Journal of Fish Diseases 2010, **33**, 865–877 doi:10.1111/j.1365-2761.2010.01189.x

Biological characteristics and pathogenicity of a highly pathogenic *Shewanella marisflavi* infecting sea cucumber, *Apostichopus japonicus*

H Li[1], G Qiao[1], Q Li[1], W Zhou[1], K M Won[2], D-H Xu[3] and S-I Park[2]

1 Key Laboratory of Mariculture & Biotechnology, Agriculture Ministry, PRC, Dalian Fisheries University, Dalian, Liaoning Province, China

2 Department of Aquatic Life Medicine, Pukyong National University, Busan, Korea

3 U.S. Department of Agriculture, Agricultural Research Service, Aquatic Animal Health Research Laboratory, Auburn, AL, USA

Abstract

Shewanella marisflavi isolate AP629 is described as a novel pathogen of sea cucumber. The LD_{50} values (14 days) in sea cucumber, mice and swordtail fish were 3.89×10^6, 6.80×10^4 and 4.85×10^4 CFU g^{-1} body weight, respectively. Studies on *S. marisflavi* were conducted, including morphology, physiological and biochemical characteristics, haemolysis, whole-cell protein and 16S rDNA gene sequence. Colonies of *S. marisflavi* appeared faint red on marine agar and green on thiosulphate–citrate–bile salt–sucrose media. *Shewanella marisflavi* had polar flagella. The cells were Gram-negative, oxidase- and catalase-positive and not sensitive to O/129. The bacterium exhibited β-haemolysis on sheep blood agar and produced H_2S. *Shewanella marisflavi* survived and grew at 4–35 °C, pH 6.0–9.2 and in the presence of 0–8% NaCl. The whole-cell proteins included 13 discrete bands, and proteins of molecular weight 87, 44 and 39 kDa were found in all five strains of *Shewanella* spp. The difference in 16S rDNA gene sequences in *S. marisflavi* was at the 446 bp site: *S. marisflavi* (KCCM 41822) – G, isolate AP629 – A. This is the first report that *Shewanella* is pathogenic to sea cucumber.

Correspondence *S-I Park, Department of Aquatic Life Medicine, Pukyong National University, Busan, Korea (e-mail: parksi@pknu.ac.kr) and H Li, Key Laboratory of Mariculture & Biotechnology, Agriculture Ministry, PRC, Dalian Fisheries University, China (e-mail: lihua@dlfu.edu.cn)*

Keywords: biological characteristics, pathogenicity, sea cucumber, *Shewanella marisflavi*.

Introduction

The genus *Shewanella* has long been studied and classified taxonomically as *Achromobacter*, *Pseudomonas*, *Alteromonas* and *Shewanella* (Venkateswaran, Moser, Dollhopf, Lies, Saffarini, MacGregor, Ringelberg, White, Nishijima, Sano, Berghardt, Stackebrandt & Nealson 1999). In 1985, *Shewanella* was defined as a new genus (Macdonell & Colwell 1985). *Shewanella* is phylogenetically (16S rDNA gene sequence) affiliated to the γ-proteobacteria (Gauthier, Gauthier & Christen 1995), included in the family Shewanellaceae (Ivanova, Flavier & Christen 2004), containing about 35 *Shewanella* species. *Shewanella* are Gram-negative, long, motile bacilli. The most important characteristic of *Shewanella* is the production of hydrogen sulphide on Kligler or triple sugar iron agar (TSIA) (Venkateswaran *et al.* 1999).

Shewanella are widely distributed in freshwater and marine environments (Bozal, Montes, Tudela, Jimenez & Guinea 2002; Ivanova, Sawabe, Hayashi, Gorshkova, Zhukova, Nedashkovskaya, Mikhailov, Nicolau & Christen 2003; Nealson, Myers & Wimpee 1991), clinical samples (Brink, Van Straden & Van Rensburg 1995; Nozue, Hayashi, Hashimoto, Ezaki, Hamasaki, Ohwada & Terawaki 1992; Vandepitte & Debois 1978), sediments (Myers & Nealson 1988) and oilfield fluids (Semple & West-

Journal of Fish Diseases

lake 1987). The bacteria are important in the turnover of organic matter and capable of reduction of various metals and other substances, such as nitrate, nitrite, thiosulphate and trimethylamine-N-oxide (Holt, Gahrn-Hansen & Bruun 2004). Many clinical cases caused by *Shewanella* spp. are reported, and the clinical syndromes caused by *Shewanella* spp. are generally similar to those caused by various species of the marine halophilic genus *Vibrio* (Dalsgaard, Frimodt-Møller, Bruun, Høi & Larsen 1996; Hornstrup & Gahrn-Hansen 1993; Leong, Mirkazemi & Kimble 2000). There are some reports associated with diseases of aquatic organisms. *Shewanella* was isolated from oyster (Richards, Watson, Crane, Burt & Bushek 2008) and ulcer disease of *Sciaenops ocellata* (Chen, Hu, Chen & Zhang 2003). In humans, the most common clinical syndrome is the infection of skin and soft tissue (Bulut, Ertem, Gökcek, Tulek, Bayar & Karakoc 2004; Chen, Lawrence, Packham & Sorrell 1991; Chen, Liu, Yen, Wang, Wann & Cheng 1997; Dominguez, Vogel, Gram, Hoffmann & Schaebel 1996; Holmes, Lapage & Malnick 1975).

There is only one report of *S. marisflavi* isolated from the Yellow Sea in South Korea (Yoon, Yeo, Kim & Oh 2004). In the current study, the biological characteristics and pathogenicity of *S. marisflavi* to sea cucumber, *Apostichopus japonicas*, were analysed.

Materials and methods

Shewanella marisflavi isolate and reference strains

Thirty-one bacterial isolates were collected from diseased sea cucumber from 2004 to 2006. *Shewanella marisflavi* strain AP629 was isolated from a skin ulcer of a sea cucumber (body weight 1.6–2.0 g) in 2006 in Dalian, using MA (marine agar; Difco), TSAS (tryptic soy agar supplemented with 2% NaCl; Acumedia Manufacturers, Inc.) and thiosulphate–citrate–bile salt–sucrose (TCBS agar; Difco). All media were incubated at 25 °C for 48–72 h. Five type strains were purchased from the Korean Culture Center of Microorganisms (KCCM) and Korean Collection for Type Cultures (KCTC) and used in this study, including *S. marisflavi* (KCCM 41822), *Shewanella aquimarina* (KCCM 41821), *Shewanella affinis* (KCTC 12234), *Shewanella waksmanii* (KCTC 12233) and *Vibrio parahaemolyticus* (KCTC 2471).

Sea cucumber

Healthy sea cucumber juveniles (body weight 1.6–2.0 g) and adults (body weight 15–20 g) were obtained from Dalianwan Hatchery. The juveniles and adults were transported to the laboratory and acclimatized for 7 days before pathogenicity assays. The water parameters in the trials were: temperature 11–15 °C, pH 8.0–8.4 and salinity 29.2–30.8 ppt.

Pathogenicity assays in sea cucumber

The pathogenicity of isolate AP269 was determined *in vivo* following published protocols (Toranzo, Barja, Colwell, Hetrick & Crosa 1983; Nieto, Toranzo & Barja 1984). Suspensions of cultures were prepared by reinoculating pure cultures on MA on test tube slants, culturing at 25 °C for 24 h, washing with sterile sea water and then diluting to the appropriate concentrations (6.1×10^8, 6.1×10^7 CFU mL^{-1}). Each group included 10 sea cucumbers. Juvenile and adult sea cucumbers were infected by intraperitoneal and body wall injection with bacterial suspensions of 0.1 mL per individual. The control group was injected with an equal volume of sterile sea water. The sea cucumbers were observed daily for 14 days post-bacterial challenge, and all mortalities were recorded. The sea cucumbers were considered to be killed by the bacteria if it was reisolated in pure culture from internal organs and skin ulcer. Four infection experiments for adults were conducted: intraperitoneal injection, body wall injection, immersion infections with individuals wounded at four sites and immersion infections of uninjured animals. Three infection methods were used for juvenile cucumbers because of their thin body wall: intraperitoneal injection, immersion with wounded individuals and normal immersion. The control groups were injected or immersed with the same volume of sterile sea water.

Pathogenicity assays in sea cucumber, mice and swordtail fish (lethal dose 50%)

To compare the difference in virulence between animals, mice (17.0–19.0 g), juvenile sea cucumber (body weight 1.6–2.0 g) and swordtail fish (*Xiphophorus helleri*, 1.6–2.0 g) were injected intraperitoneally or intramuscularly with 0.2, 0.1 and 0.02 mL of bacterial suspensions (sea cucumber:

5.06×10^8 to 5.06×10^4 CFU mL^{-1}; mice: 2.06×10^8 to 2.06×10^4 CFU mL^{-1}; swordtail: 2.27×10^7 to 2.27×10^2 CFU mL^{-1}), respectively (Figs 3–5). An equal number of sea cucumbers injected with the same volume of sterile sea water or physiological saline were used as a control. Reisolation was carried out as described earlier. At the end of the trial, the surviving sea cucumbers were killed, and the inoculated bacteria reisolated to test for a possible carrier state. Lethal dose 50% (LD_{50}) values were calculated according to Reed & Muench (1938).

Morphological observation

The purified strain AP629 and type strains of *S. marisflavi* and *V. parahaemolyticus* were streaked on culture media MA, TSAS, NAS (nutrient agar supplemented with 1.5% NaCl; Difco) and BHIAS (brain heart infusion agar supplemented with 1.5% NaCl; Difco), TCBS and MacConkey (Difco 212123), using the plate-streaking method. All media were incubated for 48–72 h at 25 °C, and colony characteristics were recorded. Cell morphogenesis was observed by transmission electron microscopy as described in *Bergey's Manual of Determinative Bacteriology* (Krieg & Holt 1994).

Physiological and biochemical characteristics

Physiological and biochemical characteristics were determined following the descriptions by Yoon *et al.* (2004), Holt, Gahrn-Hansen & Bruun (2005), Khashe & Janda (1998) and methods in *Bergey's Manual of Determinative Bacteriology* (Krieg & Holt 1994). The following tests were carried out: Gram stain; motility and cell morphology (phase-contrast microscopy after growth in marine broth for 24 h); susceptibility to 150 μg of vibriostatic agent O/129; growth in 1% peptone at 4, 10, 35 and 40 °C (7 days); growth in 1% peptone at 0%, 0.5%, 2%, 4%, 6%, 8% and 10% salt concentrations (7 days); cytochrome-oxidase; O-F test; production of H_2S on TSIA (Difco™); ONPG (β-galactosidase, API 20NE strip); gas production from glucose; indole and Voges–Proskauer tests; arginine dihydrolase; decarboxylation of lysine and ornithine; nitrate-reduction; acid production from arabinose, sucrose, lactose and mannose (Table 1). The following enzymatic activities were also determined: catalase (3% H_2O_2), urease, gelatinase, Tween-80, starch and haemolysis of sheep blood (blood agar base with 5% blood, Scoaris Dde, Colacite, Nakamura, Ueda-Nakamura, de Abreu Filho & Filho 2008; Imzilm, Lafdal & Jana 1996).

Whole-cell protein analysis

The purified strains were precultured in TSBS (tryptic soy broth supplemented with 1.5% NaCl, Bacto 211825) for 36 h at 25 °C and 170 rpm. A volume of 10 mL of each culture was transferred to 200 mL of TSBS and reincubated for 36 h at 25 °C. Cells were harvested by centrifugation at 37250 ***g*** for 10 min at 4 °C, and the pellet was washed three times in PBS (0.1 M, pH 7.2). The washed cells were suspended to 50 mg (wet weight) mL^{-1} in sample buffer (0.125 M tris base buffer containing 2.5% (vol/vol) mercaptoethanol, 2% (wt/vol) SDS and 25% glycerol (vol/vol); pH 6.8). The cell suspension was boiled for 10 min. Protein concentrations were determined by a modification of the Lowry method (Laemmli 1970; Peterson 1977). Bovine serum albumin was used as a standard. Samples were stored at −80 °C in aliquots of 50 μL (no sample was frozen more than once).

SDS-polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was carried out on a SE 600 slab gel apparatus (Hoefer Scientific Instruments) as described by Laemmli (1970). Approximately 50 μg of protein was loaded per well. Samples were electrophoresed in 4.5% stacking and 10% separation gels with 0.1% SDS at 30 mA for 40 min and 60 mA for 210 min. The temperature of electrophoresis was kept at 4 °C. Gels were stained with Coomassie brilliant blue G-250 (Sigma catalogue no. B-0770) and air dried between two sheets of cellophane. A standard marker (SDS-6H; Sigma) was included in one lane as an internal marker. Further samples of type strains were included in each gel to allow for comparison within and between gels.

16S rDNA gene sequence analysis

DNA extraction and purification was carried out following the methods of Xu, Wang, Wang & Xiao (2006) with modification: strains were cultured in TSB with 2% NaCl for 24 h at 25 °C. Cells were harvested by centrifugation (150 ***g***, 10 min) at 4 °C, and the pellet was washed twice in distilled water. The suspension and centrifugation step was

Table 1 Physiological and biochemical characteristics of *Shewanella marisflavi* isolate AP629 and type strains

Tests	*S. marisflavi* isolate AP629	*S. marisflavi* KCCM41822	*S. aquarium* KCCM41821	*Shewanella affinis* KCTC12234	*Shewanella waksmanii* KCTC12233
Gram	−	−	−	−	−
Motility	+	+	+	+	+
O/129 sensitivity	−	−	−	−	−
Haemolysis	+	+	+	+	+
Thiosulphate–citrate–bile salt–sucrose growth	+	+	+	−	+
Growth at (°C)					
4	+	+	−	−	+
15	+	+	+	−	−
20	+	+	+	+	+
35	+	+	+	+	+
40	−	−	−	−	−
Growth in NaCl (%)					
0	+	+	−	−	−
0.5	+	+	+	+	+
2	+	+	+	+	+
6	+	+	+	+	+
8	+	+	+	+	−
10	−	−	−	−	−
Oxidase	+	+	+	+	+
Catalase	+	+	+	+	+
Indole production	+	+	+	+	+
VP	+	+	+	+	+
H_2S production	+	+	+	+	+
ONPG	−	−	−	−	−
O-F test	F	F	F	F	F
TDA	+	+	+	+	+
Urease	−	−	−	−	−
Amygdalin (starch)	+	+	+	+	+
Lipase (Tween-80)	+	+	+	+	+
Gelatinase	+	+	+	+	+
Arginine dihydrolase	−	−	−	−	−
Lysine decarboxylase	−	−	−	−	−
Ornithine decarboxylase	−	−	−	−	−
NO_3 to NO_2	+	+	+	+	+
NO_2 to N_2	−	−	−	−	−
Utilization of					
Maltose	−	−	−	+	−
Sucrose	+	+	+	+	+
Lactose	+	+	+	−	−
Galactose	−	−	+	−	−
Glycerol	+	+	+	+	+
D-Fructose	+	+	+	+	+
Cellibiose	+	+	+	+	+
Arabinose	+	+	−	−	−
Raffinose	−	−	−	−	−
Rhamnose	−	−	−	−	−
Melibiose	+	+	+	+	+
Xylose	+	+	+	+	+
Salicine	−	−	−	−	−
Mannitol	−	−	−	+	+
Sorbitol	−	−	−	−	−
Inositol	−	−	+	−	+
Citrate	±	−	−	+	−

+, Positive; −, negative; O/129, 2, 4-diamino-6, 7-diso-propylpteridine.

repeated three times. The pellet was suspended and collected to extract DNA using a TIANamp Bacteria DNA kit (TIANGEN catalogue no DP302-02) following the manufacturer's instruction. DNA was purified by increasing the DNA washing times with TE buffer. Two universal bacterial primers – Eubac27F (5′-AGAGTITGATC(C/A)TGGCT-CAG-3′) and Eubac1492R (5′-TACGG(C/T) TACCTTGTTACGACTT-3′) (Weisburg, Barns, Pelletier & Lane 1991) – were synthesized by Takara

and used to amplify bacterial 16S rDNA genes. The PCR products were purified with a TIANGEN Midi' Purification kit (TIANGEN) according to the manufacturer's protocol and sequenced with a 3730DNA Analyzer (Shanghai Invitrogen Biotechnology Co. Ltd). The 16S rDNA partial sequences of *S. marisflavi* isolate AP629 have been deposited in the National Center for Biotechnology Information GenBank database under the accession number GQ 254505. Phylogenetic analysis was conducted based on its 16S rRNA sequences, which were analysed and aligned with DNA Star software. The Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov) was used to search the entire database for homologous sequences and phylogenetic analysis. ClustalX 1.83 and MEGA 4.0 programs were used to construct the phylogenetic trees (Tamura, Dudley, Nei & Kumar 2007). An unrooted evolutionary tree was inferred using the neighbour-joining (N-J) tree algorithm. The resulted tree topologies were evaluated by bootstrap analysis of the N-J method based on 1000 replicates.

Results

Pathogenicity assays in juvenile and adult sea cucumber

The clinical signs of diseased juvenile sea cucumbers infected naturally and artificially were similar: visceral ejection (Fig. 1a,c), mouth tumidity (Fig. 1c,d), skin ulceration (Fig. 1b,c,d) and death (Fig. 1c). Visceral ejection and skin ulceration were the most common signs, and mouth tumidity was also often observed. Occasionally, shaking of the anterior end could be seen. In the group exposed to a high concentration of AP629 (6.1×10^8 CFU mL^{-1}), the cumulative mortality rate of juveniles was 40% at day 1 post-infection, 80% at day 6 and 100% at day 8. In the lower concentration group (6.1×10^7 CFU mL^{-1}), clinical signs

Figure 1 Clinical signs of naturally infected (a,b) and experimentally infected (c,d) sea cucumber by *Shewanella marisflavi* isolate AP629. (a) Viscera ejection. (b) Skin ulceration. (c) Viscera ejection (arrows), mouth tumidity, skin ulceration. (d) Mouth tumidity and skin ulceration.

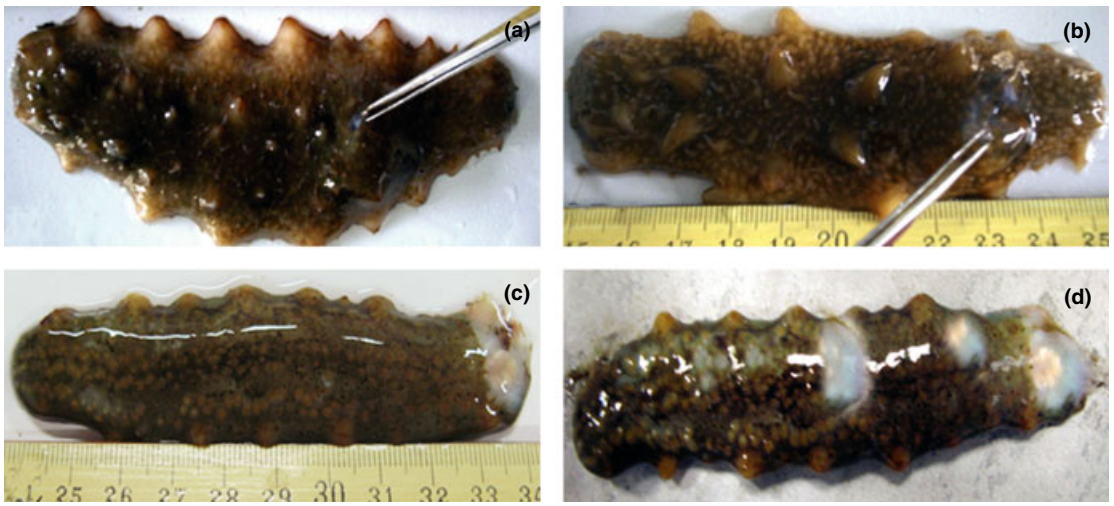


Figure 2 Clinical signs of adult sea cucumber infected by *Shewanella marisflavi* isolate AP629 by intramuscular injection. (a) Ulceration around the injection site, (b) enlarging ulceration after day 2 post-infection, (c) mouth tumidity and ulceration and (d) skin ulceration.

took a longer time to develop, and the cumulative mortality of juveniles was 60% at the end of the trial. No clinical signs were noted in the juvenile control group.

Although strain AP629 was isolated from diseased juveniles, it could also infect adults. In adults, there was body atrophy, and the skin ulceration began as small white patches at injection sites (Fig. 2a). In the lower concentration group (6.1×10^7 CFU mL^{-1}), clinical signs took a longer time to develop, and skin ulceration healed after 2–3 days. The cumulative mortality rate was 40% at the end of the trial. In the higher concentration group (6.1×10^8 CFU mL^{-1}), the sea cucumbers displayed clinical signs at day 1 post-infection, including mouth tumidity, skin ulceration at injection sites, enlarged ulcers and death (Fig. 2c,b,d). The cumulative mortality rate of adults reached 100%. Adults and juveniles both lost the ability to adhere to the wall of tanks.

Juveniles and adults could not be infected by the immersion of wounded individuals and by normal immersion. Juveniles could be infected only by intraperitoneal injection and adults by body wall or intraperitoneal injection. The adults injected intraperitoneally usually did not die until the end of the experiment (14 days) and showed mouth tumidity.

Pathogenicity assays in sea cucumber, mice and swordtail fish (LD_{50})

Sea cucumber, mice and swordtail fish were infected by *S. marisflavi* isolate AP629. For sea cucumber, cumulative mortality rates were 80% at day 2 and 100% at day 8 post-infection at high doses (5.6×10^8 CFU mL^{-1}). Mass mortalities mainly occurred at day 5 post-infection. When the concentration was below 5.6×10^4 CFU mL^{-1}, no deaths were observed (Fig. 3). The LD_{50} values

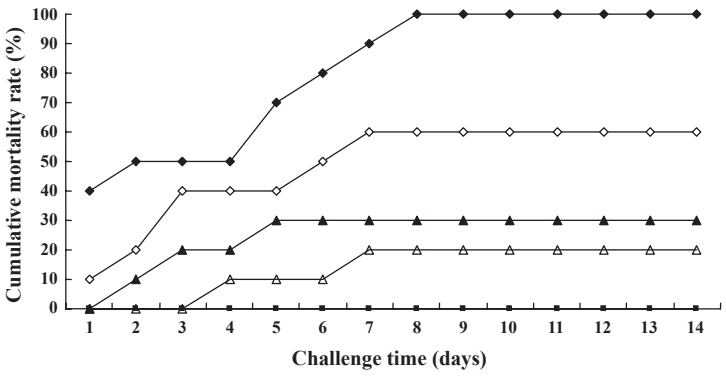


Figure 3 Cumulative mortality rate of juvenile sea cucumber infected by *Shewanella marisflavi* isolate AP629 by intraperitoneal injection at different concentrations (-◆- = 5.06×10^8 CFU mL^{-1}; -◇- = 5.06×10^7 CFU mL^{-1}; —▲— = 5.06×10^6 CFU mL^{-1}; -△- = 5.06×10^5 CFU mL^{-1}; —■— = 5.06×10^4 CFU mL^{-1}).

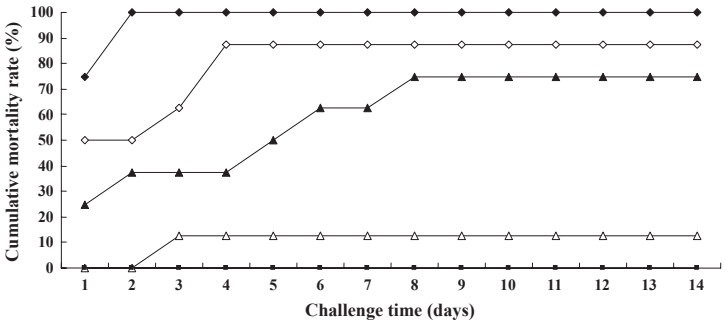


Figure 4 Cumulative mortality rate of mice infected by *Shewanella marisflavi* isolate AP629 by intraperitoneal injection at different concentrations (-♦- = 2.06×10^8 CFU mL^{-1}; -◇- = 2.06×10^7 CFU mL^{-1}; -▲- = 2.06×10^6 CFU mL^{-1}; -△- = 2.06×10^5 CFU mL^{-1}; -■- = 2.06×10^4 CFU mL^{-1}).

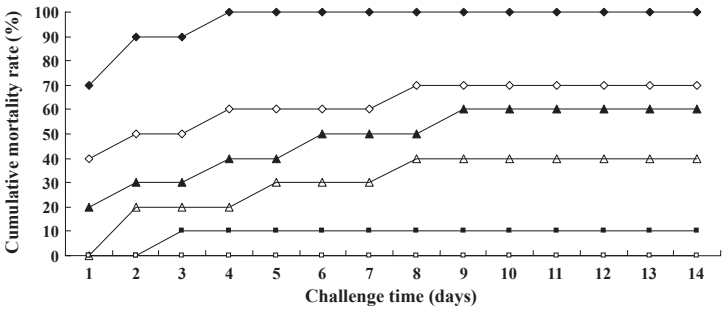
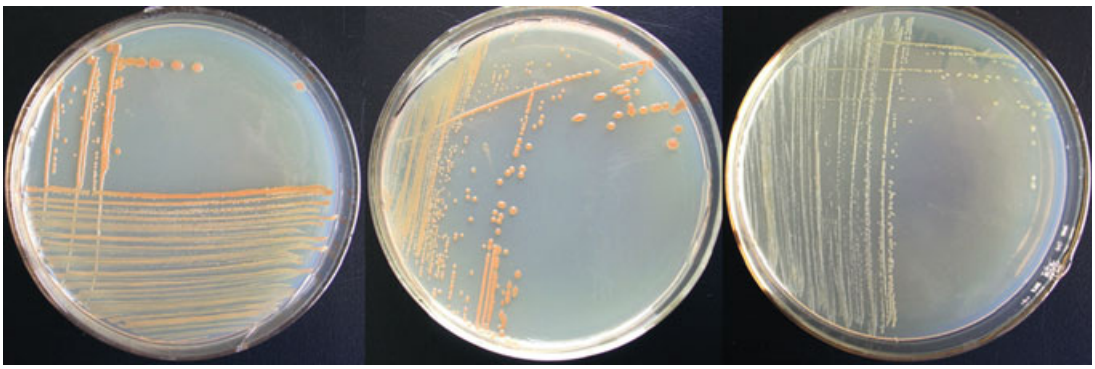


Figure 5 Cumulative mortality rate of swordtail fish infected by *Shewanella marisflavi* isolate AP629 by intramuscular injection at different concentrations (-◆- = 2.27×10^7 CFU mL^{-1}; -◇- = 2.27×10^6 CFU mL^{-1}; -▲- = 2.27×10^5 CFU mL^{-1}; -△- = 2.27×10^4 CFU mL^{-1}; -■- = 2.27×10^3 CFU mL^{-1}; -□- = 2.27×10^2 CFU mL^{-1}).



S. marisflavi KCCM41822 *S. marisflavi* (Isolate AP629) *V. parahaemolyticus* KCTC2471

Figure 6 Colony morphogenesis of *Shewanella marisflavi* isolate AP629 and type strains KCCM41822 and KCTC2471 on marine agar medium (48 h).

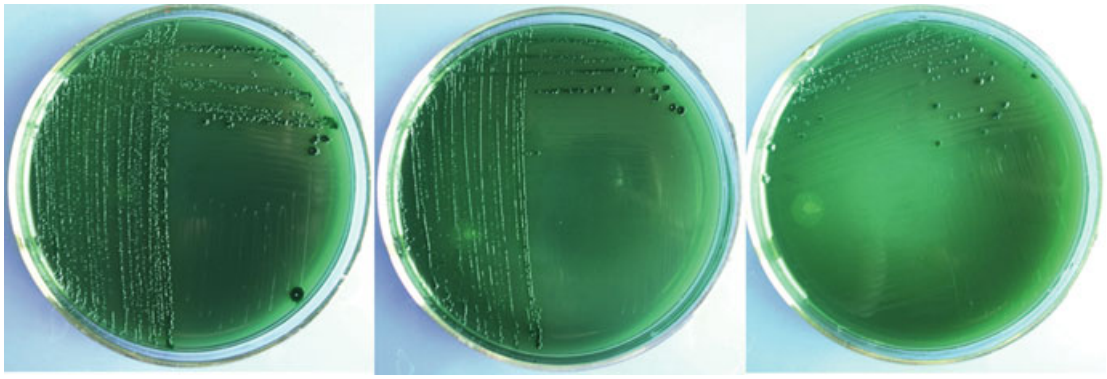
(14 days) of *S. marisflavi* to sea cucumber, mice and swordtail fish were 7.76×10^6, 6.80×10^4 and 4.85×10^4 CFU g^{-1} body weight, respectively (Figs 3–5).

Morphological features

Shewanella marisflavi isolate AP629 and type strains grew very well on MA, NAS, TSAS and BHIAS after incubation at 25 °C for 48 h. Colonies were smooth, circular, humid, sticky and faint red in colour after 48 h incubation at 25 °C on MA, NAS, TSAS and BHIAS and circular and green on TCBS (Figs 6–7). *Shewanella marisflavi* colonies were red on MacConkey Agar. TEM (Fig. 8) showed that cells of *S. marisflavi* were short rods with a single polar flagellum, 1.72×1.04 μm, with some secretion around cells and no evident boundary between the layer and cell wall.

Physiological and biochemical characteristics

Physiological and biochemical characteristics of *S. marisflavi* isolate AP629 and type strains are



S. marisflavi KCCM41822 *S. marisflavi* (Isolate AP629) *V. parahaemolyticus* KCTC2471

Figure 7 Colony morphogenesis of *Shewanella marisflavi* isolate AP629 and type strains KCCM41822 and KCTC2471 on thiosulphate–citrate–bile salt–sucrose medium (48 h).

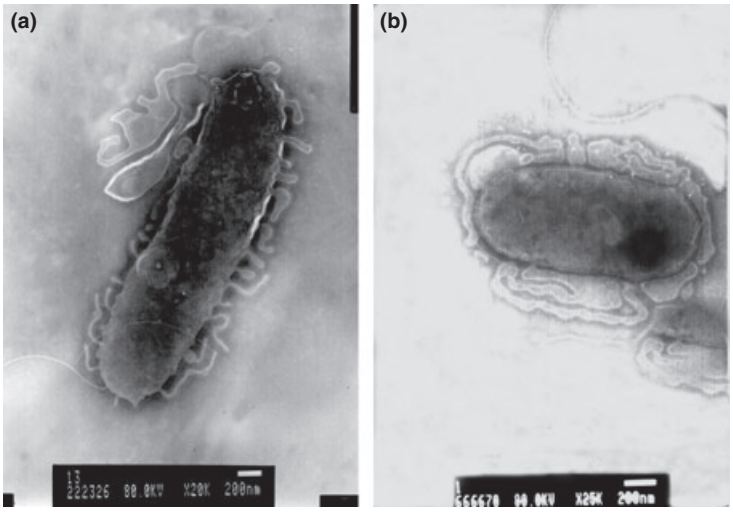


Figure 8 Electron micrograph of *Shewanella marisflavi* isolate and type strain (bar = 200 nm). (a) *Shewanella marisflavi* isolate AP629. (b) Type strain *S. marisflavi* (KCCM41822).

shown in Table 1. *Shewanella marisflavi* grew at 4 °C but not above 40 °C. Growth was observed at pH 6.0–9.2 but not at pH 5.0. Optimal growth occurred in the presence of 2–6% NaCl, and no growth was observed in the presence of more than 8% NaCl. Haemolysis on sheep blood agar was detected. *S. marisflavi* was not sensitive to O/129.

Shewanella marisflavi was Gram-negative, oxidase- and catalase-positive. The bacterium was negative in lysine decarboxylase, arginine dihydrolase and ornithine decarboxylase and positive in gelatine hydrolysis and H_2S production. *Shewanella marisflavi* produced acid from sucrose, lactose, glycerol and D-fructose but not from arabinose, raffinose, rhamnose, salicin, mannitol and inositol.

Whole-cell protein analysis

One-dimensional SDS-PAGE of whole-cell protein extracted from *S. marisflavi* isolate AP629 and type strains revealed protein profiles containing 13–16 discrete bands (Fig. 9). The regression equation was calculated as follows:

$$y = 2.2646 - 1.0772x;\ R^2 = 0.9645$$

y = log (molecular weight of standard proteins); x = mobility of protein; and R^2 = correlation coefficient.

Whole-cell proteins of *S. marisflavi* isolate AP629 and *S. marisflavi* (KCCM 41822) were identical and included 13 discrete bands. Whole-cell proteins of *S. aquimarina*, *S. waksmanii* and *S. affinis* included 16, 12 and 14 protein lanes,

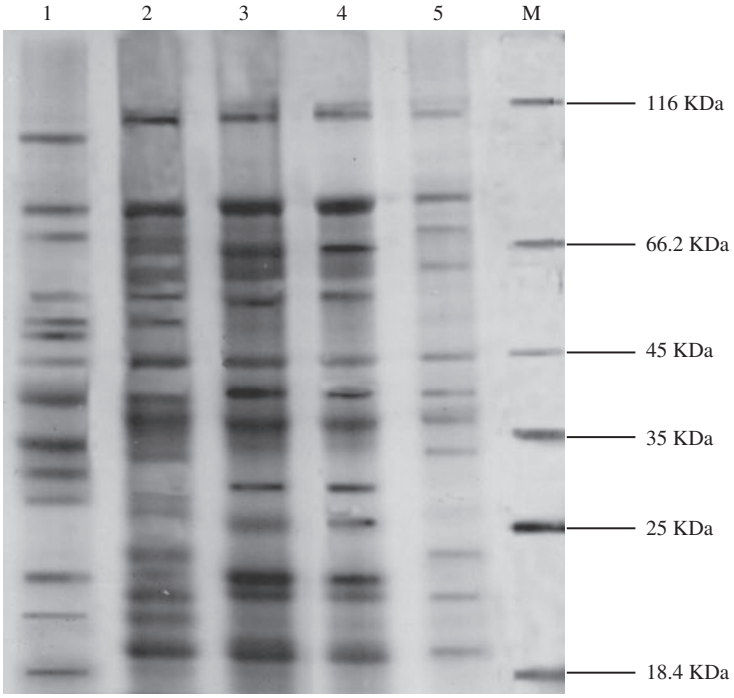


Figure 9 SDS-PAGE of whole-cell protein profiles of *Shewanella marisflavi* isolate AP629 and type strains. (M) Marker. (1) *Shewanella affinis* (KCTC12234). (2) *Shewanella aquimarina* (KCCM41821). (3) *Shewanella marisflavi* isolate AP629. (4) *Shewanella marisflavi* (KCCM41822). (5) *Shewanella waksmanii* (KCTC12233).

respectively. Three proteins of molecular weight 87, 44 and 39 kDa were found in all five strains of *Shewanella* spp.

16S rDNA gene sequence analysis

An almost complete 1460 bp 16S rDNA sequence of *S. marisflavi* isolate AP629 was obtained. In a phylogenetic tree based on the neighbour-joining algorighm, *S. marisflavi* isolate AP629 clustered with *S. marisflavi* KCCM 41822 (accession number: AY485224) with 16S rDNA gene sequence similarity levels of 99.86% and bootstrap value 100% (Fig. 10). The two strains differed at 446 bp: i.e. *Shewanella marisflavi* (KCCM 41822) – G, isolate AP629 – A.

Discussion

In this study, *S. marisflavi* was isolated from diseased sea cucumber as a novel pathogen. *Shewanella marisflavi* caused disease in juvenile and adult sea cucumbers and showed high virulence compared with *Vibrio splendidus* and *Pseudoalteromonas tetraodonis* (data not shown). Juveniles and adults of sea cucumber did not show any mortality after infection by *S. marisflavi* by immersion and by wounding and immersion. Adults could also be infected by body wall and intraperitoneal injection. The body wall of juveniles was too thin to be challenged by injection. According to the criteria of Santos, Toranzo, Barja, Nieto & Villa (1988), a bacterial strain is considered as highly virulent if LD_{50} values are from 1.7×10^4 to 1×10^6 CFU g^{-1} body weight; moderately virulent, from 1.4×10^6 to 1.8×10^7 CFU g^{-1} body weight; and non-virulent, if more than 10^8 CFU g^{-1} body weight. LD_{50} values showed that *S. marisflavi* was highly virulent to mice and swordtail and moderately virulent to sea cucumber. Sea cucumber had a relatively strong resistance to bacterial infection, which may be related to the habitat of sea cucumber which live on sediment and feed on organic detritus (Zhang, Wang, Rong, Sun & Dong 2004). Bacterial numbers can reach 10^7 CFU mL^{-1} in the digestive tract of sea cucumber (Sun & Chen 1989) and 10^6–10^7 CFU mL^{-1} in the whole body of healthy sea cucumber (data not shown).

Shewanella marisflavi species were originally isolated from the Yellow Sea in 2004, and most *Shewanella* spp. have been isolated from the marine environment. The most obvious source for human infection is exposure to sea water (Bulut *et al.* 2004; Heller, Tortora & Burger 1990; Rosenthal, Zuger & Apollo 1975; Leong *et al.* 2000). In a Danish study of ear infections, it was noted that more than

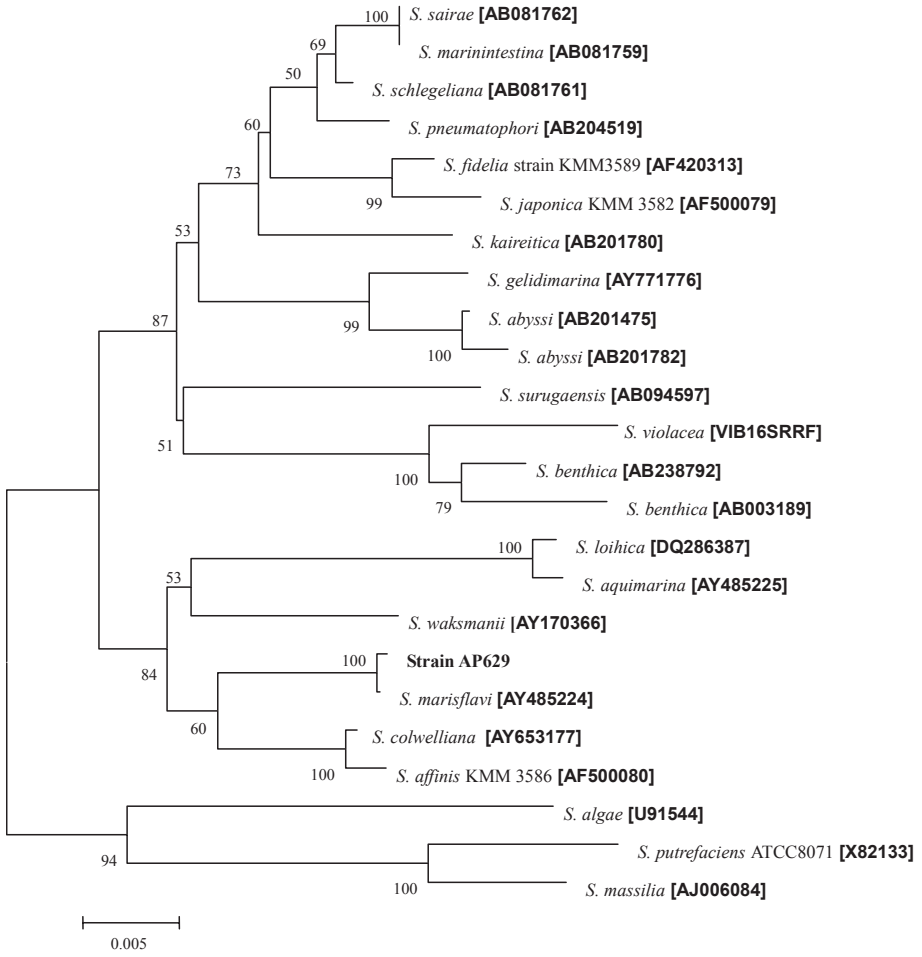


Figure 10 Phylogenetic tree of *Shewanella marisflavi* isolate AP629 based on 16S rDNA gene sequence. Bootstrap values (expressed as percentages of 1000 replicates) > 50% are shown at the branch points. Bar = 0.005 substitution per nucleotide position.

80% of patients had been swimming in the sea shortly before signs developed (Holt *et al.* 1997). In Denmark, environmental and clinical isolates of *Shewanella algae* showed great clonal variability when determined by ribotyping and random amplified polymorphic DNA profiling. However, no systematic differences were found between environmental and clinical isolates (Vogel, Holt, Gerner-Smidt, Bundvad, Sogaard & Gram). A Danish seawater isolate was found to be identical to one of the clinical isolates by all typing methods (Vogel, Holt, Gerner-Smidt, Bundvad, Sogaard & Gram 2000), supporting the theory of a marine source of infection. *Shewanella marisflavi* may pose a health threat to humans through the ingestion of contaminated seafood, by cuts or abrasions acquired in the marine environment, or by swimming and other recreational activities.

Cells of *Shewanella* spp. are straight or curved, Gram-negative and motile with polar filaments and produce H_2S (Khashe & Janda 1998). However, it is difficult to identify *Shewanella* accurately and quickly because phenotypic characteristics are obscure (Krieg & Holt 1994). This problem is compounded by the fact that the semi-automated and automated identification systems used for the identification of *Shewanella* spp. (API 20E and 20NE; API ID 32 GN and Vitek) include only *Shewanella putrefaciens* but not other species of *Shewanella.* In this study, *S. marisflavi* has been analysed using phenotypic characteristics, whole-cell protein and 16S rDNA gene sequence. *Shewanella marisflavi* was isolated and initially identified by physiological–biochemical characteristics, major fatty acids, DNA G+C content, 16S rDNA gene

sequence and DNA–DNA hybridization (Yoon *et al.* 2004).

The colony colours of *V. parahaemolyticus* (ivory) and *S. marisflavi* (faint red) are different on MA, NAS, TSAS and BHIAS. Cells of *S. marisflavi* can produce H_2S and are resistant to O/129, but *V. parahaemolyticus* does not produce H_2S and is sensitive to O/129. It is interesting that the substance surrounding the cells of AP629 is neither biofilm nor capsule (data not shown). Further study is needed to identify its functions in adhesion or colonization.

Shewanella species may be mesophilic (*S. algae*, *Shewanella amazonensis*, *Shewanella colwelliana*, *Shewanella oneidensis* and *S. putrefaciens*), psychrotrophic (*S. putrefaciens*, *Shewanella baltica*, *Shewanella frigidimarina*, *Shewanella woodyi* and *Shewanella dinitrificans*), psychrophilic (*Shewanella gelidimarina* and *Shewanella hanedai*), or psychrophilic and barophilic (*Shewanella benthica*) (Bozal *et al.* 2002; Ivanova *et al.* 2003; Hayashi, Frolova, Sergeev, Pavel, Mikhailov & Nicolau 2003; Venkateswaran *et al.* 1999). *Shewanella marisflavi* grew at 4 °C but not above 40 °C. Optimal growth occurs from 10 to 30 °C in other studies (Yoon *et al.* 2004), and the species should be considered as psychrophilic. Growth of *S. marisflavi* occurred at pH 6.0–9.2 but not at pH 5.0, which is different from the report of Yoon *et al.* (2004) that growth occurs at pH 5.0. Optimal growth occurs with 2–6% NaCl and not above 8% as with other *Shewanella* spp. Haemolysis on sheep blood agar was detected in *S. marisflavi*. In this study, O/F tests of *S. marisflavi* (strain AP629 and type strain) were fermentative by traditional methods (Krieg & Holt 1994) and a semi-automated system (API 20NE), but most studies of *Shewanella* spp. demonstrate that O/F tests are oxidative. The major fatty acid is iso-$C_{15:0}$, and the DNA G+C content is 51 mol% (determined by HPLC; Yoon *et al.* 2004).

In summary, this study, for the first time, showed *S. marisflavi* to be a potentially highly pathogenic species to sea cucumber, mice and swordtail. In respect of the potential pathogenicity of *Shewanella* spp. to humans and fish, more attention should be paid to the consumption of seafood, especially the increasing consumption of sea cucumber in China.

Acknowledgements

This research was supported by grants from the National Key Projects, National Science & Technology Pillar Programme during the eleventh Five-Year-Plan period (2006BAD09A01), National 908 Special Projects (908-ZH-03), National Natural Science Foundation of China (30800853) and Science & Technology Project of Dalian (2004B1NC030; 2006B11NC109).

References

Bozal N., Montes M.J., Tudela E., Jimenez F. & Guinea J. (2002) *Shewanella frigidimarina* and *Shewanella livingstonensis* sp. nov. isolated from Antarctic coastal areas. *International Journal of Systematic and Evolutionary Microbiology* **52,** 195–205.

Brink A.J., Van Straden A. & Van Rensburg A.J. (1995) *Shewanella* (*Pseudomonas*) *putrefaciens* bacteremia. *Clinical Infectious Diseases* **20,** 1327–1332.

Bulut C., Ertem G.T., Gökcek C., Tulek N., Bayar M.A. & Karakoc E. (2004) A rare cause of wound infection: *Shewanella putrefaciens*. *Scandinavian Journal of Infectious Diseases* **36,** 692–694.

Chen S.C., Lawrence R.H., Packham D.R. & Sorrell T.C. (1991) Cellulitis due to *Pseudomonas putrefaciens:* possible production of exotoxins. *Reviews of Infectious Diseases* **13,** 642–643.

Chen Y.S., Liu Y.C., Yen M.Y., Wang J.H., Wann S.R. & Cheng D.L. (1997) Skin and soft-tissue manifestations of *Shewanella putrefaciens* infection. *Clinical Infectious Diseases* **25,** 225–229.

Chen C., Hu C., Chen X. & Zhang L. (2003) Identification and characterization of *Shewanella algae* as a novel pathogen of ulcer disease of fish *Sciaenops ocellata*. *Oceanologia et Limnologia Sinica* **34,** 1–8 (Chinese, abstract in English).

Dalsgaard A., Frimodt-Møller N., Bruun B., Høi L. & Larsen J.L. (1996) Clinical manifestations and molecular epidemiology of *V. vulnificus* infections in Denmark. *European Journal of Clinical Microbiology & Infectious Diseases* **15,** 227–232.

Dominguez H., Vogel B.F., Gram L., Hoffmann S. & Schaebel S. (1996) *Shewanella alga* bacteremia in two patients with lower leg ulcers. *Clinical Infectious Diseases* **22,** 1036–1039.

Gauthier G., Gauthier M. & Christen R. (1995) Phylogenetic analysis of the genera *Alteromonas*, *Shewanella,* and *Moritella* using genes coding for small-subunit rRNA sequences and division of the genus *Alteromonas* into two genera, *Alteromonas* (emended) and *Pseudoalteromonas* gen. nov., and proposal of twelve new species combinations. *International Journal of Systematic Bacteriology* **45,** 755–761.

Hayashi K., Frolova G.M., Sergeev A.F., Pavel K.G., Mikhailov V.V. & Nicolau D.V. (2003) Occurrence and diversity of mesophilic *Shewanella* strains isolated from the North-West Pacific Ocean. *Systematic and Applied Microbiology* **26,** 293–301.

Heller H.M., Tortora G. & Burger H. (1990) *Pseudomonas putrefaciens* bacteremia associated with shellfish contact. *American Journal of Medicine* **88,** 85–86.

Holmes B., Lapage S.P. & Malnick H. (1975) Strains of *Pseudomonas putrefaciens* from clinical material. *Journal of Clinical Pathology* **28,** 149–155.

Holt H.M., Søgaard P. & Gahrn-Hansen B. (1997) Ear infections with *Shewanella alga* — a bacteriologic, clinical and epidemiologic study of 67 cases. *Clinical Microbiology and Infection* **3,** 329–334.

Holt H.M., Gahrn-Hansen B. & Bruun B. (2004) *Shewanella* species: infections in Denmark and phenotypic characterization. *Clinical Microbiology and Infection* **10**(Suppl 3)**,** 348–349.

Holt H.M., Gahrn-Hansen B. & Bruun B. (2005) *Shewanella algae* and *Shewanella putrefaciens*: clinical and microbiological characteristics. *Clinical Microbiology and Infection* **11,** 347–352.

Hornstrup M.K. & Gahrn-Hansen B. (1993) Extraintestinal infections caused by *V. parahaemolyticus* and *V. alginolyticus* in a Danish county, 1987–1992. *Scandinavian Journal of Infectious Diseases* **25,** 735–740.

Imzilm B., Lafdal Y.M.O. & Jana M. (1996) Effect of wastewater stabilization ponds on antimicrobial susceptibility and haemolysin occurrence among motile *Aeromonas* strains. *World Journal of Microbiology and Biotechnology* **12,** 385–390.

Ivanova E.P., Sawabe T., Hayashi K., Gorshkova N.M., Zhukova N.V., Nedashkovskaya O.I., Mikhailov V.V., Nicolau D.V. & Christen R. (2003) *Shewanella fidelis* sp. nov., isolated from sediments and sea water. *International Journal of Systematic and Evolutionary Microbiology* **53,** 577–582.

Ivanova E.P., Flavier S. & Christen R. (2004) Phylogenetic relationships among marine *Alteromonas*-like proteobacteria: emended description of the family *Alteromonadaceae* and proposal of *Pseudoalteromonadaceae* fam. nov., *Colwelliaceae* fam. nov., *Shewanellaceae* fam. nov., *Moritellaceae* fam. nov., *Errimonadaceae* fam. nov., *Idiomarinacea*e fam. nov. and *Psychromonadaceae* fam. nov. *International Journal of Systematic and Evolutionary Microbiology* **54,** 1773–1788.

Khashe S. & Janda J.M. (1998) Biochemical and pathogenic properties of *Shewanella alga* and *Shewanella putrefaciens*. *Journal of Clinical Microbiology* **36,** 783–787.

Krieg N.R. & Holt J.G. (1994) *Bergey's Manual of Systematic Bacteriology*, 9th edn. Williams and Wilkins Company, Baltimore, MD.

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Leong J., Mirkazemi M. & Kimble F. (2000) *Shewanella putrefaciens* hand infection. *Australian and New Zealand Journal of Surgery* **70,** 816–817.

Macdonell M.T. & Colwell R.R. (1985) Phylogeny of the *Vibrionaceae* and recommendation for two new genera, *Listonella* and *Shewanella*. *Systematic and Applied Microbiology* **6,** 171–182.

Myers C.R. & Nealson K.H. (1988) Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. *Science* **240,** 1319–1321.

Nealson K.H., Myers C.R. & Wimpee B. (1991) Isolation and identification of manganese-reducing bacteria, and estimates of microbial Mn(IV)-reducing potential in the Black Sea. *Deep Sea Research* **38**(Suppl 2)**,** S907–S920.

Nieto T.P., Toranzo A.E. & Barja J.L. (1984) Comparison between the bacterial flora associated with fingerling rainbow trout cultured in two different hatcheries in the Northwest of Spain. *Aquaculture* **42,** 193–206.

Nozue H., Hayashi T., Hashimoto Y., Ezaki T., Hamasaki K., Ohwada K. & Terawaki Y. (1992) Isolation and characterization of *Shewanella alga* from human clinical specimens and emendation of the description of *S. alga*. *International Journal of Systematic Bacteriology* **42,** 628–634.

Peterson G.L. (1977) A simplification of protein assay method of Lowry *et al.* which is more generally applicable. *Analytical Biochemistry* **83,** 346–356.

Reed L.J. & Muench H. (1938) A simple method of estimating fifty percent end points. *American Journal of Hygiene* **27,** 493–497.

Richards G.P., Watson M.A., Crane E.J. III, Burt I.G. & Bushek D. (2008) *Shewanella* and *Photobacterium* spp. in oysters and seawater from the Delaware Bay. *Applied and Environmental Microbiology* **74,** 3323–3327.

Rosenthal S.L., Zuger J.H. & Apollo E. (1975) Respiratory colonization with *Pseudomonas putrefaciens* after near-drowning in salt water. *American Journal of Clinical Pathology* **64,** 382–384.

Santos Y., Toranzo A.E., Barja J.L., Nieto T.P. & Villa T.G. (1988) Virulence properties and enterotoxin production of *Aeromonas* strains isolated from fish. *Infection and Immunity* **56,** 3285–3293.

Scoaris Dde O., Colacite J., Nakamura C.V., Ueda-Nakamura T., de Abreu Filho B.A. & Filho B.P.D. (2008) Virulence and antibiotic susceptibility of *Aeromonas* spp. isolated from drinking water. *Antonie van Leeuwenhoek* **93,** 111–122.

Semple K.M. & Westlake D.W.S. (1987) Characterization of iron-reducing *Alteromonas putrefaciens* strains from oil field fluids. *Canadian Journal of Microbiology* **33,** 366–371.

Sun Y. & Chen D. (1989) The microbial composition of *Stichopus japonicus* and its physiological property. *Oceanologia et Limnologia Sinica* **20,** 300–307 (in Chinese).

Tamura K., Dudley J., Nei M. & Kumar S. (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24,** 1596–1599.

Toranzo A.E., Barja J.L., Colwell R.R., Hetrick F.M. & Crosa J.H. (1983) Haemagglutinating, haemolytic and cytotoxic activities of *Vibrio anguillarum* and related vibrios isolated from striped bass on the Atlantic coast. *Federation of European Microbiological Society Microbiology Letters* **18,** 257–262.

Vandepitte J. & Debois J. (1978) *Pseudomonas putrefaciens* as a cause of bacteremia in humans. *Journal of Clinical Microbiology* **7,** 70–72.

Venkateswaran K., Moser D.P., Dollhopf M.E., Lies D.P., Saffarini D.A., MacGregor B.J., Ringelberg D.B., White D.C., Nishijima M., Sano H., Berghardt J., Stackebrandt E. & Nealson K.H. (1999) Polyphasic taxonomy of the genus *Shewanella* and description of *Shewanella oneidensis* sp. nov. *International Journal of Systematic Bacteriology* **49,** 705–724.

Vogel B.F., Holt H.M., Gerner-Smidt P., Bundvad A., Sogaard P. & Gram L. (2000) Homogeneity of Danish environmental and clinical isolates of *Shewanella algae*. *Applied and Environmental Microbiology* **66,** 443–448.

Weisburg W.G., Barns S.M., Pelletier D.A. & Lane D.J. (1991) 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173,** 697–703.

Xu M., Wang P., Wang F.P. & Xiao X. (2006) Microbial diversity at a deep-sea station of the Pacific nodule province. *Biodiversity and Conservation* **14,** 3363–3380.

Yoon J.H., Yeo S.H., Kim I.G. & Oh T.K. (2004) *Shewanella marisflavi* sp. nov. and *Shewanella aquimarina* sp. nov., slightly halophilic organisms isolated from sea water of the Yellow Sea in Korea. *International Journal of Systematic and Evolutionary Microbiology* **54,** 2347–2352.

Zhang C.Y., Wang Y.G., Rong X.J., Sun H.L. & Dong S.G. (2004) Natural resources, culture and problems of sea cucumber worldwide. *Marine Fisheries Resources* **25,** 89–93.

Received: 12 September 2009
Revision revised: 6 January 2010
Accepted: 26 January 2010

