NOTE

***Clostridium felsineum* and *Clostridium acetobutylicum* are two distinct species that are phylogenetically closely related**Elena Tamburini,¹ Simona Daly,¹ Ulrike Steiner,² Claudia Vandini¹ and Giorgio Mastromei¹¹ Department of Animal Biology and Genetics, University of Florence, Via Romana 17, 50125 Florence, Italy² DSMZ—German Collection of Microorganisms and Cell Cultures GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany

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The gene sequences encoding the 16S rRNA of *Clostridium felsineum* DSM 794^T and NCIMB 10690^T were determined. Both sequences exhibited a relatively very low degree of similarity to the previously determined 16S rRNA gene sequence from *C. felsineum* DSM 794^T. *C. felsineum* is a member of the major *Clostridium* cluster, cluster I, and is phylogenetically closely related to *Clostridium acetobutylicum*. DNA–DNA hybridization results clearly indicated that *C. felsineum* and *C. acetobutylicum* belong to distinct species.

Keywords: phylogeny, *Clostridium felsineum*, *Clostridium acetobutylicum*, 16S rRNA analysis

The genus *Clostridium* is a large and phenotypically heterogeneous taxon including anaerobic bacteria that form endospores and do not carry out dissimilatory sulfate reduction (Cato *et al.*, 1986). Great advances have been made in elucidating the phylogenetic relationships among clostridia by using 16S rRNA gene (16S rDNA) analysis (Stackebrandt, 1992; Collins *et al.*, 1994; Keis *et al.*, 1995). Molecular data reveal that phenotype-based distinctions (e.g. proteolytic versus saccharolytic) between members of the genus have no phylogenetic basis (Rainey & Stackebrandt, 1993); in addition, there is some phylogenetic incoherence in the genus, formed by several deeply branched lineages, some of which also include non-clostridial species (Johnson & Francis, 1975; Collins *et al.*, 1994). The largest and phylogenetically best-defined cluster [group I in Johnson & Francis (1975) and cluster I in Collins *et al.* (1994)], which includes *Clostridium butyricum*, the type species of the genus, has been considered the basis of a redefined genus *Clostridium*, whereas the remaining clostridia have lost their status as members of the genus (Collins *et al.*, 1994).

The species *Clostridium felsineum* has been classified in this genus on the basis of its phenotypic traits and has been included in the saccharolytic *Clostridium* group because of its ability to ferment carbohydrates, such as

pectin (Gottschalk *et al.*, 1981). This bacterium plays an important role in the pectinolytic degradation of plant material during retting (Potter & McCoy, 1952). *C. felsineum* strains have been isolated from retting flax, from soil in the United States (Smith, 1975) and Antarctica (Miwa, 1975a, b) and from human faeces (Finegold *et al.*, 1983). However, 16S rDNA sequence analysis included *C. felsineum* in the suprageneric association designated cluster XI by Collins *et al.* (1994); as a result, *C. felsineum* was considered as a species distinct from the genus *Clostridium sensu stricto*.

In this work, the sequences of genes encoding the 16S rRNA of two equivalent strains of *C. felsineum*, DSM 794^T and NCIMB 10690^T, were determined. Analysis of the almost complete 16S rDNA showed that *C. felsineum* DSM 794^T is a member of cluster I and phylogenetically closely related to *Clostridium acetobutylicum*. Thus, this species should be considered as a member of the genus *Clostridium*, not as distinct from it.

Two equivalent strains of *C. felsineum* were studied. Strain DSM 794^T was obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) and strain NCIMB 10690^T was from the NCIMB (National Collections of Industrial, Food and Marine Bacteria, Aberdeen, UK). Bacterial strains were cultured on agar plates of medium C (0.5% yeast extract, 0.5% peptone, 1%, w/v, tryptone, 0.05% cysteine) sup-

Abbreviation: RDP, Ribosomal Database Project.

The GenBank accession numbers for the 16S rRNA sequences of *Clostridium felsineum* DSM 794^T and NCIMB 10690^T are AF270502 and AF270501, respectively.

Table 1. Sequence similarity between the 16S rRNA genes of *C. felsineum* DSM 794^T and representative species of the genus *Clostridium*

Abbreviations: ATCC, American Type Culture Collection, Manassas, VA, USA; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; NCDO, National Collection of Dairy Organisms, Reading, UK (now at NCIMB); NCIMB, National Collections of Industrial, Food and Marine Bacteria, Aberdeen, UK.

Species	Strain	Sequence similarity (%) to <i>C. felsineum</i>
<i>C. acetobutylicum</i>	ATCC 824 ^T	99.1
<i>C. acetobutylicum</i>	DSM 1731	98.7
<i>C. acetobutylicum</i>	NCDO 1712	98.0
<i>Clostridium sardiniense</i>	ATCC 33455 ^T	94.7
<i>Clostridium collagenovorans</i>	DSM 3089 ^T	93.7
<i>Clostridium</i> sp.	DSM 1975	92.8
<i>Clostridium pascui</i>	DSM 10365 ^T	92.7
<i>Clostridium argentinense</i>	ATCC 27322 ^T	92.6
<i>Clostridium tetanomorphum</i>	NCIMB 11547	92.5
<i>Clostridium subterminale</i>	ATCC 25774 ^T	92.1
<i>Clostridium pasteurianum</i>	ATCC 6013 ^T	92.0
' <i>Clostridium kainantoi</i> '	DSM 523	91.5
<i>C. acetobutylicum</i>	NCP 262	91.2
' <i>Clostridium corinoform</i> '	DSM 5906	91.1
' <i>Clostridium saccharoperbutylacetonicum</i> '	ATCC 13564	91.1
<i>Clostridium beijerinckii</i>	NCIMB 8052 ^T	91.0
<i>Clostridium puniceum</i>	DSM 2619 ^T	90.9
<i>C. butyricum</i>	NCIMB 8082	90.8
<i>C. butyricum</i>	ATCC 43755	90.6
<i>Clostridium cellulovorans</i>	DSM 3052 ^T	90.5

plemented with 2% (w/v) glucose at 37 °C in a CO₂ atmosphere (Oxoid AnaeroGen kit AN25).

Bacterial strains were isolated on agar plates; a single colony was picked and dissolved in 100 µl sterile water and the cells were then incubated for 30 min at 37 °C with 1 mg lysozyme (Sigma) and thereafter for 45 min at 37 °C with 50 µg Pronase E (Sigma) and 1% (w/v) SDS. DNA was extracted with phenol/chloroform/isoamyl alcohol and precipitated with sodium chloride and ethanol. Finally, DNA was dissolved in 10 µl sterile water and used for the PCR. The 16S rDNA amplification was performed using primers P0f and P6r, designed on the basis of conserved eubacterial sequences and corresponding to positions 7–27 (P0f) and 1514–1495 (P6r) (*Escherichia coli* 16S rRNA numbering) (Lane, 1991). The volume of the PCR was 20 µl, containing 2 µl DNA sample, 1 U *Taq* DNA polymerase (Gibco BRL), 250 µM each dNTP, 1 µM each PCR primer, 1.5 mM MgCl₂ and 2 µl 10 × PCR buffer (Gibco BRL). A 35-cycle touch-down PCR program (30 s denaturation at 95 °C, 30 s annealing at temperatures reduced by 5 °C from 60 to 50 °C every five cycles, 4 min elongation at 72 °C and two 10 min final elongation steps at 72 and 60 °C) was performed using the GenAmp PCR System 9600 (Perkin-Elmer). The amplified 16S rDNA was purified from the reaction mixture by agarose gel (1.2%, w/v) electro-

phoresis and was extracted from the agarose by using the CONCERT Rapid Gel Extraction System (Gibco BRL). Determination of the 16S rDNA nucleotide sequence was performed by the sequencing service Centro Genoma Vegetale (ENEA, Italy). The sequencing primers used were the universal eubacterial primers P0f and P6r, described above.

A total of 1439 bp of the 16S rDNA sequences from *C. felsineum* DSM 794^T and NCIMB 10690^T were compared using the program CLUSTAL W (Thompson *et al.*, 1994). The two sequences showed 100% sequence identity. However, the calculated similarity value was 85.0% when these sequences were compared with the 16S rDNA sequence for *C. felsineum* DSM 794^T present in the GenBank database (accession no. X77851; Collins *et al.*, 1994). (Similarity values were calculated in the overlapping region, and no gaps – insertions or deletions – were included in the match/mismatch count in the analysis.) Since the 16S rDNA sequences of *C. felsineum* DSM 794^T and NCIMB 10690^T determined in this study were identical and different from that obtained by Collins *et al.* (1994), we conclude that this inconsistency is probably due to the fact that Collins *et al.* (1994) sequenced a different strain.

The 16S rDNA sequence of *C. felsineum* DSM 794^T was compared with the prokaryotic small-subunit

Fig. 1. Unrooted phylogenetic tree based on 16S rDNA comparisons showing the position of *C. felsineum* DSM 794^T and representative species of the genus *Clostridium*. The branching pattern was generated by neighbour-joining methods and the bootstrap values, shown at the nodes, were calculated from 1000 replicates. Bootstrap probabilities that were less than 50% are omitted from the figure. Bar, 0.01 substitutions per nucleotide position. The GenBank accession numbers for the 16S rRNA sequences are given after the strain numbers. *C.*, *Clostridium*.

rRNA sequence database of the Ribosomal Database Project II (RDP II) by using the program SIMILARITY MATRIX version 1.1. Only the 20 most similar RDP sequences that were more than 80% complete were included in the analysis. A total of 1405 alignment positions were analysed. The most similar RDP sequence was the 16S rDNA sequence of *C. acetobutylicum* ATCC 824^T, which showed a similarity value of 99.1% (Keis *et al.*, 1995; Wilkinson *et al.*, 1995). *C. felsineum* DSM 794^T exhibited a high level of 16S rDNA similarity to other *C. acetobutylicum* strains: DSM 1731 and NCDO 1712 showed similarity values of 98.7% and 98.0%, respectively. All of the best-matching RDP sequences (Table 1) belonged to the *Clostridium* species included in cluster I by Collins *et al.* (1994).

Estimation of the phylogenetic position of *C. felsineum* DSM 794^T was performed by constructing phylogenetic trees based on 16S rDNA comparisons. Sequences from *C. felsineum* DSM 794^T and other members of the genus *Clostridium* were aligned by the CLUSTAL W software (Thompson *et al.*, 1994). The alignment was corrected manually, eliminating two ambiguous portions from the dataset. The final dataset included a total of 1286 positions between positions 100 and 1431 (according to *E. coli* numbering). The 5' end of the 16S rRNA gene was omitted to avoid possible misalignment of variable region VI of the clostridial 16S rDNA (Collins *et al.*, 1994; Keis *et al.*, 1995).

Phylogenetic trees were inferred using three algorithms, namely the neighbour-joining (Saitou & Nei, 1987), least-squares (Fitch & Margoliash, 1967) and maximum-parsimony (Kluge & Farris, 1969) methods. An evolutionary distance matrix for distance methods was generated as described by Jukes & Cantor (1969). The PHYLIP package was used for making trees with the three algorithms (Felsenstein, 1993). Bootstrap analysis (1000 replicates) was used to test the tree topology of the neighbour-joining-method data (Felsenstein, 1993). The position of *C. felsineum* DSM 794^T in the phylogenetic tree was not affected by the tree-making algorithms: this species clustered together with three *C. acetobutylicum* strains (NCDO 1712, DSM 1731 and ATCC 824^T) in all trees (Fig. 1). This close relationship was supported by a high bootstrap value (100%).

The 16S rDNA sequence analysis and estimation of phylogenetic relationships showed that *C. felsineum* was erroneously included in the heterogeneous cluster, cluster XI (Collins *et al.*, 1994). In fact, our data place this species unequivocally in cluster I, it being closely related to *C. acetobutylicum*. This phylogenetic clade was proposed by Collins *et al.* (1994) as the basis for a redefined genus *Clostridium*. Thus, molecular data confirm the previous physiology-based classification of this species as a member of the genus *Clostridium*. Because of the high 16S rDNA similarity value (99.1%) between *C. felsineum* and *C. acetobutylicum*, DNA–DNA similarity studies were performed to clarify the species status of the two clostridia.

DNA was isolated by chromatography on hydroxyapatite by the procedure of Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970), with the modification described by Huß *et al.* (1983) and Escara & Hutton (1980), using a Gilford System model 2600 spectrometer equipped with a Gilford model 2527-R thermo-programmer and plotter. Renaturation rates were computed with the TRANSFER.BAS program (Jahnke, 1992). *C. acetobutylicum* DSM 792^T had reassociation values of 33.8% with *C. felsineum* DSM 794^T and of 30.7% with *C. felsineum* NCIMB 10690^T. The DNA–DNA hybridization results clearly indicated that *C. felsineum* and *C. acetobutylicum* belong to distinct species.

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