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# *Clostridium gasigenes* sp. nov., a psychrophile causing spoilage of vacuum-packed meat

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**Two psychrophilic *Clostridium* strains, DB1A$^T$ and R26, were isolated from incidences of 'blown-pack' spoilage of vacuum-packed chilled lamb. Vacuum packs of meat inoculated with these strains developed gas bubbles and pack distension within 14 d storage at 2 °C. The two main gases responsible for pack distension were carbon dioxide and hydrogen. 1-Butanol, butyric and acetic acid and butyl esters were the major volatile compounds produced by the strains in the artificially inoculated packs. The unknown strains were Gram-positive motile rods producing elliptical subterminal spores during the late-stationary growth phase. At pH 7·0, they grew from −1·5 to 26 °C, and their optimum growth temperature was 20–22 °C. At 20 °C, the pH range for growth was 5·4–8·9 and the optimum pH for growth was 6·2–8·6. In peptone/yeast extract broth, the organisms grew little or not at all in the absence of fermentable carbohydrates. Both strains hydrolysed gelatin, aesculin and starch. The fermentation products formed in peptone yeast extract glucose starch broth were ethanol, acetate, butyrate, lactate, butanol, carbon dioxide and hydrogen. The G+C contents of the DNA of strains DB1A$^T$ and R26 were 29·4 and 28·3 mol%, respectively. Phylogenetic analyses indicated that the strains belong to cluster I of the genus *Clostridium* (*sensu* Collins *et al.* 1994). The new strains differed from the phylogenetically related clostridia in cellular fatty acid composition, soluble protein profiles and phenotypic properties. On the basis of rDNA analysis and phenotypic and phylogenetic characterization, the strains were assigned to a new species for which the name *Clostridium gasigenes* is proposed. Strain DB1A$^T$ (= DSM 12272$^T$) is designated as the type strain.**

**Keywords:** *Clostridium gasigenes*, psychrophile, 'blown-pack' spoilage, food spoilage

## INTRODUCTION

'Blown-pack' spoilage is an increasingly reported spoilage condition of vacuum-packed chilled meats. This type of meat spoilage is characterized by gross, gas-induced distension of packs and the production of offensive odours after 4–6 weeks at refrigeration temperatures (−1·5 to 2 °C). Two psychrophilic species, *Clostridium estertheticum* (Collins *et al.*, 1992) and *Clostridium laramiense* (Kalchayanand *et al.*, 1989; Trüper & de' Clari, 1997), have been confirmed as the causative agents of 'blown-pack' spoilage. Eight other psychrotolerant clostridial isolates have been associated with New Zealand incidences of this type of spoilage (Broda *et al.*, 1996). However, none of these isolates has characteristics similar to those described for *C. estertheticum* or *C. laramiense* (Collins *et al.*, 1992; Kalchayanand *et al.*, 1993). Consequently, either the clostridial isolates obtained in the New Zealand spoilage incidents were not responsible for pack 'blowing', or they were responsible, in which case 'blown-pack' spoilage can be caused by clostridial species other than *C. estertheticum* or *C. laramiense*.

This study describes the characteristics of an unknown psychrophilic *Clostridium* sp. shown to cause gas production and pack distension of vacuum-packed chill-stored meats. These data will allow the identification of the micro-organism that causes 'blown-pack' spoilage. Once identified, such organisms can be

**Abbreviations:** FAME, fatty acid methyl ester; MP, maximum parsimony.

The GenBank accession numbers for the 16S rRNA sequences of strains DB1A$^T$ and R26 reported in this paper are AF092548 and AF143692, respectively.

traced back to contamination reservoirs within the abattoir, which, ultimately, will enable control of 'blown-pack' spoilage.

## METHODS

**Bacteria.** Strains DB1A$^T$ (the type strain) and R26 were isolated from 'blown' vacuum-packed lamb legs that spoiled during a period of chilled storage of less than 8 weeks, in the absence of temperature abuse. Additional isolates similar to these meat strains were obtained on different occasions from cervine and bovine slaughter stock. In a preliminary study, phenotypic properties and total soluble cell-protein profiles of these additional isolates were compared with those of the original strains. All isolates resembled either strain DB1A$^T$ or strain R26. These two strains, termed 'meat strains' in this paper, were therefore the only ones characterized in this study. The results showing the homogeneity of DB1A$^T$-like or R26-like isolates from different sources will be published elsewhere.

The two meat strains, maintained as freeze-dried cultures, were revived in Peptone Yeast Extract Glucose Starch (PYGS) broth (Lund *et al.*, 1990), subcultured on to Columbia Blood Agar (CBA; Oxoid) containing 5% (v/v) sheep blood, and incubated at 20 °C for 72 h.

Reference strains of *Clostridium vincentii* (DSM 10228$^T$), *C. estertheticum* (DSM 8809$^T$ = NCIMB 12511$^T$) and *Clostridium carnis* (DSM 1293$^T$ = ATCC 25777$^T$) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Reference strains of *Clostridium septicum* (NZRM 18$^T$ = ATCC 12464$^T$) and *Clostridium chauvoei* (NZRM 99$^T$ = ATCC 10092$^T$) were obtained from the New Zealand Culture Collection, Communicable Disease Centre (Porirua, New Zealand). Reference strains of psychrotolerant, non-proteolytic *Clostridium botulinum* type B (17B = ATCC 25765) and *C. botulinum* type E (Beluga) were obtained from Dr E. A. Szabo, CSIRO (Sydney, Australia). With the exception of *C. estertheticum*, reference strains were revived from freeze-dried material in PYGS broth and plated on to CBA supplemented with 5% (v/v) sheep blood. *C. estertheticum* was revived in sterile, anaerobic milk. *C. vincentii* and *C. estertheticum* were grown at 10 °C, and both of the psychrotolerant *C. botulinum* strains were grown at 30 °C. The remaining reference strains were grown at 37 °C.

Anaerobic techniques and pre-reduced media (Holdeman *et al.*, 1977) were used for culturing all *Clostridium* strains.

**Confirmation of 'blowing' ability.** The ability to cause 'blown-pack' spoilage in a laboratory meat model was demonstrated for strains DB1A$^T$, R26 and reference strain *C. estertheticum* (DSM 8809$^T$). Each strain was grown in 10 ml PYGS broth until a heavy suspension (approx. $10^8$ ml$^{-1}$) was obtained. Cells of each strain were harvested by centrifugation at 6000 ***g*** for 15 min and washed five times with ice-cold saline (0·85%, w/v, NaCl). These washed suspensions were used as inocula.

Chilled, boneless lamb chumps were obtained 1 d after boning at a local meat plant and were placed individually into barrier bags (Cryovac BB4L) pending inoculation. Duplicate packs were prepared for each strain tested. The packs were inoculated with 0·5 ml of a cell suspension of each strain to obtain a meat-surface concentration of approximately $10^3$ c.f.u. cm$^{-2}$. Inoculated packs and two uninoculated controls were immediately vacuum-packed using a controlled-atmosphere packaging machine (Securepack 10) and stored at 2 °C for up to 84 d (the maximum shelf-life expected for vacuum-packed chilled lamb). Packs were examined regularly for the presence of gas bubbles in the meat drip, followed by loss of vacuum and substantial pack distension.

**Composition of headspace volatiles.** The headspace volatiles from packs inoculated with strains DB1A$^T$ and R26 were analysed using GC and MS. Chilled, whole lamb legs, obtained from a local meat plant, were placed individually into barrier bags (Cryovac BB4L) pending inoculation. Duplicate packs were prepared for each strain tested. Each inoculum was prepared and packs were inoculated as described in the previous section. In addition, two uninoculated control packs were also prepared. Inoculated packs and uninoculated controls were immediately vacuum-packed using a controlled-atmosphere packaging machine (Securepack 10) and were stored at 2 °C for 84 d.

At the end of the storage trial, headspace volatiles produced in each inoculated vacuum pack and in the uninoculated control packs (released by introducing ultrapure oxygen-free nitrogen into the packs) were collected using pre-conditioned Tenax TA (Alltech Associates) traps and analysed, as described previously (Broda *et al.*, 1996). Briefly, traps carrying volatiles were thermally desorbed; then volatile compounds were cryofocused at the head of the free fatty acid phase column and separated on a Fisons 8000 series gas chromatograph fitted with a Fisons MD800 mass spectral detector (Fisons Instruments). Unknown compounds were identified by comparison of their mass spectra with those of known compounds in the National Institute of Standards and Technology library (Gaithersburg, MD, USA) and quantified against retention times and peak areas of authentic compounds.

The oxygen content of the headspace in each pack was measured with an oxygen meter (Gaspace Systech Instruments). The carbon dioxide and hydrogen contents were determined on a Varian gas chromatograph using a Porapak Q 80/100-mesh column (Supelco) and a thermal conductivity detector.

**Phenotypic characterization.** Colony morphology was described for strains DB1A$^T$ and R26 grown on the surface of CBA with 5% (v/v) sheep blood at 20 °C for 72 h. Descriptions of vegetative cell and spore morphology of the meat strains grown in PYGS broth at 20 °C for 48 h and 3 months, respectively, were based on phase-contrast microscopy (Leitz Orthoplan). Vegetative cell and spore ultrastructure was determined for each meat strain grown in PYGS broth at 20 °C for 48 h and 3 months, respectively, using a transmission electron microscope (EM 400; Philips). The presence of flagella was determined in negatively stained 48 h cultures of strains DB1A$^T$ and R26 by transmission electron microscopy.

Vegetative cells of strains DB1A$^T$ and R26 were Gram-stained using the method of Johnson *et al.* (1995). The Gram-type was determined using a KOH test (Powers, 1995). The cell wall type was determined using transmission electron microscopy.

For physiological tests, growth in anaerobic Peptone Yeast Extract (PY) broth (Holdeman *et al.*, 1977) containing 1% (w/v) yeast extract, the glucose concentration having been adjusted to 0·5% (w/v), was determined by monitoring the optical density at 550 nm relative to three uninoculated controls. The potential for growth at 20 pH values ranging from 5·13 to 9·12 was determined at 20 °C. Broths were inoculated with 2% (v/v) of culture growing exponentially at pH 7·0. The pH of the medium was kept constant ($\pm$0·1

***Table 1.*** Concentrations of some major headspace volatile compounds in vacuum-packed meat inoculated with pure cultures of strain DB1A$^T$, strain R26 or *C. estertheticum*, compared with uninoculated control packs, after storage for 84 d at 2 °C

TR, Trace; —, none detected.

| Compound* | DB1A$^T$† | R26† | *C. estertheticum*‡ | Uninoc. cntrl† |
|---|---|---|---|---|
| Acetic acid | 0·4 | 0·3 | 0·5 | 0·8 |
| Butyric acid | 1·8 | 2·5 | 2·5 | — |
| 1-Butanol | 224 | 123 | 38 | TR |
| 1-Pentanol | 0·3 | 0·2 | TR | — |
| 3-Methylbutanal | 2·1 | 4·0 | TR | — |
| 1-Butyl acetate | 0·4 | 0·4 | 4·5 | — |
| 1-Butyl butyrate | 4·4 | 1·4 | 20·5 | — |
| Carbon dioxide (%) | 68 ± 1 | 69 ± 1 | 68·5 ± 1 | — |
| Hydrogen (%) | 30 ± 1 | 30 ± 1 | 28·5 ± 1 | — |

* Concentrations in ng ml$^{-1}$ gas, unless otherwise stated.

† With packs inoculated with pure cultures of strains DB1A$^T$ and R26 and with uninoculated control packs, 1-hexanol, 1-octen-3-ol, hexanal, benzaldehyde, ethyl butyrate, 1-butyl formate, 1-butyl propionate and oxygen were not detected and the presence of nitrogen was not determined; these compounds, however, were present in vacuum packs inoculated with the pure culture of *C. estertheticum* (Dainty *et al.*, 1989).

‡ Data from Dainty *et al.* (1989); means of duplicate tests.

unit) by adjustment with potassium phosphate buffer. The temperature range for growth was determined at pH 7·0. Growth rates were obtained for 24 temperatures ranging from −1·5 to 30·2 °C. Hungate tubes containing medium were inoculated with 2% (v/v) of culture growing exponentially at 20 °C and incubated in a temperature gradient incubator. For both physiological tests, tubes were incubated for up to 21 d to determine whether or not growth had occurred.

Substrate utilization by strains DB1A$^T$ and R26 was tested anaerobically at 20 °C in PY broth containing 0·1% (w/v) yeast extract with an initial pH of 7·0. Fermentation of 21 different substrates, gelatin hydrolysis, the presence of lecithinase and lipase activities, milk reaction, meat and casein digestion, indole production, nitrate reduction, the presence of oxidase and catalase activities, urea hydrolysis, ammonia and hydrogen sulphide production, aesculin and starch hydrolysis, and growth in the presence of bile, and Tween 80, were tested according to the methods of Holdeman *et al.* (1977). The substrates were as follows [concentrations were 1% (w/v) except where otherwise 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.

Fermentation products were determined for strains DB1A$^T$ and R26 grown in PYGS broth at 20 °C for 10 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) and analysed as described previously (Broda *et al.*, 1999).

Strain DB1A$^T$ was tested for toxicity in mice using procedures described in the Bacteriological Analytical Manual of the US Food and Drug Administration (Solomon *et al.*, 1995).

**DNA base composition.** Strains DB1A$^T$ and R26 were grown in PYGS broth at 20 °C for 48 h. The G+C content of the DNA (mol%) was determined for both meat strains by using the method of Mesbah *et al.* (1989).

**Relatedness to other taxa.** The relatedness of strains DB1A$^T$ and R26 to other (eu)bacterial taxa was assessed by comparing 16S rRNA gene sequences of the unknown meat isolates with those of other (eu)bacteria and by conducting phylogenetic analyses.

For 16S rRNA gene sequence determinations, strains DB1A$^T$ and R26 were grown in PYGS broth at 20 °C for 48 h, then cells were harvested by centrifugation at 12000 ***g*** for 15 min. DNA was isolated using the method of Marmur (1961).

The 16S rRNA gene sequences were determined from PCR-amplified 16S rDNA fragments from each meat strain. The PCR fragments were purified with the Wizard purification kit (Promega, Dade Diagnostics) and sequenced directly in an ABI 377 automated sequencer (Perkin Elmer, Applied Biosystems). Sequence analysis was performed on a Silicon Graphics Indigo 2 analyser, using the GCG package (Devereux *et al.*, 1984). Sequences were aligned using the program PILEUP (Feng and Doolittle, 1987) with a low gap weighting. The alignment was unequivocal and no manual adjustments were required; 1408 sites of the aligned sequences were used and gaps were treated as missing data. Phylogenetic reconstruction was performed with maximum-parsimony (MP) as the optimality criterion, using the program PAUP* (Swofford, 1996). A single most parsimonious tree was generated using a heuristic search of 1000 replicates with random addition of sequences. The stability of the grouping was estimated using MP bootstrap analysis of 2000 replicates. The branching patterns of the phylogenetic tree were further assessed using the neighbour-joining method as described previously (Lawson *et al.*, 1996) and maximum-likelihood as the optimality criterion with the program PAUP* (Swofford, 1996). When the maximum-

likelihood criterion was selected, the HKY85 (Hasegawa *et al.*, 1985) substitution model was used. Rates were assumed to be equal and only 10 heuristic replicates were performed because of time limitations with this number of taxa.

The relatedness of strains DB1A$^T$ and R26 to phylogenetically related clostridia was assessed using SDS-PAGE of total soluble cell proteins and by cellular fatty acid analysis. For these tests, cells of each meat isolate were grown in 50 ml volumes of PYGS broth at 20 °C for 48 h. Cells were harvested at the end of the exponential growth phase by centrifugation at 6000 ***g*** for 15 min. The cells were then washed and suspended in sterile ultrapure water.

For SDS-PAGE, 0·3–0·5 ml of each bacterial suspension with an optical density of 1·5 at 550 nm was sonicated, then mixed 1:1 (v/v) with an SDS Laemmli sample buffer (Bio-Rad). Low- and broad-range protein standards (Bio-Rad) were mixed with the sample SDS buffer (1:20, v/v). Samples and standards were denatured by boiling for 5 min. Portions (30 µl) of these denatured samples and 10 µl of the standards were loaded into the wells of a 4–15 %-gradient Tris/HCl polyacrylamide gel (Bio-Rad) and subjected to electrophoresis. SDS-PAGE was performed using the discontinuous buffer system of Laemmli (1970) in a Ready Gel Cell vertical gel apparatus (Bio-Rad). Electrophoresis was carried out at constant voltage of 150 V for 1 h at 15 °C and the gel was stained with Coomassie brilliant blue R-250 (BDH).

For cellular fatty acid analysis, fatty acid methyl esters (FAMEs) were extracted as described by Kuykendall *et al.* (1988). Briefly, 40 mg wet weight cells of each strain was saponified by heating at 100 °C for 30 min with 1 ml NaOH (15 %, w/v) in aqueous methanol (50 %, v/v). Samples were cooled at ambient temperature, then 1·5 ml HCl (25 %, v/v) in aqueous methanol (50 %, v/v) was added and the mixture heated at 100 °C for 15 min. FAMEs 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 by means of a Hewlett Packard 6890 gas chromatograph, as described previously (Broda *et al.*, 1999).

## RESULTS

### Confirmation of 'blowing' ability

In packs inoculated with vegetative cells of strains DB1A$^T$ and R26, small gas bubbles were first present in the meat drip after 14 d storage at 2 °C. In comparison, gas production in packs inoculated with vegetative cells of the reference strain of *C. estertheticum* (DSM 8809$^T$) was first evident at 11 d storage. There were differences in the quantity of gas sub-

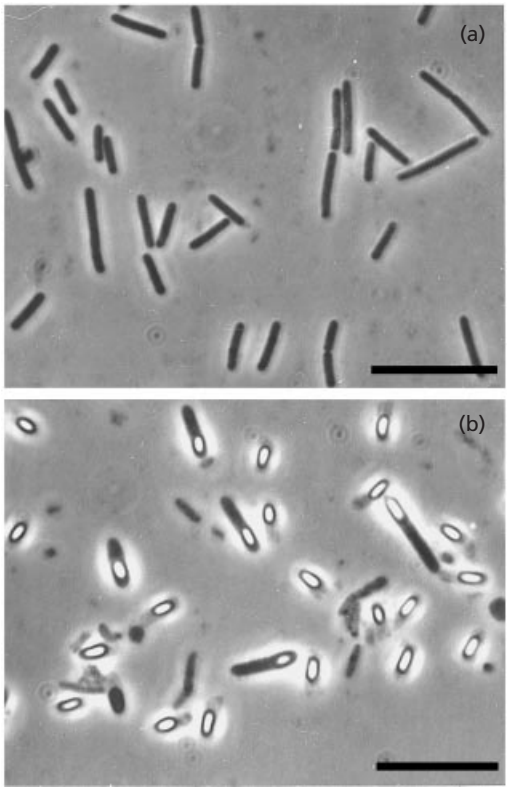


***Fig. 1.*** Phase-contrast micrographs of vegetative (a) and sporulated (b) cells of strain DB1A$^T$. Bars, 10 µm.

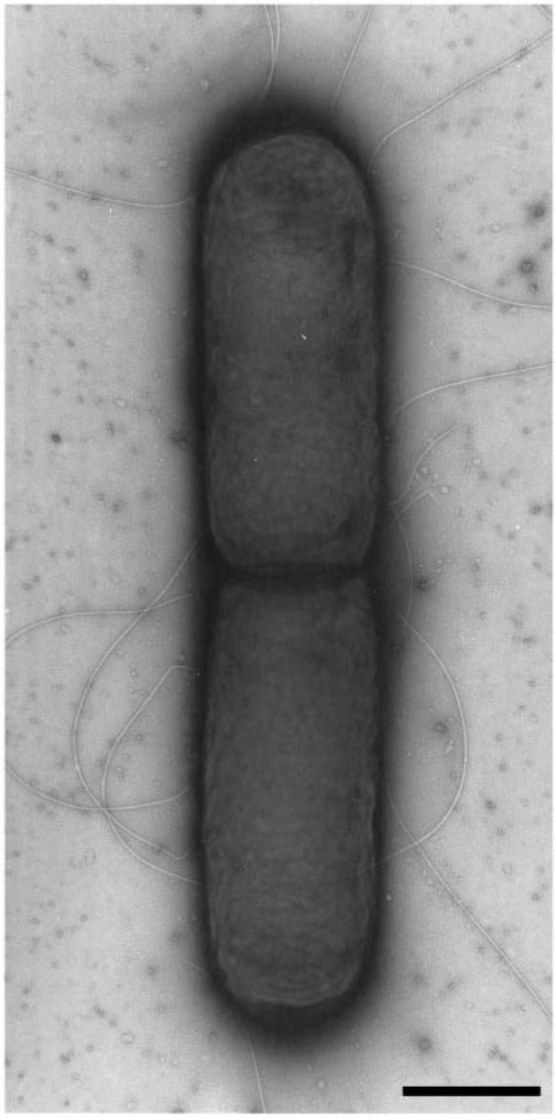

***Fig. 2.*** Electron micrographs of a negatively stained cell of strain DB1A$^T$ showing peritrichous flagella. Bar, 0·3 µm.

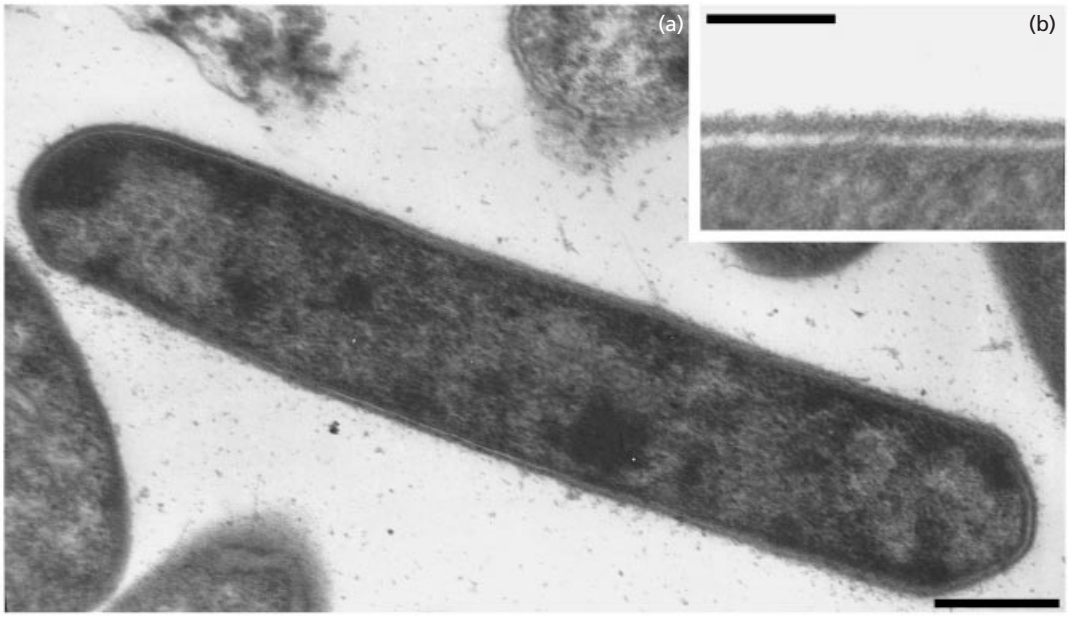


***Fig. 3.*** Electron micrographs of thin sections of vegetative cells of strain DB1A$^T$ showing (a) cell shape and (b) the Gram-positive-type cell wall. Bars, 0·3 μm (a) and 0·1 μm (b).

sequently produced in the inoculated packs. With *C. estertheticum* (DSM 8809$^T$), a large volume of gas was produced, 'blowing' the packs to twice their original size after approximately 22 d storage at 2 °C and producing tightly distended packs after 35 d storage. With strains DB1A$^T$ and R26, a smaller volume of gas was formed in the packs; these packs did not reach a tightly distended state within 84 d storage. No gas was present in uninoculated control packs stored at 2 °C for 84 d.

### Composition of headspace volatiles

Both meat strains produced a headspace gas of similar composition (Table 1). The major gases produced were carbon dioxide and hydrogen and the major volatile compounds were 1-butanol, butyl butyrate and butyric acid. There were differences, however, in the relative proportions of some volatiles produced by the two strains. The headspace volatiles of packs inoculated with strain DB1A$^T$ contained almost twice the concentration of butanol and three times the concentration of butyl butyrate compared with packs inoculated with strain R26. Acetic acid and traces of butanol were the only volatiles detected in the artificially created headspace of uninoculated control packs.

### Phenotypic characterization

Colonies of strains DB1A$^T$ and R26 on CBA agar were circular with an entire margin, raised, convex, shiny, smooth and $\beta$-haemolytic. The colonies of both meat strains were 0·7–3·0 mm in diameter. The colonies of strain DB1A$^T$ were grey/white and opaque with translucent edges; colonies of strain R26 were grey and semi-translucent. With both strains, cells in the exponential growth phase were relatively thin, straight rods 2·0–7·5 μm long and 0·4 μm wide (Fig. 1a). These rods occurred singly or in pairs and were motile by means of peritrichous flagella (Fig. 2). Electron micrographs of ultrathin sections of vegetative cells showed that the organism was a round-ended rod (Fig. 3a) and that cell division may involve both a septation and a pinching mechanism (Fig. 4). A Gram-positive-type, single-layer cell wall was observed in ultrathin sections of vegetative cells of both strains (Fig. 3b). Elliptical, subterminal spores were formed in the late stationary growth phase (Figs 1b and 5a). The spores caused slight swelling of the maternal cells (Fig. 5a). The mature spores had a fully developed endospore structure (Fig. 5b).

With both strains, DB1A$^T$ and R26, cells in the exponential growth phase stained as Gram-positive. The KOH reaction was always negative (i.e. characteristic of Gram-positive cells) irrespective of growth medium or age of culture. This Gram-positive type was confirmed by the presence of a single-layer cell wall, as observed in electron micrographs of ultrathin sections (Fig. 3b).

Strains DB1A$^T$ and R26 grew optimally at 20–22 °C (Fig. 6a). The lowest tested temperature at which growth of strain DB1A$^T$ was observed was −1·5 °C and the highest temperature at which growth was observed was 26 °C. The optimal pH for growth of both strains was 6·2–8·6 and growth occurred at pH values between 5·4 and 8·9 (Fig. 6b). No growth of meat strains was observed on blood plates incubated aerobically at 20 °C for 14 d.

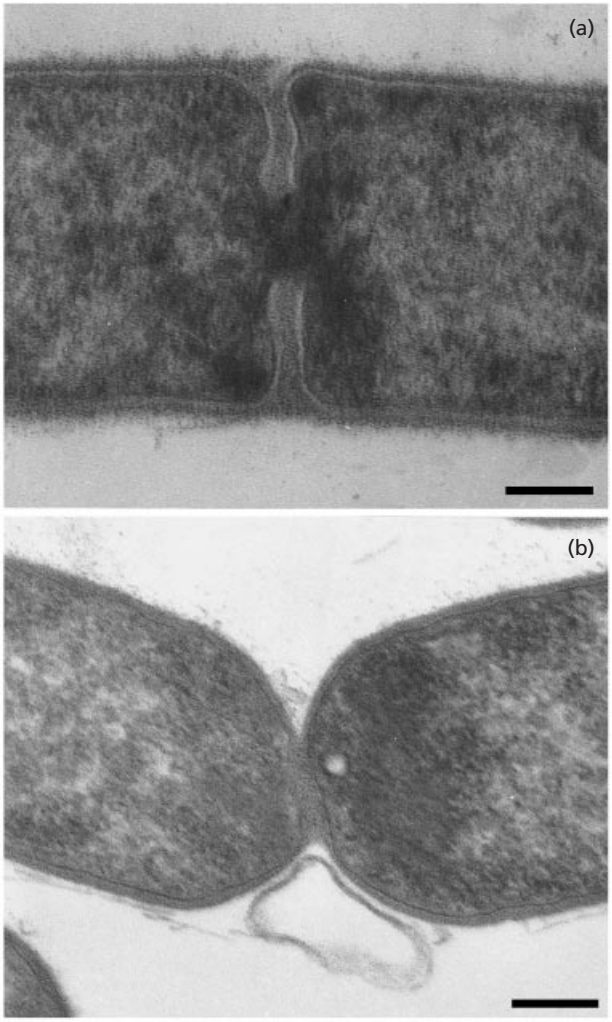


***Fig. 4.*** Electron micrographs of thin sections of a dividing cell of strain DB1A$^T$ indicating a septation (a) or pinching (b) mechanism of cell division. Bars, 0·1 μm.

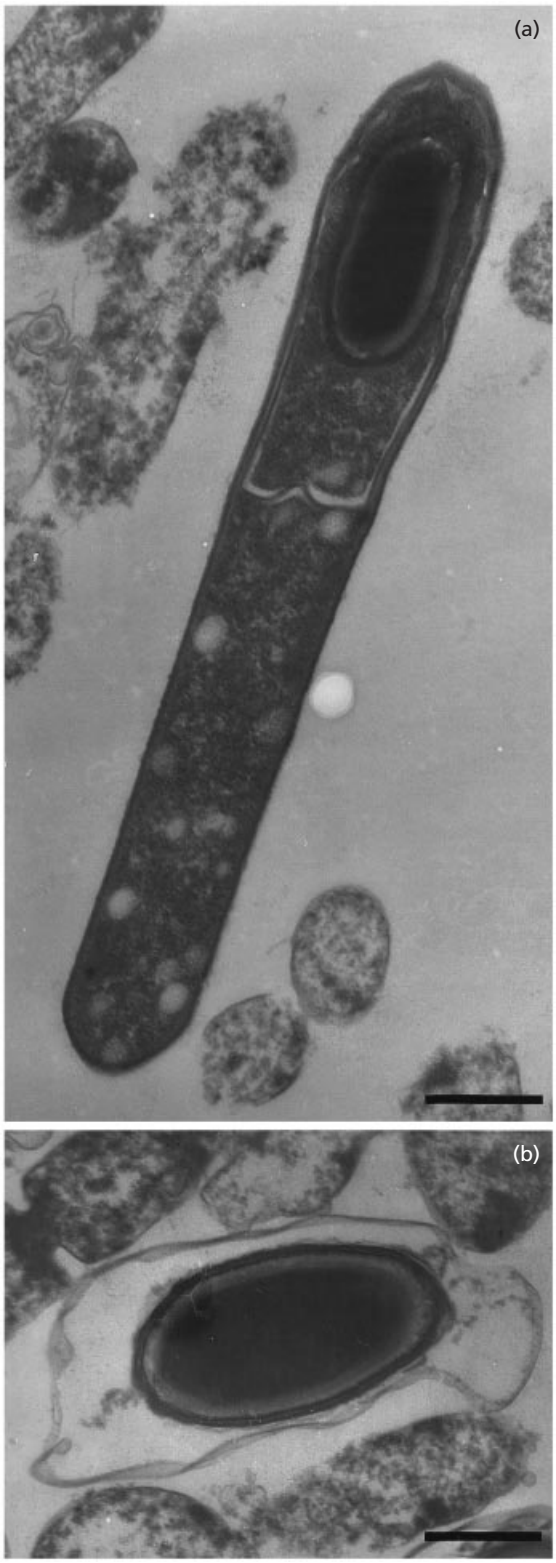


***Fig. 5.*** Electron micrographs of thin sections of a sporulated cell of strain DB1A$^T$ (a) and of a mature spore (b). Bars, 0·5 μm.

Very little or no growth of either meat strain occurred in the PY broth containing 0·1 % (w/v) yeast extract in the absence of fermentable carbohydrate. The substrates fermented by strain DB1A$^T$ in this broth were: cellobiose, fructose, glucose, inositol, maltose, mannose, salicin and trehalose. The substrates not fermented were: adonitol, arabinose, dextran, galactose, inulin, lactose, mannitol, rhamnose, raffinose, sorbitol, sorbose, sucrose and xylose. Gelatin, starch and aesculin were hydrolysed. The milk reaction was positive for curd formation. Addition of Tween 80 did not stimulate growth. The remaining tests were negative. Strain R26 was positive or negative for the same biochemical tests as strain DB1A$^T$, with the exception that sucrose was fermented, galactose was weakly fermented and inositol was not fermented.

The major fermentation products formed by strains DB1A$^T$ and R26 in PYGS broth were as follows: ethanol (26·1 and 20·9 mM, respectively), acetate (8·9 mM for each strain), butyrate (6·5 and 6·6 mM, respectively), lactate (5·1 and 5·6 mM, respectively),

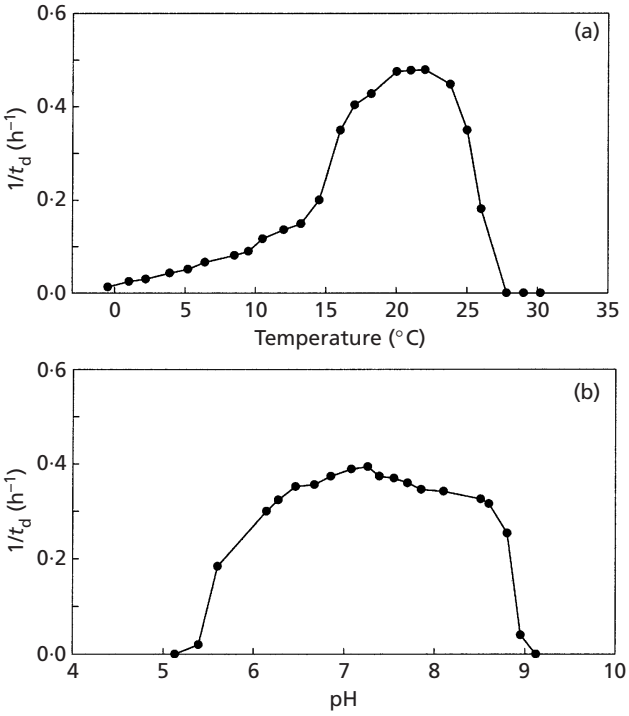


***Fig. 6.*** Growth of strain DB1A$^T$ at various incubation temperatures at pH 7·0 (a) and at various pH values at 20 °C (b); $t_d$, doubling time.

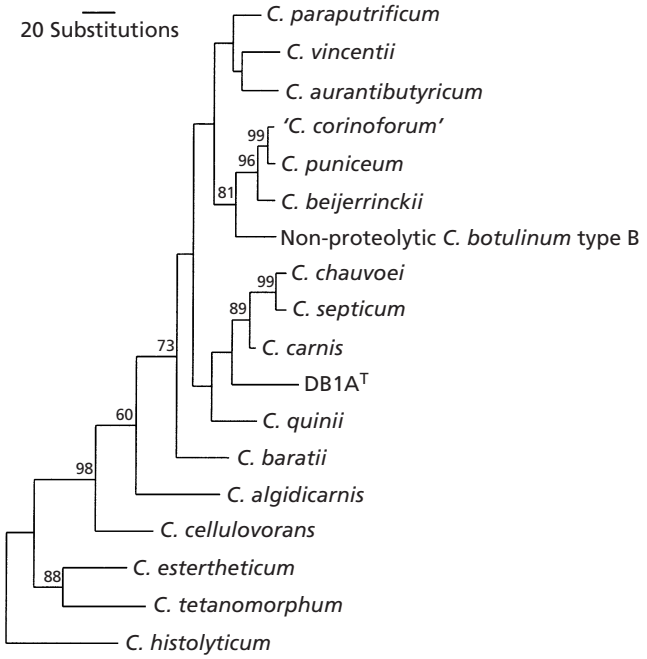


***Fig. 7.*** Most parsimonious tree inferred from aligned 16S rRNA gene sequences using a heuristic search under the criterion of maximum-parsimony. The values on the branches indicate the level of support derived from bootstrap analyses of 2000 replicates. Where no number is shown, bootstrap values are less than 50 %. The following *Clostridium* reference strains were used in phylogenetic analyses: *C. algidicarnis* NCFB 2931$^T$ (X77676), *C. aurantibutyricum* NCIMB 10659$^T$ (X68183), *C. baratii* ATCC 43756 (X68175), *C. beijerinckii* DSM 791$^T$ (X68179), non-proteolytic *C. botulinum* type B (17B) ATCC 25765 (X68173), *C. carnis* ATCC 25777$^T$ (M59091), *C. cellulovorans* DSM 3052$^T$ (X73438), *C. chauvoei* ATCC 10092$^T$ (U51843), *'C. corinoforum'* DSM 5906 (X76742), *C. estertheticum* NCIMB 12511$^T$ (X68181), *C. histolyticum* ATCC 19401$^T$ (M59094), *C. paraputrificum* DSM 2630$^T$ (X73445), *C. puniceum* DSM 2619$^T$ (X71857), *C. quinii* DSM 6736$^T$ (X57262), *C. septicum* ATCC 12464$^T$ (U59278), *C. tetanomorphum* NCIMB 11547 (X68184) and *C. vincentii* DSM 10228$^T$ (X97432). The sequence of strain R26 was excluded from the analysis because of its close similarity to strain DB1A$^T$. The *Clostridium* cluster II species *C. histolyticum* was used as an outgroup.

butanol (1·2 and 0·3 mM, respectively), carbon dioxide and hydrogen.

The supernatant of strain DB1A$^T$ was not toxic to mice.

### DNA base composition

The G+C DNA content was 29·4 mol% for strain DB1A$^T$ and 28·3 mol% for strain R26.

### Relatedness to other taxa

To determine the relatedness of the two meat strains to each other and to other (eu)bacteria, the 16S rRNA genes of strains DB1A$^T$ and R26 were sequenced and the resulting sequences used to search the 16S rRNA GenBank database. Similarity analysis of the almost complete gene sequences of the two meat isolates showed them to be nearly identical. Pairwise comparison of the sequences from strains DB1A$^T$ and R26 demonstrated three mismatches within 1483 nucleotides used in the comparison, corresponding to a 99·8% 16S rRNA gene sequence similarity between the two meat isolates. Sequence searches of the GenBank database revealed that the unknown Gram-positive rod was most closely associated with the *Clostridium* subphylum (data not shown) and, in particular, to cluster I clostridia (*sensu* Collins *et al.*, 1994). The results of the sequence-similarity calculations indicate that the nearest relatives of both strains are *C. vincentii* (95·5% sequence similarity), *C. septicum* (95·4%), *C. chauvoei* (95·2%), non-proteolytic *C. botulinum* types B and E (95·0%) and *C. carnis* (94·7%).

The aligned sequences provided 181 parsimony-informative sites. A heuristic search using maximum-parsimony as the optimality criterion found a single most parsimonious tree of length 690 (Fig. 7). The consistency index of this tree was 0·575 and the retention index was 0·525. The major branching order indicated by maximum-parsimony was confirmed by both the neighbour-joining and maximum-likelihood trees (data not shown). These trees were also in accord with that of Collins *et al.* (1994). Within cluster I, strains DB1A$^T$ and R26 were placed in the monophyletic unit containing sublines represented by *C. carnis*, *C. chauvoei*, *C. septicum* and *Clostridium quinii* (Fig. 7). Within this unit, however, strain DB1A$^T$

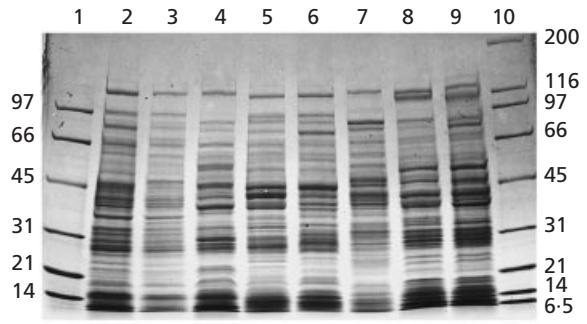


***Fig. 8.*** SDS-PAGE of soluble cell proteins from strains DB1A$^T$ and R26 and their nearest relatives. Lanes: 1, low-range protein standard; 2, strain DB1A$^T$; 3, strain R26; 4, *C. vincentii* (DSM 10228$^T$); 5, *C. septicum* (NZRM 18$^T$); 6, *C. chauvoei* (NZRM 99$^T$); 7, *C. carnis* (DSM 1293$^T$); 8, non-proteolytic *C. botulinum* type B (17B); 9, non-proteolytic *C. botulinum* type E (Beluga); 10, broad-range protein standard. The values on the left of lane 1 and on the right of lane 10 indicate the positions of protein standards (kDa).

branched deeply, indicating a relatively low level of phylogenetic relatedness to other clostridia within this phylogenetic clade.

Gel electrophoresis of soluble cell proteins demonstrated that strains DB1A$^T$ and R26 had similar profiles for major protein bands. In the protein profiles, the major bands for these strains occurred at approximately 120, 46–38, 35, 33–27 and 16 kDa. However, as shown in Fig. 8, strain R26 produced additional minor bands at approximately 34 and 47 kDa, whereas these bands were not detected in the pattern of strain DB1A$^T$. There were distinct differences in the concentrations and positions of major and minor protein bands between the profiles of these two meat isolates and those of their phylogenetic relatives *C. vincentii* (DSM 10228$^T$), *C. septicum* (NZRM 18$^T$), *C. chauvoei* (NZRM 99$^T$), non-proteolytic *C. botulinum* types B (17B) and E (Beluga) and *C. carnis* (DSM 1293$^T$).

The major cellular fatty acid identified in cell extracts of both meat strains were palmitic, oleic, myristic and palmitoleic acids (Table 2). The cellular fatty acid pattern of *C. vincentii* (DSM 10228$^T$) was easily distinguished from the patterns of the two meat isolates by the absence of lauric acid (C12:0) and the presence of only a small quantity of myristic (C14:0) and oleic (C18:1) acids. The cellular fatty acid patterns of *C. septicum* (NZRM 18$^T$) and *C. chauvoei* (NZRM 99$^T$) differed from the patterns of strains DB1A$^T$ and R26 by the absence of lauric acid. In addition, the cellular fatty acid composition of *C. septicum* (NZRM 18$^T$) had only 1·3% myristic acid, while the two meat strains had 5·6 and 6·7% of this acid. The patterns of *C. botulinum* type B (17B) and *C. botulinum* type E (Beluga) contained pentadecanoic acid (C15:1) and lower proportions of palmitoleic (C16:1), oleic (C18:1) and linoleic (C18:2) acids than those of strains DB1A$^T$ and R26. The pattern of *C. carnis* (DSM 1293$^T$) was distinguished from the patterns of the two meat strains by a lack of lauric and myristic acids (which were present in the patterns of meat isolates) and the presence of heptadecanoic (C17:0) and *cis*-10-heptadecanoic (C17:1) acids (which were absent from the patterns of meat isolates).

## DISCUSSION

With 'blown-pack' spoilage, which probably involves surface contamination of lamb carcasses and/or cuts, partial fulfilment of Koch's postulates may be obtained

***Table 2.*** Differences in the cellular fatty acid composition of strains DB1A$^T$ and R26 and phylogenetically related clostridia (each value is given as a percentage of the total peak area)

–, Not detected or at a concentration of less than 1%.

| Equivalent chain length | FAME* | DB1A$^T$ | R26 | *C. vincentii* DSM 10228$^T$ | *C. septicum* NZRM 18$^T$ | *C. chauvoei* NZRM 99$^T$ | *C. botulinum* type B (17B) | *C. botulinum* type E (Beluga) | *C. carnis* DSM 1293$^T$ |
|---|---|---|---|---|---|---|---|---|---|
| 12·000 | 12:0 | 1·0 | 1·2 | – | – | – | – | 1·0 | – |
| 14·000 | 14:0 | 5·6 | 6·7 | 2·3 | 1·3 | 5·1 | 5·7 | 7·5 | – |
| 15·525 | 15:1 | – | – | – | – | – | 1·2 | 1·5 | – |
| 15·672 | Unknown | – | – | 2·1 | – | – | 1·2 | 1·6 | – |
| 16·000 | 16:0 | 8·8 | 9·7 | 9·1 | 11·7 | 10·9 | 5·8 | 8·3 | 9·5 |
| 16·288 | Unknown | 3·5 | 4·0 | 2·8 | – | – | – | 1·6 | 1·8 |
| 16·398 | 16:1 | 5·5 | 6·0 | 3·1 | – | 0·8 | 1·2 | 1·0 | 2·2 |
| 17·000 | 17:0 | – | – | – | – | – | – | – | 1·4 |
| 17·411 | 17:1 *cis-10* | – | – | – | – | – | – | – | 1·0 |
| 17·538 | Unknown | – | – | 3·8 | – | – | – | 2·0 | – |
| 18·000 | 18:0 | 3·2 | 3·4 | 1·8 | 8·8 | 5·9 | 2·9 | 3·1 | 4·7 |
| 18·207 | 18:1 *cis-9* | 1·8 | 2·2 | 1·5 | 1·2 | 1·0 | 1·9 | 2·7 | 2·2 |
| 18·402 | 18:1 | 7·7 | 9·1 | 3·4 | 10·8 | 11·6 | 3·3 | 2·6 | 10·6 |
| 18·410 | Unknown | – | – | 2·1 | 1·0 | – | – | – | – |
| 18·446 | Unknown | 2·5 | 2·6 | 1·7 | – | – | – | – | – |
| 19·031 | 18:2 | 2·4 | 2·9 | 2·2 | 3·1 | 2·4 | 1·0 | 1·0 | 1·1 |

* Only C10–C20 fatty acids occurring in concentrations above 1% are listed. Concentrations of individual fatty acids are expressed as percentages of the total peak area that might have included peaks of straight chain and/or branched fatty acids with an effective chain length of less than 10, as well as some unknown compounds.

***Table 3.*** Some properties that differentiate strains DB1A$^T$ and R26 from their nearest relatives

NR, Not reported; +, positive; −, negative; w, weak reaction (at pH 5·5–5·9); c, 40–60 % of strains positive; d, 61–89 % of strains positive; A, acetate; B, butyrate; F or f, formate; L or l, lactate; p, propionate; s, succinate; 2, ethanol; 4, butanol. Upper-case and lower-case letters indicate major and minor fermentation products, respectively.

| Phenotypic property | DB1A$^T$/R26 | *C. vincentii** | *C. septicum*† | *C. chauvoei*† | *C. botulinum*† non-proteolytic B, E, F | *C. carnis*† |
|---|---|---|---|---|---|---|
| Fermentation products | A, B, L, 2, 4 | A, F, B | B, A, (F, p, l, 2) | A, B, F, 4, (l, s) | B, A, (l) | B, A, L, f, (s) |
| Aesculin hydrolysis | + | + | + | + | − | + |
| Starch hydrolysis | + | − | − | − | d | − |
| Fermentation of: | | | | | | |
| Cellobiose | + | + | +(w) | − | − | +(w) |
| Lactose | − | + | + | +(w) | − | c |
| Salicin | + | NR | c | − | − | w |
| Trehalose | + | NR | +(w) | − | w(+) | − |
| Xylose | − | + | − | − | − | − |

* Results from Mountfort *et al.* (1997).

† Results from Cato *et al.* (1986). Results in parentheses are for some strains of the species.

by isolation of the presumptive causative micro-organism and induction of this type of spoilage in vacuum-packed lamb inoculated with the suspect micro-organism during chilled storage in a laboratory. This study demonstrated that the unknown meat isolates, initially isolated from 'blown' vacuum-packed chilled lamb, caused gas production and pack distension of surface-inoculated, vacuum-packed, chilled lamb within 14 d storage at 2 °C. Therefore strains DB1A$^T$ and R26, along with *C. estertheticum* and *C. laramiense*, may be considered causative micro-organisms of the 'blown-pack' spoilage condition. The spoilage caused by the two meat strains in this study is, however, characterized by slower and less abundant gas production than spoilage caused by either *C. estertheticum* or *C. laramiense*.

Psychrotolerant clostridia have been associated with instances of chilled meat spoilage where initial microbiological examination failed to detect a causative micro-organism. Difficulties with the isolation of the causative clostridia have been reported (Dainty *et al.*, 1989; Kalchayanand *et al.*, 1989; Broda *et al.*, 1996). It has been suggested, therefore, that the composition of headspace volatiles from 'blown packs', as analysed by GC and MS, may indicate the bacterial species likely to be involved in the spoilage, prior to lengthy and unreliable isolation of causative psychrotolerant clostridia (Dainty *et al.*, 1989; Broda *et al.*, 1996). Butanol, butyric acid, carbon dioxide and hydrogen are products of the butyric-type fermentative metabolism typical of mesophilic, saccharolytic clostridia (Jones & Woods, 1986, 1989). Similar products with a unique range of butyl esters were found in the headspaces of both naturally spoiled vacuum packs and those artificially contaminated by psychrophilic *C. estertheticum* (Dainty *et al.*, 1989), and also in packs naturally spoiled by other psychrotolerant clostridia (Broda *et al.*, 1996). In the present study, a similar composition of volatiles, including butyl esters, was detected in the headspaces of 'blown' lamb packs inoculated with pure cultures of psychrophilic strains DB1A$^T$ and R26 (Table 1). This finding confirms that the ability to produce butanol, butyric acid, butyl esters, carbon dioxide and hydrogen may be considered a feature of many clostridial species, including psychrophilic strains. Moreover, this 'fingerprint' for headspace volatiles may serve as a useful indicator of clostridial species responsible for 'blown-pack' spoilage.

The results of 16S rRNA gene sequence similarity and phylogenetic analyses conducted in this study demonstrate that although strain DB1A$^T$ is phylogenetically related to micro-organisms in cluster I of the genus *Clostridium* (Collins *et al.*, 1994), it represents a distinct branch within this cluster. Within cluster I, strain DB1A$^T$ was firmly placed in the monophyletic unit that also contains *C. carnis*, *C. chauvoei*, *C. septicum* and *C. quinii* (Fig. 7). The robustness of this phylogenetic unit was supported by the results of maximum-parsimony, neighbour-joining and maximum-likelihood analyses. Sequence divergence values of 4·5–5 % between the meat strain and its nearest relatives (*C. vincentii*, *C. septicum*, *C. chauvoei*, non-proteolytic *C. botulinum* types B and E and *C. carnis*) and the distinct phylogenetic position of the meat strain in cluster I, together with its relatively low level of phylogenetic relatedness to cluster I clostridia, indicate that strain DB1A$^T$ should be assigned to a new species (Stackebrandt & Goebel, 1994).

Strains DB1A$^T$ and R26 were easily differentiated from their nearest relatives by soluble protein profiles, cellular fatty acid profiles and phenotypic properties (Table 3). The two meat strains were the only clostridia

***Table 4.*** Some properties that differentiate strains DB1A$^T$ and R26 from other psychrophilic clostridia and *C. putrefaciens*

ib, Isobutyrate; for remaining abbreviations and symbols, see Table 3.

| Phenotypic property | DB1A$^T$/R26 | *C. vincentii** | *C. estertheticum*† | *C. fimetarium*‡ | *C. laramiense*§ | *C. putrefaciens*¶ |
|---|---|---|---|---|---|---|
| Maximum growth temp. (°C) | 26 | 20 | 15 | 30 | 20 | < 37 |
| Optimum growth temp. (°C) | 20–22 | 12 | 10–12 | 20–25 | 15 | 15–22 |
| Fermentation products | A, B, L, 2, 4 | A, F, B | B, A | A, F, L, 2 | A, B, ib, p | a, f, l, (s) |
| Aesculin hydrolysis | + | + | + | NR | − | − |
| Starch hydrolysis | + | − | + | − | + | − |
| Fermentation of: | | | | | | |
| Arabinose | − | NR | + | + | − | − |
| Cellobiose | + | + | + | + | − | − |
| Mannitol | − | NR | + | − | + | − |
| Raffinose | − | NR | NR | − | + | − |
| Sorbitol | − | − | + | − | NR | − |
| Xylose | − | + | + | + | − | − |

* Results from Mountfort *et al.* (1997).

† Results from Collins *et al.* (1992).

‡ Results from Kotsyurbenko *et al.* (1995).

§ Results from Kalchayanand *et al.* (1993).

¶ Results from Cato *et al.* (1986).

in this group to produce both ethanol and butanol and to hydrolyse both aesculin and starch. *C. vincentii*, the only psychrophile among the nearest relatives of the meat strains, ferments lactose and xylose (Mountfort *et al.*, 1997). Similarly, *C. septicum* is lactose-positive (Cato *et al.*, 1986). In contrast, strains DB1A$^T$ and R26 did not utilize or ferment these two carbohydrates. The two meat isolates were readily distinguished from *C. chauvoei* and non-proteolytic *C. botulinum* types B and E by their ability to ferment cellobiose and salicin. In addition, *C. chauvoei* is unable to ferment trehalose, which was fermented by the meat strains, and *C. botulinum* types B and E are the only reference strains that are lipase-positive. Of the five reference strains used in the comparison, only *C. carnis* cannot hydrolyse gelatin. In addition, this reference strain is unable to ferment trehalose – a carbohydrate that is utilized and fermented by the two meat strains.

The two meat strains differed from other psychrophilic clostridia in their physiological and biochemical characteristics (Table 4). Of all the psychrophilic strains used for comparison in this study, only the meat strains formed both ethanol and butanol as their fermentation products. These strains differed from *C. estertheticum*, *C. laramiense* and *C. vincentii* by their maximum and optimum growth temperatures and substrate fermentation pattern (Collins *et al.*, 1992; Kalchayanand *et al.*, 1993; Mountfort *et al.*, 1997). With respect to their physiological characteristics, the meat strains showed closest similarity to *Clostridium fimetarium* (Kotsyurbenko *et al.*, 1995). However, *C. fimetarium* cannot hydrolyse starch and produces acid from arabinose and xylose, two carbohydrates that are not utilized or fermented by strains DB1A$^T$ and R26. Unlike *Clostridium putrefaciens*, which cannot ferment any carbohydrates, strains DB1A$^T$ and R26 were obligately saccharolytic.

A high level of 16S rRNA homogeneity and only small differences in phenotypic characteristics were found between strains DB1A$^T$ and R26, suggesting that the two isolates should be classified as the same species. The results of phenotypic characterization, as well as 16S rRNA gene sequence similarity, SDS-PAGE and cellular fatty acid analyses conducted in this study demonstrate that strains DB1A$^T$ and R26 represent a new species within the genus *Clostridium*, for which we propose the name *Clostridium gasigenes*.

### Description of *Clostridium gasigenes* sp. nov.

*Clostridium gasigenes* (ga.si′ge.nes. M.L. neut. n. *gasum* gas; Gr. v. *gennaio* to produce; N.L. gen. n. *gasigenes* gas-producing).

Colonies on sheep blood agar measure 0·7–3·0 mm in diameter and are circular with an entire margin, grey/white to grey, convex, shiny and *β*-haemolytic. Cells are Gram-positive, motile rods producing elliptical subterminal spores during the late-stationary growth phase. The micro-organism is psychrophilic and grows between −1·5 and 26 °C (the optimum growth temperature is 20–22 °C). At 20 °C, the pH range for growth is 5·4–8·9 and the optimum pH for

growth is 6·2–8·6. In PY broth with 0·1 % (w/v) yeast extract, the organism grows little or not at all in the absence of fermentable carbohydrate. Cellobiose, fructose, glucose, inositol, maltose, mannose, salicin and trehalose are fermented. Gelatin, aesculin and starch are hydrolysed. The fermentation products formed in PYGS broth are ethanol, acetate, butyrate, lactate, butanol, carbon dioxide and hydrogen. The G+C content of the DNA is 28·3–29·4 mol %. The microorganism was isolated from vacuum-packed, chilled lamb exhibiting 'blown-pack' spoilage. It causes gas production and pack distension in vacuum-packed, chilled lamb. The type strain is DB1A$^T$. This strain has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, under the culture collection number DSM 12272$^T$.

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