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Polyphasic taxonomy of the genus *Shewanella* and description of *Shewanella oneidensis* sp. nov.

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The genus *Shewanella* has been studied since 1931 with regard to a variety of topics of relevance to both applied and environmental microbiology. Recent years have seen the introduction of a large number of new *Shewanella*-like isolates, necessitating a coordinated review of the genus. In this work, the phylogenetic relationships among known shewanellae were examined using a battery of morphological, physiological, molecular and chemotaxonomic characterizations. This polyphasic taxonomy takes into account all available phenotypic and genotypic data and integrates them into a consensus classification. Based on information generated from this study and obtained from the literature, a scheme for the identification of *Shewanella* species has been compiled. Key phenotypic characteristics were sulfur reduction and halophilicity. Fatty acid and quinone profiling were used to impart an additional layer of information. Molecular characterizations employing small-subunit 16S rDNA sequences were at the limits of resolution for the differentiation of species in some cases. As a result, DNA–DNA hybridization and sequence analyses of a more rapidly evolving molecule (*gyrB* gene) were performed. Species-specific PCR probes were designed for the *gyrB* gene and used for the rapid screening of closely related strains. With this polyphasic approach, in addition to the ten described *Shewanella* species, two new species, *Shewanella oneidensis* and '*Shewanella pealeana*', were recognized; *Shewanella oneidensis* sp. nov. is described here for the first time.

Keywords: *Shewanella*, *shewanella oneidensis* sp. nov. MR-1, polyphasic taxonomy, 16S rDNA, gyrase B

INTRODUCTION

Bacteria currently classified under the generic name of *Shewanella* have been a subject for scientific scrutiny for at least 65 years. Members of this genus have long been associated with the spoilage of proteinaceous foods (Shewan, 1977; Jorgensen & Huß, 1989) and have been implicated as opportunistic pathogens of humans and aquatic animals (Brink *et al.*, 1995; Aguirre *et al.*, 1994). The potential of these organisms to mediate the co-metabolic bioremediation of halogenated organic pollutants (Petrovskis *et al.*, 1994) as well as the destructive souring of crude petroleum (Semple & Westlake, 1987) have also been considered.

More recently, *Shewanella* strains have been studied for their involvement in a variety of anaerobic processes including the dissimilatory reduction of manga-

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Abbreviations: FAME, fatty acid methyl ester; TMAO, trimethylamine *N*-oxide.

The GenBank accession numbers for the sequences reported in this paper are given in Table 1.

Table 1. Bacterial strains used in this study

Bacterium	Strain no.*	Place of isolation	Source	Reference†	Accession no.	
					gyrB‡	16S rDNA
S. algae	ACAM 4733			GenBank		AF00669
S. algae	ATCC 51181	Great Bay, NH, USA	Sediment	This study	AF005691	X81622
S. algae	ATCC 51192^T	Japan	Red algae	This study	AF005686	AF005249‡
S. algae	BCM-8	Chesapeake Bay, MD, USA	Water	This study	AF005687	
S. algae	IAM 14159	Japan	Red algae	GenBank		U91546
S. algae	SP-1		Clinical isolate	This study	AF005688	
S. algae	SP-5		Clinical isolate	This study	AF005689	
S. algae	SP-8		Clinical isolate	This study	AF005690	
S. algae	Tϕ8	Denmark, 1994	Seawater	Fonnesbech-Vogel *et al.* (1997)		U91547
S. algae	14.80-A	France, 1980	Flamingo	Fonnesbech-Vogel *et al.* (1997)		U91554
S. algae	189-tr	Italy	Fresh chicken breast	Fonnesbech-Vogel *et al.* (1997)		U91554
S. algae	43940	Denmark, 1994	Human blood	Fonnesbech-Vogel *et al.* (1997)		U91545
S. algae	68872	Denmark, 1994	Human ear infection	Fonnesbech-Vogel *et al.* (1997)		U91548
S. amazonensis	ATCC 700329^T	Amazon	Water	Venkateswaran *et al.* (1998)	AF005257	AF005248‡
S. baltica	NCTC 10735^T	Japan	Oil brine	Ziemke *et al.* (1998)		AJ000214
S. baltica	OS 155	Baltic Sea	Water	Ziemke *et al.* (1998)		AJ000215
S. baltica	OS 195	Baltic Sea	Water	Ziemke *et al.* (1998)		AJ000216
S. baltica	F137	Sweden, 1990	Pike	Fonnesbech-Vogel *et al.* (1997)		U91552
S. baltica	NCTC 10695	Formerly *Pseudomonas rubescens*	Oil emulsion	GenBank		U91553
S. benthica	ATCC 43991			GenBank		U91594
S. benthica	ATCC 43992^T			This study	AF014949	X82131
S. benthica	F1A			GenBank		U91592
S. frigidimarina	A173	Antarctica		GenBank		U85902
S. frigidimarina	ICO10^T; ACAM 591^T	Antarctica		This study	AF014947	U85906
S. frigidimarina	ICP1	Antarctica		GenBank		U85903
S. frigidimarina	ICP4	Antarctica		GenBank		U85905
S. frigidimarina	ICP12	Antarctica		GenBank		U85904
S. frigidimarina	NCIMB 400			GenBank		Y13699
S. gelidimarina	ACAM 456^T	Antarctica		This study	AF014946	U85907
S. hanedai	ATCC 33224^T	Arctic	Sediment	This study	AF005693	U91590
S. hanedai	CIP103207			GenBank		X82132
S. hanedai	ICO50	Antarctica		GenBank		U85908
S. hanedai	35256			GenBank		U91589
S. oneidensis	DLM-7	Green Bay, Lake Michigan, WI, USA	Sediment	This study	AF005697	
S. oneidensis	ATCC 700550^T; MR-1	Oneida Lake, NY, USA	Sediment	This study	AF005694	AF005251‡
S. oneidensis	MR-4	Black Sea	Water column 5 m depth	This study	AF005695	AF005252‡
S. oneidensis	MR-7	Black Sea	Water column 60 m depth	This study	AF005696	AF005253‡
S. oneidensis	MR-8			This study		AF005254‡
S. oneidensis	SP-3		Clinical isolate	This study	AF005698	
S. oneidensis	SP-7		Clinical isolate	This study	AF039060	AF039054‡
S. oneidensis	SP-22		Clinical isolate	This study	AF039058	AF039055‡
S. oneidensis	SP-32		Clinical isolate	This study	AF039059	AF039056‡
'*S. pealeana*'	ATCC 700345^T			This study	AF014945	AF011335
'*S. pealeana*'	ANG-SQ2			This study	AF039061	
S. putrefaciens	ACAM 122	Antarctica		GenBank		U39398
S. putrefaciens	ACAM 574	Antarctica		GenBank		AF006670
S. putrefaciens	ATCC 8071^T	England, 1931	Butter	This study	AF005669	U91550
S. putrefaciens	ATCC 8072	England, 1931	Butter	This study	AF005670	Unpublished‡
S. putrefaciens	BC-1	Blue Clay, IN, USA	Karst stream water	This study	AF005684	
S. putrefaciens	CE-1	Melt Pool, Near Cape Evans, Ross Island, Antarctica	Water	This study	AF005676	
S. putrefaciens	CE-10	Melt Pool, Near Cape Evans, Ross Island, Antarctica	Water	This study	AF005677	
S. putrefaciens	CG-1	KinnicKinnick River, WI, USA	Water	This study	AF005671	
S. putrefaciens	CG-3	KinnicKinnick River, WI, USA	Water	This study	AF005672	
S. putrefaciens	DLM-1	Western Lake Michigan, WI, USA	Plankton	This study	AF005678	
S. putrefaciens	DLM-2	Green Bay, Lake Michigan, WI, USA	Sediment	This study	AF005679	
S. putrefaciens	DLM-13	Mid-Lake Michigan, WI, USA	Sediment	This study	AF005680	
S. putrefaciens	LMP-1	Chemocline, Lower Mystic Pond, MA, USA	Water	This study	AF005673	
S. putrefaciens	LMP-9	Chemocline, Lower Mystic Pond, MA, USA	Water	This study	AF005674	
S. putrefaciens	LW-1	Lemonweir River, WI, USA	Water	This study	AF005683	
S. putrefaciens	NCIMB 12577	Canada, 1983; strain 200	Crude oil, Pembina oilfield	This study	AF014948	U91557
S. putrefaciens	MR-30	Green Bay, Lake Michigan, WI, USA	Sediment	This study	AF005675	AF005255‡
S. putrefaciens	SP-10		Clinical isolate	This study	AF039057	
S. putrefaciens	WAB-1	Wabash River, IN, USA	Water	This study	AF005685	
S. putrefaciens	1M-1	One Mile River, WI, USA	Creek water	This study	AF005681	
S. putrefaciens	7M-1	Seven Mile River, WI, USA	Creek water	This study	AF005682	
S. woodyi	ATCC 51908^T	Persian Gulf	Water	This study	AF014944	AF003548
Shewanella sp.	ATCC 8073	England, 1931	Butter	This study	Unpublished	Unpublished‡
Shewanella sp.	A6	Denmark, 1986	Whole gutted cod	Fonnesbech-Vogel *et al.* (1997)		U91549
Shewanella sp.	ACAM 576	Antarctica		GenBank		AF006671
Shewanella sp.	ACAM 577	Antarctica		GenBank		AF006672

* ACAM, Australian Collection of Antarctic Micro-organisms, Australia; ATCC, American Type Culture Collection, USA; IAM, Institute of Applied Microbiology, Japan; NCIMB, National Collection of Industrial and Marine Bacteria, UK; NCTC, National Collection of Type Cultures, UK. All other acronyms are strain numbers designated by individual researchers.

† Various data and cultures are either generated in this study or obtained from the mentioned bibliography, database or researcher.

‡ Nucleotide sequence data generated in this study.

nese and iron oxides (Myers & Nealson, 1988), iron in clays (Kostka *et al.*, 1996), uranium (Lovely & Phillips, 1988), thiosulfate (Perry *et al.*, 1993) and elemental sulfur (Perry *et al.*, 1993; Moser & Nealson, 1996). While long regarded as physiologically feasible, it was only in 1988 that the coupling of bacterial energy generation and growth to the reduction of iron and manganese oxides was demonstrated in *Shewanella* sp. MR-1 (Myers & Nealson, 1988; Nealson & Saffarini, 1996). Strain MR-1, formerly identified as *Shewanella putrefaciens*, is of particular interest because its total genome is currently being sequenced by The Institute of Genomic Research (TIGR, Bethesda, MD, USA).

Despite the applied microbiological relevance and diversity of function embodied by this group, the phylogenetic breadth and organization of the shewanellae remains incompletely addressed (Farmer, 1992; Stenstrom & Molin, 1990; Fonnesbech-Vogel *et al.*, 1997). Introduced as *Achromobacter putrefaciens* in 1931 (Derby & Hammer, 1931), the genus has undergone several name changes. In 1960, Shewan *et al.* (1960) reassigned *Achromobacter putrefaciens* to the genus *Pseudomonas* and, in 1977, Lee *et al.* (1977) further changed the genus designation to *Alteromonas*. The species *Alteromonas putrefaciens* remained the sole described member of the genus until the introduction of the bioluminescent *Alteromonas hanedai* in 1980 (Jensen *et al.*, 1980). In 1985, MacDonell & Colwell (1985) argued for the reclassification of these organisms into a new genus on the basis of 5S rRNA sequence data and proposed the name *Shewanella*.

The deep sea isolate, *Shewanella benthica* (MacDonell & Colwell, 1985), the marine non-H_2S-producing bacterium, *Shewanella colwelliana* (Weiner *et al.*, 1988; Coyne *et al.*, 1989) and the clinically important *Shewanella algae* (Simidu *et al.*, 1990; Nozue *et al.*, 1992; species name changed from *Shewanella alga* to *Shewanella algae* by Trüper & de' Clari, 1997) were included in *Shewanella* genus. To this day, *S. putrefaciens* remains, almost by default, the phenotypic resting place for a growing conglomerate of non-fermentative, mostly H_2S-producing aerobes loosely associated with aquatic habitats. The phenotypic boundaries of and within this group remain ill-defined for lack of a diagnostically informative pattern of phenotypic characteristics. However, with the recent improvements in the applicability of molecular approaches for microbial phylogeny, efforts are under way to clarify the phylogenetic organization of this genus. One result has been the reassessment of the distinctions between *S. putrefaciens* and *S. algae* (Fonnesbech-Vogel *et al.*, 1997). Likewise, Ziemke *et al.* (1998) showed that members of Owen's genomic group II (Owen *et al.*, 1978) of *S. putrefaciens* are phenotypically, genotypically and phylogenetically distinct enough from the rest of the *S. putrefaciens* members to be classified as a new species, *Shewanella baltica*. Also, a number of novel species have been recently introduced, including: the luminous species, *Shewanella woodyi* (Makemson *et al.*, 1997); two marine Antarctic taxa, *Shewanella gelidimarina* and *Shewanella frigidimarina* (Bowman *et al.*, 1997); *Shewanella amazonensis* (Venkateswaran *et al.*, 1998b); and a possibly symbiotic representative, '*Shewanella pealeana*' (Leonardo *et al.*, 1999).

Whereas the gene sequence of the small subunit of the 16S rRNA molecule is accepted for the definition of phylogenetic relationships between organisms (Woese, 1987), this molecule, at times, lacks the specificity required for the differentiation of close relatives (Venkateswaran *et al.*, 1998a; Fox *et al.*, 1992). To circumvent this limitation, the more rapidly evolving *gyrB* gene, (encoding the B subunit of the DNA gyrase, topoisomerase type II) (Edgell & Doolittle, 1997), has been employed as a high-resolution molecular identification marker for distinguishing strains of *Vibrio* (Venkateswaran *et al.*, 1998a), *Acinetobacter*, *Pseudomonas*, (Yamamoto & Harayama, 1995, 1996) and now *Shewanella*.

In this work, a reorganization of the genus *Shewanella* is proposed using a combination of two types of nucleotide sequence data, DNA–DNA reassociation, molecular probing, fatty acid and quinone profiling, and classical physiological testing. To assemble this polyphasic data set, the published works of other authors have been used, a variety of unpublished contributions from co-authors has been assembled and a large volume of data for established strains and new isolates collected expressly for this study has been generated. A new species, *Shewanella oneidensis* sp. nov. (type strain ATCC 700550^T; formerly known as *Shewanella* sp. MR-1 or *Shewanella putrefaciens* MR-1), is also described.

METHODS

Bacterial strains and growth conditions. Micro-organisms included in this study were isolated from various environmental sources, purchased from culture collections or were the gifts of others (Table 1). The isolation of *Shewanella*-like organisms was carried out by employing a variation of a previously described sulfur-agar-plate method (Moser & Nealson, 1996). Water or sediment slurry samples were collected in sterile containers and stored at 4 °C until bacteriological examinations were carried out. Typically, 100 µl of a given sample was spread with a flamed, glass rod over the surface of plates of half-strength LB agar (Miller, 1972) supplemented with sulfur and lactate at 40 and 30 mM, respectively (LB/S^0/Lac; Moser & Nealson, 1996). Plates were first incubated under aerobic conditions at room temperature (ca. 25 °C) for several days to obtain a collection of aerobic isolates. Colonies obtained in this manner were then screened for sulfur reduction after transfer of the plates into an anaerobic chamber (Coy Laboratory Products) maintained at 2 % H_2, with the remainder being N_2. Salmon-coloured colonies displaying cleared zones characteristic of sulfur-reducing bacteria (Moser & Nealson, 1996) were regarded as potential *Shewanella* candidates and subcultured by several rounds of streaking for isolation onto fresh plates.

Pure cultures obtained from other sources (Table 1) were likewise screened for sulfur reduction by streaking for isolation onto LB/S^0/Lac medium and following the same

two-step incubation procedure. For routine cultivation, cells were grown aerobically either in liquid with agitation or on LB agar media at 30 °C without additions. '*S. pealeana*' ATCC 700345^T was originally isolated on LB/S^0/Lac and subsequently grown on a defined medium called M1N (Leonardo *et al.*, 1999), an adaptation of M1 minimal medium (Myers & Nealson, 1988), supplemented with 0·5 M NaCl or on Marine agar (Difco).

Phenotypic characterization. Routine biochemical tests were carried out according to established procedures (Venkateswaran *et al.*, 1989; West & Colwell, 1984; Baumann *et al.*, 1984). The ability to grow at an NaCl concentration of 1–10% was determined in T_1N_1 liquid medium (Venkateswaran *et al.*, 1989), and the ability to grow without NaCl was determined in 1% sterile tryptone water. Sugars and amino acids were tested on LB broth at a concentration of 1% as described elsewhere (West & Colwell, 1984). Haemolytic activity was recorded on Trypticase soy agar supplemented with 5% defibrinated sheep blood. Additional phenotypic characteristics were determined by the BIOLOG microbial identification system. For the determination of cell shape and size and the detection of flagella, cells were negatively stained with osmium chloride according to the methods of Cole & Popkin (1981) and observed with a Hitachi transmission electron microscope (H-600).

Iron reduction. Bacterial colonies were streaked onto LM medium (0·02% yeast extract, 0·01% peptone, 0·6% NaCl, 10 mM sodium bicarbonate, 10 mM HEPES) supplemented with carbon substrates as appropriate (5 mM lactate, 5 mM succinate, 5 mM glycerol, 1 mM acetate), 50 mM ferric citrate, 5 mM sodium molybdate and the colour reagent ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4 triazine, pH 7·2]. Plates were incubated anaerobically at room temperature until colonies appeared (after ca. 7 d). Colonies displaying cleared zones were scored as positive for iron reduction. *Shewanella* sp. MR-1 and *Escherichia coli* ATCC 25922 were included as positive and negative controls, respectively.

Fatty acid analysis. Cells were cultivated overnight in liquid LB medium at 30 °C with vigorous shaking. Cellular fatty acids were extracted from lyophilized cells, methylated and analysed by GC (Ringelberg *et al.*, 1994). Fatty acid methyl esters (FAME) were analysed on a cross-linked 5% phenyl silicone capillary column (0·2 mm i.d.; 25 m long; Hewlett Packard) with a HP 5890A gas chromatograph. The column was kept at an initial temperature of 80 °C for 2 min and the temperature was programmed to increase from 80 to 150 °C at 10 °C min^{-1}, then to 282 °C at 3 °C min^{-1} and finally held at 282 °C for 5 min. The temperature of the injector was maintained at 250 °C and the detector was kept at 290 °C. The FAME peaks were identified by retention time comparisons against authentic standards and quantified by integration of peak areas. In addition, FAME peaks were also identified by MS (electron impact at 70 eV) including verification of double-bond position via dimethyl disulfide derivatization (Ringelberg *et al.*, 1994).

Quinone analysis. Fully grown overnight cultures were diluted 100-fold into 25 ml fresh LB and incubated at 30 °C with vigorous shaking until OD_{600} of 1 was reached. Aliquots from this culture were spread-plated on LB agar and incubated at 30 °C for 6 h. Cells were harvested by scraping, pooled, centrifuged and frozen prior to lyophilization (Lies *et al.*, 1996). Quinones were twice extracted with chloroform/methanol (2:1, v/v) from lyophilized ground cells. After centrifugation, the solvent phase was collected, evaporated to dryness and then resuspended in a known volume of acetone to normalize with respect to original biomass. Samples were quantified by comparison to a standard curve prepared with two known quinones (MK-4 and Q-10; Sigma). Quinones were separated by HPLC and individually identified by MS (Nishijima *et al.*, 1997). The relative molar ratios of quinone homologues were determined by reference to standard mixtures containing known amounts of MK-4 and Q-10 compounds.

Molecular characterization

Purified genomic DNA (Johnson, 1981) from liquid-grown cultures was used as the template for PCR amplification unless otherwise specified. PCR assays were performed in a DNA Thermal Cycler (Perkin-Elmer).

(i) DNA isolation and characterization. The DNA was isolated by chromatography on hydroxyapatite by the procedure of Cashion *et al.* (1977) for G+C (mol%) and DNA–DNA hybridization analyses. The G+C content of the DNA was determined by HPLC as described by Mesbah *et al.* (1989). DNA–DNA hybridization was carried out according to De Ley *et al.* (1970) with modifications as described by Huß *et al.* (1983) and Escara & Hutton (1980) using a Gilford System 2600 spectrophotometer equipped with a Gilford 2527-R thermoprogrammer and plotter. Renaturation rates were computed by the program TRANSFER.BAS (Jahnke, 1992). Strains included in the DNA–DNA hybridization experiments were: *S. algae* ATCC 51192^T, *S. amazonensis* ATCC 700329^T, *S. hanedai* DSM 6066^T, *S. benthica* DSM 8812^T, *Shewanella oneidensis* sp. nov. ATCC 700550^T, '*S. pealeana*' ATCC 700345^T, *S. putrefaciens* ATCC 8071^T and *S. woodyi* ATCC 51908^T.

(ii) 16S rDNA. Universal primers (Bact 11 and 1492) were used to amplify the 1·4 kb PCR fragment according to the protocols established by Ruimy *et al.* (1994). Amplicons thus generated were purified and sequenced as described below.

(iii) *gyrB* gene. Primers (UP-1 and UP-2r) within the known DNA sequence (Yamamoto & Harayama, 1995) were added to the PCR mixture at a concentration of 1 μM and the solution was subjected to 30 cycles of PCR (Venkateswaran *et al.*, 1998a). Amplified *gyrB* fragments (1·2 kb) from *S. putrefaciens* ATCC 8071^T and *S. hanedai* ATCC 33224^T were cloned in pGEM-ZF(+) (Promega) by conventional recombinant methods (Sambrook *et al.*, 1989). Expansion of the probes was carried out as documented previously (Sambrook *et al.*, 1989). After the ligation of PCR fragments into the vector, *E. coli* cells were transformed with the ligation mixture by the calcium chloride method and transformants were cultured under conditions which promote growth. Plasmids were recovered from transformants by the alkaline lysis method. The purified intact plasmids were then utilized as probes. DNA sequences were determined for both strands by extension from vector-specific [T7 and SP-6 primers from pGEM-ZF(+)] priming sites and by primer walking. For all of the other strains, the cloning step was eliminated and PCR-amplified 1·2 kb *gyrB* products were sequenced directly following purification on Qiagen columns.

(iv) Sequencing. The identity of a given PCR product was verified by sequencing using the dideoxy chain-termination method with the Sequenase DNA Sequencing kit (United States Biochemical) and an ABI 373A automatic sequencer as recommended by the manufacturer (Perkin-Elmer Applied Biosystems).

(v) Molecular identification of *S. putrefaciens*. By comparing the *gyrB* sequences of four *Shewanella* species (*S. putrefaciens* ATCC 8071^T, *S. algae* ATCC 51192^T, *S. hanedai* ATCC 33224^T and *S. benthica* ATCC 43992^T) a suitable PCR primer set (SP-1, 5′ TTC GTC GAT TAT TTG AAC AGT 3′ and SP-2r, 5′ TTT TCC AGC AGA TAA TCG TTC 3′) was designed from within the *gyrB* sequence of type strain ATCC 8071^T to specifically identify *S. putrefaciens*. The generation of a predicted 422 bp amplicon using these primers was inferred to indicate the presence of *S. putrefaciens* template DNA in a given sample. Amplification was performed using 30 PCR cycles, each consisting of 1 min at 94 °C, 1·5 min at 58 °C, 2·5 min at 72 °C and a final extension step at 72 °C for 7 min. The 422 bp amplicon was analysed by agarose gel electrophoresis (Venkateswaran *et al.*, 1998a). Suitable molecular size markers were included in each gel.

Phylogenetic analysis and sequence alignment. The phylogenetic relationships of organisms covered in this study were determined by comparison of individual 16S rDNA sequences with the approximately 10700 sequences that already exist in the ribosomal database for other members of the γ-subclass of the *Proteobacteria*. *gyrB* sequences were compared with ca. 300 other *gyrB* sequences in the database maintained by our laboratory and the Marine Biotechnology Institute, Kamaishi, Japan. Evolutionary trees were constructed with the PAUP (Swofford, 1990), PHYLIP (Felsenstein, 1990) and ARB program packages (Strunk & Ludwig, 1995). Nucleotide sequence accession numbers are given in Table 1.

RESULTS

Twelve species of the genus *Shewanella* have been reported or proposed as of this writing (based on 16S rDNA data in the GenBank database). Although the Committee on Reconciliation of Approaches to Bacterial Systematics validated *S. colwelliana* as a recognized *Shewanella* species (Weiner *et al.*, 1988), this species has not been considered because its 16S rRNA sequence has never been reported.

Morphological characteristics

All *Shewanella* strains examined were Gram-negative, rod-shaped and non-spore-forming (Table 2). Cells were 2–3 μm in length and 0·4–0·7 μm in diameter and were motile by means of a single unsheathed polar flagella. Cells grown on suitable agar media produced young colonies which were circular, 1–4 mm in diameter, smooth, convex and with regular edges. *S. algae*, *S. amazonensis*, *S. frigidimarina*, *S. gelidimarina*, *S. oneidensis* and *S. putrefaciens* showed pinkish coloration on LB agar whereas *S. benthica*, *S. hanedai*, '*S. pealeana*' and *S. woodyi* exhibited less intensively coloured colonies on marine agar.

Physiological properties

S. benthica grew optimally at ca. 4 °C, *S. hanedai* at 15–25 °C, *S. gelidimarina* at 15 °C and the other species at 25–35 °C. *S. algae*, *S. amazonensis* and some of the strains of *S. oneidensis* exhibited growth at 40 °C. In this report, a halotolerant strain is defined as one that grows optimally in media containing 0·0–0·3 M NaCl (Gilmour, 1990). Although the halotolerant strains grew even in the absence of NaCl, growth was accelerated in the presence of 0·2 M NaCl. Of the species considered here, *S. algae*, *S. amazonensis*, *S. frigidimarina*, *S. oneidensis* and *S. putrefaciens* fit this criterion. Alternatively, the halophiles [strains that grow optimally in media containing 0·2–2·0 M NaCl (Gilmour, 1990)], *S. benthica*, *S. hanedai*, *S. gelidimarina*, '*S. pealeana*' and *S. woodyi* grew on marine agar or any of a number of media supplemented with sufficient salts.

All strains were positive for cytochrome oxidase and catalase and negative for the production of amylase, alginase, arginine dihydrolase and lysine as well as ornithine decarboxylases. Gelatinase was produced by *S. algae*, *S. amazonensis*, *S. hanedai*, *S. frigidimarina*, *S. gelidimarina* and *S. oneidensis* and lipase was produced by *S. hanedai* and '*S. pealeana*'. Indole and acetoin were not produced by any strains. Visible bioluminescence was observed only in *S. hanedai* and *S. woodyi*.

The inability to ferment glucose has traditionally been employed as a defining characteristic of the shewanellae. However, Bowman *et al.* (1997) have reported glucose fermentation in *S. benthica* and *S. frigidimarina*.

Anaerobic electron acceptor reduction

All known shewanellae reduced trimethylamine *N*-oxide (TMAO) and the majority produced H_2S from thiosulfate. All *Shewanella* strains reduced nitrate to nitrite. Elemental sulfur reduction, as determined by the agar-plate-clearing assay (Moser & Nealson, 1996) was seen in *S. algae*, *S. amazonensis*, *S. oneidensis*, '*S. pealeana*' and *S. putrefaciens*, whereas *S. hanedai* and *S. woodyi* were negative. The same pattern was noted for iron reduction. Key diagnostic traits for the differentiation of all known 11 shewanellae are given in Table 2.

Phenotypic differentiation of *Shewanella* into its species

Shewanella strains are defined as being Gram-negative, oxidase- and catalase-positive and capable of reducing TMAO; the majority produce H_2S from thiosulfate. Glucose fermentation readily distinguishes *S. frigidimarina* and *S. benthica* from the remaining eight known shewanellae (Bowman *et al.*, 1997). Whereas the dissimilatory reduction of iron differentiates these shewanellae from other carbohydrate-fermenting organisms, the absence of growth at 20 °C distinguishes *S. benthica* from *S. frigidimarina*. Among the eight remaining shewanellae, the halophiles, *S. gelidimarina*, *S. hanedai*, '*S. pealeana*' and *S. woodyi* and the halotolerant strains, *S. algae*, *S. amazonensis*, *S. oneidensis* and *S. putrefaciens* form distinct clusters. The halophiles can be further separated on the basis of bioluminescence with the *S. gelidimarina*/'*S. pealeana*' group being non-luminous. The lipase test can be

Table 2. Phenotypic characterization of various *Shewanella* species

1, *S. algae* ($n=4$); 2, *S. amazonensis* ATCC 700329^T; 3, *S. baltica* NCTC 10735^T [data reproduced from Ziemke *et al.*, (1998)]; 4, *S. benthica* ATCC 43922^T [data reproduced from MacDonell & Colwell (1985) and Bowman *et al.* (1997)]; 5, *S. hanedai* ATCC 33224^T [data reproduced from Jensen *et al.* (1980)]; 6, *S. frigidimarina* ACAM 591^T [data reproduced from Bowman *et al.* (1997)]; 7, *S. gelidimarina* ACAM 456^T [data reproduced from Bowman *et al.* (1997)]; 8, *S. oneidensis* ($n=5$); 9, '*S. pealeana*' ATCC 700345^T [data reproduced from Leonardo *et al.* (1999)]; 10, *S. putrefaciens* ($n=10$); 11, *S. woodyi* ATCC 51908^T [data reproduced from Makemson *et al.* (1997)]. All strains showed the presence of a single polar flagellum, growth in the presence of 3 % NaCl, oxidase and catalase production. All strains were Gram-negative and did not produce alginase, arginine dihydrolase, lysine or ornithine decarboxylase. None of the strains produced indole or acetoin. Apart from the values given in the first five rows, numbers denote the percentage of strains giving a positive reaction.

Character	1	2	3	4	5	6	7	8	9	10	11
Cell shape	Straight rod	Straight rod	Straight rod	Curved rod	Straight rod	Straight or curved rod	Straight or curved rod	Straight rod	Straight rod	Straight rod	Straight rod
Luminescence	–	–	–	–	+	–	–	–	–	–	+
DNA G+C (mol %)	52–55	52	46	47	45	40–43	48	45	45	43–47	39
Optimal growth temp. (°C)	25–35	25–35		4–15	⩽25	20–22	15–17	25–35	25–30	25–35	25
Optimal pH for growth	7–8	7–8						7–8	7–8	7–8	7–8
Growth at:											
4 °C in 24 h	0	+	+	+	+	+	+	20	–	100	+
35 °C	100	+		–	–	–	–	100	–	80*	–
40 °C	100	+	–	–	–			60	–	0	–
NO_3 to NO_2	100	+	+	+	+	+	+	80	+	100	+
NO_2 to N_2	0	+						0	–	0	–
Production of:											
Amylase	0	–		–	+	–	–	0	–	0	+
Gelatinase	50	+		+	+	+	+	100	–	0	+
Lipase	0	–	+	–	+	+	+	0	+	0	–
Chitinase				+	+	–	+				–
Haemolysis	75	+						0	–	0	
H_2S production from $S_2O_3^{2-}$	100	+	+	+	–	+	+	100	+	100	–
Growth in NaCl (%):											
0	100	+		–	–	+	–	100	–	100	–
6	100	–		–	–	+	+	40	–	100	–
10	100	–		–	–	–	–	0	–	0	–
Utilization of:											
D-Galactose	75	+			+	–	–	80	+	100	+
D-Fructose	0	–				–	–	0	–	0	
Sucrose	0	–	+		–	+	–	0	+	40	–
Maltose	25	–	+			+	–	0	+	60	
Lactose	0	–						0	–	30	
Succinate	50	+			–	+	–	100	–	60	+
Fumarate	75	+				+	–	80	–	60	
Citrate	0	–	+		–			0	–	0	–
D-Mannitol	0	–				+	–	0	–	0	
Glycerol	0	–			–			0	–	0	–
D-Sorbitol	0	–						0	–	0	
DL-Malate	0	–	+					0	–	0	
DL-Lactate	100	+						100	–	60	
Reduction of:†											
Iron oxide	100	+	+	+	–	+	+	100	+	100	–
Manganese oxide	100	+		+	–			100	+	100	–

* One strain showed a positive reaction after 48 h incubation.

† Most of the strains were examined in this study.

employed to discriminate *S. hanedai* (positive) from *S. woodyi* (negative). The gelatin liquefaction test differentiates *S. gelidimarina* (positive) from '*S. pealeana*' (negative). The halotolerant strains can be divided on the basis of gelatin liquefaction. In this regard, *S. putrefaciens* can be separated from the remainder by virtue of being gelatinase-negative. *S. oneidensis* can be distinguished from *S. algae* and *S. amazonensis* by its inability to haemolyse sheep blood. Finally, *S. amazonensis* can be differentiated from *S. algae* by growth at 4 °C.

Fatty acid analysis

The FAME compositions of various *Shewanella* species are shown in Table 3. The FAME profiles display only those fatty acids comprising ⩾ 0·1 % of the total. The elimination of minor fatty acids from consideration had no effect on any of the relationships described here. All of the values presented here were recalculated to show the relative abundance of the various fatty acids.

Straight-chain saturated FAME (20–41 %), terminally

Table 3. Fatty acid composition (%) of various *Shewanella* species

1, *S. algae* ATCC 51192^T; 2, *S. amazonensis* ATCC 700329^T; 3, *S. benthica* ($n=2$) [data reproduced from Bowman *et al.* (1997)]; 4, *S. frigidimarina* ($n=8$) [data reproduced from Bowman *et al.* (1997)]; 5, *S. hanedai* ($n=2$) [data reproduced from Bowman *et al.* (1997)]; 6, *S. gelidimarina* ($n=2$) [data reproduced from Bowman *et al.* (1997)]; 7, *S. oneidensis* ATCC 700550^T; 8, '*S. pealeana*' ATCC 700345^T; 9, *S. putrefaciens* ATCC 8071^T; 10, *S. woodyi* ($n=2$) [data reproduced from Makemson *et al.* (1997)]. Both *S. frigidimarina* and *S. gelidimarina* produce eicosapentaenoic acid, 20:5ω3.

Fatty acid	1	2	3	4	5	6	7	8	9	10
Straight chain saturated fatty acids:										
14:0	1·3	1·4	14·3	3·7	11·5	5·1	2·6	6·1	2·3	6·7
15:0	6·5	9·2	0·8	2·5	4·2	5·1	4·7	2·8	3·2	5·2
16:0	16·8	6·1	13·8	11·8	17·6	10·9	14·8	20·3	19·1	27·5
17:0	4·1	3·9	5·4	1·2	1·0	0·6	2·8	1·6	1·5	1·6
18:0	0·4	0·1	0·3	0·1	0·3	0·2	1·1	1·1	2·1	
Terminally branched saturated fatty acids:										
13:0-iso	0·5	4·7	7·3	6·3	8·3	13·1	2·5	15·1	2·5	12·4
14:0-iso	1·4	1·5	1·1	0·6	0·3	0·4	2·3	1·2	0·3	0·3
15:0-iso	27·4	26·7	6·6	9·0	10·4	12·3	25·4	17·5	21·1	20·1
16:0-iso	0·5	1·4					1·4		0·1	
17:0-iso	1·4	1·8	1·0	0·3	2·1	0·3	1·7	1·1	1·7	1·4
Monounsaturated fatty acids:										
15:1ω6*c*	0·2	0·8	0·1	1·2	0·3	0·1	0·3		0·2	
16:1ω7*c*	15·3	14·7	42·3	51·1	37·7	39·9	23·3	22·0	29·6	19·9
16:1ω9*c*	2·8	0·7		2·2		0·4	2·1	1·1	3·5	1·4
17:1ω6*c*	0·9	2·4	0·7			1·1	1·5	1·0	0·9	
17:1ω8*c*	10·9	23·4	5·1	3·0	1·3	3·8	8·0	3·2	6·7	2·3
18:1ω7*c*	5·2	4·5	1·1	5·3	3·8	5·8	5·7	3·2	6·0	1·2
18:1ω9*c*	4·9	1·4	0·1	1·7	0·6	0·7	2·9	2·6	3·8	

branched saturated FAME (16–36%) and monounsaturated FAME (25–65%) were the major lipid classes detected. *S. benthica*, *S. frigidimarina*, *S. hanedai* and *S. putrefaciens* were high in terms of monounsaturates followed by straight-chain saturates and terminally branched saturates. As seen in the above group, *S. algae*, *S. amazonensis*, *S. gelidimarina* and *S. oneidensis* produced large amounts of monounsaturates but terminally branched saturates were more abundant than straight-chain saturates. All three groups of FAME were equally evident in '*S. pealeana*' whereas *S. woodyi* synthesized high amounts of straight-chain saturates followed by terminally branched saturates and monounsaturates.

Palmitoleic acid (16:1ω7*c*) was the most abundant monounsaturate found in all *Shewanella* species, except for *S. amazonensis*, in which 17:1ω8*c* was more abundant. Pentadecanoic acid (15:0) was the most abundant straight-chain saturated FAME in *S. amazonensis*, as compared to palmitic acid (16:0) which was most abundant in most of the other species. *S. benthica* and *S. hanedai*, however, produced proportionately more 14:0 than 16:0. Among terminally branched FAME, iso-pentadecanoic acid was the most abundant in all *Shewanella* species. However, 13:0-iso was also produced in considerable amounts in *S. benthica*, *S. frigidimarina*, *S. hanedai*, *S. gelidimarina*, '*S. pealeana*' and *S. woodyi*.

Quinone analysis

The isoprenoid quinone composition for the type strains of three of the *Shewanella* species is given in Table 4. Under aerobic growth conditions, ubiquinones (predominantly Q-7 and Q-8) were present at much higher concentrations than menaquinones (predominantly MK-7). *S. oneidensis* 700550^T and *S. putrefaciens* ATCC 8071^T exhibited very similar overall quinone compositions, whereas *S. hanedai* ATCC 33224^T had relatively lower menaquinone (MK-7) levels and lacked MMK-7. Although the type strain of *S. algae* has not been tested, strains of '*S. putrefaciens*' group IV (Owen *et al.*, 1978) (now classified as *S. algae*) have been examined (Akagawa-Matsushita *et al.*, 1992; Moule & Wilkinson, 1987). The quinone compositions of group IV strains strongly resemble those of *S. oneidensis* and *S. putrefaciens*.

Molecular phylogenetic analysis

(i) 16S rDNA sequence analysis. The 1·4 kb nucleotide sequences of the 16S rDNA covering base positions 11–1492 (*E. coli* numbering), were determined for the

Table 4. Isoprenoid quinone composition (%) of *Shewanella* species

Strain	Q-6	Q-7	Q-8	Q-9	MK-7	MK-8	MMK-7	Reference
S. oneidensis ATCC 700550^T	2	25	27		41		6	This work
S. putrefaciens ATCC 8071^T	2	36	42	1	11		7	This work
S. putrefaciens ATCC 8071^T	1	11	34		44	2	6	Akagawa-Matsushita *et al.* (1992)
S. hanedai ATCC 33224^T		35	58	1	6			Akagawa-Matsushita *et al.* (1992)

Table 5. 16S rDNA nucleotide sequence similarities (%) among known *Shewanella* species

Shewanella species: 1, *S. algae* ATCC 51192^T; 2, *S. amazonensis* ATCC 700329^T; 3, *S. baltica* NCTC 10735^T; 4, *S. benthica* ATCC 43992^T; 5, *S. frigidimarina* ACAM 591^T; 6, *S. gelidimarina* ACAM 456^T; 7, *S. hanedai* ATCC 33224^T; 8, *S. oneidensis* ATCC 700550^T; 9, '*S. pealeana*' ATCC 700345^T; 10, *S. putrefaciens* ATCC 8071^T; 11, *S. woodyi* ATCC 51908^T.

Species	1	2	3	4	5	6	7	8	9	10
1	–									
2	92·9	–								
3	93·7	94·0	–							
4	91·6	91·2	92·5	–						
5	93·0	91·9	96·7	92·1	–					
6	93·8	92·1	93·7	94·5	94·0	–				
7	93·5	91·3	87·1	95·5	93·2	95·0	–			
8	94·0	93·0	97·6	91·3	96·2	93·9	93·0	–		
9	93·0	92·1	93·0	94·1	93·3	97·0	94·1	92·8	–	
10	93·9	93·7	97·7	92·5	96·5	94·5	93·1	97·0	93·9	–
11	93·3	91·0	92·0	92·9	91·9	93·8	97·5	92·8	93·1	91·9

Table 6. 16S rDNA nucleotide sequence similarities (%) in *S. algae*

S. algae strain: 1, ATCC 51192^T; 2, IAM 14159; 3, ACAM 4733; 4, ATCC 51181; 5, Tϕ8; 6, 14.80-A; 7, 189-tr; 8, 68872.

Strain	GenBank no.	1	2	3	4	5	6	7
1	AF005249	–						
2	U91546	98·7	–					
3	AF00669	97·7	97·9	–				
4	X81622	99·6	99·1	98·1	–			
5	U91547	98·8	98·8	98·7	99·3	–		
6	U91554	99·0	99·3	98·3	99·5	99·2	–	
7	U91554	96·7	97·0	96·8	97·1	97·4	97·2	–
8	U91548	99·3	99·1	98·4	99·7	99·3	99·5	97·1

type strains of 11 *Shewanella* species. The percentage of nucleotide substitutions in the sequences varied from 2·5 to 8·8 % (Table 5). It should be noted that the pairs '*S. pealeana*'/*S. gelidimarina*, *S. putrefaciens*/*S. oneidensis* and *S. woodyi*/*S. hanedai* had dissimilarities in their 16S rDNA nucleotide sequences of only 2·5–3·0 %.

Variation in 16S rDNA sequences, generated in this study or retrieved from GenBank, among strains was examined for *S. algae* ($n=8$), *S. putrefaciens* ($n=7$), *S. oneidensis* ($n=8$) and *S. frigidimarina* ($n=6$). The relationships between *S. algae* strains as revealed by 16S rDNA sequences are depicted in Table 6. Although strains from various environmental, animal and clini-

Table 7. 16S rDNA nucleotide sequence similarities (%) in *S. putrefaciens*

S. putrefaciens strain: 1, ATCC 8071^T; 2, NCIMB 12577; 3, MR-30; 4, ACAM 122; 5, ACAM 574.

Strain	GenBank no.	1	2	3	4
1	X82133	–			
2	U91551	99·2	–		
3	AF005255	98·6	98·7	–	
4	U39398	99·6	99·1	98·6	–
5	AF006670	99·3	98·7	97·9	99·0

Table 8. 16S rDNA nucleotide sequence similarities (%) in *S. oneidensis*

S. oneidensis strain: 1, ATCC 700550^T; 2, LMG 2369; 3, SP-7; 4, SP-22; 5, SP-32; 6, ACAM 576; 7, MR-4; 8, MR-7; 9, MR-8.

Strain	GenBank no.	1	2	3	4	5	6	7	8
1	AF005251	–							
2	AJ000213	98·6	–						
3	AF039054	97·6	98·2	–					
4	AF039055	97·1	97·2	98·7	–				
5	AF039056	96·0	96·6	97·3	97·3	–			
6	AF006671	97·2	97·7	96·5	96·3	95·3	–		
7	AF005252	97·7	97·4	96·6	95·7	95·2	95·7	–	
8	AF005253	97·4	97·5	96·8	96·4	95·3	96·2	98·6	–
9	AF005254	96·9	96·9	96·2	95·8	94·7	95·7	98·1	98·9

Table 9. 16S rDNA nucleotide sequence similarities (%) in *S. frigidimarina*

S. frigidimarina strain: 1, ICO10^T: 2, ICP1; 3, ICP4; 4, ICP12; 5, NCIMB 400; 6, A173.

Strain	GenBank no.	1	2	3	4	5
1	U85906	–				
2	U85903	98·9	–			
3	U85905	99·6	99·1	–		
4	U85904	98·8	98·2	98·7	–	
5	Y13699	99·3	98·3	99·1	98·3	–
6	U85902	99·1	98·7	99·1	98·3	98·6

cal sources were sequenced (Table 1), all were quite homogeneous except for strain 189-tr which forms its own branch on the tree generated by the maximum-likelihood method (see Fig. 2). On the basis of their proximity to type strain ATCC 8071^T, the five strains shown in Table 7 were chosen as being representative of *S. putrefaciens*. Strains ATCC 8071^T, NCIMB 12577 and MR-30 were screened against the SP-1/SP-2r primer set and generated the expected amplicon. Strain NCTC 10695, which was formerly classified as *Pseudomonas rubescens* and recently renamed *S. putrefaciens*, was the most highly divergent of the group with similarities of 96·3–98·1 %. Further 16S rDNA analysis of the NCTC 10695 strain with recently established *S. baltica* revealed high similarity between them (97·3 %).

A group of strains isolated from Oneida Lake (NY, USA), the Black Sea, the Southern Ocean and some clinical strains, although somewhat heterogeneous in 16S rDNA sequence (94·7–98·9 % similarities) does appear to form a cluster (Table 8). Within this cluster, several subgroups appear. The group of environmental isolates collectively known as the MR strains (Myers & Nealson, 1988; Nealson *et al.*, 1991) and the clinical isolates partition into two groups, while ACAM 576 remains distinct. The 16S rDNA similarities between the six strains of *S. frigidimarina* are shown in Table 9.

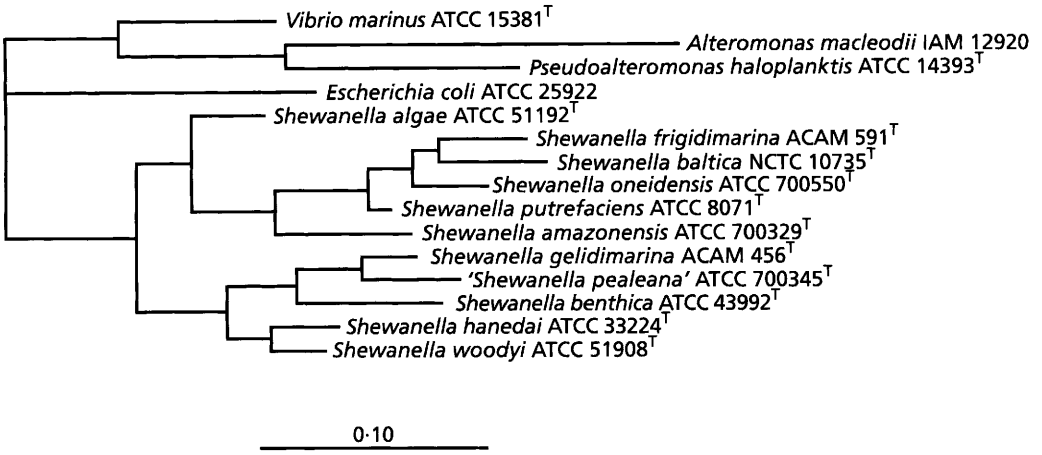

Fig. 1. Phylogenetic tree including type strains of *Shewanella* based on 16S rDNA sequence comparison by maximum-likelihood analysis.

This group forms a tight cluster, including one strain (NCIMB 400) which was formerly designated *S. putrefaciens* and has been renamed as *S. frigidimarina* (Reid & Gordon, 1999).

A phylogenetic tree based on type strain 16S rDNA sequences is shown in Fig. 1. The branching order of the 16S rDNA-based tree shows two distinct clusters corresponding to halophilic ('*S. pealeana*', *S. gelidimarina*, *S. benthica*, *S. hanedai* and *S. woodyi*) and halotolerant (*S. algae*, *S. amazonensis*, *S. putrefaciens*, *S. frigidimarina* and *S. oneidensis*) *Shewanella* groupings. The 16S rDNA sequences of 47 strains from various sources (Table 1) were aligned and a neighbour-joining phylogenetic tree was constructed (Fig. 2). 16S rDNA analysis indicates that the luminous species, *S. hanedai* and *S. woodyi*, form a group that is independent of the others in which *S. woodyi* ATCC 51908^T branches most deeply of all. *S. benthica*, '*S. pealeana*' and *S. gelidimarina* also form a distinct independent group. In this phylogeny, the *S. algae* group forms a distinct clade with the exception of strain 189-tr. *S. putrefaciens*, *S. oneidensis*, *S. baltica* and *S. frigidimarina* occupy a position adjoining *S. amazonensis*.

The clinical strains of *S. oneidensis* (SP-7, SP-22 and SP-32) along with *S. oneidensis* ATCC 700550^T and ACAM 576 form a single group. The other three strains of *S. oneidensis* (MR-4, MR-7 and MR-8) comprise a distinct cluster. Among strains that were until recently called *S. putrefaciens*, three distinct clusters are noticed. They are: type strain ATCC 8071^T cluster, *S. baltica* cluster and *S. oneidensis* cluster. Apparently these clusters correspond to Owen's hybridization groups I, II and III, respectively. However, NCTC 10695, which is the reference strain for Owen's group I, formed a cluster with *S. baltica* (Owen's group II). Although the strain ATCC 8073 (Owen's group III) appeared to form a cluster with the *S. oneidensis* group, strain 8073 branches most deeply among the others in the group. In addition, 16S rDNA nucleotide sequence dissimilarities are very high (5·6%) between strains 8073 and ATCC 700550^T. Furthermore, the strain LMG 2369 (synonym of ATCC 8073) showed only 95·4% similarity in its 16S rDNA sequences with ATCC 8073. Because of these inconsistencies in Owen's hybridization grouping and recent classification of *S. putrefaciens* based on 16S rDNA sequencing, a detailed DNA–DNA hybridization study is necessary to unearth the problem, if any, by including as many strains as possible.

(ii) *gyrB* sequence analysis. The 1·2 kb nucleotide sequences of the *gyrB* gene covering base positions 274–1525 (*E. coli* numbering, accession number P06982), were determined for ten *Shewanella* type strains. The percentage of nucleotide substitutions in the *gyrB* genes varied from 12·7 to 27·3% (Table 10), whereas the percentage of substitutions in the amino acid sequences was 7·2–20·6%. Among halophilic shewanellae, the range of *gyrB* substitutions was 16–21%, whereas the halotolerant strains exhibited greater heterogeneity (14–23%).

One of the major conclusions drawn from this work is that the species *S. putrefaciens* needs to be better defined. Our first suspicions in this regard derived from an unexpected result in a molecular probing experiment. A primer set (SP-1/SP-2r) was designed to specifically recognize the *gyrB* sequence of *S. putrefaciens* type strain ATCC 8071^T. As predicted, the primer set failed to produce the 422 bp amplicon when checked against 77 other species, including members of the genera *Shewanella*, *Vibrio*, *Alteromonas*, *Deleya*, *Marinomonas* and some representative of enteric bacteria (data not shown). When the PCR primer set was tested against the type strains of all ten known shewanellae (Fig. 3), a 422 bp amplicon was generated only for *S. putrefaciens*. The universal *gyrB* primer set (UP-1/UP-2r) was used as a control and generated the predicted 1·2 kb PCR product for every organism tested in this study. When the primer set was tested against 100

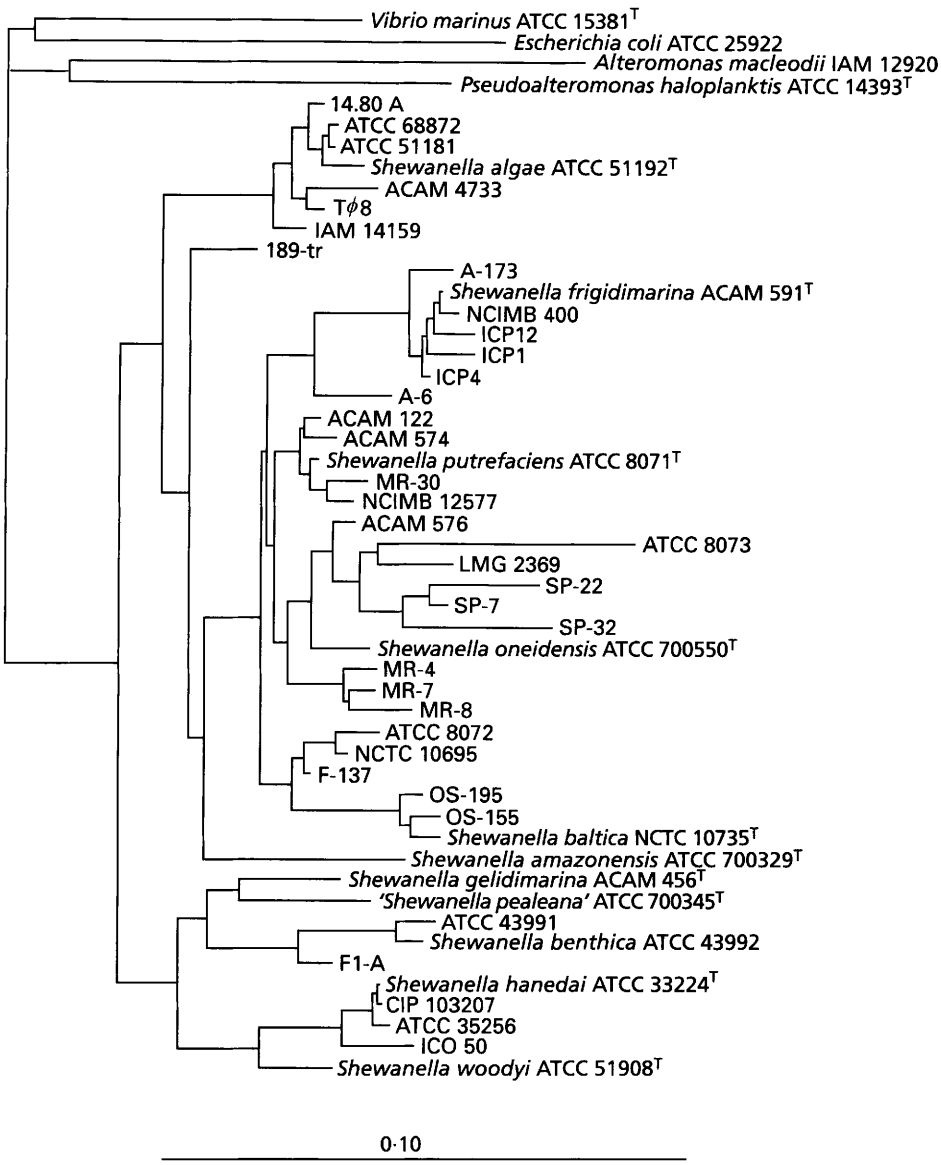

Fig. 2. Phylogenetic tree including various strains of *Shewanella* based on 16S rDNA sequence comparison by maximum-likelihood analysis.

putative *S. putrefaciens* isolates from a variety of sources, 23 strains produced no amplicon. Of the 73 strains that showed a *S. putrefaciens*-specific amplicon, 19 strains, as well as 23 that were negative for the PCR product, were selected for 1·2 kb *gyrB* gene sequencing. Of the subset for which the amplicon was generated, DNA sequence analysis revealed a close phylogenetic relationship between these strains and *S. putrefaciens* type strain ATCC 8071^T. In the case of those which failed to generate the PCR product, all could be reassigned to the known species *S. algae* or represented novel species within the genus *Shewanella* (*S. amazonensis* and *S. oneidensis*), thus verifying the utility of the probe for the identification of *S. putrefaciens* and suggesting that the name *S. putrefaciens* must be applied more critically. The primer set established here may then be used as a convenient and highly specific diagnostic tool for the identification of *S. putrefaciens*. Questions resulting from these experiments became the point of departure for the remainder of the study.

Variation in *gyrB* sequences among strains was examined for *S. algae* ($n=6$), *S. putrefaciens* ($n=19$) and *S. oneidensis* ($n=8$). The relationships between *S. algae* strains as revealed by *gyrB* sequences are depicted in Table 11. Although three strains each from environmental and clinical sources were sequenced, no patterns were observed except for between ATCC 51181 and the clinical isolates.

Dissimilarity among the 19 strains of *S. putrefaciens*

Table 10. *gyrB* nucleotide sequence similarities (%) among known *Shewanella* species

Shewanella species: 1, *S. algae* ATCC 51192^T; 2, *S. amazonensis* ATCC 700329^T; 3, *S. benthica* ATCC 43992^T; 4, *S. frigidimarina* ACAM 591^T; 5, *S. gelidimarina* ACAM 456^T; 6, *S. hanedai* ATCC 33224^T; 7, *S. oneidensis* ATCC 700550^T; 8, '*S. pealeana*' ATCC 700345^T; 9, *S. putrefaciens* ATCC 8071^T; 10, *S. woodyi* ATCC 51908^T.

Species	1	2	3	4	5	6	7	8	9
1	–								
2	80·5	–							
3	75·2	77·9	–						
4	74·3	76·3	77·1	–					
5	73·3	76·1	80·1	80·8	–				
6	74·4	77·4	83·6	80·4	87·3	–			
7	76·3	77·9	77·8	79·1	79·3	79·9	–		
8	72·7	75·3	78·7	79·3	79·7	83·2	76·8	–	
9	77·1	79·1	81·0	81·8	81·1	82·6	85·4	78·9	–
10	73·5	74·2	80·7	74·7	79·2	82·4	78·6	79·2	77·5

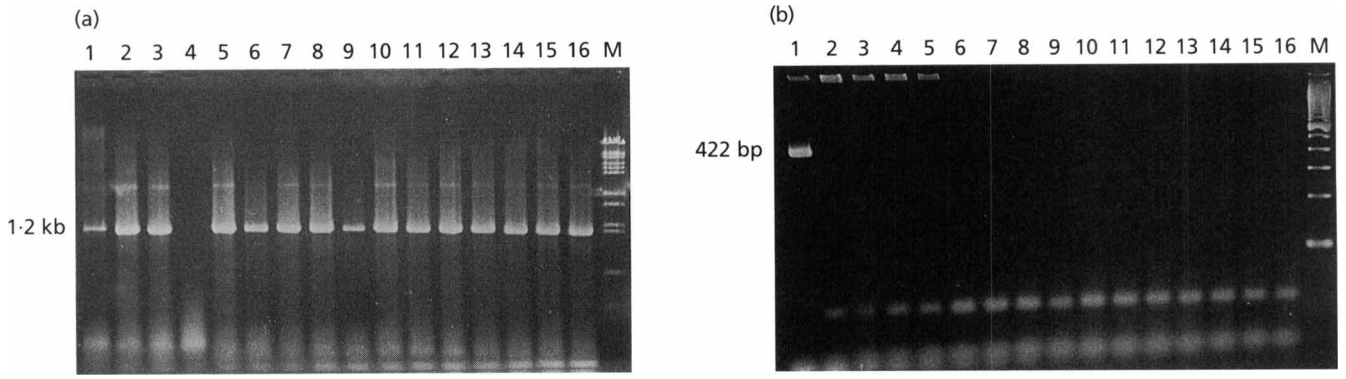

Fig. 3. Sensitivity of SP-1 and SP-2r PCR primers for the amplification of the *S. putrefaciens*-specific amplicon. (a) 1·2 kb *gyrB* fragment; (b) 422 bp *S. putrefaciens*-specific fragment. Lanes: M, DNA ladder; 1, *S. putrefaciens* ATCC 8071^T; 2, *S. algae* ATCC 51181; 3, *S. amazonensis* ATCC 700329^T; 4, bacterial DNA-free PCR mixture (negative control); 5, *S. benthica* ATCC 43992^T; 6, *S. frigidimarina* ACAM 594; 7, *S. gelidimarina* ACAM 456^T; 8, *S. hanedai* ATCC 33224^T; 9, *S. oneidensis* 700550^T; 10, '*S. pealeana*' ATCC 700345^T; 11, *S. woodyi* ATCC 51908^T; 12, *E. coli* ATCC 25992; 13, *Vibrio parahaemolyticus* ATCC 17802^T; 14, *Vibrio marinus* ATCC 15381^T; 15, *Alteromonas macleodii* ATCC 27126^T; and 16, *Pseudoalteromonas haloplanktis* ATCC 14393^T.

Table 11. *gyrB* nucleotide sequence similarities (%) in *S. algae*

S. algae strain: 1, ATCC 51192^T; 2, ATCC 51181; 3, BCM-8; 4, SP-1; 5, SP-5; 6, SP-8.

Strain	GenBank no.	1	2	3	4	5
1	AF005686	–				
2	AF005691	90·9	–			
3	AF005687	96·5	91·1	–		
4	AF005688	96·4	90·0	95·1	–	
5	AF005689	96·0	89·3	93·6	97·2	–
6	AF005690	95·0	88·6	92·8	96·0	98·0

Table 12. *gyrB* nucleotide sequence similarities (%) in *S. putrefaciens*

S. putrefaciens strain: 1, ATCC 8071^T; 2, ATCC 8072; 3, BC-1; 4, CE-1; 5, CE-10; 6, CG-1; 7, CG-3; 8, DLM-1; 9, DLM-2; 10, DLM-13; 11, LMP-1; 12, LMP-9; 13, LW-1; 14, NCIMB 12577; 15, MR-30; 16, SP-10; 17, WAB-1; 18, 1M-1; 19, 7M-1.

Strain	GenBank no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	AF005669	–																	
2	AF005670	91·0	–																
3	AF005684	88·2	90·4	–															
4	AF005676	88·3	89·6	87·0	–														
5	AF005677	87·3	89·2	86·3	98·5	–													
6	AF005671	91·2	93·6	92·5	90·4	91·3	–												
7	AF005672	89·7	93·6	91·3	89·6	90·0	95·9	–											
8	AF005678	92·4	92·8	91·7	89·6	88·3	95·5	93·2	–										
9	AF005679	93·2	93·8	92·1	90·7	89·4	95·9	93·5	97·2	–									
10	AF005680	93·6	93·8	92·4	90·4	89·4	95·9	94·0	97·1	98·8	–								
11	AF005673	91·2	95·0	90·9	89·9	90·1	95·1	94·3	93·3	94·2	94·3	–							
12	AF005674	91·2	95·6	91·1	90·4	91·2	96·5	95·1	93·4	94·6	94·5	98·3	–						
13	AF005683	88·5	90·5	97·1	87·5	86·9	93·2	91·4	91·8	93·0	93·0	91·2	91·2	–					
14	AF014948	97·5	92·0	88·6	89·1	87·9	91·6	90·1	92·3	92·9	93·1	92·1	92·0	89·0	–				
15	AF005675	92·0	95·1	91·7	89·6	89·4	95·4	93·6	94·1	95·9	96·2	94·7	95·4	92·1	92·8	–			
16	AF039057	97·6	91·6	88·6	89·1	87·9	91·7	90·1	91·9	92·5	92·7	91·7	91·7	88·7	97·1	92·3	–		
17	AF005685	87·5	88·9	96·5	86·4	85·8	91·4	89·7	90·9	91·9	92·1	89·7	89·9	95·5	88·0	90·9	87·4	–	
18	AF005681	88·6	89·9	94·9	86·6	85·9	91·0	89·3	91·7	92·4	92·4	90·9	90·6	94·6	88·7	91·0	88·6	94·0	–
19	AF005682	88·9	90·3	96·7	87·5	86·8	92·7	90·2	91·6	92·6	92·6	91·3	91·3	97·8	89·0	92·1	89·2	95·6	96·0

Table 13. *gyrB* nucleotide sequence similarities in *S. oneidensis*

S. oneidensis strain: 1, ATCC 700550^T; 2, MR-4; 3, MR-7; 4, DLM-7; 5, SP-3; 6, SP-7; 7, SP-22; 8, SP-32.

Strain	GenBank no.	1	2	3	4	5	6	7
1	AF005694	–						
2	AF005695	90·4	–					
3	AF005696	86·9	94·4	–				
4	AF005697	92·0	87·6	86·1	–			
5	AF005698	89·8	89·8	88·1	86·9	–		
6	AF039060	86·9	88·4	90·0	86·8	92·3	–	
7	AF039058	87·3	89·0	90·5	87·2	92·6	99·0	–
8	AF039059	87·3	88·9	90·5	87·3	92·9	98·6	99·1

examined was 1·2–12·0% (Table 12). The strain isolated from the inner harbour of Milwaukee (CG-1) showed the greatest consensus, being $\geqslant 90\%$ homologous to all of the others. The isolates from Lake Michigan (DLM strains) and Lower Mystic Pond, MA, USA (LMP strains) displayed at least 90% identity with 16–17 of the others. Wisconsin isolates (LW-1, 1M-1 and 7M-1) were homologous to 11–13 strains. Among the strains tested, the Antarctic strains (CE-1 and CE-10) exhibited the highest dissimilarity (8·7–14·2%) when their *gyrB* sequences were compared to others. Strains SP-10 (clinical isolate), NCIMB 12577 (oilfield isolate) and ATCC 8071^T (type strain), although derived from very different source materials, formed a defined cluster, exhibiting a similarity of $>97·5\%$. The dissimilarities in the *gyrB* sequences of the group *S. oneidensis* are shown in Table 13. Although a large within-group variation was observed (1·0–13·9%), no definite patterns with respect to the sources of these isolates was apparent.

The relative phylogenetic positions occupied by each of the *Shewanella* type strains, based on *gyrB* nucleotide sequences, are shown in Fig. 4. An unrooted tree was generated by the maximum-likelihood method (Fig. 4). The results indicate that *S. putrefaciens* and *S. oneidensis*, as well as *S. algae* and *S. amazonensis*, form distinct clusters. The branching order of the *gyrB*-based tree differs somewhat from that of a similar tree based on 16S rDNA nucleotide sequences. The *gyrB* sequences of 41 strains from various sources were aligned and the resulting unrooted tree diverged into two major branches corresponding to the halophilic and halotolerant species (Fig. 5). The halotolerant strains, *S. putrefaciens* ($n=19$), *S. oneidensis* ($n=8$), *S. algae* ($n=6$) and *S. amazonensis* clustered into four distinct groups of perfect integrity with regard to species. Within the *S. putrefaciens* group, five clusters are evident and at least partially consistent with geographical groupings. These clusters include: the deep-branching Antarctic isolates (CE-1 and CE-10);

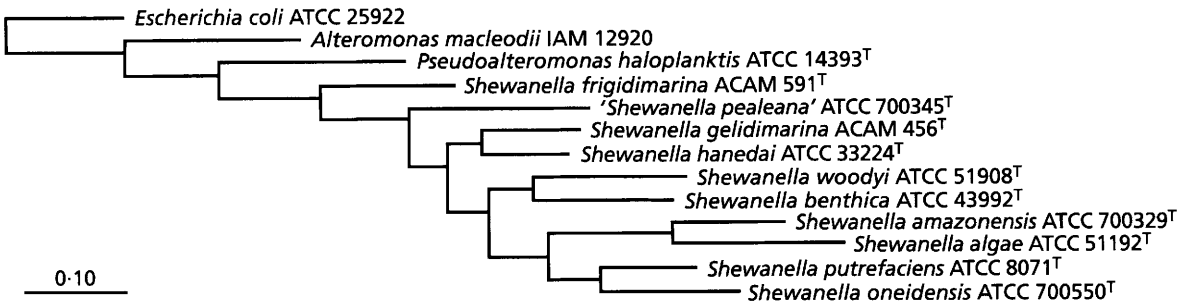

Fig. 4. Phylogenetic tree including type strains of *Shewanella* based on *gyrB* nucleotide sequence comparison by maximum-likelihood analysis.

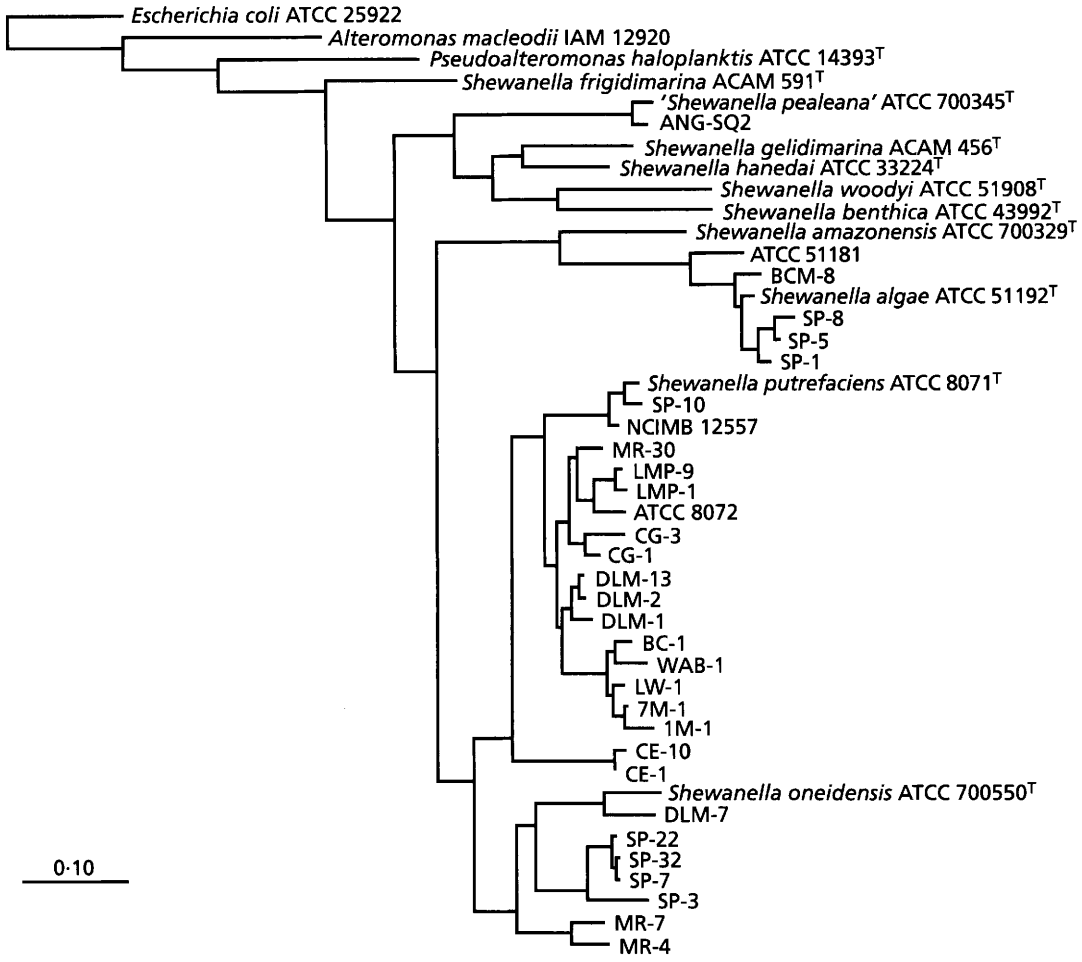

Fig. 5. Phylogenetic tree including various strains of *Shewanella* based on *gyrB* nucleotide sequence comparison by maximum-likelihood analysis.

the type strain cluster, comprised ATCC 8071^T, NCIMB 12557 and one clinical strain, SP-10; the ATCC 8072 cluster containing strains isolated from the inner harbour of Milwaukee, Lower Mystic Pond, MA, and Green Bay, WI, USA; an off-shore Lake Michigan cluster (DLM-1, DLM-2, DLM-13); and a cluster derived from agricultural streams of Wisconsin and Indiana (1M-1, 7M-1, LW-1, WAB-1, BC-1). Among the *S. oneidensis* isolates, strains ATCC 700550^T and DLM-7 form a cluster, MR-4 and MR-7 form another and the clinical isolates, SP-3, SP-7, SP-22 and SP-32, form yet another. The *S. algae* group

Table 14. DNA–DNA hybridization (%) of various *Shewanella* species

Shewanella species: 1, *S. algae* ATCC 51192^T; 2, *S. amazonensis* ATCC 700329^T; 3, *S. benthica* ATCC 43992^T; 4, *S. hanedai* ATCC 33224^T; 5, *S. oneidensis* ATCC 700550^T; 6, '*S. pealeana*' ATCC 700345^T; 7, *S. putrefaciens* ATCC 8071^T; 8, *S. woodyi* ATCC 51908^T.

Species	1	2	3	4	5	6	7
1	–						
2	30·1	–					
3	23·0	31·2	–				
4	15·0	29·3	31·3	–			
5	17·6	40·3	30·5	24·4	–		
6	37·6	27·3	29·4	26·4	20·4	–	
7	16·6	42·7	24·6	15·7	35·7	21·5	–
8	25·6	24·6	33·5	27·4	39·0	23·0	29·4

clusters tightly, with the exception of strain ATCC 51181 (BrY), which bifurcates at some distance from the others. The other major branch, comprising the halophiles, can be divided into five distinct groups.

(iii) DNA–DNA hybridization. DNA–DNA hybridization was performed to examine relatedness between the eight species considered here. The percentage similarities of *Shewanella* strains examined are given in Table 14. None of the values were above the 70% similarity value which would place the strains within the same species (Stackebrandt & Goebel, 1994). Among the halophilic shewanellae, the DNA reassociation percentage range was 23·0–33·5%, with *S. benthica* and *S. woodyi* being most closely related (33·5%) and the two luminous species, *S. hanedai* and *S. woodyi*, being less closely related (27·4%). Among the halotolerant strains, DNA–DNA hybridization values varied between 16·6 and 42·7%. DNA–DNA similarity values between *S. putrefaciens* and *S. oneidensis* were 35·7%; *S. algae* and *S. amazonensis*, 30·1%; and *S. putrefaciens* and *S. algae*, 16·6%.

DISCUSSION

As discussed previously, Farmer (1992) divided the family *Vibrionaceae* into four major genera, *Aeromonas*, *Photobacterium*, *Plesiomonas* and *Vibrio*, and a dozen minor genera, including *Shewanella*. In spite of this humble treatment, the shewanellae represent bacteria of economic significance. Perhaps because these organisms have been studied largely on the basis of applied microbiology, the phylogeny of this group may have been neglected.

Until recently, bacterial taxonomists have had little option but to rely on patterns of physiological traits to classify their subjects. Colony appearance, characteristic smells, pigmentation and other attributes of organisms in culture can be of dubious value in terms of useful taxonomic insight and can potentially lead to the assignment of misguided associations between loosely affiliated or unrelated organisms. While it is possible to systematize any group of isolates on the basis of phenotypic characters, the comparative value of a given trait or group of traits over another remains difficult to assess. Even when consensus provides a set of biochemical criteria with which to define a given organism, in the absence of exhaustive quality assurance measures, variations in the approaches used for the culture and testing of isolates can produce very different results, potentially leading to spurious identifications.

For example, *Shewanella* species display an ability to adjust their membrane phospholipid fatty acid content depending on whether they are grown under aerobic or anaerobic conditions (Nichols *et al.*, 1997). Ester-linked phospholipid fatty acid patterns of strain ATCC 700550^T were monitored over an 8 h incubation period under two sets of conditions: an aerobic to anaerobic transition and an anaerobic to aerobic one. The relative amounts of terminally branched saturated FAME increased by 13–25% when the organisms were shifted from an aerobic to anaerobic environment. This switch also resulted in a decrease in the relative percentage of the monounsaturated forms from 65 to 55%. In the opposite case, anaerobic to aerobic transition, little change in either class of FAME was detected. Monounsaturated FAME remained at a relative percentage of 54% while a small increase, 21 to 26%, in the terminally branched saturates was observed (data not shown). The occurrence of ubiquinones and menaquinones in the membranes of a number of the species of this genus (Lies *et al.*, 1996) coincides with the ability of these organisms to proliferate under both aerobic and anaerobic conditions.

These differences clearly point out the need for standardized growth conditions for biomolecular analyses and make comparisons of data from different laboratories difficult. This is not meant to infer that phenotypic considerations have lost standing in the determination of taxonomic relationships. Indeed, the Ad Hoc Committee on the Reconciliation of Approaches to Bacterial Systematics concluded that phenotypic consistency must remain the bottom line in polyphasic taxonomy (Wayne *et al.*, 1987). Rather, the limitations of classical phenotypic taxonomy argue for the inclusion of independent measures for the validation of bacterial relationships.

Although it is possible to differentiate all 11 shewanellae based on physiological traits, the required biochemical testing is labour intensive, costly and potentially prone to experimental error. Molecular methods are tacitly less susceptible to artefactual misinterpretation than culture-based approaches. With the advent of these techniques, a well-defined series of criteria for the designation of taxa now exist that function independently of sometimes phylogenetically arbitrary physiological and morphological boundaries. The basic unit of taxonomy is the species,

which is defined as a group of strains, including the type strain, sharing $\geqslant 70\%$ relatedness over the entire genome (Wayne *et al.*, 1987). An extensive review of the literature by Stackebrandt & Goebel (1994) revealed that organisms with less than 97% similarity over the 16S rRNA gene do not yield DNA reassociation values of more than 60%; hence, rDNA sequence analysis may be used as a surrogate for DNA reassociation. On the other hand, rDNA similarity of greater than 97% does not necessarily indicate that any two isolates are of the same species. For example, although *Vibrio parahaemolyticus* and *Vibrio alginolyticus* show very high rDNA similarity (99·9%; Ruimy *et al.*, 1994), phenotypically they are easily distinguished and regarded as distinct species (Venkateswaran *et al.*, 1989). Similarly, *Bacillus cereus* and *Bacillus thuringiensis* cannot be differentiated at the molecular level though the latter can be physiologically distinguished by virtue of its insecticidal crystal protein (Turnbull & Kramer, 1991). For these reasons, any taxonomic scheme must show phenotypic as well as molecular consistency (Wayne *et al.*, 1987).

At the time of submission, GenBank contained 39 16S rDNA sequences for organisms denoted as *S. putrefaciens*. Members of this rather heterogeneous group possessed sequence similarities as low as 94%. For example, strains 189-tr, NCIMB 400 and ACAM 576, which are placed within *S. putrefaciens* (95, 96·1, 95·3% similarity) actually have higher similarities to *S. algae* (96·7%), *S. frigidimarina* (99·3%) and *S. oneidensis* (97·2%), respectively. In accordance with the consensus molecular definition of the species (Wayne *et al.*, 1987), we have defined the subset of strains within this group containing 97% or greater similarity to the type strain ATCC 8071^T as legitimate *S. putrefaciens*.

Previously, Owen *et al.* (1978) reported the existence of four groups in *S. putrefaciens* based on the DNA–DNA reassociation values. *S. putrefaciens* ATCC 8071^T, 8072 and 8073 strains are representative of Owen's group I, II and III, respectively. Group IV is already defined as *S. algae* (Simidu *et al.*, 1990). Group I strains that are highly related to the type strain ATCC 8071^T are considered as the true *S. putrefaciens*. Ziemke *et al.* (1998) established *S. baltica* as a new species to represent group II. However, strain ATCC 8072 has low DNA–DNA reassociation value with the type strain of *S. baltica* NCTC 10735^T (58%; Owen *et al.*, 1978). Additionally, in our study the *gyrB* primers specific for ATCC 8071^T do amplify the *S. putrefaciens*-specific fragment with the DNA extracted from the ATCC 8072 strain. Since other strains from group II, including those now classified as *S. baltica*, have not been examined, it is not known whether these primers are specific for group I or also recognize group II strains, or whether ATCC 8072 should be reclassified as a group I strain.

No-one has reported any definite species belonging to Owen's proposed group III. However, Ziemke *et al.* (1998) cited in their recent publication that strain LMG 2369 might represent group III. Our study showed that *S. oneidensis* has high 16S rDNA similarities with LMG 2369 (98·6%) whereas the ATCC 8073 strain that represents group III had very low 16S rDNA homologies with both LMG 2369 (95·4%) and ATCC 700550^T (94·4%) strains. The phylogenetic tree constructed by including these strains showed a single cluster with a deep branch for strain ATCC 8073. Since the NCIMB strain catalogue lists LMG 2369 as a synonym for ATCC 8073, it is unclear why the 16S rDNA sequences for these strains are different and this also complicates the assignment of these strains to a given species or hybridization group. As a DNA–DNA reassociation study with *S. oneidensis* ATCC 700550^T and LMG 2369 was not performed, it cannot be concluded here that *S. oneidensis* represents group III.

Owen *et al.* (1978) also claimed that DNA–DNA reassociation values are variable between the members of all four groups within the shewanellae and noted high reassociation values (>80–100%) only for groups I and IV. In contrast, groups II and III showed ranges of 57–83% and 41–72%, respectively. Because of these inconsistencies within the *S. putrefaciens*, Owen concluded that it is difficult to relate DNA relatedness to the taxonomic hierarchy of this species. While our approach is not based on the Owen groupings, we agree with the report of Owen's that DNA relatedness is not the decisive factor deciding the taxonomic hierarchy in *S. putrefaciens*. Further studies are warranted.

DNA–DNA hybridization was used to verify our assertion that each of the 11 groups described here deserves species status. Although *S. oneidensis* strains (formerly the MR group) have traditionally been placed within the species *S. putrefaciens* (Myers & Nealson, 1988) and phenotypic data fail to reveal any major distinctions between them, DNA–DNA reassociation results (35·7%) clearly indicate that these should be regarded as distinct. It should be noted that the recently reported luminous species *S. woodyi* exhibited 97·5% 16S rDNA sequence similarities with its luminous counterpart *S. hanedai*, whereas DNA–DNA reassociation and *gyrB* sequence similarities were 27·4 and 82·4 %, respectively. Although *S. amazonensis* and *S. algae* appear to be closely related phenotypically, they display a low level of DNA–DNA similarity (30·1%); whereas *S. amazonensis* and *S. putrefaciens* show the highest similarity of all (42·7%). While this manuscript was in proof, our recent DNA hybridization study revealed that '*S. pealana*' ANG-SQ1^T and *S. gelidimarina* ACAM 456^T had only 25.3% similarity and deserve a separate species status (Leonardo *et al.*, 1999).

The use of *gyrB* as a measure of bacterial relatedness has been established in the genera *Pseudomonas*, *Acinetobacter* (Yamamoto & Harayama, 1995, 1996), *Vibrio* (Venkateswaran *et al.*, 1998a), *Bacillus* and *Clostridium* (unpublished results). The base substitution frequency of *gyrB* is much higher than that of the 16S rRNA gene, making it a more appropriate

choice for differentiating close phylogenetic relationships (Ochman & Wilson, 1987). When the *gyrB* sequences of 17 *S. putrefaciens* isolates were compared, 121 out of 136 total combinations showed similarities of $\geqslant 90\%$. A similar pattern was also noted for *S. algae* and thus we propose a species cut-off value of 90% for *gyrB* sequences. Even though their 16S rDNA similarities are inconclusive at 97%, when the *gyrB* criteria are applied to the *S. putrefaciens*/*S. oneidensis*, *S. woodyi*/*S. hanedai* and '*S. pealeana*'/*S. gelidimarina* couples, similarities of 85, 82 and 80% were noted, respectively. These values are below the 90% threshold, suggesting that *S. putrefaciens*, *S. oneidensis*, *S. woodyi*, *S. hanedai*, '*S. pealeana*' and *S. gelidimarina* are indeed separate species. Similarly, even though the 16S rDNA similarity between *S. baltica* and ATCC 8071^T is very high (97·7%), the status of species is given to *S. baltica* (NCTC 10735^T) based on the low DNA relatedness between these two strains (28%). Even so, there are still intergenic dissimilarities among group II strains (ATCC 8072 and *S. baltica* DNA relatedness is only 58%).

The work at hand represents a comprehensive effort to impose a logical phylogenetic framework on the growing collection of *Shewanella* isolates. While it appears likely that additional isolates representing novel species will be discovered in the future, the 11 species considered here already indicate that this genus contains formerly unappreciated diversity. This study illustrates the utility of molecular and chemotaxonomic approaches for determining phylogenetic relationships in phenotypically ambiguous groups. It is possible to build upon the taxonomic foundations established by the polyphasic approach to design gene probes for the rapid and efficient screening of isolates.

Reassignment of *Shewanella putrefaciens* MR-1 to *Shewanella oneidensis* sp. nov.

Since its introduction in 1988, the metal-reducing strain MR-1 has been identified as *S. putrefaciens* on the basis of both conventional phenotypic taxonomy and chemotaxonomy. When 100 putative *S. putrefaciens* isolates were screened against the SP-1 and SP-2r *gyrB* probes, no PCR amplification product was observed for 23 of the strains, including MR-1. The physiology and genetics of strain MR-1 are perhaps better studied than any of the other shewanellae (Myers & Nealson, 1988). This led us to perform a phylogenetic analysis for this group.

The phylogenetic position of MR-1 was examined by the comparison of its 16S rDNA sequence to those of all known eubacterial phyla (Woese, 1987). All phylogenetic analyses confirmed the placement of MR-1 within the γ-subclass of the *Proteobacteria*. The phylogenetic relationships of MR-1 were then analysed, and this study was repeated with several different subdomains of the 16S rDNA sequence and bootstrapping analysis performed to avoid sampling artefacts. Both 16S rDNA and *gyrB* nucleotide sequences of MR-1 indicate that it shares a close phylogenetic relationship with the species *Shewanella*, *Alteromonas* and *Vibrio*.

Neighbour-joining, parsimony and maximum-likelihood analyses were then performed on this subgroup of bacteria, using several subdomains of the 16S rDNA. The results of these analyses are summarized in Fig. 1 and Table 5. In all cases, MR-1 was most closely associated with members of the genus *Shewanella*. The 16S rDNA sequence of MR-1 was compared with the nine other *Shewanella* species for which 16S rDNA sequences are available in GenBank, and dissimilarities in their 16S rDNA nucleotide sequences are shown (Table 5). Very high variation (9%) was noted between MR-1 and *S. benthica* ATCC 43992^T. Otherwise, the values were mostly between 3 and 7%. The dissimilarity between MR-1 and *S. frigidimarina* ACAM 591^T (U85906) was 3·8% and that between MR-1 and *S. putrefaciens* ATCC 8071^T (X81623) was 3%. This 97% similarity between MR-1 and the *S. putrefaciens* type strain is insufficient to prove or disprove a species relationship.

Because the *gyrB* probe data failed to support a species relationship between MR-1 and *S. putrefaciens* and rDNA similarity data were inconclusive in this regard, DNA–DNA hybridization and *gyrB* sequence analysis were carried out. Strain MR-1 showed DNA reassociation values of between 17·6 and 40% with the other species tested (Table 14). The percentage similarity of DNA between MR-1 and *S. putrefaciens* was only 36%, well below the 70% threshold for the inclusion within a species (Stackebrandt & Goebel, 1994).

The dissimilarities in the *gyrB* gene sequences of all 11 shewanellae along with MR-1 are given in Table 10. A phylogenetic tree based on *gyrB* nucleotide sequences is shown in Fig. 4. Unlike the situation with the 16S rDNA, variation between *gyrB* genes was very high (>20 %). A very high variation (22·7%) was noted between *S. oneidensis* and *S. algae* ATCC 51192^T. Unlike 16S rDNA analysis, a clear conclusion could be drawn from *gyrB* sequence analysis. Although the 85% similarity seen between MR-1 and *S. putrefaciens* indicates a closer relationship than for any other pairs, this value is still well below the 90% species cut-off. Hence, strain MR-1 is distinct and deserves the status of species.

Description of *Shewanella oneidensis*

S. oneidensis (o.nei.da. M.L. n. *oneidensis* named after Oneida Lake, NY, USA, where the bacterium was isolated).

The cells of the type strain are rod-shaped. They are 2–3 μm in length and 0·5–0·6 μm in diameter, Gram-negative, facultatively anaerobic, polarly flagellated bacteria. Neither endospores nor capsules are formed. Colonies on LB agar medium are circular, smooth and convex with an entire edge, and beige to pinkish depending on age. Sodium ions are not essential for

growth. Cells are able to grow at mesophilic temperatures. Optimal growth is observed at 30 °C. Cells are able to reduce nitrate to nitrite and nitrite to nitrous oxide. They exhibit cytochrome oxidase, catalase and gelatinase activities, and produce H_2S from thiosulfate. They do not haemolyse sheep erythrocytes and show weak growth at NaCl concentrations above 3%. They utilize lactate, succinate and fumarate as sole carbon sources and reduce transition metal oxides (Fe, Mn, U) and elemental sulfur. Based on both 16S rDNA and *gyrB* nucleotide sequences, this bacterium belongs to the γ-subclass of the class *Proteobacteria* and is a member of the genus *Shewanella*. The type strain MR-1 (=ATCC 700550^T) was isolated from sediments.

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