Vagococcus carniphilus sp. nov., isolated from ground beef

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Nine enterococcus-like strains were referred to the *Streptococcus* Laboratory at the Centers for Disease Control and Prevention (CDC) for further identification from the National Antimicrobial Resistance Monitoring System Laboratory at the CDC. The cultures were isolated from ground beef purchased from retail in Oregon in 2000. Conventional biochemical testing and analysis of whole-cell protein electrophoretic profiles distinguished these strains from known species of enterococci and vagococci. Comparative 16S rRNA gene sequencing studies revealed that these strains were most closely related to the species *Vagococcus fluvialis*. DNA–DNA reassociation studies confirmed that these nine strains represented a new taxon. The relative binding ratio was 87 % or greater at the optimal temperature, and the divergence was less than 1 % for strains hybridized against the isolate designated the type strain. DNA–DNA relatedness was 25 % to *V. fluvialis* and 9 % or less to the other three species of *Vagococcus*. DNA–DNA relatedness was 33 % or less to the 25 currently described species of *Enterococcus*. On the basis of this evidence, it is proposed that these strains be classified as *Vagococcus carniphilus* sp. nov. The type strain of *V. carniphilus* is 1843-02^T (= ATCC BAA-640^T = CCUG 46823^T). The clinical significance (if any) of these strains is yet to be determined.

There are currently four recognized species in the genus *Vagococcus*. *Vagococcus fluvialis* was the first-described species when it was shown that a group of motile lactococcus-like bacteria formed a distinct line of descent by 16S rRNA sequencing (Collins *et al.*, 1989). Later, *Vagococcus salmoninarum* was isolated from a trout, *Vagococcus lutrae* was isolated from an otter, and *Vagococcus fessus* from a seal and a porpoise (Hoyles *et al.*, 2000; Lawson *et al.*, 1999; Wallbanks *et al.*, 1990). These previous studies have shown that the vagococci are phylogenetically and phenotypically similar to the enterococci and are often difficult to differentiate based on conventional physiological testing.

Over the past year or so, several isolates have been received for identification in the *Streptococcus* Reference Laboratory from the National Antimicrobial Resistance Monitoring System Laboratory at the Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA. Some isolates were preliminarily identified as enterococci-like based on positive tests for hydrolysis of pyrrolidonyl- β -naphthylamide and hydrolysis of aesculin in the presence of 40 % bile salts (bile-aesculin), and growth in 6.5 % NaCl broth, but could not be identified to the species level using any of the schemes currently used for the phenotypic identification of enterococci (Facklam & Sahm, 1995; Facklam *et al.*, 2002). Nine of these strains, which were isolated from ground beef purchased from retail in Oregon in 2000, formed a phenotypically similar group distinct from any of the previously described enterococci. Evaluation of these isolates through a polyphasic taxonomic approach as suggested by the International Committee for the Systematics of Prokaryotes (Stackebrandt *et al.*, 2002) and comparison with reference strains of vagococci and enterococci revealed that these strains represent a new species in the genus *Vagococcus*, for which the name *Vagococcus carniphilus* is proposed.

Published online ahead of print on 27 February 2004 as DOI 10.1099/ij.s.0.02908-0.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Vagococcus carniphilus* 1843-02^T (= SS-1714^T = ATCC BAA-640^T = CCUG 46823^T) is AY179329.

PFGE profiles for *Vagococcus carniphilus* and phenotypic characteristics for distinguishing all currently described *Enterococcus* and *Vagococcus* species that initially cluster into Group IV are provided as supplementary material in IJSEM Online.

The strains were characterized phenotypically using conventional biochemical tests as described previously (Facklam & Elliott, 1995) and the Rapid ID 32 Strep (bioMérieux) (Freney *et al.*, 1992). The AccuProbe *Enterococcus* Identification Test was performed following the manufacturer's instructions. The Rapid ID 32 Strep system was used according to the manufacturer's instructions and the generated profile number was faxed to the manufacturer to obtain their reported identification. The profiles generated for the type strains of the four species of *Vagococcus* described to date and for all of the *V. carniphilus* isolates were identified as having an 'Unacceptable Profile'. This would be the correct response, since *Vagococcus* species are not in their database. The phenotypic characteristics for *V. carniphilus* are described in the species description.

The phenotypic identification scheme used initially subdivides the enterococci and vagococci species into groups based on the combinations of results obtained for three key tests: acid production from mannitol and sorbose, and hydrolysis of arginine (Facklam & Sahm, 1995; Facklam *et al.*, 2002). *V. salmoninarum*, *V. fessus* and *V. carniphilus* are placed in Group IV, since they are negative for all three key tests. The acidification of mannitol readily distinguishes *V. lutrae* and *V. fluvialis* from *V. carniphilus* and the other members of Group IV. Given that these isolates were originally misidentified as '*Enterococcus* species', the phenotypic characteristics for distinguishing all currently described *Enterococcus* and *Vagococcus* species that initially cluster into Group IV are provided as supplementary material in IJSEM Online. The key tests for distinguishing Group IV organisms are as follows. The acidification of trehalose and methyl α -D-glucopyranoside by *V. carniphilus* readily distinguishes it from *V. fessus*. *V. salmoninarum* is distinguished from other Group IV species due to its inability to grow at 37 °C and inability to grow in broth containing 6.5 % NaCl. The remaining species in this group are enterococci (*Enterococcus asini*, *Enterococcus cecorum*, *Enterococcus phoeniculicola* and *Enterococcus sulfureus*). They are all positive for lactose acidification, whereas *Vagococcus* species are negative for this test. Most of the *V. carniphilus* isolates were motile, except for two cultures (1846-02 and 2261-02) that were negative upon repeat testing. Similar variability in motility has been shown in *Enterococcus gallinarum* and *Enterococcus casseliflavus* (Carvalho *et al.*, 1998).

For DNA–DNA hybridization studies, all strains were grown in 2 litre flasks containing 1 litre Todd–Hewitt broth for 24 h at 35 °C (except *V. salmoninarum* which required incubation at 30 °C). Cells were harvested by centrifugation and lysed as described by Teixeira *et al.* (1995). DNA extraction, purification and reassociation using the hydroxyapatite method were performed as described previously (Brenner *et al.*, 1982). The DNA of the designated type strain (1843-02^T) was labelled with [³²P]dCTP by using a nick-translation reagent kit (Gibco-BRL Life Technologies). DNA reassociation experiments

were performed at 55 °C (optimal temperature) and 70 °C (stringent temperature). DNA from strain 1843-02^T was hybridized with the eight phenotypically similar isolates, the type strains of the four currently described *Vagococcus* species, and the type strains of the 25 currently described *Enterococcus* species (data not shown) at the optimal temperature. The relative binding ratio at the optimal temperature was 87 % or greater and the divergence was less than 1 % for the unidentified strains hybridized with the designated type strain. Based on these two criteria, these nine strains met the definition of species relatedness (Wayne *et al.*, 1987). In addition, when strain 1843-02^T was hybridized against the four *Vagococcus* species, DNA–DNA relatedness was 25 % to *V. fluvialis* and 9 % or less to the other three *Vagococcus* species. DNA–DNA relatedness of strain 1843-02^T hybridized against the 25 *Enterococcus* species showed that the relative binding ratio was 33 % or less at the optimal temperature. These results indicate that the nine isolates are clearly genetically distinct from these physiologically similar bacteria. DNA–DNA reassociation values can be considered only as standards for establishing bacterial species; they do not refer to the determination of proximity levels among species from different genera (Stackebrandt *et al.*, 2002; Wayne *et al.*, 1987).

The G + C content was determined by thermal denaturation (Mandel *et al.*, 1970). The G + C content for strain 1843-02^T was 34 mol%, which is close to that reported for *V. salmoninarum*, 36.0–36.5 % (Wallbanks *et al.*, 1990).

Genomic DNA isolated from the *Vagococcus* strains was prepared for PFGE based on the procedure of Elliott *et al.* (1998) with a few modifications. Bacteria were grown for 18–24 h at 35 °C on plates containing trypticase soy agar supplemented with 5 % defibrinated sheep blood. The bacteria were removed from the plate with a sterile loop and suspended in 0.5 ml of Tris/NaCl buffer (1.0 M NaCl in 10 mM Tris/HCl, pH 7.6). The chromosomal digests were separated by PFGE with a switch time of 0.2–25 s for 20 h. The PFGE profiles for the *V. carniphilus* isolates (available as supplementary material in IJSEM Online) demonstrated that five (1843-02^T, 1844-02, 1845-02, 1847-02 and 2214-02) of the nine had identical banding patterns and two (1846-02 and 2261-02) of the nine isolates had only one difference each and were probably variants of the same strain (Tenover *et al.*, 1995). Two isolates (2218-02 and 2219-02) showed five and four differences in banding pattern and most likely represent distinct strains. PFGE profile variation among the nine strains of the proposed new taxon fulfil the recommendation of the International Committee for the Systematics of Prokaryotes for basing a species description on more than a single strain (Stackebrandt *et al.*, 2002).

Extract preparation and whole-cell protein profile analysis using one-dimensional SDS-PAGE were performed as described previously (Merquior *et al.*, 1994). Dice indexes were determined for each isolate by using the Molecular Analyst Fingerprint Plus Software Package Version 1.6 (Bio-Rad)

and a dendrogram was constructed by using the UPGMA algorithm. The SDS-PAGE profiles of whole-cell protein extracts of *V. carniphilus* isolates and of strains belonging to the other species of *Vagococcus* and of physiologically related enterococcal species are shown in Fig. 1. Lanes 2–8 contain the *V. carniphilus* isolates and formed a tight cluster, indicating that they are the same species. These profiles were significantly different from the profiles of the other *Vagococcus* species and physiologically related enterococci.

Purified genomic DNA was amplified using the Expand High Fidelity PCR System (Roche), using primers FD1 and RD1 (Segonds *et al.*, 1999). Amplification conditions were 94 °C for 5 min, 35 cycles of 94 °C for 15 s, 50 °C for 15 s, 72 °C for 90 s, with a single final extension at 72 °C for 5 min followed by a 4 °C hold. Products were detected after electrophoresis on 1.2 % (w/v) agarose gels and staining with ethidium bromide. Excess dNTPs and primers were removed from products with the QIAquick 8 PCR Purification Kit (Qiagen). Cycle sequencing was performed using primers and standard protocols suggested by Sacchi *et al.* (2002). Excess dyes were removed with magnetic carboxylate beads (Agencourt Bioscience) and reaction products were sequenced on an ABI 3100 (Applied Biosystems). Sequences were assembled in GCG's Seqmerger (Wisconsin

Package, Version 10.2) and trimmed to a minimum of two bidirectional (both strands) confirming reads. Pairwise comparisons of related sequences were performed against each other and blasted against the GenBank sequence database. Related entries were aligned in the PILEUP program (Devereux *et al.*, 1984), trimmed to consensus, and further analysis was performed in BIOEDIT and TREECON. In BIOEDIT, sequences were realigned by the CLUSTAL W method (Thompson *et al.*, 1994) with 1000 bootstraps, and a distances matrix was created. In TREECON, distances of aligned sequences were estimated with the Jukes–Cantor scheme (Jukes & Cantor, 1969), bootstrapped 1000 times and the tree topology was determined by the neighbour-joining method. The final phylogenetic tree was rooted with an outgroup.

The almost-complete 16S rRNA gene sequence (1514 bp) of strain 1843-02^T (=ATCC BAA-640^T) was deposited in the GenBank/EMBL/DDBJ sequence database under the accession number AY179329. Previous studies have shown that the vagococci are phylogenetically most closely related to the enterococci and *Carnobacterium* (Collins *et al.*, 1989; Wallbanks *et al.*, 1990). A multiple sequence alignment analysis trimmed to consensus comparing 1324 bp of the 16S rRNA gene sequences stored in GenBank for the closest, and/or representative species for these genera were

Fig. 1. (a) SDS-PAGE profiles of whole-cell protein extracts of *Vagococcus carniphilus* strains and of strains belonging to other species of *Vagococcus* and physiologically related enterococcal species. Lanes: 1 and 18, molecular mass markers (in kDa); 2, *V. carniphilus* 1843-02^T; 3, *V. carniphilus* 1844-02; 4, *V. carniphilus* 1846-02; 5, *V. carniphilus* 1847-02; 6, *V. carniphilus* 2218-02; 7, *V. carniphilus* 2219-02; 8, *V. carniphilus* 2261-02; 9, *V. fessus* ATCC BAA-289^T; 10, *V. fluvialis* NCDO 2497^T; 11, *V. lutrae* CCUG 39187^T; 12, *V. salmoninarum* NCFB 2777^T; 13, *E. asini* ATCC 700915^T; 14, *E. cecorum* ATCC 43198^T; 15, *Enterococcus columbae* NCIMB 13013^T; 16, *E. sulfureus* NCDO 2370^T; 17, *Enterococcus faecalis* LMG 7937^T. (b) Dendrogram resulting from the computer-assisted analysis of the protein profiles in (a). Scale represents mean percentages of similarity.

used to construct the phylogenetic tree with *Lactococcus lactis* as the outgroup (Fig. 2). PILEUP alignment of the sequences from strain 1843-02^T and from the type strains of *V. lutrae* and *V. fluvialis* with ends trimmed and Ns removed resulted in an alignment of 1407 bp. Sequence 1843-02^T was 96 % similar to that of *V. lutrae*, and 99 % similar to the sequence of *V. fluvialis*, suggesting that *V. carniphilus* lies between these two species with a closer relationship to *V. fluvialis*. The CLUSTAL W realigned matrix also showed that 1843-02^T was approximately 94 % similar to the closest enterococci and 92 % to the closest *Carnobacterium* species.

It is not known if the new bacterial species identified in the present study plays a role in the pathogenesis of infections or in the transmission of antimicrobial resistance and food contamination, as it has been described for the enterococci (Donabedian *et al.*, 2003). Although specific clinical manifestations in humans for *V. fluvialis* have yet to be confirmed, it has been identified from the following human clinical sources: peritoneal fluid, bite wounds, blood and cerebrospinal fluid (Teixeira *et al.*, 1997). Therefore, it is

possible that *V. carniphilus* could be associated with opportunistic infections in man or animals.

Description of *Vagococcus carniphilus* sp. nov.

Vagococcus carniphilus (car.ni'phi.lus. L. n. *caro* meat, gen. *carnis* of meat; Gr. adj. *philos* friendly, loving; N.L. adj. *carniphilus* meat-loving).

Cells are Gram-positive cocci and form single cells, pairs, or short chains. Catalase-negative, does not produce haemolysis or pigment when grown on tryptic soy agar supplemented with 5 % defibrinated sheep blood, and susceptible to vancomycin. Strains are positive for pyrrolidonyl arylamidase activity, leucine aminopeptidase activity, hydrolysis of aesculin in the presence of bile, growth in broth containing 6.5 % NaCl, pyruvate utilization and growth at 10 °C. Growth at 45 °C is variable. The majority of strains are motile. Gas is not produced in *Lactobacillus* de Man–Rogosa–Sharpe broth. Arginine, hippurate and urea are not hydrolysed. Acetoin is not produced and tellurite (0.4 %) is

Fig. 2. Phylogenetic tree, based on 16S rRNA gene sequences, showing relationships among *Vagococcus carniphilus* and other species of *Vagococcus*, and other related genera (namely, *Enterococcus* and *Carnobacterium*). *Lactococcus lactis* was used as the outgroup, and bootstraps values at the nodes are displayed as percentages.

not tolerated. Strains produce acid from glycerol, maltose, ribose, trehalose and methyl α -D-glucopyranoside. Acid from sucrose is variable. Acid is not produced from arabinose, inulin, lactose, mannitol, melibiose, raffinose, sorbitol or sorbose. Strains are positive with the AccuProbe *Enterococcus* genetic probe. Using the Rapid ID 32 Strep system, acid is produced from ribose, trehalose, cyclodextrin, maltose and methyl β -D-glucopyranoside, and pyroglutamic acid arylamidase and glyl-tryptophan arylamidase are produced. Arginine dihydrolase, β -glucosidase, β -galactosidase (both substrates), β -glucuronidase, α -galactosidase, alkaline phosphatase, ala-phenylalanine-proline-arylamidase, *N*-acetyl- β -glucosaminidase, β -mannosidase and urease are not produced. Hippurate is not hydrolysed. Acetoin is not produced. Acid is not produced from mannitol, sorbitol, lactose, raffinose, saccharose/sucrose, L-arabinose, D-arabitol, glycogen, pullulan, melibiose, melezitose or tagatose. The natural habitat is unknown. Distinguished by whole-cell protein profiling and 16S rRNA gene sequencing.

The type strain is 1843-02^T (= ATCC BAA-640^T = CCUG 46823^T); its G + C content is 34.0 mol%.

Acknowledgements

We are grateful to H. G. Trüper for assisting with the species epithet. M. G. S. C. was supported by a 'Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq' post-doctoral fellowship. This study was supported in part by CNPq and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Brazil.

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