Reclassification of *Clostridium proteoclasticum* as *Butyrivibrio proteoclasticus* comb. nov., a butyrate-producing ruminal bacterium

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It is proposed that *Clostridium proteoclasticum* be reclassified as *Butyrivibrio proteoclasticus* comb. nov. on the basis of phylogenetic position, DNA G+C content and physiological traits. Phylogenetic analyses based on 16S rRNA gene sequences from an extensive range of taxa within clostridial rRNA subcluster XIVa grouped *C. proteoclasticum* together with isolates of the genus *Butyrivibrio*, though this species was genetically distinct from the extant *Butyrivibrio* species examined. The DNA G+C content of *C. proteoclasticum* was originally erroneously reported as 28 mol%. However the genome sequence of the type strain of *C. proteoclasticum*, strain B316^T, and HPLC analysis estimate the DNA G+C content as 40 mol%, which is within the range reported for strains of *Butyrivibrio*. *C. proteoclasticum* was distinguishable from other species of the genus *Butyrivibrio* as the 16S rRNA gene from strain B316^T shared less than 97% sequence similarity with sequences from the type strains of *Butyrivibrio* species. *C. proteoclasticum* was also able to convert linoleic acid to stearic acid, in contrast to other species of *Butyrivibrio*. Physiological characteristics, including carbon source utilization, volatile fatty acid production and proteinase activities, were assessed for a panel of representative strains of the genera *Butyrivibrio* and *Pseudobutyribrio* and *C. proteoclasticum*. These data, together with the phylogenetic analyses, support the reclassification of *Clostridium proteoclasticum* as a separate species within the genus *Butyrivibrio*, *Butyrivibrio proteoclasticus* comb. nov. (type strain B316^T=ATCC 51982^T=DSM 14932^T).

The original principal characteristics of members of the genus *Butyrivibrio* (family *Lachnospiraceae*) (Bryant & Small, 1956; Bryant, 1986) are that they possess curved, strictly anaerobic, non-spore-forming, rod-shaped cells that are generally motile by means of one or more polar to subpolar flagella. They possess an atypical Gram-positive ultrastructure, although stain Gram-negative (Bryant, 1986), and have a fermentative metabolism with butyrate

as the main product. Strains of the genus *Butyrivibrio* are commonly isolated from the rumen and the mammalian gastrointestinal tract and have a DNA G+C content of 36–42 mol% (Bryant, 1986). Phylogenetic analyses have revealed that the genus *Butyrivibrio* is a diverse polyphyletic assemblage (Willems *et al.*, 1996) for which three species: *Butyrivibrio fibrisolvens* (the type species), *Butyrivibrio crossotus* and *Butyrivibrio hungatei* are currently recognized (Bryant & Small, 1956; Kopečný *et al.*, 2003; Moore *et al.*, 1976). *Clostridium proteoclasticum*, exemplified by the type strain B316^T (=ATCC 51982^T=DSM 14932^T), was first proposed in 1996 (Attwood *et al.*, 1996) to describe a strictly anaerobic Gram-positive bacterium with straight to slightly curved rod-shaped cells bearing a single subpolar flagellum, isolated from the rumen of a New Zealand cow (Attwood & Reilly, 1995). Accordingly, this highly proteolytic bacterium was originally described as *Butyrivibrio*-like (Attwood & Reilly, 1995). However, its apparently much lower DNA G+C content (originally

Abbreviation: VFA, volatile fatty acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Butyrivibrio fibrisolvens* C219a and *Pseudobutyribrio ruminis* CF1b are EU346756 and EU346757, respectively.

Supplementary tables detailing the strains used in this study and the 16S rRNA gene sequence similarity for strains of the genera *Butyrivibrio* and *Pseudobutyribrio* and a supplementary figure showing a multi-sequence alignment of partial 16S rRNA genes from strains of the genera *Butyrivibrio*, *Pseudobutyribrio*, *Clostridium*, *Ruminococcus* and *Bacillus* generated using CLUSTAL W are available with the online version of this paper.

reported as 28 mol%, which is typical of the *Clostridium* type species, *Clostridium butyricum*, lack of motility and lack of dextrin utilization (Attwood *et al.*, 1996) distinguished strain B316^T from *B. fibrisolvens*. Furthermore, phenotypic characterization and phylogenetic analyses of 16S rRNA genes showed that strain B316^T was most closely related to *Clostridium aminophilum* within clostridial subcluster XIVa, a strongly supported cluster defined on the basis of phylogenetic analysis of 16S rRNA genes from an extensive range of clostridial strains (Collins *et al.*, 1994). The same analysis demonstrated that the genus *Clostridium* is highly diverse, with strains often intermixed with members of many different genera (Collins *et al.*, 1994). However, it is generally accepted that the genus *Clostridium* should be retained only for organisms belonging to cluster I, which contains the type species (Collins *et al.*, 1994). It was initially proposed that strain B316^T be assigned to a novel species of the genus *Clostridium*, *C. proteoclasticum*, with a view that classification was likely to change upon review of clostridial subcluster XIVa (Attwood *et al.*, 1996).

Since the original description of *C. proteoclasticum*, a body of evidence to support its reclassification to the genus *Butyrivibrio* has arisen. It has been found that the DNA G+C content originally reported for strain B316^T, 28 mol% (Attwood *et al.*, 1996), was erroneous and recent estimates now place the value in the range of that characteristic for species of the genus *Butyrivibrio*. Furthermore, phylogenetic analyses involving 16S rRNA gene sequences from a wider range of taxa that fit into clostridial subcluster XIVa, including those from the genera *Butyrivibrio* and *Pseudobutyrvibrio*, have been performed (Forster *et al.*, 1996; Kopečný *et al.*, 2001, 2003; Wallace *et al.*, 2006; Willems *et al.*, 1996). Such analyses show that *C. proteoclasticum* sequences cluster closely with sequences of the genus *Butyrivibrio*, where it is most closely related to *B. hungatei* (Kopečný *et al.*, 2003; Wallace *et al.*, 2006). In addition, PCR primers designed specifically to amplify 16S rRNA gene sequences from species of the genus *Butyrivibrio* (Mrázek & Kopečný, 2001) were able to amplify sequences from *C. proteoclasticum* isolates (Kopečný *et al.*, 2003). Despite its close relationship with *B. hungatei*, *C. proteoclasticum* can be distinguished from this species by its ability to form stearic acid from linoleic acid (Paillard *et al.*, 2007; Wallace *et al.*, 2006), a feature that has not been observed in other strains of the genera *Butyrivibrio* or *Pseudobutyrvibrio* (Paillard *et al.*, 2007).

To support the reclassification of *C. proteoclasticum* to *B. proteoclasticus* comb. nov., we present phylogenetic analyses and a phenotypic characterization of strains of the genera *Butyrivibrio* and *Pseudobutyrvibrio* (listed in Supplementary Table S1 in IJSEM Online), including the type strains *B. crossotus* DSM 2876^T, *B. fibrisolvens* ATCC 19171^T, *B. hungatei* JK 615^T, *Pseudobutyrvibrio ruminis* DSM 9787^T and *Pseudobutyrvibrio xylanivorans* Mz 5^T. Hereafter, we refer to *C. proteoclasticum* as *B. proteoclasticus*.

The evolutionary positions of strains of *B. proteoclasticus* [B316^T, UC142, H17c(SA), DSM 10301 and DSM 10304] relative to other strains of the genera *Butyrivibrio*, *Pseudobutyrvibrio*, *Clostridium* and *Ruminococcus* from clostridial cluster I and subcluster XIVa (Collins *et al.*, 1994) were inferred by 16S rRNA gene sequence-based phylogenetic analyses. Near full-length 16S rRNA gene sequences (>1400 bp) were obtained by PCR amplification and automated sequencing or were retrieved from GenBank and the Ribosomal Database II databases (Cole *et al.*, 2007). Sequence alignment and phylogenetic analyses were implemented in MEGA4 (Tamura *et al.*, 2007). A multi-sequence alignment was generated using CLUSTAL W and corrected manually for alignment ambiguities (see Supplementary Fig. S1 in IJSEM Online). A phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) with the Kimura two-parameter model (transition to transversion ratio=2.0) for distance estimation (Kimura, 1980). Bootstrap values (Felsenstein, 1985) were determined from 10 000 replicates. The resulting phylogenetic tree (Fig. 1) clearly shows that *B. proteoclasticus* sequences are located in clostridial subcluster XIVa (rather than cluster I), and group together in a well-supported clade (88% bootstrap support), which is closely related to clades containing *B. hungatei* strains and *Butyrivibrio* strains NCDO 2222 and NCDO 2434. Despite this close relationship, the 16S rRNA gene sequence similarity between the type strains of *B. proteoclasticus* and *B. hungatei* is 95.7% (see Supplementary Table S2 in IJSEM Online), which is below the cut off value (97%) that is generally accepted for two strains to be considered members of the same species (Stackebrandt & Goebel, 1994; Staley, 2006). Interestingly, strains NCDO 2222 and NCDO 2434 were also considered to be isolates of *C. proteoclasticum* on the basis of their ability to convert linoleic acid to stearic acid (Paillard *et al.*, 2007), and NCDO 2222 was earlier considered to be a *B. hungatei* strain (Kopečný *et al.*, 2003). However, the distinct position of these two strains within the 16S rRNA gene phylogeny (Fig. 1 and Wallace *et al.*, 2006) and <97% 16S rRNA gene sequence similarity to other type strains of the genus *Butyrivibrio* (see Supplementary Table S2) suggests that strains NCDO 2222 and NCDO 2434 are neither representatives of *B. proteoclasticus* nor *B. hungatei*, and possibly represent a novel species of the genus *Butyrivibrio* (and thus are referred to only as *Butyrivibrio* strains in this study).

Estimation of DNA G+C content was determined by enzymic hydrolysis of total genomic DNA, followed by HPLC analyses as previously described (Kopečný *et al.*, 2003). For each strain, the mean G+C content value was determined from two replicate experiments. Values for *B. proteoclasticus* strains (B316^T, 40.04 mol%; DSM 10301, 40.09 mol%; DSM 10304, 39.72 mol%; DSM 10305, 42.67 mol%; JK 669, 39.79 mol%, and UC 142, 41.06 mol%) were within the general range specified for species of the genus *Butyrivibrio*, in contrast to the original

Fig. 1. 16S rRNA gene phylogeny using near full-length sequences from clostridial cluster I and subcluster XIVa strains, with *Bacillus subtilis* subsp. *subtilis* as an outgroup. The phylogeny was inferred by neighbour-joining with distances calculated using the Kimura two-parameter method, implemented in MEGA4 (Tamura *et al.*, 2007). There was a total of 1525 positions in the final dataset and all positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons. The rate variation among sites was modelled with a gamma distribution (shape parameter=1). The optimal tree with the sum of branch lengths=0.95873951 is shown. Percentage bootstrap values were calculated from 10 000 replicates and are shown at nodes if >50%. GenBank accession numbers are shown in parentheses. Bar, 0.02 nucleotide substitutions per site.

reported G+C value for strain B316^T of 28 mol% (Attwood *et al.*, 1996). These values were further supported by the recent availability of the complete genome sequence of strain B316^T, where the G+C content of the main chromosome is 40.21 mol% and an estimated value for the whole genome (considering one copy of each extra-chromosomal element) is 40.01 mol% (W. J. Kelly, E. Altermann, S. C. Leahy, C. J. Yeoman, C. D. Moon and G. T. Attwood, unpublished information).

A panel of fifteen strains of the genera *Butyrivibrio* and *Pseudobutyrvibrio* was assessed for carbon source utilization, proteinase activity and volatile fatty acid (VFA) production. The ability to utilize 30 different carbon sources was tested by growth in DSM Medium 330 broth (modified by omission of all carbohydrates and containing four times the specified amount of yeast extract and double the specified volume of mineral solution), with addition of

each specific carbon source at 0.5% w/v final concentration. Cultures were inoculated in duplicate and grown under anaerobic conditions overnight at 39 °C, with results shown in Table 1. Additionally, the substrate utilization abilities of the *B. proteoclasticus* strains DSM 10301, DSM 10304, DSM 10305, JK 669, JK 722, UC 142 and X2D62 were tested in triplicate using API 20A test kits (bioMérieux; Table 1). In comparison to its closest relatives, *B. hungatei* and *Butyrivibrio* sp. NCDO 2222, *B. proteoclasticus* differed from *B. hungatei* in terms of its ability to utilize amygdalin, glycogen, mannose and melezitose. *B. proteoclasticus* also differed from strain NCDO 2222 in its ability to utilize fructose, inulin, mannose, melezitose, rhamnose and trehalose (Table 1). In contrast to the original description, strain B316^T grew on dextrin and trehalose, but growth on mannose and rhamnose was not observed as originally reported

Table 1. Carbon source utilization of strains of the genera *Butyrivibrio* and *Pseudobutyrivibrio*

Taxa: 1, *B. proteoclasticus* strain B316^T (or where indicated by asterisk, strains B316^T, DSM 10301, DSM 10304, DSM 10305, JK 669, JK 722, UC 142 and X2D62 analysed); 2, *Butyrivibrio* sp. NCDO 2222; 3, *B. hungatei* strains JK 615^T, AR10 and DSM 10295; 4, *B. fibrisolvens* strains D1^T, C211, C219a and WV1; 5, *B. crossotus* DSM 2876^T; 6, *P. ruminis* strains DSM 9787^T, DSM 10317, CF1b and CF3; 7, *P. xylanivorans* Mz 5^T. Results are expressed as a percentage of positive results.

Substrate	1	2	3	4	5	6	7
Aesculin	100	100	100	100	0	100	100
Amygdalin	100	100	0	25	0	100	100
Arabinose	75*	100	100	75	0	100	100
Cellobiose	100	100	67	100	0	100	100
Dextrin	100	100	67	100	100	100	100
Fructose	100	0	100	100	0	100	100
Galactose	100	100	67	100	0	100	100
Glucose	100	100	100	100	0	100	100
Glycerol	0*	0	0	0	0	0	0
Glycogen	100	100	0	25	100	75	100
Inositol	0	0	0	0	0	0	0
Inulin	100	0	100	100	0	100	100
Lactose	100*	100	67	100	100	100	100
Maltose	100	100	67	100	100	100	100
Mannitol	25*	0	0	0	0	0	0
Mannose	88*	0	0	50	0	50	100
Melezitose	100	0	0	50	0	25	0
Melibiose	50*	100	33	75	0	100	100
Pectin	100	100	100	100	0	100	100
Raffinose	100*	100	33	75	0	100	100
Rhamnose	75*	0	33	50	0	0	0
Ribose	0	0	0	0	0	0	0
Rutin	100	100	100	100	100	75	100
Salicin	88*	100	100	100	0	100	100
Sorbitol	0*	0	0	25	0	0	0
Starch	100	100	67	100	100	75	100
Sucrose	100*	100	100	100	0	100	100
Trehalose	62*	0	33	50	0	25	0
Xylan	100	100	100	100	100	75	100
Xylose	75*	100	100	75	0	100	100

(Attwood *et al.*, 1996). This may be due to differences in the base medium used, as carbon source utilization was previously assessed in CC medium (Attwood *et al.*, 1996; Leedle & Hespell, 1980), or it may illustrate plasticity in the expression of these utilization phenotypes. The general ability to use substrates within a single species was variable, as previously observed (Kopečný *et al.*, 2003).

Proteinase activities were assessed by monitoring azocasein degradation by bacterial cells grown in DSM Medium 704 as previously described (Attwood & Reilly, 1996). Experiments were performed in duplicate. Proteinase activity was observed from all strains examined except *B. hungatei* AR10 and *P. ruminis* DSM 9787^T. *B. proteoclasticus* displayed moderate levels of proteinase activity, along

with all strains of *B. fibrisolvens*, *B. hungatei* JK 615^T and DSM 10295, and *P. xylanivorans* DSM 14809^T.

VFA production was determined from two replicate broth cultures of each strain grown overnight in DSM Medium 704 and assessed for acetate, propionate, *iso*-butyrate, *n*-butyrate, *iso*-valerate and *n*-valerate concentrations on a HP 6890 series GC (Hewlett Packard) with α -caproate as the internal standard. All strains tested were net producers of *n*-butyrate, with strain B316^T producing the highest concentration, 15.3 mM. All strains were net utilizers of the remaining VFAs that were tested for.

Phylogenetic, genetic, morphological and physiological data all support the reclassification of *C. proteoclasticum* to the genus *Butyrivibrio* where it is genetically and physiologically distinct from extant species of the genus. Hence it is proposed that *Clostridium proteoclasticum* be reclassified as *Butyrivibrio proteoclasticus* comb. nov.

Description of *Butyrivibrio proteoclasticus* comb. nov.

Butyrivibrio proteoclasticus (pro.te.o.clas'ti.cus. N.L. n. *proteinum* protein; N.L. adj. *clasticus* breaking; N.L. adj. *proteoclasticus* protein breaking).

Basonym: *Clostridium proteoclasticum* Attwood *et al.* 1996.

Physiological data and phylogenetic analysis based on 16S rRNA gene sequences support the reclassification of *Clostridium proteoclasticum* as *Butyrivibrio proteoclasticus* comb. nov. The type strain (B316^T) has the following properties in addition to those given in the original description: growth is supported by dextrin, aesculin, pectin, raffinose, rutin and trehalose, but not by ribose. Converts linoleic acid to stearic acid. The DNA G+C content of strain B316^T is 40.01 mol% (as determined from the genome sequence).

The type strain, B316^T (=ATCC 51982^T=DSM 14932^T), was isolated from the rumen of a New Zealand cow fed a fresh, predominantly ryegrass and clover diet.

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