***Marinobacter aquaeolei* sp. nov., a halophilic bacterium isolated from a Vietnamese oil-producing well**

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Several strains of moderately halophilic and mesophilic bacteria were isolated at the head of an oil-producing well on an offshore platform in southern Vietnam. Cells were Gram-negative, non-spore-forming, rod-shaped and motile by means of a polar flagellum. Growth occurred at NaCl concentrations between 0 and 20%; the optimum was 5% NaCl. One strain, which was designated VT8^T, could degrade n-hexadecane, pristane and some crude oil components. It grew anaerobically in the presence of nitrate on succinate, citrate or acetate, but not on glucose. Several organic acids and amino acids were utilized as sole carbon and energy sources. The major components of its cellular fatty acids were C_{12:0} 3-OH, C_{16:1} ω9c, C_{16:0} and C_{18:1} ω9c. The DNA G+C content was 55.7 mol%. 16S rDNA sequence analysis indicated that strain VT8^T was closely related to *Marinobacter* sp. strain CAB (99.8% similarity) and *Marinobacter hydrocarbonoclasticus* (99.4% similarity). Its antibiotic resistance, isoprenoid quinones and fatty acids were similar to those of *Marinobacter hydrocarbonoclasticus* and *Pseudomonas nautica*. However, the whole-cell protein pattern of VT8^T differed from that of other halophilic marine isolates, including *P. nautica*. DNA–DNA hybridization indicated that the level of relatedness to *Marinobacter hydrocarbonoclasticus* was 65% and that to *P. nautica* was 75%. Further differences were apparent in Fourier-transformed IR spectra of cells and lipopolysaccharide composition. It is proposed that VT8^T should be the type strain of a new species and should be named *Marinobacter aquaeolei*. *P. nautica* may have been misclassified, as suggested previously, and may also belong to the genus *Marinobacter*.

Keywords: *Marinobacter aquaeolei*, 16S rRNA gene analysis, Fourier-transformed IR spectroscopy, lipopolysaccharide, *Pseudomonas nautica*

INTRODUCTION

The oil/gas fields are new and special ecosystems because of their physico-chemical and geochemical conditions. Environmental parameters such as temperature, pressure, pH, salinity, heavy metal concentration and petroleum composition vary widely from reservoir to reservoir (Bernard *et al.*, 1992; Bhupathiraju *et al.*, 1993). Recently, several reports

have described halophilic bacteria obtained from such environments (Adkins *et al.*, 1993; Dang *et al.*, 1996; Tardy-Jacquenod *et al.*, 1996a, b). In our microbial investigation of offshore oil/gas fields near the coastal town of Vung Tau in southern Vietnam, several novel micro-organisms have been isolated, including halophilic sulfate-reducing bacteria (Dang *et al.*, 1996). In this study, properties of a new moderately halophilic aerobic isolate (strain VT8^T) are reported which, on the basis of genotypic and phenotypic analyses, should be placed in the recently created genus *Marinobacter* (Gauthier *et al.*, 1992). Strain VT8^T shared several phenotypic properties with *Marinobacter hydrocarbonoclasticus* and *Pseudomonas nautica*, but

Abbreviation: FT-IR, Fourier-transformed IR.

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differences in whole-cell protein pattern, Fourier-transformed IR (FT-IR) spectra and lipopolysaccharide (LPS) composition justified its designation as a distinct species. It has been pointed out by several authors that *P. nautica* could be clearly separated from other *Pseudomonas* species, i.e. that it was probably misclassified (De Ley, 1992; De Vos *et al.*, 1989; Zumft, 1992). Our data suggest that *P. nautica* may also belong to the genus *Marinobacter*.

METHODS

Isolation, cultivation and maintenance of bacterial strains. Samples were collected at the head of an oil-producing well on the offshore oil/gas platform near the coastal town of Vung Tau in southern Vietnam. For enrichments and isolation, a culture medium similar to that described in American Petroleum Institute Research Publication 38 (1975) was used. It contained (l⁻¹ distilled water): peptone, 10 g; meat extract, 1 g; glucose, 5 g; NaCl, 100 g; MgCl₂·6H₂O, 3 g; and phenol red, 0.018 g. The pH was adjusted to 6.9–7.3 with NaOH before autoclaving. When necessary, agar was added at 2% (w/v). Samples of 2 ml each were inoculated into 12 ml vials containing 7 ml liquid medium. Following growth of the community by incubation at ambient temperature (30–37 °C), samples were transferred to agar plates by spreading. Single colonies were picked and purified further by streaking repeatedly on agar plates. Pure cultures were maintained by suspending cells in a small volume of 25% glycerol and 5% NaCl in screw-capped plastic vials and keeping them frozen at –70 °C.

Reference strains. The following strains were used for biochemical and morphological comparisons and obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ): *Halomonas elongata* DSM 2581^T, *Marinobacter hydrocarbonoclasticus* DSM 8798^T, *Marinomonas communis* DSM 5604, *Marinomonas vaga* DSM 5605^T, *Pseudomonas nautica* DSM 50418^T, *Salinivibrio* (formerly *Vibrio*) *costicola* DSM 11403^T. Sequence data from the following additional strains were used for phylogenetic comparisons (see also Fig. 4): *Marinobacter* sp. strain CAB, *Marinobacterium georgiense* ATCC 700074^T, *Oceanospirillum japonicum* ATCC 19191, *Oceanospirillum commune* ATCC 27118, *Oceanospirillum jannaschii* ATCC 27135^T, Str. clone SAR92 (Sargasso Sea bacterioplankton), Str. clone agg. 53 (marine snow-associated), *Chromohalobacter marismortui* DSM 6770^T and *Piscirickettsia salmonis* ATCC VR-1361.

Nutritional and growth characteristics. Marine broth 2216 (ZoBell, 1941; Difco), *Halomonas* complex (HMC) or defined (HMD) medium (Vreeland & Martin, 1980; Vreeland *et al.*, 1980) with slight modifications were used. HMC medium contained (l⁻¹ distilled water): Casamino acids (Difco), 7.5 g; peptone (Roth), 5 g; yeast extract (Difco), 1 g; NaCl, 50 g; MgSO₄·7H₂O, 20 g; sodium citrate, 3 g; K₂HPO₄, 0.5 g; and FeSO₄(NH₄)₂SO₄, 0.05 g. The pH was adjusted to 7.3 with KOH or HCl before autoclaving. HMD medium contained (l⁻¹ distilled water): NaCl, 50 g; MgCl₂·6H₂O, 5.3 g; KCl, 0.75 g; FeSO₄(NH₄)₂SO₄·6H₂O, 0.04 g; K₂HPO₄, 0.87 g; (NH₄)₂SO₄, 4 g. When required, agar was added at 2% (w/v) concentration. Cultures in liquid media were grown in 100 ml side-arm flasks containing 20 ml medium. Growth was monitored by measuring OD₆₀₀ in a Novaspec II

instrument (Pharmacia). The temperature range for growth was between 4 and 55 °C in HMC medium. The requirement for NaCl was determined in the same medium, which was supplemented with 0–32% NaCl (w/v). The pH range for growth was determined in Marine broth 2216, which was supplemented with 50 g NaCl and whose pH was adjusted to 4.0–10.0 with HCl or KOH. The utilization of carbohydrates as sole carbon and energy sources was tested in HMD medium (Vreeland & Martin, 1980), which was supplemented with 5% NaCl and whose pH was adjusted to 7.3 with KOH. Carbohydrates were added at 10 mM final concentration. The utilization of pristane (2,6,10,14-tetramethylpentadecane) and n-hexadecane (both from Sigma) was tested by inoculating strain VT8^T into HMD medium, which was supplemented with 1% (v/v) of the hydrocarbon compounds. Growth was scored by measuring OD₆₀₀. Degradation of crude oil fractions was tested by inoculating strain VT8^T into mineral medium, which contained (l⁻¹ tap water): KNO₃, 3 g; NaCl, 50 g; MgSO₄·7H₂O, 0.4 g; KH₂PO₄, 0.3 g; Na₂HPO₄, 0.7 g. Crude oil (5%, v/v) from White tiger oil-field (Vietnam) was added and incubation proceeded for 7 d at 30 °C. The residual oil was analysed by GC. Control experiments were performed under identical conditions, but omitting the bacterial inoculum.

Ion specificity determination. KCl, LiCl and NH₄Cl were substituted for NaCl at concentrations of 0.85 M in HMC medium to test for a specific ion requirement.

Phenotypic analysis. Standard tests (Gram staining, cytochrome oxidase, catalase, gelatinase) were performed as described previously (Smibert & Krieg, 1994). The Analytical Profile Index system (API 20NE and API ZYM; bioMérieux) was used for analysis of additional enzyme activities (Humble *et al.*, 1977) and for assimilation tests, respectively. Strips were inoculated with a bacterial suspension in minimal medium and incubated for up to 2 weeks. Antibiotic susceptibility was tested by spreading bacterial suspensions on agar plates and applying filter-paper disks (Schleicher and Schüll; 6 mm diameter) on which the following antibiotics were dispensed (µg per disk shown in parentheses): ampicillin (20), anisomycin (15), bacitracin (20), chloramphenicol (30), erythromycin (15), gentamicin (30), kanamycin (30), novobiocin (30), oleandomycin (25), streptomycin (20) and tetracycline (20). Zones of inhibition were measured after 2 d incubation at 30 °C.

Analysis of isoprenoid quinones. Lipoquinones were extracted from lyophilized cells with methanol:hexane (2:1, v/v) as described by Tindall (1990). The hexane phase contained the lipoquinones, which were separated by TLC on silica gel plates (Kieselgel F₂₅₄; Merck), using hexane:diethylether (85:15, v/v) as the developing solvent, and compared with lipoquinones of type strains based on R_F values (Collins, 1994).

Electron microscopy. Cells were harvested following 1 d incubation in HMC medium and prepared for scanning (Denner *et al.*, 1994) or transmission electron microscopy (Dang *et al.*, 1996) as described previously.

Cellular fatty acid analysis. Fatty acid methyl esters were prepared and analysed by GC as described by Kuykendall *et al.* (1988). This work was performed by R. Kroppenstedt, DSMZ, Braunschweig, Germany.

Whole-cell protein pattern analysis. SDS-PAGE of whole-cell proteins was performed as described previously (Stan-Lotter *et al.*, 1989). Briefly, approximately 50 mg cells (wet

weight) ml⁻¹ were lysed by boiling in SDS sample buffer (Laemmli, 1970) for 10 min, and then centrifuged at 10 000 *g* for 15 min, to remove any precipitates. The gel system of Laemmli (1970) was used. Proteins were visualized by staining with Coomassie blue. Marker proteins of 2.5–200 kDa were from Novex. Protein gels were run at least in triplicate.

Preparation of cell envelopes and analysis of LPS. Cell envelopes were prepared by passing cells through a French press and subsequent centrifugation at 200 000 *g* as described previously (Stan-Lotter & Sanderson, 1981), except that a buffer consisting of 50 mM Tris/HCl, 10 mM MgCl₂, pH 7.4, was used in all operations. Cell envelopes were suspended in SDS sample buffer (Laemmli, 1970) and 0.25% proteinase K (Sigma) was added (Seltman *et al.*, 1994). Following digestion at 37 °C for 1 h, samples were separated on the same gel system as that used for whole-cell proteins and stained with silver (Bio-Rad kit) according to the manufacturer's instructions.

FT-IR spectrometry. Bacterial strains were streaked on agar plates containing HMC medium and incubated overnight. Bacterial films were prepared by suspending one loopful (1 mm diameter) in 80 µl distilled water and applying an aliquot of 35 µl to a preformed sample area on a zinc selenite optical plate. Following drying of samples under reduced pressure, spectra were recorded between wave numbers 4000 and 500 cm⁻¹ in an FT-IR spectrometer type IFS 28/B (Bruker) as described by Helm *et al.* (1991a, b). Data analysis was carried out using the OPUS 3.0 software for bacterial identification from the same manufacturer. At least six independent preparations of each investigated strain were measured and a mean spectrum was calculated.

DNA base composition. Cells were harvested in the late exponential phase of growth and G+C contents were determined by the Identification Service of the DSMZ. DNA was isolated by the procedure of Visuvanathan *et al.* (1989) and was analysed by HPLC according to Mesbah *et al.* (1989) and Tamaoka & Komagata (1984).

DNA–DNA hybridization. DNA was isolated as described by Cashion *et al.* (1977). Levels of DNA–DNA hybridization between isolate VT8^T and two type strains were determined spectrophotometrically by the renaturation method of De Ley *et al.* (1970), with modifications by Huß *et al.* (1983) and Escara & Hutton (1980). Renaturation rates were computed by the program TRANSFER.BAS (Jahnke, 1992). These experiments were carried out by the Identification Service of the DSMZ.

16S rRNA sequence analysis. Sequencing of the 16S rRNA gene was carried out by C. Spröer at the DSMZ (see under DNA base composition). Extraction of the genomic DNA, amplification of the 16S rDNA by PCR and purification of the products were performed as described by Rainey *et al.* (1996). The purified 16S rDNA was sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) as indicated by the manufacturer. The DNA fragments of the sequencing reactions were separated and analysed using an Applied Biosystems 373 DNA Sequencer. The resulting sequences were read into the Alignment Editor ae2 (Maidak *et al.*, 1996), aligned manually and compared with 16S rRNA gene sequences of representative organisms of the gamma subclass of the *Proteobacteria*. Sequences which were used for comparisons were obtained from the EMBL or the RDP (Maidak *et al.*, 1996) databases.

RESULTS

Cellular and colonial morphology

Several rod-shaped isolates were obtained from the head of an oil-producing well on the offshore oil/gas platform at Vung Tau; all were Gram-negative, formed yellowish colonies on HMC medium and exhibited a wide range of NaCl tolerances. Strain VT8^T was characterized further; it was a short rod-shaped bacterium of 0.4–0.5 µm in diameter and 1.4–1.6 µm in length (Fig. 1). It was characteristically found growing singly or in pairs. Cells were actively motile; transmission electron microscopy (not shown) revealed a single, frequently polar flagellum, which is also visible in some areas of Fig. 1. Spore formation was not observed under any of the growth conditions described in this work. Colonies on HMC medium containing 5% NaCl were yellow, circular, convex, smooth with entire margins and 1.5–2.0 mm in diameter after 2 d cultivation at 30 °C.

Cultural and biochemical characteristics

Strain VT8^T grew at 0.0–3.4 M NaCl and growth was optimal at 0.85 M NaCl. K⁺, Li⁺ or NH₄⁺ ions were not able to replace Na⁺ ions. The optimum growth temperature was 30 °C. The temperature range for growth was 13–50 °C; no growth occurred at 55 or 4 °C. The pH range for growth was 5–10, with an optimum pH of 7.3. Growth was aerobic; catalase and oxidase activities were present. VT8^T could grow anaerobically with nitrate on succinate, citrate or acetate, but not on glucose. DL-Lactate, acetate, succinate, fumarate, citrate, L-glutamate and butyrate could be used as sole carbon and energy sources, but not D(–)arabinose, D(+)fructose, D(+)galactose, D(+)glucose, D(+)mannose, cellobiose, lactose, melibiose, sucrose, maltose, raffinose, glycerol, mannitol, sorbitol, benzoate, glycerate, oxalate, salicin, N-acetylglucosamine or amino acids (except L-Pro, L-Leu, L-Ile, L-Gln, L-Glu and L-Ala). On HMC medium,

Fig. 1. Scanning electron micrograph of strain VT8^T grown on *Halomonas* complex medium containing 5% NaCl. Bar, 1 µm.

Table 1. Fatty acid compositions of strain VT8^T, *Marinobacter hydrocarbonoclasticus* and *P. nautica*

Fatty acid	Total fatty acid (%) in:		
	Strain VT8 ^T	<i>Marinobacter hydrocarbonoclasticus</i>	<i>P. nautica</i>
10:0	0.57	1.02	0.96
12:0	7.89	4.66	5.88
11:0 3-OH	0.62	0.32	0.35
13:0	0.62	0.50	0.46
12:0 3-OH	9.94	7.74	9.03
14:0	2.60	2.24	2.07
15:0	2.24	1.36	1.16
16:0 iso	0.38	—	—
16:1 ω 9c	11.62	7.40	8.46
16:1 ω 5c	0.46	0.56	0.41
16:0	22.63	23.59	25.55
16:0 10-methyl	2.84	2.78	6.61
17:1 ω 8c	4.54	3.60	2.79
17:0	3.78	3.43	2.49
17:0 10-methyl	0.46	—	0.88
18:1 ω 9c	19.82	29.44	24.46
18:0	1.44	1.87	1.33
Summed feature 4*	6.78	8.29	4.99
Summed feature 7*	0.77	1.18	0.81

* Summed features are groups of fatty acids which were not resolved chromatographically; they consisted of fatty acids 16:1 ω 7t and 15:0 iso 2-OH (feature 4) and isomers 18:1 ω 7c, 18:1 ω 9t and 18:1 ω 12t (feature 7), respectively.

strain VT8^T was susceptible to the following antibiotics (diameter of inhibition zone is given in mm): bacitracin (12), chloramphenicol (22), erythromycin (18), gentamicin (13), kanamycin (10) and streptomycin (9); it was resistant to ampicillin, anisomycin, novobiocin, oleandomycin and tetracycline. Antibiotic susceptibility results for *Marinobacter hydrocarbonoclasticus* and *P. nautica* were similar to those of strain VT8^T. The following enzymes were present in strain VT8^T when assayed with the API ZYM system: alkaline phosphatase, esterase (C4), lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol AS-BI phosphohydrolase and *N*-acetyl- β -glucosaminidase. These enzymes were also found in *P. nautica*. The following enzymes were not present in either strain VT8^T or *P. nautica* when assayed for with the API ZYM system: cystine arylamidase, trypsin, chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, α -mannosidase or α -fucosidase. The following tests were positive when assayed with the API 20NE system: nitrate reduction to nitrite, urease activity and gelatin liquefaction. Except for urease activity, these tests were also positive for *P. nautica*. The following tests were negative for both VT8^T and *P. nautica* when assayed for with the API 20NE system: nitrite reduction to N₂, indole production, glucose oxidation, glucose fermentation, arginine dihydrolase and aesculin hydrolysis.

Utilization of hydrocarbons and crude oil

Strain VT8^T was able to utilize pristane, n-hexadecane and crude oil components as sole carbon sources. Preliminary results showed that saturated hydrocarbons were reduced by 41 %, aromatic hydrocarbons by 52 % and asphaltene by 20 % over a course of 7 d shaking at 30 °C in minimal medium (Dang T. C. H. & Nguyen B. H., unpublished data).

Isoprenoid quinones

The lipoquinones of strain VT8^T were compared with lipoquinones of *P. nautica* and *Marinobacter hydrocarbonoclasticus*, following separation by TLC on silica gel. All three strains displayed similar levels of fluorescence quenching bands with *R_F* values of about 0.3 (data not shown); this indicated the presence of ubiquinones, which are typical for many Gram-negative bacteria and are clearly distinct from menaquinones, which occur predominantly in Gram-positive bacteria and archaea, and whose *R_F* values are about 0.7–0.8 (Collins, 1994).

Fatty acid composition

The fatty acid compositions of VT8^T isolate and the reference strains are shown in Table 1. The major fatty acids of strain VT8^T were C_{12:0} 3-OH, C_{16:1} ω 9c, C_{16:0}

Fig. 2. Whole-cell proteins from strain VT8^T and several type strains of the gamma *Proteobacteria*. Cells were lysed and proteins were separated by SDS-PAGE and stained with Coomassie blue (see Methods). Approximately 15–20 µg protein was applied per lane. Lanes: 1, molecular mass markers; 2, *H. elongata*; 3, strain VT8^T; 4, *C. marismortui*; 5, *Marinobacter hydrocarbonoclasticus*; 6, *Marinomonas vaga*; 7, *Marinomonas communis*; 8, *S. costicola*; and 9, *P. nautica*.

and C_{18:1} ω9c. The results of cellular fatty acid analysis confirmed that strain VT8^T, *Marinobacter hydrocarbonoclasticus* and *P. nautica* had similar profiles in general, but strain VT8^T differed from both strains by lower levels of the fatty acids C_{16:1} ω9c and C_{18:1} ω9c.

Gel electrophoresis of whole-cell proteins

SDS-PAGE of whole-cell proteins is a rapid method for distinguishing bacterial species and has a similar level of discrimination to DNA–DNA hybridization (Jackman, 1987). Bacterial cells which are grown under identical conditions produce constant protein patterns which greatly facilitate the identification of strains under investigation (Vauterin *et al.*, 1993). This method has been widely used in the systematics of numerous bacterial strains (Kersters & De Ley, 1980); Gram-negative bacteria with very similar protein patterns in particular exhibit unusually high DNA–DNA reassociation values (Vandamme *et al.*, 1997, 1998, and references therein). Cells of strain VT8^T were compared with several type strains of the gamma *Proteobacteria* with similar morphological and biochemical characteristics (Fig. 2). Strain VT8^T showed a unique protein profile which did not resemble the profiles of *H. elongata*, *C. marismortui*, *Marinobacter hydrocarbonoclasticus*, *Marinomonas vaga*, *Marinomonas communis*, *S. costicola* or *P. nautica*.

FT-IR spectrometry

FT-IR spectra of bacteria are recorded from whole cells, without any preparation other than drying, and provide fingerprint-like, highly reproducible patterns which can distinguish strains at the subspecies level

Fig. 3. Electrophoretic analysis of LPS. Following separation by SDS-PAGE, LPS was stained with silver. LPS from strain VT8^T (lane 1), *Marinobacter hydrocarbonoclasticus* (lane 2) and *P. nautica* (lane 3) are shown. The arrow indicates the positions of the polysaccharide chains of the LPS from strain VT8^T.

(Helm *et al.*, 1991a; Naumann *et al.*, 1991). The spectra of strain VT8^T and *P. nautica* showed differences in terms of prominent additional peaks or shoulders between wave numbers 1200 and 900 (data not shown); this window represents the polysaccharide

region (Helm *et al.*, 1991a). Some additional differences between the two isolates were apparent between wave numbers 900 and 700 cm^{-1} ; according to Helm *et al.* (1991a), this region is the true fingerprint region, where remarkable spectral patterns often become apparent, although the chemical groups responsible for the spectra in this region have not yet been assigned.

LPS content

Since FT-IR spectra indicated possible differences in the polysaccharide composition of strains VT8^T and *P. nautica*, the LPS content of these strains was examined by SDS-PAGE (Fig. 3). The LPS patterns from strain VT8^T, *P. nautica* and *Marinobacter hydrocarbonoclasticus* appeared as the typical series of regularly spaced bands, which represent LPS molecules with varying lengths of *O*-linked side-chains and where different distances between the bands are due to different repeating sugar units in the polysaccharide chains (Palva & Mäkelä, 1980). While the LPS from *Marinobacter hydrocarbonoclasticus* and *P. nautica* (Fig. 3, lanes 2 and 3) showed a wide range of chain lengths, albeit with different spacing, that from strain VT8^T was apparently more homogeneous (Fig. 3, arrow); in addition, the distances between bands in the LPS of strain VT8^T were shorter than those of the LPS from the other two strains, suggesting less sugar molecules per repeating unit. While not strictly proven, these results would be consistent with the observed differences in the polysaccharide region of the FT-IR spectra of *P. nautica* and strain VT8^T.

G + C content

The DNA G+C content of VT8^T was 55.7 ± 0.1 mol%. This value was higher than that of *Marinobacter hydrocarbonoclasticus* (52.7 mol%; Gauthier *et al.*, 1992) and lower than that of *P. nautica* (59.2%; De Vos *et al.*, 1989).

DNA-DNA hybridization

The measurement of DNA-DNA hybridization showed the following homology values: 65% between strain VT8^T and *Marinobacter hydrocarbonoclasticus*; 75.4% between strain VT8^T and *P. nautica*; 85.1% between *Marinobacter hydrocarbonoclasticus* and *P. nautica*. A DNA homology value of 65% is well below the usual criterion of approximately 70% or higher, which justifies a different species (Wayne *et al.*, 1987). The homology values of approximately 75 and 85% between VT8^T and *P. nautica*, and *P. nautica* and *Marinobacter hydrocarbonoclasticus*, respectively, could indicate a closer relationship on the DNA level. However, the variation in several methods used for DNA-DNA hybridization is considered to range from ± 4 –5% or higher (Balkwill *et al.*, 1997; DSMZ Identification Service).

Fig. 4. Phylogenetic dendrogram indicating the position of strain VT8^T within the radiation of representatives of the gamma subclass of the *Proteobacteria*. The position of the root was determined by including *Piscirickettsia salmonis* in the analysis. The scale bar represents 10 nucleotide substitutions per 100 nucleotides.

Phylogeny

A matrix of sequence similarity of strain VT8^T and selected reference organisms from marine habitats belonging to the gamma *Proteobacteria* was constructed. Pairwise evolutionary distances were computed using the correction of Jukes & Cantor (1969). From these numbers, a phylogenetic dendrogram (Fig. 4) was drawn using the neighbour-joining method (Saitou & Nei, 1987) and the PHYLIP program (Felsenstein, 1993). Results of 16S rRNA sequence analysis suggested that strain VT8^T was more closely related to *Marinobacter* sp. strain CAB (99.8% similarity) and *Marinobacter hydrocarbonoclasticus* (99.4% similarity) than to *Marinobacterium georgiense* (90.1% similarity), *Marinomonas vaga* (89.2% similarity) and strains in the genus *Oceanospirillum* (similarity levels of 88.8–91.7%). It also appeared to be closely related to *P. nautica* (99.8% similarity; C. Spröer, unpublished data).

DISCUSSION

Strain VT8^T grew optimally at 0.85 M (5%) NaCl and could thus be classified as a moderately halophilic bacterium according to Kushner & Kamekura (1988). It utilized a range of sugars, organic and amino acids, and other carbon sources that might be available in marine waters. The ability to grow over a range of salinities and moderate temperatures as well as to utilize a variety of substrates appear characteristic of

numerous marine isolates. Strain VT8^T was also able to degrade hydrocarbons and some crude oil components; this was consistent with its origin from an oil-field brine. Analysis of its 16S rRNA sequence placed strain VT8^T in the gamma subclass of the *Proteobacteria*, which includes, among others, the genera *Oceanospirillum*, *Marinobacter*, *Marinomonas*, *Halomonas* and *Pseudomonas*. The genus *Marinobacter* was created by Gauthier *et al.* (1992), and recently Rontani *et al.* (1997) discovered a new *Marinobacter* sp., strain CAB. Growth characteristics, nutritional properties and 16S rRNA sequence data suggested that VT8^T should be placed in this genus. Since the results of phylogenetic analysis indicated that strain VT8^T was related to *Marinobacter hydrocarbonoclasticus* and also to *P. nautica* (C. Spröer, unpublished data), several experiments were performed to compare strain VT8^T with these two species. The overall antibiotic susceptibility of strain VT8^T was similar to those of *Marinobacter hydrocarbonoclasticus* and *P. nautica*. The fatty acid composition of the three strains (Table 1) indicated a somewhat lower amount of both C_{16:1} ω9c and C_{18:1} ω9c in strain VT8^T, as compared to *Marinobacter hydrocarbonoclasticus* and *P. nautica*. The G+C contents of *P. nautica*, strain VT8^T and *Marinobacter hydrocarbonoclasticus* were different (59.2, 55.7 and 52.7%, respectively). The DNA–DNA hybridization value of 65% between strain VT8^T and *Marinobacter hydrocarbonoclasticus* was below the value of approximately 70%, which has been suggested for delineating a species (Wayne *et al.*, 1987). The DNA homology values were higher between VT8^T and *P. nautica* (75%) and between *P. nautica* and *Marinobacter hydrocarbonoclasticus* (85%), respectively; this could partially be due to the known inherent experimental variation. Even so, high DNA–DNA hybridization values, approaching the range for species identity, should be reflected in very similar whole-cell protein patterns (Vandamme *et al.*, 1998). The comparison of whole-cell protein patterns following separation by SDS-PAGE (Fig. 2) showed, however, that strain VT8^T differed markedly from other selected strains of the gamma *Proteobacteria*, including *Marinobacter hydrocarbonoclasticus* and *P. nautica*; protein patterns of the latter two were also very different. Other differences in cell constituents were apparent from the LPS patterns of the three strains. The different spacing of bands suggested not only different repeating sugar units, but also a pronounced heterogeneity of the polysaccharide chain lengths of *P. nautica* and *Marinobacter hydrocarbonoclasticus* (Fig. 3), in contrast to strain VT8^T. In addition, FT-IR spectra of strain VT8^T and *P. nautica* showed numerous differences in the polysaccharide and 'fingerprint' regions of wave numbers (Helm *et al.*, 1991a), which would be consistent with differences in the LPS composition. Together with the differences in the whole-cell protein patterns, these results reflect differences in the genomic content of the organism, thus suggesting that VT8^T constitutes a novel species. Taking into account the results of the phenotypic

characterization and analysis of the 16S rRNA sequence, it can be concluded that strain VT8^T belongs to the genus *Marinobacter*.

Phylogenetic analysis placed strain VT8^T close to the recently described isolate *Marinobacter* sp. strain CAB (Rontani *et al.*, 1997). This strain is not yet available for direct comparisons. However, several properties of strain CAB suggested that it differed from strain VT8^T: optimal growth was at 0.6 M NaCl (VT8^T was 0.85 M); it could not grow beyond 2.5 M NaCl (VT8^T could grow up to 3.4 M); it could not liquefy gelatin; of the 20 amino acids tested, only Glu, Leu and Pro were utilized as carbon and energy sources (VT8^T could utilize L-Ile, L-Ala, L-Gln, in addition to L-Glu, L-Leu and L-Pro).

Marine bacteria belonging to the species *P. nautica* are Gram-negative heterotrophs; their taxonomic position remains to be established (Baumann *et al.*, 1972; De Vos *et al.*, 1989; Kita-Tsukamoto *et al.*, 1993; Zumft, 1992). Kita-Tsukamoto *et al.* (1993) suggested that halophilic, marine pseudomonads should be separated from the genus *Pseudomonas* and should be placed in newly created groups. Results from the comparison of nutritional requirements, antibiotic susceptibility, isoprenoid quinones, fatty acid composition, FT-IR spectra and 16S rRNA sequence data, which were obtained during our studies with strain VT8^T, would be consistent with a placement of *P. nautica* into the genus *Marinobacter*.

Description of *Marinobacter aquaeolei* sp. nov.

Marinobacter aquaeolei (a.quae.o'le.i. L. n. *aqua* water; L. n. *oleum* oil; M.L. gen. n. *aquaeolei* from water of oil, isolated from an oil-field brine).

Cells are Gram-negative, rod-shaped, non-spore-forming and motile by means of a polar flagellum. Cells are 0.4–0.5 µm wide and 1.4–1.6 µm long. Colonies on *Halomonas* complex medium are circular, yellow, convex, with entire margins; diameter is 1.5–2.0 mm after 2 d incubation at 30 °C. Growth occurs at 13–50 °C and pH 5–10; optimal growth occurs at 30 °C and pH 7.3. The range of NaCl for growth is 0–20%; optimal concentration is 5%. Anaerobic growth occurs in the presence of nitrate on succinate, citrate or acetate, but not on glucose. DL-Lactate, acetate, succinate, fumarate, citrate, L-glutamate, butyrate and the amino acids L-Pro, L-Leu, L-Ile, L-Gln, L-Glu and L-Ala are utilized as sole carbon and energy sources. n-Hexadecane, pristane and some crude oil components are degraded. Susceptible to chloramphenicol, erythromycin, streptomycin, kanamycin, gentamicin and bacitracin; not susceptible to tetracycline, novobiocin, oleandomycin, anisomycin or ampicillin. The DNA G+C content is 55.7±0.1 mol% (as determined by HPLC). The type strain is VT8^T, which was isolated from an oil-producing well on an offshore platform in southern Vietnam. It has been deposited in the Deutsche

Sammlung von Mikroorganismen und Zellkulturen as DSM 11845^T and in the American Type Culture Collection as ATCC 700491^T.

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