NOTE

Carnobacterium viridans sp. nov., an alkaliphilic, facultative anaerobe isolated from refrigerated, vacuum-packed bologna sausage

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A facultatively anaerobic, non-spore-forming, psychrophilic, Gram-positive, non-aciduric but alkaliphilic, rod-shaped bacterium (MPL-11^T) was found to be responsible for green discoloration of refrigerated vacuum-packaged bologna upon opening of the package. Although *Aerococcus viridans*, which had been implicated earlier in causing the same problem, was also found, this is the first report of discoloration caused by an organism shown to be a species of *Carnobacterium*. Bacterial discoloration was caused by H_2O_2 production upon exposure of the meat to air. Strain MPL-11^T is catalase- and oxidase-negative. It is not motile and does not reduce nitrate to nitrite or produce ammonia from arginine. It does not grow in acetate-containing broth or agar (Rogosa) or produce H_2S. The peptidoglycan is of the *meso*-diaminopimelic acid type and it produces predominantly L(+)-lactic acid from glucose. It grows from at least 2 to 30 °C over a pH range from 5·5 to 9·1. Ribotyping suggested that strain MPL-11^T could be a species of either *Lactobacillus* or *Carnobacterium*, but analysis using DNA sequences from the 16S rRNA gene showed conclusively that the organism belonged to the genus *Carnobacterium*. Since acid is not produced from amygdalin, inulin, mannitol, methyl α-D-glucoside or D-xylose, the organism differs from the seven described species of *Carnobacterium*. In addition, strain MPL-11^T is the first member of the genus found that does not produce acid from ribose. It is capable of acid production/growth on galactose, glucose, fructose, mannose, *N*-acetylglucosamine, aesculin, cellobiose, maltose, lactose, sucrose, trehalose and tagatose. Although extremely salt tolerant, it does not grow in ⩾ 4% NaCl. On the basis of phenotypic and genotypic data, it is concluded that this isolate represents a separate, novel species. Accordingly, the name *Carnobacterium viridans* sp. nov. is proposed. The type strain is strain MPL-11^T (= ATCC BAA-336^T = DSM 14451^T).

Keywords: *Carnobacterium viridans*, cured meat spoilage, psychrophile, meat discoloration, alkaliphile

Green discoloration of vacuum-packed cooked cured meats is a periodic problem that can be caused by bacterial production of H_2S or can result from oxidation of di-nitrosyl haemochrome (pink) by bacterial peroxides (H_2O_2) upon opening the meat package and exposure to oxygen. The former yields greenish sulfmyoglobin, while the latter causes the formation of green cholemyoglobin (Lawrie, 1998) or oxidized porphyrins, which are also green. Peroxide-based discoloration can arise from the presence of thermally tolerant organisms like *Weissella viridescens*, *Enterococcus faecium* or *Enterococcus faecalis*. These organisms can cause greening in the core or central portions

Published online ahead of print on 19 April 2002 as DOI 10.1099/ijs.0.02216-0.

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Abbreviations: APT, all-purpose Tween; BHI, brain/heart infusion; CTSI, cresol red/thallium acetate/sucrose/inulin; DAP, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of *Carnobacterium viridans* MPL-11^T is AF425608.

of the cooked cured meat. Just as commonly, if post-heat process contamination occurs (e.g. at slicing or packaging), a variety of Gram-positive, catalase-negative, H_2O_2 producers such as the homo- and heterofermentative lactobacilli or leuconostocs may be involved (Borch *et al*., 1996). Grant *et al*. (1988) suspected *Pediococcus* species could be responsible, but low temperature limits for their growth (8 °C) and our recent work (M. Peirson and R. A. Holley, unpublished) has shown that *Aerococcus viridans* (which can be confused with pediococci) was also responsible for this colour defect in cured meat stored at $\geqslant$ 4 °C. Isolation of *Aerococcus viridans* from meat curing brines and its production of similar discoloration reactions in cured meats were reported much earlier by Deibel & Niven (1960). Borch *et al*. (1996) noted that carnobacteria also have the potential to cause H_2O_2-based colour defects in cooked cured meats, but this observation has not been reported in the literature. Carnobacteria have become of increasing interest to food microbiologists because they can represent a significant proportion of the microbiota in perishable foods stored at low temperature for extended periods under reduced oxygen tension (Lai & Manchester, 2000). Some are capable of producing potent antimicrobial compounds that inhibit more noxious organisms (Barakat *et al*., 2000; Jöborn *et al*., 1999). Originally described as non-aciduric, atypical lactobacilli (Collins *et al*., 1987), carnobacteria appear to be regularly present in vacuum- or modified-atmosphere-packaged pork (McMullen & Stiles, 1993; Nadon *et al*., 2001; Wasney *et al*., 2001), poultry (Grant & Patterson, 1991; Barakat *et al*., 2000) and lamb (Nissen *et al*., 1994).

In this study, we report the morphological, phenotypic and phylogenetic characteristics of a *Carnobacterium* strain, MPL-11^T (isolated from commercially prepared vacuum-packaged bologna sausage), that was responsible for causing green discoloration of the product after package opening. Phenotypic and phylogenetic characteristics were different from those of all known species of *Carnobacterium*, indicating that this organism represents a novel species. Strain MPL-11^T has been designated *Carnobacterium viridans* sp. nov.

Strain MPL-11^T was isolated from sliced bologna in a vacuum-sealed retail package obtained directly from the federally inspected plant where it was manufactured. Two other similar strains (MPL-2 and MPL-14) were isolated from the same package. Strain MPL-2 was lost during subculture. The packages were held at 10 °C until the product expiry code was exceeded by 30 days. A sample of meat (10 g) from one aseptically opened pack showing green discoloration was homogenized (Stomacher), diluted in 0·1% peptone and plated on all-purpose Tween (APT, Difco), M5 (Zuniga *et al*., 1993) and MRS (Hammes *et al*., 1991) agars. Plates were incubated anaerobically at 25 or 30 °C for 48 h (BBL Gaspak). The isolates were routinely grown in APT broth and preserved in APT broth containing 20% (w/v) glycerol, stored at −80 °C. Carnobacteria and aerococci dominated the microbiota of the commercial package of discoloured bologna and were each present at 10^7 c.f.u. bacteria cm^{-2}. Ten of 15 isolates produced a defect typical of that seen in commercial products and all 10 were from these two genera. Two of the three commercial lots of product did not discolour.

Isolates were tested for their ability to grow on peptonized agar medium (PTM) containing $MnSO_4$ and TMBZ (Berthier, 1993), lactobacillus selective (LBS) agar (Difco), KF streptococcus agar (Difco), brain/heart infusion (BHI) broth (Difco), the meat-based medium of Grant *et al*. (1988) and cresol red/thallium acetate/sucrose/inulin (CTSI) agar (Wasney *et al*., 2001). All agar plates were incubated anaerobically at 30 °C for 48 h, except for CTSI plates, which were incubated for 2 days at 25 °C plus 2 days at 8 °C (anaerobically).

To determine whether isolates caused green discoloration of meat, they were grown in APT broth for 48 h at 30 °C, centrifuged and the pellet resuspended in sterile 0·1% peptone to contain about 10^7 c.f.u. bacteria ml^{-1}. Commercial, 500 g packages of sliced all-beef bologna were treated with e-beam irradiation ($\geqslant$ 3 KGy) to eliminate the background microflora. Individual slices of bologna (10 cm diameter) were transferred to O_2-barrier bags (Winpak, Deli*1) and 0·4 ml of the test bacterial suspension was spread evenly over the meat surface with a sterile glass rod or by massaging the plastic film. This yielded 10^5 c.f.u. bacteria cm^{-2} of bologna. A vacuum was drawn (Bizerba model GM 2002) and the bags were heat-sealed and stored at 4 or 9 °C for up to 40 days. Packages were opened and half were treated with 0·4 ml of an aqueous, sterile 3% (w/v) solution of catalase (Sigma-Aldrich). Unsealed, but still wrapped slices were held at 4 °C for $\leqslant$ 3 days and inspected visually for discoloration.

Morphological examination of the isolates was by phase-contrast microscopy. Cell measurements were made by bright-field examination of crystal violet-stained cells. Gram reactions were done using 3% KOH (Gregersen, 1978); catalase and oxidase activities were measured by standard methods. Growth over the range 2–45 °C and in $\leqslant$ 14% NaCl, ammonia production from arginine and gas production from glucose were monitored in modified MRS (acetate omitted) (Hammes *et al*., 1991) at 25 °C following inoculation with late exponential phase cells. Survival in APT broth supplemented with 26·4% NaCl was examined for up to 37 days at 4, 10 and 25 °C. Survivors were plated on APT agar using the hydrophobic grid membrane filtration technique (Entis & Boleszczuk, 1986).

Carbohydrate fermentation tests were done using the API 50CHL (bioMérieux) and Biolog AN MicroPlate systems. Production of D- and L-lactic acid was quantified using D(−)- and L(+)-lactate dehydrogenases (Boehringer Mannheim). Whole-cell hydro-

Table 1. Characteristics useful in differentiating *Carnobacterium* species

Modified from Collins *et al.* (1987), Franzmann *et al.* (1991), Holt *et al.* (1994), Jöborn *et al.* (1999) and Lai & Manchester (2000). Strains: 1, *Carnobacterium divergens* NCDO 2763^T; 2, *Carnobacterium gallinarum* NCFB 2766^T; 3, *Carnobacterium mobile* NCFB 2765^T; 4, *Carnobacterium piscicola* NCDO 2762^T; 5, *Carnobacterium funditum* DSM 5970^T; 6, *Carnobacterium alterfunditum* DSM 5972^T; 7, *Carnobacterium inhibens* CCUG 31728^T; 8, *Carnobacterium viridans* sp. nov. MPL-11^T. +(−), Occasional strain negative; −(+), occasional strain positive; ±, variable; W, weakly positive; NT, not tested.

Characteristic	1	2	3	4	5	6	7	8
Motility	−	−	+	−	+	+	+	−
Acid from:								
Amygdalin	+	+	−	+	−	+	+	−
Inulin	−	−	+	+(−)	−	−	W	−
D-Lactose	−	+	±	(−)	−	−	W	+
Mannitol	−	−	−	+	+	−	+	−
Methyl α-D-glucoside	−	+	−	+	NT	NT	−	−
D-Xylose	−	+	−	−	−	−	−	−
D-Tagatose	−	+	−(+)	−	NT	NT	−	+
D-Ribose	+	+	+	+	+	+	+	−
D-Trehalose	+	+	+	+	+	−	+	+
Aesculin hydrolysis	+	+	+	+	−	±	+	+
Growth at 37 °C	+	+	±	+	−	−	−	−
Voges–Proskauer	+	+	−(+)	+	NT	NT	NT	−

lysates were used for the detection of *meso*-diaminopimelic acid (*meso*-DAP) in the peptidoglycan by TLC (Bousefield *et al.*, 1985).

The thermal resistance of strain MPL-11^T and other isolates was characterized using an immersion-sealed capillary tube (ISCT) procedure (Foegeding & Leasor, 1990).

Several isolates were obtained and their identity to MPL-11^T was examined using PFGE (Zhang & Holley, 1999). Strain MPL-11^T and others were ribotyped using the RiboPrinter microbial characterization system following the manufacturer's procedures (Bruce, 1996). The nearly complete sequence of 16S rDNA for MPL-11^T (1465 bp) and a partial sequence for MPL-14 (> 600 bp) were obtained using a PCR amplification strategy outlined by Kim *et al.* (2000). PCR products were sequenced at the UC DNA Services facilities (University of Calgary). The resultant sequences were aligned to the small-subunit rRNA sequences from the Ribosomal Database Project (Maidak *et al.*, 2001). Positions that were aligned unambiguously were used in the construction of a evolutionary distance matrix using the maximum-likelihood method (Bratina *et al.*, 1998). An unrooted evolutionary tree was constructed from the distance matrix using a neighbour-joining method (Saitou & Nei, 1987). The phylogenetic analysis was conducted using the PHYLIP software package (Felsenstein, 1993).

Strain MPL-11^T grew on bologna inoculated with 10^5 c.f.u. cells cm^{-2} and reached 10^7 c.f.u. cm^{-2} within 25 days at 10 °C. Slices of pasteurized bologna, each inoculated with either of two carnobacterium isolates, as well as those inoculated with each of the several strains of *Aerococcus viridans*, turned greenish within 3 days of package opening. When slices were treated with catalase immediately after opening the package, discoloration was prevented.

Results from the API 50CHL and Biolog fermentations showed that there were differences between strain MPL-11^T and other known strains of *Carnobacterium* (Table 1). The isolate was consistent in its ability to produce acid from those substrates normally fermented by carnobacteria except for ribose. Like other species of carnobacteria, strain MPL-11^T did not produce acid from arabinose, arabitol, dulcitol, erythritol, fucose, glycogen, inositol, raffinose, L-rhamnose, L-sorbose, xylitol or L-xylose (Hammes *et al.*, 1991). Strain MPL-11^T produced small colonies with light colour (beige to grey) on CTSI agar (Wasney *et al.*, 2001) with slight yellowing of the medium following anaerobic incubation (25 °C for 2 days plus 8 °C for 2 days). The organism produced 97·8 % of lactic acid as the L(+) enantiomer.

The organism was not thermally resistant and did not survive heating for 1 min at 60 °C, so a D-value could not be calculated at this temperature. In the same test, *Carnobacterium piscicola* ATCC 43224 had a D_{60} value of 0·54 min, with no survivors at 4 min.

Restriction endonuclease digestion of DNA (*Sma*I) followed by PFGE showed that the two viable isolates from bologna (MPL-11^T and MPL-14) were genetically similar, sharing eight electrophoretic bands. MPL-14 had an additional band at 242·5 kb, near the

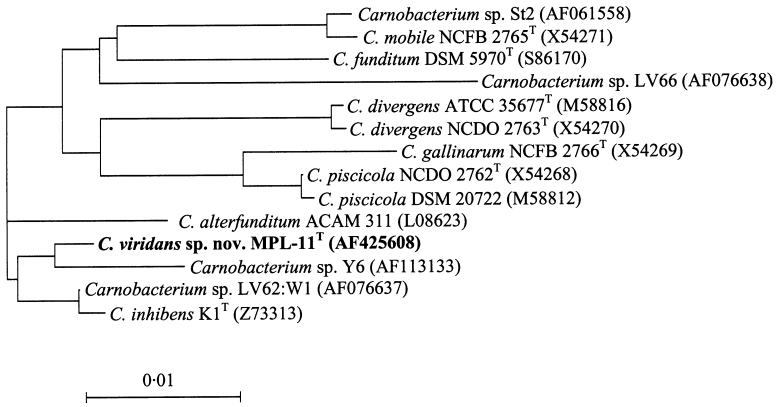

Fig. 1. Unrooted neighbour-joining tree based on alignment of 16S rDNA sequences between strain MPL-11^T and members of the genus *Carnobacterium*. Bar, 0·01 substitutions per nucleotide position.

top of the gel, but this still indicated significant similarity (Tenover *et al.*, 1995).

Comparison of the RiboPrint patterns obtained from the two isolates revealed a similarity index of $0{\cdot}99 \pm 0{\cdot}1$. Comparison of these profiles against those of six known *Carnobacterium* species [*Carnobacterium inhibens*, a motile species (Jöborn *et al.*, 1999), was not included] and all lactobacilli strains held in the DuPont Identification Database generated matches with $\leqslant 85\%$ similarity. Although identification was not possible using this method, the profiles of the unknowns in terms of conservation of intensity and positioning of the bands were taken to indicate that they were members of the genus *Lactobacillus* or *Carnobacterium*.

The 16S rDNA sequence data showed that strain MPL-11^T is a member of the genus *Carnobacterium* in a clade separate from the known species of this genus (Fig. 1). Strain MPL-11^T is related most closely to *Carnobacterium* sp. Y6, a clinical strain isolated from a patient with multi-bacterial synergistic gangrene, and is clearly different from all previously named species of this genus.

Strain MPL-11^T exhibited major characteristics that define membership of the genus *Carnobacterium* (Lai & Manchester, 2000; Stiles & Holzapfel, 1997; Holt *et al.*, 1994; Hammes *et al.*, 1991; Montel *et al.*, 1991; Collins *et al.*, 1987). However, the organism exhibited characteristics that did not allow assignment to any of the currently accepted species of this genus. A principal phenotypic difference from all other *Carnobacterium* species was that strain MPL-11^T did not produce acid from ribose (Table 1). It should be noted that in Jöborn *et al.* (1999), *Carnobacterium funditum* DSM 5970^T and *Carnobacterium alterfunditum* DSM 5972^T were reported, incorrectly, to be ribose-negative. Their observation arose from a tabular error by Franzmann *et al.* (1991) that also involved *Carnobacterium mobile*. The latter authors showed that both *C. funditum* DSM 5970^T and *C. alterfunditum* DSM 5972^T produced moderate amounts of formic and acetic acids plus ethanol in addition to lactic acid from D(−)-ribose. *C. mobile* NCFB 2765^T produced acid from ribose (Collins *et al.*, 1987).

16S rDNA analysis of strain MPL-11^T showed that it and strain MPL-14 belonged in a separate phylogenetic group from other species of *Carnobacterium* (Fig. 1). Interestingly, its closest relatives are clinical and aquatic environmental isolates, while *Carnobacterium* species from meat and meat products are more distantly related. In addition, ribotyping results indicated no match with known strains of either *Carnobacterium* or *Lactobacillus* in the DuPont Identification Database. Perhaps this is not surprising, since Lai & Manchester (2000) found in their study of 73 carnobacteria isolates that several strains represented single-membered clusters. They suggested that the genus *Carnobacterium* is underspeciated because of this observation and because of the recognition that only a very few phenotypic traits are traditionally used for their speciation. Accordingly, and on the basis of phenotypic results, we have named isolate MPL-11^T *Carnobacterium viridans* sp. nov.

Description of *Carnobacterium viridans* sp. nov.

Carnobacterium viridans (vi′ri.dans. N.L. adj. *viridans* from L. v. *viridare* to make green, referring to the production of a green colour in cured meat by the organism).

Gram-positive, non-motile, non-spore-forming, facultatively anaerobic organism that occurs as slightly curved rods, singly or in pairs, or as straight rods ($0{\cdot}8 \times 3{\cdot}6 \pm 0{\cdot}6$ μm) in chains sometimes 20 μm long. Grows satisfactorily in BHI, APT, M5 and CTSI media, but poorly on a variety of media including MRS. Does not grow on Rogosa agar. It grows over a range of pH from 5·5 to 9·1 and from 2 to 30 °C, but the range may be slightly greater. No growth at 37 °C. Does not produce catalase or oxidase and is *β*-haemolytic on blood-agar base with 0·8 % sheep blood. Ammonia is not produced from arginine. No gas is produced from glucose, nitrate is not reduced and H_2S is not produced. The Voges–Proskauer reaction is negative. Acid is not produced from amygdalin, inulin, mannitol, methyl α-D-glucoside, ribose or D-xylose. Thus, the organism differs from the seven described species of *Carnobacterium*. It is capable of acid production/growth on: galactose, glucose, fructose,

mannose, *N*-acetylglucosamine, aesculin, cellobiose, maltose, lactose, sucrose, trehalose and tagatose (API 50CHL). The organism also metabolizes *N*-acetyl D-mannosamine, arbutin, dextrin, gentiobiose, glucose 6-phosphate, maltotriose, 3-methyl D-glucose, salicin, α-hydroxybutyric acid, α-ketovaleric acid, pyruvic acid and uridine (Biolog AN). Negative for all other substrates used in the API 50CHL and Biolog AN panels. Does not grow in 4% (w/v) NaCl but will tolerate 26·4% (w/v) NaCl (saturated brine) for long periods at 4 °C. Does not grow on KF streptococcus agar. Produces predominantly L(+)-lactic acid from glucose and the cell wall peptidoglycan contains *meso*-DAP.

The type strain is strain MPL-11^T (= ATCC BAA-336^T = DSM 14451^T). The GenBank accession number for its 16S rDNA sequence is AF425608.

Acknowledgements

We are grateful to A. J. McCardell, Qualicon Inc., Wilmington, DE, USA, for ribotyping analyses and to Maple Leaf Consumer Foods, Mississauga, ON, Canada, and the Agri-Food Research Development Initiative (Manitoba) for financial support.

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