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Shewanella upenei sp. nov., a Lipolytic Bacterium Isolated from Bensasi Goatfish *Upeneus bensasi*

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A Gram-staining-negative, motile, non-spore-forming and rod-shaped bacterial strain, 20-23R^T, was isolated from intestine of bensasi goatfish, *Upeneus bensasi*, and its taxonomic position was investigated by using a polyphasic study. Phylogenetic analyses based on 16S rRNA gene sequences revealed that strain 20-23R^T belonged to the genus *Shewanella*. Strain 20-23R^T exhibited 16S rRNA gene sequence similarity values of 99.5, 99.2, and 97.5% to *Shewanella algae* ATCC 51192^T, *Shewanella haliotis* DW01^T, and *Shewanella chilikensis* JC5^T, respectively. Strain 20-23R^T exhibited 93.1-96.0% 16S rRNA gene sequence similarity to the other *Shewanella* species. It also exhibited 98.3-98.4% *gyrB* sequence similarity to the type strains of *S. algae* and *S. haliotis*. Strain 20-23R^T contained simultaneously both menaquinones and ubiquinones; the predominant menaquinone was MK-7 and the predominant ubiquinones were Q-8 and Q-7. The fatty acid profiles of strain 20-23R^T, *S. algae* KCTC 22552^T and *S. haliotis* KCTC 12896^T were similar; major components were iso-$C_{15:0}$, $C_{16:0}$, $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH and $C_{17:1}$ ω8c. The DNA G+C content of strain 20-23R^T was 53.9 mol%. Differential phenotypic properties and genetic distinctiveness of strain 20-23R^T, together with the phylogenetic distinctiveness, revealed that this strain is distinguishable from recognized *Shewanella* species. On the basis of the data presented, strain 20-23R^T represents a novel species of the genus *Shewanella*, for which the name *Shewanella upenei* sp. nov. is proposed. The type strain is 20-23R^T (=KCTC 22806^T =CCUG 58400^T).

***Keywords*:** *Shewanella upenei*, taxonomy, new species

The genus *Shewanella* was first described by MacDonell and Colwell (1985) through the reclassification of *Alteromonas putrefaciens* and *Alteromonas hanedai* as *Shewanella putrefaciens* and *Shewanella hanedai*, respectively. Subsequently, continuous descriptions of novel species have increased considerably the number of species belonging to the genus *Shewanella*, and at the time of writing, the genus *Shewanella* comprises at least 54 species with validly published names (Euzéby *et al*., 1997). Many species belonging to the genus *Shewanella* have been isolated from marine environments, including intertidal sediment and sand (Yoon *et al*., 2004a; Chang *et al*., 2008), seawater (Ivanova *et al*., 2004; Yoon *et al*., 2004b), marine and deep-sea sediments (Miyazaki *et al*., 2006; Yang *et al*., 2007; Zhao *et al*., 2007) and marine organisms (Lee *et al*., 2006; Satomi *et al*., 2006, 2007; Kim *et al*., 2007). In this study, we describe a bacterial strain, designated 20-23R^T, which was isolated from intestine of bensasi goatfish, *Upeneus bensasi*. Comparative 16S rRNA gene sequence analysis indicated that strain 20-23R^T is most phylogenetically closely affiliated to the genus *Shewanella*. The aim of the present work was to determine the exact taxonomic position of strain 20-23R^T by using a polyphasic characterization that included determination of the phenotypic and chemotaxonomic properties, a phylogenetic investigation based on 16S rRNA gene sequences and genetic analysis.

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Materials and Methods

Bacterial strains and culture conditions

Bensasi goatfish, *Upeneus bensasi*, collected from the South Sea in Korea was used for the isolation of bacterial strains. Strain 20-23R^T was isolated from intestine of bensasi goatfish, *Upeneus bensasi*, by means of the standard dilution plating technique at 25°C on marine agar 2216 (MA; Difco). For short-term preservation, strain 20-23R^T was maintained on MA at 4°C. For long-term preservation, cells of strain 20-23R^T was harvested from agar plates and transferred to cryo-tube containing 20% (w/v) glycerol solution. The tube is stored at -80°C. Strain 20-23R^T has been deposited in the Korean Collection for Type Cultures (KCTC, Korea) and Culture Collection, University of Göteborg (CCUG; Sweden) as KCTC 22806^T and CCUG 58400^T, respectively. The type strains of three *Shewanella* species were used as reference strains for phenotypic characterization, fatty acid analysis and sequencing of DNA gyrase B subunit gene (*gyrB*) and/or DNA-DNA hybridization. *Shewanella algae* KCTC 22552^T, *Shewanella haliotis* KCTC 12896^T, and *Shewanella chilikensis* KCTC 22540^T were obtained from the Korean Collection for Type Cultures, Daejeon, Korea. Cell mass of strain 20-23R^T for DNA extraction and for isoprenoid quinone analysis and cell mass of *S. algae* KCTC 22552^T, *S. haliotis* KCTC 12896^T, and *S. chilikensis* KCTC 22540^T for DNA

extraction were obtained from cultures grown for 3 days in marine broth 2216 (MB; Difco) at 30°C. For fatty acid methyl ester analysis, cell mass of strain 20-23R^T, *S. algae* KCTC 22552^T and *S. haliotis* KCTC 12896^T was harvested from MA plates after incubation for 3 days at 30°C.

Phenotypic characterization

The morphological, physiological and biochemical characteristics of strain 20-23R^T were investigated using routine cultivation on MA at 30°C. Cell morphology was examined by light microscopy (Nikon E600) and transmission electron microscopy. Flagellation was determined by using a Philips CM-20 transmission electron microscope with cells from an exponentially growing culture. For this purpose, the cells were negatively stained with 1% (w/v) phosphotungstic acid and the grids were examined after being air-dried. The Gram reaction was determined by using the bioMérieux Gram stain kit according to the manufacturer's instructions. Growth at various temperatures (4, 10, 20, 25, 28, 30, 35, 37, 40, and 45°C) was measured on MA. Growth in the absence of NaCl and in the presence of 0.5, 1.0, 2.0, and 3.0% (w/v) NaCl was investigated in trypticase soy broth prepared according to the formula of the Difco medium except that NaCl was excluded and that 0.45% (w/v) $MgCl_2 \cdot 6H_2O$ or 0.06% (w/v) KCl was added. Growth at various NaCl concentrations (2.0-15.0%, w/v, at increments of 1.0%) was investigated in MB. The pH range for growth was determined in MB adjusted to pH 4.5-9.5 (using increments of 0.5 pH unit) by using sodium acetate/acetic acid and Na_2CO_3 buffers. Growth under anaerobic conditions was determined after incubation in a Forma anaerobic chamber on MA and on MA supplemented with potassium nitrate (0.1%, w/v), both of which had been prepared anaerobically under nitrogen atmosphere. Catalase and oxidase activities were determined as described by Cowan and Steel (1965). Lipolytic activity was investigated on tricaprylin (TCN)-MA, which was prepared as follows: a TCN emulsion was made by emulsifying 5 ml TCN in 45 ml gum arabic solution (200 mM NaCl, 10 mM $CaCl_2$ and 5%, w/v, gum arabic) for 2 min using a Waring blender, and the TCN emulsion (50 ml) was mixed with 450 ml MA. Nitrate reduction and hydrolysis of esculin, gelatin, urea and Tweens 20, 40, 60, and 80 were investigated as described previously (Lanyi, 1987) with the modification that artificial seawater was used for preparation of media. Hydrolysis of casein, starch, hypoxanthine, tyrosine, and xanthine was tested on MA, using the substrate concentrations described previously (Cowan and Steel, 1965). DNase activity was examined by using DNase test agar with methyl green (Difco) with the modification that artificial seawater was used for the preparation of the medium. The artificial seawater contained (per L distilled water) 23.6 g NaCl, 0.64 g KCl, 4.53 g $MgCl_2 \cdot 6H_2O$, 5.94 g $MgSO_4 \cdot 7H_2O$, and 1.3 g $CaCl_2 \cdot 2H_2O$ (Bruns *et al*., 2001). H_2S production was tested as described previously (Bruns *et al*., 2001). Acid production from carbohydrates was determined as described by Leifson (1963). Utilization of substrates as sole carbon and energy sources was tested as described by Baumann and Baumann (1981) using supplementation with 2% (v/v) Hutner's mineral base (Cohen-Bazire *et al*., 1957) and 1% (v/v) vitamin solution (Staley, 1968). Susceptibility to antibiotics was tested on MA plates using antibiotic discs containing the following concentrations; polymyxin B (100 U), streptomycin (50 μg), penicillin G (20 U), chloramphenicol (100 μg), ampicillin (10 μg), cephalothin (30 μg), gentamicin (30 μg), novobiocin (5 μg), tetracycline (30 μg), kanamycin (30 μg), lincomycin (15 μg), oleandomycin (15 μg), neomycin (30 μg), and carbenicillin (100 μg). Other physiological and biochemical tests were performed with the API 20E and API ZYM systems (bioMérieux).

Molecular systematics

Chromosomal DNA was isolated and purified according to the method described previously (Yoon *et al*., 1996), with the exception that RNase T1 was used in combination with RNase A to minimize contamination with RNA. The 16S rRNA gene was amplified by PCR using two universal primers as described previously (Yoon *et al*., 1998). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al*. (2003). PCR amplification of the *gyrB* was performed by using two primers, UP-1 and UP-2r, according to the method described previously (Yamamoto and Harayama, 1995). The PCR product was purified with the QIAquick PCR Purification kit (QIAGEN). The amplified *gyrB* was cloned into pGEM T-easy vector (Promega) according to the manufacturer's instructions. Sequence of the *gyrB* was determined for both strands by extension from vector-specific priming sites (T7 and SP-6 primers from pGEM T-easy vector). DNA-DNA hybridization was performed fluorometrically by the method of Ezaki *et al*. (1989) using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained in each sample were excluded, and the means of the remaining three values were quoted as DNA-DNA relatedness values.

Chemosystematic characterization

Isoprenoid quinones were extracted according to the method of Komagata and Suzuki (1987) and analyzed using reversed-phase HPLC and a YMC ODS-A (250×4.6 mm) column. The fatty acid methyl esters were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The DNA G+C content was determined by the method of Tamaoka and Komagata (1984) with the modification that DNA was hydrolyzed and the resultant nucleotides were analyzed by reversed-phase HPLC.

Nucleotide sequence accession numbers

The 16S rRNA gene sequence of strain 20-23R^T has been deposited in the GenBank database under accession number GQ260190. The *gyrB* sequences of strain 20-23R^T, *Shewanella algae* KCTC 22552^T, and *Shewanella haliotis* KCTC 12896^T have been deposited in the GenBank database under accession numbers GQ260193, GQ260194, and GQ260195, respectively.

Results and Discussion

Morphological, cultural, physiological, and biochemical characteristics

Strain 20-23R^T was facultatively anaerobic, Gram-staining-negative, non-spore-forming and rods (0.3-0.7×1.0-4.5 μm). It was motile by means of a single polar flagellum. Colonies were circular to slightly irregular, raised, smooth, glistening and moderate orange-yellow in colour on MA. Strain 20-23R^T grew at 10 and 40°C with an optimum temperature of 30°C. It grew optimally at pH 7.0-8.0 and in the presence of 2.0-5.0% (w/v) NaCl. Strain 20-23R^T showed catalase and oxidase activities and reduced nitrate to nitrite. Morphological, cultural, physiological, and biochemical characteristics of strain 20-23R^T are given in the species description (see below) or in Table 1.

Table 1. Differential phenotypic characteristics of *Shewanella upenei* 20-23R^T and two phylogenetically closely related *Shewanella* species Strains: 1, *S. fistulariae* 20-23R^T; 2, *S. algae* KCTC 22552^T; 3, *S. haliotis* KCTC 12896^T. All data from this study except for motility, nitrate reduction, H_2S production, urease, catalase, oxidase and DNA G+C content; these data are from Nozue *et al*. (1992) and Kim *et al*. (2007). +, positive reaction; –, negative reaction; w, weakly positive reaction. All species are Gram-negative and rod-shaped. All species are positive for motility; catalase; oxidase; nitrate reduction; hydrolysis of casein, DNA, gelatin, tyrosine and Tweens 20, 40, 60, and 80; utilization of D-glucose, acetate, L-malate, pyruvate and succinate; activity of alkaline phosphatase, esterase (C 4), esterase lipase (C 8), leucine arylamidase (weak), α-chymotrypsin; and susceptibility to gentamicin, kanamycin, neomycin and streptomycin. All species are negative for urease; hydrolysis of esculin, agar, starch, hypoxanthine and xanthine; utilization of L-arabinose, D-cellobiose, D-galactose, D-fructose, maltose, D-mannose, sucrose, D-trehalose, D-xylose, citrate, salicin, benzoate, formate and L-glutamate; acid production from L-arabinose, D-cellobiose, D-fructose, D-galactose, *myo*-inositol, lactose, maltose, D-mannitol, D-mannose, D-melezitose, melibiose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose and D-xylose; activity of lipase (C 14), valine arylamidase, cystine arylamidase, trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase; and susceptibility to novobiocin, cephalothin, lincomycin, penicillin G, polymyxin B and tetracycline.

Characteristic	1	2	3
H_2S production	-	-	+
Acid production from			
D-Glucose	+	-	-
D-Ribose	+	-	+
Enzyme activity (API ZYM)			
Acid phosphatase	-	+	+
Naphthol-AS-BI-phosphohydrolase	-	+	+
N-Acetyl-β-glucosaminidase	-	w	-
Susceptibility to			
Ampicillin	-	+	w
Carbenicillin	-	+	+
Oleandomycin	-	w	+
DNA G+C contents (mol%)	53.9	54.0	53.7

Phylogenetic analysis

The almost complete 16S rRNA gene sequence of strain 20-23R^T determined in this study comprised 1468 nucleotides, representing approximately 96% of the *Escherichia coli* 16S rRNA sequence. In the neighbor-joining phylogenetic tree based on 16S rRNA gene sequences, strain 20-23R^T fell within the clade comprising *Shewanella* species, forming a coherent cluster with the type strains of *Shewanella algae* ATCC 51192^T and *Shewanella haliotis* DW01^T (Fig. 1). The clustering of strain 20-23R^T, *S. algae* ATCC 51192^T and *S. haliotis* DW01^T was also found in the trees constructed using the maximum-likelihood and maximum-parsimony algorithms (data not shown). Strain 20-23R^T exhibited 16S rRNA gene sequence similarity values of 99.5, 99.2, and 97.5% to *S. algae* ATCC 51192^T, *S. haliotis* DW01^T, and *S. chilikensis* JC5^T, respectively, and 93.1-96.0% to the other *Shewanella* species used in the phylogenetic analysis. Strain 20-23R^T exhibited *gyrB* sequence similarity values of 98.3 and 98.4% to *S. algae* KCTC 22552^T and *S. haliotis* KCTC 12896^T, respectively. The *gyrB* sequence similarity value between *S. algae* KCTC 22552^T and *S. haliotis* KCTC 12896^T was 98.0%.

DNA-DNA relatedness

Strain 20-23R^T exhibited mean DNA-DNA relatedness values of 54, 53, and 18% to *S. algae* KCTC 22552^T, *S. haliotis* KCTC 12896^T and *S. chilikensis* KCTC 22540^T, respectively, when DNAs of the four strains were used individually as lebelled DNA probes for cross-hybridization.

Chemosystematic characteristics

Strain 20-23R^T contained simultaneously both menaquinones (MK) and ubiquinones (Q) as isoprenoid quinones. The predominant menaquinone detected in strain 20-23R^T was MK-7 and the predominant ubiquinones detected in strain 20-23R^T were Q-8 and Q-7 at a peak area ratio of approximately 59 and 38%, respectively. The cellular fatty acid profile of strain 20-23R^T is shown in Table 2, together with those of *S. algae* KCTC 22552^T and *S. haliotis* KCTC 12896^T analyzed also in this study. Strain 20-23R^T had a fatty acid profile containing large amounts of straight-chain, branched, unsaturated and hydroxy fatty acids; major components (> 10% of total fatty acids) were iso-$C_{15:0}$, $C_{16:0}$, $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH and $C_{17:1}$ ω8c (Table 2). The fatty acid profiles of the three strains were essentially similar, although there were differences in the proportions of some fatty acids. The DNA G+C content of strain 20-23R^T was 53.9 mol%. The results obtained from chemotaxonomic analyses support the result of phylogenetic analysis, i.e. that strain 20-23R^T belongs to the genus *Shewanella*.

Conclusion

Strain 20-23R^T was differentiated from the two phylogenetically closely related *Shewanella* species by differences in several phenotypic characteristics (Table 1). DNA-DNA related data indicate that strain 20-23R^T differs genetically from the three reference strains, according to a widely accepted criterion stating that strains with a level of DNA-DNA relatedness less than 70% are considered as being different species (Wayne *et al*., 1987). The phylogenetic and genetic distinctiveness and differential phenotypic properties of strain 20-23R^T are sufficient to show that this strain is separate from *Shewanella* species (Stackebrandt and Goebel, 1994). Therefore, on the basis of

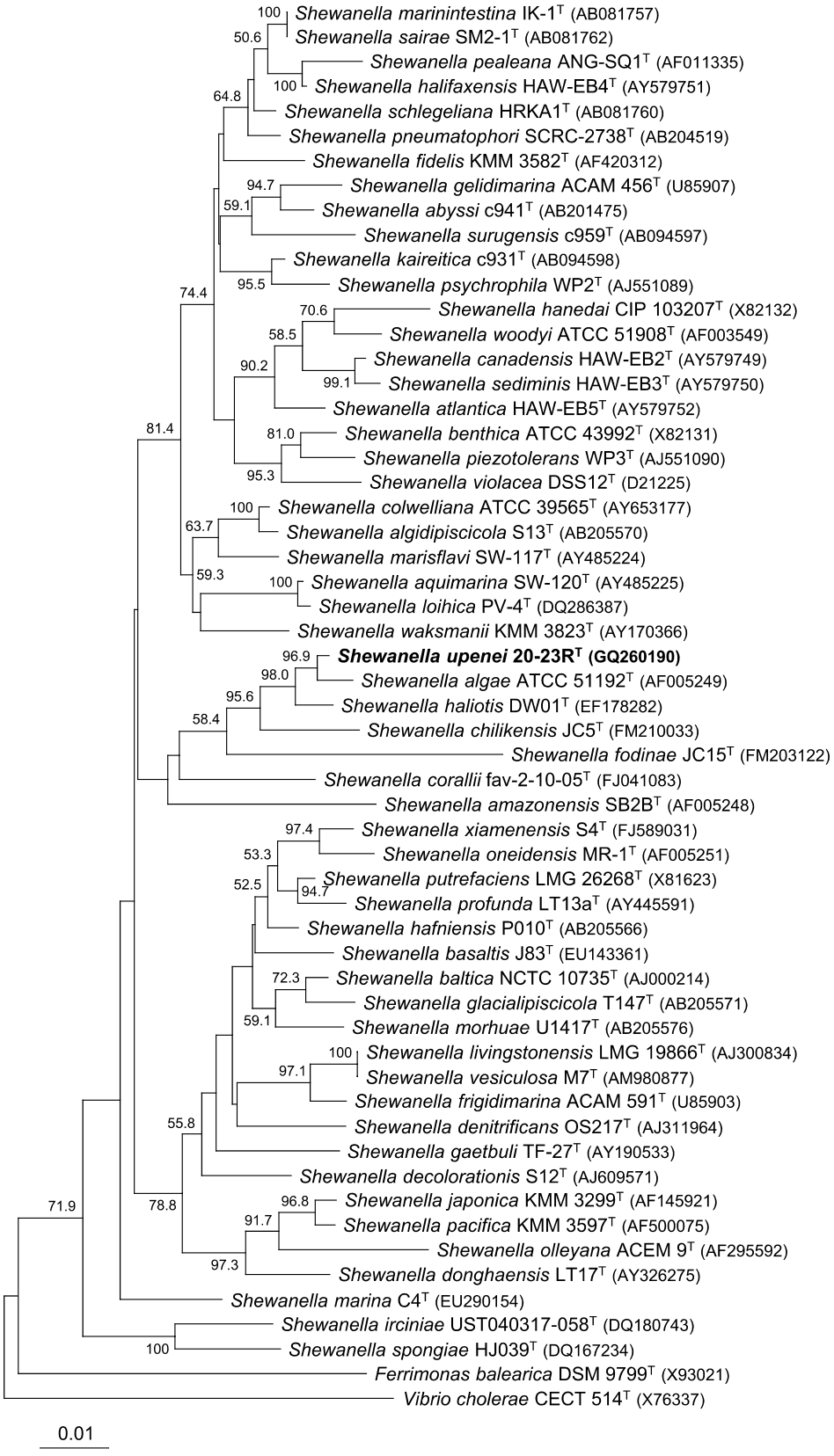

Fig. 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of *S. upenei* 20-23R^T and some other related taxa. Bootstrap values (expressed as percentages of 1,000 replications) of >50% are shown at branching points. *V. cholerae* CECT 514^T (GenBank accession no. X76337) was used as an outgroup. Scale bar, 0.01 substitutions per nucleotide position.

Table 2. Cellular fatty acid compositions (%) of *Shewanella upenei* 20-23R^T and the type strains of two phylogenetically related *Shewanella* species. Strains: 1, *S. upenei* 20-23R^T; 2, *S. alae* KCTC 22552^T; 3, *S. halotis* KCTC 12896^T. All data from this study. Fatty acids that represented <1.0% in all strains were omitted.

Fatty acid	1	2	3
Straight-chain fatty acids			
$C_{12:0}$	2.8	2.6	2.1
$C_{13:0}$	1.2	1.1	1.1
$C_{15:0}$	5.0	4.7	5.1
$C_{16:0}$	16.7	14.2	11.8
$C_{17:0}$	4.0	3.3	4.4
$C_{18:0}$	1.5	1.1	1.0
Branched fatty acids			
iso-$C_{13:0}$	3.8	3.7	4.5
iso-$C_{15:0}$	18.2	19.9	22.1
iso-$C_{17:0}$	2.2	5.1	2.9
Unsaturated fatty acids			
$C_{17:1}$ ω8c	10.5	9.2	12.6
$C_{18:1}$ ω7c	3.6	3.2	3.0
$C_{18:1}$ ω9c	3.1	3.3	2.7
Hydroxy fatty acids			
$C_{12:0}$ 3-OH	2.2	1.9	1.7
$C_{15:0}$ 3-OH	0.2	1.8	0.3
iso-$C_{13:0}$ 3-OH	2.0	2.0	2.6
Summed features*			
1	2.0	1.6	1.8
2	2.0	1.9	1.5
3	11.5	11.9	10.6

* Summed features represent groups of two or three fatty acids which could not be separated by GLC with the MIDI system. Summed feature 1 contained iso-$C_{15:1}$ and/or $C_{13:0}$ 3-OH. Summed feature 2 contained iso-$C_{16:1}$ and/or $C_{14:0}$ 3-OH. Summed feature 3 contained $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH.

the phenotypic, phylogenetic and genetic data, strain 20-23R^T is considered to represent a novel species of the genus *Shewanella*, for which the name *Shewanella upenei* sp. nov. is proposed.

Description of *Shewanella upenei* sp. nov.

Shewanella upenei (u.pe'ne.i. N.L. gen. n. *upenei* of Upeneus, the generic name of bensasi goatfish *Upeneus bensasi*, from which the type strain was isolated).

Cells are Gram-negative, non-spore-forming and rods (0.3-0.7×1.0-4.5 μm). Motile by means of a single polar flagellum. Colonies on MA are circular to slightly irregular, raised, smooth, glistening, moderate orange-yellow in colour and 3.0-5.0 mm in diameter after incubation for 3 days at 30°C. Optimal growth occurs at 30°C; growth occurs at 10 and 40°C, but not at 4 and 45°C. Optimal pH for growth is between 7.0 and 8.0; growth occurs at pH 5.0, but not at pH 4.5. Optimal growth occurs in the presence of 2-5% (w/v) NaCl; growth occurs at 0-11.0% (w/v) NaCl. Growth occurs under anaerobic conditions on MA and on MA supplemented with nitrate. Catalase- and oxidase-positive. Nitrate reduction is positive. Both menaquinones (MK) and ubiquinones (Q) are present; the predominant menaquinone is MK-7 and the predominant ubiquinones are Q-8 and Q-7. The major fatty acids (>10% of total fatty acids) are iso-$C_{15:0}$, $C_{16:0}$, $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH and $C_{17:1}$ ω8c. The DNA G+C content is 53.9 mol% (HPLC). Other phenotypic properties are shown in Table 1.

The type strain, 20-23R^T (=KCTC 22806^T =CCUG 58400^T), was isolated from the intestine of bensasi goatfish, *Upeneus bensasi*.

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