Phylogenetic analysis of psychrophilic bacteria isolated from the Japan Trench, including a description of the deep-sea species *Psychrobacter pacificensis* sp. nov.

Akihiko Maruyama,¹ Daisuke Honda,^{†1,2} Hiroyuki Yamamoto,³ Keiko Kitamura¹ and Takanori Higashihara¹

Author for correspondence: Akihiko Maruyama. Tel: +81 298 61 6062. Fax: +81 298 61 6412.
e-mail: maruyama@nibh.go.jp

¹ National Institute of Bioscience and Human-Technology, Tsukuba, Ibaraki 305-8566, Japan

² Marine Biotechnology Institute Co. Ltd, Kamaishi, Iwate 026-0001, Japan

³ St Marianna University School of Medicine, Kawasaki, Kanagawa 216-8511, Japan

Phylogenetic positions of psychrophilic bacteria isolated from the Japan Trench were determined by sequencing analysis of PCR-amplified bacterial small subunit (16S) rRNA genes. Between surface and deep-sea psychrophiles, distinct positions clearly differed within the γ -Proteobacteria. In phylogenetic analysis using neighbour-joining, maximum-parsimony and maximum-likelihood, strains from surface seawater were inferred to be located in the *Halomonas aquamarina-meridiana* clade within the family Halomonadaceae. Strains from deep seawater (5000–6000 m), however, formed a novel monophyletic clade within the *Moraxella-Psychrobacter* branch in the family Moraxellaceae, showing separation from terrestrial and Antarctic relatives. These deep-sea strains were also discriminated from other known *Psychrobacter* species in phenotype, e.g. limited growth in the absence of NaCl (optimum at about 3% NaCl), positive urease activity, acid production from xylose and arabinose, and the presence of multiple fimbriae. DNA relatedness values among six deep-sea strains were >85% in DNA–DNA hybridization experiments and >98% in aligned 16S rDNA sequences. From this evidence, a new species, *Psychrobacter pacificensis*, is proposed for these deep-sea psychrophiles; the type strain of *Psychrobacter pacificensis* is strain NIBH P2K6^T (= IFO 16270^T). Occurrence of psychrobacters in cold Japan Trench deep seawater and at the Antarctic sea surface suggests that deep-sea bacterial habitation and evolution have been mediated by global deep-ocean circulation linked to the sinking of cooled seawater in polar regions.

Keywords: marine bacteria, psychrophiles, *Psychrobacter pacificensis*, *Moraxella*, *Halomonas*

INTRODUCTION

Most bacteria found in surface to deep marine environments remain to be characterized because of difficulties in cultivation and isolation. Knowledge on the origins and habitats of even culturable microbes is still limited, especially for those from the deep ocean.

These microbes appear to have adapted to or survived in deep-sea environments under physico-chemical parameters that differ from those at the ocean surface, e.g. dark, cold, high pressure, relatively stable salinity and richness in inorganic nutrients but ordinarily a paucity of organic nutrients.

Many psychrophilic bacteria, which grow at as low as 4 °C and at more than 20 °C, have been previously isolated from the surface and deep-sea environments of the Japan Trench (Maruyama *et al.*, 1997). These psychrophiles appear to be distributed in various marine environments, although their predominance differs in the Antarctic (Simidu *et al.*, 1986), ocean

[†] **Present address:** Faculty of Science, Konan University, 8-9-1 Okamoto, Higashinada, Kobe, Hyogo 658-8501, Japan.

Abbreviations: ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining; PNPG, *p*-nitrophenyl β -D-galactopyranoside.

Table 1. Bacterial species, strains, sources and 16S rDNA sequence information

Species	Strain*	Sequence accession number (length)	Original source and other names
Halomonadaceae and related species			
<i>Halomonas aquamarina</i>	ATCC 14400 ^T	M93352 (1466 bp)	' <i>Achromobacter aquamarinus</i> ', ' <i>Deleya aquamarina</i> '
<i>Halomonas cupida</i>	DSM 4740 ^T	L42615 (1471 bp)	' <i>Deleya cupida</i> '
<i>Halomonas elongata</i>	ATCC 33173 ^T	M93355 (1479 bp)	
<i>Halomonas halmophila</i>	ATCC 19717 ^T	M59153 (1540 bp)	' <i>Flavobacterium halmephilum</i> '
<i>Halomonas halodenitrificans</i>	ATCC 13511 ^T	L04942 (1531 bp)	' <i>Paracoccus halodenitrificans</i> '
<i>Halomonas halodurans</i>	DSM 5160 ^T	L42619 (1474 bp)	
<i>Halomonas halophila</i>	DSM 4770 ^T	M93353 (1453 bp)	' <i>Deleya halophila</i> '
<i>Halomonas marina</i>	ATCC 25374 ^T	M93354 (1446 bp)	' <i>Arthrobacter marinus</i> ', ' <i>Deleya marina</i> '
<i>Halomonas meridiana</i>	DSM 5425 ^T	M93356 (1475 bp)	
<i>Halomonas pacifica</i>	DSM 4742 ^T	L42616 (1480 bp)	' <i>Deleya pacifica</i> '
<i>Halomonas pantelleriensis</i>	AAP	X93493 (1477 bp)	
<i>Halomonas salina</i>	ATCC 49509 ^T	X87217 (1478 bp)	' <i>Deleya salina</i> '
<i>Halomonas subglaciescola</i>	DSM 4683 ^T	M93358 (1481 bp)	
<i>Halomonas variabilis</i>	DSM 3051 ^T	M93357 (1470 bp)	' <i>Halovibrio variabilis</i> '
<i>Halomonas venusta</i>	DSM 4743 ^T	L42618 (1479 bp)	' <i>Deleya venusta</i> '
<i>Marinomonas vaga</i>	ATCC 27119 ^T	X67025 (1467 bp)	' <i>Alteromonas vaga</i> ', ' <i>Oceanospirillum vagum</i> '
<i>Oceanospirillum linum</i>	ATCC 11336 ^T	M22365 (1517 bp)	
<i>Halomonas eurihalina</i>	ATCC 49336 ^T	L42620 (1490 bp)	' <i>Volcaniella eurihalina</i> '
<i>Zymobacter palmae</i>	T109	D14555 (1503 bp)	
<i>Halomonas</i> sp.	NIBH P1H8	AB016049 (1517 bp)	Japan Trench surface seawater (Station P1; 0 m)
<i>Halomonas</i> sp.	NIBH P1H13	AB016050 (1525 bp)	Japan Trench surface seawater (Station P1; 0 m)
<i>Halomonas</i> sp.	NIBH P1H14	AB016051 (1526 bp)	Japan Trench surface seawater (Station P1; 0 m)
<i>Halomonas</i> sp.	NIBH P1H22	AB016052 (1528 bp)	Japan Trench surface seawater (Station P1; 0 m)
<i>Halomonas</i> sp.	NIBH P1H25	AB016053 (1519 bp)	Japan Trench surface seawater (Station P1; 0 m)
Moraxellaceae			
' <i>Acinetobacter anitratus</i> '	ATCC 15308	U10874 (1528 bp)	' <i>Acinetobacter anitratus</i> ', ' <i>Acinetobacter baumannii</i> '
<i>Acinetobacter baumannii</i>	DSM 30007 ^T	X81660 (1459 bp)	' <i>Bacterium anitratus</i> '
<i>Acinetobacter calcoaceticus</i>	33604	M34139 (1536 bp)	' <i>Moraxella calcoacetica</i> ', ' <i>Neisseria winogradskyi</i> ', ' <i>Micrococcus calcoaceticus</i> ', <i>Acinetobacter</i> sp.
<i>Acinetobacter haemolyticus</i>	DSM 6962 ^T	X81662 (1460 bp)	
<i>Acinetobacter johnsonii</i>	DSM 6963 ^T	X81663 (1460 bp)	
<i>Acinetobacter junii</i>	DSM 1532	X81658 (1459 bp)	
<i>Acinetobacter lwoffii</i>	ATCC 17925	U10875 (1538 bp)	' <i>Acinetobacter lwoffii</i> '
<i>Acinetobacter radioresistens</i>	DSM 6976 ^T	X81666 (1459 bp)	
<i>Moraxella catarrhalis</i>	ATCC 25238 ^T	U10876 (1511 bp)	' <i>Branhamella catarrhalis</i> '
<i>Moraxella lacunata</i>	ATCC 17967 ^T	D64049 (1519 bp)	
<i>Psychrobacter frigidicola</i>	ACAM 304 ^T	U46143 (1484 bp)	Antarctic ornithogenic soil
<i>Psychrobacter glacincola</i>	ACAM 483 ^T	U46145 (1467 bp)	Antarctic sea ice; ' <i>Psychrobacter endoglaciicola</i> '
<i>Psychrobacter immobilis</i>	ATCC 43116 ^T	U39399 (1477 bp)	Seawater, fish, pork sausage, ground beef, etc.

Table 1 (cont.)

Species	Strain*	Sequence accession number (length)	Original source and other names
<i>Psychrobacter immobilis</i>	ACAM 286	U46139 (1476 bp)	Antarctic ornithogenic soil
<i>Psychrobacter phenylpyruvicus</i>	ATCC 23333 ^T (= ACAM 535 ^T)	U46144 (1433 bp)	Blood; ' <i>Moraxella phenylpyruvica</i> ', ' <i>Psychrobacter phenylpyruvicum</i> '
<i>Psychrobacter urativorans</i>	ACAM 534 ^T (= ATCC 15174 ^T)	U46141 (1485 bp)	Pork sausage; ' <i>Micrococcus cryophilus</i> ', ' <i>Psychrobacter uratovorans</i> '
<i>Psychrobacter pacificensis</i>	NIBH P2J2	AB016054 (1536 bp)	Japan Trench deep seawater (Station P2; 5000 m)
<i>Psychrobacter pacificensis</i>	NIBH P2J3	AB016055 (1531 bp)	Japan Trench deep seawater (Station P2; 5000 m)
<i>Psychrobacter pacificensis</i>	NIBH P2J13	AB016056 (1525 bp)	Japan Trench deep seawater (Station P2; 5000 m)
<i>Psychrobacter pacificensis</i>	NIBH P2K6 ^T (= IFO 16270 ^T)	AB016057 (1526 bp)	Japan Trench deep seawater (Station P2; 6000 m)
<i>Psychrobacter pacificensis</i>	NIBH P2K17	AB016058 (1530 bp)	Japan Trench deep seawater (Station P2; 6000 m)
<i>Psychrobacter pacificensis</i>	NIBH P2K18	AB016059 (1520 bp)	Japan Trench deep seawater (Station P2; 6000 m)
Others			
<i>Bacillus subtilis</i>	168	D26185 (1553 bp)	
<i>Escherichia coli</i>	Unknown	J01695 (1542 bp)	
<i>Pseudomonas aeruginosa</i>	DSM 50071 ^T	X06684 (1537 bp)	
<i>Pseudoalteromonas</i> sp.	NIBH PS2M11	AB016267 (1522 bp)	Japan Trench deep-sea sediment (Station PS2; 3569 m)
<i>Pseudoalteromonas</i> sp.	NIBH PS1M3	AB016268 (1524 bp)	Japan Trench deep-sea sediment (Station PS1; 5974 m)
<i>Bacillus</i> sp.	NIBH PS3M5	AB016269 (1542 bp)	Japan Trench deep-sea sediment (Station PS3; 2169 m)
<i>Bacillus</i> sp.	NIBH PS3M26	AB016270 (1540 bp)	Japan Trench deep-sea sediment (Station PS3; 2169 m)
<i>Vibrio</i> sp.	NIBH P2K5	AB016271 (1533 bp)	Japan Trench deep seawater (Station P2; 6000 m)

* ATCC, American Type Culture Collection, Manassas, VA, USA; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; ACAM, Australian Collection of Antarctic Micro-organisms, University of Tasmania, Australia; NIBH, National Institute of Bioscience & Human-Technology, Tsukuba, Japan; IFO, Institute for Fermentation, Osaka, Japan.

upwelling (Rüger, 1989) and hydrothermal environments (Maruyama *et al.*, 1993). From growth-temperature experiments with psychrophilic bacteria from the Japan Trench, higher degrees of psychrophily were generally found in deep-sea strains than in surface strains (Maruyama *et al.*, 1997). These marine psychrophiles were also characterized by an abundance of *Moraxella*-like bacteria, especially in deep seawater. RFLP analysis of bacterial 16S rRNA genes suggests that these deep-sea *Moraxella*-like bacteria are not assignable to representative species in the genera *Moraxella* and *Psychrobacter* of the family *Moraxellaceae* (Maruyama *et al.*, 1997).

Moraxella-like bacteria characterized by Gram-negative, aerobic, oxidase-positive, non-motile and non-pigmented coccobacilli have been isolated from natural environments and cold-storage meat products such

as fish, poultry, lamb and beef (Juni & Heym, 1980, 1986; Maruyama *et al.*, 1986, 1990; Prieto *et al.*, 1992; Shaw & Latty, 1988; Shewan *et al.*, 1960; Simidu *et al.*, 1977, 1980). Among these, psychrophilic moraxellas, previously termed *Achromobacter*, have been classified in the genus *Psychrobacter* (Juni & Heym, 1986). In addition to the genospecies *Psychrobacter immobilis*, Bowman *et al.* (1996, 1997) recently proposed some new species of Antarctic origin, i.e. *Psychrobacter urativorans* and *Psychrobacter frigidicola* from Antarctic ornithogenic soils and *Psychrobacter glacicola* from Antarctic sea ice, indicating that members of the genus *Psychrobacter* are widespread in cold Antarctic environments.

Some members of the genera *Halomonas*, *Deleya* and *Halovibrio* in the family *Halomonadaceae* that are characterized by aerobic, motile or non-motile, mod-

Fig. 1. Phylogenetic relatedness between psychrophilic bacteria from surface seawater and members of the family *Halomonadaceae*. The phylogenetic tree was inferred by ML using a gap-free aligned 16S rDNA sequence profile (27 operational taxonomic units, total 1036 nt). The alpha/beta parameter was set at 2.158. Bootstrap probabilities less than 50% are shown by '<' and those estimated by NJ and MP are given in parentheses, e.g. ML (NJ, MP). *Bacillus subtilis* was used as an outgroup taxon.

erately or strictly halophilic, Gram-negative rods (Holt *et al.*, 1994) may be assigned as *Moraxella*-like in diagnostic identification. Since difficulties exist in differentiating these genera using chemotaxonomic and phylogenetic parameters, Dobson & Franzmann (1996) recently proposed unification of the genera *Deleya*, *Halomonas* and *Halovibrio* and the species *Paracoccus halodenitrificans* into a single genus, *Halomonas*. Although little data on their psychrophily exists, it is plausible that some non-motile *Halomonas* members have been identified as *Moraxella*-like bacteria among heterotrophs culturable at temperatures below 10 °C.

The genus *Moraxella* was established with bacteria isolated from warm-blooded animals (Krieg & Holt, 1984), but more attention must be paid to these *Moraxella*-like bacteria because of their wide distribution in natural and artificial environments from cold to moderate temperatures. In this study, phylogenetic positions of psychrophilic moraxellas of

oceanic-surface and deep-sea origins were defined, their motility was cross-checked and appendages were observed. Together with phenotypic and chemotaxonomic results, a new species, *Psychrobacter pacificensis*, is proposed for these psychrophilic moraxellas of deep-sea origin.

METHODS

Bacterial strains. The 16 strains studied were selected from 67 strains isolated from surface and deep-sea environments of the Japan Trench (Table 1); of these, 11 were tentatively identified as *Moraxella*-like bacteria. They were purified on different agar media including artificial-seawater-based 1/2 TZ containing polypeptone and yeast extracts (Maruyama *et al.*, 1993), marine agar 2216 (Difco) and nutrient agar (Difco). Each strain was incubated at 20 °C and cells in the middle to late exponential growth phases were used for experiments unless otherwise noted. A 1/2 TZ semi-solid agar medium containing 0.3% agar instead of the original concentration of 1.5% was used for preservation at 4 °C. For long-term preservation, cells were suspended in a sterilized 1/2 SW preservative [12% (w/v) sucrose, 50% (v/v) horse serum and 50% (v/v) aged seawater], dried from the liquid state in an aseptically glass tube under a vacuum at room temperature, enclosed in the tube under the vacuum, and stored at 4 °C (Suzuki & Yamasato, 1986).

DNA sequencing and alignment. For *Moraxella*-like strains isolated from deep seawater, the sequence of the 16S rRNA gene was determined as follows. Cells were harvested by centrifugation, washed and resuspended in TE buffer (10 mM Tris/HCl, 1 mM EDTA; pH 8.0). Genomic DNA was extracted by a standard protocol using cetyltrimethylammonium bromide, phenol, chloroform and isoamyl alcohol (Murray & Thompson, 1980). PCR for the almost complete 16S rRNA gene was conducted by GeneAmp PCR 9600 (Perkin-Elmer) using primers 27f and 1525r (Lane, 1991) and a previously described PCR cycle (Maruyama *et al.*, 1997). Excess primers and dNTP were removed from PCR products using Suprec 02 (Takara). Purified PCR products were sequenced directly by an automated DNA sequencer (ALFred; Pharmacia) in cycle sequencing according to the manufacturer's instructions, using appropriate forward and reverse primers (Lane, 1991): 342r, 359f (5'-TCC TAC GGG AGG CAG CAG TG; 20-mer), 519r, 803r (5'-CAT CGT TTA CGG CGT GGA C; 19-mer), 821f (5'-GTC CAC GCC GTA AAC GAT G; 19-mer), 1104r (5'-TTG CGC TCG TTG CGG GAC; 18-mer) and 1111f (5'-GTC CCG CAA CGA GCG CAA; 18-mer) in the *Escherichia coli* numbering system. Sequences for each fragmental 16S rDNA region were determined over both strands and were connected by GENETYX software (version 8; Software Development). Except for deep-sea strains, bacterial 16S rDNA was extracted, amplified and subcloned as described previously (Maruyama *et al.*, 1997). Sequences were aligned using a multiple alignment program in CLUSTAL W (version 1.71; Thompson *et al.*, 1994). Our determined sequences were fitted to known aligned sequences obtained from the rRNA server at the University of Antwerp (<http://rrna.uia.ac.be/>; Van de Peer *et al.*, 1997) using a CLUSTAL W profile alignment option. From this aligned data matrix, all positions with gaps, i.e. undetermined and ambiguous sequences, were removed. The plain, aligned sequence data set was then used for phylogenetic analysis. Species names, accession numbers and sequence lengths used are shown in Table 1.

Table 2. Motility and appendages of psychrophilic bacteria from surface and deep seawater in the Japan Trench

Strain	Motility*		Appendage†	Phylogenetic location
	Microscopic	Agar plate		
Surface seawater				
P1H8	—	—	Flagella‡	<i>Halomonadaceae</i>
P1H13	—	—	None	<i>Halomonadaceae</i>
P1H14	—	—	None	<i>Halomonadaceae</i>
P1H22	+	+	Flagella	<i>Halomonadaceae</i>
P1H25	+	+	Flagella	<i>Halomonadaceae</i>
Deep seawater				
P2J2	—/w	—	Fimbriae	<i>Moraxellaceae</i>
P2J3	—/w	—	Fimbriae	<i>Moraxellaceae</i>
P2J13	—/w	—	Fimbriae	<i>Moraxellaceae</i>
P2K6 ^T	—/w	—	Fimbriae	<i>Moraxellaceae</i>
P2K17	—/w	—	Fimbriae	<i>Moraxellaceae</i>
P2K18	—/w	—	Fimbriae	<i>Moraxellaceae</i>

* Microscopic motility was assessed by light microscopy with Nomarski apparatus; agar plate motility was assessed on semi-solid agar plates with nutrient gradient. —, Negative; +, positive; w, weak twitching motility.

† EM observation.

‡ Flagellum attachment was occasionally observed.

Phylogenetic analysis. Maximum-likelihood (ML; Felsenstein, 1981), neighbour-joining (NJ; Saitou & Nei, 1987) and maximum-parsimony (MP; Fitch, 1971) were used to construct phylogenetic trees. ML analysis was implemented with NUCML and NJDIST programs in MOLPHY (version 2.3b; Adachi & Hasegawa, 1996). Using a distance matrix estimated by ML, an NJ tree was first inferred as the starting tree. The NJ tree was then analysed by local rearrangement (Adachi & Hasegawa, 1996) using the HKY85 model (Hasegawa *et al.*, 1985) to search for the ML tree. NJ was analysed using Kimura's two-parameter procedure (Kimura, 1980) and PHYLIP software (version 3.57c; Felsenstein, 1995). MP was analysed by a heuristic search with options of random stepwise addition (100 replicates), a tree-bisection-reconnection branch swapping algorithm, and MULPARS, using PAUP* (test version 4.0d63; David L. Swofford). A strict consensus tree was selected from 35 equal parsimonious trees in *Halomonadaceae* analysis or 40 equal ones in *Moraxellaceae* analysis. The statistical significance of tree branches was assessed by bootstrap analysis involving the construction of 1000 trees from resampled data in NJ and 100 trees in MP. All ML, NJ and MP trees were further analysed by NUCML and the best ML tree with the highest likelihood and the lowest estimate of Akaike's information criterion (Akaike, 1974) was selected. Local bootstrap probabilities in the best ML tree were calculated by resampling estimated log-likelihood (Hasegawa & Kishino, 1994; Kishino *et al.*, 1990).

DNA–DNA hybridization. Bacterial DNA was purified by the general extraction method using phenol mixture [phenol, chloroform and 3-methyl-1-butanol (25: 24: 1)] and ethanol. To increase DNA purity, cells in TE buffer were also pretreated with lysozyme (0.1 mg ml⁻¹) and 3% SDS at 60 °C for 10 min. After extraction, the ethanol precipitate was treated with RNase A (10 µg ml⁻¹) at 35 °C for 30 min, incubated with proteinase K (10 µg ml⁻¹) and 0.3% SDS at

50 °C for 60 min, and purified again. Purified DNA was then dissolved in TE buffer, immobilized on a microplate (Ezaki *et al.*, 1989) and labelled using a photoactivatable form of biotin (PHOTOPROBE Long Arm Biotin, SP-1020; Vector Laboratories). The relative relatedness among the DNA samples was determined by colorimetric microplate hybridization (Kusunoki *et al.*, 1991). Blocking solution [2 × SSC (0.3 M NaCl, 0.03 M sodium citrate) containing 2% blocking reagent (Roche Diagnostics)] was added to DNA plate wells and kept at room temperature for 30 min. After discarding this, 100 µl hybridization solution (2 × SSC, 50% formamide, 1% blocking reagent; pH 7.2–7.6) containing labelled single-stranded DNA was distributed to wells. After 16 h hybridization at 55 °C, wells were washed three times with 1 × SSC solution. Alkaline phosphatase–streptavidin (Vector Laboratories) was diluted 1000 times with PBS(–) (sodium chloride, 8 g; potassium chloride, 0.2 g; disodium phosphate, 1.15 g; monopotassium phosphate, 0.20 g; distilled water, 1000 ml; pH 7.3–7.6) containing blocking agent (Block Ace TM Powder; Dai Nippon Seiyaku), and 100 µl of the dilution was distributed to wells. After 30 min incubation at 35 °C, wells were washed three times with PBS(–) and filled with 100 µl substrate solution (BluePho TM Microwell Phosphatase Substrate system; Kirkegaard & Perry Laboratories). The plate was incubated at room temperature and the A_{630} value of each well was measured. Homology values among strains were calculated from triplicate DNA–DNA hybridization experiments.

Motility tests and microscopic observation. Bacterial cells in the early exponential growth phase were used for motility tests. Bacterial motility was observed under a microscope (Axiophot; Zeiss) using Nomarski differential interference contrast. Motility was also tested on a semi-solid agar plate made by pouring plain agar containing 0.3% purified agar

Fig. 2. Phylogenetic relatedness between psychrophilic bacteria from deep seawater and members of the family *Moraxellaceae*. The phylogenetic tree was inferred by ML using a gap-free aligned 16S rDNA sequence profile (24 operational taxonomic units, total 1174 nt). The alpha/beta parameter was set at 2.152. Bootstrap probabilities less than 50% are shown by '<' and those estimated by NJ and MP are given in parentheses, e.g. ML (NJ, MP). *Bacillus subtilis* was used as an outgroup taxon. Thicker lines show the *Moraxella*–*Psychrobacter* branch. *, The same phylogenetic position as *P. immobilis* ATCC 43116^T.

(Difco) and 90% artificial seawater (Kester *et al.*, 1967) onto small amounts of marine agar 2216 (Difco) solidified at the corner of a sterilized plate. Cells were inoculated onto the semi-solid agar surface 3–4 cm from the nutrient-rich corner and incubated at both 4 and 20 °C. For electron microscopic observation, cells were fixed with 5% (v/v) glutaraldehyde (TAAB) in HM buffer (100 mM HEPES, 2 mM MgCl₂; pH 6.8) and resuspended in 3% NaCl solution. Alternatively, fixed cells were harvested by slow centrifugation at 2000–2300 g for 5 min and washed with pure water, recentrifuged, then a small portion of cell pellets was resuspended with pure water. The cell suspension was mixed with an equal volume of 5% glutaraldehyde in 0.2 M cacodylate buffer. Cells were finally placed on EM grids covered with polyvinyl Formvar film, treated with a drop of 1% uranyl acetate for negative staining and dried at room temperature. A Hitachi H-7000 EM was used to observe bacterial cells and their appendages.

Phenotypic tests. Phenotypic tests for key taxonomic items such as Gram reaction, oxidase, catalase, glucose fermentation and acidification of carbohydrates were examined based on a standard manual (Smibert & Krieg, 1994). Nitrate reduction and urease activity were determined by the method of Yamasato *et al.* (1982). Phenylalanine and

tryptophan deaminase were determined according to Bøvre & Henriksen (1976). Tolerance to different concentrations of NaCl was defined at 20 °C on 1/2 TZ agar medium, which replaced artificial seawater with pure water. The API 20NE system (bioMérieux) was used for tests of oxidase, nitrate reduction, indole production, glucose fermentation, arginine dihydrolase, urease, aesculin decomposition, gelatin hydrolysis and β -galactosidase using *p*-nitrophenyl β -D-galactopyranoside (PNPG), and the use of different substrates as sole carbon and energy sources. Substrate use was also determined by the API ID 32GN system (bioMérieux).

Fatty acid, quinone and GC content analyses. Cells were hydrolysed and methylated with methanol containing 5% HCl and fatty acids were extracted with *n*-hexane. Fatty acid profiles were determined with a GC (GC-14A; Shimadzu) having a methyl silicone-coated capillary column (0.25 mm in diameter, 15 m long, 0.25 μ m depth of film) and a flame-ionization detector under increasing temperatures of 150–240 °C at 4 °C min⁻¹, using helium carrier gas at a flow rate of 2.3 ml min⁻¹ and a split ratio of 1:30. Cellular quinones were extracted with chloroform:methanol (2:1, v/v), purified by TLC, and determined by HPLC (LC-6A; Shimadzu) with a Zorbax-ODS column (4.6 mm in diameter, 250 mm long) and a UV detector (275 nm), using methanol:isopropylether (3:1, v/v) eluent at a flow rate of 1 ml min⁻¹. For DNA G+C content, bacterial DNA was digested into nucleotides with nuclease P1 (Yamasa) and then into nucleosides with alkaline phosphatase. The relative content of both components to total nucleosides (mol%) was estimated by HPLC using a Cosmosil C18 column (4.6 mm in diameter, 150 mm long; Cosmo Bio), 0.2 M ammonium phosphate:acetonitrile (30:1, v/v) eluent (1 ml min⁻¹) and a UV detector (270 nm).

RESULTS

The phylogenetic relatedness of *Moraxella*-like bacteria isolated from surface seawater in the Japan Trench was studied by ML (Fig. 1) in which almost complete 16S rDNA sequence data from approved species belonging to the family *Halomonadaceae* was used. All five surface strains (P1H8, P1H13, P1H14, P1H22 and P1H25) were located around *Halomonas aquamarina* and *Halomonas meridiana*. Along with these known species, they made an independent clade on the *Halomonas* sub-branch, i.e. the former *Deleya*–*Halomonas*–*Halovibrio* branch, at a bootstrap probability of 91% (Fig. 1). NJ at a bootstrap probability of 82% and MP at 63% also supported this primary structure, although slightly different branching was found downstream. Pairwise distances between taxa also indicated high sequence similarities between this clade's members, >98% in aligned 16S rDNA sequences, and clear discrimination of these strains from the closest neighbour, *Halomonas venusta*. Based on these data, all surface strains were phylogenetically assigned as members of the *Halomonas aquamarina*–*meridiana* group in the family *Halomonadaceae*.

Two of the five strains, P1H22 and P1H25, possessed weak motility as determined by microscopic observation and a cultivation experiment using semi-solid agar plates with a nutrient gradient (Table 2). After

Table 3. DNA relatedness among *Psychrobacter* species and strains by DNA–DNA hybridization

The percentage homology is shown.

Strain	<i>P. immobilis</i> ATCC 43116 ^T	<i>P. glacincola</i> ACAM 483 ^T	<i>P. pacificensis</i>						<i>E. coli</i>
			P2J2	P2J3	P2J13	P2K6 ^T	P2K17	P2K18	
<i>P. immobilis</i> ATCC 43116 ^T	100	22	27	15	22	21	24	28	0
<i>P. glacincola</i> ACAM 483 ^T	16	100	17	9	12	14	14	22	0
<i>P. pacificensis</i> P2J2	20	17	100	96	98	98	94	103	0
<i>P. pacificensis</i> P2J3	8	6	94	100	91	90	85	100	0
<i>P. pacificensis</i> P2J13	17	14	95	93	100	93	91	97	0
<i>P. pacificensis</i> P2K6 ^T	19	17	96	94	94	100	94	100	0
<i>P. pacificensis</i> P2K17	27	22	94	93	93	93	100	97	0
<i>P. pacificensis</i> P2K18	22	21	92	91	88	89	86	100	0

EM, the presence of peritrichous flagella was confirmed for these two sluggishly motile strains (Fig. 3A, B). All five strains were also found to be halophilic, growing on 12% NaCl-based nutrient agar. This group of psychrophilic bacteria from surface seawater is thus characterized as aerobic, Gram-negative, non-pigmented, oxidase-positive, motile or non-motile, halophilic, rod-shaped cells – characteristic of the genus *Halomonas*.

All six *Moraxella*-like strains from deep seawater (P2J2, P2J3, P2J13, P2K6^T, P2K17 and P2K18) were located phylogenetically on the *Moraxella*–*Psychrobacter* branch in the family *Moraxellaceae*, which includes the genera *Moraxella*, *Acinetobacter* and *Psychrobacter* (Fig. 2). The best phylogenetic tree inferred from ML showed that these strains formed a monophyletic clade separate from the *Moraxella* clade on the *Moraxella*–*Psychrobacter* branch at a probability of 100% (Fig. 2). This deep-sea bacterial clade could also be differentiated from the *Psychrobacter* clade of *P. immobilis*, *P. urativorans*, *P. frigidicola*, *Psychrobacter phenylpyruvicus* and *P. glacincola*, all of which, except for *P. phenylpyruvicus*, were widespread in Antarctic environments (Table 1). Two other topologies inferred from NJ and MP gave the same three clades on the *Moraxella*–*Psychrobacter* branch at probabilities of 100%. Phylogenetically, these deep-sea moraxellas appear to form a sister group to the *Moraxella* and *Psychrobacter* groups in the family *Moraxellaceae*.

In quantitative DNA–DNA hybridization experiments, genomic relatedness among these six deep-sea strains exceeded 85%, whereas genomic relatedness was less than 30% with *P. immobilis* or *P. glacincola* (Table 3). Pairwise distance analysis of aligned 16S rDNA sequences also showed > 97% similarity between deep-sea members, but lower similarity to any known *Psychrobacter* species. From these types of relatedness, the six deep-sea strains appeared to form a single independent species.

Under light microscopy, occasional and very weak motility was observed among these deep-sea strains, but no distinct motility was found in the culture experiment using semisolid agar plates (Table 2). Many fimbria-like appendages were detected around cells by EM, but no flagella were found (Fig. 3C, D). Most strains grew at 4–35 °C in the presence of 3% NaCl, but produced only poor growth in the absence of NaCl. They were not fermentative in the O-F test. The following tests were positive for all three strains studied: catalase, oxidase and urease; tolerance to 6.5% NaCl; and use of L-proline, L-histidine and DL-malate as sole carbon and energy sources. All strains were capable of producing acid from carbohydrates such as glucose, xylose and arabinose. Tests negative for all strains studied are shown in Table 4. In key phenotypic characteristics, these deep-sea strains were almost identical to members of the genus *Psychrobacter*, characterized by aerobic, Gram-negative, non-pigmented, oxidase-positive, non-motile, psychrophilic coccobacilli, but not identical in detail to any *Psychrobacter* phenon type (Table 4).

DNA G + C contents determined by HPLC for three representative deep-sea strains were 44–45 mol% (Table 4). Whole-cell fatty acid profiles were very similar, indicating 16:0, 16:1 ω 7c, 18:0 and 18:1 ω 9c as major components (Table 5). More than half of the total content was attributable to the unsaturated fatty acid 18:1 ω 9c. The major quinone type for these strains was Q8 (Table 5).

Two other *Moraxella*-like strains isolated from deep-sea sediments, PS2M11 and PS1M3 (Table 1), clustered on the *Alteromonas* branch and were finally identified as members of the genus *Pseudoalteromonas* with phenotypic mismatch in motility. Strains PS3M5 and PS3M26 from deep-sea sediments were on the *Bacillus* branch and strain P2K5 from deep seawater was on the *Vibrio* branch (Table 1), indicating no contradiction between phylogenetic positions and conventional identification results. No psychrobacters were detected in isolates from deep-sea sediments.

Fig. 3. Electron micrographs of psychrophilic bacteria, *Halomonas* sp. [A, P1H22 (bar, 1 µm); B, P1H25 (bar, 1 µm)] from surface seawater and *Psychrobacter pacificensis* [C, P2J3 (bar, 1 µm); D, P2K18 (bar, 1 µm)] from deep seawater. Enlarged photos of appendages are shown for surface (flagella: a, b; bars, 0.1 µm) and deep-sea strains (fimbriae: c, d; bars, 0.1 µm).

DISCUSSION

In a previous report (Maruyama *et al.*, 1997), almost half of the psychrophilic bacteria isolated from the Japan Trench were tentatively identified as members of the *Moraxella*-like group. The deep-sea bacterial community was then characterized by the abundance of these moraxellas (> 90%), and growth-temperature experiments showed that deep-sea microbes possessed a lower predicted minimum growth temperature (T_{min}) than surface microbes at the community level, in which T_{min} is a good indicator of low-temperature adaptation by bacteria. Our study indicated that these deep-sea psychrophiles with lower T_{min} were clearly differentiated from surface psychrophiles phylogenetically, i.e. the *Halomonas* group from the oceanic surface water group and the *Psychrobacter* group from the deep seawater group.

All *Moraxella*-like psychrophiles from deep seawater were phylogenetically located near the *Psychrobacter* clade in the family *Moraxellaceae*, which included three genera – *Moraxella*, *Acinetobacter* and *Psychrobacter* (Rossau *et al.*, 1991). Key phenotypic features of our deep-sea psychrophiles also coincided with the

original description of the genus *Psychrobacter*, i.e. psychrophilic, Gram-negative, aerobic, oxidase-positive, non-motile and non-pigmented coccobacilli (Juni & Heym, 1986), although no information exists about appendages. These were clearly distinct from known *Psychrobacter* species phylogenetically and genotypically, e.g. in tree topology, bootstrap probability, sequence similarity of 16S rDNA and genomic DNA relatedness.

Significant differences between our strains and the known *Psychrobacter* species were also found in the following phenotypic characteristics: NaCl requirement for growth, acid production from carbohydrates, enzyme activities, and compounds used as sole carbon and energy sources. All *Psychrobacter* species are reported to grow well in a wide range of NaCl concentrations of 0–8% (Bowman *et al.*, 1996, 1997), whereas deep-sea strains seldom or never grow at an NaCl concentration of 0 or 8%. This feature of deep-sea strains appears to come from the stable salinity of the original habitat, i.e. salt concentration in the 5000–6000 m deep seawater of about 3.6%. Unlike deep-sea strains, four of the five *Psychrobacter* species

Table 4. Phenotypic characteristics of *Psychrobacter pacificensis* and related species

All species and strains were positive for oxidase, catalase, growth at 4–15 °C, tolerance to 6.5 % NaCl, and use of L-proline as sole carbon and energy source. The following carbon and energy sources were not used by any species or strain: *N*-acetylglucosamine, adipate, L-arabinose, caprate, L-fucose, 2-ketogluconate, 5-ketogluconate, D-glucose, *myo*-inositol, itaconate, maltose, D-mannitol, D-melibiose, L-rhamnose, D-ribose, salicin (D), D-sorbitol, sucrose [(D/L) showed the type of optical isomer used by Bowman *et al.* (1996)]. All species and strains were negative for the following (as determined by the API 20NE test): glucose fermentation, indole production, hydrolysis of aesculin and gelatin, and arginine dihydrolase. Frequency of positive strains in the *P. pacificensis* columns: +, positive; v, variable; –, negative; w, weak reaction. Frequency of positive strains in other columns: +, 90–100 % of the strains are positive; v+, 11–89 % are positive and the type strain is positive; v–, 11–89 % are positive and the type strain is negative; –, 0–10 % are positive. Species: 1, *P. immobilis* (phenon 1); 2, *P. urativorans* (phenon 2); 3, *P. frigidicola* (phenon 3); 4, *P. phenylpyruvicus* ACAM 535^T; 5, *P. glacincola* ACAM 483^T.

Characteristic	<i>P. pacificensis</i>				Species				
	NIBH strain no.			Species character	1*	2*	3*	4*	5†
	P2J3	P2K6 ^T	P2K18						
Urease activity	+	+	+	+	v+	v+	–	+	v–
Phenylalanine deaminase	–	–	–	–	+	–	+	+	–
Tryptophan deaminase	–	–	–	–	v–	–	+	–	–
Nitrate reduction	–	–	–	–	v–	v–	–	–	v+
Growth in NaCl (%):									
0	w	–	–	–	+	+	+		+
1	+	–	–	v	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+
8	–	–	–	–	+	+	+	+	+
Growth at (°C):									
30	+	+	+	+	+	–	–	+	–
35	+	w	+	+	v+	–	–	+	–
40	–	–	–	–					
Acid production from:									
D-Glucose	+	w	+	+					–
D-Xylose	+	w	+	+	+	–	–	–	–
D-Arabinose	+	+	+	+	+	–	–	–	–
PNPG test	–	–	–	–					
Use as sole carbon and energy sources:									
Acetate	+	–	–	v	+	v+	+	+	+
L-Alanine	+	–	–	v	+	–	–	+	v+
<i>p</i> -Hydroxybenzoate	–	–	–	–	–	v–	–	–	–
3-Hydroxybutyrate	+	–	+	v	+	+	–	+	v+
Citrate	–	–	–	–	v–	–	–	+	v+
Gluconate‡	–	–	–	–	v–	–	–	–	–
L-Histidine	+	+	+	+	+	–	–	–	v+
Lactate	+	–	+	v	+	v+	–	+	v+
Malate‡	+	+	+	+	+(L)	v+(L)	+(L)	+(L)	–(L)
Malonate	–	+	–	v	–	–	–	–	–
Propionate	–	–	–	–	v+	–	–	+	+
L-Serine	–	–	–	–	–	v–	–	–	–
Suberate	+	–	–	v	v–	–	+	–	–
<i>n</i> -Valerate	–	–	–	–	+	v+	+	+	+
Others§	–	–	–	–					
DNA G+C content (mol %)	44	44	45	44–45	44–47	44–46	41–42	43	43–44

* Data from Bowman *et al.* (1996).

† Data from Bowman *et al.* (1997). Use of compounds was estimated with the API ID 32GN test.

‡ Determined by the API 20NE test.

§ *N*-Acetyl-D-glucosamine, m-hydroxybenzoate, glycogen and phenylacetate.

Table 5. Fatty acid composition and major quinone type of *P. pacificensis* and *P. immobilis* ATCC 43116†

Tr, Trace amount (< 0.1 %). The major quinone type in all strains shown was Q8.

Composition	<i>P. pacificensis</i>			<i>P. immobilis</i> ATCC 43116 ^T	
	Average content*				
	NIBH strain no.				
	P2J3	P2K6 ^T	P2K18		
Fatty acid:					
10:0	1.3	Tr	1.2	0.8 (0.7)	0.9
11:0	0.1	Tr	0.2	0.1 (0.1)	Tr
12:0	2.2	0.8	2.3	1.8 (0.8)	Tr
14:0	0.7	0.6	0.5	0.6 (0.1)	0.3
14:1 (X1) [†]	0.1	0.2	Tr	0.1 (0.1)	0.1
15:0	0.4	Tr	0.4	0.3 (0.2)	0.2
16:0	7.3	8.7	6.5	7.5 (1.1)	4.3
16:1 (<i>ω</i> 7 <i>c</i>)	9.7	15.8	6.6	10.7 (4.7)	3.8
16:1 (X2) [†]	0.4	0.4	0.3	0.4 (0.1)	0.4
17:0	2.7	5.6	4.8	4.4 (1.5)	4.2
i17:0	0.6	Tr	0.4	0.3 (0.3)	Tr
17:1 (X3) [†]	5.1	1.5	5.1	3.9 (2.1)	6.8
18:0	9.4	5.6	13.1	9.4 (3.8)	8.0
18:1 (<i>ω</i> 7 <i>c</i>)	1.2	0.8	0.7	0.9 (0.3)	0.8
18:1 (<i>ω</i> 9 <i>c</i>)	50.1	52.8	50.9	51.3 (1.4)	63.1
18:2	3.4	2.4	1.8	2.5 (0.8)	3.5
19:0	0.4	0.4	0.6	0.5 (0.1)	0.9
20:0	Tr	Tr	Tr	Tr	0.1
Total	95.1	95.6	95.4	95.5	97.4
Total unsaturated	70.0	73.9	65.4	69.8	75.0
Hydroxy fatty acid:					
3-OH 12:0	4.1	3.6	3.9	3.9 (0.3)	2.2
3-OH 14:0	0.8	0.8	0.7	0.8 (0.1)	0.4
Total	4.9	4.4	4.6	4.6 (0.3)	2.6

* Numbers in parentheses are standard deviations ($n = 3$).

† X1–X3, exact double bond positions are not known.

(*P. urativorans*, *P. frigidicola*, *P. glacincola* and *P. phenylpyruvicus*) cannot produce acid from carbohydrates, and *P. immobilis* is positive in tests for phenylalanine deaminase and *n*-valerate use (Table 3). From this evidence, a new species, *Psychrobacter pacificensis*, is proposed for these deep-sea strains.

Ecologically, it is worth noting that almost the same phylogenetic and phenotypic group of bacteria dominated in extremely cold, remote environments such as the Antarctic surface and Japan Trench deep seawater. Other than the temperature similarity, does any close relationship exist between the two environments? Since a major but very slow deep water current is known to run constantly toward the Japan Trench from the Antarctic Ocean (Stommel, 1958), it is highly probable that some micro-organisms in Japan Trench deep seawater originate in polar regions such as the Antarctic Ocean. After the entrainment of Antarctic microbes from the surface to the deep sea via the

sinking of cooled seawater and/or faecal pellets excreted by zooplankton, further selection and evolution may occur in the deep-ocean current directed toward the Japan Trench. This hypothesis is also supported by the physiological characteristics of such deep-sea bacteria, i.e. cell membranes adapting to low temperatures at least partially preadapt to high hydrostatic pressure (Delong & Yayanos, 1985, 1986). Other than having biotechnological applicability such as useful lipids and enzymes, marine bacteria such as deep-sea psychrobacters could be indicators for evaluating global deep-ocean circulation, regional upwelling and food chains in the ocean.

As shown by Boivin-Jahns *et al.* (1995), who studied many bacterial isolates from deep-underground water both phenotypically and phylogenetically, more attention must be paid to motility tests in the conventional identification of Gram-negative, aerobic, oxidase-positive, non-pigmented bacteria. Our study

on marine bacteria also indicates that phylogenetic analysis has great advantages in the rapid, precise identification and classification of bacteria whose motility is obscure. Both motile and non-motile *Halomonas* strains were found in surface seawater and many fimbriae were observed in deep-sea psychrobacters. Since bacterial fimbriae are considered to correlate with cell attachment and gene transformation (in the latter case, called pili), occasional, very weak motility may be attributable to these appendages (Henrichsen, 1972). The relationship between motility and fimbria attachment has not yet been well characterized, but such very weak 'twitching motility' has been reported for different types of bacteria (Henrichsen, 1975), including *Moraxella* and *Acinetobacter* species (Krieg & Holt, 1984). Such appendages may contribute to cell flotation, flocculation and particle formation in nature, and flotation appears to favour the movement of these psychrobacters in the deep-ocean current. Further comprehensive studies on marine, polar and deep subsurface bacteria at the molecular phylogenetic level should present new findings in microbial systematics and in the adaptation, evolution and dispersion of microbes.

Description of *Psychrobacter pacificensis* sp. nov.

Psychrobacter pacificensis (pa.ci.fi.cen'sis. M.L. adj. *pacificensis* pertaining to the Pacific Ocean).

Strains are aerobic, Gram-negative, non-motile, non-pigmented, non-sporulating, oxidase-positive, catalase-positive coccobacilli 1.0–1.5 µm long and about 1 µm wide. Strains produce many fimbriae as appendages, but no flagella. Circular, convex, off-white colonies with a complete edge occur on agar plates containing polypeptone and yeast extract. No fluorescent pigments form. Seawater or NaCl at about 3% is required for optimal growth, with most strains not growing at 0 or 8% NaCl. It takes 1–2 weeks to reach the stationary phase at 4 °C, but isolates give growth yields at 4 °C comparable to those at 20 °C. Optimal growth occurs at about 25 °C, with the maximum growth temperature being 38 °C. Acid is formed aerobically from glucose, xylose and arabinose. Urease is positive, but phenylalanine deaminase and tryptophan deaminase are negative. The species is negative for the following biochemical tests: glucose fermentation, indole production, aesculin hydrolysis and gelatin and arginine dihydrolase. Strains use L-histidine and DL-malate as sole carbon and energy sources. Some strains use acetate, L-alanine, 3-hydroxybutyrate, lactate, malonate and suberate, but none use *p*-hydroxybenzoate, citrate, gluconate, propionate, L-serine or *n*-valerate. The major fatty acid is 18:1 ω 9c and Q8 is the major quinone. DNA G+C content is 43–44 mol% as determined by HPLC. The type strain is NIBH P2K6^T (= IFO 16270^T), isolated at 4 °C from 6000 m deep seawater in the Japan Trench off Hachijo Island, Japan.

ACKNOWLEDGEMENTS

This work was part of an Industrial Science and Technology Frontier Program supported by the Agency of Industrial Science and Technology, Japan. We thank Project Leader Ryuichiro Kurane, NIBH, for supporting the marine microbiological study. Thanks are also due to S. Yoshida, Japan Food Research Laboratories, for biochemical examination, and K. Kawasaki and H. Kanazaki, NIBH, for electron microscopy observation.

REFERENCES

- Adachi, J. & Hasegawa, M. (1996). *Computer science monographs*, no. 28. MOLPHY version 2.3 – programs for molecular phylogenetics based on maximum likelihood. Tokyo: Institute of Statistical Mathematics.
- Akaike, H. (1974). A new look at the statistical model identification. *IEEE Trans Automat Contr AC* **19**, 716–723.
- Boivin-Jahns, V., Bianchi, A., Ruimy, R., Garcin, J., Daumas, S. & Christen, R. (1995). Comparison of phenotypical and molecular methods for the identification of bacterial strains isolated from a deep subsurface environment. *Appl Environ Microbiol* **61**, 3400–3406.
- Bøvre, K. & Henriksen, S. D. (1976). Minimal standards for description of new taxa within the genera *Moraxella* and *Acinetobacter*: proposal by the subcommittee on *Moraxella* and allied bacteria. *Int J Syst Bacteriol* **26**, 92–96.
- Bowman, J. P., Cavanagh, J., Austin, J. J. & Sanderson, K. (1996). Novel *Psychrobacter* species from Antarctic ornithogenic soils. *Int J Syst Bacteriol* **46**, 841–848.
- Bowman, J. P., Nichols, D. S. & McMeekin, T. A. (1997). *Psychrobacter glacincola* sp. nov., a halotolerant, psychrophilic bacterium isolated from Antarctic sea ice. *Syst Appl Microbiol* **20**, 209–215.
- Delong, E. F. & Yayanos, A. A. (1985). Adaptation of the membrane lipids of a deep-sea bacterium to changes in hydrostatic pressure. *Science* **228**, 1101–1103.
- Delong, E. F. & Yayanos, A. A. (1986). Biochemical function and ecological significance of novel bacterial lipids in deep-sea procaryotes. *Appl Environ Microbiol* **51**, 730–737.
- Dobson, S. J. & Franzmann, P. D. (1996). Unification of the genera *Deleya* (Baumann et al. 1983), *Halomonas* (Vreeland et al. 1980), and *Halovibrio* (Fendrich 1988) and the species *Paracoccus halodenitrificans* (Robinson and Gibbons 1952) into a single genus, *Halomonas*, and placement of the genus *Zymobacter* in the family *Halomonadaceae*. *Int J Syst Bacteriol* **46**, 550–558.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric DNA–DNA hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.
- Felsenstein, J. (1995). PHYLIP (Phylogeny Inference Package) and manual, version 3.57c. Department of Genetics, University of Washington, Seattle, WA, USA.
- Fitch, W. M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* **20**, 406–416.

- Hasegawa, M. & Kishino, H. (1994). Accuracies of the simple methods for estimating the bootstrap probability of a maximum likelihood tree. *Mol Biol Evol* **11**, 142–145.
- Hasegawa, M., Kishino, H. & Yano, T. (1985). Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J Mol Evol* **22**, 160–174.
- Henrichsen, J. (1972). Bacterial surface translocation: a survey and a classification. *Bacteriol Rev* **36**, 478–503.
- Henrichsen, J. (1975). The occurrence of twitching motility among Gram-negative bacteria. *Acta Pathol Microbiol Scand Sect B* **83**, 171–178.
- Holt, J. G., Krieg, N. R., Sneath, P. H., Staley, J. T. & Williams, S. T. (editors) (1994). Gram-negative aerobic/microaerophilic rods and cocci. In *Bergey's Manual of Determinative Bacteriology*, 9th edn, pp. 71–174. Baltimore: Williams & Wilkins.
- Juni, E. & Heym, G. A. (1980). Transformation assay for identification of psychrophilic achromobacters. *Appl Environ Microbiol* **40**, 1106–1114.
- Juni, E. & Heym, G. A. (1986). *Psychrobacter immobilis* gen. nov., sp. nov.: genospecies composed of Gram-negative, aerobic, oxidase-positive coccobacilli. *Int J Syst Bacteriol* **36**, 388–391.
- Kester, D. R., Duedall, I. W., Connors, D. N. & Pytkowicz, R. M. (1967). Preparation of artificial seawater. *Limnol Oceanogr* **12**, 176–179.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Kishino, H., Miyata, T. & Hasegawa, M. (1990). Maximum likelihood inference of protein phylogeny and the origin of chloroplasts. *J Mol Evol* **31**, 151–160.
- Krieg, N. R. & Holt, J. G. (editors) (1984). Genus *Moraxella* and genus *Acinetobacter*. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 296–307. Baltimore: Williams & Wilkins.
- Kusunoki, S., Ezaki, T., Tamesada, M., Hatanaka, Y., Asano, K., Hashimoto, Y. & Yabuuchi, E. (1991). Application of colorimetric microdilution plate hybridization for rapid genetic identification of 22 *Mycobacterium* species. *J Clin Microbiol* **29**, 1596–1603.
- Lane, D. L. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.
- Maruyama, A., Maeda, M. & Simidu, U. (1986). Occurrence of plant hormone (cytokinin)-producing bacteria in the sea. *J Appl Bacteriol* **61**, 569–574.
- Maruyama, A., Maeda, M. & Simidu, U. (1990). Distribution and classification of marine bacteria with the ability of cytokinin and auxin production. *Bull Jpn Soc Microb Ecol* **5**, 1–8.
- Maruyama, A., Mita, N. & Higashihara, T. (1993). Particulate materials and microbial assemblages around the Izena black smoking vent in the Okinawa Trough. *J Oceanogr* **49**, 353–367.
- Maruyama, A., Taniguchi, R., Tanaka, H., Ishiwata, H. & Higashihara, T. (1997). Low-temperature adaptation of deep-sea bacteria isolated from the Japan Trench. *Mar Biol* **128**, 705–711.
- Murray, M. G. & Thompson, W. F. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res* **8**, 4321–4325.
- Prieto, M., Garcia-Armesto, M. R., Garcia-Lopez, M. L., Otero, A. & Moreno, B. (1992). Numerical taxonomy of Gram-negative, nonmotile, nonfermentative bacteria isolated during chilled storage of lamb carcasses. *Appl Environ Microbiol* **58**, 2245–2249.
- Rossau, R., Van Landschoot, A., Gillis, M. & De Ley, J. (1991). Taxonomy of *Moraxellaceae* fam. nov., a new bacterial family to accommodate the genera *Moraxella*, *Acinetobacter*, and *Psychrobacter* and related organisms. *Int J Syst Bacteriol* **41**, 310–319.
- Rüger, H.-J. (1989). Benthic studies of the northwest African upwelling region: psychrophilic and psychrotrophic bacterial communities from areas with different upwelling intensities. *Mar Ecol Prog Ser* **57**, 45–52.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Shaw, B. G. & Latty, J. B. (1988). A numerical taxonomic study of nonmotile nonfermentative Gram-negative bacteria from foods. *J Appl Bacteriol* **65**, 7–21.
- Shewan, J. M., Hobbs, G. & Hodgkiss, W. (1960). The *Pseudomonas* and *Achromobacter* groups of bacteria in the spoilage of marine white fish. *J Appl Bacteriol* **23**, 463–468.
- Simidu, U., Kaneko, E. & Taga, N. (1977). Microbiological studies of Tokyo Bay. *Microb Ecol* **3**, 173–191.
- Simidu, U., Taga, N., Colwell, R. R. & Schwartz, J. R. (1980). Heterotrophic bacterial flora of the seawater from the Nansei Shoto (Ryukyu Retto) area. *Bull Jpn Soc Sci Fish* **46**, 505–510.
- Simidu, U., Kogure, K., Fukami, K. & Imada, C. (1986). Heterotrophic bacterial flora of the Antarctic ocean. *Mem Natl Inst Polar Res Japan (Spec Issue)* **40**, 405–412.
- Smibert, R. M. & Krieg, N. R. (1994). Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607–654. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.
- Stommel, H. (1958). The abyssal circulation. *Deep-Sea Res* **5**, 80–82.
- Suzuki, M. & Yamasato, K. (1986). Factors affecting the viability of L-dried cells and frozen cells of marine bacteria. *Jpn J Freez Dry* **32**, 39–46 (in Japanese).
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties, and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Van de Peer, Y., Jansen, J., De Rijk, P. & De Wachter, R. (1997). Database on the structure of small ribosomal subunit RNA. *Nucleic Acids Res* **25**, 111–116.
- Yamasato, K., Akagawa, M., Oishi, N. & Kuraishi, H. (1982). Carbon substrate assimilation profiles and other taxonomic features of *Alcaligenes faecalis*, *Alcaligenes ruhlandii*, and *Achromobacter xylosoxidans*. *J Gen Appl Microbiol* **28**, 195–213.