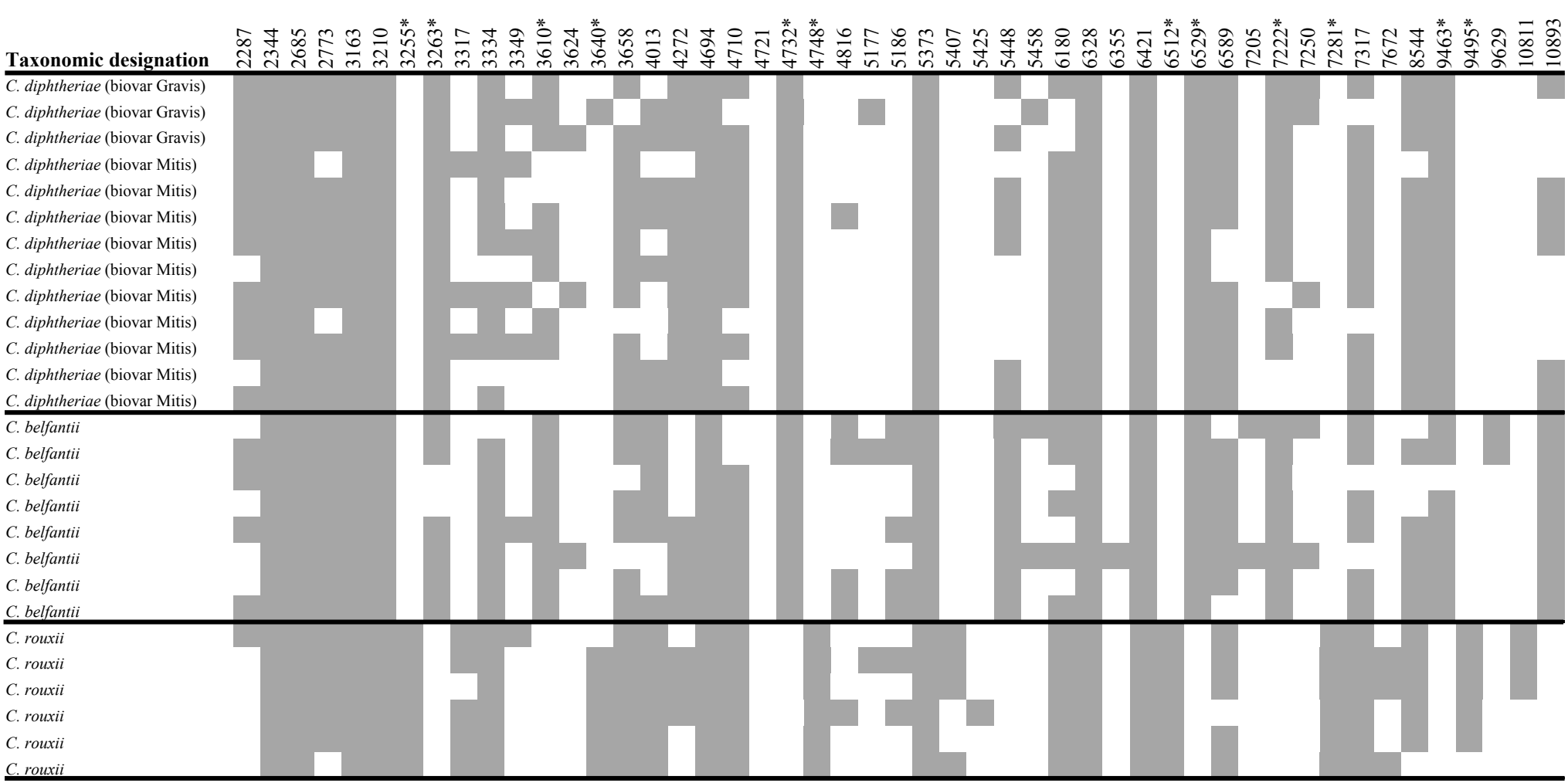

Figure S5. Amino acid sequence alignments and the respective molecular weight of the proteins presumptively associated with specific MALDI-TOF MS peaks detected in *Corynebacterium diphtheriae, C. belfantii* and *C. rouxii.*

L30 ribosomal protein alignment

```
Crouxii_FRC0190T          (M)ALKITQHKGLVGANPKQRKNIAALGLKHINHSVVHQDTPVVRGMVNVVRHMVSVEEVAGE* 61 Mw = 6513 Da
Cbelfantii_FRC0043T       (M)ALKITQHKGLVGANPKQRKNMAALGLKHINHSVVHQDTPVVRGMVNVVRHMVSVEEVAGE* 61 Mw = 6531 Da
Cdiphtheriae_NCTC11397T   (M)ALKITQHKGLVGANPKQRKNMAALGLKHINHSVVHQDTPVVRGMVNVVRHMVSVEEVAGE* 61 Mw = 6531 Da
                          (*)********************:****************************************
```

CsbD stress response protein alignment

```
Crouxii_FRC0190T          (M)SDFENKIEEFGGKAKEAVGEATENEHLADEGRADQTKADIKQAVSDAGDKIKGAADKVL 60
Cbelfantii_FRC0043T       (M)SDFENKIEELGGKAKEAVGDATENEQLADEGRADQTKADVKQAISDAGDKIKGAADKVL 60
Cdiphtheriae_NCTC11397T   (M)SDFENKIEELGGKAKEAVGEATENEQLADEGRADQTKADVKQAVSDAGDKIKGAADKVL 60
                          (*)*********:*********:*****:*************:***:***************

Crouxii_FRC0190T           GSFQKDEEN* 69  Mw = 7282 Da
Cbelfantii_FRC0043T        GSFQKDEEN* 69  Mw = 7225 Da
Cdiphtheriae_NCTC11397T    GSFQKDEEN* 69  Mw = 7225 Da
                           *********
```

S20 ribosomal protein alignment

```
Crouxii_FRC0190T           (M)ANIKSQKKRILTNEKARQRNQAIRSAVRTEIRKFRAAVAAGDKAAAETQLRVASRALDK 60
Cbelfantii_FRC0043T        (M)ANIKSQKKRILTNEKARQRNQAIRSAVRTEIRKFRAAVAAGDKAAAEAQLRVASRALDK 60
Cdiphtheriae_NCTC11397T    (M)ANIKSQKKRILTNEKARQRNQAIRSAVRTEIRKFRAAVAAGDKAAAEAQLRVASRALDK 60
                           (*)*********************************************:************

Crouxii_FRC0190T            SVTKGVFHRNNAANKKSNMAHALNKMA* 87  Mw = 9493 Da
Cbelfantii_FRC0043T         SVTKGVFHRNNAANKKSNMAHALNKMA* 87  Mw = 9468 Da
Cdiphtheriae_NCTC11397T     SVTKGVFHRNNAANKKSNMAHALNKMA* 87  Mw = 9468 Da
                            ***************************
```

Amino acid substitutions leading to mass changes are represented in bold.
Mw, molecular weight.

Table S1 : Strains used in this study and their characteristics

Isolate &	Species	biovar #	Isolation year	Country	Geographic origin @	Isolation source	Disease	Polymicrobial infection	*tox* gene
FRC0043^{T}	*C. belfantii*	Belfanti	2009	France	Corrèze, Brives	Pharyngeal membrane	Laryngitis		Negative
06-4305	*C. belfantii*	Belfanti	2006	France	Rhône, Lyon	Expectoration	Bronchopathy		Negative
00-0744	*C. belfantii*	Belfanti	2000	France	Calvados, Caen	Expectoration	Cystic fibrosis		Negative
FRC0074	*C. belfantii*	Belfanti	2011	France	Côte d'Or, Dijon	Expectoration	Cystic fibrosis		Negative
FRC0223	*C. belfantii*	Belfanti	2014	France	Pas-de-Calais, Coquelles	Sinusal swab	Sinusitis		Negative
05-3187	*C. belfantii*	Belfanti	2005	France	Seine-Maritime, Rouen	Nasal swab	Rhinitis		Negative
FRC0250	*C. belfantii*	Belfanti	2014	France	Bas-Rhin, Strasbourg	Bronchoalveolar wash	Pneumonia		Negative
FRC0301	*C. belfantii*	Belfanti	2015	France	Calvados, Lisieux	Expectoration	n.a.		Negative
NCTC 13129	*C. diphtheriae*	Gravis	1997	United Kingdom	unknown	Pharyngeal membrane	Diphtheria		Positive
NCTC 11397^{T}	*C. diphtheriae*	Gravis	1969	USA	New York, USA	n.a.	n.a.		Negative
FRC0336	*C. diphtheriae*	Gravis	2015	France	Ille-et-Vilaine, Rennes	Cutaneous	Leishmaniasis	*Leishmania spp.*	Positive
FRC0304	*C. diphtheriae*	Gravis	2015	France	La Réunion, St Denis	Cutaneous	Bullous skin lesion		Negative
FRC0375	*C. diphtheriae*	Mitis	2015	France	Oise, Creil	Cutaneous	Ankle ulceration		Positive
FRC0432	*C. diphtheriae*	Mitis	2016	France	Seine-et-Marne, Vaires sur Marne	Cutaneous	Purulent scalp skin injury		Negative
FRC0157	*C. diphtheriae*	Mitis	2013	France	Paris	Cutaneous	Left ankle wound	*S. pyogenes*	Negative
FRC0132	*C. diphtheriae*	Mitis	2012	France	Yvelines, Le Chesnay (return from Mali)	Cutaneous	Necrotic lesions		Negative
FRC0036	*C. diphtheriae*	Mitis	2009	France	Mayotte, Mamoudzou	Cutaneous	Burn wound		Negative
FRC0154	*C. diphtheriae*	Mitis	2012	France	Haut-Rhin, Colmar	Cutaneous	Cutaneous infection	*S. pyogenes*	Positive
FRC0049	*C. diphtheriae*	Mitis	2009	France	Mayotte, Mamoudzou	Cutaneous	Genital lesion		Positive
FRC0430	*C. diphtheriae*	Mitis	2016	France	Rhône, Bron	Cutaneous	Leg ulcerations	*S. pyogenes*	Positive
FRC0436	*C. diphtheriae*	Mitis	2016	France	Ille-et-Vilaine, Rennes	Cutaneous	Cutaneous infection	*S. pyogenes*	Positive
FRC0071	*C. rouxii*	Belfanti	2011	France	Haute-Garonne, Toulouse	Cutaneous	Leg ulceration on chronic arteritis - diabetes		Negative
FRC0190^{T}	*C. rouxii*	Belfanti	2013	France	Lot, Cahors	Cutaneous	Foot ulceration, chronic arteritis		Negative
FRC0284	*C. rouxii*	Belfanti	2015	France	Rhône, Lyon	Cutaneous	Limb amputation - vasculitis		Negative
FRC0297	*C. rouxii*	Belfanti	2015	France	Herault, Beziers	Ascitic fluid	Spontaneous peritonitis		Negative
FRC0412	*C. rouxii*	Belfanti	2016	France	Lot, Cahors	Cutaneous	Purulent orbital cellulitis (dog)		Negative
FRC0527	*C. rouxii*	Belfanti	2017	France	Savoie, Chambery	Cutaneous	Foot ulceration on chronic arteritis		Negative
ATCC 19410^{T}	*C. pseudotuberculosis*	not applicable	1931	n.a.	South America	Infected gland (sheep)	n.a.		Negative
NCTC 7910^{T}	*C. ulcerans*	not applicable	1948	United Kingdom	n.a.	Throat	n.a.		Negative

Biovar of *C. diphtheriae* as classically defined

& FRC: collection of the French National Reference Center for the Corynebacteria of the C. diphtheriae complex; NCTC: National Collection of Type Cultures (Public Health England); ATCC: American Type Culture Collection

@ Geographic origin for French isolates is given as "French Department, city"

$ Sequence type is defined at https://pubmlst.org/cdiphtheriae/

n.a.: not available

a 'T' at the end of the strain name indicates taxonomic type strains

£: negative using API coryne (bioMerieux); intermediate using Rosco Dignostica test (see supplementary Figure)

Sequence type $	Nitrate	Glycogen	Maltose	Urease	Genome size	G+C%	Number of contigs	Contig N50	Coverage depth (%)	Genome sequence accession number	Reference
106	Negative	Negative	**Positive**	Negative	2609417	53.6	156	40698	66	ERS1867695	Dazas *et al.* 2018 IJSEM
177	Negative	Negative	**Positive**	Negative	2629949	53.7	165	32494	91	ERS1867696	Dazas *et al.* 2018 IJSEM
106	Negative	Negative	**Positive**	Negative	2736533	53.9	140	42875	92	ERS1867697	Dazas *et al.* 2018 IJSEM
208	Negative	Negative	**Positive**	Negative	2642382	53.7	159	35757	88	ERS1867698	Dazas *et al.* 2018 IJSEM
365	Negative	Negative	**Positive**	Negative	2765539	53.7	185	33077	76	ERS1867699	Dazas *et al.* 2018 IJSEM
107	Negative	Negative	**Positive**	Negative	2748440	53.8	146	37603	91	ERS1867700	Dazas *et al.* 2018 IJSEM
92	Negative	Negative	**Positive**	Negative	2720797	53.7	159	39690	90	ERS1867701	Dazas *et al.* 2018 IJSEM
106	Negative	Negative	**Positive**	Negative	2756747	53.9	190	38471	63	ERS1867702	Dazas *et al.* 2018 IJSEM
8	**Positive**	**Positive**	**Positive**	Negative	2488635	53.5	1	2488635	100	BX248353.1	Dazas *et al.* 2018 IJSEM
26	**Positive**	**Positive**	**Positive**	Negative	2463666	53.5	1	2463666	100	LN831026.1	Dazas *et al.* 2018 IJSEM
412	**Positive**	**Positive**	**Positive**	Negative	2356118	53.4	30	153402	91	ERS1867704	Dazas *et al.* 2018 IJSEM
102	**Positive**	**Positive**	**Positive**	Negative	2378091	53.5	31	164345	92	ERS1867705	Dazas *et al.* 2018 IJSEM
389	**Positive**	Negative	**Positive**	Negative	2547821	53.6	65	127569	86	ERS1867703	Dazas *et al.* 2018 IJSEM
432	**Positive**	Negative	**Positive**	Negative	2523954	53.7	34	251189	85	ERS1867706	Dazas *et al.* 2018 IJSEM
167	**Positive**	Negative	**Positive**	Negative	2443018	53.6	39	142469	87	ERS1867707	Dazas *et al.* 2018 IJSEM
251	**Positive**	Negative	**Positive**	Negative	2422441	53.7	59	88375	88	ERS1867708	Dazas *et al.* 2018 IJSEM
97	**Positive**	Negative	**Positive**	Negative	2413085	53.6	34	202424	84	ERS1867709	Dazas *et al.* 2018 IJSEM
91	**Positive**	Negative	**Positive**	Negative	2506294	53.6	46	124932	70	ERS1867710	Dazas *et al.* 2018 IJSEM
184	**Positive**	Negative	**Positive**	Negative	2426231	53.5	35	230251	92	ERS1867711	Dazas *et al.* 2018 IJSEM
217	**Positive**	Negative	**Positive**	Negative	2474468	53.7	45	171058	92	ERS1867712	Dazas *et al.* 2018 IJSEM
217	**Positive**	Negative	**Positive**	Negative	2474070	53.7	49	137997	88	ERS1867713	Dazas *et al.* 2018 IJSEM
181	Negative	Negative	Negative £	Negative	2405963	53.2	30	201570	61	ERS3795539 (rpoB: MN542347; 16S gene: MN535982)	This study
181	Negative	Negative	Negative £	Negative	2417020	53.3	31	215924	78	ERS3795540 (rpoB: MN542348; 16S gene: MN535983)	This study
181	Negative	Negative	Negative £	Negative	2414177	53.2	30	191771	68	ERS3795541 (rpoB: MN542349; 16S gene: MN535984)	This study
537	Negative	Negative	Negative £	Negative	2314268	53.2	35	174067	71	ERS3795542 (rpoB: MN542350; 16S gene: MN535985)	This study
538	Negative	Negative	Negative £	Negative	2390838	53.3	30	191901	80	ERS3795543 (rpoB: MN542351; 16S gene: MN535986)	This study
181	Positive	Negative	Negative £	Negative	2398149	53.2	31	201570	76	ERS3795544 (rpoB: MN542352; 16S gene: MN535987)	This study
NA	Negative	**Positive**	**Positive**	**Positive**	2337763	52.2	1	2337763	100	CP021251.1	PMID: 13882624 (Cummins, 1962)
NA	Negative	Negative	**Positive**	**Positive**	2463666	53.5	1	2463666	100	LN831026.1	PMID: 7729671 (Riegel et al., 1995)

Table S2: blastn results for *narKGHIJ* nitrate reduction gene cluster *																
Isolate &	Species	biovar #	*narI (DIP_RS13820)*	*narJ (DIP_RS13825)*	*narH (DIP_RS13830)*	*narG (DIP_RS13835)*	*narK (DIP_RS13845)*									
FRC0071	*C. rouxii*	Belfanti	Negative	Negative	Negative	Negative	Negative									
FRC0190^{T}	*C. rouxii*	Belfanti	Negative	Negative	Negative	Negative	Negative									
FRC0284	*C. rouxii*	Belfanti	Negative	Negative	Negative	Negative	Negative									
FRC0297	*C. rouxii*	Belfanti	Negative	Negative	Negative	Negative	Negative									
FRC0412	*C. rouxii*	Belfanti	Negative	Negative	Negative	Negative	Negative									
FRC0527	*C. rouxii*	Belfanti	Negative	Negative	Negative	Negative	Negative									
00-0744	*C. belfantii*	Belfanti	Negative	Negative	Negative	Negative	Negative									
05-3187	*C. belfantii*	Belfanti	Negative	Negative	Negative	Negative	Negative									
06-4305	*C. belfantii*	Belfanti	Negative	Negative	Negative	Negative	Negative									
FRC0043^{T}	*C. belfantii*	Belfanti	Negative	Negative	Negative	Negative	Negative									
FRC0074	*C. belfantii*	Belfanti	Negative	Negative	Negative	Negative	Negative									
FRC0223	*C. belfantii*	Belfanti	Negative	Negative	Negative	Negative	Negative									
FRC0250	*C. belfantii*	Belfanti	Negative	Negative	Negative	Negative	Negative									
FRC0301	*C. belfantii*	Belfanti	Negative	Negative	Negative	Negative	Negative									
FRC0304	*C. diphtheriae*	Gravis	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**									
FRC0336	*C. diphtheriae*	Gravis	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**									
NCTC 13129	*C. diphtheriae*	Gravis	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**									
NCTC 11397^{T}	*C. diphtheriae*	Gravis	Negative	Negative	**Positive**	**Positive**	Negative									
FRC0375	*C. diphtheriae*	Mitis	Negative	Negative	**Positive**	**Positive**	Negative									
FRC0432	*C. diphtheriae*	Mitis	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**									
FRC0157	*C. diphtheriae*	Mitis	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**									
FRC0132	*C. diphtheriae*	Mitis	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**									
FRC0036	*C. diphtheriae*	Mitis	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**									
FRC0154	*C. diphtheriae*	Mitis	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**									
FRC0049	*C. diphtheriae*	Mitis	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**									
FRC0430	*C. diphtheriae*	Mitis	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**									
FRC0436	*C. diphtheriae*	Mitis	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**									
NCTC 7910^{T}	*C. ulcerans*	not applicable	Negative	Negative	Negative	Negative	Negative									
ATCC 19410^{T}	*C. pseudotuberculosis*	not applicable	Negative	Negative	Negative	Negative	Negative									
# Biovar of *C. diphtheriae* as classically defined																
& FRC: collection of the French National Reference Center for the Corynebacteria of the C. diphtheriae complex; NCTC: National Collection of Type Cultures (Public Health England); ATCC: American Type Culture Collection																
a 'T' at the end of the strain name indicates taxonomic type strains																
* Blastn results were considered positive when identity was >70% and coverage was >90%																

Table S3. Antimicrobial susceptibility data for six *C. rouxii* isolates

Strain	Penicillin (10 IU)		Penicillin (1 IU)	
	Zone diameter (mm) / CMI (g/L)	SIR	Zone diameter (mm) / CMI (g/L)	SIR
FRC0071	28.00 / 0.125	S	N.T	
FRC0190T	25.52 / 0.125	S	N.T	
FRC0284	N.T		14.62 / 0.19	R
FRC0297	N.T		19.04 / 0.064	S
FRC0412	N.T		6 /0.19	R
FRC0527	N.T		18 /0.19	R

N.T.: Not tested; SIR: interpretation as susceptible (S), intermediate (I) or resistant (R). Note tha

Some zone diameter values were defined with an electronic reader (with two decimals); others

Amoxicillin		Oxacillin		Cefotaxime
Zone diameter (mm) / CMI (g/L)	SIR	Zone diameter (mm) / CMI (g/L)	SIR	Zone diameter (mm) / CMI (g/L)
34.00	S	27.00	S	32.00
36.28	S	21.68	S	29.94
33.90	S	21.06	S	26.64
40.48	S	29.14	S	34.06
24.00 / 0.25	S	24.00	S	25.00 / 1
31	S	26	S	37

at the CMI was determined for all resistant zone diameter values, but only for some suseptible ones

were read manually (no decimals)

	Imipenem		Erythromycin	
SIR	Zone diameter (mm) / CMI (g/L)	SIR	Zone diameter (mm) / CMI (g/L)	SIR
S	41.00	S	34.00	S
S	41.80	S	41.58	S
S	38.70	S	37.76 / <0.016	S
S	46.24	S	40.76	S
R	38.00	S	33.00	S
S	44	S	41	S

Clarithromycin		Azithromycin		Spiramycin
Zone diameter (mm) / CMI (g/L)	SIR	Zone diameter (mm) / CMI (g/L)	SIR	Zone diameter (mm) / CMI (g/L)
39.00	S	33.00	S	31.00
40.78	S	38.40	S	> 24
38.50 / <0.016	S	37.28 / 0.023	S	32.00
38.84	S	39.22	S	35.86
36.00	S	32.00	S	30.00
44	S	39	S	39

	Pristinamycin		Kanamycin	
SIR	Zone diameter (mm) / CMI (g/L)	SIR	Zone diameter (mm) / CMI (g/L)	SIR
S	36.00	S	27.00	S
S	38.18	S	23.30	S
S	35.56	S	25.08	S
S	40.06	S	28.66	S
S	33.00	S	24.00 / 1	S
S	42	S	31	S

Gentamicin		Rifampicin		Tetracycline
Zone diameter (mm) / CMI (g/L)	SIR	Zone diameter (mm) / CMI (g/L)	SIR	Zone diameter (mm) / CMI (g/L)
27.00	S	37.00	S	32.00
23.64	S	> 19	S	34.88
25.22	S	34.78	S	31.38
52.46	S	38.10	S	35.32
23.20 / 0.125	S	32.00	S	29.00
32	S	39	S	36

	Ciprofloxacin		Clindamycin	
SIR	Zone diameter (mm) / CMI (g/L)	SIR	Zone diameter (mm) / CMI (g/L)	SIR
S	30.00	S	29.00	S
S	33.96	S	30.70	S
S	33.98	S	29.54 / 0.064	S
S	41.28	S	33.74	S
S	28.00	S	26.00	S
S	38	S	30	S

Sulfonamide		Trimethoprim		Trimethoprim-Sulphamethoxazole
Zone diameter (mm) / CMI (g/L)	SIR	Zone diameter (mm) / CMI (g/L)	SIR	Zone diameter (mm) / CMI (g/L)
		N.T	N.T	28.00
29.40	S	37.28	S	36.50
28.16	S	35.20 / 0.125	S	31.26
23.62	S	36.56	S	33.18
23.00	S	29.00	S	31.00
29	S	21	S	28

	Vancomycin		Fosfomycin	
SIR	Zone diameter (mm) / CMI (g/L)	SIR	Zone diameter (mm) / CMI (g/L)	SIR
S	24.00	S	6.00	R
S	26.48	S	6.00 / 1024	R
S	19.84	S	6.00 / >1024	R
S	20.78	S	6.00 / >1024	R
S	16.00 / 0.75	R	6.00 / >1024	R
S	21	S	6.00 / >1024	R

Linezolid		Moxifloxacin	
Zone diameter (mm) / CMI (g/L)	SIR	Zone diameter (mm) / CMI (g/L)	SIR
N.T.		N.T.	
N.T.		N.T.	
36.54	S	34.00	S
43.14	S	41.28	S
28.00	S	31.00	S
37	S	41	S

Table S4. MALDI-TOF mass spectrometry peaks useful to discriminate *C. rouxii* from *C. diphtheriae* and *C. belfantii*.

Present in	Peak Position (*m/z*)[1]	Sensitivity[2] [95% CI]	Specificity[3] [95% CI]	Possible Proteins
C. diphtheriae* and *C. belfantii	**3263**[4]	19/21, 90.5% [69.6%-98.8%]	6/6, 100% [54.1%-100%]	Ribosomal protein L30
	6529	21/21, 100% [83.89%-100%]	6/6, 100% [54.1%-100%]	
	3610[4]	16/21, 76.2% [52.8%-91.8%]	6/6, 100% [54.1%-100%]	Stress response protein CsbD
	7222	18/21, 85.7% [63.7%-97%]	6/6, 100% [54.1%-100%]	
	4732[4]	21/21, 100% [83.89%-100%]	6/6, 100% [54.1%-100%]	Ribosomal protein S20
	9463	20/21, 95.2% [76.2%-99.9%]	6/6, 100% [54.1%-100%]	
C. rouxii	**3255**[4]	6/6, 100% [54.1%-100.0%]	21/21, 100% [83.9%-100.0%]	Ribosomal protein L30
	6512	6/6, 100% [54.1%-100.0%]	21/21, 100% [83.9%-100.0%]	
	3640[4]	5/6, 83.3% [35.9%-99.58%]	20/21, 95.2% [76.2%-99.9%]	Stress response protein CsbD
	7281	6/6, 100% [54.1%-100.0%]	21/21, 100% [83.9%-100.0%]	
	4748[4]	6/6, 100% [54.1%-100.0%]	21/21, 100% [83.9%-100.0%]	Ribosomal protein S20
	9495	5/6, 83.3% [35.9%-99.58%]	21/21, 100% [83.9%-100.0%]	

CI, confidence interval
[1]Position in the spectra using a tolerance of ±0.055%.
[2]Proportion of true positives that are correctly identified as such.
[3]Proportion of true negatives that are correctly identified as such.
[4]Double charged ion.