***Methylarcula marina* gen. nov., sp. nov. and *Methylarcula terricola* sp. nov.: novel aerobic, moderately halophilic, facultatively methylotrophic bacteria from coastal saline environments**

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A new genus, *Methylarcula*, with two new species, *Methylarcula marina* and *Methylarcula terricola*, are proposed for strains h1^T and h37^T of moderately halophilic facultatively methylotrophic bacteria isolated from the coastal saline habitats. These methylotrophic bacteria are aerobic, Gram-negative, asporogenous, non-motile, colourless rods that multiply by binary fission. Their cellular fatty acids profiles consist primarily of straight-chain unsaturated (C_{18:1}; 70–80%), saturated (C_{18:0}; 14–16%) and cyclopropane (C_{19:0}; 5–6%) acids. The major ubiquinone is Q-10. The dominant phospholipids are phosphatidylethanolamine and phosphatidylcholine. Both strains could use methylamine, some sugars and organic acids as carbon and energy sources. They grew well under optimal conditions (29–35 °C, pH 7.5–8.5, 0.5–1.0 M NaCl) and accumulated intracellularly poly-β-hydroxybutyrate and the compatible solute ectoine. The ectoine pool was found to increase upon increasing the external NaCl concentration and accounted for 18% of the dry cellular weight. Both strains oxidized methylamine by the *N*-methylglutamate (*N*-MG) pathway enzymes (γ-glutamylmethylamide synthetase/lyase and *N*-MG synthetase/lyase) to formaldehyde and assimilated it via the icl[−] serine pathway. The DNA G+C content was 60.4 mol% for *Methylarcula marina* h1^T and 57.1 mol% for *Methylarcula terricola* h37^T. The DNA–DNA hybridization value between strains h1 and h37 was 25–30%, although they had a low level of DNA relatedness (5–7%) with the type strains of the serine pathway methylotrophic bacteria belonging to the genera *Methylobacterium*, *Aminobacter*, *Methylorhabdus* and *Methylophaga*. A comparative 16S rDNA sequence-based phylogenetic analysis placed the two species of *Methylarcula* into a separate branch of the α-3 subclass of the *Proteobacteria*. The type strains of the new species are *Methylarcula marina* h1^T (= VKM B-2159^T) and *Methylarcula terricola* h37^T (= VKM B-2160^T).

Keywords: *Methylarcula marina* gen. nov., sp. nov., *Methylarcula terricola* gen. nov., sp. nov., serine pathway, halophilic methylotrophs, ectoine

Abbreviations: GMA, glutamylmethylamide; MTS, maximum topological similarity; N-MG, *N*-methylglutamate; PHB, poly-β-hydroxybutyrate; RuBP, ribulose biphosphate; RuMP, ribulose monophosphate.

The GenBank accession numbers for the 16S rDNA sequences of strains h1^T and h37^T are AF030437 and AF030436, respectively.

INTRODUCTION

Although reduced C₁ compounds such as methanol and methylated amines are present in various saline environments, only a few taxa of aerobic, halophilic non-methane-utilizing methylotrophs (methylotrophic bacteria) have been described. The first, genus *Methylophaga*, with two species, *Methylophaga marina* and

Methylophaga thalassica, was proposed for several strains of moderately halophilic marine methylobacteria (Janvier *et al.*, 1985; Urakami & Komagata, 1987). Two new species, *Methylophaga sulfidovorans* (de Zwart *et al.*, 1996) and '*Methylophaga limanica*' (Doronina *et al.*, 1997) have also been described. All *Methylophaga* species require NaCl and vitamin B₁₂ for growth. Their DNA composition ranges from 43.0 to 49.0 mol% G+C. As defined by 16S rDNA sequencing, these ribulose monophosphate (RuMP) pathway methylobacteria form a distinct branch within the γ -subclass of the *Proteobacteria* (Janvier & Grimont, 1995).

Over several decades the only serine pathway methylobacteria isolated from saline environments have been *Hyphomicrobium* species (Hirsch, 1980, 1989). Two novel genera of restricted facultative methylobacteria with the serine pathway have since been described: '*Marinosulfonomonas*' and '*Methylosulfonomonas*' (Holmes *et al.*, 1997). These bacteria grew well on methanesulfonic acid as the carbon and energy source. The marine strains of the genus '*Marinosulfonomonas*' were able to grow at 3% NaCl, whereas the terrestrial strains of the '*Methylosulfonomonas*' genus did not grow above 0.5% NaCl. As explained in a comprehensive review by Ventosa *et al.* (1998), the isolation and detailed characterization of new aerobic moderate halophiles is an important aspect of assessing their metabolic and ecological roles in saline environments. Here we describe the properties of newly isolated aerobic moderately halophilic facultative methylobacteria, accumulating intracellularly a compatible solute, ectoine, as the major osmoprotectant. A novel genus, *Methylarcula*, with two new species, *Methylarcula marina* and *Methylarcula terricola*, are proposed for these isolates.

METHODS

Bacterial strains and culture conditions. Strain h1^T was isolated from Azov Sea estuary water samples with a salinity of 11‰ (pH 7.1). Strain h37^T was isolated from Black Sea coastal salty (10‰) soil samples collected near the city of Alushta. *Methylobacterium extorquens* NCIB 9399^T, *Aminobacter aminovorans* NCIB 9039^T, *Methylorhabdus multivorans* ATCC 51890^T, *Methylophaga marina* ATCC 35842^T, *Methylophaga thalassica* ATCC 33146^T and *Methylopila capsulata* VKM B-1606^T were used as reference strains.

The non-halophilic methylotrophic cultures were grown on liquid mineral medium K, containing 2.0 g (NH₄)₂SO₄, 2.0 g KH₂PO₄, 0.5 g NaCl, 0.125 g MgSO₄·7H₂O, 0.002 g FeSO₄·7H₂O in 1 l distilled water, and the pH of this medium was adjusted to 7.2 prior to autoclaving. Halophilic bacteria were grown on modified mineral medium MK, containing additionally 30 g NaCl, 0.2 g MgSO₄·7H₂O, 10 µg vitamin B₁₂ in 1 l distilled water. Substrates were sterilized and added separately. Methylamine or methanol were added to a concentration of 0.3 or 0.5%, respectively. Bacto-Agar (20 g l⁻¹; Difco) was added for the preparation of solidified media.

Liquid cultures were grown at 29 °C in 750 ml Erlenmeyer flasks containing 100 ml of the medium with shaking at 180 r.p.m. The bacterial strains were stored in liquid mineral

medium for 10 d, on agar slants at 4 °C for 2 weeks or in a freeze-dried state with a protectant (skim milk) for over a year.

Identification methods. Cell morphology, Gram staining, motility and flagellation were determined by using the cultures grown on solid medium MK. Production of a fluorescent pigment was tested on King A and B agar media with 3% NaCl. Nitrate reduction was tested in liquid medium MK in which (NH₄)₂SO₄ was replaced with 0.5% KNO₃ after 1, 3 and 5 d incubation. The methyl red and Voges-Proskauer (acetoin) reactions were tested in glucose/phosphate/peptone broth with 3% NaCl. Indole production was determined with Kovacs reagent in 1% Tryptone broth. Hydrogen sulfide production was tested on triple-sugar-iron agar for 2 weeks. Gelatin hydrolysis was observed in stab cultures in yeast extract/peptone medium containing 12% gelatin and 3% NaCl after 4 weeks incubation at 22 °C. Starch hydrolysis was determined by using an iodine solution on medium MK agar containing 0.2% soluble starch after 1, 3 and 10 d incubation.

Ammonia production was tested in peptone water with 3% NaCl by Nessler's reagent. The oxidase activity test was performed with a 1% solution of tetramethyl-*p*-phenylenediamine dihydrochloride. Urease activity was observed on Christensen's medium with 3% NaCl. Catalase activity was detected by pouring a 3% H₂O₂ solution into colonies on solid medium MK or PYG agar. Halotolerance was tested by inoculating the cells into liquid medium K with various concentrations of NaCl (1–14%). Growth at different temperatures and pH values was tested in liquid medium MK. Utilization of a wide range of growth substrates (> 50) was also determined in liquid medium MK after 2 weeks cultivation with methylamine being replaced by the other carbon compounds. Organic acids and amino acids were added at concentrations of 0.05–0.3%, while carbohydrates and alcohols were added at concentrations of 0.2–0.5%. To test alternative nitrogen sources (NH₄)₂SO₄ was replaced by other nitrogen compounds.

Methane utilization was tested in an atmosphere containing methane and air (1:1, v/v) in 700 ml conical flasks containing 100 ml medium MK and fitted with rubber stoppers. Hydrogen utilization was tested by the same procedure but in an atmosphere of H₂/O₂/CO₂ (7:2:1, by vol.). Utilization of mono- and dichloromethane was tested as described previously (Doronina *et al.*, 1995). Cell lysis was estimated according to Janvier *et al.* (1985).

Chemotaxonomic properties. Phospholipid composition of the cells was determined according to methods described previously (Govorukhina & Trotsenko, 1989). To determine the cellular fatty acid composition the cultures were grown on methylamine agar medium MK on Petri plates for 48 h at 29 °C. Several milligrams of cells were transferred with a loop to a screw-top microreaction jar containing 150 µl of a 20% aqueous tetramethylammonium hydroxide solution, the mixture was heated at 100 °C for 20 min and cooled to room temperature, and 100 µl acetonitrile was added. The jar was shaken, 20 µl of the top (acetonitrile) layer was withdrawn and evaporated in a stream of nitrogen at room temperature, 20 µl 20% tetramethylammonium hydroxide solution was added, the mixture was heated at 100 °C for 1 min and a 0.2–0.5 µl portion was introduced into the chromatograph injector (heated to 385 °C). The analysis was carried out in a Hewlett Packard 5830A chromatograph, using a 15 m × 0.2 mm glass capillary column filled with silicone OV-101 sorbent.

Table 1. Reference 16S rDNA sequences used in the phylogenetic analysis

Species	Strain	Accession no.	Species	Strain	Accession no.
<i>Amaricoccus kaplicensis</i>	Ben101 ^T	U88041	<i>Paracoccus kocurii</i>	JCM 7684 ^T	E05004
<i>Amaricoccus macauensis</i>	Ben104 ^T	U88042	<i>Paracoccus marcusii</i>	MH1 ^T	Y12703
<i>Amaricoccus tamworthensis</i>	Ben103 ^T	U88044	<i>Paracoccus pantotrophus</i>	ATCC 35512 ^T	Y16933
<i>Amaricoccus veronensis</i>	Ben102 ^T	U88043	<i>Paracoccus solventivorans</i>	DSM 6637 ^T	Y07705
<i>Antarctobacter heliothermus</i>	EL-219 ^T	Y11552	<i>Paracoccus thiocyanatus</i>	TH1011 ^T	D32242
<i>Hirschia baltica</i>	IFAM 1418 ^T	X52909	<i>Paracoccus versutus</i>	ATCC 25364 ^T	Y16932
<i>Hyphomonas hirschiana</i>	VP-5 ^T	AF082794	<i>Rhodobacter azotoformans</i>	KA25 ^T	D70846
<i>Hyphomonas jannaschiana</i>	VP-2 ^T	AF082789	<i>Rhodobacter blasticus</i>	ATCC 33485 ^T	D16429
<i>Hyphomonas neptunium</i>	LE-670 ^T	AF082798	<i>Rhodobacter capsulatus</i>	ATCC 11166 ^T	M34129
<i>Hyphomonas oceanitis</i>	SCH-89 ^T	AF082797	<i>Rhodobacter sphaeroides</i>	ATCC 17023 ^T	M55498
<i>Hyphomonas polymorpha</i>	DSM 2665	AJ227813	<i>Rhodobacter veldkampii</i>	ATCC 35703 ^T	D16421
<i>Maricaulus maris</i>	ATCC 15268 ^T	AJ227802	<i>Rhodobium marinum</i>	DSM 2698 ^T	D30790
' <i>Marinosulfonomonas methylophilus</i> '	PSCH4	U62894	<i>Rhodobium orientis</i>	MB312 ^T	D30792
<i>Methylobacterium extorquens</i>	JCM 2802	D32224	<i>Rhodovulum adriaticum</i>	ATCC 35885 ^T	D13476
<i>Methylobacterium fujisawaense</i>	DSM 5686	AJ250801	<i>Rhodovulum euryhalinum</i>	DSM 4868 ^T	D16426
<i>Methylobacterium mesophilicum</i>	JCM 2829	D32225	<i>Rhodovulum iodolum</i>	N1 ^T	Y15011
<i>Methylobacterium organophilum</i>	NCIMB 11278 ^T	D32226	<i>Rhodovulum robiginosum</i>	N2 ^T	Y15012
<i>Methylobacterium radiotolerans</i>	JCM 2831	D32227	<i>Rhodovulum strictum</i>	MB-G2 ^T	D16419
<i>Methylobacterium rhodesianum</i>	JCM 2810	D32228	<i>Rhodovulum sulfidophilum</i>	DSM 1374 ^T	D16423
<i>Methylobacterium rhodinum</i>	JCM 2811	D32229	<i>Roseovarius tolerans</i>	EL 172 ^T	Y11551
<i>Methylobacterium zatmanii</i>	NCIMB 12243 ^T	L20804	<i>Roseivivax halodurans</i>	OCh 239 ^T	D85829
<i>Methylopila capsulata</i>	IMI ^T	AF004844	<i>Roseivivax halotolerans</i>	OCh 210 ^T	D85831
' <i>Methylosulfonomonas methylovora</i> '	M2	U62893	<i>Roseobacter denitrificans</i>	OCh 114 ^T	L01784
<i>Octadecabacter antarcticus</i>	307 ^T	U14583	<i>Roseobacter litoralis</i>	ATCC 49566 ^T	X78312
<i>Octadecabacter arcticus</i>	238 ^T	U73725	<i>Rubrimonas cliftonensis</i>	OCh 317 ^T	D85834
<i>Paracoccus alcaliphilus</i>	JCM 7364 ^T	D32238	<i>Ruegeria algicola</i>	ATCC 51440 ^T	X78315
<i>Paracoccus alkenifer</i>	A901/1 ^T	Y13827	<i>Ruegeria gelatinovorans</i>	IAM 12617 ^T	D88523
<i>Paracoccus aminophilus</i>	JCM 7686 ^T	D32239	<i>Sagittula stellata</i>	E-37 ^T	U58356
<i>Paracoccus aminovorans</i>	JCM 7685 ^T	D32240	' <i>Silicibacter lacuscaerulensis</i> '	ITI-1157	U77644
<i>Paracoccus carotinifaciens</i>	E-396 ^T	AB006899	<i>Sulfitobacter mediterraneus</i>	CH-B427 ^T	Y17387
<i>Paracoccus denitrificans</i>	ATCC 7741 ^T	Y16927	<i>Sulfitobacter pontiacus</i>	ChLG-10 ^T	Y13155

Poly- β -hydroxybutyrate (PHB) was analysed according to Braunegg *et al.* (1978) by using a Pye Unicam 104 gas chromatograph. Ubiquinones were extracted and purified according to Collins (1985). Their analysis was done by using a Finnigan MX-1310 mass spectrometer.

The methanol/chloroform extraction procedure was used to isolate organic solutes from lyophilized cells. Dry cells (30 mg) were suspended in 1 ml chloroform/methanol/water mixture (2:2:1) and stirred at room temperature. Cell debris was removed by centrifugation (30000 g, 15 min), followed by re-extraction of the pellet in 1 ml of the same mixture. Then 1 ml chloroform/water mixture (1:1) was added to the combined supernatant. The upper phase containing the intracellular solutes was dried in a rotary evaporator. Finally, the solutes were redissolved in 1 ml 0.3 M citrate buffer (pH 3.3) and analysed on a Biotronic LC 3000 amino acid analyser.

For ectoine analysis, the solute extracts were dissolved in 1 ml 0.1 M KOH and hydrolysed for 20 h at 50 °C. The production of *N*-acetyldiaminobutyric acids (N-Ac-DABAs) was followed by TLC with 0.4 M sodium citrate (pH 3.3) as running buffer on Fixion 50 $\times$ 8 plates (Hungary).

The spots identical to N _{α} - and N _{γ} -Ac-DABAs were visualized by ninhydrin reagent and then eluted in 1 ml ethanol saturated with CuSO₄ and adjusted to pH 3.3 with 0.2 ml 0.4 M citrate buffer. Extinction of the eluates was determined at 500 nm on a Shimadzu UV-160 spectrophotometer. Authentic ectoine and hydroxyectoine were used after hydrolysis as the standards for quantification of the N-Ac-DABAs. The ectoine intracellular content was determined by using high resolution ¹H-NMR spectroscopy on an 80 MHz WP-80SY Bruker NMR spectrometer (Doronina *et al.*, 1998a). Enzyme assays were done as described previously (Trotsenko *et al.*, 1986; Doronina *et al.*, 1995). The activity of γ -glutamylmethylamide (γ -GMA) lyase and *N*-methylglutamate (N-MG) lyase were assayed by the rate of formaldehyde formation from γ -GMA or N-MG, respectively (Kimura *et al.*, 1995). Formaldehyde was quantified colorimetrically with acetylacetone (Nash, 1953).

Electron microscopic analysis. The cell suspension was mounted on a Formvar-coated copper grid and stained with 0.2 % (w/v) phosphotungstic acid (pH 7.2). Samples were prefixed with 1.5 % (v/v) glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) and washed three times with 1 % (w/v)

OsO₄ in 0.05 M cacodylate buffer (pH 7.2) for 3 h at 20 °C for thin sectioning. After dehydration in a series of alcohols, the cells were embedded in Spurr epoxy resin and sectioned with an LKB 2128 Ultratome. The ultrathin sections were mounted on copper grids and double-stained with uranyl acetate and lead citrate. The cells were taken from plates and frozen in liquid propane cooled to -196 °C with liquid nitrogen for freeze-etching. Cells were fractured in a JEOL JEE-4X vacuum evaporator at 3×10^{-4} Pa and a sample temperature of -100 °C. The fracture faces were shadowed with a platinum/carbon mixture and coated with carbon. Negatively stained preparations, thin sections and replicas were imaged in a JEOL JEM-100B transmission electron microscope at an operating voltage of 60 kV.

DNA isolation and characterization. DNA was isolated and purified according to Marmur (1961). DNA G + C contents were determined by the thermal denaturation method with a Beckman DU-8B spectrophotometer at a heating rate of 0.5 °C min⁻¹ and were calculated according to Owen & Lapage (1976). The DNA from *Escherichia coli* K-12 was used as the standard. The DNA-DNA hybridization was done on nitrocellulose membrane filters (0.22 µm) according to the procedure of Denhardt (1966) in the following incubation mixture: 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0, with 30 % formamide at a temperature of 60 °C for 24 h. [1', 2', 5'-³H]dCTP and nick translation kit N 5500 (Amersham International) were used for labelling the probe DNA.

16S rDNA amplification and sequencing. For amplification and sequencing of 16S rRNA genes, DNA was isolated by standard phenol/chloroform extraction. The 16S rRNA gene was selectively amplified by PCR using the following primers: forward primer, 5'-AGAGTTTGATCCTGGC-TCAG-3' (8-27; *E. coli* numbering); reverse primer, 5'-TACGGTTACCTGTACGACTT-3' (1471-1492) (Lane, 1991). PCR was carried out in a final volume of 100 µl, with 1 µg DNA template, 200 µM each primer, 200 µM each dNTP, 3 U Tet-z polymerase (BioMaster, Russia) and reaction buffer (100 mM Tris/HCl pH 8.3, 500 mM KCl, 2 mM MgCl₂). The temperature cycling was done by using 30 cycles of the following thermal profile: 1 min at 94 °C, 1 min at 42 °C, 1 min at 72 °C. The final extension was carried out at 72 °C for 6 min. The PCR products were purified using the PCR-prep kit (Promega), according to the manufacturer's instruction. The 16S rRNA gene was sequenced in both directions with the use of forward and reverse primers listed by Lane (1991). DNA sequencing was carried out by using Sequenase version 2 of the VSB kit (Biochemicals Cleveland) with minor modifications.

Phylogenetic analysis. The sequences of strains h1^T and h37^T were aligned manually against the database of small subunit rRNAs (Larsen, 1986). The sequences of the final alignment set were obtained from the latest version of GenBank (Table 1). Regions that were not sequenced in one or more reference organisms were omitted from the final analysis and a total of 1308 nt was used for tree construction. Pairwise evolutionary distances (expressed as estimated changes per 100 nt) were computed by use of the correction of Jukes & Cantor (1969). A tentative unrooted phylogenetic tree was constructed by the maximum topological similarity (MTS) algorithm (Chumakov & Yushmanov, 1988) of the program TREE of the GENEBEE package (Brodsky *et al.*, 1991). The advantage of the MTS method is that it does not require any assumptions on the relative rate of evolutionary change in

different lineages and provides straightforward computationally efficient algorithms to construct a phylogenetic tree. This method defines the topology of all subtrees from the four species and joins these trees. The first step is fulfilled by the use of a four-nod rule, which allows one to find two pairs of neighbours in any subset of four species based on the distance between them. The second step, linking subtrees in a single tree, is achieved by using different heuristic algorithms. The unrooted phylogenetic tree was constructed by neighbour-joining methods (Saitou & Nei, 1987), with bootstrap analysis of 100 trees, by using the programs of the TREECON package (Van de Peer & De Wachter, 1994). Additional unrooted trees were constructed by the maximum-parsimony method by using the programs of the PHYLIP package (Felsenstein, 1990) and the maximum-likelihood method by program PUZZLE.

RESULTS

Morphology

The bacterial isolates h1^T and h37^T are Gram-negative, asporogenous, colourless, non-motile rods 0.5–0.8 × 1.0–2.0 µm in size (Fig. 1a, d). Reproduction occurred by binary fission (Fig. 1b, e). No capsules or intracellular complex membranes were formed. Both strains accumulated intracellular PHB inclusions (Fig. 1b, c, e, f).

Phenotypic characteristics

When grown on agar medium, colonies were white and mucoid. Both strains grew without aggregation and pigmentation in mineral salt liquid medium with methylamine as the carbon and energy source. Cells did not grow on nutrient broth or peptone broth supplemented with 3 % NaCl. No water-soluble fluorescent pigment was produced. Nitrate was not reduced to nitrite. Methyl red and Voges-Proskauer (acetoin) tests were negative. Ammonia, indole and hydrogen sulfide were not produced. Hydrolysis of gelatin and starch was not observed. Acid was produced from glucose oxidatively. Growth factors were not required. Ammonia and methylamine, but not nitrate, were utilized as nitrogen sources. Oxidase-positive, urease and lipase-negative. Strain h1^T did not produce catalase. Growth occurred at temperatures between 10 and 42 °C, but the optimum growth temperature was 29–35 °C. The optimum pH was 7.5–8.5; no growth was detected below pH 4.0 and above pH 10.5. The strains grew well in 3–8 % NaCl. Growth was inhibited at NaCl concentrations above 12 % (strain h1^T) and 14 % (strain h37^T). Generation time was about 5 and 3 h on medium MK with 0.3 % CH₃NH₂ and 0.1 % succinate, respectively.

When transferred into distilled water after NaCl treatment, the cells of both isolates lysed. The divalent cations Mg²⁺ and Ca²⁺ at concentrations as low as 50 mM added to the 0.5 M NaCl washing solution prevented the cell lysis. By using ¹H-NMR spectroscopy and TLC, the accumulation of a compatible solute, ectoine, in the cells of strains h1^T and h37^T was

Fig. 1. Electron micrographs of strain $h1^T$ (a, b, c) and strain $h37^T$ (d, e, f). (a, d) Negatively stained cells. (b, e) Ultrathin sections showing cell wall structure and granules of PHB. (c, f) Freeze-fractured cell. Arrows indicate PHB. Bars, 1 μ m.

Fig. 2. Influence of NaCl concentration on growth and intracellular ectoine and glutamate accumulation by *Methylarcula marina* $h1^T$ growing on methylamine. ■, Growth rate (h^{-1}); solid bars, ectoine; open bars, glutamate.

detected. The 1H -NMR spectrum showed that ectoine was the major cytosolic constituent, whereas glutamate was a minor one. Hydroxyectoine and glycine betaine were not detected. The ectoine pool was found to increase upon increasing the NaCl concentration in the growth medium (Fig. 2). The strains could use methylamine, fructose, glucose, maltose, lactose, mannose, ribose, trehalose, galactose, xylose, sucrose, succinate, pyruvate and acetate as the carbon and energy sources. Strain $h37^T$ also grew on dimethylamine. However, both strains grew very slowly on methanol. No growth occurred on citrate, oxaloacetate, *cis*-aconitate, fumarate, propionate, α -ketoglutarate, ethanol, butanol, formaldehyde, trimethylamine, dimethylformamide, dimethylacetamide, dimethylsulfoxide, acetamide, mono- and dichloromethane, methanesulfonic acid and gas mixtures of $CO_2/H_2/O_2$ or CH_4/O_2 .

As seen from Table 2, the dominant cellular fatty acids of strains $h1^T$ and $h37^T$ were straight-chain unsaturated ($C_{18:1\omega7}$), saturated ($C_{18:0}$) and cyclopropane ($C_{19:0}$) acids. Analysis of the cellular phospholipid composition revealed the presence of phosphatidyl-

Table 2. Cellular fatty acid composition of the new strains (h1^T and h37^T) grown on methylamine

Methyloctadecenoic acid (Me18:1 ω 7) was not detected.

Fatty acid	Percentage of total fatty acids	
	h1 ^T	h37 ^T
16:1	0.1	0.2
16:0	0.7	1.0
Me17	0.6	0.2
17:1	0.1	0.1
17:0	3.0	2.3
18:1 ω 7	74.2	73.4
18:0	14.7	15.6
c19:0	5.3	6.0
19:0	0.1	0.7

ethanolamine, phosphatidylcholine, phosphatidylglycerol and cardiolipin. The major ubiquinone detected in both strains was Q-10. They accumulated a large amount of PHB (10–40 % dry cell wt).

Metabolic characteristics

The results of enzymological analysis of the pathways for primary and intermediary metabolism of methylamine in both strains are given in Table 3. Methylamine dehydrogenase and amine oxidase were absent in methylamine-grown cells. Alternatively, they contained an inducible γ -GMA synthetase/lyase and N-MG synthase/lyase. Both enzymic systems produce formaldehyde which is further oxidized by glutathione-independent formaldehyde dehydrogenase to formate. The latter is ultimately oxidized to CO₂ by phenazine-methosulphate-linked and NAD-dependent formate dehydrogenases. Formaldehyde assimilation occurs

Table 3. Enzyme activities in cell extracts of new strains (h1^T and h37^T) grown on methylamine

There was no activity with any of the following enzymes: methylamine dehydrogenase (phenazine methosulphate), amine oxidase, 3-hexulosephosphate synthase, RuBP carboxylase, isocitrate dehydrogenase (NAD⁺), glutamate dehydrogenase (NADH).

Enzyme (cofactor)	Enzyme activity [nmol min ⁻¹ (mg protein) ⁻¹]	
	h1 ^T	h37 ^T
γ -GMA synthetase	50	18
N-MG synthase	13	22
γ -GMA lyase	14	17
N-MG lyase	28	11
Formaldehyde dehydrogenase		
(Phenazine methosulphate)	8	112
(NAD ⁺)	57	130
Formate dehydrogenase		
(Phenazine methosulphate)	32	37
(NAD ⁺)	14	43
Hydroxypyruvate reductase		
(NADH)	500	167
(NADPH)	381	135
Serine-glyoxylate aminotransferase		
(NADH)	290	149
(NADPH)	222	90
Malate lyase (ATP, CoA)	11	10
Isocitrate lyase	1	3
Citrate synthase	12	11
Isocitrate dehydrogenase (NADP ⁺)	78	58
α -Ketoglutarate dehydrogenase (NAD ⁺)	10	7
Glutamate dehydrogenase (NADPH)	280	170
Glutamate synthase		
(NADH)	28	43
(NADPH)	41	0
Glutamine synthetase (Mg ²⁺ , ADP)	237	182

Fig. 3. Phylogenetic tree showing the positions of strains h1^T and h37^T among members of the *Rhodobacter* and *Hyphomonas* groups and other genera of the α -3 subclass of the *Proteobacteria*. Bootstrap values (expressed as a percentage of 100 replications) are shown at branch points; values greater than 85 were considered as significant. Bar indicates the Jukes-Cantor distance (5 nt substitutions per 100 nt).

via the serine pathway as confirmed by the presence of the appropriate specific enzymes: hydroxypyruvate reductase, serine-glyoxylate aminotransferase and malate lyase. Strains h1^T and h37^T had a very low isocitrate lyase (icl) activity and consequently implemented the icl⁻ variant of the serine pathway. Both the ribulose biphosphate (RuBP) and RuMP pathways of C₁ assimilation do not operate due to the absence of the appropriate key enzymes: RuBP carboxylase and hexulose phosphate synthase. Primary assimilation of ammonia occurs both by reductive amination of α -ketoglutarate and via the glutamate

cycle. The strains contained a complete set of the enzymes of the tricarboxylic acid cycle playing primarily a biosynthetic role during methylotrophic growth.

Genotypic characteristics

The DNA G + C contents of strains h1^T and h37^T were estimated via T_m as 60.4 and 57.1 mol%, respectively. The level of DNA relatedness between these two strains did not exceed 25–30%, consistent with their assignation to separate species of the new genus.

Remarkably, they had a very low degree of DNA hybridization (2–6%) with the representatives of the serine pathway methylobacteria belonging to the genera *Methylobacterium*, *Aminobacter*, *Methylorhabdus* and *Methylopila*.

A total of 1359 and 1360 nt of sequence were determined for strains h1^T and h37^T, respectively, corresponding to positions 7–1449 of the *E. coli* sequence. The sequences were compared to representatives of the α -, β - and γ -subclasses of the *Proteobacteria*, including the validly published methylo-trophic and halophilic genera. In preliminary trials, a total of 98 sequences were used and several phylogenetic trees were generated. The phylogenetic analysis employed by different algorithms showed similar results. According to our data, strains h1^T and h37^T appeared to be related to the members of the α -subclass of the *Proteobacteria*. The affiliation of both methylo-trophic strains to the α -subclass is supported by signature analysis. Some features characteristic of 16S rDNA molecules of the α -subclass, such as a shortened helix between nt positions 184–193 (*E. coli* numbering) and all specific signature positions (Woese, 1987), were found in the sequences analysed.

More precisely, strains h1^T and h37^T were found to group with members of the phylogenetic *Rhodobacter* group within the α -3 subclass of the *Proteobacteria*. According to 16S rDNA sequence analysis, strains h1^T and h37^T were closely related with a similarity level of 95.9%. The highest degree of relationship of these halophilic strains was found with species of the genus *Paracoccus* (91.9–93.3%). The similarity values for strains h1^T and h37^T and other members of the *Rhodobacter* group, including marine species, fell into the range 87.1–92.6%. Phylogenetic positions of strains h1^T and h37^T among the type species of all genera of the *Rhodobacter* group are shown in Fig. 3. The topologies of additional phylogenetic trees constructed by using MTS, maximum parsimony and maximum-likelihood methods (data not shown) were almost the same. More distant relatedness of strains h1^T and h37^T was found with marine bacteria of the genera *Hyphomonas*, *Hirschia* and *Maricaulus* belonging to the *Hyphomonas* group of the α -3 subclass of the *Proteobacteria* (range of similarity 86.7–89.1%).

The levels of similarity with members of the other subclasses of the α -subclass (82.0–86.9%) and other subclasses of the *Proteobacteria* (76.7–81%), including marine and methylo-trophic genera, were significantly lower. The affiliation of the identified methylo-trophic strains to the α -3 subclass is supported by analysis of secondary structures of their 16S rDNAs. The significant structural difference occurs in the helix at (*E. coli*) positions 1241–1296. An obvious change in this region was the loss of the apex helix element (Fig. 4). In all members of the *Rhodobacter* and *Hyphomonas* groups of the α -3 subclass, 21 nt are missing in this region. The abbreviated version of the local structure seems to be highly characteristic of the members of the α -3 subclass and may be used for their fast detection.

Fig. 4. The unusual secondary structure element in *Methyloarcula marina* and other members of α -3 subclass of *Proteobacteria* (compared with typical versions taken from *E. coli*).

DISCUSSION

As defined by Kushner (1978) and Larsen (1986), moderately halophilic bacteria grow optimally in 0.5–3.0 M salt. Strains h1^T and h37^T fall into this group since they not only grew over a wide range of NaCl concentrations but also required sodium for growth. The moderately halophilic methylobacteria pose specific questions related to their adaptability to a wide range of salinities (Ventosa *et al.*, 1998).

By using ¹H-NMR-spectroscopy and TLC, an intracellular accumulation of the compatible solute ectoine (1,4,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) as the major osmoprotectant in both strains was detected. Indeed, the intracellular content of ectoine increased with the elevated external osmolarity and attained 18% dry cell wt at 6–10% NaCl.

Glutamate obviously plays a minor role in the osmotic balance of both species. On the other hand, according to our results, these methylobacteria metabolize methylamine via the N-MG pathway: methylamine being accepted by glutamate to produce its *N*-methylated derivatives (γ -GMA and N-MG). The specific enzyme, γ -GMA lyase, was found in the new isolates. So far, this enzyme system has been detected only in the RuMP pathway marine methylobacteria *Methylophaga* sp. AA-30 (Kimura *et al.*, 1995) and '*Methylophaga limanica*' (Doronina *et al.*, 1997). Perhaps, such a variant of the N-MG pathway operates in many other moderately halophilic methylobacteria independent of the type of C₁ assimilatory cycle. Further work on these relatively little-studied halophiles may be expected to bring the new insights into their halo-adaptation and osmoregulatory mechanisms.

Recent taxonomic and phylogenetic studies have defined a large number of species to be included within the heterogeneous group of moderately halophilic bacteria, distributed over at least half of the major phylogenetic branches of the *Proteobacteria* (Gonzales

& Moran, 1997; Ventosa *et al.*, 1998). Our moderately halophilic isolates are distinguished clearly from the genus *Methylophaga* on the basis of the following characteristics: spectrum of utilizable polycarbon compounds, C_1 assimilation via the serine pathway, quinone system, specific cellular fatty acid composition, the occurrence of ectoine as a major osmoprotectant and higher DNA base composition (Janvier *et al.*, 1985; Doronina *et al.*, 1997).

Also, the novel isolates are distinguished from all known Gram-negative serine pathway methylobacteria of the genera *Methylobacterium* (Bousfield & Green, 1985), *Aminobacter* (Urakami *et al.*, 1992), *Hyphomicrobium* (Hirsch, 1989), *Methylorhabdus* (Doronina *et al.*, 1995) and *Methylopila* (Doronina *et al.*, 1998b) on the basis of some morphological features, physiological and biochemical properties, halotolerance and low DNA–DNA hybridization values (Table 3). Strains h1^T and h37^T are clearly distinguished from the serine pathway methylobacteria of the genera ‘*Methylosulfonomonas*’ and ‘*Marinosulfonomonas*’ (Holmes *et al.*, 1997) by their distinctive morphology and inability to grow on methanesulfonic acid as the carbon and energy source.

In the phylogenetic trees derived from 16S rRNA sequences (Fig. 3), halophilic strains h1^T and h37^T consistently branched together with the α -3 subclass of the *Proteobacteria*. Most genera of the serine pathway methylobacteria belong to another subclass, α -2. On the contrary, moderately halophilic marine methylobacteria of the genus *Methylophaga* belong to the γ -subclass of the *Proteobacteria*. Recently described marine methylotroph ‘*Marinosulfonomonas*’ belongs to the α -3 subclass, but strains h1^T and h37^T were not closely related to these bacteria. It has been shown that marine bacteria belonging to the α -3 subclass, including strains of described genera, new isolates and uncultivated clones, form a single cluster within this subclass (Gonzalez & Moran, 1997). The new halophilic strains h1^T and h37^T do not belong to this cluster and appeared to be more closely related to members of genus *Paracoccus*. Although this genus includes some aerobic methylotrophic species, they are clearly distinguishable from strains h1^T and h37^T by their ability to grow autotrophically on methanol through the RuBP pathway, as well as by their significantly higher DNA G + C ratio (66–68 mol%).

The level of 16S rDNA sequence similarity between the new strains and members of the genus *Paracoccus* (91.9–93.3%) was lower than the interspecies similarity for this genus (93.7–99.8%). The *Rhodobacter* group is a very compact phylogenetic cluster with a high level (87.1–95.6%) of intergeneric 16S rDNA sequence similarity. So, the genetic distance found for both strains and other members of the *Rhodobacter* group corresponds to a generic taxonomic level of this group. Thus, strains h1^T and h37^T are phylogenetically separated from the other representatives of the α -3 subclass of the *Proteobacteria* at the generic level.

It is now generally accepted that bacteria differing by more than 5% in their G + C ratio should not be assigned to the same species and those differing by more than 10% G + C should not be classified within the same genus (Wayne *et al.*, 1987). Strains that show 70% or more DNA–DNA hybridization values are currently defined as belonging to the same species. In spite of general phenotypic similarity, strains h1^T and h37^T differed in some properties, especially by their DNA G + C contents. The level of DNA–DNA hybridization (near 25–32%) and the dissimilarity value of 16S rDNA (more than 3%) are too low to combine these strains in one species (Stackebrandt & Goebel, 1994). Based on the data obtained, both isolates might be placed in the novel genus *Methylarcula* as *Methylarcula marina* gen. nov., sp. nov. and *Methylarcula terricola* gen. nov., sp. nov. The major characteristics differentiating the genus *Methylarcula* from other related genera are summarized in Table 4. Phylogenetic data also demonstrated that the new species clearly separated from all known methylotrophic and marine bacteria.

Description of *Methylarcula* gen. nov.

Methylarcula (Me.thyl.ar.cu'la. M.L. n. *methyl* methyl group; L. fem. n. *arcula* small box; M.L. fem. n. *Methylarcula* methyl-using small box).

When grown in methylamine mineral salt medium, cells are Gram-negative, non-sporulating, colourless rods, usually 0.5–0.8 μ m in length and 0.8–2.0 μ m in diameter, which multiply by fission. Colonies on methylamine agar are white and mucoid. Aerobic and moderately halophilic and requiring NaCl for growth. Accumulate ectoine intracellularly as the main osmoprotectant, and PHB granules. Do not require vitamins for growth. Do not grow on peptone/yeast extract medium with or without NaCl. Do not grow under methane or hydrogen. Do not reduce nitrate to nitrite. Utilize methylamine, sugars and some organic acids as carbon and energy sources. Ammonium salts and methylamine are utilized as nitrogen sources. Acids are produced from sugars oxidatively. Do not form acetoin, indole, hydrogen sulfide and ammonia. Chemo-organotrophic and facultatively methylotrophic bacteria assimilating C_1 compounds via the isocitrate lyase-negative (icl⁻) serine pathway. The major ubiquinone is Q-10. Dominant phospholipids are phosphatidylethanolamine and phosphatidylcholine. The prevailing cellular fatty acids are straight-chain unsaturated ($C_{18:1}$), saturated ($C_{18:0}$) and cyclopropane ($C_{19:0}$) acids. DNA G + C content ranges from 57 to 61 mol%. The levels of DNA–DNA hybridization with members of the genera *Methylobacterium*, *Methylorhabdus*, *Aminobacter* and *Methylopila* are below 10%. The genus *Methylarcula* belongs to the α -3 subclass of the *Proteobacteria*, as revealed by comparative sequence analysis of the 16S rDNA and is phylogenetically distinct from the other methylotrophic genera. Type species is *Methylarcula marina*.

Table 4. Major differentiating characteristics of facultative serine pathway methylbacteria belonging to various genera

+, Present; —, absent; ND, not determined.

Characteristics	<i>Methylobacterium</i>	<i>Aminobacter</i>	<i>Hyphomicrobium</i>	<i>Methylorhabdus</i>	<i>Methylophila</i>	<i>Methylosulfonomonas</i>	<i>Marinosulfonomonas</i>	<i>Methyloarcula</i>
Morphology (flagella)	+	+	+	—	+	+	—	—
Reproduction by:								
Budding	—	+	+	—	—	—	—	—
Division	+	—	—	+	+	+	+	+
Hyphae formation	—	—	+	—	—	—	—	—
Oxidase	+	+	—	—	+	—	+	+
Catalase	+	+	+	+	—	+	+	+ / —
Carotenoids	+	—	—	—	—	—	—	—
Reduction of nitrate to nitrite	+	+	+	+	+	ND	ND	—
Methylamine metabolism:								
Amine dehydrogenase	+	—	—	+	+	ND	ND	—
N-MG derivatives	+	+	+	—	—	ND	ND	+
γ -GMA lyase	—	—	—	—	—	ND	ND	+
Isocitrate lyase	—	+	+	—	—	ND	ND	—
Cyclopropane acid, c19:0	TRACE	+	+	+	+	+	+	+
Methyloctadecenoic acid (Me18:1 ω 7)	—	—	+	+	+	ND	ND	—
Major ubiquinone	Q-10	Q-10	Q-9	Q-10	Q-10	ND	ND	Q-10
Tolerance to NaCl (%)	2.5	2.5	3.0	2.0	2.0	0.5	3.5	12.0
Growth at pH 10.0	—	—	—	—	—	—	—	+
Utilization of methanol	+	—	+	+	+	+	+	WEAK
G + C (mol %)	60–70	62–64	61–65	66–67	66–70	61	57	57–61

Description of *Methyloarcula marina* sp. nov.

Methyloarcula marina (ma.ri'na. L. fem. n. *mare* sea; L. fem. adj. *marina* of the sea).

Has all the characteristics which define the genus. Additionally, it has the following characteristics. Cells are non-motile rods, $0.5\text{--}0.8 \times 0.8\text{--}1.2\ \mu\text{m}$. Colonies on methylamine agar medium (4–5 d) are white or pale pink, 1–2 mm in diameter. Oxidase-positive. Urease, lipase and catalase-negative. Nitrate is not utilized. Able to grow at $10\text{--}42\ ^\circ\text{C}$, pH 5.0–10.5 and 0.05–12% NaCl. Optimal conditions for growth are $29\text{--}35\ ^\circ\text{C}$, pH 7.5–8.5 and 3–8% NaCl. Utilizable carbon sources are methylamine, fructose, glucose, maltose, lactose, mannose, ribose, trehalose, galactose, xylose, sucrose, succinate, pyruvate and acetate. Methylamine is oxidized to formaldehyde by the N-MG pathway enzymes: γ -GMA synthetase/lyase and N-MG synthase/lyase. DNA G + C content is 60.4 mol% (T_m). The type strain *Methyloarcula marina* h1^T was isolated from the Azov Sea estuary water and is deposited in the Russian Collection of Microorganisms as VKM B-2159^T.

Description of *Methyloarcula terricola* sp. nov.

Methyloarcula terricola (ter.ri'co.la. Gr. adj. *terricola* of the soil).

Has all the characteristics which define the genus. Additionally, it has the following characteristics. Cells are non-motile rods, $0.5\text{--}0.8 \times 1.6\text{--}2.0\ \mu\text{m}$. Colonies on methylamine-agar medium (4–5 d) are white, 2 mm in diameter. Oxidase and catalase-positive. Urease and lipase-negative. Nitrate is not utilized. Able to grow at $10\text{--}40\ ^\circ\text{C}$, pH 5.5–10.0 and 0.05–14% NaCl. Optimal conditions for growth are $29\text{--}32\ ^\circ\text{C}$, pH 7.5–8.5 and

3–6% NaCl. Utilizable carbon sources are mono- and dimethylamine, fructose, glucose, maltose, lactose, mannose, ribose, trehalose, galactose, xylose, sucrose, succinate, pyruvate, acetate. Methylamine is oxidized to formaldehyde by the N-MG pathway enzymes: γ -GMA synthetase/lyase and N-MG synthase/lyase. DNA G + C content is 57.1 mol% (T_m). Level of DNA–DNA hybridization with the type species *Methyloarcula marina* is around 25–30% (membrane method). The type strain *Methyloarcula terricola* h37^T was isolated from coastal salty soil of the Black Sea (Crimea) and is deposited in the Russian Collection of Microorganisms as VKM B-2160^T.

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