Shewanella pealeana* sp. nov., a member of the microbial community associated with the accessory nidamental gland of the squid *Loligo pealei

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A new, mesophilic, facultatively anaerobic, psychrotolerant bacterium, strain ANG-SQ1^T (T = type strain), was isolated from a microbial community colonizing the accessory nidamental gland of the squid *Loligo pealei*. It was selected from the community on the basis of its ability to reduce elemental sulfur. The cells are motile, Gram-negative rods (2.0–3.0 µm long, 0.4–0.6 µm wide). ANG-SQ1^T grows optimally over the temperature range of 25–30 °C and a pH range of 6.5–7.5 °C in media containing 0.5 M NaCl. 16S rRNA sequence analysis revealed that this organism belongs to the γ-3 subclass of the *Proteobacteria*. The closest relative of ANG-SQ1^T is *Shewanella gelidimarina*, with a 16S rRNA sequence similarity of 97.0%. Growth occurs with glucose, lactate, acetate, pyruvate, glutamate, citrate, succinate, Casamino acids, yeast extract or peptone as sole energy source under aerobic conditions. The isolate grows anaerobically by the reduction of iron, manganese, nitrate, fumarate, trimethylamine-*N*-oxide, thiosulfate or elemental sulfur as terminal electron acceptor with lactate. Growth of ANG-SQ1^T was enhanced by the addition of choline chloride to growth media lacking Casamino acids. The addition of leucine or valine also enhanced growth in minimal growth media supplemented with choline. The results of both phenotypic and genetic characterization indicate that ANG-SQ1^T is a *Shewanella* species. Thus it is proposed that this new isolate be assigned to the genus *Shewanella* and that it should be named *Shewanella pealeana* sp. nov., in recognition of its association with *L. pealei*.

Keywords: *Shewanella pealeana*, *Loligo pealei*, sulfur reducer, choline

INTRODUCTION

Shewanella species have been isolated from a wide range of environments. For example, *Shewanella putrefaciens* has been found in a variety of aquatic environments (freshwater, D. P. Moser, M. R. Leonardo, K. Venkateswaran & K. H. Nealson, un-

published results; and marine, Nealson *et al.*, 1991); sediments (Myers & Nealson, 1988) and in oilfield fluids (Semple & Westlake, 1987). *S. putrefaciens* and other *Shewanella* species have been implicated as causative agents in marine-animal disease (Aguirre *et al.*, 1994) and in the spoilage of proteinaceous foods (Curtiss, 1931; Parker & Levin, 1983; Stenström &

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Abbreviations: ANG, accessory nidamental gland; CAA, Casamino acids; EPS, exopolysaccharide; FAME, fatty acid methyl ester; PTA, phosphotungstic acid; RDP, Ribosomal Database Project; TEM, transmission electron microscopy; TMAO, trimethylamine-*N*-oxide. The GenBank accession number for the 16S rRNA gene fragment (1492 bp) of strain ANG-SQ1^T is AF011335.

Molin, 1990). The prevalence of *Shewanella* species in these niches may be a result of their capacity to exploit a wide variety of compounds for both aerobic and anaerobic growth. *S. putrefaciens* is capable of the dissimilatory reduction of a variety of electron acceptors, including iron, manganese, nitrate, nitrite, thiosulfate, DMSO, trimethylamine *N*-oxide (TMAO), glycine, fumarate and elemental sulfur (Moser & Nealson, 1996a; Myers & Nealson, 1988, 1990). Molecular hydrogen (Lovley *et al.*, 1989), as well as a diverse range of simple carbon compounds, can be utilized as electron donors for these reactions (Nealson *et al.*, 1991). Because of their metabolic versatility and wide distribution in a variety of aquatic habitats (Nealson & Myers, 1992), *Shewanella*-like organisms are thought to play a significant role in the cycling of organic carbon and other bionutrients (Nealson, 1997).

The accessory nidamental gland (ANG), an organ in the reproductive system of female squids (orders Sepioidea and Teuthoidea), has been shown to harbour a dense microbial community (Bloodgood, 1977; Getzel, 1934; Lum-Kong & Hastings, 1992). When the squid reaches sexual maturity, the ANG turns from almost colourless to an orange/red colour, possibly because of pigments produced by some members of the squid's associated microbiota (Bloodgood, 1977; Van den Branden *et al.*, 1979). Early efforts led to the isolation of a small number of bacterial types that were superficially characterized by their morphology and pigmentation, but no attempt was made to identify these organisms or describe their biochemical characteristics. These initial studies, some of which were performed decades apart, yielded similar bacterial types (Bloodgood, 1977; Getzel, 1934; Lum-Kong & Hastings, 1992), suggesting a specific association between the ANG and the microbial community. The role of the ANG and its microbiota in the reproductive cycle of the squid is unclear. However, it is hypothesized that the microbiota could protect the eggs from predation once laid (Barbieri *et al.*, 1997).

During the 1996 Microbial Diversity Course at the Marine Biological Laboratory (MBL, Woods Hole, MA, USA), the ANG microbial community was characterized using both culture-based and molecular approaches. A more detailed characterization of the cultivable ANG microbial community is reported elsewhere (Barbieri *et al.*, 1999). Course participants screened the ANG microbial population for the presence of elemental sulfur-reducing bacteria, using an adaptation of a previously described cultivation-based approach (Moser & Nealson, 1996a). All of the sulfur-reducing, facultative anaerobes obtained by this method appeared to be represented by a single colony and cell-morphology type. In this study, the morphological, phenotypic and phylogenetic characteristics of a representative ANG sulfur reducer are described. Initial work performed during the Microbial Diversity Course, and supported by our own analyses, suggests that these sulfur reducers represent a novel

Shewanella species. We propose the name *Shewanella pealeana* sp. nov., because of its association with the ANG of *Loligo pealei*, and designate ANG-SQ1^T the type strain.

METHODS

Bacterial strain isolation and growth conditions. For the isolation of strain ANG-SQ1^T (T = type strain), ANGs were dissected from live, mature, female squids (*n* = 6) under aseptic conditions, washed in filtered, autoclaved seawater and the fluids collected from different internal areas of the gland using sterile capillary pipettes. This fluid was streaked for isolation on to Luria-Bertani (LB) agar plates (50 µl per plate) supplemented with 30 mM sodium lactate and 40 mM elemental sulfur prepared as described by Moser & Nealson (1996a). The plates were incubated for 48 h at 25 °C to obtain a collection of cultivable microbes. The plates were then moved to a Coy anaerobic chamber (Coy Instruments), maintained with a 2% H₂ atmosphere, to screen for that subset of the isolates able to reduce elemental sulfur (Moser & Nealson, 1996a, b). Colonies with cleared zones beneath and around them were scored as positive. Six of the numerous sulfur-reducing colonies were selected at random for further study.

The sulfur-reducing ANG strains were maintained on Marine Broth Agar (Difco Laboratories). To determine the NaCl concentration that was optimal for growth of these strains, our standard *Shewanella* medium (M1; Myers & Nealson, 1988), supplemented with 20 mM sodium lactate and 0.001% Casamino acids (CAA; Difco Laboratories) was used. NaCl was added to final concentrations of 0.0, 0.125, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5 and 1.75 M. Experiments were performed in 125 ml Erlenmeyer flasks with vigorous agitation (150 r.p.m.). A final concentration of 0.5 M NaCl proved optimal; M1 supplemented at this concentration (M1N) was subsequently employed as our routine growth medium. Controlled temperature incubations were performed in either water baths or environmental chambers covering the range of 4–42 °C using M1N-lactate supplemented with 0.001% CAA as the growth media. All growth characterizations were performed in duplicate 125 ml Erlenmeyer flasks with vigorous agitation (150 r.p.m.).

Morphological and microscopic analysis. The size and shape of the ANG-SQ1^T cells were determined by phase-contrast and transmission electron microscopy (TEM). Photographs of living cells were obtained using wet mounts. Motility was ascertained by the direct microscopic observation of 24-h-old culture in M1N/lactate plus CAA. Flagellar staining was performed using the Flagella Stain (Carr-Scarborough). Cells were stained for 5 min prior to viewing at 2000× magnification. Gram staining was performed as previously described (Murray *et al.*, 1994). The methods of Fox *et al.* (1995) [a 1.0% (v/v) phosphotungstic acid (PTA) solution (pH 6.6)] were used to negatively stain and view the flagella under TEM. Cross-sections were prepared as follows: cells were fixed in 2.5/0.1% (w/w) glutaraldehyde/OsO₄ solution for 1 h (Owen *et al.*, 1990), collected by centrifugation, rinsed twice in 0.01 M HEPES (pH 6.8) and post-fixed in a 2.0% (w/v) OsO₄ solution. After two additional HEPES rinses, the cell pellet was collected by centrifugation into Point-Beam capsules and embedded in 1.5% (w/v) agar. The cells were then dehydrated using a graded acetone series and embedded in Spurr resin (Electron Microscope Sciences). Ultrathin sections were obtained with an RMC

Table 1. DNA–DNA hybridization of *S. pealeana* ANG-SQ1^T to other *Shewanella* species

Bacterium	Strain no.	Similarity (%)
<i>S. algae</i>	ATCC 51192	37.6
<i>S. amazonensis</i>	ATCC 700329 ^T	27.3*
<i>S. benthica</i>	ATCC 43992 ^T	29.4
<i>S. gelidimarina</i>	ACAM 456 ^T	25.3†
<i>S. hanedai</i>	ATCC 33224 ^T	26.4
<i>S. putrefaciens</i>	ATCC 8071 ^T	21.5
<i>S. woodyi</i>	MS-32 ^T	23.0

* Data from Venkateswaran *et al.* (1998).

† Data provided by M. Satomi (personal communication).

MT-7000 ultramicrotome fitted with a diamond knife. Sections were post-stained with uranyl acetate and Reynold's lead citrate before being viewed with a Hitachi H600 TEM at 75 keV.

Physiological studies. Catalase, oxidase and other standard phenotypic analyses were performed on strain ANG-SQ1^T at 25 °C as described elsewhere (Smibert & Krieg, 1994). Nutritional tests were performed in 5 ml batches of M1N in 18 ml culture tubes at 25 °C with vigorous agitation (150 r.p.m.). Results were recorded after 7 d incubation. The compounds listed in Table 2 were added to the media from sterile, pH-neutralized 1.0 M stock solutions and employed as sole energy sources at a final concentration of 20 mM (except formate and ethanol; 40 mM). CAA, yeast extract and peptone were tested at a 0.1 % final concentration added from sterile 10 % (w/v) stock solutions. Variations of Balch's vitamin mix (Balch *et al.*, 1979), each lacking one vitamin, were added to M1N/lactate (no CAA) to test for specific requirements. A similar method was used to test the 20 amino acids. Purines, pyrimidines and other bionutrients found in CAA (Difco technical analysis) were also examined (25 µg ml⁻¹ final concentration) in M1N/lactate. Iron- and manganese reduction were monitored in M1N/lactate agar plates containing 40 mM ferric-citrate or 40 mM MnO₂ in a soft-agar overlay, each added to the media from sterile 0.4 M stocks prepared as described previously (Myers & Nealson, 1988; 1990). Fresh cultures were streaked on to the ferric-citrate or stabbed into the MnO₂ plates and incubated at 25 °C in the Coy anaerobic chamber for 7 d. Cleared zones surrounding the colony or stab were defined as areas of metal reduction. Growth by reduction of nitrate (5 mM) or TMAO (20 mM) was monitored by noting changes in the optical density (OD₆₀₀) of a 10 ml culture over 7 d anaerobic incubation with gentle agitation (50 r.p.m.). Thiosulfate reduction was tested on Kligler Iron Agar (Difco) supplemented with 0.5 M NaCl; the production of black colonies was considered as a positive result.

Genetic analysis. Genomic DNA was purified from ANG-SQ1^T by the method of Marmur (1961). For the determination of the G+C content of the ANG-SQ1^T genome, the DNA was further purified on hydroxyapatite by the method of Cashion *et al.* (1977). The DNA was digested with P1 nuclease and the nucleotides were then dephosphorylated with bovine alkaline phosphatase (Mesbah *et al.*, 1989). The resulting deoxyribonucleosides were analysed by HPLC within the parameters stated by Tamaoka & Komagata (1984). DNA–DNA hybridization analysis (De Ley *et al.*,

1970; Escara & Hutton, 1980) and the determination of renaturation rates (Huß *et al.*, 1983; Jahnke, 1992) between *S. pealeana* and the other known *Shewanella* species were performed as previously described.

In vitro PCR amplification of the 16S rRNA gene was performed in a Minicycler (MJ Research) using the GeneAmp kit (Perkin-Elmer Cetus) and a final MgCl₂ concentration of 3 mM. Primers listed in the Oligonucleotide Probe Database (<http://www.cme.msu.edu>) (S-D-Bact-0011-a-S-17 and S-D-Bact-1492-b-A-16; Alm *et al.*, 1996) were used to generate PCR products. Conditions for gene amplification consisted of 35 cycles of 45 s at 95 °C, 45 s at 50 °C and 2 min at 72 °C, with a final extension reaction of 10 min at 72 °C. The sequences of the amplified DNA were determined directly using the IR² sequencing kit (LI-COR, Inc.) and run on an LI-COR automated DNA sequencer. Sequences thus obtained were manually aligned with closely related and representative sequences as determined with the software utilities of the Ribosomal Database Project (RDP; Maidak *et al.*, 1997). Phylogenetic trees and parsimony analyses were performed using PAUP version 3.2 (Swofford, 1993). Reference sequences were obtained from the RDP (Maidak *et al.*, 1997).

Cellular fatty acid analysis. Fatty acid methyl ester (FAME) analysis was performed at MicroCheck, Inc. The isolates were cultured in M1N/lactate or Tryptic Soy Broth (TSB) at 28 °C with gentle shaking. Fatty acids were extracted from dry cells, methylated and analysed by GC (Moss *et al.*, 1974) on a cross-linked 5 % phenyl silicone capillary column. The FAME peaks were identified and quantified by comparing the results with the patterns from other microorganisms, using MIDI System software (version 3.2). The relative amount of each fatty acid was expressed as a percentage of the total for that strain.

RESULTS

Isolation and microscopic examination of ANG-SQ1^T

Sulfur-reducing members of the ANG microbial community were isolated as stated in Methods. All of the numerous facultatively anaerobic sulfur reducers obtained in this manner were of a characteristic colonial appearance consistent with known *Shewanella* isolates (opaque salmon coloration with a mucoid surface). Cells grown in M1N/lactate and analysed by light- and phase-contrast microscopy proved to be motile, Gram-negative rods. Light microscopy also revealed that cells grown under non-optimal conditions appeared filamentous and deficient in proper segregation after division (data not shown). Flagellar staining revealed at least three polar flagella (data not shown), but the exact number could not be determined. Negative staining also presented problems. If cells were grown in liquid culture, NaCl from the media inhibited the staining. When cells were resuspended in buffer from an agar plate, the exopolysaccharide (EPS) produced by the colony clumped the cells on the grid (data not shown). Attempts to separate the cells from the EPS usually led to shearing of the flagella. When the cells could be separated from the EPS, four distinct flagella could be seen arising from the pole of each cell (data not shown). This feature differs from that of other *Shewanella* species, whose cells each have a

Fig. 1. Electron micrographs of thin sections of ANG-SQ1^T grown in M1N/lactate plus CAA. Bar, 0.5 μ m. (a) Cross-section of cells. The outer membrane (OM) and the cytoplasmic membrane (CM) are labelled with arrows. (b) Longitudinal section.

single, polar flagellum. TEM of ultrathin sections revealed that the cells were 2.0–3.0 μ m in length and 0.4–0.6 μ m in diameter (Fig. 1). The cells appear to divide by binary fission (data not shown) and no visible inclusions, storage vesicles or endospores were observed.

Genetic properties and phylogenetic analysis

The 16S rRNA gene was amplified from purified genomic DNA obtained from each of the six elemental sulfur-reducing isolates by PCR and sequenced. In order to determine their phylogenetic positions, these sequences were compared to those in the RDP (Maidak *et al.*, 1997). This query demonstrated that each of these isolates belong to the γ -3 subclass of the *Proteobacteria*. Five of the six isolates were similar to the known *Shewanella* species (> 88%), *Shewanella hanedai* (accession no. U91590) being the least similar because of a large number of ambiguous bases in the sequence. The sixth isolate was more closely affiliated with the genus *Aeromonas*. The 16S rDNA sequences of the *Shewanella* isolates differed by less than 1.5% (two being almost identical), suggesting that all are members of the same species. One isolate (ANG-SQ1^T) was then selected for further analysis.

The 16S rRNA gene sequences were further compared with other members of the γ -3 subclass of *Proteobacteria*, which includes *Alteromonas*, *Vibrio* and *Shewanella*. Their phylogenetic relationships were analysed using several different subdomains of the 16S sequence. Neighbour-joining analysis with Felsenstein distance corrections on these subsets are summarized in Fig. 2. All three sets of analysis gave similar phylogenetic topologies. Of the *Shewanella* species described, strain ANG-SQ1^T was most closely related to *Shewanella gelidimarina* (ACAM 456), at 97.0% similarity. *S. hanedai* (ATCC 33224) was the most distantly related member of this group, with a similarity level of 88.6%.

The overall DNA G+C composition for the known *Shewanella* species ranges from 40 to 54 mol% (Vogel *et al.*, 1997). ANG-SQ1^T has a G+C content of 45.0 mol%, which places it within the range that is typical for the *Shewanella putrefaciens* group of species (43.0–47.0 mol%; Vogel *et al.*, 1997). The direct comparison of the G+C content of ANG-SQ1^T to *S. gelidimarina* (48.0 mol%; Table 2) supports our view that ANG-SQ1^T and ACAM 456 are different species.

DNA–DNA hybridization analysis between *S. pealeana* and the other characterized *Shewanella* species is presented in Table 1. The percentage similarity of ANG-SQ1^T DNA ranged from 37.6% (*Shewanella algae*) to 21.5% (*S. putrefaciens*), indicating that *S. pealeana* is distinct from the other species. *S. gelidimarina* (ACAM 456), the member of this group which is the most closely related phylogenetically to ANG-SQ1^T, showed 25.3% similarity (Table 1). *Shewanella baltica* DNA was unavailable for this comparison.

Growth conditions

As strain ANG-SQ1^T is a marine isolate, the organism was tested for growth on NaCl concentrations ranging from 0.0 to 1.75 M. The isolate displayed an obligate requirement for NaCl and grew on levels of between 0.125 and 0.75 M, 0.5 M being optimal (data not shown). The strain grew when the starting pH was between 6.0 and 8.0, with optima of pH 7.0 for growth rate and pH 6.0 for total biomass (data not shown). Over the course of this experiment, the pH of the culture increased significantly (> 1.0 unit, data not shown), possibly explaining the disparity between the optima for growth rate and biomass. ANG-SQ1^T grew at temperatures of between 4 and 30 °C; 30 °C led to the fastest doubling time. However, cultures grown at 25 °C ultimately produced higher yields of cellular biomass (data not shown).

Physiological and biochemical characteristics

Initial studies indicated that these isolates grew poorly on nutrient-rich solid media, such as LB. On such plates, spontaneous mutants of a deeper orange colour

Fig. 2. Phylogenetic tree based on 16S rRNA gene comparisons between members of the γ -subclass of the class *Proteobacteria* and ANG-SQ1^T. The branching pattern was generated using the neighbour-joining method with Felsenstein distance corrections. The GenBank accession numbers are as follows: *A. caviae* ATCC15468^T (X74674), *A. hydrophila* ATCC 7966^T (X74677), *A. salmonicida* ATCC 33658^T (X74681), *A. schubertii* ATCC 43700^T (X74682), *A. macleodii* IAM 12920^T (X82145), *D. desulfuricans* ATCC 27774 (M34113), *E. coli* ATCC 25922 (X080724), *F. balearica* DSM 9799 (X93021), *M. barkeri* DSM 1538 (M59144), *Moritella* sp. HAS1123 (AB011353), *Moritella* sp. PE36 (U91587), *Moritella* sp. ODA02 (AB011353), *Moritella* sp. SC22 (AB011361), *Moritella* sp. J13 (AB011355), *M. marinus* (X82142), *M. viscosa* 486/88 (M59144), *P. atlantica* IAM 12927^T (X82134), *P. bacteriolytica* IAM14594^T (D89929), *P. haloplanktis* ATCC 14393 (X67024), *S. frigidimarina* A173 (U85902), *S. alga* ATCC 51192 (AF005249 Z95846), *S. amazonensis* ATCC 700329 (AF005248 Z95849), *S. baltica* NCTC 10735 (AJ000214), *S. benthica* ATCC 43992 (X82131), *S. frigidimarina* ICP4 (U85905), *S. frigidimarina* IC010 (U85906), *S. gelidimarina* ACAM 456 (U85907), *S. hanedai* ATCC 33224 (U91590), *S. pealeana* ATCC 700345 (AF011335), *S. putrefaciens* ATCC 8071^T (X82133), *Shewanella* sp. (DB5501 D21229), *S. woodyi* ATCC 51908 (AF003549), *V. alginolyticus* CIP 70.65 (X74691), *V. mytili* (X99761), *V. parahaemolyticus* CIP73.30 (X74721), *V. pelagius* ATCC 43700^T (X74722).

often arose which could be transferred to fresh LB agar plates, whereas the paler orange/red, wild-type colonies could not. This problem was abolished when ANG-SQ1^T was cultured on marine broth plates or grown in liquid M1N/lactate media. Strain ANG-SQ1^T was catalase-, oxidase- and lipase-positive, unable to hydrolyse starch or gelatin and did not produce indole. ANG-SQ1^T was able to utilize glucose, galactose, lactate, acetate, pyruvate, glutamate, succinate, citrate, CAA, yeast extract or peptone as sole energy source under optimal aerobic conditions (Table 2). In contrast, *S. gelidimarina* can only utilize lactate,

acetate or pyruvate as sole carbon source (Table 2; Bowman *et al.*, 1997). Fructose, sorbitol, glycerol, arabinose, formate or ethanol could not be used as sole carbon source in M1N media (data not shown). ANG-SQ1^T was able to utilize nitrate, fumarate, TMAO, iron, manganese, thiosulfate and elemental sulfur as alternative electron acceptors in M1N/lactate under anaerobic conditions (Table 2) and avoid fermentative growth (data not shown). Initial growth studies demonstrated a requirement for CAA in minimal liquid media, suggesting an auxotrophy. The addition of a mixture containing all 20 amino acids enhanced

Table 2. Differential biochemical characterization of *S. pealeana*From references Bowman *et al.* (1997), Makemson *et al.* (1997), Venkateswaran *et al.* (1998) and this study.

Characteristic	<i>S. pealeana</i> ATCC 700345 ^T	<i>S. gelidimarina</i> ACAM 456 ^T	<i>S. amazonensis</i> ATCC 700329 ^T	<i>S. hanedai</i> ATCC 33224 ^T	<i>S. woodyi</i> ATCC 51908 ^T
Growth temperature:					
4 (°C)	+	+	+	+	+
30 (°C)	+	—	+	—	—
H ₂ S production (from thiosulfate)	+	+	+	+	+
Chitinase	—	+	—	+	—
Gelatinase	—	+	+	+	+
Sole carbon source:*					
D-Glucose	+	—	—	—	—
D-Galactose	+	—	ND	+	+
Citrate	+	—	+	—	—
Succinate	+	—	+	—	+
Fumarate	—	—	+	ND	ND
Pyruvate	+	+	—	ND	ND
Lactate	+	+	+	+	+
Acetate	+	+	+	+	+
L-Glutamate	+	—	—	ND	ND
CAA	+	—	+	+	+
Yeast extract	+	ND	ND	ND	ND
Peptone	+	ND	ND	ND	ND
Anaerobic respiration	+†	+	+	+	+
G + C content (mol %)	45.0	48.0	51.7	45.2	39.0

ND, Not determined.

* All carbon sources were used at 20 mM. CAA, yeast extract and peptone were added at 0.1 %. Growth was monitored at 25 °C for 7 d.

† Screened nitrate, fumarate, TMAO, S⁰, Mn(IV) and Fe(III) with 20 mM lactate as the electron donor.

growth (final OD₆₀₀ = 0.4) over that observed for M1N/lactate alone (final OD₆₀₀ = < 0.1), but not to the same extent as the CAA-amended cultures (final OD₆₀₀ = > 1.0). This suggested that some constituent(s) of CAA, other than the amino acids *per se*, was/were responsible for the additional growth stimulation. Variations of Balch's vitamin mix were tested as supplements but failed to enhance growth. Purines, pyrimidines and inositol, which are also present in CAA, failed to enhance growth; the addition of guanine proved inhibitory (data not shown). One notable exception was the addition of choline chloride, the salt of a compound used by eukaryotic cells as a growth factor and lipid 'building block' (Jukes, 1979). Supplementation with choline chloride allowed ANG-SQ1^T to grow in M1N/lactate alone (final OD₆₀₀ = 0.4) and growth was comparable to CAA-amended cultures when the amino acid mix was also added. Given the growth requirement for choline, amino acids were tested as groups based on their chemical properties (charged, hydrophobic, etc.) in the presence of choline chloride. From this initial screen, the mixes containing either the hydrophobic amino acids (valine, isoleucine, serine and proline) or the uncharged amino acids (alanine glycine, leucine and lysine) enhanced

ANG-SQ1^T growth in the presence of choline. Two distinct biosynthetic pathways are responsible for the synthesis of most of the amino acids contained in these two groups [isoleucine, leucine, valine (ILV) and glycine, serine (GS)]. These five amino acids were tested both individually and in their biosynthetic groups to determine which are required for ANG-SQ1^T growth. From this analysis, the ILV biosynthetic group seems to be the necessary growth supplement, leucine being the one that provides the greatest enhancement (data not shown). These data, combined with the observations that *S. gelidimarina* required no nutritional supplements (Bowman *et al.*, 1997), further support our view that these strains are unique.

Cellular fatty acid composition

Following saponification, the following major fatty acids were detected in strain ANG-SQ1^T: 12:0, i13:0, i13:0 3-OH, 14:0, 15:0, i15:0, 16:0, 17:0, 17:1ω8c and 18:1ω9c (Table 3). Other major fatty acids which were present in ANG-SQ1^T (but could not be quantified owing to poor resolution of the chromatogram) were: 16:1ω7c/i15:0 2-OH and 18:1ω7c/ω9t/ω12t. No match could be determined during comparisons

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

Data from Bowman *et al.* (1997), Makemson *et al.* (1997), Venkateswaran *et al.* (1998) and this study. Tr, Trace (< 0.1%). NR, Not resolved; set of fatty acid esters which could not be resolved; the total level of these esters was 2.92% of the total profile.

Fatty acid	<i>S. pealeana</i>	<i>S. putrefaciens</i>	<i>S. algae</i>	<i>S. gelidimarina</i>	<i>S. benthica</i>	<i>S. amazonensis</i>	<i>S. hanedai</i>	<i>S. woodyi</i>
12:0	4.0	0.1–2.6	0.1	0.3–0.7	0.2–1.4		0.1–2.6	4.13
i13:0	13.8	1.0–3.6	0.5	5.4–15.8	2.8–8.9	4.7	5.1–7.7	10.04
13:0	0.9	0.0–0.5	0.1	Tr–1.3	0–0.3		0–0.3	0.87
12:0 3-OH	1.5							
i14:0	1.2	Tr–0.3	0.8	0.1–0.6	0.7–1.0	1.5	0.1–0.4	
14:1 ω 7c		0.0–Tr	Tr	0.3–0.8	0.1–0.5		0–Tr	
14:0	5.6	1.5–5.9	0.7	3.8–4.4	11.0–11.9	1.4	7.6–10.1	4.65
i13:0 3-OH	3.2							
i15:0	16.0	8.8–24.2	17.8	8.3–11.6	4.4–6.2	26.7	7.9–8.2	19.53
15:0	2.5	1.4–7.5	4.0	1.6–6.7	0.4–0.9	9.2	2.9–3.5	4.05
15:1 ω 8c		0.0–0.7	Tr	0.7–1.7			0–0.1	
15:1 ω 6c		0.0–0.3	Tr	0.4–1.7	0–0.2	0.8	0–0.5	
i16:0		0.0–Tr	1.1			1.4		
16:1 ω 9c		0.0–2.4		0–0.7		0.7		17.26
16:1 ω 7c	20.1	25.2–31.4	16.3	27.4–37.0	24.0–43.9	14.7	25.0–33.1	
16:1 ω 7t		1.0–1.7	0.2	0–0.1				
16:0	18.6	27.9–31.0	13.3	6.4–11.1	10.6–11.6	6.1	12.2–15.0	20.81
i17:0	1.1	Tr–1.6	0.5	0.1–0.4	0.1–1.5	1.8	0.8–2.4	1.66
17:1 ω 8c	2.8	2.7–3.7	14.7	1.9–4.2	1.6–6.6	23.4	0.5–1.5	
17:1 ω 6c		0.0–0.4	1.4	0.8–0.9	0–1.2	2.4	0.3–0.5	
17:0	1.5	0.6–1.6	1.4	0.4–0.5	0.5–8.1	3.9	0.6–1.0	1.3
18:1 ω 9c	2.4	1.1–3.6	6.0	0.3–0.9	0–0.2	1.4	0.2–0.8	0.98
18:1 ω 9t	NR							
18:1 ω 7c	NR	2.4–3.9	7.2	1.0–8.4	0–1.7	4.5	2.2–3.7	
18:1 ω 7t			0.2					
18:1 ω 12t	NR							
18:0	0.97	0.5–3.2	2.7	0.1–0.2	0–0.5	0.1	0–0.4	
20:5 ω 3				11.7–16.0	15.8–18.5		19.1–22.2	
Other	0.97	0.0–0.8	2.7	0–0.8			0–0.3	6.25

performed against the database at MicroCheck, Inc. The organism in this database with the closest similarity to ANG-SQ1^T was the non-type strain of *Brevibacterium acetylicum*-GC sub-group (distance coefficient, 11.9). In addition, ANG-SQ1^T lacked the polyunsaturated fatty acid eicosapentanoic acid (20:5 ω 3), a major constituent of the whole-cell fatty acids of its closest relative, *S. gelidimarina* (Table 3). This further supports our contention that these two organisms are unique species.

DISCUSSION

Six bacterial isolates representing one of the cultivable bacterial types from the ANG of the squid *L. pealei* were purified for this study. All were initially screened on the basis of elemental sulfur reduction and all shared a common colony and cell morphology. One isolate (ANG-SQ1^T) was chosen at random for detailed analysis. In this report, we make the case, based on a combination of independent molecular and

phenotypic characterizations, that the isolate ANG-SQ1^T falls within the genus *Shewanella* and represents a novel species.

Initial comparisons of the *S. pealeana* 16S rRNA sequence with those of the other known *Shewanella* species indicated that this organism had a close phylogenetic relationship to the others in this genus (88.6–97.0%). With one notable exception however, ANG-SQ1^T failed to meet the 97% homology criterion (Wayne *et al.*, 1987) for inclusion within any of the described species. The 16S rDNA sequence homology between ANG-SQ1^T and the type strain of the new species, *S. gelidimarina* (Bowman *et al.*, 1997), is in fact 97.0%, a value that can neither prove nor disprove a species relationship. In a case such as this, DNA–DNA hybridization is the preferred method for clarifying ambiguous phylogenetic relationships. These experiments were performed and *S. gelidimarina* was 25.3% similar to strain ANG-SQ1^T (Table 1). In addition, Venkateswaran *et al.* (1999) have reported that, on the basis of an independent molecular marker

(*gyrB*, the β -subunit of DNA topoisomerase II), *S. gelidimarina* and *S. pealeana* are almost certainly unique species. Physiological, fatty acid and mol% G+C profiles for these two organisms also support the conclusion that *S. pealeana* and *S. gelidimarina* are distinct from one another.

The facultatively anaerobic sulfur-reducing ANG isolates were Gram-negative, motile rods (2.0–3.0 μ m long, 0.4–0.6 μ m in diameter) with polar flagella. Growth on marine broth agar produced salmon-coloured colonies 2 mm in diameter after several days of growth. *S. pealeana* is a non-fermentative organism that can utilize glucose, galactose, lactate, acetate, pyruvate, glutamate, citrate, succinate, CAA, yeast extract or peptone as sole energy source under aerobic conditions. Anaerobically, this organism performs the dissimilatory reduction of nitrate, fumarate, iron, manganese, TMAO, thiosulfate and elemental sulfur, with lactate as the electron donor. ANG-SQ1^T can grow within a wide range of salinities at mesophilic temperatures. The morphological and physiological characteristics of these isolates closely resemble those of other *Shewanella* species, more specifically those of the *S. putrefaciens* subclass rather than the *Shewanella algae* subclass (Vogel *et al.*, 1997). 16S rRNA sequence analysis grouped these isolates with the γ -3 subclass of the *Proteobacteria*, their closest known relative being an organism (*S. gelidimarina*) isolated from the Antarctic Sea (Bowman *et al.*, 1997).

The requirement of choline chloride for cultures of *S. pealeana* in M1N/lactate media was unexpected. Choline is an important nutritional component in the diet of higher eukaryotes, which employ it in the synthesis of phosphatidylcholine, a membrane phospholipid (Jukes, 1979). However, the synthesis of phosphatidylcholine by most prokaryotes for their membranes has not been demonstrated (Moat, 1979), suggesting that this possible use for choline is possible but unlikely. The growth of several Gram-positive organisms in the absence (Horne & Tomasz, 1993) or in an excess (Podvin *et al.*, 1988) of choline was shown to result in severely affected cell shape, cell division and overall physiology. Perhaps a high choline content in the yeast extract of LB affected ANG-SQ1^T, leading to cell-division defects (data not shown) and eventual cell death. Another possibility is that choline may serve as a precursor to glycine-betaine, a compatible solute which is known to accumulate in cells in response to stressful conditions such as high salt (Csonka & Hanson, 1991) or low temperatures (Ko *et al.*, 1994). Most micro-organisms lack the ability to synthesize glycine-betaine, but it can be readily transported across the cytoplasmic membrane (Boch *et al.*, 1994). The glycine-betaine biosynthesis operon encoding the choline oxidase gene (*codA*) has been recently cloned and sequenced from a halophile (Cánovas *et al.*, 1996). It has been subsequently expressed in other organisms (Deshnium *et al.*, 1997; Hayashi *et al.*, 1997) to enhance tolerance to salt and cold stress. An excellent starting point for future studies would be to determine

whether this operon is present in the *S. pealeana* genome. Glycine-betaine also can be further metabolized into methionine, potentially to supplement an auxotrophy. However, as methionine itself did not enhance the growth of ANG-SQ1^T in M1N/lactate media, this explanation seems improbable. The lipase activity demonstrated by *S. pealeana* may be used for the extraction of choline from *Loligo* membranes for these purposes. However, there are no experimental data to support this hypothesis. Choline could also play a role in host-micro-organism interactions. Choline-binding proteins have been identified on the cell surface of several micro-organisms (Sanchez-Beato & Garcia, 1996). If these membrane proteins are present, they could provide a matrix for a specific interaction between the phosphatidylcholine present in the *Loligo* membranes and ANG-SQ1^T. This may facilitate the colonization of the ANGs by this *Shewanella* species, hence explaining the repeated isolation of related organisms from the gland (Bloodgood, 1977; Getzel, 1934).

At present, the role of the microbial community within the *Loligo* ANG remains undetermined. The function of the ANG is unknown but histological studies have shown that the gland exhibits a secretory cycle associated with maturation of the squid (Lum-Kong, 1992). Some members of the genus *Alteromonas* can produce antibiotic compounds (Gauthier, 1976; Gauthier & Breittmayer, 1979). Because the ANG is a reproductive organ and located immediately adjacent to the egg-producing nidamental glands, the antibiotic activities of *Alteromonas*-like species could provide protection for the cephalopod eggs once laid. Previous studies of other *Loligo* species have demonstrated that a dense culture of bacteria is arranged in the egg capsule sheath which appears to help to protect the embryos from animal, fungal and microbial predation during incubation (Biggs & Epel, 1991). Strains closely related to *S. pealeana* have been isolated recently from the egg sheath of *L. pealei* as well as from the ANG from the Pacific squid *Loligo opalescens* (Barbieri *et al.*, 1999). This further supports the hypothesized mutualistic role for the ANG micro-organisms in the cephalopod life cycle (Barbieri *et al.*, 1996). Because *Shewanella* species were once classified as *Alteromonas* species (Coyne *et al.*, 1989; MacDonell & Colwell, 1985), a genus known for antibiotic production, it seems plausible that ANG-SQ1^T may also produce antibiotics. Another possibility is that ANG-SQ1^T could synthesize allelochemicals that would affect its host, competitors or predators (as observed with crustacean embryo-associated micro-organisms; Gil-Turnes *et al.*, 1989). Initial assays for antimicrobial activity by ANG-SQ1^T have demonstrated a weak inhibition of several marine pathogens (Barbieri *et al.*, 1997). Whether this inhibition is the result of antibiotic or allelochemical activities is, at present, unknown. The roles of *S. pealeana* in the ANG, as well as the participation of choline in this relationship, are ideal starting points for future studies. The evidence we

present here suggests that these isolates represent a new *Shewanella* species. We propose the name *Shewanella pealeana* for this organism, reflecting its relationship with *Loligo pealei*.

Description of *Shewanella pealeana* sp. nov.

Shewanella pealeana (peal'le.ana. M.L. adj. *pealeana* from *peale* or *pealei*, the species name of the squid *Loligo pealei* with which the bacterium is associated).

Cells are Gram-negative, polarly flagellated, rods 2.0–3.0 µm long and 0.4–0.6 µm wide. Circular, opaque, salmon-coloured colonies are formed after 2 d on marine broth agar at 25 °C. Catalase-, oxidase- and lipase-positive. Amylase- and gelatinase-negative. Growth occurs at temperatures of 4–30 °C (optimal temperature for biomass, 25 °C) in the presence of NaCl concentrations ranging from 0.125–0.75 M (optimum NaCl concentration, 0.5 M) and within a pH range of 6–8 (optimum starting pH, 7.0). Glucose, galactose, lactate, acetate, pyruvate, citrate, succinate, glutamate, Casamino acids, yeast extract and peptone are used as energy sources aerobically. Fructose, glycerol, sorbitol, arabinose, formate and ethanol are not utilized. An obligate respirer using nitrate, fumarate, iron, manganese, TMAO, thiosulfate and elemental sulfur as alternate electron acceptors anaerobically with lactate. Choline chloride appears to be an essential growth factor in minimal media. The main cellular fatty acids are iso-branched acids, which account for 35.2% of the total fatty acids. The G + C content of the genome is 45.0 mol %. The type strain, *Shewanella pealeana* ANG-SQ1^T, was isolated from the accessory nidamental gland of the squid *Loligo pealei*. This isolate was a member of a microbial community harboured within the ANG and was selected for its ability to reduce elemental sulfur. Strain ANG-SQ1^T has been deposited in the American Type Culture Collection as strain ATCC 700345^T.

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