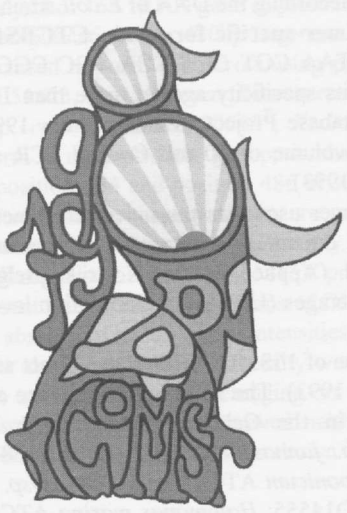
PS 10

Poster session and workshop 10

Fermented products



Thursday, September 3rd
11:15h-12:45h

Carnimonas nigrificans GEN. NOV., SPEC. NOV. A BACTERIAL CAUSATIVE AGENT FOR BLACK SPOTS FORMATION ON CURED MEAT PRODUCTS

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BACKGROUND

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 have been described in connection with miscellaneous organisms from different origins. There are some reports on organisms isolated from marine environments mainly belonging to the Proteobacteria which are capable of forming brown-black colonies.
The presence of dark spots on the surface of raw cured meat products was firstly described by Hugas & Arnau (1987). In 1993 Arnau and Garriga identified a Gram-negative bacterium as responsible for a rust like colour turning black within a few hours in cured meat products. The defect was reproduced on minced meat with salt (40 g/kg), dextrose (20 g/kg) and nitrate (0.2 g/kg) after inoculation of an overnight culture of the previously identified strain (CTCBS1). The black spots appeared under aerobic conditions, between 30-35°C as optimum temperature and in the presence of dextrose, maltose or dextrin (Arnau and Garriga, 1993).

OBJECTIVES

To provide physiological data as well as a phylogenetic characterization in order to clarify the taxonomic position of these organisms.

METHODS

Bacterial strains and cultivation. Nine strains CTCBS1 to CTCBS9 were investigated. Organisms used as reference strains were from DSMZ (Braunschwaig, Germany). All organisms were stored at -80°C in glycerol (20%) and were routinely cultured at 30°C in tryptone soja broth (Difco) supplemented with Bacto Agar (1.4%) depending on the assay.
Physiological and biochemical characterization. Gram staining, oxidase and catalase activities were performed according to standard methods. All physiological tests were performed at 30°C and incubated for 72 hours, unless otherwise stated. API 50CH strips were inoculated by using the manufacturer's instruction (API System S.A., Lyon, France) and analyzed after 11 days. Growth in different salt concentrations was carried out for 72 hours in TSB supplemented with NaCl to final concentrations of 5, 7.5, and 10% (wt/vol), respectively. The growth was determined in McConkey agar, SS-agar and Cetrimide agar (Difco). Respiratory lipochinones were extracted according to Tindall (1990) and analyzed by high-performance liquid chromatography HPLC as described by Franzmann and Tindall (1990). The cellular fatty acid composition of strain CTCBS1 were determined by gas chromatography after preparation of whole cell methanolysates (Minnikin et al., 1977). The fatty acid methylesters were analyzed using Microbial Identification System (MIS). The relative amount of fatty acids were expressed as a percentage of the total fatty acids. Polar lipids were analyzed as described in (Franzmann & Tindall, 1990).
DNA isolation. Large scale genomic DNA isolation were performed according to Marmur (1961). Small amounts of DNA used for PCR were extracted and purified as described by Lewington et al. (1987). The mol% G+C content of the DNA was determined by the thermal denaturation method using a Gilford 2600 according to Huss et al. (1983). For the calculation of the mol% G+C content the equation of DeLey (1970) was used and corrected according the DNA of *E.coli* strain K12 (mol% G+C 51.7) used as a reference.
Design and evaluation of a rRNA-targeted primer specific for strain CTCBS1. The sequence of the oligonucleotide used as specific primer for strain CTCBS1 (bs1) was 5´-TAA CGT CCT TCA TGC CGG-3´ (binding position 469 to 486 in the *E.coli* numbering system). This primer was checked for its specificity against more than 10.000 16S rRNA sequences by using the probe checking software provided in the Ribosomal Database Project (Maidak et al., 1996). The PCR reactions were carried out in an Omnigene thermocycler (Hybaid, UK) in a total volume of 50 ml. Control PCR with universal primers 616VII and 630R were performed as described previously (Springer et al., 1993).
Generation of RAPD patterns. The arbitrary primer used was the universal primer M13V. After agarose electrophoresis patterns were visualized by ethidium bromide staining and documented using a video camera. Images were stored as tiff-files and further processed by GelCompar™ software version 3.1 (Applied Maths, Kortrijk, Belgium). Cluster analysis was performed by the unweighted pair-group method using arithmetic averages (UPGMA). Correlation levels are expressed using percentual 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 done as previously described (Springer et al. 1993). The strains and sequence accession numbers used for calculations were as follows (for sequences which are not present in the GenBank database the Ribosomal Database Project code is given): *Oceanospirillum comune* ATCC27118, 686; *Osp. jannaschii* ATCC 27135^T, 682; *Osp. linum* ATCC 11336, M22365; *Osp. beijerinckii* ATCC 12754^T, 678, M34133; *Osp. japonicum* ATCC19191^T, 661; *Osp. minutulum* ATCC 19193^T, 662; *P. aeruginosa* ATCC25330, *Zymobacter palmae* ATCC 51621, D14555; *Halomonas marina* ATCC25374^T, M93354; *H. elongata* ATCC33173^T, M93355; *H. halmophila* ATCC19717^T, M59153; *H. halophila* DSM 4770, M93353; *H. halodurans* DSM 5160, L42619; *H. aquamarina* DSM 30161^T, M93352; *Chromohalobacter marismortui* ATCC17056^T, X87219.
Sequence data analysis. The newly determined 16S rRNA primary structure was added to an alignment of more than 6000 homologous bacterial sequences. Sequence analysis were performed with the ARB software package (Ludwig & Strunk, 1996) For determination of similarity values all positions were included except of uncertain positions. The construction of the phylogenetic tree was based on a maximum likelihood analysis as implemented in the program fastDNAml of Olsen (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 which 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 10.000 sequences (Van den Peer et al., 1994).

RESULTS AND DISCUSSION

Morphological and physiological characteristics. On Tryptone Soja agar colonies of all of the nine strains CTCBS1 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 mm in width and 1.0-1.7 mm in length, occurring singly or as pairs. Cells were not motile and spores could not be detected. The physiological characteristics of strain CTCBS1 were as follows: cells are strictly aerobic. No gas is produced from glucose. Optimum temperature for growth is 28-30°C. No growth occurs at 5°C and 37°, respectively. Growth occurs in 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 esculin were hydrolyzed, casein, gelatine and DNA were not decomposed. All strains produced acid from glucose, xylose, melibiose, maltose and saccharose. Growth occurs on McConkey agar, and Cetrimid agar but not on SS-agar.

Biochemical characterization. Biochemical tests were performed with strain CTCBS1. The main component of respiratory quinones was menaquinone 9. The main components in the polar lipid composition were diphosphatidylglycerin, phosphatidylglycerin and phosphatidylethanolamin. 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 cycolpropanic acid 19:0cyc that occurred at high amounts of 21.07%. Oleic acid 18:1trans9 occurred with 7.45% in addition with 12.9% of a summed in feature (18:1cis11/t9/t6). Moreover 16:1 could be detected at an amount of 6.7% 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 base composition of strain CTCBS1 analyzed by the thermal denaturation method was 56.0 ± 0.3 mol% G+C. The mol %G+C content of 56 %, major respiratory lipochinone and the lack of C19cyc in the non polar lipid profile are sufficient for excluding these organisms from *Oceanospirillum*.

Phylogenetic relationship. The 16S rDNA sequence of the strain CTCBS1 (1519 nucleotides) was determined and deposited in EMBL Nucleotide Sequence Database. Accession number is Y13299. Sequence similarity values were in the range of 84.4% to 93.3% and placed the strain CTCBS1 within the γ-subgroup of Proteobacteria. The phylogenetic tree shows the closest relationship to *Zymobacter palmae* ATCC 51623^T (sequence similarity of ca. 93.3%) and with a lower extent species of the genus *Halomonas* (at least 91.9%) and *Chromohalobacter* (91.5%). *Zymobacter palmae* is the single species of the genus *Zymobacter* and the closest phylogenetic relative to strain CTCBS1 (Okamoto et al., 1993). Moreover, the most chemotaxonomic similarity to CTCBS1 is shown in this case. The G+C content of its DNA is 55.8 mol% and in the range of that determined for CTCBS1. Their profiles of the major fatty acids are similar, however a significant amount of 12:0 (5%) that might be unique to *Zymobacter palmae* was not detected in strain CTCBS1 as well in the other closely related genera. There also were a couple of physiological and morphological properties e.g. their relationship to oxygen, the growth temperature, hydrolysis of starch, oxidase reaction as well as flagellation and motility, that are useful in differentiation between these two organisms. A common characteristic of all genera mentioned in this study is their more or less pronounced salt requirement or tolerance explainable by their isolation from saline environments including the surface of cured meat products from which strains CTCBS1-9 have been isolated. CTCBS1 optimal growth occurs at 4% NaCl and its maximum is 8%.

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 480bp fragment using primer bs1 and 616V occurs only in CTCBS strains. Amplification of a 1535bp fragment occurred in positive controls using primer 616V and 630R in all strains tested including the reference strains *Zymobacter palmae* ATCC51623, *Pseudomonas fluorescens* DSM 50106, *Escherichia coli* K12, *Enterococcus faecalis* DSM20478; *Lactococcus lactis* subsp. *cremoris* DSM 20069^T, *Pediococcus damnosus* DSM 20331^T. Although strain CTCBS1 and *Zymobacter palmae* actually form a monophyletic group, it shares only 13 of 15 signatures defining the family *Halomonadaceae*. 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 successfully used in this study as a diagnostic sequence for a specific identification and differentiation by a PCR assay.

RAPD patterns. RAPD PCR from DNA´s of all organisms tested using M13V primer generated up to 10 distinct bands ranging from 3.3 to 0.55 kb. Most of the bands were of different intensities. The patterns of DNAs of the nine isolates CTCBS1 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 were high e.g. the fragments of 3.3 kb and 0.55 kb for CTCBS1. The fingerprints obtained from the reference strains belonging to other genera *Zymobacter palmae* ATCC 51623, *Pseudomonas aeruginosa* DSM 50106 and *Escherichia coli* K12 showed neither common bands to each other nor to the meat isolates. From the UPGMA cluster a highly genetic similarity is obvious as shown in the correlation coefficient of ca. 80%.

CONCLUSIONS

All data presented here support the status of strains CTCBS1 to 9 in a new genus, for which we propose the name *Carnimonas* considering their source. Due to their genetically homogeneity, the nine strains CTCBS1 to CTCBS9 should be combined into one species named *Carnimonas nigrificans*. This name points to their blackening effect on meat products.

LITERATURE

Garriga, M., Ehrmann, M.A., Arnau, J., Hugas, M. and Vogel, R.F. *Carnimonas nigrificans* gen. nov., spec. nov. a bacterial causative agent for black spots formation on cured meat products. Int. J. Syst. Bacteriol. (in press)