Clostridium proteoclasticum: a ruminal bacterium that forms stearic acid from linoleic acid

R. John Wallace, Lal C. Chaudhary, Nest McKain, Neil R. McEwan, Anthony J. Richardson, Philip E. Vercoe, Nicola D. Walker & Delphine Paillard

Rowett Research Institute, Bucksburn, Aberdeen, UK

Correspondence: R.J. Wallace, Rowett Research Institute, Bucksburn, Aberdeen, AB21 9SB, UK. Tel.: + 44 1224 716656; fax: + 44 1224 716687; e-mail: john.wallace@rowett.ac.uk

Present addresses: Lal C. Chaudhary, Centre of Advanced Studies in Animal Nutrition, Indian Veterinary Research Institute, Izatnagar - 243 122, India.
Neil R. McEwan, Institute of Rural Science, University of Wales, Llanbadarn Campus, Aberystwyth, SY23 3AL, UK.
Philip E. Vercoe, Animal Science Group, University of Western Australia, Crawley, WA 6009, Australia.
Nicola D. Walker, l'Unité de Microbiologie, INRA de Clermont-Ferrand-Theix, 63122 St Genès Champanelle, France.

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Abstract

The aim of this study was to identify ruminal bacteria that form stearic acid (18 : 0) from linoleic acid (*cis*-9,*cis*-12-18 : 2). One 18 : 0-producing isolate, P-18, isolated from the sheep rumen was similar in morphology and metabolic properties to '*Fusocillus*' spp. isolated many years ago. Phylogenetic analysis based on nearly full-length 16S rRNA gene sequence (> 1300 bp) analysis indicated that the stearate producer was most closely related to *Clostridium proteoclasticum* B316^T. *Clostridium proteoclasticum* B316^T was also found to form 18 : 0, as were other bacteria isolated elsewhere, which occurred in the same family subclass of the low G+C% Gram-positive bacteria, related to *Butyrivibrio fibrisolvens*. These bacteria are not clostridia, and the ability to form 18 : 0 was present in all strains in contrast to proteolytic activity, which was variable. Production of 18 : 0 occurred in growing, but not in stationary-phase, bacteria, which made detection of biohydrogenating activity difficult, because of the inhibitory effects of linoleic acid on growth.

Introduction

Comparison of national dietary habits reveals that the consumption of saturated fatty acids in dairy products is directly correlated to the incidence of death by heart attack in different countries (Menotti *et al*., 1999). Saturation of fatty acids (biohydrogenation) occurs primarily in the rumen (Dawson & Kemp, 1970; Viviani, 1970). *Butyrivibrio fibrisolvens* was identified many years ago to undertake biohydrogenation (Polan *et al*., 1964), but was observed not to form stearic acid (18 : 0) from linoleic acid (LA, *cis*-9,*cis*-12-18 : 2). The bacteria responsible for 18 : 0 formation were later identified as *Fusocillus* spp. (Kemp *et al*., 1975), a genus description that, like the cultures themselves, has not survived. The aim of the present study was to assess the biohydrogenating activity of fresh isolates from grass-fed sheep and to reisolate and identify *Fusocillus*-like bacteria by modern phylogenetic techniques.

Materials and methods

Fresh bacterial isolates were made nonselectively immediately *postmortem* from strained ruminal digesta of four grazing mature sheep. These were diluted in the basal medium of Bryant & Robinson (1961), plated onto petri dishes of complete Bryant & Robinson medium and

incubated at 39 °C for 48 h. Colonies from 10^4 to 10^8 dilutions were selected on the basis of differing colony morphology and were maintained in the liquid form of M2 medium (Hobson, 1969). In all growth experiments, culture tubes were 12.5 × 1.6 cm, closed with screw caps fitted with butyl rubber septa (Bellco Biotechnology, Vineland, NJ). All transfers were carried out under O_2-free CO_2. Incubations were at 39 °C. Inoculum volumes were 5% (v/v) of a fresh culture.

Bacterial strains other than P-18 were obtained from the Rowett collection (SH1, SH13 and JW11), purchased from culture collections (DSM or ATCC) or gifted by international colleagues. Most cultures were obtained from J. Kopečný and they are described in Kopečný *et al.* (2003). *Clostridium proteoclasticum* 'C-proteo' was a gift from G.T. Attwood and is among the strains described by Attwood *et al.* (1996). Strain Su6 was a gift from K.N. Joblin and is described in van de Vossenberg & Joblin (2003).

In order to determine whether bacteria produced 18 : 0 from LA or α-linolenic acid (LNA, *cis*-9,*cis*-12,*cis*-15-18 : 3), the isolates were inoculated into M2 liquid medium containing 50 μg LA or LNA mL^{-1} and incubated for 48 h. Cultures were then extracted and analysed for free fatty acid concentrations. Where cultures failed to grow but grew on medium with no added LA, the culture was inoculated into medium containing 20 μg LA mL^{-1} and the process was repeated. Extraction and derivatization of fatty acids were carried out by procedures based on those described by Christie (2003) and modified by Wąsowska *et al.* (2006), using samples (1 mL) of fresh or more usually resuspended, freeze-dried cultures. Proteolytic activity was measured in triplicate 6-h incubations of stationary-phase cultures with [^{14}C]-labelled casein (Wallace & Brammall, 1985).

Isolate P-18 was characterized by conventional methods based on those of Holdeman *et al.* (1977). The presence of spores was investigated by incubating M2 cultures at 85 °C for 15 min, and then reinoculating fresh tubes of M2 liquid medium. Fermentation products were determined by derivatization and capillary gas-liquid chromatography of supernatants from cultures grown in M2 liquid with no added LA (Richardson *et al.*, 1989). Some fermentation tests were carried out using API 20 A strips (bioMérieux, Lyon, France) under anaerobic conditions to determine sugar utilization and indole production. Most were done by inoculating strain P-18 into Bryant & Robinson basal medium with added individual sugars (5 g L^{-1}). Sugar utilization was considered to be positive when there was a growth response as measured turbidimetrically at 650 nm using a Novaspec II spectrophotometer (Amersham Pharmacia Biotech, Little Chalfont, Bucks). Gelatin liquefaction was carried out as described by Holdeman *et al.* (1977), except that liquid M2 was the basal medium. Staining for cytoplasmic inclusions and chemical analysis for storage polysaccharide and poly-β-hydroxybutyrate were carried out as described in the ASM technical manual (Hanson & Phillips, 1981). G+C% content was measured by the Deutsche Sammlung von Mikroorganismen und Zellculturen GmbH (Braunschweig, Germany) using reverse-phase HPLC (Mesbah & Whitman, 1989). In order to determine the influence of growth phase on fatty acid metabolism, LA was added to the medium, to a final concentration of 50 μg mL^{-1}, before inoculation, or when the OD had reached 0.3 (mid-exponential phase), or when the stationary phase was reached. In each case, cultures were incubated for 48 h after adding LA, and then cultures were freeze-dried and analysed for fatty acids. Electron microscopy was carried out on bacteria taken from a colony on M2 agar medium, the cultures being stained using ammonium molybdate as described previously (Wallace *et al.*, 2003).

Bacterial DNA was extracted using the Wizard Genomic DNA purification kit (Promega). A full sequence of 16S rRNA gene was amplified using the universal primers P0124 5′-CACGGATCCGGACGGGTGAGTAACACG-3′ and C1400 5′-TGGTGTGACGGGCGGTGTGT-3′ in a mixture containing 1 μM of each of the primers, 200 μM of each of the dNTPs, 50 mM KCl and 1.5 M $MgCl_2$ in 10 mM Tris HCl pH 8.3 buffer. 0.1 μg of DNA and 2.5 U of Taq DNA polymerase were added to 50 μL of this mixture and amplification was carried out using 35 cycles at 94 °C for 1 min followed by 55 °C for 2 min and 72 °C for 2 min. Amplification was confirmed on an agarose gel. Amplified DNA was cloned into the PCR®2.1-TOPO vector (Invitrogen BV, Leek, the Netherlands) following the manufacturer's instructions. Plasmids were isolated from recombinant colonies using the Wizard Plus SV miniprep kit (Promega), and the nucleotide sequence of the insert was determined using an ABI Prism™ 377XL DNA sequencer. The 16S rRNA gene sequences were aligned with the CLUSTALX program. Distance matrices were calculated with the DNADIST program using the algorithm of Kimura-2 within the PHYLIP package (Felsenstein, 1989). The phylogenetic trees were inferred from evolutionary distances with the Neighbor program of the same package. The confidence level of the phylogenetic tree topology was evaluated by performing 1000 bootstrap replications with the programs SEQBOOT and CONSENSE of the same package. The P-18 sequence used in the present work was deposited with EMBL under accession number AM039827. Others included strain Mz3 (AM039822), strain Mz9 (AM039823), strain CE51 (AM039824), strain JK612 (AM039825) and strain JK684 (AM039826).

Results and discussion

One hundred and fifteen bacteria were isolated nonselectively from individual colonies from four grazing sheep.

Only one strain, P-18, isolated from a 10^4 dilution, formed $> 5\,\mu g$ 18 : 0 mL^{-1}, with three others producing traces (*c.* 2 $\mu g\,mL^{-1}$). The growth of strain P-18 was subject to a lag period of 96 h when 50 μg LA mL^{-1} was present in the medium, and to shorter lag times at lower concentrations. Isolate P-18 was a motile, strictly anaerobic Gram-negative staining bacillus with morphology indistinguishable from *Fusocillus babrahamensis* (Fig. 1). Some of the cells were spindle-shaped, as was described for *F. babrahamensis* (Kemp *et al.*, 1975). No spores were visible, and bacteria were killed by incubation at 80 °C. Cells were motile, with a single subpolar flagellum. Electron microscopy indicated that, despite the staining reaction being negative, the cell envelope morphology was Gram-positive. The concentrations of fermentation products were higher from strain P-18 than two *Fusocillus* isolates from Kemp *et al.* (1975), but other fermentation properties were closely similar (Table 1). Properties were generally similar to the stearate-producing strain Su6 designated by van de Vossenberg & Joblin (2003) to be a *Butyrivibrio hungatei*. Like the *Fusocillus* strains, but unlike *B. hungatei* Su6, P-18 was xylanolytic. Conversely, *B. hungatei* Su6 and strain P-18 hydrogenated LNA to 18 : 0, while the early *Fusocillus* isolates did not.

A phylogenetic tree was constructed using the 16S rRNA gene sequences of 15 type strains of close relatives of P-18, together with *Butyrivibrio crossotus* NCDO 2416, *B. hungatei* Su6 (van de Vossenberg & Joblin, 2003) and *Pseudobutyrivibrio ruminis* JK205 (Kopečný *et al.*, 2003), with an *Escherichia coli*-type strain as the outlier (Fig. 2). The tree divided into three main branches. The first one contained

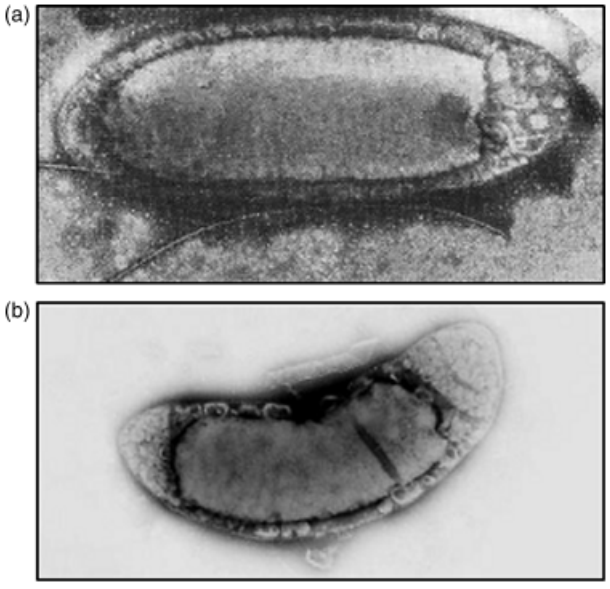

Fig. 1. Comparative morphology of stearate-producing isolate P-18 and the original '*Fusocillus*' isolate. (a) Scanned from the original paper by Kemp *et al.* (1975). (b) New isolate, strain P-18.

Table 1. Characteristics of stearate-producing strain P-18 in comparison with other published stearate producers from the rumen

Property	Strain P-18	'*F. babrahamensis*' P2/2*	'*Fusocillus*' sp. T344*	*B. hungatei* Su6†
Substrate				
Arabinose	–	+	+	+
Xylose	+	+	+	+
Rhamnose	+	+	–	
Glucose	+	+	+	+
Fructose	+	+	+	
Mannose	+	+	+	
Galactose	+	+	+	
Sucrose	+	+	+	+
Maltose	+	+	+	
Lactose	–	+	–	+
Cellobiose	+	+	+	+
Melibiose	+	+	+	
Trehalose	–	–	–	
Raffinose	–	–	–	
Starch	+	+	–	
Inulin	+	+	+	
Dextrin	–	+	+	
Glycerol	–	–	–	
Erythritol	–	–	–	
Adonitol	–	–	–	
Mannitol	–	–	–	–
Dulcitol	–	–	–	
Sorbitol	–	–	–	
Salicin	+	+	+	
Aesculin	+	+	+	
Inositol	–	+	–	–
Lactate	–	–	–	
Succinate	–	–	–	
Pyruvate	+	+	–	
Citrate	–	–	–	
Acetate	–	–	–	
Fumarate	–	–	–	
Pectin	+	+	+	+
Xylan	+	+	+	–
Cellulose	–			–
Other				
G+C%	39.2			
Glucose+5 μM monensin	–			
Stearate from α-linolenic acid	+	–		+
Flagellum	Sub-polar	Sub-polar	Sub-polar	
Gelatin liquefaction	–	–	–	
Catalase		–	–	
Fermentation products (mM)				
Butyrate	10.5	2.9	4.6	Major
Propionate	0	0	0	
Acetate	3.1	0.6	0.1	Major
Formate	23.2			Major
Lactate	7.7	0.1	0.2	
Succinate	0	0		

*Properties recorded by Kemp *et al.* (1975).
†From van de Vossenberg & Joblin (2003).
Where there is a space rather than a number or symbol, the property was not reported.

Ruminococcus type strains, the second contained *Clostridium xylanolyticum*, *Butyrivibrio* type strains and strains P-18 and JK205 and the third branch had type strains of *Lachnospira*, *Eubacterium rectale*, *Roseburia* and *Pseudobutyrivibrio*. The sequence of P-18 was located in a cluster comprising *B. fibrisolvens* D1^T, *B. hungatei* JK615^T, *C. proteoclasticum* B316^T, Su6 and JK 205, but was much closer to B316, Su6 and JK205 sequences than to the others, with a high level of confidence. This phylogenetic grouping and the absence of spores, also observed in the type strain of *C. proteoclasticum* (Attwood *et al.*, 1996) and '*Fusocillus*' (Kemp *et al.*, 1975), imply that the nomenclature of these bacteria as *Clostridium* is probably incorrect.

A more detailed positioning of different P-18-related strains was carried out by comparing its 16S rRNA gene sequence with those of *Butyrivibrio* and related isolates obtained from around the world (Fig. 3). Many of the full-length sequences used in this study were obtained by us and have been placed in sequence databases. Selected strains from across the tree were investigated for their ability to form 18 : 0 from LA and for their proteolytic activity (Fig. 3). As before, strain P-18 clustered on a branch most closely with isolates Su6 and JK205. The closely related strain JK669 was identified as *C. proteoclasticum* based on 16S rRNA gene sequence analysis (Kopečný *et al.*, 2003). Strains P-18, Su6, Bu43, JK205, JK669 and JK668 formed 18 : 0 from LA. The nearest branch contained the type strain of *C. proteoclasticum* B316^T, which also formed 18 : 0, whereas bacteria from elsewhere in the tree did not, including a neighbouring branch containing the type strain JK615 of *B. hungatei* (Kopečný *et al.*, 2003). All the strains producing 18 : 0 were distantly related to the *B. fibrisolvens* type strain D1^T (ATCC 19171), the *P. ruminis* type strain (DSM 9787^T) and the *Pseudobutyrivibrio xylanivorans* type strain Mz5 (DSM 14809^T; Kopečný *et al.*, 2003). Proteolytic activity was high in several strains, including SH13 and DSM 10317, but very low in most strains tested (Fig. 3). Unlike 18 : 0 production, proteolytic activity was not correlated with phylogenetic position.

The influence of growth phase on 18 : 0 production by P-18 was determined by adding LA to mid-exponential and stationary-phase cultures as well as LA being present in the medium at inoculation. When LA was added to stationary-phase cells, conjugated linoleic acids (CLA) accumulated (Table 2), predominantly as *cis*-9,*trans*-11–18 : 2. No 18 : 1 or 18 : 0 was formed. Adding LA before

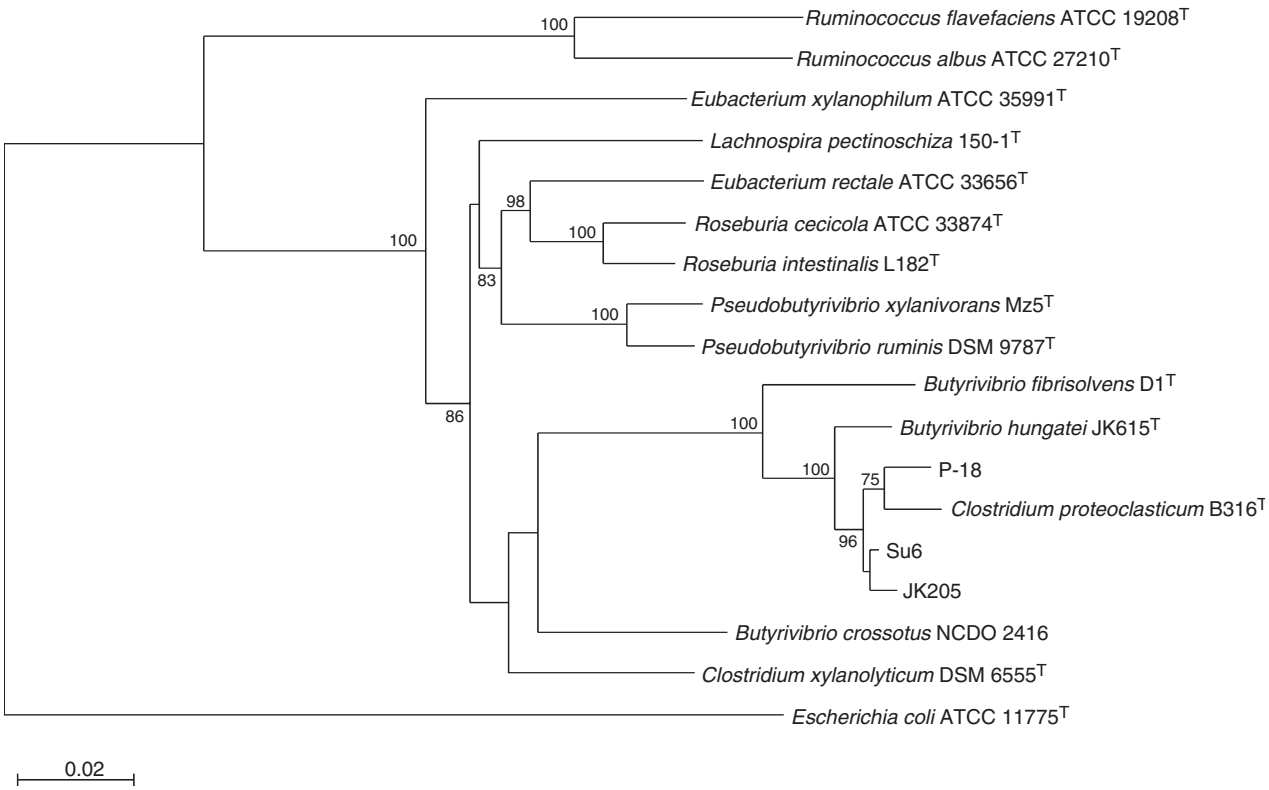

Fig. 2. Neighbour-joining phylogenetic tree of P-18 and related bacteria. The tree was constructed using nearly full-length 16S rRNA gene sequences (> 1300 bp). Numbers represent the significance (percent of outcomes) of the branches (bootstrap analysis). Only numbers higher than 70 are shown. The bar indicates 2% difference in nucleotide sequence. T: Type strain.

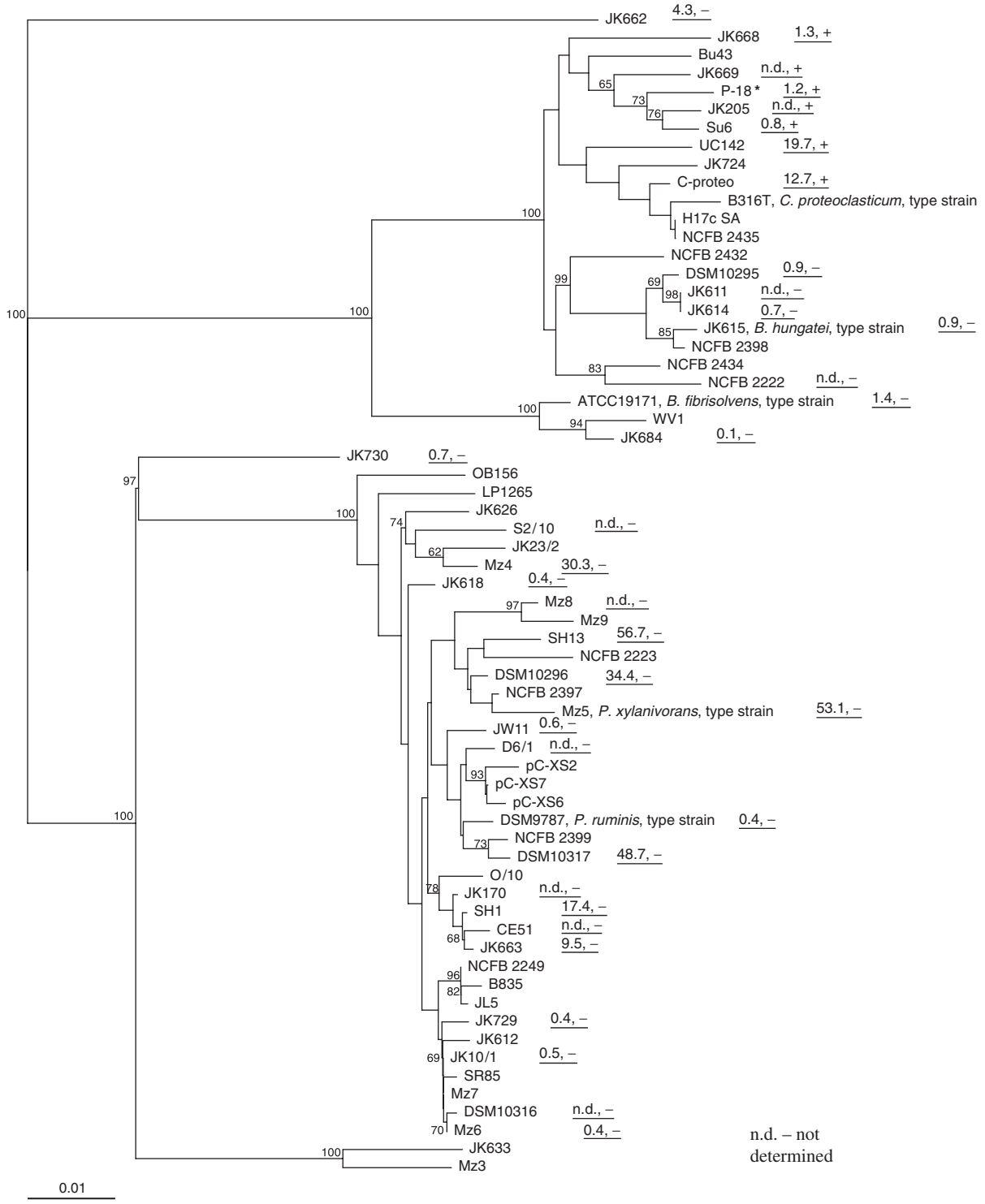

Fig. 3. Proteolytic activity and stearic acid formation from individual strains in a Neighbour-joining phylogenetic tree of *Butyrivibrio fibrisolvens* and related bacteria. The tree was constructed using nearly full-length 16S rRNA gene sequences (> 1300 bp). The numbers in the tree indicate the significance (percent of outcomes) of the branches (bootstrap analysis). Only numbers higher than 60 are shown. The underlined text alongside many of the bacterial strains describes proteolytic activity and the ability to form 18 : 0 from LA. The first number is proteolytic activity in units μg ^{14}C-casein hydrolysed h^{-1} mg protein^{-1}, the second 18 : 0 production (+) or no 18 : 0 production (–). Measurements were carried out on three replicate cultures.

Table 2. Influence of growth phase on the metabolism of LA by strain P-18

	Products ($\mu g\,mL^{-1}$)		
Treatment	18 : 0	*trans*-11-18 : 1	*cis*-9,*trans*-11-18 : 2
LA added before inoculation	11.6	14.3	0.5
LA added in exponential phase	11.2	12.7	0.2
LA added in stationary phase	1.1	0	28.2

inoculation or in the exponential phase resulted in the formation of roughly similar concentrations of 18 : 1 and 18 : 0, with minimal formation of CLA (Table 2). Kepler & Tove (1967) demonstrated that, in *B. fibrisolvens*, isomerization of LA to CLA did not require cofactors and indeed occurred aerobically in whole cells or cell extracts. In contrast, the subsequent conversion of CLA to VA was NADH-dependent (Hunter *et al.*, 1976). Thus, it seems probable that the absence of 18 : 0 formation in stationary-phase *C. proteoclasticum* was caused by a limited supply of reducing equivalents once the energy sources in the medium had been depleted.

The growth-phase results also mean that, in order to demonstrate the formation of 18 : 0 by P-18, it is necessary to grow the bacteria in the presence of LA. Given the growth inhibition that occurs when LA is added to the medium, 18 : 0 formation by P-18 can sometimes be difficult to demonstrate. In our initial studies, which preceded the experiments reported here, we were unsuccessful in isolating stearic acid-forming bacteria. The reasons for this are now obvious, namely that bacteria must be growing in order to carry out biohydrogenation; yet, the unsaturated fatty acids themselves have a strong tendency to inhibit growth. These properties undoubtedly must have hampered the isolation of more stearate producers by ourselves and others.

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