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International Journal of Systematic and Evolutionary Microbiology
September 2010, Volume 60, Pages 2159-2164
http://dx.doi.org/10.1099/ijs.0.012872-0
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Vagococcus penaei sp. nov., isolated from spoilage microbiota of cooked shrimp (*Penaeus vannamei*)

Emmanuel Jaffrès[1, 2], Hervé Prévost[1], Albert Rossero[1], Jean-Jacques Joffraud[2] and Xavier Dousset[1, *]

[1] UMR INRA 1014 SECALIM ENITIAA, rue de la Géraudière, BP 82225, 44322 Nantes Cedex 3, France
[2] Ifremer, Laboratoire Science et Technologie de la Biomasse Marine, BP 21105, 44311 Nantes Cedex 3, France

*: Corresponding author : X. Dousset, email address : dousset@enitiaa-nantes.fr

Abstract:

A polyphasic taxonomic study, using phenotypic, phylogenetic and genotypic characterization, was performed on five Gram-stain-positive, catalase-negative, coccus-shaped *Vagococcus*-like bacteria isolated from the spoilage microbiota of cooked shrimp. Comparative 16S rRNA gene sequence analysis indicated that the isolates belonged to the genus *Vagococcus*. The five isolates shared 100% 16S rRNA gene sequence similarity, and representative strain CD276^T formed a branch that was distinct from the type strains of the six recognized species of the genus *Vagococcus* (*Vagococcus fluvialis* CCUG 32704^T, *V. salmoninarum* NCFB 2777^T, *V. lutrae* CCUG 39187^T, *V. fessus* M2661/98/1^T, *V. carniphilus* ATCC BAA-340^T and *V. elongatus* PPC9^T). The taxonomic position of strain CD276^T was clarified using DNA–DNA hybridization, pulsed-field gel electrophoresis of whole-genome DNA, G+C content determination, cell-wall peptidoglycan typing, fatty acid analysis and biochemical characterization. On the basis of this evidence, a novel species, *Vagococcus penaei* sp. nov., is proposed. The type strain is CD276^T (=LMG 24833^T =CIP 109914^T).

Abbreviations: PFGE, pulsed-field gel electrophoresis

39 The bacterial genus *Vagococcus* was proposed twenty years ago by (Collins *et al.*, 1989) in
40 order to improve the taxonomy of Gram-positive, catalase-negative, motile, coccus-shaped
41 bacteria that were close to the *Lactococci* in terms of reacting with Lancefield group N
42 antisera, but phylogenetically distant. Phylogenetic studies have shown that the closest
43 relatives of *Vagococcus* are the genera *Enterococcus* and *Carnobacterium* (Wallbanks *et al*.,
44 1990). To date, the *Vagococcus* genus consists of six species. The first described was *V.*
45 *fluvialis*, isolated from chicken faeces and sea water (Hashimoto *et al.*, 1974) and from
46 various internal organs of domestic animals such as pigs, cattle, cats and horses (Pot *et al.*,
47 1994). *V. fluvialis* strains have also been isolated from human clinical samples such as blood,
48 peritoneal fluid and wounds (Teixeira *et al.*, 1997) and, more recently, from a root-filled tooth
49 with periradicular lesions (Al-Ahmad *et al.*, 2008). *V. salmoninarum* was recognized as the
50 second species of the genus by Wallbanks *et al.* (1990). It was isolated from diseased fish
51 such as Atlantic salmon, rainbow and brown trout (Michel *et al.*, 2007, Ruiz-Zarzuela *et al.*,
52 2005, Schmidtke & Carson, 1994, Wallbanks *et al.*, 1990). Two further species were
53 subsequently found in several marine mammals and allotted to the genus. These were *V.*
54 *lutrae* (Lawson *et al*., 1999) and *V. fessus* (Hoyles *et al.*, 2000) isolated from the common
55 otter (*Lutra lutra*) and from a seal and harbour porpoise, respectively. In the last few years,
56 two more species have been isolated; *V. carniphilus* from ground beef (Shewmaker *et al.*,
57 2004) and *V. elongatus* from a swine manure storage pit (Lawson *et al.*, 2007). Recently, we
58 have isolated five strains of *Vagococcus*-like bacteria from the microbiota of spoiled cooked
59 shrimps (Jaffres *et al.*, 2009). In the present work, we describe the polyphasic taxonomic
60 study of one representative strain, CD276^T, from the group of five. The phenotypic and
61 phylogenetic evidence is presented to support the description of a novel species of the
62 *Vagococcus* genus, for which the name *Vagococcus penaei* sp. nov. is proposed.
63

64 The five strains, named CD276, CD279, CD285, CD310 and CD380, were collected from a
65 batch of cooked, peeled, brined, drained shrimps (*Penaeus vannamei*, harvested in the Pacific
66 Ocean) and packaged in modified atmosphere, which were considered as spoiled by a trained
67 panel, experienced in the sensory evaluation of seafood. All details of the sensory and
68 microbiological monitoring, strain collection and identification can be found in the previous
69 study (Jaffres *et al.*, 2009).
70
71 The phylogenetic position of the five strains was determined by complete 16S rRNA gene
72 sequence analysis. Genomic DNA extraction and purification were carried out using the
73 Qiagen DNeasy blood and tissue kit (Qiagen, S.A., Courtaboeuf, France). The 16S rRNA
74 gene amplification, purification and sequencing were performed as described previously
75 (Valcheva *et al.*, 2006). A sequence analysis using the BioEdit sequence alignment software
76 (Hall, 1999), revealed that the five strains presented a 100% sequence similarity in their 16S-
77 rRNA gene. The complete 16S rRNA gene sequence (1550 bp) of the strain CD276^T was
78 deposited in the GenBank/EMBL/DDBJ database, under the accession number FJ360897. The
79 CD276^T 16S rRNA gene sequence was combined with those of the most closely related
80 species of *Vagococcus* and *Enterococcus*, from the GenBank (http://www.ncbi.nlm.nih.gov/)
81 and from the ribosomal database project (RDP, USA, http://rdp.cme.msu.edu/) (Cole *et al.*,
82 2003). These sequences were aligned with CLUSTAL in MEGA software, V4.0., and a
83 neighbour-joining phylogenetic tree was created (Tamura *et al.*, 2007) (Fig 1). The topology
84 analysis of the tree showed the phylogenetic clustering of *Vagococcus* and *Enterococcus*
85 sequences into two distinct monophyletic groups. The close association of the strain CD276^T
86 with members of the genus *Vagococcus* was confirmed, and the clustering together occurred
87 with a bootstrap value of 99%. Strain CD276^T formed with the four others strains CD279,
88 CD285, CD310 and CD380, a distinct branch based on this comparative 16S rRNA gene

89 sequence analysis with a common branching node (bootstrap value of 99%) with *V. carniphilus* and *V. fluvialis*, which presented 97.5% and 97.3% sequence similarity, respectively, after a realigned matrix by CLUSTAL W. For the other *Vagococcus* type strains, the realigned matrix showed 96.2, 95.6, 95.1 and 94.2% sequence similarity with *V. lutrae*, *V. elongatus*, *V. salmoninarum* and *V. fessus*, respectively. These phylogenetic results indicated that the group of strains CD276^T, CD279, CD285, CD310 and CD380 was phylogenetically distinct from the six *Vagococcus* species already recognized.

96

97 The designated type strain CD276^T was characterized phenotypically using conventional biochemical tests. All phenotypical tests were performed at 30°C except for temperature growth tests at 10 and 45°C. Strain CD276^T was analyzed for colony appearance and, by using phase contrast microscopy, for cell morphology and motility. Motility was also examined in mannitol nitrate mobility medium (MNM, BioRad) inoculated by stabbing the centre of the tube. Growth was studied in BHI broth with 0.1% glucose and bromcresol purple indicator, incubated at various temperatures (10 and 45°C) or supplemented with NaCl (6.5%) to determine the resistance to NaCl. Haemolysis was assessed in Columbia Agar supplemented with 5% defibrinated horse blood (BioRad, Marne-la-Coquette, France) with 5-10% CO_2. Gas production from glucose was examined in Man Rogosa Sharp medium (MRS, Biokar Diagnostic) using Durham tubes and sealed by melted petroleum jelly. The cells strain CD276^T recovered from the spoilage microbiota of tropical cooked shrimp (*Penaeus vannamei*) consisted of Gram-positive, catalase-negative, coccus-shaped bacteria (0.5 to 1µm in diameter), non-motile by microscopic observation and MNM medium, occurring singly, in pairs or in a short chain, elongated in the direction of the chain. It produced small, smooth and white colonies (0.5 to 1 mm in diameter) on BHI agar at 30°C. Facultatively anaerobic, it produced lactic acid but not gas from glucose. The sugar fermentation patterns and enzymatic

114 activities were characterized for the type strain CD276^{T} and also for the strains CD279,
115 CD285, CD310 and CD380, by using the API Rapid ID32S and API ZYM systems,
116 respectively, according to the manufacturer's instructions (API-BioMerieux, Craponne,
117 France). All tests for biochemical characterization were carried out at least in duplicate.
118 Within the five strains group, the sugar fermentation patterns and enzymatic activities showed
119 a variability between strains. Indeed, CD279, CD285 and CD380 exhibited an identical
120 profile but by contrast six and eight reactions were different regarding respectively the strain
121 CD76^{T} (α cyclodextrin, α-chymotrypsin, α-glucosidase, Arginine dihydrolase, Glycyl-
122 tryptophan arylamidase, Leucine arylamidase) and CD310 (D-raffinose, D-sorbitol, methyl-
123 ßD-glucopyranoside, α-cyclodextrin, ß-glucosidase, ß-mannosidase, Arginine dihydrolase,
124 Glycyl-tryptophan arylamidase). Type Strain CD276^{T} was the second most reactive compared
125 to the *Vagococcus* species already known with 15 positive reactions and 1 weak positive
126 reactions, behind *V. lutrae* with 18 positive reactions and 5 weak positive reactions. However,
127 four reactions were positive for CD276^{T} exclusively and not for any other *Vagococcus*
128 species. These were the acidification of D-melezitose and D-raffinose, the enzymatic activity
129 for arginine dihydrolase and acetoin production (Voges Proskauer). Furthermore, acid was
130 produced from several other carbohydrates, such as D-maltose, D-ribose, D-saccharose, D-
131 trehalose and α cyclodextrin, and activity was displayed for acid phosphatase, glycyl-
132 tryptophan arylamidase, naphthol-AS-BI-phosphohydrolase, and pyroglutamic acid
133 arylamidase as well as a weak activity for ß-glucosidase, esterase (C4) and esterase lipase
134 (C8). The detailed biochemical characteristics of the strain CD276^{T} are presented in the
135 description of this novel species and in Table 1.
136
137 An analysis of the peptidoglycan structure of the strain CD276^{T} was carried out as described
138 by Schleifer (1985) and Schleifer & Kandler (1972) with the modification that TLC on

139 cellulose was applied instead of paper chromatography. Strain CD276^T possesses cell-wall
140 peptidoglycan of type A4alpha L-Lys-D-Asp (type A11.31 according to the DSMZ-Catalogue
141 of strains, seventh edition, 2001).
142
143 For the quantitative analysis of cellular fatty acids, strain CD276^T was grown for 24h on the
144 medium described by Fischer & Arneth-Seifert (1998). Cells were harvested and cellular fatty
145 acids were saponified, methylated and extracted as described by the Sherlock Microbial
146 Identification System (MIDI, 1999). Fatty acids were analyzed by GC (Hewlett Packard
147 6890) and identified using the Microbial Identification software package (Sasser, 1990). The
148 predominant cellular fatty acids of strain CD276^T were cis-9-octadecenoic acid (18:1 ω9c),
149 hexadecanoic acid (16:0) and tetradecanoic acid (14:0). The complete fatty acid composition
150 of strain CD276^T is shown in Table 2.
151
152 The analysis of chromosomal DNA restriction patterns by pulsed-field gel electrophoresis
153 (PFGE) was carried out as described by Teixeira *et al.* (1997). Genomic DNA digested by
154 *smaI* in agarose plugs was electrophoresed on a 1.3% agarose gel in TBE using a CHEF-DR
155 III system (Bio-Rad). The band patterns were visualized and photographed under UV light.
156 The PFGE profiles for the *V. penaei* isolates (Fig. 2) displayed two different banding patterns:
157 one for CD276^T and CD279 and the other for CD285, CD310 and CD380. The latter pattern
158 differed from that of CD276^T and CD279 in eight positions, thus both groups most probably
159 represent distinct strains of the *V. penaei* species. The variation in PFGE profiles among the
160 five strains of the proposed new taxon fulfils the recommendation of the International
161 Committee for the Systematics of Prokaryotes to base a species description on more than a
162 single strain (Stackebrandt *et al.*, 2002).
163

164 For the determination of G+C content, genomic DNA was prepared according to Gevers *et al*.
165 (2001). The DNA G+C contents were determined in triplicate by using high performance
166 liquid chromatography (Mesbah *et al.*, 1989). The DNA G+C content of the strain CD276^T
167 was 35.4 mol%. This value is within the range (33.6-44.5 mol%) obtained by compiling the
168 G+C content of the six other recognized species of *Vagococcus*.
169 DNA-DNA hybridizations were carried out by the BCCM/LMG Bacteria Collection
170 (University of Gent, Belgium) with a genomic DNA prepared according to a modification of
171 the procedure of Gevers *et al.* (2001). They were performed at 35°C using the fluorometric
172 method in duplicate, according to a modification of the method described by Ezaki *et al.*
173 (1989). The two most closely related strains, based on the previous 16S rRNA sequence
174 analysis (*V. fluvialis* and *V. carniphilus*), were included in the tests. Table 3 shows the values
175 of DNA-DNA relatedness obtained. The DNA homology percentages reported are the means
176 of 2 hybridizations. Thus, DNA-DNA hybridization experiments confirmed the grouping
177 found with the phylogenetic data, with DNA-DNA reassociation values of 14 and 18% with
178 *V. carniphilus* and *V. fluvialis*, respectively. These values are below the threshold of 70%
179 suggested for species delineation (Stackebrandt & Goebel, 1994, Wayne *et al.*, 1987),
180 indicating that the strain CD276^T represents a separate genomic species.
181

182 **Description of *Vagococcus penaei* sp. nov.**

183 *Vagococcus penaei* (pe.na'e.i. N.L. gen. n. *penaei*, of *Penaeus*, pertaining to the spoilage
184 microbiota of cooked shrimp *Penaeus vannamei*, from which the first described strain of this
185 species was isolated).
186 Cells are Gram-positive, coccus-shaped, 0.5 to 1 μm in diameter, occurring singly, in pairs or
187 in a short chain, elongated in the direction of the chain. Cells are non-motile and non-spore
188 forming, facultatively anaerobic and catalase and oxidase-negative. Small, smooth and white

189 colonies, up to 1 mm in diameter, are formed by cells when grown on BHI agar at 30°C.
190 When grown on Columbia agar supplemented with 5% defibrinated horse blood, cells do not
191 produce haemolysis or pigment. Lactic acid but not gas is produced from glucose
192 fermentation. The strain is able to grow at 10°C but no growth is detected at 45°C, nor in
193 broth containing 6.5% NaCl. Using API systems, acid is produced from D-maltose, D-
194 melezitose, D-raffinose, D-ribose, D-saccharose, D-trehalose and α cyclodextrin. Acid is not
195 produced from L-arabinose, D-arabitol, D-lactose, D-mannitol, D-melibiose, D-sorbitol, D-
196 tagatose, glycogen, methyl-ßD-glucopyranoside and pullulan. Acid phosphatase, arginine
197 dihydrolase, esterase (C4), esterase lipase (C8), glycyl-tryptophan arylamidase, naphthol-AS-
198 BI-phosphohydrolase and pyroglutamic acid arylamidase activity is detected. No activity is
199 detected for α-chymotrypsin, α-glucosidase, α-fucosidase, α-galactosidase, α-mannosidase, ß-
200 galactosidase, β-glucosidase, ß-glucuronidase, ß-mannosidase, alanyl-phenylalanyl-proline-
201 arylamidase, alkaline phosphatase, cystine arylamidase, leucine arylamidase, lipase (C14), N-
202 acetyl-ß-glucosaminidase, trypsin, urease and valine arylamidase. Hippurate is not
203 hydrolysed. Nitrate is not reduced. Acetoin is produced (Voges-Proskauer). Cell-wall
204 peptidoglycan is of type A4alpha L-Lys-D-Asp, and the predominant cellular fatty acids were
205 cis-9-octadecenoic acid, hexadecanoic acid and tetradecanoic acid. The DNA G+C content is
206 35.4 mol%. The type strain is CD276^T (= LMG 24833^T = CIP 109914^T), which was isolated
207 from the spoilage microbiota of cooked shrimp *Penaeus vannamei*.
208

209 **Acknowledgements**
210 This study was supported by the regions Bretagne et Pays de La Loire. We thank Albert
211 Rossero for the PFGE analysis and also Florian Torselli for the molecular characterization of
212 *Vagococcus* strains. We are grateful to Jean-Paul Euzéby for consultation regarding the
213 species epithet. Emmanuel Jaffrès received a Ph.D grant awarded by Ifremer and ENITIAA.

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339

340 **Table 1.** Phenotypic characteristics of *Vagococcus penaei* sp. nov. strain CD276^{T} and closely
341 related *Vagococcus* species.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Acid from:											
D-maltose	+	+	+	+	+	+	-	+	+	-	-
D-melezitose	+	+	+	+	+	-	-	-	-	-	-
D-raffinose	+	+	+	+	-	-	-	-	-	-	-
D-ribose	+	+	+	+	+	+	-	+	+	-	-
D-saccharose	+	+	+	+	+	+	-	-	+	-	-
D-sorbitol	-	-	-	-	+	$+^{w}$	-	-	+	-	-
D-trehalose	+	+	+	+	+	+	+	+	+	-	-
methyl-ßD-glucopyranoside	-	-	-	-	+	-	-	+	+	-	-
α cyclodextrin	+	-	-	-	+	+	-	+	+	-	-
Enzymatic activity :											
α-chymotrypsin	-	+	+	+	+	$+^{w}$	+	-	+	+	-
α-glucosidase	-	+	+	+	+	$+^{w}$	-	-	+	-	-
α-galactosidase	-	-	-	-	-	-	-	-	+	-	+
ß-glucosidase	-	-	-	-	+	+	$+^{w}$	-	+	-	$+^{w}$
ß-mannosidase	-	-	-	-	+	$+^{w}$	-	-	+	-	-
Acid phosphatase	+	+	+	+	+	+	+	-	+	-	-
Alkaline phosphatase	-	-	-	-	-	-	$+^{w}$	$+^{w}$	$+^{w}$	-	$+^{w}$
Arginine dihydrolase	+	-	-	-	+	-	-	-	-	-	-
Esterase (C4)	+	+	+	+	+	$+^{w}$	$+^{w}$	-	$+^{w}$	$+^{w}$	$+^{w}$
Esterase lipase (C8)	+	+	+	+	+	+	$+^{w}$	-	+	$+^{w}$	$+^{w}$
Glycyl-tryptophan arylamidase	+	-	-	-	+	+	+	+	+	$+^{w}$	+
Leucine arylamidase	-	+	+	+	+	-	+	+	+	+	-
Naphthol-AS-BI-phosphohydrolase	+	+	+	+	+	+	-	-	$+^{w}$	$+^{w}$	-
Pyroglutamic acid arylamidase	+	+	+	+	+	$+^{w}$	+	+	+	+	-
Acetoin production (Voges Proskauer)	+	+	+	+	+	-	-	-	-	-	-

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343 +, positive reaction; -, negative reaction; $+^{w}$, weak positive reaction. Strains: 1, *Vagococcus*
344 *penaei* sp. nov. strain CD276^{T} ; 2, 3, 4, 5, respectively strain CD279, CD285, CD380,
345 CD310 ; 6, *V. fluvialis* CCUG 32704^{T} ; 7, *V. salmoninarum* CCUG 33394^{T} ; 8, *V. carniphilus*
346 CCUG 46823^{T} ; 9, *V. lutrae* CCUG 39187^{T} ; 10, *V. fessus* CCUG 41755^{T} ; 11, *V. elongatus*
347 CCUG 51432^{T}. Data for reference strains were taken from the CCUG database
348 (http://www.ccug.se).

349 **Table 2.** Fatty acid content (%) of type strain CD276^T

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Fatty acid	Systematic name	Shorthand Name	Abundance (mol%)
1	decanoic acid	10:0	2.30
2	dodecanoic acid	12:0	1.79
3	3-hydroxydodecanoic acid	12:0 3OH	0.64
4	tetradecanoic acid	14:0	9.80
5	2-hydroxy-tridecanoic acid	13:0 2OH	0.51
6	13-Methyl tetradecenoic acid isomer F	15:1 iso F	1.11
7	cis-9-Hexadecenoic acid	16:1 ω9c	8.45
8	hexadecanoic acid	16:0	16.37
9	15-methyl cis-9-hexadecenoic acid	iso 17:1 ω9c	1.06
10	15-methyl cis-5-hexadecenoic acid	iso 17:1 ω5c	1.27
11	cis-9-octadecenoic acid	18:1 ω9c	30.43
12	cis-7-octadecenoic acid	18:1 ω7c	4.20
13	Octadecanoic acid	18:0	6.29
14	nonadecenoic acid	19:1	3.49
15	cis-9-eicosenoic acid	20:1 ω9c	5.88
16	cis-7-eicosenoic acid	20:1 ω7c	0.83

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364 **Table 3**. DNA relatedness between *Vagococcus penaei* sp. nov. strain CD276^T and
365 phylogenetically closely related *Vagococcus* species (values are means of 2 minimum
366 hybridizations).

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Species	**% DNA homology**		
	V. penaei CD276^T	*V. carniphilus* CIP 108561^T	*V. fluvialis* LMG 9664^T
V. penaei CD276^T	100%	/	/
V. carniphilus CIP 108561^T	(14 ± 2)%	100%	/
V. fluvialis LMG 9664^T	(18 ± 2)%	(23 ± 0)%	100%

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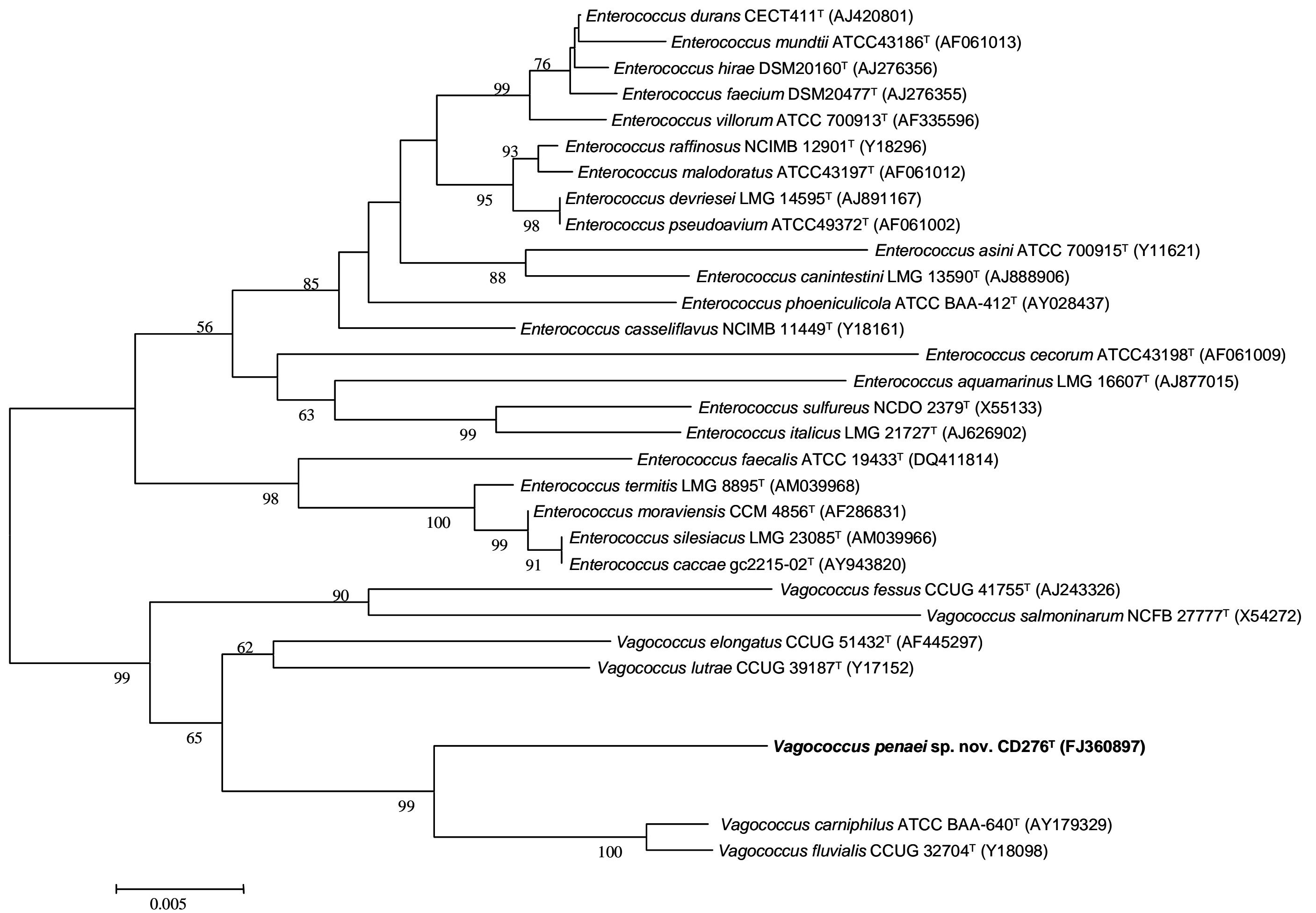

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence analysis, showing the relationships of *Vagococcus penaei* sp. nov. strains CD276^T, CD279, CD285, CD310 and CD380, with the other *Vagococcus* species and some selected *Enterococcus* species. The tree was constructed using the neighbour-joining method, with Kimura's 2-parameter (Gascuel, 1997, Kimura, 1980), on 1425 gap-free sites. Horizontal branch lengths are proportional to evolutionary distance. Bootstrap values (Felsenstein, 1985), expressed as a percentage of 1000 replicates, appear next to the corresponding branching node.

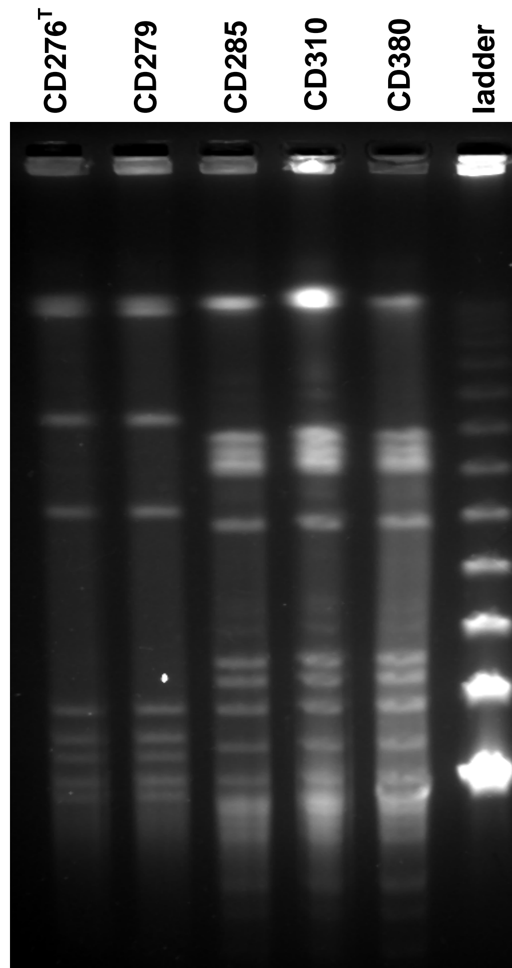

Fig. 2. Restriction patterns by PFGE of genomic DNA of *Vagococcus penaei* sp. nov. strains after *smaI* digestion. Lanes show strains and size ladder.