Echinicola pacifica gen. nov., sp. nov., a novel flexibacterium isolated from the sea urchin *Strongylocentrotus intermedius*

Olga I. Nedashkovskaya,[1] Seung Bum Kim,[2] Marc Vancanneyt,[3] Anatoly M. Lysenko,[4] Dong Sung Shin,[2] Myung Soo Park,[5] Kang Hyun Lee,[6] Won Jin Jung,[6] Natalia I. Kalinovskaya,[1] Valery V. Mikhailov,[1] Kyung Sook Bae[6] and Jean Swings[3,7]

Correspondence
Olga I. Nedashkovskaya
olganedashkovska@piboc.dvo.ru
or
olganedashkovska@yahoo.com

[1]Pacific Institute of Bioorganic Chemistry of the Far-Eastern Branch of the Russian Academy of Sciences, Pr. 100 Let Vladivostoku 159, 690022, Vladivostok, Russia

[2,5]Department of Microbiology[2] and Department of Applied Microbiology, College of Agriculture and Life Sciences[5], Chungnam National University, 220 Gung-dong, Yusong, Daejon 305-764, Republic of Korea

[3,7]BCCM/LMG Bacteria Collection[3] and Laboratory of Microbiology[7], Ghent University, Ledeganckstraat 35, B-9000 Ghent, Belgium

[4]Institute of Microbiology of the Russian Academy of Sciences, Pr. 60 let October 7/2, Moscow, 117811, Russia

[6]Korea Institute of Bioscience and Biotechnology, 52 Oun-dong, Yusong, Daejon 305-333, Republic of Korea

The taxonomic position of three novel marine, heterotrophic, pigmented and agarolytic bacteria with gliding motility, isolated from the sea urchin *Strongylocentrotus intermedius*, was investigated. 16S rRNA gene sequence analysis revealed that strains KMM 6166, KMM 6172^T and KMM 6173 are members of the phylum *Bacteroidetes*; their nearest neighbours were *Belliella baltica* and *Hongiella marincola* (similarities of 94·5 and 93·6 %, respectively). The DNA G+C content of the strains was 44–45 mol%. The predominant fatty acids were $C_{15:0}$ iso, $C_{16:1}\omega5c$, $C_{17:1}$ iso $\omega9c$, $C_{17:0}$ iso 3-OH and summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{15:0}$ iso 2-OH). The major respiratory quinone was MK-7. Results of molecular experiments supported by phenotypic and chemotaxonomic data enabled the isolates to be classified as representatives of a novel species in a new genus, for which the name *Echinicola pacifica* gen. nov., sp. nov. is proposed. *Echinicola pacifica* is the type species of the genus *Echinicola*, and its type strain is KMM 6172^T (=KCTC 12368^T=LMG 23350^T).

A phylogenetic survey on the culturable bacteria associated with the sea urchin *Strongylocentrotus intermedius* revealed rich species diversity. Previously undescribed bacteria belonging to the *Proteobacteria*, low- and high-G+C-content Gram-positive bacteria and *Bacteroidetes* were found. Up to now, only some of the novel species have been assigned validly published names (Nedashkovskaya *et al.*, 2005a, b, c, d).

In the present paper, the heterotrophic, Gram-negative, pink-coloured, gliding agarolytic strains KMM 6166, KMM 6172^T and KMM 6173 were selected for further study on the basis of their significant molecular divergence from described taxa. Results of genotypic, chemotaxonomic and phenotypic analyses confirmed that the strains had a distinct taxonomic position in a new genus.

Strains KMM 6166, KMM 6172^T and KMM 6173 were isolated from the sea urchin *Strongylocentrotus intermedius* collected in Troitsa Bay, Gulf of Peter the Great, East Sea (also known as the Sea of Japan), during September 2002. After primary isolation and purification on marine agar 2216 (Difco), the strains were cultivated on the same medium at 25 °C for 48 h and stored at −80 °C in marine broth (Difco) supplemented with 20 % (v/v) glycerol.

Genomic DNA extraction, PCR and 16S rRNA gene sequencing were carried out as described previously (Kim

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Echinicola pacifica* KMM 6172^T, KMM 6166 and KMM 6173 are DQ185611, DQ186987 and DQ186988, respectively.

et al., 1998). Sequence data obtained were aligned with those of representative members of the phylum *Bacteroidetes* using PHYDIT version 3.2 (http://plaza.snu.ac.kr/~jchun/phydit/). Phylogenetic trees were inferred using suitable programs of the PHYLIP package (Felsenstein, 1993). Phylogenetic distances were calculated using the Kimura two-parameter model (Kimura, 1980) and trees were constructed on the basis of the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Kluge & Farris, 1969) and maximum-likelihood (Felsenstein, 1993) algorithms. Bootstrap analysis was performed with 1000 resampled datasets using the SEQBOOT and CONSENSE programs of the PHYLIP package.

Phylogenetic analysis of the almost-complete 16S rRNA gene sequences revealed that the three sea-urchin isolates occupied a distinct lineage within the phylum *Bacteroidetes* (Fig. 1). The nearest neighbours of the strains studied were *Belliella baltica* BA134^T, *Hongiella marincola* SW-2^T and *Cyclobacterium marinum* LMG 13164^T, with similarity values of 94·5, 93·6 and 93·1 %, respectively.

DNA was isolated according to the method of Marmur (1961) and its G+C content was determined using the thermal denaturation method (Marmur & Doty, 1962). The G+C content of the DNA of the strains under study was 44·2–44·6 mol%. DNA–DNA hybridization experiments were performed using the method of De Ley *et al.* (1970). DNA–DNA hybridization values between strains KMM 6166, KMM 6172^T and KMM 6173 were 93–98 %. Therefore, the strains can be placed in the same species according to criteria defined by Wayne *et al.* (1987).

Analysis of fatty acid methyl esters was carried out according to the standard protocol of the Sherlock Microbial Identification System (Microbial ID). The predominant cellular fatty acids of strains KMM 6166 and KMM 6172^T were $C_{15:0}$ iso (17·3–18·0 %), $C_{16:1}\omega5c$ (6·7–7·8 %), $C_{17:1}$ iso $\omega9c$ (6·3–6·9 %), $C_{17:1}\omega6c$ (4·3–4·8 %), $C_{15:0}$ iso 3-OH (3·4–5·0 %), $C_{17:0}$ iso 3-OH (9·4–10·0 %) and summed feature 3 (30·7–30·8 %), comprising $C_{16:1}\omega7c$ and/or $C_{15:0}$ iso 2-OH (Table 1). Isoprenoid quinones were extracted from lyophilized cells and analysed as described previously (Nedashkovskaya *et al.*, 2004b). The main isoprenoid quinone of the novel isolates was MK-7.

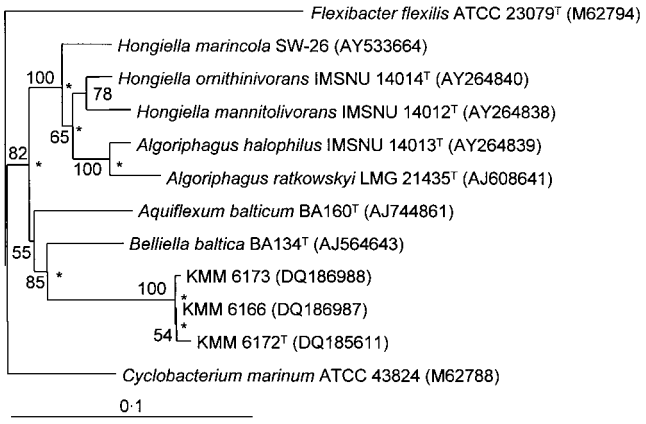

Fig. 1. Phylogenetic tree based on the 16S rRNA gene sequences of KMM strains and representative members of the family '*Flexibacteraceae*'. Asterisks indicate branches that were also recovered using maximum-likelihood and maximum-parsimony algorithms. Numbers at nodes indicate bootstrap values (%). Bar, 0·1 substitutions per nucleotide position.

The absorption spectrum of pigments extracted using 7 : 2 (v/v) acetone/methanol was determined between 300 and 700 nm with UV spectrophotometer CECIL, CE 7250, 7000 series. Cells produced pink-coloured carotenoid pigments with maximum absorption at 472·8 nm.

Physiological and biochemical properties of strains KMM 6166, KMM 6172^T and KMM 6173 were examined as described by Nedashkovskaya *et al.* (2004a, b). Physiological and biochemical properties of KMM 6172^T were also determined using the API 20E, API 20NE, API ZYM and API 50CH galleries (bioMérieux) and the Biolog GN2 Microplate system according to the manufacturers' instructions. Susceptibility to antibiotics was tested as described previously (Nedashkovskaya *et al.*, 2004a) using additional discs containing chloramphenicol (30 μg), doxycycline (10 μg) and erythromycin (15 μg). The ability to grow under anaerobic conditions was observed using the Oxoid Anaerobic System. Gliding motility was determined as described by Bowman (2000).

Strains isolated in this study were Gram-negative, chemoorganotrophic, pink-coloured and motile by gliding. The main physiological and biochemical characteristics are given in Tables 2 and 3 and the species description. The strains tested differed from their nearest neighbour, *B. baltica*, by their ability to move by gliding, to grow with 12 % NaCl and to produce hydrogen sulfide. Other distinctive features between these taxa included hydrolysis of agar and gelatin, acid production from D-fructose, D-melibiose and L-rhamnose, enzyme activities and utilization of a number of organic compounds (Table 2). The differential features of the strains studied and other related members of the phylum *Bacteroidetes* are shown in Table 3. It should be noted that the sea-urchin isolates can be clearly distinguished from all close relatives by their moving by means of gliding and their ability to ferment D-glucose. The latter characteristic of the strains, together with the absence of growth under strictly anaerobic conditions, indicates that the strains may grow in a wide range of oxygen concentrations. Phenotypic divergence between the strains studied and their relatives is supported by significant distinctiveness in the cellular fatty acid profiles (Table 2). For example, the presence of a substantial amount of $C_{15:0}$ 3-OH, the absence of $C_{17:1}$ anteiso and very low levels of $C_{15:1}$ iso G fatty acids were noted in extracts of KMM 6166 and KMM 6172^T, in contrast with their nearest neighbour, *B. baltica*. Consequently, the combination of all data obtained allows the strains to be

Table 1. Cellular fatty acid composition (%) of *Echinicola pacifica* gen. nov., sp. nov. and related taxa of the phylum *Bacteroidetes*

Taxa: 1, *Echinicola pacifica* gen. nov., sp. nov.; 2, *Algoriphagus*; 3, *Aquiflexum*; 4, *Belliella*; 5, *Cyclobacterium*; 6, *Hongiella*. Data from Brettar *et al.* (2004a, b); Nedashkovskaya *et al.* (2004b, 2005e) and this study. Values of less than 1 % for all strains are not shown. Predominant fatty acids are shown in bold. Summed features consist of one or more fatty acids that could not be separated by the Microbial Identification System. Summed feature 3 is $C_{15:0}$ iso 2-OH and/or $C_{16:1}\omega7c$; summed feature 4 is $C_{17:1}$ iso I and/or $C_{17:1}$ anteiso B.

Fatty acid	1	2	3	4	5	6
$C_{11:0}$ anteiso	–	0·6–2·1	–	–	–	0·0–0·9
$C_{13:1}$ AT	–	–	–	<1·4	–	–
$C_{14:0}$ iso	0·1–0·2	0·3–1·4	**4·8**	2·0	–	0·6–2·3
$C_{15:1}$ iso	0·0–0·6	0–2·9	**9·4**	**10·2**	**8·4–8·5**	0·2–0·4
$C_{15:0}$ iso	**17·3–18·0**	**28·4–38·9**	**22·6**	**20·7**	**22·2–25·5**	**26·5–32·7**
$C_{15:0}$ anteiso	2·1–2·8	1·6–3·6	**18·5**	**4·5**	**6·3–9·2**	**3·7–6·1**
$C_{15:0}$	0·8–1·0	1·0–2·5	–	2·9	0·0–0·8	2·7–3·0
$C_{15:1}\omega6c$	1·1–1·2	1·0–2·3	–	2·1	0·5–1·3	1·1–4·1
$C_{16:1}\omega5c$	**6·7–7·8**	**3·5–5·8**	2·0	3·3	–	0·6–1·1
$C_{16:1}$ iso	0·8–1·0	1·5–3·5	**9·5**	3·5	–	**3·5–6·4**
$C_{16:0}$ iso	1·1–1·2	2·4–7·7	**4·2**	2·6	–	**6·5–12·3**
$C_{16:0}$	0·8–0·9	0–2·9	–	–	0·5–0·7	–
$C_{15:0}$ iso 3-OH	**3·4–5·0**	1·6–2·9	1·6	2·2	3·5–3·7	1·8–2·7
$C_{15:0}$ 3-OH	2·5–2·6	–	–	–	–	–
$C_{17:1}$ anteiso	–	–	2·6	3·7	–	–
$C_{17:1}$ iso $\omega9c$	**6·3–6·9**	1·5–9·0	**5·2**	**8·4**	**4·3–5·6**	**6·0–12·2**
$C_{17:1}$ anteiso $\omega9c$	–	–	1·1	–	–	0·0–1·0
$C_{17:1}\omega8c$	–	–	–	1·2	–	–
$C_{17:1}\omega6c$	**4·3–4·8**	0·5–3·4	3·0	**7·3**	1·3–1·4	**4·5–7·0**
$C_{16:0}$ iso 3-OH	0·6–0·7	0·9–3·1	2·0	1·9	0·0–1·0	3·4–4·7
$C_{16:0}$ 3-OH	0·9–1·4	0·5–1·9	–	<1·1	1·3–1·7	–
$C_{17:0}$ iso 3-OH	**9·4–10·0**	**5·9–9·2**	1·4	3·2	**10·3–10·7**	**6·4–6·7**
$C_{17:0}$ 2-OH	0·4	0·0–0·6	–	–	1·5–2·9	0·4–1·8
$C_{18:1}\omega5c$	0·2	–	–	–	1·2–1·4	–
$C_{19:1}$ iso	–	–	1·5	–	–	–
Summed feature 3	**30·7–30·8**	**19·0–24·6**	**6·1**	**9·2**	**24·3–25·1**	**6·0–7·4**
Summed feature 4	–	0·9–2·7	–	–	2·5–4·4	2·0–2·5

discriminated from their close relatives. Low sequence similarities of the strains tested with other members of the family '*Flexibacteraceae*' described to date (81·1–92·5 %) demonstrate clearly that the strains isolated in this study represent a novel genus.

Thus, the polyphasic data presented in this study support the conclusion that strains KMM 6166, KMM 6172^T and KMM 6173 could not be affiliated to any taxa currently included in the phylum *Bacteroidetes*. Based on this fact, it is proposed that these strains should be placed in a novel genus as *Echinicola pacifica* gen. nov., sp. nov.

Description of *Echinicola* gen. nov.

Echinicola (E.chi.ni.co′la. L. masc. n. *echinus* -*i* sea urchin; L. suff. -*cola* derived from L. masc. or fem. n. *incola* a dweller; N.L. fem. n. *Echinicola* a sea-urchin dweller).

Rod-shaped cells, motile by gliding. Gram-negative. Do not form endospores. Can ferment D-glucose. Produce non-diffusible carotenoid pigments. Chemo-organotrophs. Positive for cytochrome oxidase, catalase and alkaline phosphatase. The major respiratory quinone is MK-7. The main cellular fatty acids are straight-chain unsaturated and branched-chain unsaturated fatty acids $C_{15:0}$ iso, $C_{16:1}\omega5c$, $C_{17:1}$ iso $\omega9c$, $C_{17:1}\omega6c$, $C_{15:0}$ iso 3-OH, $C_{17:0}$ iso 3-OH and summed feature 3, comprising $C_{15:0}$ iso 2-OH and/or $C_{16:1}\omega7c$. As determined by 16S rRNA gene sequence analysis, the genus *Echinicola* is a member of the phylum *Bacteroidetes*. The type species is *Echinicola pacifica*.

Description of *Echinicola pacifica* sp. nov.

Echinicola pacifica (pa.ci′fi.ca. N.L. fem. adj. *pacifica* referring to the Pacific Ocean, from which the type strain was isolated).

Main characteristics are those given for the genus. In addition, cells are 0·3–0·4 × 1·2–1·9 μm. On marine agar, colonies are circular, 2–3 mm in diameter, convex, shiny, smooth, pink-coloured and sunken into agar.

Table 2. Phenotypic characteristics that differentiate between *Echinicola pacifica* gen. nov., sp. nov. and *Belliella baltica* BA134^T

Taxa: 1, *Echinicola pacifica* ($n=3$); 2, *Belliella baltica* BA134^T. Data from Brettar *et al.* (2004a) and this study.

Characteristic	1	2
Gliding motility	+	−
Nitrate reduction	−	+
H_2S production	+	−
Growth with 12 % NaCl	+	−
Esterase (C4), *N*-acetylglucosaminidase, α-galactosidase, α-mannosidase, α-fucosidase	+	−
Hydrolysis of agar	+	−
Acid production from:		
L-Rhamnose, D-fructose	+	−
D-Melibiose	−	+
Utilization of:		
Dextrin, α-cyclodextrin, glycogen, *N*-acetyl-D-glucosamine, D-fructose, L-fucose, α-lactose, D-mannose, D-melibiose, methyl β-D-glucoside, psicose, D-raffinose, sucrose, turanose, D-galacturonic acid, D-glucuronic acid, alaninamide, L-alanyl glycine, L-aspartic acid, L-rhamnose, L-alanine, L-asparagine, hydroxy-L-proline, L-threonine	+	−
Acetic acid, α-ketoglutaric acid, α-ketovaleric acid	−	+
DNA G+C content (mol%)	44–45	35·4

β-Galactosidase-positive. Does not require Na^+ ions or sea water for growth. Growth occurs at 6–41 °C. Optimal temperature for growth is 25–28 °C. Growth occurs with 0–12 % NaCl. No flexirubin-type pigments are formed. Degrades agar, gelatin (weakly), aesculin, Tween 40 and starch. Can decompose Tween 20 and 80. Does not hydrolyse casein, DNA, cellulose (carboxymethyl-cellulose or filter paper) or chitin. Produces acid from L-arabinose, D-cellobiose, D-glucose, D-lactose, D-maltose, D-mannose, L-rhamnose, DL-xylose and *N*-acetylglucosamine. Can oxidize D-galactose and D-sucrose. Does not form acid from fucose, melibiose, raffinose, sorbose, glycerol, adonitol, dulcitol, inositol or mannitol. Can ferment D-glucose. According to the API 20E gallery (bioMérieux), the type strain (KMM 6172^T) utilizes citrate, forms acid from amygdalin and is negative for arginine dihydrolase, lysine

Table 3. Phenotypic characteristics that differentiate between *Echinicola pacifica* gen. nov., sp. nov. and its close relatives in the family '*Flexibacteraceae*'

Taxa: 1, *Echinicola pacifica* gen. nov., sp. nov.; 2, *Algoriphagus*; 3, *Aquiflexum*; 4, *Belliella*; 5, *Cyclobacterium*; 6, *Hongiella*. Data from Bowman *et al.* (2003), Brettar *et al.* (2004a, b), Nedashkovskaya *et al.* (2004b, 2005e), Van Trappen *et al.* (2004), Yi & Chun (2004), Yoon *et al.* (2004, 2005) and this study. Abbreviations: V, variable; ND, Not detected.

Characteristic	1	2	3	4	5	6
Cell morphology	Regular rods	Regular rods	Regular rods	Regular rods	Ring-like/ horseshoe-shaped	Regular rods
Cell size (μm)	0·4–0·6 × 0·8–2·3	0·3–0·7 × 1·0–10·0	0·3–0·6 × 1·1–4·8	0·3–0·5 × 0·9–3·0	0·3–0·7 × 0·8–1·5	0·3–0·6 × 0·8–3·0
Gliding motility	+	−	−	−	−	−
Nitrate reduction	−	V	+	+	−	V
Fermentation of D-glucose	+	−	−	−	−	−
Hydrogen sulfide	+	−	ND	ND	−	−
Salinity range (%)	0–12	0–10	0–6	0–6	0–10	0–8
Growth at 42 °C	−	V	−	−	V	+
Hydrolysis of:						
Agar	+	V	−	−	−	−
Gelatin	+	V	+	−	−	V
Starch	+	V	+	+	−	V
DNA G+C content (mol%)	44–45	35–42	38·4	35·4	41–42	38–43

decarboxylase and ornithine decarboxylase. Results of Biolog GN2 (Biolog) testing show that strain KMM 6172^T utilizes α-cyclodextrin, dextrin, glycogen, α-D-glucose, D-fructose, L-fucose, D-galactose, gentibiose, α-lactose, α-D-lactose, lactulose, D-mannose, D-melibiose, methyl β-D-glucoside, psicose, D-raffinose, sucrose, D-trehalose, turanose, D-galacturonic acid, D-glucuronic acid, α-ketobutyric acid, alaninamide, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, hydroxy-L-proline and L-threonine. Does not utilize Tween 80, *N*-acetyl-D-galactosamine, adonitol, L-arabitol, *i*-erythritol, *myo*-inositol, D-mannitol, D-sorbitol, xylitol, methyl pyruvate, monomethyl succinate, acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-galactonic acid, D-gluconic acid, D-glucosaminic acid, α-, β- and γ-hydroxybutyric acids, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketoglutaric acid, α-ketovaleric acid, DL-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, D-alanine, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, L-serine, DL-carnitine, γ-aminobutyric acid, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2, 3-butanediol, glycerol, DL-α-glycerol phosphate, glucose 1-phosphate and glucose 6-phosphate. Nitrate is not reduced to nitrite. Hydrogen sulfide is produced. Indole and acetoin (Voges–Proskauer reaction) production are negative. According to the API ZYM gallery (bioMérieux), produces α- and β-galactosidases, alkaline and acid phosphatases, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, α- and β-glucosidases, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase, but not lipase (C14) or β-glucuronidase. Susceptible to lincomycin. Resistant to ampicillin, benzylpenicillin, chloramphenicol, doxycycline, erythromycin, gentamicin, kanamycin, carbenicillin, oleandomycin, neomycin, polymixin B, streptomycin and tetracycline. Predominant fatty acids are $C_{15:0}$ iso (17·3–18·0 %), $C_{16:1}\omega5c$ (6·7–7·8 %), $C_{17:1}$ iso $\omega9c$ (6·3–6·9 %), $C_{17:1}\omega6c$ (4·3–4·8 %), $C_{15:0}$ iso 3-OH (3·4–5·0 %), $C_{17:0}$ iso 3-OH (9·4–10·0 %) and summed feature 3 (30·7–30·8 %), comprising $C_{16:1}\omega7c$ and/or $C_{15:0}$ iso 2-OH (Table 1). The G+C content of the DNA is 44–45 mol%.

The type strain is KMM 6172^T (=KCTC 12368^T=LMG 23350^T), isolated from the sea urchin *Strongylocentrotus intermedius* collected in Troitsa Bay, Gulf of Peter the Great, the East Sea (also known as the Sea of Japan).

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