***Colwellia demingiae* sp. nov., *Colwellia hornerae* sp. nov., *Colwellia rossensis* sp. nov. and *Colwellia psychrotropica* sp. nov.: psychrophilic Antarctic species with the ability to synthesize docosahexaenoic acid (22:6 ω 3)**

John P. Bowman,^{1,2} John J. Gosink,³ Sharee A. McCammon,¹ Tom E. Lewis,^{1,2} David S. Nichols,^{1,2} Peter D. Nichols,^{1,4} Jenny H. Skerratt,¹ Jim T. Staley³ and Tom A. McMeekin^{1,2}

Author for correspondence: John P. Bowman. Tel: +61 3 6226 2776. Fax: +61 3 6226 2642.
e-mail: john.bowman@utas.edu.au

^{1,2} Antarctic CRC¹ and Department of Agricultural Science², University of Tasmania, GPO Box 252-80, Hobart, Tasmania 7001, Australia

³ Department of Microbiology, University of Washington, Seattle, WA 98195, USA

⁴ CSIRO Marine Research Division, Castray Esplanade, Hobart, Tasmania, 7001, Australia

As part of a general survey of the biodiversity and inherent ecophysiology of bacteria associated with coastal Antarctic sea-ice diatom assemblages, eight strains were identified by 16S rRNA sequence analysis as belonging to the genus *Colwellia*. The isolates were non-pigmented, curved rod-like cells which exhibited psychrophilic and facultative anaerobic growth and possessed an absolute requirement for sea water. One isolate was able to form gas vesicles. All strains synthesized the ω 3 polyunsaturated fatty acid (PUFA) docosahexaenoic acid (22:6 ω 3, DHA) (0.7–8.0% of total fatty acids). Previously, DHA has only been detected in strains isolated from deep-sea benthic and faunal habitats and is associated with enhanced survival in permanently cold habitats. The G+C content of the DNA from the Antarctic *Colwellia* strains ranged from 35 to 42 mol% and DNA–DNA hybridization analyses indicated that the isolates formed five genospecies, including the species *Colwellia psychrerythraea* (ACAM 550^T). 16S rRNA sequence analysis indicated that the strains formed a cluster in the γ -subclass of the *Proteobacteria* with *Colwellia psychrerythraea*. Sequence similarities ranged from 95.2 to 100% between the various Antarctic *Colwellia* isolates. Phenotypic characterization confirmed distinct differences between the different genospecies. These studies indicate that the DHA-producing Antarctic isolates consist of five different *Colwellia* species: *Colwellia psychrerythraea* and four novel species with the proposed names *Colwellia demingiae* sp. nov. (ACAM 459^T), *Colwellia psychrotropica* sp. nov. (ACAM 179^T), *Colwellia rossensis* sp. nov. (ACAM 608^T) and *Colwellia hornerae* sp. nov. (ACAM 607^T).

Keywords: Antarctic sea ice, polyunsaturated fatty acids, docosahexaenoic acid, psychrophilic bacteria, *Colwellia*

INTRODUCTION

Colwellia-related clones and isolates have been obtained from aggregates of particulate organic material and coastal marine water samples collected offshore of the United States (DeLong *et al.*, 1993;

Suzuki *et al.*, 1997) and from the Marianas Trench (DeLong & Yayanos, 1986). Phylogenetic analysis based on 5S (Deming *et al.*, 1984, 1988) and 16S rRNA sequences (DeLong *et al.*, 1993; Gosink & Staley, 1995; Bowman *et al.*, 1997a) has indicated that this genus is most closely related to other Gram-negative marine genera such as *Shewanella*, *Ferrimonas*, *Pseudoalteromonas* and *Alteromonas* (Gauthier *et al.*, 1995; Rossello-Móra *et al.*, 1995). Species of the genus *Colwellia* are facultative anaerobes, characteristically

Abbreviations: DHA, docosahexaenoic acid (22:6 ω 3); EPA, eicosapentaenoic acid (20:5 ω 3); PUFA, polyunsaturated fatty acid; TMAO, trimethylamine *N*-oxide.

psychrophilic (no growth at 20 °C, optimum growth at < 15 °C), sometimes barophilic and possess a requirement for elevated sodium and magnesium ions to maintain cell wall and cytoplasmic membrane integrity (D'Aoust & Kushner, 1972). Recent studies indicate that strains of *Colwellia* and [*Vibrio*] *marinus* synthesize ω 3 polyunsaturated fatty acids (PUFAs), in particular docosahexaenoic acid (22:6 ω 3, DHA) (DeLong & Yano, 1986; DeLong *et al.*, 1997). PUFAs such as DHA and eicosapentaenoic acid (20:5 ω 3, EPA) are essential for human and animal health in a variety of ways. For example, PUFAs are believed to lower the levels of blood plasma cholesterol and triacylglycerols, prevent cardiovascular disease and reduce the risk of some cancers (Kelly, 1991). They are also required for normal embryonic development, particularly optical and neural development (Craigschmidt *et al.*, 1996; Farkas *et al.*, 1996; Linko & Hayakawa, 1996). ω 3 PUFAs are important food supplements for rearing larval fish in the aquaculture industry. In most cases fish obtain PUFA from 'live feed', including rotifers or *Artemia* spp. fed on PUFA-rich microalgae, or from PUFA-amended artificial feed (Ostrowski & Divakaran, 1990; Southgate & Lou, 1995). Bacteria are recognized as an alternative source of PUFAs for the development of less expensive aquaculture fish feeds (Nichols *et al.*, 1996).

Currently, the genus *Colwellia* contains two species: *Colwellia psychroerythrus* and *Colwellia hadaliensis* (Deming *et al.*, 1988). *Colwellia psychroerythrus* was named following the observation that the species '*Vibrio psychroerythrus*' (D'Aoust & Kushner, 1972) was phylogenetically distinct from the rest of the genus *Vibrio* (Deming *et al.*, 1988). The obligately barophilic species *Colwellia hadaliensis* was also described at that time. The epithet *psychroerythrus* has been changed to *psychrerythraea* (L. adj. *erythraeus*, -a, -um red) as the name *Colwellia* is feminine (Euzéby, 1998).

Recently, it was found that a number of Antarctic fast sea-ice isolates and a single strain from an Antarctic meromictic marine salinity lake belonged to the genus *Colwellia* (Bowman *et al.*, 1997a). In addition, a gas-vesicle-forming isolate from the ice/water interface of sea ice in McMurdo Sound, Antarctica, was also shown to be closely related to *Colwellia psychrerythraea* (Gosink & Staley, 1995). In this study, these isolates were investigated and found to represent several novel species of the genus *Colwellia*: *Colwellia demingiae* sp. nov., *Colwellia rossensis* sp. nov., *Colwellia hornerae* sp. nov. and *Colwellia psychrotropica* sp. nov. All species of the genus *Colwellia* were found to form DHA, the level of which increases with decreasing optimum growth temperature and possibly represents an adaptation to continually cold marine environments.

METHODS

Bacterial strains. The strains investigated in this study (Table 1) were routinely grown at 5–10 °C on marine 2216 agar

(Difco) or on R2A agar (Oxoid) prepared with sea water salts (Ocean Nature, Aquasonic).

Phenotypic characterization. Most of the phenotypic tests used in this study have been described previously (Bowman *et al.*, 1997b). Sodium, magnesium and calcium ion requirement was tested in a basal medium derived from marine 2216 agar containing (per litre distilled water) 5 g Bacto peptone, 2 g yeast extract and 10 mg ferric phosphate. Chloride salts were tested at concentrations found typically in sea water: 0.4 M NaCl, 25 mM MgCl₂ and 8 mM CaCl₂ (ZoBell, 1946).

Dissimilatory iron reduction was tested in a medium adapted from Widdel & Bak (1992) and prepared using the Hungate technique (Hungate, 1968). The medium was prepared with artificial sea water and contained 10 mM amorphous ferric oxide/neutralized ferric chloride or 10 mM soluble ferric oxide/ferric pyrophosphate (Lovely & Phillips, 1986) as the electron acceptors and 10 mM sodium acetate as the electron donor and carbon source. Uninoculated controls lacking the iron electron acceptors were also prepared. Iron reduction was confirmed by the appearance of a black precipitate (magnetite) and by use of the Merckoquant Iron Test (Merck).

Anaerobic growth with 10 mM trimethylamine *N*-oxide (TMAO) was tested in mineral salts medium containing (per litre sea water) 2 g ammonium chloride, 2 mM potassium phosphate buffer, pH 7.0, 2 ml SL-10 trace element solution (Overmann & Pfennig, 1989), 1 g yeast extract and 10 ml vitamin solution no. 6 (Staley *et al.*, 1992). The medium pH was adjusted to 7.0 using 1 M KOH and was solidified with 1.3% (w/v) purified agar (Oxoid). Sodium acetate (20 mM) was used as the electron donor and carbon source. Plates were incubated in an anaerobic jar using Anaerogen Gaspaks (Oxoid). Growth on the plates was compared with control plates lacking TMAO.

Additional biochemical tests were performed using the API 20E test kit (bioMérieux) prepared according to the manufacturer's specifications except that bacterial strains were suspended in chilled artificial sea water. Carbon and energy source screening utilized a 0.1% concentration of test compounds, except carbohydrates, which were tested at a concentration of 0.2%. The basal medium used was the same as for the TMAO reduction test. Media lacking a carbon source were prepared as negative controls to account for any background growth.

Growth rate analysis. The growth rates of strains ACAM 605, ACAM 607^T and ACAM 179^T were determined in a temperature gradient incubator (Toyokagaku Sangyo). The temperature gradient used ranged from 0 to 20–27 °C and tubes, containing 10 ml marine 2216 broth, were inoculated with 0.5 ml cells taken from cultures in late-exponential growth phase. Growth was measured by a decrease in transmittance at 550 nm for up to 14 d. Growth was considered to have occurred if the culture transmittance decreased by more than 20%. The data were fitted to the square-root growth model of Ratkowsky *et al.* (1983) and cardinal temperatures determined.

Whole-cell fatty acid analysis. Representative strains were grown on marine 2216 agar at 10 °C for 2–7 d, harvested into a small amount of artificial sea water and then lyophilized using a vacuum freeze-drier (Dynavac). Profiles of total extractable fatty acids were quantitatively determined using GC and GC-MS procedures (Nichols *et al.*, 1993). The geometry and position of double bonds in monounsaturated fatty acids was confirmed using dimethyl-

Table 1. *Colwellia* strains and reference strains investigated in this study

Received as*	ACAM No.	Name	Isolation site
ATCC 27364 ^T	550 ^T	<i>Colwellia psychrerythraea</i>	Flounder eggs
IC062	604	<i>Colwellia psychrerythraea</i>	Sea ice, Prydz Bay, Antarctica
IC064	—	<i>Colwellia psychrerythraea</i>	Sea ice, Prydz Bay, Antarctica
IC072	605	<i>Colwellia psychrerythraea</i>	Sea ice, Prydz Bay, Antarctica
ACAM 179 ^T	179 ^T	<i>Colwellia psychrotropica</i> sp. nov.	Burton Lake pycnocline, Antarctica
ICP11 ^T	459 ^T	<i>Colwellia demingiae</i> sp. nov.	Sea ice, Prydz Bay, Antarctica
IC068	606	<i>Colwellia demingiae</i> sp. nov.	Sea ice, Prydz Bay, Antarctica
IC035 ^T	607 ^T	<i>Colwellia hornerae</i> sp. nov.	Sea ice, Ellis Fjord, Antarctica
S51-W(gv)1 ^T	608 ^T	<i>Colwellia rossensis</i> sp. nov.	Sea ice, McMurdo Sound, Antarctica

* ATCC, American Type Culture Collection, Rockville, MD, USA; ACAM, Australian Collection of Antarctic Micro-organisms, Antarctic Cooperative Research Centre, University of Tasmania, Hobart, Tasmania, Australia.

Fig. 1. Temperature growth curves of four Antarctic *Colwellia* species based on the square-root growth model of Ratkowsky *et al.* (1983). (a) *Colwellia psychrerythraea* ACAM 605, (b) *Colwellia demingiae* ACAM 459^T, (c) *Colwellia hornerae* ACAM 607^T and (d) *Colwellia psychrotropica* ACAM 179^T. Baseline points ($T_{\min}$ and $T_{\max}$) were extrapolated from the growth model.

disulfide derivatization and GC-MS analysis (Nichols *et al.*, 1986). The double bond positions are numbered from the methyl (ω) end of the fatty acid.

DNA base composition. Cells were grown in the same way as for fatty acid analysis. Genomic DNA was extracted and purified using the procedure of Marmur & Doty (1962). The DNA G+C content was then determined from thermal denaturation profiles (Sly *et al.*, 1986).

DNA-DNA hybridization. The spectrophotometric renaturation rate kinetic procedure, as adapted by Huss *et al.* (1983), was used to determine DNA-DNA reassociation values between genomic DNA of different strains. Genomic DNA was sheared to a mean size of 1 kb by sonication, dialysed overnight at 4 °C in $2 \times$ SSC buffer ($2 \times$ SSC is 0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) and adjusted in concentration to approximately $75 \mu\text{g ml}^{-1}$. Following denaturation of the DNA samples, hybridization was

performed at the optimum temperature for renaturation (T_{or}) which was 25 °C below the DNA melting temperature and was calculated from the equation: $T_{\text{or}} (\text{°C}) = 48.5 + (0.41 \times \%G + C)$. The decreases in absorbance over a 40 min interval of DNA mixtures and control DNA samples were used to calculate DNA hybridization values from the equation: $\text{DNA hybridization (\%)} = (4AB - A - B) / 2\sqrt{(A \times B)} \times 100$ (Huss *et al.*, 1983), where A and B represent the change in absorbance for two DNA samples being compared and AB represents the change in absorbance for equimolar mixtures of A and B . DNA hybridization values equal to or below 25% are considered to represent background hybridization and are thus not considered significant (Huss *et al.*, 1983).

Phylogenetic analysis. The 16S rRNA sequences for the Antarctic *Colwellia* strains have been determined previously (Gosink & Staley, 1995; Bowman *et al.*, 1997a). The

Table 2. Phenotypic properties differentiating *Colwellia psychrerythraea* and novel Antarctic *Colwellia* species

+, All strains positive for test; v+, variable reactions for test, type strain is positive; v-, variable reactions for test, type strain is negative; -, all strains negative for test; w, weak or delayed reaction; F, acid (but no gas) was formed from these substrates in Leifson oxidation/fermentation medium; NG, no growth occurred on test medium.

Characteristic*	<i>Colwellia psychrerythraea</i>	<i>Colwellia demingiae</i>	<i>Colwellia rossensis</i>	<i>Colwellia hornerae</i>	<i>Colwellia psychrotropica</i>
Prodigiosin-like pigments	v+	—	—	—	—
Tolerance of 200% sea salts	v—	—	—	+	+
Susceptibility to vibriostatic agent O/129 (100 µg ml ⁻¹)	v—	—	—	+	—
Hydrolysis of:					
Urea	v+	—	+	—	+
Uric acid	—	—	—	—	+
Tween 80	+	—	NG	+	+
Casein	+	+	—	+	+
Gelatin	v+	—	—	—	—
Aesculin	+	+	NG	+	—
Chitin	+F	+F	+F	—	+F
Starch	+	+	+	+	—
Tyrosine	v— w	—	—	—	+†
Production of acid from:					
L-Rhamnose	—	—	—	+F	—
D-Glucose	+F	—	+F	—	—
D-Galactose	—	—	+	—	—
N-Acetylglucosamine	+F	+F	+F	—	+F
Cellobiose	v+ w	—	—	—	—
Maltose	+F	—	—	—	—
Glycerol	—	—	+	+	—
Utilization of:					
D-Glucose	v+	—	+	—	—
L-Arabinose, D-fructose, D-gluconate	—	—	+	—	—
N-Acetylglucosamine	+	+	+	—	+
Glycerol	—	—	+	+	—
Glycogen	+	v—	+	—	—
Propionate	v—	+	—	+	—
Isobutyrate	—	v+	—	—	+
Valerate, caproate, γ-aminobutyrate	—	+	+	+	+
Heptanoate	—	v+	—	+	—
Caprylate	—	v+	—	—	—
Malonate	—	v+	+	—	—
Glutarate	—	+	—	+	—
Azelate	—	v+	—	+	—
Citrate	—	+	+	+	—
2-Oxoglutarate	—	—	—	—	+
3-Hydroxybutyrate	+	—	+	—	+
L-Malate	—	—	+	—	+
DL-Lactate	v+	+	—	+	+
Oxaloacetate	+	+	—	+	+
L-Alanine, L-aspartate	—	v+	+	—	+
L-Asparagine	—	+	+	—	+
L-Phenylalanine	—	v+	—	—	—
Hydroxy-L-proline	—	v+	—	+	—
L-Serine	—	+	—	—	—

* The following tests were positive for all strains: catalase, cytochrome-*c* oxidase, nitrate reduction, alkaline phosphatase, growth at 0–15 °C and utilization of acetate, butyrate, succinate, fumarate, pyruvate, L-glutamate and L-proline as sole sources of carbon and energy. The following tests were all negative: Fe(III) reduction; TMAO reduction, growth on 0–25% sea salts or ≥ 400% sea salts, hydrolysis of ONPG, DNA and dextran, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase activity, production of indole from L-tryptophan, H₂S production, production of acid from arabinose, xylose, melibiose, fructose, lactose, mannitol, sorbitol, inositol, mannose, sucrose and trehalose, and utilization of D-galactose, D-xylose, cellobiose, lactose, maltose, D-melibiose, trehalose, sucrose, L-raffinose, D-adonitol, D-arabitol, *m*-inositol, D-mannitol, D-sorbitol, D-gluconate, D-glucuronate, saccharate, α-glycerophosphate, isovalerate, nonanoate, adipate, pimelate, aconitate, L-threonine, L-valine and putrescine.

† Brown diffusible pigment formed during L-tyrosine hydrolysis.

nucleotide sequences were 1373–1503 bases long and stretched between positions 8 and 1508 (*Escherichia coli* numbering). Sequence data were manually aligned to various 16S rRNA sequences of representatives of the γ -subclass of the *Proteobacteria*. Software from PHYLIP version 3.57c (Felsenstein, 1993) was used to further analyse the sequence data set. DNADIST (using the maximum likelihood option) was used to determine sequence similarities and NEIGHBOR (using the neighbourliness option) to create a phylogenetic tree. The 16S rRNA sequence of *Marinobacter hydrocarbonoclasticus* was used as the outgroup. Reference sequences utilized in the phylogenetic analysis were obtained directly from GenBank and included aggregate clone AGG53 (accession no. L10950), *Alteromonas macleodii* IAM 12920^T (X82145), *Colwellia*-like strain S51-W(gv)1^T = ACAM 608^T (U14581); barophilic *Colwellia* sp. MT41 (U91595); *Ferrimonas balearica* DSM 9799^T (X93021), *Pseudoalteromonas haloplanktis* ATCC 14393^T (X67024), *Shewanella putrefaciens* ATCC 8071^T (X82123), *Marinobacter hydrocarbonoclasticus* ATCC 49840^T (X67022) and [*Vibrio*] *marinus* ATCC 15381^T (X82142). Sequences obtained from the study of Bowman *et al.* (1997a) and utilized in this study include the following: *Colwellia psychrerythraea* ACAM 550^T (AF001375), *Colwellia* sp. ACAM 604 (U85841), *Colwellia* sp. IC064 (U85842), *Colwellia* sp. ACAM 605 (U85843), *Colwellia* sp. ACAM 606 (U85844), *Colwellia* sp. ACAM 459^T (U85845), *Colwellia* sp. ACAM 179^T (U85846) and *Colwellia* sp. ACAM 607^T (U85847).

RESULTS

Growth and morphological characteristics

Most of the *Colwellia* strains investigated were psychrophilic when grown on agar medium, unable to grow at 20 °C. Selected strains (ACAM 179^T, ACAM 459^T, ACAM 605 and ACAM 607^T) were investigated in more detail with cardinal growth temperatures determined using the square-root growth model (Fig. 1). In liquid medium the cardinal growth temperatures were higher than what could be estimated from growth on agar medium. The optimum growth temperatures (T_{opt}) ranged from 10 to 18 °C. ACAM 179^T proved to be the most thermotolerant strain, growing at 25 °C in liquid medium and at 20 °C on solid medium. In liquid medium the maximum growth temperature (T_{max}) values of the other strains fell between 18 and 23 °C. Theoretical minimum growth temperature (T_{min}) values ranged from –10 to –20 °C, similar to T_{min} values found for other psychrophiles such as *Shewanella* spp. (Nichols & Russell, 1996; Bowman *et al.*, 1997b) and *Psychrobacter* spp. (Bowman *et al.*, 1996).

All strains possessed a requirement for sea salts, with no growth occurring if the sea salt concentration was less than 25%. ACAM 179^T was more halotolerant than the other isolates and grew in the presence of three times the normal concentration of sea salts, while most other strains failed to grow at sea salt concentrations two times greater than normal. Scanty or no growth occurred in medium with NaCl added alone. The best growth occurred when sodium, magnesium

and calcium ions (chloride salts) were added at concentrations typical for sea water.

Except for ACAM 608^T, all strains investigated were motile and the morphology of the cells varied from spherical to curved to straight rod-shaped cells with occasional short filaments also present. Strain ACAM 608^T was the only strain that appeared capable of forming gas vesicles (Gosink & Staley, 1995). *Colwellia psychrerythraea* ACAM 550^T produced a prodigiosin-like, brilliant red compound which increased in concentration in cells during the exponential growth stage (D'Aoust & Kushner, 1972). Other strains did not produce pigments. The colonial morphology of the Antarctic strains were similar, forming chalk-white to off-white convex colonies, possessing a low or raised elevation, with entire to lobate edges and butyrous consistency.

Biochemical and nutritional characteristics

All strains were facultative anaerobes able to grow by fermentation of a suitable carbohydrate as indicated by acid production in Leifson oxidation/fermentation medium. No gas was detected during fermentation. Carbohydrate oxidation and fermentation varied between strains with *N*-acetylglucosamine, chitin and D-glucose being the preferred substrates (Table 2). All of the strains were capable of nitrate reduction. Anaerobic growth by dissimilatory Fe(III) and TMAO reduction was not observed. Most strains were chitinolytic and able to degrade starch (Table 2). The strains varied somewhat in the hydrolysis of other substrates. For instance, only ACAM 179^T was capable of degrading uric acid (Table 2). Most of the strains examined did not require an organic nitrogen source, amino acids or vitamins for growth; however, ACAM 608^T required yeast extract (0.1%) for good growth. The addition of a vitamin solution resulted in an observable stimulation of growth for most of the strains. As a result, carbon utilization studies were performed in a medium supplemented with yeast extract and vitamins. Results for the carbon source utilization tests are shown in Table 2.

Genotypic analyses

The DNA G+C content of the *Colwellia* isolates ranged from 35 to 42 mol% (Table 3). DNA–DNA hybridization indicated the presence of five genospecies amongst the strains investigated (Table 3): 1, *Colwellia psychrerythraea* (strains ACAM 550^T, ACAM 604, ACAM 605 and IC064); 2, strains ACAM 459^T and ACAM 606 (*Colwellia demingiae*); 3, strain ACAM 608^T (*Colwellia rossensis*); 4, strain ACAM 607^T (*Colwellia hornerae*); and 5, strain ACAM 179^T (*Colwellia psychrotropica*). *Colwellia psychrerythraea* and *Colwellia demingiae* were closely related with DNA–DNA reassociation levels ranging from 35 to 40% between the strains. Other combinations of genospecies exhibited DNA–DNA hybridization

Table 3. DNA–DNA reassociation values between *Colwellia psychrerythraea* and the Antarctic *Colwellia* strains

G + C content and DNA–DNA hybridization values are based on the mean of triplicate analyses (SD $\pm$ 0.3–0.6 mol % and $\pm$ 5–15 %, respectively).

Species	G + C (mol %)	Percentage DNA–DNA hybridization				
		ACAM 550 ^T	ACAM 605	ACAM 459 ^T	ACAM 608 ^T	ACAM 607 ^T
<i>Colwellia psychrerythraea</i> ACAM 550 ^T	38	100	85	35	13	12
<i>Colwellia psychrerythraea</i> ACAM 605	36	85	100	40	14	14
<i>Colwellia psychrerythraea</i> ACAM 604	36	79	85	–	–	–
<i>Colwellia psychrerythraea</i> IC064	35	93	91	–	–	–
<i>Colwellia demingiae</i> ACAM 459 ^T	37	35	40	100	21	21
<i>Colwellia demingiae</i> ACAM 606	37	38	–	79	–	–
<i>Colwellia rossensis</i> ACAM 608 ^T	38	13	14	21	100	22
<i>Colwellia hornerae</i> ACAM 607 ^T	39	12	14	21	22	100
<i>Colwellia psychrotropica</i> ACAM 179 ^T	42	12	–	–	24	18

Table 4. Whole-cell fatty acid profiles of *Colwellia psychrerythraea* and novel Antarctic *Colwellia* species

Fatty acid*	Fatty acid composition (% of total)†				
	<i>Colwellia psychrerythraea</i>	<i>Colwellia demingiae</i>	<i>Colwellia rossensis</i>	<i>Colwellia hornerae</i>	<i>Colwellia psychrotropica</i>
14:0	5.1–7.8	7.6–8.0	4.6	3.0	0.8
14:1 ω 7c	5.1–7.3	9.1–9.3	2.8	2.8	2.0
15:1 ω 8c	0–2.3	1.9–2.6	4.1	20.3	4.2
15:1 ω 6c	0–0.4	–	–	1.1	0.1
15:0	1.7–11.0	0.9–1.4	2.9	14.3	2.7
i16:0	0–0.2	–	–	10.3	–
16:1 ω 9c	6.2–8.8	9.5–11.8	1.8	2.0	–
16:1 ω 7c	31.4–36.3	37.5–37.8	43.4	15.4	56.8
16:0	26.8–33.2	21.9–23.6	27.1	13.5	21.9
17:1 ω 8c	0–1.3	TR	0.5	5.6	4.5
17:1 ω 6c	0–0.9	–	–	1.9	0.9
17:0	0–1.3	TR	0.1	2.5	1.5
18:1 ω 9c	0–1.7	TR–0.2	0.8	1.4	0.3
18:1 ω 7c	0.3–2.1	1.3–1.4	4.2	–	1.9
18:0	0.1–2.4	0.2–0.5	TR	2.0	0.4
20:5 ω 3	0–1.5	–	TR	–	0.1
22:6 ω 3	5.5–8.0	1.7–2.2	6.0	2.1	0.7
Other	1.7–2.6	3.6–4.1	1.6	1.2	1.3

* The following fatty acids were present at levels of < 1 % of total fatty acid content in all species: i13:0, i14:0, 14:1 ω 5c, i17:0 and i17:1 ω 7c.

† TR, Trace fatty acid component making up 0.1 % or less of total fatty acid content.

equivalent to background reassociation levels (Table 3).

Whole-cell fatty acid profiles

The predominating fatty acid components detected in extracts are shown in Table 4. C₁₄–C₁₆ mono-unsaturated and saturated fatty acids made up the

bulk of total fatty acids. Relative levels of several fatty acids varied considerably among strains, including 15:1 ω 8c, 15:0 and 16:1 ω 9c. These fatty acids may represent labile pools within cells. The relative abundance of these fatty acid pools may be strongly influenced by the overall metabolic state and age of the culture. All strains contained DHA with some strains also forming trace levels of EPA (Table 4).

Fig. 2. Phylogenetic tree based on 16S rRNA sequence comparison showing the positions of members of the genus *Colwellia* within the *Colwellia* assemblage radiation of the γ -subclass of the *Proteobacteria*. Bar is equivalent to 5% sequence dissimilarity.

Phylogenetic analyses

16S rRNA sequence data indicated the Antarctic isolates were closely related to *Colwellia psychrerythraea* (Fig. 2). The next closest relatives were species of the genera *Pseudoalteromonas* and *Alteromonas* with sequence similarities of 87.1–89.3%. The sequence similarity levels reflected DNA–DNA reassociation values with *Colwellia psychrerythraea* strains ACAM 550^T, ACAM 604, ACAM 605 and IC064 and the strain pair ACAM 459^T and ACAM 606 (*Colwellia demingiae*) each forming tight clusters with similarities of 99.0–99.1 and 99.8%, respectively. The remaining strains, including ACAM 607^T, ACAM 608^T and ACAM 179^T, possessed sequence similarities of 95.1–97.8% with other *Colwellia* strains (Fig. 2).

DISCUSSION

Bacterial communities associated with Antarctic fast sea-ice diatom assemblages are dominated by Gram-negative, psychrophilic bacterial species (Gosink & Staley, 1995; Bowman *et al.*, 1997a) and the majority of sea-ice bacterial taxa so far cultivated are concentrated in the γ -subclass of the *Proteobacteria* and in the family *Flavobacteriaceae*. Non-pigmented, facultatively anaerobic strains were amongst those isolated and included novel species belonging to the genus *Shewanella* (Bowman *et al.*, 1997b) and a number of *Vibrio*-like strains. Several of the *Vibrio*-like strains were later revealed by 16S rRNA sequence analysis to belong to the genus *Colwellia* (Bowman *et al.*, 1997a).

Colwellia species appear to be widely distributed in coastal (DeLong *et al.*, 1993; Bowman *et al.*, 1997a; Suzuki *et al.*, 1997) and abyssal oceanic regions (Deming *et al.*, 1984; DeLong *et al.*, 1997) charac-

terized by permanently low temperatures. The colonization of constantly low temperature and/or high-pressure marine environments by *Colwellia* species has been proposed to be made possible by their ability to synthesize DHA (DeLong & Yayanos, 1986). DHA, along with other PUFAs, help to maintain the homeoviscosity of cellular membranes under low temperature and high hydrostatic pressure (Nichols *et al.*, 1995). It was generally believed up to 5–10 years ago that prokaryotes did not contain PUFAs. DHA is now known to be formed by bacterial strains isolated from deep-sea habitats, including water columns, benthic sediments, amphipods and in deep-sea fish intestines (DeLong & Yayanos, 1986; Yano *et al.*, 1994; Hamamoto *et al.*, 1995; DeLong *et al.*, 1997). EPA- and DHA-producing bacteria often occupy the same low temperature/high pressure environmental niches, such as sea ice and the deep-sea benthos. In these environments they may supply PUFAs necessary for the normal development of metazoa (DeLong & Yayanos, 1986; Craigschmidt *et al.*, 1996), which generally lack the ability to synthesize C₂₀ and C₂₂ PUFAs *de novo*.

Preliminary evidence suggests that with a decrease in T_{opt} there is a corresponding increase in the level of DHA. ACAM 179^T, which possesses the highest determined T_{opt} of the strains in this study, has a DHA level of 0.7%, while the more highly psychrophilic strains, including *Colwellia psychrerythraea* ACAM 550^T, ACAM 604, ACAM 605, IC064 and *Colwellia rossensis* ACAM 608^T (T_{opt} 10–12 °C) have DHA levels ranging from 5 to 8% (Table 4). The inherent T_{opt} and relative levels of EPA found in *Shewanella* species correlate in a similar way (Bowman *et al.*, 1997b). This hypothesis can be indirectly supported by available information. To begin with, cold adaptation

Table 5. Characteristics useful in differentiating *Colwellia* species and [*Vibrio*] *marinus*

+, All strains positive for test; v, variable reactions for test; —, all strains negative for test; ND, no data available; NG, no growth on test medium.

Species	Growth at 25 °C	Hydrolysis of:						Production of acid from:		Gas vesicles	Barophilic growth	G + C (mol %)
		Chitin	Starch	Uric acid	Urease	Tween 80	Gelatin	Glucose	Maltose			
<i>Colwellia psychrerythraea</i>	—	+	+	—	—	+	v	+	+	—	—	35–38
<i>Colwellia hadaliensis</i> *	—	+	ND	ND	ND	ND	ND	+	ND	—	+	46
<i>Colwellia demingiae</i>	—	+	+	—	—	—	—	—	—	—	—	37
<i>Colwellia rossensis</i>	—	+	+	—	+	NG	—	+	—	+†	—	38
<i>Colwellia hornerae</i>	—	—	+	—	—	+	—	—	—	—	—	39
<i>Colwellia psychrotropica</i>	+	+	—	+	+	+	—	—	—	—	—	42
[<i>Vibrio</i>] <i>marinus</i> ‡	—	+	—	ND	ND	+	+	+	—	—	v	42

* Data from Deming *et al.* (1988).

† *Colwellia rossensis* is also non-motile.

‡ Data from Baumann *et al.* (1984) and DeLong & Yayanos (1986).

is independent of growth rate, i.e. psychrotrophs grow just as well as psychrophiles at the same low temperatures (Nichols *et al.*, 1995). In sea-ice environments the *in situ* temperature is constantly subzero, so cold adaptation in sea-ice *Colwellia* species may manifest (amongst other effects) as elevated DHA levels. *Colwellia psychrotropica* differs from the other *Colwellia* species in that it was isolated from Burton Lake which has an annual *in situ* temperature of about 5 °C (Franzmann *et al.*, 1990). This has presumably resulted in less cold adaptation and thus lower DHA levels in *Colwellia psychrotropica*. Recent data indicates that lowering the incubation temperature results in a proportionally similar increase in PUFA levels in various bacterial strains (Hamamoto *et al.*, 1995; Nichols & Russell, 1996; Nichols *et al.*, 1997). Recent evidence also shows that *Colwellia* species possess exoenzyme and ectoenzyme (proteases, amylases, lipases, β -galactosidase, etc.) catalytic rates with low T_{opt} values and that *Colwellia psychrotropica* possesses uniformly higher enzyme T_{opt} values compared to other tested *Colwellia* species (Buia, 1997).

16S rRNA-based phylogenetic analysis revealed that the sequence similarities between the *Colwellia* strain groups, defined by DNA hybridization experiments (Table 3) range from 95.1 to 97.8% (Fig. 2). This places a number of strains outside the estimated level of 16S rRNA sequence similarity ($\geq 97\%$), potentially indicative of a species level relationship (Stackebrandt & Goebel, 1994). The genospecies can also be differentiated clearly by phenotypic tests and DNA base composition (Table 5). It is thus proposed that each genospecies making up the genus *Colwellia* as defined in this study on the basis of polyphasic taxonomic analyses represents a distinct and novel species. On this basis four new species are proposed to be included in the genus *Colwellia* with the following names: *Colwellia demingiae* sp. nov., *Colwellia hornerae* sp. nov., *Colwellia rossensis* sp. nov. and *Colwellia psychrotropica* sp. nov. The genus *Colwellia* also

includes the type species *Colwellia psychrerythraea* and the obligately barophilic species *Colwellia hadaliensis*.

Description of *Colwellia demingiae* sp. nov.

Colwellia demingiae (de.ming'i.ae. L. adj. *demingiae* in honour of Jody W. Deming, an American microbiologist who has expanded knowledge of deep-sea bacteria).

Gram-negative. Individual cells are straight to curved rods, with spherical and short filamentous cells occasionally occurring. Length 1.5–4.5 μ m, width 0.4–0.6 μ m. Gas vesicles not formed. Motile. Colonies are off-white, have a mucoid consistency, raised elevation and convex circular shape with entire to lobate edges. Psychrophilic. In liquid media T_{opt} is approximately 10–12 °C and T_{max} is about 18 °C. Requires sea salts for growth. Growth factors are not required. Facultatively anaerobic chemoheterotroph. Acid (with no gas) is formed fermentatively and oxidatively from chitin and *N*-acetylglucosamine. Casein, aesculin, chitin and starch are hydrolysed. The following compounds are utilized as sole sources of carbon and energy: *N*-acetylglucosamine, acetate, propionate, butyrate, valerate, caproate, succinate, glutarate, citrate, fumarate, pyruvate, DL-lactate, oxaloacetate, L-asparagine, L-glutamate, L-proline, L-serine and γ -aminobutyrate. Some strains can also utilize glycogen, isobutyrate, heptanoate, caprylate, malonate, azelate, L-alanine, L-aspartate, L-phenylalanine and hydroxy-L-proline. G + C content is 37 mol% (T_m). Isolated from fast sea ice of the Prydz Bay coast, Antarctica. Type strain is ACAM 459^T.

Description of *Colwellia hornerae* sp. nov.

Colwellia hornerae (hor.ner'ae. L. adj. *hornerae* in honour of Rita Horner, an American biologist who pioneered studies on sea-ice microbiota).

Gram-negative. Individual cells are straight to curved rods, with spherical cells occasionally occurring. Length 1.5–3.0 µm, width 0.4–0.8 µm. Gas vesicles not formed. Motile. Colonies are off-white, have a mucoid consistency, raised elevation and convex circular shape with entire to lobate edges. Psychrophilic. In liquid media T_{opt} is approximately 12 °C and T_{max} is about 23–24 °C. Requires sea salts for growth. Growth factors not required. Sensitive to vibriostatic agent O/129 (100 µg ml⁻¹). Facultatively anaerobic chemoheterotroph. Acid (but no gas) is formed fermentatively and oxidatively from L-rhamnose. Acid is formed oxidatively from glycerol. Tween 80, casein, aesculin and starch are hydrolysed. The following compounds are utilized as sole sources of carbon and energy: glycerol, acetate, propionate, butyrate, valerate, caproate, heptanoate, succinate, glutarate, azelate, citrate, fumarate, pyruvate, DL-lactate, oxaloacetate, L-glutamate, L-proline, hydroxy-L-proline and γ -aminobutyrate. G+C content is 39 mol% (T_m). Isolated from fast sea ice of the Prydz Bay coast, Antarctica. Species is monotypic. Type strain is ACAM 607^T.

Description of *Colwellia rossensis* sp. nov.

Colwellia rossensis (ross.en'sis. M.L. fem. adj. *rossensis* from the Ross Sea, Antarctica).

Gram-negative. Individual cells are straight to curved rods, with spherical cells occasionally occurring. Length 1.5–3.0 µm, width 0.4–0.8 µm. Gas vesicles are formed. Non-motile. Colonies are chalky-white, have a raised elevation and convex circular shape with entire edges. Psychrophilic. In liquid media T_{opt} is approximately 10 °C and T_{max} is about 15 °C. Requires sea salts for growth. Yeast extract required for growth. Facultatively anaerobic chemoheterotroph. Acid (but no gas) is formed fermentatively and oxidatively from chitin, *N*-acetylglucosamine and D-glucose. Also produces acid oxidatively from D-galactose and glycerol. Urea, chitin and starch are hydrolysed. The following compounds are utilized as sole sources of carbon and energy: glycogen, L-arabinose, *N*-acetylglucosamine, D-fructose, D-glucose, glycerol, D-gluconate, acetate, butyrate, valerate, caproate, malonate, succinate, citrate, 3-hydroxybutyrate, L-malate, fumarate, pyruvate, L-alanine, L-aspartate, L-asparagine, L-glutamate, L-proline and γ -aminobutyrate. G+C content is 38 mol% (T_m). Isolated from the sea/ice interface of fast sea ice, McMurdo Sound Antarctica (Gosink & Staley, 1995). Species is monotypic. Type strain is ACAM 608^T.

Description of *Colwellia psychrotropica* sp. nov.

Colwellia psychrotropica (psy.chro.tro.pi'ca. Gr. adj. *psychros* cold; Gr. n. *tropica* tropic, circle; M.L. fem. adj. *psychrotropica* having an affinity for cold).

Gram-negative. Individual cells are straight to curved rods, with spherical cells occasionally occurring. Length 1.5–3.0 µm, width 0.4–0.8 µm. Motile. Colonies

are off-white, have a mucoid consistency, raised elevation and convex circular shape with entire to lobate edges. In liquid media T_{opt} is approximately 18 °C and T_{max} is about 26 °C. Requires sea salts for growth. Growth factors not required. Growth occurs in the presence of sea salts at three times normal concentration and is resistant to vibriostatic agent O/129 (100 µg ml⁻¹). Facultatively anaerobic chemoheterotroph. Acid (but no gas) is formed fermentatively and oxidatively from chitin and *N*-acetylglucosamine. Urea, uric acid, Tween 80, casein, chitin and L-tyrosine are degraded. The following compounds are utilized as sole sources of carbon and energy: *N*-acetylglucosamine, acetate, butyrate, valerate, caproate, succinate, 2-oxoglutarate, 3-hydroxybutyrate, L-malate, fumarate, pyruvate, DL-lactate, oxaloacetate, L-alanine, L-aspartate, L-asparagine, L-glutamate, L-proline and γ -aminobutyrate. G+C content is 42 mol% (T_m). Isolated from the pycnocline of an Antarctic marine salinity meromictic lake (Burton Lake). Species is monotypic. Type strain is ACAM 179^T.

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