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Carnimonas nigrificans gen. nov., sp. nov., a bacterial causative agent for black spot formation on cured meat products

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Nine different strains, CTCBS1^T to CTCBS9, were identified to be the causative agents of black spots on the surface of raw cured meat products. The formation of black spots under aerobic conditions is reproducible upon reinoculation of meat products with any of these strains, indicating that they are the causative agent. The strains were Gram-negative, catalase-positive and obligately aerobic rods. The G C content of DNA of strain CTCBS1^T is 56·0 0·3 mol%. The content of non-polar main fatty acids were 16:0, 16:1, 18:1 and 19:0 cyc. Its phylogenetic position was elucidated by comparative sequence analysis of the 16S rRNA gene. Overall sequence similarity to other bacteria does not exceed 93·3%. Isolate CTCBS1^T clustered phylogenetically within the γ-subclass of the *Proteobacteria* and is closely related to members of *Halomonas* (90·5–91·9%) and to *Zymobacter palmae* (93·3%). A genetic homogeneity of the nine strains was demonstrated by M13 random amplified polymorphic DNA-PCR, whereas differentiation from other genera, e.g. *Zymobacter* and *Pseudomonas*, could easily be achieved by their chemotaxonomic characteristics. Taxonomic data revealed the status of a separate genus for which the name *Carnimonas* gen. nov., sp., nov. is proposed. Despite chemotaxonomic and physiological similarities, the new genus is at present not a member of the family *Halomonadaceae* because of the lack of two out of 15 descriptive 16S rRNA signature sequences. The first member of the new genus is *Carnimonas nigrificans*. The use of a specific, 16S rRNA-targeted oligonucleotide primer allowed the identification of all nine strains of *C. nigrificans* in a PCR assay. Toxicological studies showed no pathogenic potential for *C. nigrificans* strain CTCBS1^T (CECT 4437^T).

Keywords: *Carnimonas nigrificans* gen. nov., sp. nov., cured meat products

INTRODUCTION

The browning of foods is a major problem, which can cause deleterious changes in its appearance, texture and flavour, resulting in a shorter shelf life and decreased market value.

The presence of black spots on the surface of food products has been described in connection with miscellaneous organisms of different origin. Some fungal species, such as *Cladosporium cladosporoides*, *Cladosporium herbarum*, *Penicillium hirsutum* and *Aureobasidium pullulans*, develop black-coloured colonies on raw meat stored at −1 °C (Gill & Lowry, 1982). *Colletotrichum coccodes* (Wallr.) Hughes produces black spot tubers of potato (Read, 1991), whereas *Alternaria alternata* is the causal agent of black spot disease on mango fruits (Prusky *et al.*, 1993). Within bacteria, pigmentation is a well-known phenomenon. There are some reports on organisms isolated from marine environments belonging mainly to the *Proteobacteria*, which are capable of forming brown–black colonies. In the case of *Alteromonas nigrifaciens*, the chemical structure of the dark pigment was identified as an indigoidine-like compound, the production of

Abbreviation: RAPD, random amplified polymorphic DNA.

which has already been described for *Clavibacter michiganense* subsp. *insidiosum*, formerly *Corynebacterium insidiosum* (Starr, 1958; Norton & Jones, 1969). Within the *Proteobacteria*, there is also a variety of organisms known for pigmentation, e.g. *Halomonas subglaciescola* (Franzmann *et al*., 1987), *Chromohalobacter* (Ventosa *et al*., 1989) and members of the recently described genus *Pseudoalteromonas* (Gauthier *et al*., 1992), which includes both pigmented and non-pigmented species. *Chromobacterium* produces violacein, which is only produced freely on media containing tryptophan (Sneath, 1984).

The presence of dark spots on the surface of raw cured meat products was first described by Hugas & Arnau (1987). In 1993, a Gram-negative bacterium was identified as being responsible for a rust-like colour, turning black within a few hours, in cured meat products (Arnau & Garriga, 1993). The defect was reproduced on minced meat with salt (40 g kg^{-1}), dextrose (20 g kg^{-1}) and nitrate (0·2 g kg^{-1}) after inoculation of an overnight culture of the previously identified strain (CTCBS1^T). The black spots appeared under aerobic conditions, between 30 and 35 °C as optimum temperature and in the presence of dextrose, maltose or dextrin. Sodium nitrite and potassium bisulphide were the most efficient among the preservatives assayed in preventing the defect (Arnau & Garriga, 1993).

This contribution provides physiological data as well as a phylogenetic characterization to clarify the taxonomic position of these organisms. We propose a new genus, the genus *Carnimonas* gen. nov, containing one species, *Carnimonas nigrificans* sp. nov., which includes nine different strains.

METHODS

Bacterial strains and cultivation. Nine strains, CTCBS1^T to CTCBS9, isolated by Arnau & Garriga in 1993 were investigated. Organisms used as reference strains were from the DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany). All organisms were stored at −80 °C in glycerol (20%) and were cultured routinely at 30 °C in tryptone soya broth (Difco) supplemented with bacto agar (1·4%) depending on the assay.

Physiological and biochemical characterization. Gram staining and determination of oxidase and catalase activities were by standard methods. The presence of endospores was tested using the Schaeffer–Fulton method. The mobility of cells was observed at 1000-fold magnification (resolution of 0·26 μm) in a phase-contrast microscope. All physiological tests were performed at 30 °C and incubated for 72 h, unless otherwise stated. API 50CH strips were inoculated according to the manufacturer's instructions (API System) and analysed after 11 d. Growth in different salt concentrations was carried out for 72 h in tryptone soya broth supplemented with NaCl to final concentrations of 5, 7·5 and 10% (w/v) respectively. Growth was determined in MacConkey agar, SS agar and cetrimide agar (Difco). Bacto OF basal medium (Difco) was used for the determination of acid production from different carbohydrates added to a final concentration of 1% (w/v). Respiratory lipochinones were extracted according to Tindall (1990) and analysed by HPLC as described by Franzmann & Tindall (1990). The cellular fatty acid composition of strain CTCBS1^T was determined by GC after the preparation of whole-cell methanolysates (Minnikin *et al*., 1977). The fatty acid methyl esters were analysed using a Microbial Identification System (MIS; Microbial ID). The relative amount of fatty acids was expressed as a percentage of the total fatty acids. Polar lipids were analysed as described by Franzmann & Tindall (1990).

DNA isolation. Large-scale genomic DNA isolation was performed according to Marmur (1961). Small amounts of DNA used for PCR were extracted and purified as described by Lewington *et al*. (1987).

DNA base composition. The G+C content of the DNA was determined by the thermal denaturation method using a Gilford 2600 spectrophotometer according to Huss *et al*. (1983). For the calculation of the G+C content, the equation of DeLey (1970) was used and corrected according to the DNA of *Escherichia coli* strain K-12 (G+C 51·7 mol%) used as a reference.

Design and evaluation of an rRNA-targeted primer specific for strain CTCBS1^T. The sequence of the oligonucleotide used as a specific primer for strain CTCBS1^T (bs1) was 5′-TAACGTCCTTCATGCCGG-3′ (binding position 469–486 in the *Escherichia coli* numbering system). This primer was checked for its specificity against more than 10000 16S rRNA sequences by using the probe-checking software provided in the Ribosomal Database Project (Maidak *et al*., 1996). The sequence of the universal primers 616VII and 630R used for PCR controls were 5′-AGAGTTTGATYMTGGCTCAG-3′ and 5′-CAKAAAGGAGGTGATCC-5′. PCR and cycle conditions for the species-specific reaction with primer bs1 and universal primer 616V were as follows: one initial cycle of 94 °C for 120 s, followed by 32 cycles of 94 °C for 45 s, 50 °C for 90 s and 72 °C for 120 s. The PCR reactions were carried out in an Omnigene thermocycler (Hybaid) in a total volume of 50 μl. Master mixes were prepared with reaction buffer containing 10 $mmol^{-1}$ Tris/HCl, 1·5 $mmol^{-1}$ $MgCl_2$, 50 $mmol^{-1}$ KCl, pH 8·3, 200 $nmol^{-1}$ dNTP, 0·5 pmol each primer and *Taq* DNA polymerase (Boehringer Mannheim). Control PCR with universal primers 616VII and 630R was performed as described previously (Springer *et al*., 1993).

Generation of random amplified polymorphic DNA (RAPD) patterns. RAPD-PCR was performed in a total volume of 50 μl. The arbitrary primer used was the universal primer M13V with the following sequence, 5′-GTTTTCCCAGTCACGACGTTG-3′. The reaction mixture contained 20 $pmol^{-1}$ primer, 1·5 U *Taq* polymerase (Boehringer Mannheim), 5 μl *Taq* polymerase buffer (Boehringer Mannheim), 200 $nmol^{-1}$ each dNTP, 100 ng template DNA and 7 μl 25 $mmol^{-1}$ $MgCl_2$, resulting in a final concentration of 5 $mmol^{-1}$ $MgCl_2$. Cycling conditions were set as follows: three cycles of 94 °C for 3 min, 40 °C for 5 min and 72 °C for 5 min. This was followed by 32 cycles of 94 °C for 1 min, 60 °C for 2 min and 72 °C for 3 min. After agarose electrophoresis, patterns were visualized by ethidium bromide staining and documented using a video camera. Images were stored as tiff-files and processed further by GelCompar software version 3.1 (Applied Maths). Cluster analysis was performed by the unweighted pair–group method using arithmetic averages (UPGMA). Correlation levels are expressed using percentage values of the Pearson product–moment coefficient.

16S rRNA sequence analysis. *In vitro* amplification of 16S rRNA genes and direct sequencing of the amplified 16S rDNA fragments were carried out as described previously (Springer *et al*., 1993). The strains and sequence accession numbers used for the calculations were as follows (for sequences that are not present in the GenBank database, the Ribosomal Database Project code is given): *Oceanospirillum commune* ATCC 27118, 686; *Oceanospirillum jannaschii* ATCC 27135^T, 682; *Oceanospirillum linum* ATCC 11336, M22365; *Oceanospirillum beijerinckii* ATCC 12754^T, 678, M34133; *Oceanospirillum japonicum* ATCC 19191^T, 661; *Oceanospirillum minutulum* ATCC 19193^T, 662; *Pseudomonas aeruginosa* ATCC 25330, *Zymobacter palmae* ATCC 51621, D14555; *Halomonas marina* ATCC 25374^T, M93354; *Halomonas elongata* ATCC 33173^T, M93355; *Halomonas halmophila* ATCC 19717^T, M59153; *Halomonas halophila* DSM 4770, M93353; *Halomonas halodurans* DSM 5160, L42619; *Halomonas aquamarina* DSM 30161^T, M93352; *Chromohalobacter marismortui* ATCC 17056^T, X87219.

Analysis of sequence data. The newly determined 16S rRNA primary structure was added to an alignment of more than 6000 homologous bacterial sequences. Sequence analysis was performed with the ARB software package (Ludwig & Strunk, 1996) For the determination of similarity values, all positions were included, except those of uncertain positions. The construction of the phylogenetic tree was based on a maximum-likelihood analysis as implemented in the program fastDNAml (Maidak *et al*., 1996) using a subset of 16S rRNA sequences of *Proteobacteria*, including selected members of the γ subclass of the *Proteobacteria*. Only sequences of at least 90% completeness were included. Positions that are not shared by individual residues in at least 50% of sequences were omitted. The topology of the tree was evaluated and corrected according to the results obtained by distance matrix and parsimony analyses based on nearly 10000 sequences (Van den Peer *et al*., 1994).

Evaluation of pathogenicity. The strain CTCBS1^T was tested for pathogenicity in male mice weighing 25–30 g, using three pathogenic strains isolated from clinical sources. The bacterial strains used as controls were *Escherichia coli* HM-42, *Pseudomonas aeruginosa* HS-116 and *Staphylococcus aureus* HS-93. To establish the lethal doses, six lots of mice (60 mice per bacterial strain) were injected intraperitoneally with concentrated and serially diluted suspensions of the cultured strains. The survival of mice was controlled for up to 8 d.

Scanning electron microscopy. For examination by scanning electron microscope (Zeiss DSM960A), samples were fixed in a solution containing 2·5% glutaraldehyde, 0·1 mol^{-1} cacodylate buffer followed by 1% OsO_4 in the same buffer. The samples were processed through a series of ethanol solutions (50, 70, 90 and 100%), dried by CO_2 critical point and coated with gold.

RESULTS

Morphological characteristics

On tryptone soya agar, colonies of all of the nine strains CTCBS1^T to CTCBS9 appeared white, convex, shiny and circular. No pigmentations were visible in any culture medium. All strains were Gram-negative, straight or slightly curved rods, 0·5–0·6 μm in width and 1·0–1·7 μm in length, occurring singly or as pairs. Fig. 1 shows a scanning electron microscopic picture of strain CTCBS1^T. Cells were not motile, and spores could not be detected.

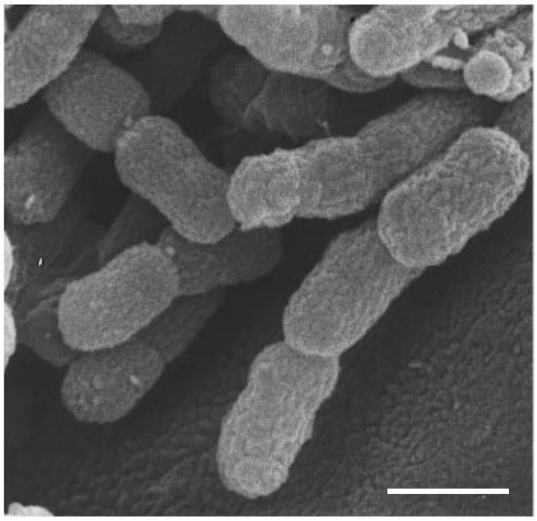

Fig. 1. Scanning electron micrograph of *Carnimonas nigrificans* strain CTCBS1^T. Bar, 758 nm.

Physiological characteristics

The physiological characteristics of strain CTCBS1^T were as follows: cells are obligately aerobic. No gas is produced from glucose. Optimum temperature for growth is 28–30 °C. No growth occurs at 5 or 37 °C. Growth occurs in the presence of up to 8% sodium chloride. Oxidase and catalase activity were positive; indole production, reduction of nitrate and Voges–Proskauer were negative. Neither urease nor lecithinase activity were detected. Starch and aesculin were hydrolysed; casein, gelatin and DNA were not decomposed. All strains produced acid from glucose, xylose, melibiose, maltose and saccharose. Growth occurs on MacConkey agar and cetrimide agar but not on SS agar. Additional physiological data are provided in Table 1.

Biochemical characterization

Biochemical tests were performed with strain CTCBS1^T. The main component of respiratory quinones was menaquinone 9. The main components in the polar lipid composition were diphosphatidylglycerine, phosphatidylglycerine and phosphatidylethanolamine. In addition, there were three unidentified components.

The fatty acid profile contained major amounts of several saturated and unsaturated straight-chain fatty acids and only minor fractions of 3-hydroxylated fatty acids. The main fatty acids were palmitic acid (16:0) with 40% and the cyclopropanic acid 19:0cyc that occurred at the high level of 21·07%. Oleic acid 18:1*trans*9 occurred at the rate of 7·45% in addition

Table 1. Differential characteristics between *Carnimonas nigrificans* CTCBS1^T (CECT 4437) and *Z. palmae* ATCC 51623^T

Characteristic	*C. nigrificans*†	*Z. palmae*
Morphology	Short rod	Short rod
Cell length (μm)	1·0–1·7	1·3–2·4
Cell width (μm)	0·5–0·6	0·7–0·9
Gram reaction	Negative	Negative
Motility	–	+
Flagella	–	Peritrichous
Chromogenicity	–	–
Relation to oxygen	Strictly aerobic	Facultative anaerobic
Growth at 37 °C	–	+
Hydrolysis of:		
Aesculin	+	ND
Gelatin	–	–
Casein	–	–
Starch	+	–
Tween 80	–	ND
Acid production from:		
Amygdalin	+(3/9)	ND
L-Arabinose	–(0/9)	–
Aesculin	+(9/9)	ND
Fructose	+(7/9)	+
Galactose	w (9/9)	+
Glucose	+(9/9)	+
Mannose	+(7/9)	–
Mannitol	+(7/9)	+
Melibiose	+(9/9)	+
Melezitose	–(2/9)	ND
Raffinose	w (3/9)	+
Ribose	w (4/9)	ND
Saccharose	+(9/9)	+
Salicin	w (9/9)	ND
Trehalose	w (6/9)	–
D-Xylose	+(9/9)	–
Utilization of:‡		
L-Arabinose	–	–
Mannose	+	+
Mannitol	–	+
N-acetylglucosamine	–	ND
Maltose	+	+
Gluconate	+	ND
Caprate	–	ND
Adipate	–	ND
Malate	–	ND
Citrate	+	–
Oxidase	+	–
Voges–Proskauer	–	+
β-Galactosidase (ONPG)	+	–
α-Glucosidase (PNPG)	+	+
Phenylalanine deaminase	+	–
Arginine dihydrolase	–	–
Catalase	+	+
Nitrate reduction	–	–
Urease	–	ND
DNase	–	ND
Lecithinase	–	ND
Production of indole	–	–
Max. [NaCl] (w/v)	8–10%	ND

ND, Not determined.

* Data taken from Okamoto *et al.* (1993).

† Numbers in parentheses indicate the number of strains that showed weak or positive reactions against the number of strains tested.

‡ On media containing vitamins and yeast extract.

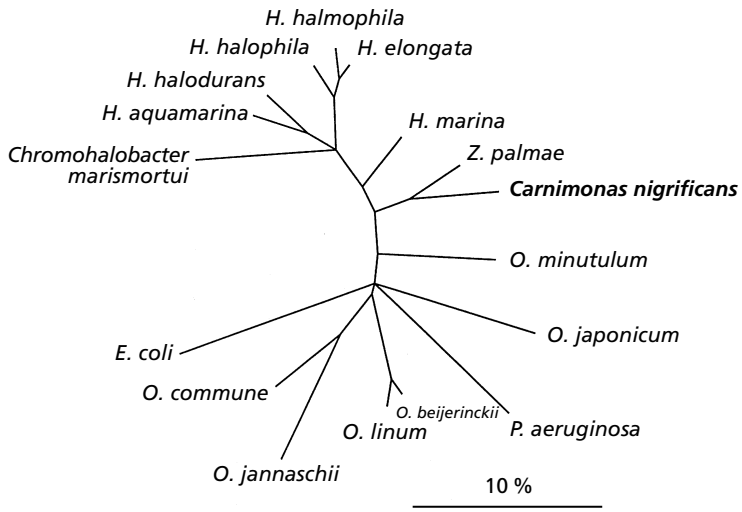

Fig. 2. Phylogenetic relationships of *Carnimonas nigrificans* strain CTCBS1^T and selected representatives of the γ-subclass of *Proteobacteria*. The topology of the tree is based on the results of maximum-likelihood analyses and was corrected according to maximum-parsimony and distance methods. The scale bar indicates 10% estimated substitutions per sequence.

with 12·9% of a summed in feature (18:1*cis*11/*trans*9/*trans*6). Moreover, 16:1 could be detected at the 6·7% level, whereas only minor amounts of 3-OH12:1 (1·85%) and traces of 3-OH16:0 (0·46%) were found. No match was found after comparison with the TSBA library.

The DNA G+C content of strain CTCBS1^T analysed by the thermal denaturation method was 56·0 ± 0·3 mol%.

Phylogenetic relationships

The 16S rDNA sequence of the strain CTCBS1^T (1519 nucleotides) was determined and deposited in the EMBL database.

Sequence similarity values were in the range 84·4–93·3% and placed strain CTCBS1^T within the γ-subclass of *Proteobacteria*. The phylogenetic tree (Fig. 2) shows the closest relationship to *Z. palmae* ATCC 51623^T (sequence similarity of about 93·3%).

Specific PCR assay

Comparative sequence analysis of 16S rRNA gene sequences revealed a diagnostic sequence that can be used as a target site for specific amplification. Specific amplification of a 480 bp fragment using primer bs1 and 616V occurs only in CTCBS strains (Fig. 4).

Amplification of a 1535 bp fragment occurred in positive controls using primer 616V and 630R in all strains tested, including the reference strains *Zymobacter palmae* ATCC 51623^T, *Pseudomonas fluorescens* DSM 50106, *Escherichia coli* K-12, *Enterococcus faecalis* DSM 20478, *Lactococcus lactis* subsp. *cremoris* DSM 20069^T, *Pediococcus damnosus* DSM 20331^T (Fig. 4).

RAPD patterns

RAPD-PCR from DNAs of all the organisms tested using M13V primer generated up to 10 distinct bands ranging from 3·3 to 0·55 kb (Fig. 3a). Most of the bands were of different intensities. The patterns of DNAs of the nine isolates CTCBS1^T to CTCBS9 were very similar and differed only in the presence or absence of bands whose intensities were low.

In general, full reproducibility was given only for those bands whose intensity was high, e.g. the fragments of 3·3 and 0·55 kb for CTCBS1^T. The fingerprints obtained from the reference strains belonging to other genera, *Zymobacter palmae* ATCC 51623^T, *Pseudomonas aeruginosa* DSM 50106 and *Escherichia coli* K-12, showed bands neither common to each other nor to the meat isolates. From the UPGMA cluster, a high genetic similarity is obvious, as shown in the correlation coefficient of about 80% (Fig. 3b).

Evaluation of pathogenicity

After intraperitoneal injection of up to $1·9 \times 10^{10}$ c.f.u. of strain CTCBS1^T, no pathogenic effect was detectable in mice after 8 d. Under the same conditions, bacteria used as controls showed a concentration-dependent lethality. The most pathogenic effect was observed for *Escherichia coli* HM-42. This organism killed 30% of mice at $3·5 \times 10^6$ c.f.u. and 90% at $3·5 \times 10^8$ c.f.u. No mice survived at $3·5 \times 10^9$ c.f.u. In the case of *Pseudomonas aeruginosa* HS-116, 10% of mice were killed at $6·3 \times 10^8$ c.f.u. and 90% at $6·3 \times 10^9$ c.f.u. The application of $3·7 \times 10^{11}$ c.f.u. of *Staphylococcus aureus* HS-93 killed 90% of mice, whereas no pathogenic effect was observed with $3·7 \times 10^9$ c.f.u.

DISCUSSION

One of the problems encountered in the production of raw cured meat products is the occurrence of a rust-like colour on the surface of these products (Hugas & Arnau, 1987). This defect was found to be caused by the presence of Gram-negative bacteria and shown to be fully reproducible by an inoculation with these organisms (Arnau & Garriga, 1993).

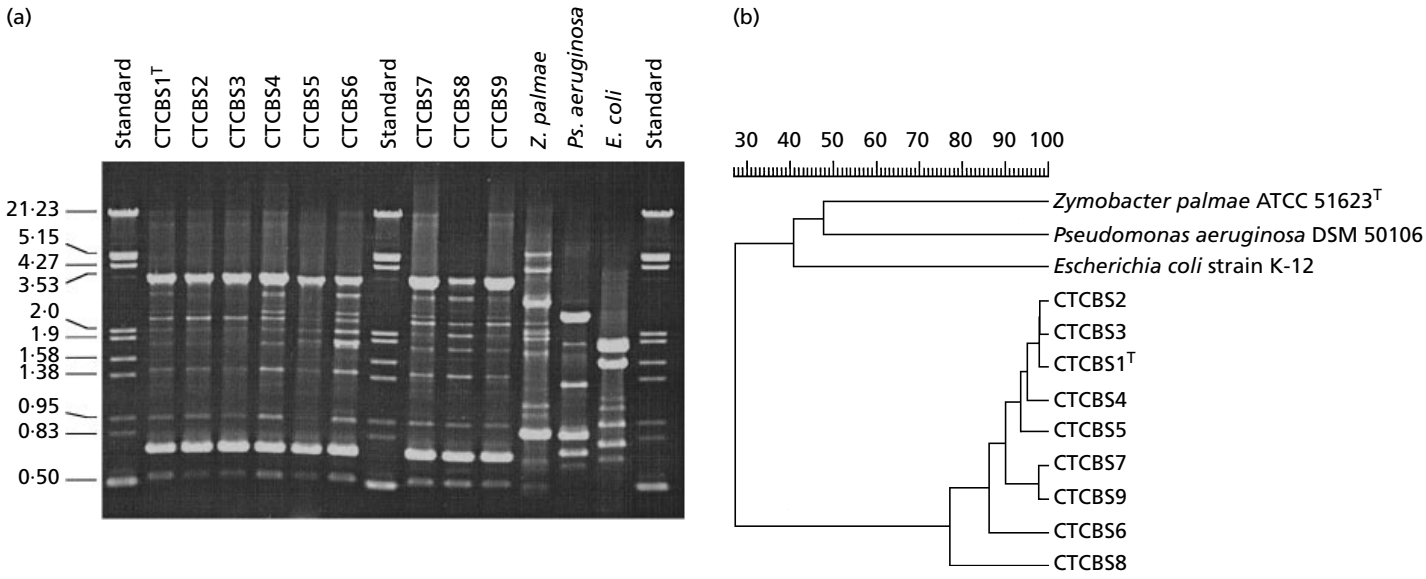

Fig. 3. (a) RAPD fingerprint with M13V primer of *Carnimonas nigrificans* strains CTCBS1^T to CTCBS9, reference organisms *Z. palmae* ATCC 51623^T, *Pseudomonas fluorescens* DSM 50106, *Escherichia coli* strain K-12. Standard molecular mass marker III (Boehringer Mannheim). (b) UPGMA cluster analysis of RAPD patterns from part (a) of *Carnimonas nigrificans* strains CTCBS1^T to CTCBS9, reference organisms *Z. palmae* ATCC 51623^T, *Pseudomonas fluorescens* DSM 50106, *Escherichia coli* strain K-12. Correlation levels are expressed using percentage values of the Pearson product–moment coefficient.

In this paper, nine different isolates were investigated according to their physiological and biochemical characteristics to clarify their phylogenetic positions.

The cells are non-spore-forming, straight or slightly curved, Gram-negative rods, oxidase- and catalase-positive. Their colonies on tryptone soya agar were not pigmented. All strains were obligately aerobic, and their optimum temperature for growth was 28–30 °C. They grew well on cetrimide agar, which is selective for *Pseudomonas aeruginosa*, but also in MacConkey agar, which is selective for coliform organisms. The strains were not able to grow at 5, 37 or 41 °C or in SS agar. Despite some differences regarding the use of carbohydrates, e.g. ribose, fructose and mannose, a high genetic uniformity of the nine strains was demonstrated when using the M13 primer for a RAPD-PCR analysis. RAPD has already been evaluated as a potential tool for the differentiation of individual strains within certain species belonging to the γ-subclass of the *Proteobacteria* (Aznar *et al*., 1993; Martin-Kearley *et al*., 1994). However, its differential power for species, strains or even lower clearly depends on the primer used or the combination of results obtained with various primers (Parent *et al*., 1996). In this investigation, when the M13 primer was applied, a differentiation from other species could easily be achieved at a correlation value of less than 30%, whereas within the nine isolates highly similar patterns were observed (Fig. 3). The small differences proved not to be reproducible and, hence, it is likely that, in this case, a reliable differentiation at strain level needs the choice of additional primers.

The topology of all organisms illustrated in the tree based on maximum-likelihood analysis of 16S rRNA sequences (Fig. 2) is consistent with that in a tree published by Dobson & Franzmann (1996). The closest relatives of strain CTCBS1^T were *Z. palmae* with 93·3% sequence similarity and, to a lesser extent, species of the genus *Halomonas* (at least 91·9%) and *Chromohalobacter* (91·5%).

The physiological data indicated, in agreement with the results of chemotaxonomical data discussed later, that these organisms belonged to the γ-subclass of the *Proteobacteria*. The 16S rRNA sequence analysis placed these organisms in the γ-3 subclass of the *Proteobacteria*, which includes the fluorescent pseudomonads, the genera *Oceanospirillum*, *Chromohalobacter* and *Marinobacter* (Gauthier *et al*., 1992; Gosink & Stanley, 1995), as well as all genera of the family *Halomonadaceae* (Dobson *et al*., 1993; Franzmann *et al*., 1988). They formed a genetically homogeneous group, which is phylogenetically distinct from all other organisms actually present in the mentioned taxa. All data presented here support the status of strains CTCBS1^T to CTCBS9 belonging in a new genus, for which we propose the name *Carnimonas* due to their source of isolation.

Owing to their genetic homogeneity, the nine strains CTCBS1^T to CTCBS9 should be combined into one species named *Carnimonas nigrificans*. This name reflects their blackening effect on meat products.

At present, DNA–DNA hybridization data for *Halomonadaceae* and related genera are limited. More-

Table 2. Differential characteristics of strain CTCBS1^T and the genera *Oceanospirillum* and genera belonging to the family *Halomonadaceae*

Data from this study and Ventosa *et al.* (1989), Franzmann & Tindall (1990), Okamoto *et al.* (1993) and Sakane & Yokota (1994).

Characteristic	CTCBS1^T	Family *Halomonadaceae*:			*Oceanospirillum*
		Halomonas	*Chromohalobacter*	*Zymobacter*	
G+C content (mol%)	56±0·3	60·5–66·7	62–64·9	55·4–56·2	42–51
Major respiratory lipochinone	Ubiquinone 9	Ubiquinone 9	ND	Ubiquinone 9	Ubiquinone 8
Number of 16S rRNA signature characteristics shared with *Halomonas*	13	19	19	15	10
Pigments	None	White/yellow	Yellow/blue	None	None*
Major non-polar fatty acids	16:0, 16:1, 18:1†,19cyc	16:0,16:1, 17cyc, 18:1†, 19cyc	ND	12:0, 16:0, 18:0, 18:1, 19cyc	16:0, 16:1, 18:0
Motility	−	D	+	+	+
Flagella	None	Peritrichous/polar	Peritrichous	Peritrichous	Polar
Max. [NaCl] (%)	8	32·5	>30	ND	9·75
Relation to oxygen	Aerobic	Aerobic‡	Aerobic	Fac. anaerobic	Aerobic
Oxidase	+	D	−	−	+

ND, Not determined; D, different.

* Some strains produces a water-soluble, yellow–green fluorescent pigment on PSS seawater agar.

† Summed feature containing 18:1, *cis*11, *trans*9 and *trans*6.

‡ Some strains are capable of anaerobic growth in the presence of nitrate.

over, it has already been shown that it is unlikely that two species exhibiting less than 99% 16S rRNA sequence similarity would have a DNA–DNA hybridization value of $\geqslant 70\%$, this being the criterion for delineating species (Amann *et al.*, 1992; Fox *et al.*, 1992). The level of sequence similarity between *Halomonas euryhalina* and *Halomonas elongata* is 99·1% (Dobson *et al.*, 1996) and the level of DNA–DNA hybridization is only 7% (Valderama *et al.*, 1991). As 16S rRNA sequence similarity values of strain CTCBS1^T to all other organisms does not exceed 93·3%, no DNA–DNA hybridization data are provided.

In contrast to the species definition, there is no widely accepted level of phylogenetic relationship for defining bacterial genera expressed in concrete values of DNA–DNA hybridization or sequence similarity (Wayne *et al.*, 1987; Stackebrandt, 1992). Therefore, the delineation of genera solely on the basis of phylogenetic data is completely impracticable (Murray *et al.*, 1990).

The description of a new taxon requires not only that a group of organisms be phylogenetically homogeneous as inferred from genotypic data, but that there be chemotaxonomic or phenotypic features characteristic of the group that differentiate it from closely related genera. Likewise, in Table 2, there were sufficient phenotypical differences to support the assignment of strains CTCBS1^T to CTCBS9 to a new taxon at genus level.

The G+C content of 56 mol%, major respiratory lipochinone and the lack of C19cyc in the non-polar lipid profile are sufficient to exclude these organisms from *Oceanospirillum*.

The recent unification of the genera *Deleya*, *Halomonas* and *Halovibrio* into the single genus *Halomonas* arose from the realization that a couple of phenotypic features failed to delineate phylogenetically meaningful taxa within these organisms (Dobson *et al.*, 1996). In fact, in this case, differences in fatty acid profiles, major respiratory lipochinones and G+C contents are too small to delineate different subgroups. On the other hand, criteria such as morphology, salt tolerance, motility and oxidase were found to be too diverse within these organisms and, hence, more useful for describing species than genera (Dobson *et al.*, 1996). Nevertheless, these criteria become more useful when comparing taxa that are phylogenetically distinct at a lower level.

The G+C content of *Halomonas* and *Chromohalobacter* differs by at least 4·2 mol% from strain CTCBS1^T, and distinct differences in the fatty acid profile were as follows. The levels of 16:0 in the profiles of members of the genus *Halomonas* range from 15·5 to 32%, whereas the level in CTCBS1^T is 40%. Additionally, in contrast to *Halomonas*, in CTCBS1^T no 17cyc could be detected. Even if conversion of monosaturated fatty acids to its cyclopropane derivative is sensitive to culture conditions

Table 3. 16S rRNA sequence signature characteristics of the genus *Halomonas* which are different from *Zymobacter* and/or *Carnominas nigrificans* strain CTCBS1^T

Data for Halomonas are from Dobson *et al.* (1996). Positions are according to the *Escherichia coli* numbering system.

Position	*Halomonas*	*Zymobacter*	*Carnimonas*
76–93	6 bp stem	6 bp stem	6 bp stem
484	A	A	**G**
486	C	C	**U**
640	G	G	G
660	A	A	A
668	A	A	A
669	A	A	A
737	U	U	U
738	U	U	U
745	U	U	U
776	U	U	U
1124	U	U	U
1297	U	U	U
1298	C	C	C
1423	A	A	A
1424	C	U	U
1439	U	C	C
1462	A	C	C
1464	C	U	C

and the addition of 16:0 plus 17cyc may be a more stable characteristic (Jantzen & Bryn, 1985), the difference remained significant (CTCBS1^T about 6·7% and *Halomonas* 7·7–43·1%). No difference was found in their major respiratory lipochinone, which is ubiquinone 9 in the case of *Halomonas* and *Zymobacter*. Unfortunately, in the case of members of *Chromohalobacter*, neither lipid profile nor respiratory lipochinone data exist, although they can easily be distinguished by their production of pigments (Ventosa *et al.*, 1989).

Z. palmae is the single species of the genus *Zymobacter* and the closest phylogenetic relative to strain CTCBS1^T (Okamoto *et al.*, 1993). Moreover, the greatest chemotaxonomic similarity to CTCBS1^T is shown in this case. The G+C content of its DNA is 55·8 mol%, which is within the range determined for CTCBS1^T. Their profiles of the major fatty acids are similar; however, a significant amount of 12:0 (5%), which might be unique to *Z. palmae*, was not detected in strain CTCBS1^T or in the other closely related genera. In addition, some physiological and morphological properties, e.g. relationship to oxygen, growth temperature, hydrolysis of starch, oxidase reaction as well as flagellation and motility, are useful in differentiating between these two organisms.

A common characteristic of all the genera mentioned in this study is their more or less pronounced salt requirement or tolerance, which is explicable by their isolation from saline environments, including the surface of cured meat products, from which strains CTCBS1^T–9 were isolated. CTCBS1^T growth is optimal at 4% NaCl and inhibited at 8% NaCl. To our knowledge, no study has been conducted on the salt tolerance of *Z. palmae*, but high osmotolerance has been shown by the fact that it can grow in the presence of 50% maltose (Okamoto *et al.*, 1993).

At present, the family *Halomonadaceae* is based on a pattern or core of phenotypical characteristics that would also be shared by strain CTCBS1^T. However, this grouping is not supported by phylogenetic data. In the emendation of the family *Halomonadaceae*, *Z. palmae* was included as a member of this family mainly because of the common occurrence of signature characteristics in its 16S rRNA sequence (Dobson & Franzmann, 1996). To achieve this, the number of diagnostic signature characteristics was reduced from 19 to 15, which are retained as descriptive characteristics of the members of the family *Halomonadaceae*.

Although strain CTCBS1^T and *Z. palmae* actually form a monophyletic group, it shares only 13 of 15 signatures defining the family *Halomonadaceae*. As detailed in Table 3, these substitutions were at position 484 and position 486, at which an adenine is replaced by a guanine and a rare cytosine is replaced by guanine, respectively. Furthermore, these positions are within the region used successfully in this study as a diagnostic sequence for a specific identification and differentiation by a PCR assay (Fig. 4).

As both *Zymobacter* and the newly described genus *Carnimonas* are genera containing one species up to now, we propose to avoid a hasty change of the family description that would necessarily include a further reduction in the signature sequences from 15 to 13 and, hence, not to include *Carnimonas* before more species are found.

All newly described strains were isolated from cured meat products. Therefore, strain CTCBS1^T was subjected to toxicological studies. No pathogenic potential of the strain CTCBS1^T was determined compared with the three reference strains assayed.

As these organisms were not pigmented and no pigments were released in the culture media, the visible colouration on meat seems rather to be a non-enzymic browning reaction of special components derived from the meat surface. The chemical mechanism has not yet been elucidated but, as far as we know, some sugars and/or specific amino acids are involved (Arnau & Garriga, 1993).

Description of *Carnimonas* gen. nov.

Carnimonas (Car.ni′mo.nas. L. gen. n. *carnis* of meat; Gr. n. *monas* a unit, monad).

Non-spore-forming, Gram-negative, straight or slightly curved rod, occurring singly or as pairs, non-

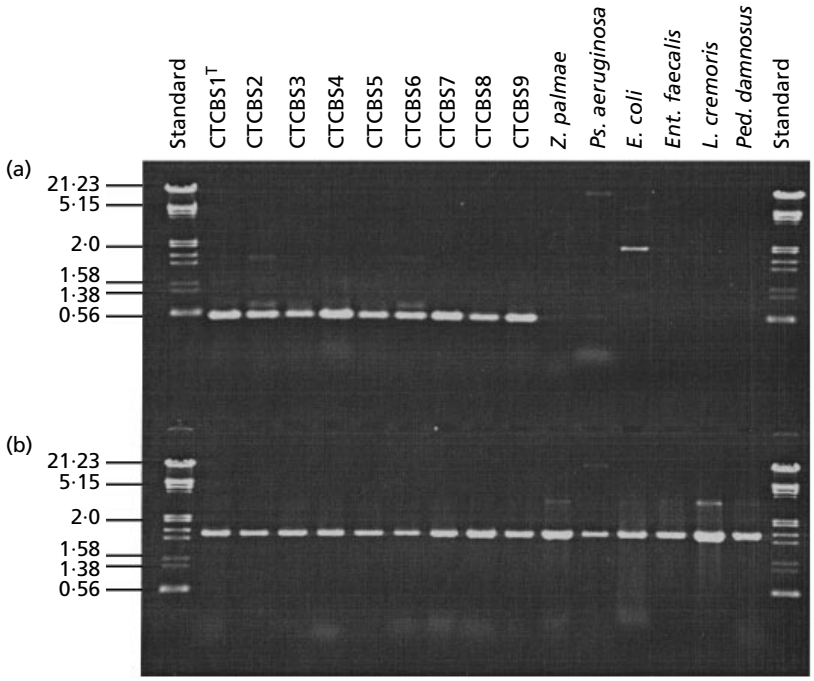

Fig. 4. Specific PCR assay for identification of the newly described *Carnimonas nigrificans* strains, reference organisms *Z. palmae* ATCC 51623^T, *Pseudomonas fluorescens* DSM 50106, *Escherichia coli* strain K-12, *Enterococcus faecalis* DSM 20478; *Lactococcus lactis* subsp. *cremoris* DSM 20069^T, *Pediococcus damnosus* DSM 20331^T. S, Molecular mass marker III (Boehringer Mannheim). (a) The specific PCR using primer bs1 and universal primer 97K resulting in a 480 bp fragment. (b) To exclude false-negatives, a PCR was performed using universal primers 616V and 630R, resulting in a 1535 bp fragment.

motile, oxidase and catalase activity positive. Cells are obligately aerobic and slightly or moderate halophile. No growth occurs in the presence of more than 8% NaCl. Optimum temperature for growth is 28–30 °C. No growth occurs at 5 or 37 °C. Chemo-organotrophic. The main component of respiratory quinones is ubiquinone 9. The main components in the polar lipid composition are diphosphatidylglycerine, phosphatidylglycerine and phosphatidylethanol-amine. The major fatty acids are 16:0, 16:1, 18:1 plus 19cyc. The type species is *Carnimonas nigrificans*.

Description of *Carnimonas nigrificans* sp. nov.

Carnimonas nigrificans (nig.rif'i.cans. L. adj. *niger* black; L. v. *facere* to make; L. n. *nigrificans* black-making).

The description of the species corresponds to that of the genus. Additional characteristics are given as follows. Colonies are non-pigmented, white, convex, shiny and circular. Acid is produced from glucose, fructose, maltose, xylose, melibiose and saccharose. Aesculin and starch are hydrolysed. Gelatin, casein and DNA are not hydrolysed. Voges–Proskauer negative. Arginine dihydrolase, urease, lecithinase and phenylalanine deaminase negative. Indole is not produced. Nitrate is not reduced. Strain CTCBS1^T (CECT 4437^T) is the type strain.

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