Clostridium frigidicarnis sp. nov., a psychrotolerant bacterium associated with 'blown pack' spoilage of vacuum-packed meats

D. M. Broda,[1,2] P. A. Lawson,[3] R. G. Bell[2] and D. R. Musgrave[1]

Author for correspondence: D. M. Broda. Tel: +64 7 854 8550. Fax: +64 7 854 8560.
e-mail: d.broda@mirinz.org.nz

[1,2] Department of Biological Sciences, University of Waikato[1], and MIRINZ Food Technology & Research, PO Box 617[2], Hamilton, New Zealand

[3] Department of Food Science and Technology, University of Reading, Reading RG6 6BZ, UK

Two strains of a psychrotolerant *Clostridium*, isolated from vacuum-packed, temperature-abused beef, were characterized using a multiphasic approach. The strains were Gram-positive motile rods producing elliptical subterminal spores during early stationary growth phase. The strains were psychrotolerant. At pH 7·0, they grew between 3·8 and 40·5 °C; their optimum growth temperature was 30·0–38·5 °C. At 30 °C, the pH range for growth was between 4·7 and 9·5; the optimum pH for growth was 6·4–7·2. The organisms were proteolytic and saccharolytic, lecithinase-positive and hydrolysed gelatin. The fermentation products formed in peptone/yeast extract/glucose/starch broth were acetate, ethanol, butyrate, isovalerate, butanol, isobutyrate, oxalacetate, lactate, hydrogen and carbon dioxide. The DNA G+C compositions of the two meat strains were 27·3 and 28·4 mol %. Phylogenetic analyses indicated that the strains belong to Cluster I of the genus *Clostridium* (*sensu* Collins *et al.*, 1994). The new strains differed from phylogenetically related clostridia in terms of cellular fatty acid composition, soluble protein profiles and phenotypic properties. On the basis of phenotypic and genotypic characterization data, the strains were assigned to a new species for which the name *Clostridium frigidicarnis* is proposed; strain SPL77A^T (= DSM 12271^T) is the type strain.

Keywords: *Clostridium frigidicarnis*, psychrotolerance, meat spoilage

INTRODUCTION

Historically, *Clostridium* species were believed to play only a minor role in meat spoilage (Gill, 1979; Roberts & Mead, 1986). Early reports (Ingram, 1952; Callow & Ingram, 1955; Ross, 1965) regarded mesophilic clostridia and *Clostridium putrefaciens*, which is atypical with respect to its growth temperature range, as the causative agents of deep tissue spoilage ('bone taint') of beef and/or cured hams. *Clostridium*-mediated 'bone taint' spoilage was believed to have been eliminated by the development of chilled storage and modern packaging technologies (Roberts & Mead, 1986). However, more recently, psychrotolerant (psychrophilic) *Clostridium* species have been recog-

nized as the causative agents of 'blown pack' spoilage of vacuum-packed meat (Dainty *et al.*, 1989; Kalchayanand *et al.*, 1989; Broda *et al.*, 1996). Similarly, production of offensive, sickly odours in vacuum-packed cooked pork has been attributed to the growth of a psychrotolerant *Clostridium* species (Lawson *et al.*, 1994). These types of meat spoilage occur during chilled storage in the absence of temperature abuse.

To date, three psychrotolerant meat spoilage-causing species of clostridia have been described: *Clostridium estertheticum* (Collins *et al.*, 1992), *Clostridium laramiense* (Kalchayanand *et al.*, 1993; Trüper & De' Clari, 1997) and *Clostridium algidicarnis* (Lawson *et al.*, 1994). However, the results obtained from a slaughter stock survey that included faecal, hide and tonsil samples (J. A. Boerema, D. M. Broda & R. G. Bell, unpublished results), as well as examination of spoiled vacuum packs (Broda *et al.*, 1996), indicate that numerous species of psychrotolerant clostridia,

including some spoilage-causing strains, may be commonly associated with slaughter stock and vacuum-packed meat. It appears, therefore, that the detection of psychrotolerant *Clostridium* species as a group in vacuum-packed meat may no longer be sufficient to account for premature meat spoilage at refrigeration temperatures. Consequently, to effect spoilage control it is necessary first to determine which clostridial species are causing the spoilage and second to trace the causative micro-organism back to its reservoir of contamination in the meat plant.

The aim of this study was to describe and characterize a psychrotolerant *Clostridium* species associated with a meat spoilage incident. These data may also provide a means to differentiate this new species from other psychrotolerant clostridia, both spoilage-causing and benign, that may be encountered in spoiled vacuum packs and the meat plant environment. Such differentiation will facilitate tracing spoilage-causing clostridia to their reservoirs within the abattoir.

METHODS

General procedures. Unless stated otherwise, stringent anaerobic procedures (Holdeman *et al.*, 1977) were used for culturing all *Clostridium* strains and for physiological and substrate utilization tests. All anaerobic media that contained reducing agents were pre-reduced by cooling after sterilization inside an anaerobic chamber (Forma Scientific) operating with an anaerobic gas mixture (10%, v/v H_2; 5%, v/v CO_2; 85%, v/v N_2). Only fully reduced media, as indicated by the colourless state of resazurin indicator in the medium, were used for tests.

Bacteria. Strains SPL77A^T (T = type strain) and SPL77B were initially isolated from the same 'blown' temperature-abused vacuum pack of raw beef using methods described previously (Broda *et al.*, 1996). After their initial isolation, strains SPL77A^T and SPL77B were maintained as freeze-dried cultures. Before characterization, both strains were revived from freeze-dried material in peptone/yeast extract/glucose/starch (PYGS) broth (Lund *et al.*, 1990), subcultured onto Columbia Blood Agar (CBA; Oxoid) containing 5% (v/v) sheep blood (Life Technologies) and incubated at 30 °C for 48 h. Strains were checked for purity before use.

Reference strains of *Clostridium cadaveris* (DSM 1284^T), *Clostridium fallax* (DSM 2631^T), *Clostridium intestinale* (DSM 6191^T) and *Clostridium carnis* (DSM 1293^T) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. A reference strain of *C. algidicarnis* (NCFB 2931^T) was obtained from the National Collection of Food Bacteria, Reading, UK. All reference strains were revived from freeze-dried material in PYGS broth and checked for purity by plating onto CBA supplemented with 5% (v/v) sheep blood. With the exception of *C. algidicarnis*, which was grown at 30 °C, reference strains were grown at 37 °C.

Phenotypic characterization

(i) Morphology and ultrastructure. For description of colony morphology, strains SPL77A^T and SPL77B were grown on the surface of CBA with 5% (v/v) sheep blood at 30 °C for 48 h. The morphology of well-isolated colonies was described.

For descriptions of vegetative cell and spore morphology, meat strains were grown in PYGS broth at 30 °C for 18 h and 5 d, respectively. A light microscope (Leitz Orthoplan) in phase-contrast mode was used to examine and photograph vegetative cells and spores of each bacterium. Presence of flagella was determined in 18-h-old cultures of strains SPL77A^T and SPL77B. For this determination, the cultures were washed with water, allowed to settle onto Formvar-coated copper grids and negatively stained with 1–2% (w/v) uranyl acetate (Beveridge *et al.*, 1994). Stained grids were dried and examined with a transmission electron microscope (Philips EM 400).

The ultrastructure of vegetative cells and spores was determined for the isolates grown in PYGS broth at 30 °C for 18 h and 5 d, respectively. The bacteria were fixed overnight at 4 °C in a solution containing 2·5% (v/v) glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7·2). The fixed cells were washed in 100 mM sodium cacodylate buffer and postfixed with 1% (w/v) aqueous osmium tetroxide. The cells were then stained en bloc with 4% (w/v) aqueous uranyl acetate, dehydrated and embedded in Spurr embedding medium. Ultra-thin sections were then prepared and stained with a 4% (w/v) uranyl acetate and lead citrate solution (Beveridge *et al.*, 1994). The grids containing sections were examined with a transmission electron microscope.

(ii) Gram reaction, Gram-type and cell wall type. Exponentially growing (at 30 °C for 18 h) cells of strains SPL77A^T and SPL77B were Gram stained using the method of Johnson *et al.* (1995). A KOH test (Powers, 1995) was additionally conducted to determine the Gram-type of cells grown on the surface of CBA with 5% (v/v) sheep blood at 30 °C for 18 h. Cell wall type was determined using transmission electron microscopy, as described in the previous section.

(iii) Physiological tests. For physiological tests, growth was determined by monitoring an increase in OD_{550}, as measured with a spectrophotometer (Novaspec II; Pharmacia), against three uninoculated controls.

The potential for growth at 25 pH values ranging from 4·23 to 9·82 was determined at 30 °C in peptone/yeast extract (PY) broth (Holdeman *et al.*, 1977) with glucose concentration adjusted to 0·5% (w/v). Broths were inoculated with 2% (v/v) culture growing exponentially at pH 7·0. The pH of the broth was kept constant ($\pm$0·1 unit) by adjustment with potassium phosphate buffer. The broth pH was measured with a pH meter (Schott CG 837; Labsupply Pierce) equipped with a combination electrode. Cultures were incubated for up to 10 d to determine whether growth occurred.

Growth rates were measured for 43 temperatures ranging from −1·5 to 47·0 °C. The temperature range for growth was determined at pH 7·0 in PY broth with 0·5% (w/v) glucose. Hungate tubes containing medium were inoculated with 2% (v/v) culture growing exponentially at 30 °C and incubated in a temperature gradient incubator of local manufacture. Throughout the study, temperatures were monitored with copper constatan thermocouples and a Comark 1624 electronic thermometer. Tubes were incubated for up to 21 d to determine whether growth occurred.

(iv) Biochemical characteristics. All biochemical tests were conducted in duplicate. Unless otherwise stated, consensus

duplicate results were required for the final results to be recorded as positive or negative.

Substrate utilization was tested according to Holdeman *et al.* (1977) at 30 °C and initial pH 7·0 in PY broth containing 0·1 % (w/v) yeast extract. The substrates were (1 %, w/v concentration, except where noted): adonitol (0·5 %, w/v), arabinose (0·5 % w/v), cellobiose, dextran, fructose, galactose, glucose, inositol, inulin, lactose, maltose, mannitol, rhamnose, raffinose, salicin, sorbitol, sorbose, sucrose, trehalose (0·5 %, w/v) and xylose. Hungate tubes containing the medium with substrate and duplicate controls with no substrate were inoculated with 2 % (v/v) culture exponentially growing in PYGS broth at 30 °C. Changes in OD_{550} and pH were monitored. Tubes showing an increase in OD of more than 0·1 OD unit, as compared with controls, were recorded as positive with respect to substrate utilization. Tubes showing a pH drop of more than 1·0 pH unit, as compared with controls, were recorded as positive with respect to substrate fermentation.

Gelatin hydrolysis, lecithinase and lipase activities, milk reaction, meat and casein digestion, indole production, nitrate reduction, oxidase and catalase production, urea hydrolysis, ammonia and hydrogen sulphide production, aesculin and starch hydrolysis, and growth in the presence of bile and Tween 80 were tested following methods of Holdeman *et al.* (1977). The Rapid ID 32 A system (bioMérieux) was used to study an additional 29 enzymic reactions: urea hydrolysis, arginine dihydrolase activity, fermentation of 11 sugars, glutamic acid decarboxylase activity, nitrate reduction, indole production, alkaline phosphatase activity and arylamidase activity towards 12 amino acids. Test strips were inoculated according to the manufacturer's instructions and read after aerobic incubation at 37 °C for 4 h.

Fermentation products were determined for strains SPL77A^T and SPL77B grown in PYGS broth at 30 °C for 5 d. Ether extracts of volatile fatty acids and alcohols, and methyl esters of non-volatile fatty acids were prepared according to Holdeman *et al.* (1977). Extracts were analysed on a Hewlett Packard 5890/II GC equipped with a 30 m × 0·53 mm, 1·0 μm FFAP capillary column (J&W Scientific), a flame ionization detector (FID) and a Hewlett Packard model 7673 automatic sampler. The GC parameters were as follows: column head pressure, 47·4 kPa; split vent flow of helium carrier gas, 50 ml min^{-1}; column flow measured at 60 °C, 9·9 ml min^{-1}; resultant split ratio, 5:1; split/splitless injector and FID detector temperature, 260 °C. Run conditions were: initial temperature 60 °C for 2 min, raised to 180 °C at 5 °C min^{-1}, final temperature of 240 °C held for 2 min after raising at a rate of 20 °C min^{-1}. Peaks were automatically integrated (Chemstation v.4) and fatty acids were identified and quantified against retention times and peak areas of known standards. Results of duplicate experiments were recorded as a concentration range for a product. Production of H_2 and CO_2 was determined on a Varian GC equipped with a Porapak Q 80/100-mesh column (Supelco) and a thermal conductivity detector. This test was qualitative only.

(v) Toxicity test. To test for lethality of the culture supernatant of strain SPL77A^T, procedures described in the FDA Bacteriological Analytical Manual (Solomon *et al.*, 1995) for detection of botulinal neurotoxin were followed. National Animal Ethics Committee approval was granted before testing began. Strain SPL77A^T was grown for 5 d at 30 °C in cysteine- and resazurin-deficient PYGS broth. An undiluted, trypsin- and heat-treated culture supernatant was prepared and injected intraperitoneally into mice. For each sample, a separate pair of mice was injected. Mice were observed periodically over 72 h.

Genotypic characterization

(i) DNA isolation. Meat isolates were grown in PYGS broth at 30 °C for 18 h and were harvested by centrifugation at 12000 *g* for 15 min. Cells were washed twice with saline/EDTA (0·15 M NaCl, 1 mM EDTA, pH 8·0). DNA was isolated using the method of Marmur (1961) modified as described by Johnson (1994). Briefly, cells were lysed with lysozyme at a final concentration (FC) of 50 mg ml^{-1}, followed by SDS (FC 2·5 %, w/v) and proteinase K (FC 50 μg ml^{-1}) lysis. The lysate was repeatedly deproteinized with phenol, phenol/chloroform (1:1) and chloroform, DNA was precipitated with absolute ethanol and spooled onto a glass rod. DNA was washed, dried and dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8·0). RNase A treatment (FC 50 μg ml^{-1}) was used to remove RNA from the solution, followed by repeated deproteinization. The DNA solution was dialysed against ice-cold TE and stored at −20 °C.

DNA concentration was measured by a fluorimetric dye-binding method (Hoechst 33258) using a fluorimeter (Hoeffer DynaQuant 200; Biolab Scientific). The procedure recommended by the fluorimeter's manufacturer was followed. Calf thymus DNA (Sigma) was used as a standard.

(ii) Determination of DNA base composition. G + C composition was determined in DNA from strains SPL77A^T and SPL77B, and in DNA from *Escherichia coli* B (51·0 mol % ; Sigma) by the method of Mesbah *et al.* (1989). DNA was denatured and digested enzymically with P1 nuclease (FC 36 ng μl^{-1}) and alkaline phosphatase (FC 0·01 U μl^{-1}). The resulting nucleosides were separated using a reverse-phase HPLC equipped with a 1·5 m × 4·6 mm, 3 μm Econosphere C18 column (Alltech Associates), and fractions were quantified. G + C composition was calculated using the mole fractions of deoxyguanosine and thymidine against the *E. coli* standard.

(iii) 16S rRNA sequence determination. The 16S rRNA sequences of strains SPL77A^T and SPL77B were determined by direct sequencing of the purified PCR-amplified 16S rDNA fragment.

Genomic DNA was used as the PCR template. PCR was performed with universal (eu)bacterial primers complementary to conserved regions of the 5′ and 3′ ends of the 16S rRNA gene. The primer sequences were (Hutson *et al.*, 1993): pA (forward) 5′-AGA GTT TGA TCC TGG CTC AG-3′ (approx. positions 8–28 in *E. coli* numbering) and pH* (reverse) 5′-AAG GAG GTG ATC CAG CCG CA-3′ (approx. positions 1542–1522 in *E. coli* numbering).

The PCR mix was prepared according to the manufacturer's (Boehringer Mannheim) recommendation and contained: PCR buffer 10 × (10 μl), 0·2 mM each deoxynucleoside triphosphate, 0·5 μM each primer, 2·5 U *Taq* polymerase and 10 μl template DNA (100 ng) in a total volume of 100 μl. Amplifications were performed in a heated lid thermal cycler (Techne Genius; John Morris Scientific). After initial denaturation for 3 min at 93 °C, target DNA was amplified in 30 cycles. Each cycle consisted of denaturation for 1 min at 92 °C, annealing for 1 min at 55 °C and extension for 2 min at 72 °C. The final extension was for 3 min at 72 °C.

The PCR reaction tubes, each containing an amplified 16S rDNA fragment were held at 4 °C until further analysis.

The PCR products were purified with the Wizard purification kit (Promega; Dade Diagnostics) according to the manufacturer's instructions. 16S rDNA fragments were sequenced directly using dideoxy terminator cycle sequencing chemistry in an ABI 377 automated sequencer (Perkin Elmer; Applied Biosystems). PCR reactions were performed with AmpliTaq DNA polymerase (Perkin Elmer). In addition to primers pA and pH*, the following sequencing primers, complementary to internal regions of the 16S rRNA gene, were used (Hutson et al., 1993): Sef 1 (forward) 5'-CGT GCC AGC AGC CGC GGT AAT-3'; Sef 2 (forward) 5'-GGA GCA TGT GGT TTA ATT CG-3'; Ser 1 (reverse) 5'-CAC GAC ACG AGC TGA CGA CAA C-3'; Ser 2 (reverse) 5'-CTA CGC ATT TCA CCG CTA CAC-3'; and Ser 3 (reverse) 5'-GCC GTG TCT CAG TCC CAA TGT-3'. The sequences were aligned using Sequence Navigator (Perkin Elmer) and corrected manually in agreement with the electropherograms and the reading of complementing and overlapping fragments.

(iv) Phylogenetic analysis. To find the nearest relative of strains SPL77A^T and SPL77B, sequences of these strains were compared with sequences of micro-organisms in the EMBL and GenBank databases using the program FASTA (Pearson & Lipman, 1988). To determine the phylogenetic position of the isolates, their sequences and the sequences of their closest relations belonging to the Clostridium subphylum were aligned using the PILEUP program (Devereux et al., 1984). The alignment was corrected manually and approximately 100 nucleosides at the 5' end of the molecule were omitted from further analyses because of sequence ambiguities. 16S rRNA sequence similarities were calculated and corrected for substitution rates according to Kimura (1980). A phylogenetic tree was constructed using pairwise analysis and the neighbour-joining method (Saitou & Nei, 1987). The stability of relationships was assessed using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE of the PHYLIP package (Felsenstein, 1989). At least 1000 bootstrap trees were generated for each data set.

Differentiation from phylogenetically related clostridia

For SDS-PAGE of total soluble cell proteins and for cellular fatty acid (CFA) analysis, strains SPL77A^T, SPL77B and the reference strain of C. algidicarnis (NCFB 2931^T) were grown in PYGS broth at 30 °C for 24 h. The remaining reference strains of phylogenetically related clostridia were grown in PYGS broth at 37 °C for 24 h. Cells were harvested by centrifugation at 6000 g for 15 min, washed and suspended in sterile ultra-pure water to obtain a bacterial suspension with OD$_{550}$ of 1·5 units.

(i) Soluble cell protein profiles. For SDS-PAGE, a 0·3–0·5 ml volume of bacterial suspension was sonicated on ice with an ultrasound probe (Kontes CV 18; Biolab Scientific) at a maximum output of 90 W. Three 1-min bursts, with cooling on ice between each burst, were used to disrupt the cells. Sonicated samples were mixed in a 1:1 ratio with an SDS sample buffer containing Tris/HCl, 20% (w/v) SDS, 2-mercaptoethanol, bromophenol blue and water. Low- and broad-range protein standards (Bio-Rad) were mixed with the sample SDS buffer in the ratio 1:20. Samples and standards were denatured by boiling for 5 min. Portions (30 μl) of these denatured samples and 10 μl standards were loaded in the wells of a 4–15% gradient Tris/HCl poly-

acrylamide gel (Bio-Rad) and subjected to electrophoresis. SDS-PAGE was performed using the discontinuous buffer system of Laemmli (1970) in a Mini Protean II vertical gel apparatus (Bio-Rad). Electrophoresis was carried out at constant voltage of 150 V for 1 h at 15 °C. The gel was stained with 0·125% (w/v) Coomassie blue R-250 (BDH) dissolved in 50% (v/v) aqueous methanol and 10% (v/v) aqueous acetic acid. The gel was destained in a solution containing 50% (v/v) aqueous methanol and 10% (v/v) aqueous acetic acid. To assess similarities between protein profiles of individual strains, a scanned image of the destained gel was analysed with a Molecular Imager and Molecular Analyst software (Bio-Rad).

(ii) CFA analysis. For CFA analysis, fatty acid methyl esters (FAME) were extracted as described by Kuykendall et al. (1988). Briefly, 40 mg (wet wt) cells of each strain were saponified by heating at 100 °C for 30 min with 1 ml 15% (w/v) NaOH in 50% (v/v) aqueous methanol. Samples were cooled at ambient temperature, then 1·5 ml 25% (v/v) HCl in 50% (v/v) aqueous methanol was added and the mixture was heated at 100 °C for 15 min. FAME were extracted with a mixture of hexane and methyl-tert-butyl ether (1:1, v/v). The C10–C20 fatty acids were identified and quantified using a Hewlett Packard 6890 GC equipped with a 60 m × 0·25 mm, 0·25 μm BPX 70 column (GBC Scientific), an FID and a Hewlett Packard model 7673 automatic sampler. The GC parameters were as follows: mean linear velocity, 22 cm s^{-1}; column head pressure, 210 kPa; split vent flow of helium carrier gas, 44 ml min^{-1}; constant column flow, 5·9 ml min^{-1}; resultant split ratio, 50:1; split/splitless injector and FID detector temperature, 250 °C. Run conditions were: initial temperature 100 °C for 4 min, raised to 130 °C at 10 °C min^{-1}, then raised to 150 °C at 1 °C min^{-1}, raised to 200 °C at 2·5 °C min^{-1}, final temperature of 240 °C held for 2 min after raising at a rate of 25 °C min^{-1}. Peaks were automatically integrated (Chemstation v.4) and FAME were identified and quantified against retention times and peak areas of known standards. Peaks that did not have retention times identical to those of the standards were reported as unknown compounds at their respective equivalent chain-length (Christie, 1989).

RESULTS

While strains SPL77A^T and SPL77B were initially isolated from the 'blown' temperature-abused vacuum pack of raw beef, an additional 34 SPL77A^T-like and SPL77B-like isolates were subsequently obtained from faeces, hides and tonsils of bovine slaughter stock (J. A. Boerema, D. M. Broda & R. G. Bell, unpublished results). Preliminary morphological and biochemical characteristics were determined for these additional isolates and they were found to be identical to those of either SPL77A^T or SPL77B. Consequently, only characteristics of the two original meat-derived strains are described in this study.

Phenotypic characterization

The colonies of strain SPL77A^T grown on the surface of CBA containing 5% sheep blood were circular to irregular with undulate to lobate margins, creamy grey, raised, low convex, shiny and opaque. The
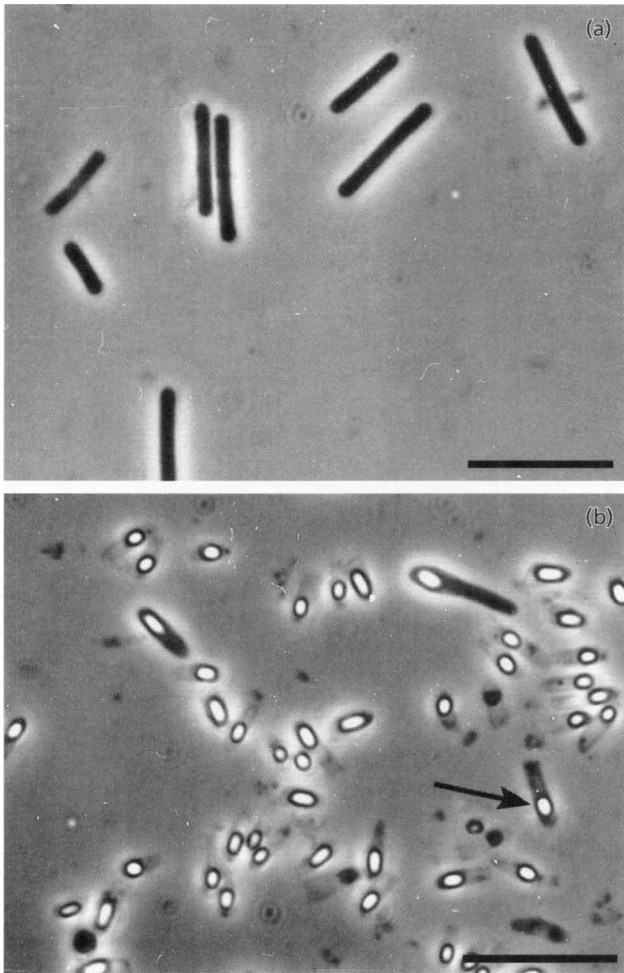
Fig. 1. Phase-contrast micrographs of vegetative (a) and sporulated (b) cells of strain SPL77A^T. The subterminal position of the spore in the cell is indicated with an arrow. Bar, 10 μm.
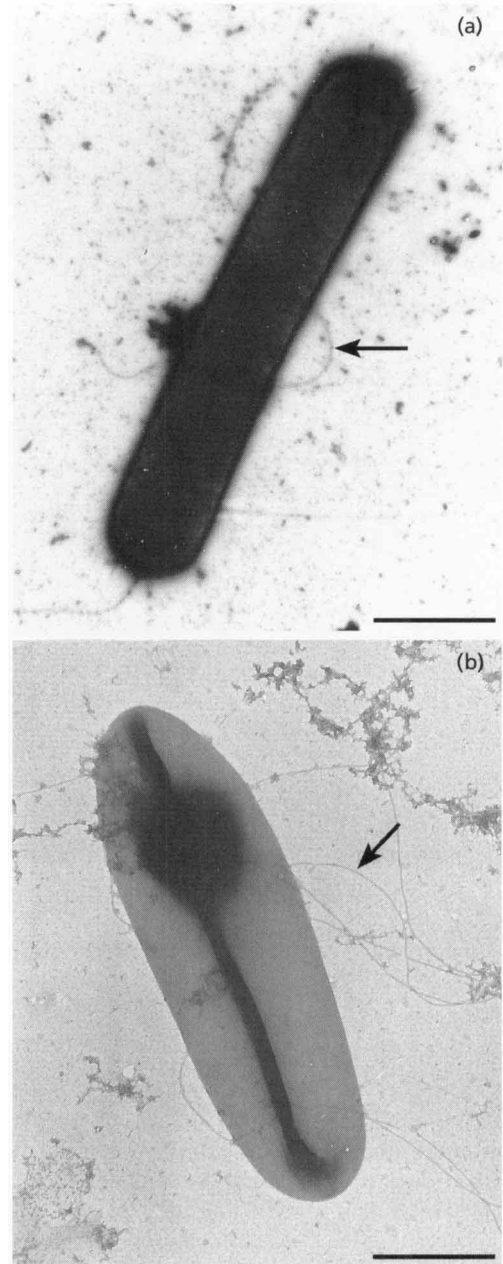

Fig. 2. Electron micrographs of negatively stained cells of strain SPL77A^T showing peritrichous flagella (arrow) in both a vegetative (a) and a sporulated (b) cell. Bar, 1·5 μm.

colonies measured 2·2–3·2 mm in diameter. The colonies of strain SPL77B were irregular with erose margins, grey, flat to raised, shiny, semi-opaque to translucent and measured 3·3–7·2 mm. Both strains produced large zones of *β*-haemolysis. With both strains, cells in exponential growth phase were round-ended, straight to slightly bent rods 4·5–9·4 μm long and 1·3–1·6 μm wide (Fig. 1a). These rods occurred singly or in pairs and were motile by means of three to seven peritrichous flagella per cell (Fig. 2a). Electron micrographs of ultra-thin sections from vegetative cells showed that the organism was rod-shaped (Fig. 3a) and frequently produced excess membranes during cell division (Fig. 3b). The Gram-positive-type, single layer cell wall was observed in ultra-thin sections of vegetative cells of both strains (Fig. 3c). Elliptical, subterminal spores were formed, starting during early stationary growth phase (Figs 1b and 4a). The sporulated cells were motile and carried peritrichous flagella (Fig. 2b). The spores caused swelling of the maternal cells. As observed in electron micrographs,

the mature spores had a fully developed endospore structure (Fig. 4b).

With both strains SPL77A^T and SPL77B, cells in exponential growth phase stained Gram-positive. The KOH reaction was always negative (i.e. characteristic of Gram-positive cells) irrespective of growth medium or age of the culture. This Gram-positive type was confirmed by the single layer cell wall, as observed in electron micrographs of ultra-thin sections (Fig. 3c).

Both strains grew optimally at 30·0–38·5 °C (Fig. 5a
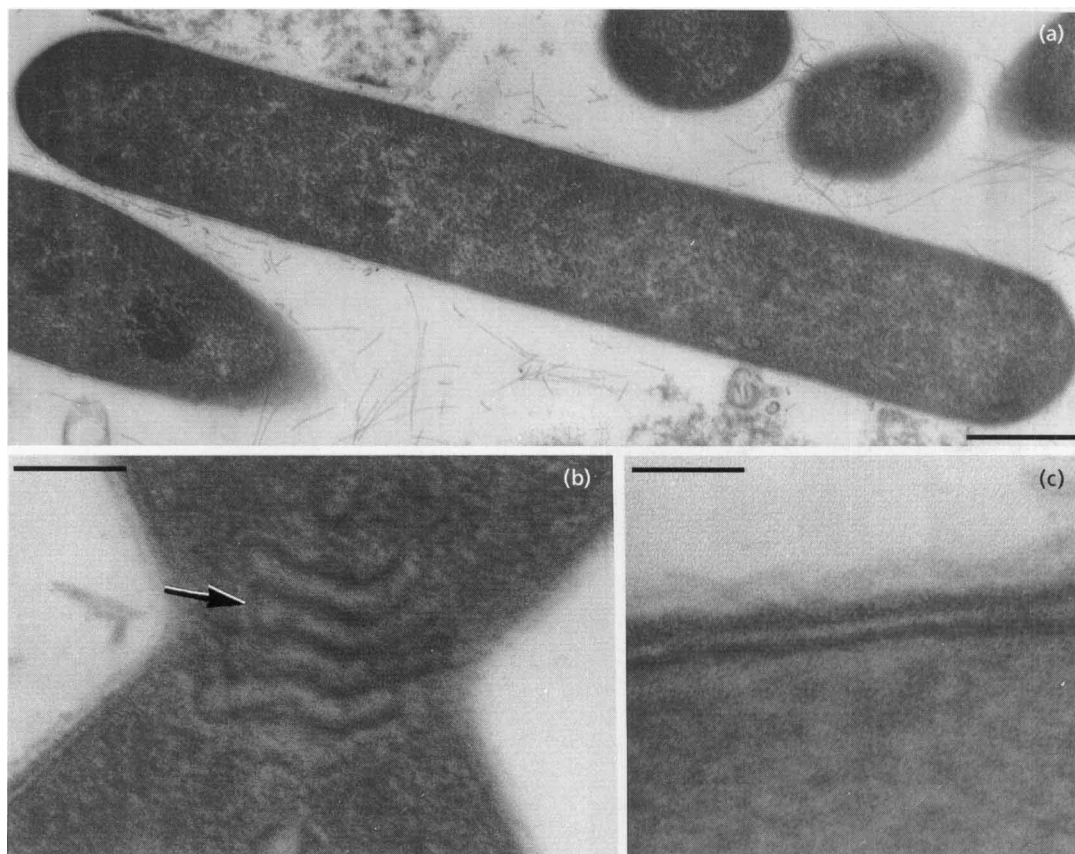
Fig. 3. Electron micrographs of thin sections of vegetative cells of strain SPL77A^T showing (a) cell shape (bar, 0·5 μm), (b) the excess of membranes at the site of cell division (arrow; bar, 150 nm) and (c) Gram-positive type cell wall (bar, 50 nm).

and data not shown). The lowest temperature at which growth of strain SPL77A^T was observed was 3·8 °C and the highest temperature at which growth was observed was 40·5 °C. The optimal pH for growth of both strains was 6·4–7·2 and growth occurred at pH values between 4·7 and 9·5 (Fig. 5b and data not shown). Strains SPL77A^T and SPL77B required anaerobic conditions for growth.

The substrates that supported growth resulting in acidification of the growth medium by strain SPL77A^T in the presence of 0·1% (w/v) yeast extract were: fructose, glucose, maltose, mannose, sorbitol and trehalose. Cellobiose supported growth but the growth medium was only weakly acidified. Gelatin was hydrolysed, lecithinase activity was present, casein and meat were digested, and ammonia and hydrogen sulphide were produced. The milk reaction was positive for both digestion and curd formation. Addition of Tween 80 did not stimulate growth. The remaining substrate utilization tests were negative. With the Rapid ID 32 A, positive reactions were observed for β-N-acetylglucosaminidase and proline arylamidase and weak reactions were observed for arginine arylamidase and pyroglutamic acid arylamidase. Strain SPL77B was positive or negative for the same biochemical tests

as strain SPL77A^T, with the exception that meat, casein and milk were only weakly digested and β-N-acetylglucosaminidase activity was not present in strain SPL77B.

The major fermentation products formed by strain SPL77A^T in PYGS broth were: acetate (43–45 mM), ethanol (19–20 mM), butyrate (18–20 mM), isovalerate (6–7 mM), butanol (4–5 mM), isobutyrate (2–3 mM), oxalacetate (1–2 mM), lactate (1 mM), hydrogen and carbon dioxide. The major fermentation products formed by strain SPL77B were: ethanol (22–26 mM), acetate (23–25 mM), butyrate (16–17 mM), isovalerate (2–3 mM), butanol (1–2 mM), isobutyrate (1 mM), oxalacetate (1 mM), lactate (1 mM), hydrogen and carbon dioxide.

As determined by the botulinal neurotoxin test, the supernatant of strain SPL77A^T was non-toxic to mice.
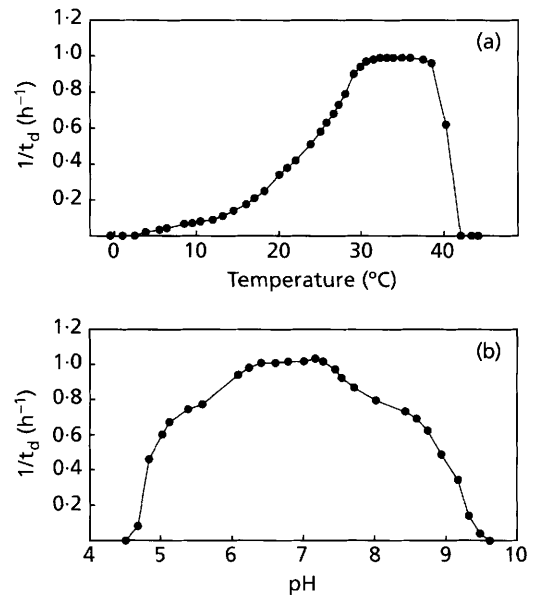
Genotypic characterization

The DNA G+C composition was 27·3 mol% for strain SPL77A^T and 28·4 mol% for strain SPL77B.

To establish the phylogenetic position of the unknown bacteria, the 16S rRNA genes of strains SPL77A^T and
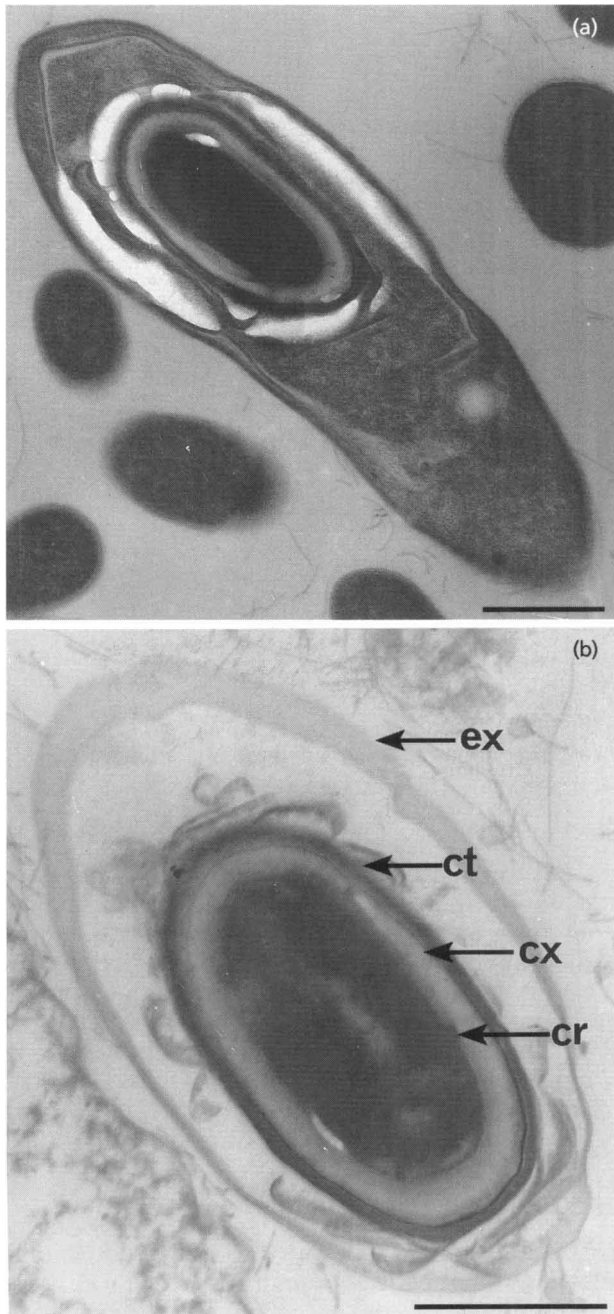
Fig. 4. Electron micrographs of thin sections of a sporulated cell of strain SPL77A^T (a) and of a mature spore (b). Bar, 0·5 µm. ex, Exosporium; ct, spore coat; cx, cortex; and cr, spore core.

Fig. 5. Growth of strain SPL77A^T at various incubation temperatures at pH 7·0 (a) and at various pH values at 30 °C (b). t_d, Doubling time.

SPL77B were amplified by PCR and characterized by sequence analysis. The 16S rRNA sequences for both strains consisted of a continuous stretch of 1481 and 1486 nt, respectively. This length of sequences indicates that approximately 96% of the sequence of the 16S rRNA molecule was determined. Pairwise analysis of the almost complete gene sequences of the two meat isolates showed them to be genealogically homogeneous (100% 16S rRNA sequence similarity). Se-

quence searches of GenBank and the Ribosomal Database Project databases revealed that the unknown Gram-positive rod was phylogenetically most closely associated with the *Clostridium* subphylum (data not shown) and, in particular, to Cluster I clostridia (*sensu* Collins *et al.*, 1994). The sequences of the nearest relatives of the unknown organism were retrieved and subjected to comparative analysis to determine the phylogenetic position of the strains. A tree depicting the phylogenetic affinity of the unknown bacterium within the Cluster I clostridia is shown in Fig. 6. Results of sequence similarity calculations indicate that the nearest relatives of both strains are *C. fallax* (94·5% sequence similarity), *C. algidicarnis* (94·1%), *C. intestinale* (93·9%), *C. cadaveris* (93·6%) and *C. carnis* (93·5%). Detailed phylogenetic analysis not only confirmed the position of strains SPL77A^T and SPL77B within Cluster I of the genus *Clostridium* (Fig. 6), but also the relatively low level of phylogenetic relatedness of both strains to other clostridia within this cluster.

Differentiation from phylogenetically related clostridia

Gel electrophoresis of soluble cell proteins demonstrated that strains SPL77A^T and SPL77B had indistinguishable profiles. In the protein profiles of both strains, major bands occurred at approximately 130, 45, 40, 31, 19 and 16 kDa. However, as shown in Fig. 7, there were distinct differences in the protein profiles, as evidenced by changes in the concentration and positions of major and minor protein bands between profiles of these two meat isolates and their phylo-
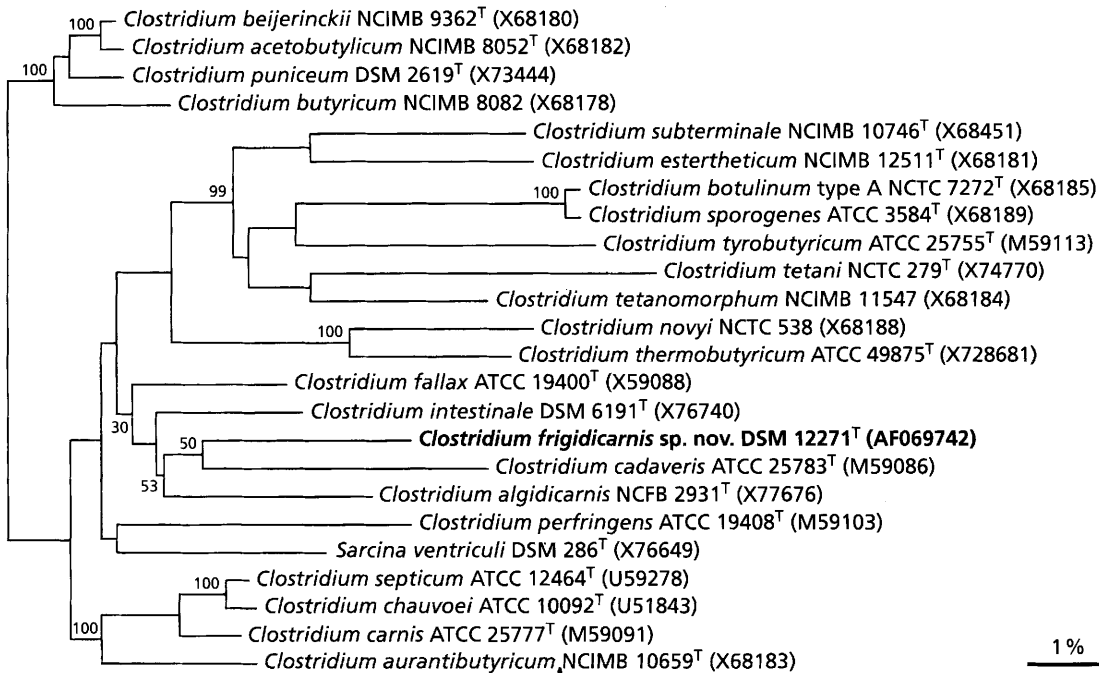
Fig. 6. Phylogenetic tree showing the relationship of strain SPL77A^T (*C. frigidicarnis* sp. nov. DSM 12271^T) within Cluster I of the genus *Clostridium* (Collins *et al.*, 1994). Bar, 1% sequence divergence. Bootstrap values are given at the branching points.
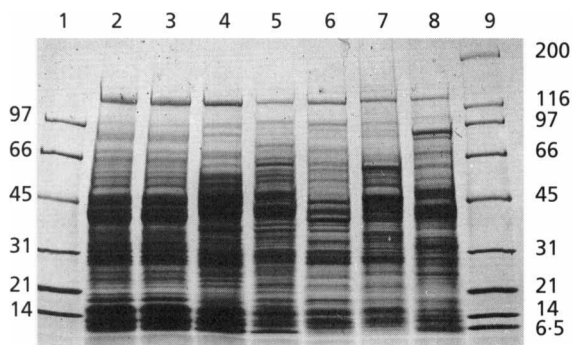
Fig. 7. SDS-PAGE of soluble cell proteins from strains SPL77A^T and SPL77B and other phylogenetically related clostridia. Lane 1, low range protein standard; lane 2, strain SPL77A^T; lane 3, strain SPL77B; lane 4, *C. fallax* (DSM 2631^T); lane 5, *C. algidicarnis* (NCFB 2931^T); lane 6, *C. intestinale* (DSM 6191^T); lane 7, *C. cadaveris* (DSM 1284^T); lane 8, *C. carnis* (DSM 1293^T); and lane 9, broad range protein standard. The values on the left of lane 1 and on the right of lane 9 indicate the positions of protein standards in kDa.

genetic relatives *C. fallax* (DSM 2631^T), *C. algidicarnis* (NCFB 2931^T), *C. intestinale* (DSM 6191^T), *C. cadaveris* (DSM 1284^T) and *C. carnis* (DSM 1293^T).

The major CFA identified in cell extracts of strain SPL77A^T were myristic (C14:0, 14·7%), palmitic (C16:0, 11·6%) and oleic (C18:1, 6·1%) acids. A number of minor fatty acids were also detected (Table 1). Strain SPL77B had a CFA pattern very similar to

that of strain SPL77A^T. However, some differences were observed in the presence and proportions of minor fatty acids. As shown in Table 1, there were distinct differences in the CFA profiles, as evidenced by changes in the presence and proportions of major and minor fatty acids, between profiles of each of the two meat strains and their phylogenetic relatives *C. fallax* (DSM 2631^T), *C. algidicarnis* (NCFB 2931^T), *C. intestinale* (DSM 6191^T), *C. cadaveris* (DSM 1284^T) and *C. carnis* (DSM 1293^T).

DISCUSSION

When strains SPL77A^T and SPL77B were initially isolated from spoiled vacuum-packed meat, their taxonomic status was uncertain. According to the phenotypic criteria and identification keys of Cato *et al.* (1986) and Holdeman *et al.* (1977), these strains could not be assigned to any of the clostridial species described at that time. Similarly, the Rapid ID system designed for identification of medically important anaerobes proved unsatisfactory with these micro-organisms. Strains SPL77A^T and SPL77B were unable to produce abundant gas in artificially inoculated chill-stored packs (S. M. Moorhead, D. M. Broda & R. G. Bell, unpublished results) and did not seem to resemble known psychrotolerant 'blown pack'-causing clostridia. In addition, the phenotypic characteristics for other psychrotolerant clostridial species discovered between 1986 and 1998 differed from those described here for strains SPL77A^T and SPL77B (Collins *et al.*, 1992; Kalchayanand *et al.*, 1993; Lawson *et al.*, 1994;

Table 1. Differences in CFA composition of strains SPL77A^T, SPL77B and phylogenetically related clostridia

Values are given as percentages of total peak area. Strains: 1, SPL77A^T; 2, SPL77B; 3, *C. fallax* DSM 2631^T; 4, *C. algidicarnis* NCFB 2931^T; 5, *C. intestinale* DSM 6191^T; 6, *C. cadaveris* DSM 1284^T; 7, *C. carnis* DSM 1293^T.

Equivalent chain-length	FAME*	1	2	3	4	5	6	7
12·000	12:0	–	–	1·0	–	1·2	–	–
12·206	Unknown	–	–	–	2·6	–	–	–
14·000	14:0	14·7	13·6	6·7	1·7	5·2	12·5	–
14·527	14:1	2·8	2·0	–	2·5	–	–	–
14·716	Unknown	2·0	1·4	–	–	–	–	–
15·350	Unknown	1·4	–	5·1	6·7	7·6	2·4	2·0
15·525	15:1	–	–	1·0	–	1·0	–	–
15·672	Unknown	3·8	2·3	1·1	–	–	–	–
15·868	Unknown	–	–	2·3	3·2	3·7	1·1	–
16·000	16:0	11·6	10·2	21·3	9·2	11·7	13·0	9·5
16·288	Unknown	–	–	2·2	–	2·0	1·6	1·8
16·336	Unknown	1·7	1·8	–	–	–	–	–
16·398	16:1	2·9	3·6	1·3	1·5	5·6	1·4	2·2
17·000	17:0	–	–	–	–	–	–	1·4
17·411	17:1 *cis*-10	–	–	–	–	–	–	1·0
17·538	Unknown	2·8	–	2·2	1·8	3·9	1·5	–
18·000	18:0	3·5	3·0	5·1	1·3	4·7	5·6	4·7
18·207	18:1 *cis*-9	3·0	–	–	–	–	1·0	2·2
18·402	18:1	6·1	4·2	6·5	11·0	8·0	9.6	10·6
18·446	Unknown	–	–	3·1	–	3·1	–	–
19·031	18:2	1·2	1·7	11·2	6·4	1·8	3.8	1·1
19·426	18:3	–	–	–	1·6	–	–	–

* Only fatty acids occurring at concentrations over 1% are listed.

Table 2. Some properties which differentiate strain SPL77A^T from phylogenetically related clostridia

Strains: 1, SPL77A^T; 2, *C. fallax*; 3, *C. algidicarnis*; 4, *C. intestinale*; 5, *C. cadaveris*; 6, *C. carnis*. Results in parentheses are for some strains of the species. +, Positive; –, negative; w, weak reaction (pH 5·5–5·9); NR, not reported.

Phenotypic property	1	2*	3†	4‡	5*	6*
Fermentation products§	A, B, ib, iv, l, o, 2, 4	A, B, L (s, 2, 4)	A, B	A, B (l, f, s)	B, A, 2, 4 (f, p, l, s)	B, A, L, f (s)
Gelatin hydrolysis	+	–	–	–	+	–
Meat digestion	+	–	–	NR	+	–
Indole production	–	–	–	–	+	–
Lecithinase activity	+	–	–	–	–	–
Aesculin hydrolysis	–	+	–	+	–	+
Fermentation of:						
Mannose	+	+	+	+	– (w)	+ (w)
Sorbitol	+	– (w)	–	+	–	–
Trehalose	+	–	–	+	–	– (w)

* Data from Cato *et al.* (1986).

† Data from Lawson *et al.* (1994)

‡ Data from Lee *et al.* (1989).

§ A, Acetate; B, butyrate; L or l, lactate; ib, isobutyrate; iv, isovalerate; f, formate; p, propionate; s, succinate; o, oxalacetate; 2, ethanol; 4, butanol. Upper-case letters indicate major and lower-case letters indicate minor fermentation products.

Table 3. Some properties which differentiate strain SPL77A^T from other psychrotolerant clostridia

Strains: 1, SPL77A^T; 2, *C. arcticum*; 3, *C. algidicarnis*; 4, *C. botulinum* non-proteolytic types B, E, F; 5, *C. estertheticum*; 6, *C. fimetarium*; 7, *C. laramiense*; 8, *C. putrefaciens*; 9, *C. vincentii*. See Table 2 for abbreviations and symbols.

Phenotypic property	1	2*	3†	4*	5‡	6§	7‖	8*	9¶
Maximum growth temperature (°C)	40·5	37	37	<45	15	30	20	<37	20
Optimum growth temperature (°C)	30–38·5	22–25	25–30	25–30	10–12	20–25	15	15–22	12
Fermentation products	A, B, ib, iv, l, o, 2, 4	P, A (b)	A, B	B, A (l)	B, A	A, F, L, 2	A, B, ib, p	a, f, l (s)	A, F, B
Gelatin hydrolysis	+	−	−	+	NR	NR	−	−	NR
Meat digestion	+	−	−	−	NR	NR	+	−	NR
Lipase activity	−	NR	NR	+	NR	NR	+	−	NR
Lecithinase activity	+	NR	−	−	NR	NR	−	−	NR
Starch hydrolysis	−	−	−	+	+	−	+	−	−
Fermentation of:									
Cellobiose	−	W	−	−	+	+	−	−	+
Galactose	−	NR	NR	−	+	+	+	−	+
Lactose	−	−	−	−	NR	−	−	−	+
Maltose	+	−	−	+	+	+	+	−	+
Mannitol	−	W	−	−	+	−	+	−	NR
Sorbitol	+	−	−	+	+	−	NR	−	−
Trehalose	+	−	−	+	NR	−	−	−	NR
Xylose	−	+	+	−	+	+	−	−	+

*Data from Cato *et al.* (1986).
† Data from Lawson *et al.* (1994).
‡ Data from Collins *et al.* (1989).
§ Data from Kotsyurbenko *et al.* (1995).
‖ Data from Kalchayanand *et al.* (1993).
¶ Data from Mountfort *et al.* (1997).

Kotsyurbenko *et al.*, 1995; Mountfort *et al.*, 1997). Consequently, in phenotypic terms, the meat strains were either a new species or were previously described clostridial species that had been phenotypically modified by growth conditions in the laboratory.

The 16S rRNA sequence analysis demonstrated that strains SPL77A^T and SPL77B are phylogenetically related to micro-organisms in Cluster I of the genus *Clostridium* (Collins *et al.*, 1994). The nearest phylogenetic relatives of these meat strains are *C. fallax* (DSM 2631^T), *C. algidicarnis* (NCFB 2931^T), *C. intestinale* (DSM 6191^T), *C. cadaveris* (DSM 1284^T) and *C. carnis* (DSM 1293^T). Sequence divergence values of the meat strains SPL77A^T and SPL77B and their phylogenetic relatives of approximately 5·5–6·4 % indicate that these meat strains should be assigned to a new species (Stackebrandt & Goebel, 1994).

Strains SPL77A^T and SPL77B were easily differentiated from their phylogenetic neighbours by soluble protein profiles, CFA profiles and phenotypic properties (Table 2). During growth in PYGS broth, both strains produced a complex mixture of eight alcohols and volatile and non-volatile fatty acids, including ethanol, butanol, isovalerate, isobutyrate and oxalacetate. None of their near phylogenetic relatives are known to produce this combination of compounds (Cato *et al.*, 1986; Lee *et al.*, 1989; Lawson *et al.*, 1993). The meat strains were lecithinase-positive, whereas their relatives are lecithinase-negative. Of five reference strains used in the comparison, only *C. cadaveris* (DSM 1284^T) hydrolyses gelatin and digests meat. This reference strain, however, produces indole, whereas strains SPL77A^T and SPL77B do not, and it is unable to ferment mannose, sorbitol and trehalose – three carbohydrates fermented by the two meat strains.

Strains SPL77A^T and SPL77B were able to grow at 4 °C. Therefore, by the definition of Eddy (1960) they can be described as psychrotolerant. However, the phenotypic characteristics of these strains differed from those of the other psychrotolerant clostridia (Table 3). Of all the psychrotolerant strains used for comparison in this study, only the meat strains formed ethanol, butanol, isovalerate, isobutyrate and oxalacetate as fermentation products. These strains were readily distinguished from *Clostridium arcticum*, *C. estertheticum*, *C. laramiense* and *Clostridium vincentii* by their maximum and optimum growth temperature

and substrate utilization pattern (Cato *et al.*, 1986; Collins *et al.*, 1992; Kalchayanand *et al.*, 1993; Mountfort *et al.*, 1997). In addition, *C. laramiense* exhibits lipase but not lecithinase activity, reduces nitrate but does not hydrolyse gelatin (Kalchayanand *et al.*, 1993). Unlike *C. putrefaciens*, strains SPL77A^T and SPL77B were both proteolytic and saccharolytic. With respect to the optimum growth temperature, the meat strains showed closest similarity with *C. algidicarnis* and non-proteolytic *Clostridium botulinum* types B, E or F. However, *C. algidicarnis* cannot hydrolyse gelatin or digest meat and utilizes a different range of substrates than strains SPL77A^T and SPL77B (Lawson *et al.*, 1994). Non-proteolytic *C. botulinum* types B, E or F exhibit lipase but not lecithinase activity, can hydrolyse starch but do not digest meat or milk (Cato *et al.*, 1986). In contrast, strains SPL77A^T and SPL77B showed lecithinase but not lipase activity, did not hydrolyse starch but digested meat and milk.

Only small differences in colony morphology, physiological and biochemical reactions, DNA base composition and minor CFA were found between strains SPL77A^T and SPL77B, suggesting that the two isolates are different strains of the same species.

Based on both phenotypic and phylogenetic findings, we consider that strains SPL77A^T and SPL77B merit classification as a new species within the genus *Clostridium*, for which we propose the name *Clostridium frigidicarnis*.

Description of *Clostridium frigidicarnis* sp. nov.

Clostridium frigidicarnis (fri.gi.di.car'nis. L. adj. *frigidus* cool; L. n. *carno* meat; N.L. gen. n. *frigidicarnis* of cool meat).

Colonies on sheep blood agar measure from 2·2–7·2 mm in diameter and are: circular to irregular; with undulate, lobate or erose margins; creamy-grey to grey; opaque or semi-opaque to translucent; and β-haemolytic. Cells are Gram-positive, motile rods (4·5–9·4 μm long and 1·3–1·6 μm wide) occurring singly or in pairs. Elliptical spores are produced in early stationary growth phase. Psychrotolerant and grows between 3·8 and 40·5 °C (optimum growth temperature is 30·0–38·5 °C). At 30 °C, it grows between pH 4·7 and 9·5; optimum pH for growth is 6·4–7·2. Fructose, glucose, mannose, mannitol and trehalose are fermented, lecithinase activity is present and gelatin is hydrolysed. Fermentation products formed in PYGS broth are acetate, ethanol, butyrate, isovalerate, butanol, isobutyrate, oxalacetate, lactate, hydrogen and carbon dioxide. Major CFA are myristic, palmitic and oleic acids. DNA G+C composition is 27·3–28·4 mol%. Isolated from vacuum-packed temperature-abused beef and from faeces, hides and tonsils of bovine slaughter stock. Possibly forms a part of a normal flora of vacuum-packed chilled meats. The type strain is strain SPL77A^T (= DSM 12271^T).

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