Psychrotrophic clostridia mediated gas and botulinal toxin production in vacuum-packed chilled meat

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S.M. MOORHEAD AND R.G. BELL. 1999. A cocktail of washed spores from six psychrotrophic *Clostridium* strains isolated from blown vacuum-packed meats was inoculated onto lamb chumps. A second washed spore cocktail of four toxigenic reference *Cl. botulinum* strains, types A, B (two strains) and E, and a *Cl. butyricum* type E strain, was similarly inoculated onto lamb chumps. All inoculated lamb chumps were individually vacuum-packed and placed into storage at various temperatures typical of good to grossly abusive chilled storage (−1 °C to 15 °C). All packs were observed for gas production (pack-'blowing') over a 12 week storage period. On gas production, or after 12 weeks of storage, packs were examined by mouse bioassay for botulinum toxin production. The packs inoculated with the meat isolate cocktail showed evidence of gas production earlier than packs inoculated with reference strains. No botulinum toxin was recovered from the meat isolate inoculated packs, while botulinal toxin was detected in reference strain inoculated packs down to a nominal storage temperature of 2 °C.

INTRODUCTION

Psychrotrophic/psychrophilic clostridia-mediated spoilage of vacuum-packed chilled meats, characterized by copious gas production causing pack 'blowing', was first reported in 1989 (Dainty *et al*. 1989; Kalchayanand *et al*. 1989). The association of this group of micro-organisms with the spoilage of commercial vacuum-packed meats and semi-preserved dog rolls has been confirmed in New Zealand (Broda *et al*. 1996a, b, 1997).

To date, blown pack spoilage has not been regarded as presenting a health hazard. This belief, despite the ability of known toxigenic species in *Clostridium botulinum* to produce large amounts of hydrogen (Cato *et al*. 1967), is founded on the absence of any reported association between clinical illness and the consumption of meat from blown packs. Consumption of such meat is, however, unlikely because of the obnoxious odours that characterize such spoilage. The absence of botulinal toxins has been confidently assumed because commercial storage temperatures for chilled meat are below 13 °C, the generally recognized minimum temperature for toxin production by psychrotrophic non-proteolytic *Cl. botulinum* strains (Eklund *et al*. 1967b).

Complacency with respect to botulinal toxin formation in vacuum packs of chilled meat has been questioned in this laboratory by the results obtained in an ongoing taxonomic and phylogenetic investigation of psychrotrophic/psychrophilic clostridia, isolated from blown vacuum packs. The 16S rRNA gene sequences of some of these strains closely resemble *Cl. botulinum* (unpublished data), and these strains are termed 'industry isolates' in this paper. The present study was undertaken to determine the distension and botulinal toxin production in vacuum packs inoculated with a cocktail of six of these *Cl. botulinum*-like industry isolates and a cocktail of five toxigenic *Clostridium* sp. reference strains.

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MATERIALS AND METHODS

Clostridia

The strains used in this study are listed in Table 1. All six *Cl. botulinum*-like industry isolates were obtained from 'blown' vacuum-packed meats and had been maintained under anaerobic conditions at 10 or 15 °C in PYGS (peptone, yeast extract, glucose, starch) broth (Lund *et al*. 1990). Before use, both industry and reference isolates were subcultured onto CBA (Columbia Blood Agar, Oxoid) containing 5% sterile defibrinated sheep blood, incubated at 30 °C in Oxoid HPII anaerobic jars for 48 h, then checked for purity.

Table 1 Industry isolates and reference strains used in challenge study cocktails

Taxon	Strain	Toxin type	Source
Industry isolates			
Clostridium sp.	PP1	–*	'Blown' vacuum-packed venison
Clostridium sp.	DB2	–	'Blown' vacuum-packed lamb
Clostridium sp.	SPL242	–	'Blown' vacuum-packed beef
Clostridium sp.	M14	–	'Blown' vacuum-packed venison
Clostridium sp.	KDL358	–	'Blown' plastic-wrapped dog roll
Clostridium sp.	SPL17	–	'Blown' vacuum-packed beef
Reference strains			
Cl. botulinum	ATCC 25763 (ACM 3691)	A	ACM†
Cl. botulinum	ATCC 25765 (ACM 3857)	B	ACM
Cl. botulinum	17B	B	CSIRO‡
Cl. botulinum	Beluga	E	CSIRO
Cl. butyricum	ATCC 43755 (ACM 3587)	E	ACM

* –, Toxin status unknown.
† ACM, Australian Collection of Micro-organisms, University of Queensland, Australia.
‡ CSIRO, Commonwealth Scientific and Industrial Research Organization, North Ryde, Australia.

Spore suspensions

Spores were obtained by inoculating each strain into a two-phase sporulation medium, as described by Peck *et al*. (1992). The lower solid phase consisted of single-strength cooked meat medium (CMM; Oxoid) prepared by mixing 30 g meat granules in a flask with 300 ml distilled water. Prior to autoclaving, 4·5 g agar and 0·3 g glucose were added. The medium was pre-reduced inside an anaerobic chamber (Forma Scientific, Marietta, OH, USA) under a head-space of 85% N_2, 10% H_2 and 5% $C0_2$ for 24 h before the upper liquid phase of 40 ml deoxygenated sterile distilled water was added to each flask. Individual flasks were inoculated with 5 ml of a 24 h/15 °C PYGS broth culture of one of the test strains.

Inoculated flasks were incubated anaerobically at 15 °C. At intervals during incubation, cultures were examined by phase contrast microscopy for the presence of spores. Once significant numbers of spores were observed, the culture was concentrated by centrifugation at 4300 *g* for 15 min. The spores were washed four times with ice-cold physiological saline (0·85% w/v NaCl) and resuspended in 10 ml ice-cold saline. The spore suspensions were then sonicated in an ultrasonic water-bath (Unisonics Pty Ltd, Manly Vale, NSW, Australia) at 40 Hz 100 W output at 10 °C to release the spores from their sporangia. Each spore suspension received three 10 min sonication treatments. Spores were washed with ice-cold saline between each treatment. After final centrifugation, the washed spores of each isolate were suspended in 5 ml ice-cold saline and stored at 4 °C.

A 0·5 ml sub-sample of each spore suspension was heat shocked at 60 °C for 10 min, then enumerated by preparing serial dilutions in sterile diluent (0·1% peptone, 0·85% NaCl) and plating onto CBA containing 5% defibrinated sheep blood. All plates were incubated anaerobically at 15 °C for 4 d. Once individual spore suspensions were enumerated, appropriate dilutions of each suspension were made so that equal volumes of the six industry strains or of the five reference strains could be combined to give final inoculation suspension cocktails containing 10^7 spores ml^{-1}. Immediately prior to inoculation of the test packs, the spore suspension cocktails were heat-shocked at 60 °C for 10 min then rapidly cooled to 4 °C.

Inoculation and packaging

Chilled boneless lamb chumps were obtained on the day after slaughter from the boning room of a local meat plant, and placed individually into pre-labelled barrier bags (Cryovac BB4L; W.R. Grace, Porirua, New Zealand) pending inoculation.

The packs were divided into three groups; one group was inoculated with the industry isolate cocktail, the second group (positive control) was inoculated with the reference strain cocktail, and the third group remained uninoculated (negative

control). For inoculation, packs received 0·5 ml of the appropriate spore suspension cocktail to obtain an end concentration of approximately 10^4 spores cm^{-2} of meat surface. Inoculated and negative control packs were then vacuum-packed using a Securepak 10, controlled atmosphere packaging machine (Securefresh Pacific, Auckland, New Zealand). The packs were then heat shrunk for 2–3 s in an 80–85 °C water-bath to simulate standard industry practice, and duplicate packs from each group were placed into storage at a range of incubation temperatures (15, 12, 10, 8, 6, 4, 2, 0 and −1·5 °C).

Storage

The storage trial lasted 84 d (12 weeks), which is a reasonable shelf-life expectation for chilled, commercial, vacuum-packed lamb. Sanyo MIR 252 incubators (Sanyo Electric Trading Co., Ltd, Osaka, Japan) were used as storage cabinets, and their operating temperatures were monitored using Delphi data loggers (Trutest, Auckland, New Zealand). Delphi loggers were also used to monitor the meat surface temperature of selected 'vacuum-packed' lamb chumps stored at 2 °C by puncturing the bag and positioning the temperature probe tip immediately below the meat surface. To simulate commercial practice, regular weekly defrost cycles were performed without removing the stored product from the cabinets.

The vacuum packs were examined regularly for the first sign of 'blown pack' spoilage, i.e. the presence of small bubbles in the meat drip. The next stage in 'pack blowing', noticeable gas production, is accompanied by loss of vacuum. On reaching this stage, the packs were removed to storage at −1·5 °C, pending toxin extraction.

Toxin extraction

On removal from pre-extraction storage at −1·5 °C, each pack was opened and subjected to a toxin extraction process based on that of Kautter *et al.* (1992). Briefly, 25 ml meat drip and 25 g surface tissue were taken and combined in a sterile stomacher bag. Each combined drip and surface tissue sample was homogenized for 2 min in 50 ml gelatin phosphate buffer (an equal-parts mixture of 0·05 mol l^{-1} sodium phosphate and 0·4% w/v gelatin, pH 6·2) using a Colworth Stomacher (Seward, London, UK).

Approximately 10 ml of each gelatin-phosphate buffer homogenate was centrifuged under refrigeration (9000 *g* for 30 min at 4 °C). Volumes (8 ml) of supernatant fluid were packed on ice and couriered to ESR Health, Auckland, New Zealand, for mouse bioassay toxin testing. The remainder of each sample was stored at −20 °C.

Toxin testing

Separate pairs of mice were injected intraperitoneally with 0·5 ml undiluted meat extract supernatant fluid, 0·5 ml trypsin-treated supernatant fluid or 0·5 ml heat-treated (100 °C, 10 min) supernatant fluid. The mice were observed periodically over 72 h for symptoms of botulism toxicity, e.g. ruffling of fur, laboured breathing and limb weakness followed by total paralysis and death due to respiratory failure (Kautter *et al.* 1992).

RESULTS

Storage temperature

Except during defrost cycles, temperature loggers indicated that product temperature was maintained within ±0·5 °C of the set point temperature. During the 2 °C storage cabinet defrost cycles, the meat surface temperature increased on average to 3·1 °C above the set point temperature but returned to the set point temperature within 2·5 h of the start of the defrost cycle.

Gas and toxin production

The results of the gas production trial and mouse toxicity test are presented in Table 2. Time to gas production is reported for each inoculation/storage temperature combination to show signs of vacuum loss. At temperatures below 6 °C, the test packs inoculated with the industry isolates produced gas earlier than the positive control packs. However, no industry isolate inoculated packs produced botulinal toxin at any of the temperatures within the storage range. Reference strain cocktail inoculated packs stored between 15 and 2 °C contained botulinal toxin. Gas was produced in the uninoculated (negative control) packs at temperatures of 6 °C and above, i.e. under conditions of abusive chilled storage.

DISCUSSION

Once commercial vacuum-packed chilled meat shows loss of vacuum, it can reasonably be expected to be withdrawn from distribution. Subsequent phases of blown-pack spoilage (flaccid, then turgid pack distension) are inappropriate as determinants of effective storage life because both are symptomatic of advanced product spoilage. Production of gas in the uninoculated (negative control) packs stored at abusive temperatures (≥6 °C) can be attributed to the growth of members of the Enterobacteriaceae (Hanna *et al.* 1979). When loss of vacuum was observed in the 6 °C negative control packs, aerobic plate counts, which were predominantly

Table 2 Time to pack blowing, in days, and toxin production (+ / −) of vacuum-packed lamb chumps inoculated with clostridial isolates

		Storage temperature (°C)								
Packs		−1·5	0	2	4	6	8	10	12	15
Industry	a*	>84 (−)	45 (−)	40 (−)	17 (−)	17 (−)	11 (−)	6 (−)	3 (−)	3 (−)
cocktail	b*	>84 (−)	73 (−)	55 (−)	20 (−)	20 (−)	11 (−)	6 (−)	3 (−)	3 (−)
Reference	a	>84 (−)	>84 (−)	55 (+)	27 (+)	17 (+)	11 (+)	6 (+)	3 (+)	3 (+)
cocktail	b	>84 (−)	>84 (−)	55 (+)	27 (+)	20 (+)	11 (+)	7 (+)	3 (+)	3 (+)
Negative	a	>84 (−)	>84 (−)	>84 (−)	>84 (−)	48 (−)	52 (−)	38 (−)	38 (−)	7 (−)
control	b	>84 (−)	>84 (−)	>84 (−)	>84 (−)	48 (−)	52 (−)	39 (−)	38 (−)	12 (−)

* Duplicate packs.

Enterobacteriaceae, exceeded 10^7 cfu cm^{-2} (results not shown).

Over the trial storage temperature range, −1·5–15 °C, no botulinal toxin was detected in industry isolate inoculated packs after either 84 d storage or when vacuum loss first became evident. This result is entirely consistent with the demonstrated absence of BoNT genes in five of the six isolates (Broda *et al*. 1998). Strain SPL17 has not been tested for the presence of BoNT genes.

As was expected, botulinal toxin was produced at storage temperatures between 4 and 15 °C, in packs inoculated with the toxigenic reference strains. The detection of toxins in reference strain packs stored at 2 °C, 1·3 °C below the generally accepted limit for botulinal toxin production of 3·3 °C (Eklund *et al*. 1967a,b; Solomon *et al*. 1977; Notermans *et al*. 1981; Solomon *et al*. 1982; Lund *et al*. 1985; Garcia *et al*. 1987) was, however, unexpected. It should be appreciated that the 3·3 °C temperature minimum for toxin production applies only to a relatively few strains growing under otherwise optimum growth conditions (Dodds 1994), although growth and toxin production at a marginally lower temperature, 2·9 °C, has recently been reported under laboratory culture conditions (Graham *et al*. 1997).

As the 2 °C storage cabinet was subject to weekly defrost cycles (simulating commercial practice), it could be argued that growth, with concomitant toxin production, occurred during temperature excursions above the set point. In the 2 °C storage cabinet the highest average meat surface temperature reached during any defrost cycle was 5·1 °C. Over the 55 d storage period, the seven defrost cycles allowed the average meat surface temperature to be maintained at or above 3·3 °C for only 7 h and above 2·5 °C (upper limit for storage cabinet temperature control fluctuation) for 11 h 45 min. Irrespective of the actual minimum growth temperature on a given substrate, toxin production at close to the lower temperature limitgenerally requires several weeks (Eklund *et al*. 1967b; Solomon *et al*. 1977). Consequently, it appears unlikely that toxin was produced only during the 11 h 45 min the meat surface temperature exceeded 2·5 °C.

The toxigenic reference cocktail contained, in addition to *Cl. botulinum* strains, *Cl. butyricum* ATCC 43755 (Aureli *et al*. 1986). The minimum toxin production temperature for this organism has not, to the best of the authors' knowledge, been recorded in the literature. Consequently, the presence of botulinal toxin in our packs stored at a nominal temperature of 2 °C could be attributed to *Cl. butyricum* and not *Cl. botulinum* growth. This avenue is currently being explored.

The present study has not demonstrated whether product spoilage (loss of vacuum) follows, or is synchronous with, toxin production. Consequently, to assure product safety, storage conditions must preclude the possibility of toxin production within the period of commercial chilled storage. Such assurance would be provided by a storage regimen that maintains product temperature at or below 0 °C. It would appear from Table 2 that such storage should also prevent blown pack spoilage. This may not, however, be the case, as isolate DB2 and the majority of psychrotrophic clostridia associated with blown packs, i.e. those that do not resemble *Cl. botulinum* (Collins *et al*. 1962; Broda *et al*. 1996a), grow rapidly in laboratory media and produce copious quantities of gas at that temperature.

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