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 VT8⁷, 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 C₁₂:₀ 3-OH, C₁₆:₁ ɷ₉c, C₁₆:₀ and C₁₈:₁ ɷ₉c. The DNA G+C content was 55.7 mol%. 16S rDNA sequence analysis indicated that strain VT8⁷ 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 VT8⁷ 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 VT8⁷ 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 VT8⁷) 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 VT8⁷ shared several phenotypic properties with Marinobacter hydrocarbonoclasticus and Pseudomonas nautica, but
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 American Petroleum Institute Research Publication 38 was monitored by measuring OD₆₆₀ in a Novaspec I (Zumft, 1992). Our data suggest 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*.

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weight) ml⁻¹ were lysed by boiling in SDS sample buffer (Laemmli, 1970) for 10 min, and then centrifuged at 10000 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 25–200 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 200000 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 (Selman 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 Viswanathan 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 isolates VT8T 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 VT8T 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 VT8T 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. VT8T 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, benzote, 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 VT8T grown on Halomonas complex medium containing 5% NaCl. Bar, 1 μm.
Table 1. Fatty acid compositions of strain VT8T, *Marinobacter hydrocarbonoclasticus* and *P. nautica*

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Total fatty acid (%) in:</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Strain VT8T</td>
</tr>
<tr>
<td>10:0</td>
<td>0.57</td>
</tr>
<tr>
<td>12:0</td>
<td>7.89</td>
</tr>
<tr>
<td>11:0 3-OH</td>
<td>0.62</td>
</tr>
<tr>
<td>13:0</td>
<td>0.62</td>
</tr>
<tr>
<td>12:0 3-OH</td>
<td>9.94</td>
</tr>
<tr>
<td>14:0</td>
<td>2.60</td>
</tr>
<tr>
<td>15:0</td>
<td>2.24</td>
</tr>
<tr>
<td>16:0 iso</td>
<td>0.38</td>
</tr>
<tr>
<td>16:1 ω9c</td>
<td>11.62</td>
</tr>
<tr>
<td>16:1 ω5c</td>
<td>0.46</td>
</tr>
<tr>
<td>16:0</td>
<td>22.63</td>
</tr>
<tr>
<td>16:0 10-methyl</td>
<td>2.84</td>
</tr>
<tr>
<td>17:1 ω8c</td>
<td>4.54</td>
</tr>
<tr>
<td>17:0</td>
<td>3.78</td>
</tr>
<tr>
<td>17:0 10-methyl</td>
<td>0.46</td>
</tr>
<tr>
<td>18:1 ω9c</td>
<td>19.82</td>
</tr>
<tr>
<td>18:0</td>
<td>1.44</td>
</tr>
<tr>
<td>Summed feature 4*</td>
<td>6.78</td>
</tr>
<tr>
<td>Summed feature 7*</td>
<td>0.77</td>
</tr>
</tbody>
</table>

* Summed features are groups of fatty acids which were not resolved chromatographically; they consisted of fatty acids 16:1 ω7t and 15:0 iso 2-OH (feature 4) and isomers 18:1 ω7c, 18:1 ω9t and 18:1 ω12t (feature 7), respectively.

Strain VT8T 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 VT8T. The following enzymes were present in strain VT8T 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 VT8T 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 VT8T 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 VT8T 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 VT8T 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 VT8T isolate and the reference strains are shown in Table 1. The major fatty acids of strain VT8T were C₁₂:0 3-OH, C₁₆:₁ ω9c, C₁₆:₀.
and C<sub>18:1ω9c</sub>. The results of cellular fatty acid analysis confirmed that strain VT8<sup>T</sup>, *Marinobacter hydrocarbonoclasticus* and *P. nautica* had similar profiles in general, but strain VT8<sup>T</sup> differed from both strains by lower levels of the fatty acids C<sub>16:1ω9c</sub> and C<sub>18:1ω9c</sub>.

**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<sup>T</sup> were compared with several type strains of the gamma Proteobacteria with similar morphological and biochemical characteristics (Fig. 2). Strain VT8<sup>T</sup> 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 (Helm *et al.*, 1991a; Naumann *et al.*, 1991). The spectra of strain VT8<sup>T</sup> 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 VT8T and P. nautica, the LPS content of these strains was examined by SDS-PAGE (Fig. 3). The LPS patterns from strain VT8T, 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 VT8T was apparently more homogeneous (Fig. 3, arrow); in addition, the distances between bands in the LPS of strain VT8T 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 VT8T.

G + C content

The DNA G+C content of VT8T 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 VT8T and Marinobacter hydrocarbonoclasticus; 75.4% between strain VT8T 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 VT8T 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).

Phylogeny

A matrix of sequence similarity of strain VT8T 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 VT8T 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 VT8T 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 differences in the polysaccharide and heterogeneity of the polysaccharide chain lengths of protein patterns (Vandamme et al., 1993b). The different spacing of bands suggested not only high DNA-DNA hybridization values, approaching the range for species delineation by SDS-PAGE (Fig. 2) showed, however, that strain VT8T, as compared to Marinobacter hydrocarbonoclasticus and P. nautica. The G + C contents of P. nautica, strain VT8T and Marinobacter hydrocarbonoclasticus were different (59.2 and 52.7%, respectively). The DNA-DNA hybridization value of 65% between strain VT8T 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 VT8T 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 VT8T 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 heterogeneity of the polysaccharide chain lengths of P. nautica and Marinobacter hydrocarbonoclasticus (Fig. 3), in contrast to strain VT8T. In addition, FT-IR spectra of strain VT8T and P. nautica showed numerous differences in the polysaccharide and ‘fingerprint’ regions of wave numbers (Helms 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 VT8T 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 VT8T belongs to the genus Marinobacter.

Phylogenetic analysis placed strain VT8T 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 VT8T: optimal growth was at 0-6 M NaCl (VT8T was 0-85 M); it could not grow beyond 2-5 M NaCl (VT8T 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 (VT8T 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 VT8T, 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-spor-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 VT8T, 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 als DSM 11845\textsuperscript{T} and in the American Type Culture Collection as ATCC 700491\textsuperscript{T}.

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