Vibrio casei sp. nov., isolated from the surfaces of two French red smear soft cheeses

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Three Gram-negative, rod-shaped, catalase- and oxidase-positive, facultatively anaerobic and motile bacteria, strains WS 4538, WS 4539^T and WS 4540, were isolated from the surfaces of two fully ripened French red smear soft cheeses. Based on 16S rRNA gene sequence similarity, all three strains were shown to belong to the genus *Vibrio*. They are most closely related to *Vibrio rumoiensis* S-1^T (96.3% similarity) and *Vibrio littoralis* MANO22D^T (95.9%). DNA–DNA hybridization confirmed that all three isolates belong to the same species and clearly separated strain WS 4539^T from *V. rumoiensis* DSM 19141^T (38–42% relatedness) and *V. littoralis* DSM 17657^T (28–37%). In contrast to their nearest relatives, the strains exhibited β -galactosidase and aesculin hydrolase activities. A 14 bp insertion in the 16S rRNA gene sequence forms an elongated structure at helix 10 in the rRNA molecule and provides a tool for PCR-based identification of the novel species. Partial sequences of the housekeeping genes *atpA*, *recA*, *rpoA* and *pyrH* supported the conclusion that the three isolates constitute a separate species within the genus *Vibrio*. The name *Vibrio casei* sp. nov. is proposed for the novel taxon. Strain WS 4539^T (=DSM 22364^T =LMG 25240^T; DNA G+C content 41.8 mol%) is the type strain and WS 4540 (=DSM 22378 =LMG 25241) is a reference strain.

The family *Vibrionaceae* (Baumann & Schubert, 1984) belongs to the class *Gammaproteobacteria* and includes, amongst others, the genera *Photobacterium* (Baumann & Baumann, 1984), *Salinivibrio* (Mellado *et al.*, 1996) and *Vibrio*. At the time of writing, the genus *Vibrio* consists of 72 recognized species that form nine phylogenetically distinct clades (Association of *Vibrio* Biologists; <http://www.vibriobiology.net/>). The clearly separated *rumoiensis* clade consists of *Vibrio rumoiensis* (Yumoto *et al.*, 1999) and *Vibrio littoralis* (Nam *et al.*, 2007) and forms a distant lineage within the genus. Micro-organisms belonging to the genus *Vibrio* are known to live either freely or associated as symbionts with aquatic animals in marine or estuarine waters (Browne-Silva & Nishiguchi, 2008) or as parasites of fish, crustaceans and molluscs (Thompson *et al.*, 2004). *Vibrio* species have been reported less frequently as members of microbial consortia on cheese

surfaces (Feurer *et al.*, 2004), but some studies have suggested that they play a role in the ripening process (El-Baradei *et al.*, 2007).

The composition of the surface microbial consortia of two French smear-ripened cheeses, Petit Munster Ermitage and Reflets de France Petit Livarot, was analysed using Fourier-transform infrared (FT-IR) spectroscopy (Kümmerle *et al.*, 1998; Naumann *et al.*, 1991). After 5 days of aerobic incubation at 10–30 °C, isolates were picked randomly from fully grown plate count agar plates, supplemented with 3% NaCl (PC3+). The cell densities of the surface consortia were 6×10^9 c.f.u. ml⁻¹ (Munster) and 4×10^{10} c.f.u. ml⁻¹ (Livarot). Strains WS 4538 and WS 4539^T were isolated from the Munster cheese at the Fromagerie de l'Ermitage, Bulgnéville, France (48° 12' 16" N 5° 49' 17" E), and strain WS 4540 was isolated from the Livarot cheese at Les Fromages de Tradition, Mondeville, France (49° 10' 31" N 0° 19' 22" W). The novel species represented by the isolated strains was estimated to contribute approximately 10^8 c.f.u. ml⁻¹ to the surface consortia of the two cheeses.

Unless otherwise stated, cells were cultured in marine broth (MB; Difco) at 30 °C with shaking at 180 r.p.m. All experiments were conducted at least twice. Tolerance towards temperature (2, 4, 10, 14, 30, 37 and 43 °C) was recorded for 5 days. Growth occurred between 2 and 30 °C. Tolerance towards pH as well as requirement for

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene, *atpA*, *recA*, *rpoA* and *pyrH* gene sequences of strains WS 4539^T and WS 4540 and *V. littoralis* DSM 17657^T are FJ968710–FJ968723.

DNA–DNA hybridization results, detailed methods for phylogenetic analysis of housekeeping genes and the resulting trees and details of members of the *Vibrionaceae* showing a similar 16S rRNA gene insert to the novel strains and models of 16S rRNA secondary structure with and without the insert are available as supplementary material with the online version of this paper.

and tolerance of sodium ions were tested in 2 % (w/v) peptone broth [15 g peptone from casein (Oxoid) and 5 g soy peptone (BD) l⁻¹] adjusted to pH 3–11 or supplemented with NaCl (2, 4, 6, 8, 10, 12 and 14 %, w/v). To compensate for effects due to osmolarity in the absence of NaCl, the basal medium according to Baumann & Baumann (1981) was used for inoculation, where Na⁺ is replaced by K⁺. Growth was observed between pH 6 and 8.5 and at 2–10 % NaCl.

The Gram reaction was determined by addition of 3 % KOH to a colony grown for 1 day on marine agar (MA; Difco) and by Gram staining. Catalase and oxidase activities were documented by gas production in a 3 % hydrogen peroxide solution and by using Bactident Oxidase test strips (Merck), respectively. Sensitivity towards the vibriostatic agent O/129 was determined using Oxoid discs (150 mg per disc). Motility was tested on freshly poured MA plates dried for 1 h; swarming of the cells was checked after incubation at 30 °C overnight. The ability to grow under anaerobic conditions was determined over a 3-day period in an anaerobic jar containing the catalyst Anaerocult IS (Merck), prepared according to the manufacturer's instructions.

API 20NE and API ZYM test strips (bioMérieux) were used to analyse substrate utilization from sole carbon sources, acid production from carbohydrates and enzyme activities. Since misidentification using API systems has been reported when resuspending marine organisms in the solutions provided by the manufacturer (Martinez-Urtaza *et al.*, 2006), the following modifications were introduced to ensure optimal growth conditions. NaCl was added to a final concentration of 1.5 % to the API 20NE suspension medium and the AUX solution. Cell suspensions with a standard opacity equivalent to McFarland 3 were used to inoculate the strips, which were incubated for 24 h at 30 °C prior to evaluation. For API ZYM, the incubation conditions were modified (6 h at 30 °C instead of 4 h at 37 °C).

Bacteria were grown for 24 h at 28 °C on MA to analyse cellular fatty acid composition. Fatty acids were extracted and analysed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) as described previously (Verborg *et al.*, 2008). Analysis of the fatty acid content showed the predominance of three major fatty acids (Table 1), and the profiles of the novel strains were similar but not identical to those of *V. rumoiensis* DSM 19141^T and *V. litoralis* DSM 17657^T. No significant differences were found between the three new isolates.

The G+C content of the genomic DNA was examined by the Identification Service of the DSMZ using HPLC as described by Mesbah & Whitman (1989). The values obtained were 41.8 mol% for WS 4539^T and 40.7 mol% for WS 4538 and WS 4540.

Table 2 summarizes the phenotypic differences between strain WS 4539^T and its two closest phylogenetic relatives.

Table 1. Major fatty acids of the three novel isolates and their closest phylogenetic relatives

Strains: 1, *V. casei* sp. nov. WS 4539^T; 2, *V. casei* sp. nov. WS 4538; 3, *V. casei* sp. nov. WS 4540; 4, *V. litoralis* DSM 17657^T; 5, *V. rumoiensis* DSM 19141^T. Values are percentages of total fatty acids and were obtained in this study.

Fatty acid	1	2	3	4	5
16:1 ω 7c and/or iso-15:0 2-OH	41.0	41.3	40.8	43.4	40.3
16:0	22.0	21.2	20.4	23.9	23.2
18:1 ω 7c	15.6	15.9	15.4	12.0	14.0
14:0 3-OH and/or iso-16:1 I	3.2	3.3	3.2	7.7	7.0
14:0	1.5	1.8	1.7	1.8	4.0
12:0	3.8	3.8	3.6	4.0	3.8

DNA–DNA hybridization was performed between the strains isolated from smear cheeses and *V. litoralis* DSM 17657^T as well as *V. rumoiensis* DSM 19141^T. The studies were carried out by the Identification Service of the DSMZ based on renaturation curves. DNA was isolated using a French pressure cell (Thermo Spectronic) and purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). Hybridization studies were performed as described by De Ley *et al.* (1970) with the modifications introduced by Huß *et al.* (1983), using a model Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier temperature-controlled 6 × 6 multicell changer with an *in-situ* temperature probe (Varian) in 2 × SSC at 67 °C. Following the recommendations of Wayne *et al.* (1987), the threshold value of DNA–DNA relatedness is 70 % for the definition of a species. DNA–DNA hybridization showed that strains WS 4539^T, WS 4538 and WS 4540 belong to the same species (data not shown). This is also corroborated by the fact that we were unable to find physiological or biochemical differences between the three isolates. DNA pairing of isolate WS 4539^T with *V. litoralis* DSM 17657^T and *V. rumoiensis* DSM 19141^T revealed values for DNA–DNA reassociation

Table 2. Differential phenotypic characteristics of the three novel isolates and their phylogenetically closest relatives

Strains: 1, *V. casei* sp. nov. WS 4539^T; 2, *V. litoralis* DSM 17657^T; 3, *V. rumoiensis* DSM 19141^T. Data were obtained in this study.

Characteristic	1	2	3
Expression of:			
β -Galactosidase	+	–	–
Aesculin hydrolase	+	–	+
Growth in 12 % NaCl	–	+	–
Growth at 37 °C	–	+	+
Growth at pH 5	–	+	+
Fatty acid content (%)			
14:0	1.5	1.8	4.0
14:0 3-OH and/or iso-16:1 I	3.2	7.7	7.0

between 28 and 42 % (Supplementary Table S1, available in IJSEM Online). Therefore, the three strains isolated from the surface of two smear-ripened cheeses constitute a novel species within the genus *Vibrio*.

The phylogenetic position of the three isolates within the *Vibrionaceae* was determined by 16S rRNA, *atpA*, *recA*, *rpoA* and *pyrH* gene sequence analyses. Genomic DNA was extracted as described by Wenning *et al.* (2006). Cycle-sequencing PCR of the 16S rRNA gene was performed by Sequiserve (Vaterstetten, Germany) using the 5'-primer 609V (5'-GGATTAGATACCCBDGTA-3', corresponding to positions 785–802 in the *Escherichia coli* 16S rRNA gene) and the 3'-primer 907R (5'-CCGTCAATTCMTTGTG-AGTTT-3'; positions 907–926). A 1519 bp amplicon revealed the presence of 11 heterogenic positions scattered over the 16S rRNA gene, indicating the existence of at least two *rrn* copies. A sequence similarity of 96.3 % was found to the closest relative, *V. rumoiensis* S-1^T. The sequences were aligned using CLUSTAL_X version 2.09 (Larkin *et al.*, 2007). A rooted phylogenetic tree was calculated according to Kimura's two-parameter model using TREECON version 1.3b (Van de Peer & De Wachter, 1997) based on 16S rRNA gene sequences using the neighbour-joining algorithm (Fig. 1). A bootstrap analysis based on 1000 replicates was performed to test the stability of individual branches (Felsenstein, 1985). As suggested by Thompson *et al.* (2005), *Campylobacter jejuni* subsp. *jejuni* NCTC 11168^T was used as an outgroup. The isolates clustered with the clearly separated *V. rumoiensis* group. UPGMA and

maximum-likelihood calculations produced the same grouping of the novel isolates (not shown).

Primer sequences and thermal cycling conditions for amplification and sequencing of the *atpA*, *recA*, *rpoA* and *pyrH* genes were chosen according to Thompson *et al.* (2005, 2007). Phylogenetic trees based on these house-keeping genes supported the status of a novel species and are available in Supplementary Fig. S1.

All three isolates shared a 14 bp insertion in their 16S rRNA genes, located between positions 188 and 189 in the *E. coli* 16S rRNA gene sequence (Cannone *et al.*, 2002; GenBank accession no. J01695). The presence of this fragment in the mature rRNA could be demonstrated by PCR amplification of cDNA samples using the universal primer 27f and the insertion-specific primer 188r (for a detailed description, see supplementary material). This insertion results in an elongated structure at helix 10 (calculations carried out using the ARB software; Ludwig *et al.*, 2004) (Supplementary Fig. S2). The insertion further distinguishes the isolates from their closest phylogenetic neighbours and provides a useful tool for quick identification. Such an insertional fragment may move between species through horizontal gene transfer followed by recombination. Among the family *Vibrionaceae*, comprising 105 species, a 14 or 15 bp fragment was also detected in five other species, which are marked by asterisks in Fig. 1 (see also Supplementary Table S2). Since these species inhabit different environments, the opportunity for horizontal gene transfer must remain speculative.

Fig. 1. 16S rRNA gene sequence-based consensus neighbour-joining phylogenetic tree illustrating the position of strains WS 4539^T and WS 4540 within the *Vibrionaceae*. The sequence of *Campylobacter jejuni* subsp. *jejuni* NCTC 11168^T was chosen as an outgroup. Bootstrap values, expressed as percentages of 1000 replications, are given at branching points. Bar, 10% sequence divergence. Strains that contain a 14 or 15 bp insert in their 16S rRNA gene sequence are marked by asterisks (see text).

Based on these results, we propose the name *Vibrio casei* sp. nov. for the novel species. *Vibrio* sp. R-27449 (GenBank accession no. AJ967016) shares 100 % identity of its 16S rRNA gene sequence with *V. casei* sp. nov.; this strain was found by Mounier *et al.* (2005) on the surface of the Irish smear-ripened cheese Milleens. While this report adds emphasis to the incidence of *V. casei* sp. nov. on cheese surfaces, this isolate was not described as representing a novel species.

Description of *Vibrio casei* sp. nov.

Vibrio casei (ca'sei. l. gen. masc. n. *casei* of/from cheese, because the first strains were isolated from the surfaces of smear-ripened cheeses).

Cells are Gram-negative, straight rods, 0.6–1.2 µm wide and 1.5–1.8 µm long. Colonies grown aerobically on MA are cream, smooth, round or slightly irregular in shape and 1–2 mm in diameter after 3 days of culture at 30 °C. Growth occurs at 2–30 °C and pH 6–8.5. Isolates require sodium ions and grow in the presence of 2–10 % NaCl. Motile but not swarming. Bioluminescence is not observed. Susceptible to the vibriostatic agent O/129. Catalase- and oxidase-positive. Facultatively anaerobic. Nitrate is reduced to nitrite. The following substrates can be utilized as sole energy and carbon sources: glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose, gluconate, malate and citrate. β-Galactosidase, aesculin hydrolase, alkaline phosphatase, acid phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase activities are present. The predominant fatty acids are 16:1ω7c and/or iso-15:0 2-OH, 18:1ω7c and 16:0. The DNA G+C content of the type strain is 41.8 mol%.

The type strain, WS 4539^T (=DSM 22364^T =LMG 25240^T) was isolated from the surface of Petit Munster cheese from the Fromagerie de l'Ermitage, Bulgnéville, France. The reference strain WS 4540 (=DSM 22378 =LMG 25241) was isolated from the surface of Petit Livarot cheese at Les Fromages de Tradition, Mondeville, France.

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