Flavivirga eckloniae sp. nov. and Flavivirga aquimarina sp. nov., isolated from seaweed Ecklonia cava

Ji Hee Lee,¹ Joo Won Kang,¹ Han Na Choe² and Chi Nam Seong¹,*

Abstract

Two Gram-stain-negative, non-spore-forming, rod-shaped, aerobic and yellow-coloured bacterial strains, designated strains ECD14T and EC2D5T, were isolated from a seaweed Ecklonia cava. The isolates required sea salts for growth. Flexirubin-type and carotenoid pigment was produced. The 16S rRNA gene sequence similarity between the two new strains was 95.5 %. Flavivirga amylovorans JC268T was the nearest neighbour of strains ECD14T and EC2D5T with 96.5 and 96.8 % 16S rRNA gene sequence similarity, respectively. The common major fatty acids and respiratory quinones were iso-C15:0, iso-C15:0 3-OH and unknown 13.565 and menaquinone 6 (MK-6), respectively. The common major polar lipids were phosphatidylethanolamine, an unknown amino lipid and an unknown lipid. The DNA G+C contents of strains ECD14T and EC2D5T were 33 and 31 mol%, respectively. On the basis of the polyphasic characterization of the two strains, it is suggested that the two isolates represent novel species of the genus Flavivirga, for which the names Flavivirga eckloniae sp. nov. (type strain, ECD14T=KCTC 52352T=JCM 31797T) and Flavivirga aquimarina sp. nov. (type strain, EC2D5T=KCTC 52353T=JCM 31796T) are proposed.

The genus Flavivirga was first proposed by Yi et al. [1], as a member of the family Flavobacteriaceae, class Flavobacteria, phylum Bacteroidetes and encompasses only two species with validly published names. The two species, Flavivirga jejuensis (type species of the genus) and F. amylovorans, were isolated from seawater. Members of the genus Flavivirga are Gram-stain-negative, aerobic, non-spore-forming and rod-shaped with DNA G+C contents of 27–33 mol% and require sea salts for growth. The major respiratory quinone of Flavivirga species is menaquinone 6 (MK-6). The major polar lipids are phosphatidylethanolamine and a varying number of unknown amino lipids, phospholipids and unknown lipids [1]. In the course of our study, two yellow-pigmented and rod-shaped bacterial strains, designated ECD14T and EC2D5T, were isolated and subjected to taxonomic investigation.

Strains ECD14T and EC2D5T were isolated from seaweed Ecklonia cava obtained from the South Sea (GPS position: 34° 02' 58" N, 127° 20' 00" E), Republic of Korea, by using the standard dilution plating technique. Isolation was achieved on a marine agar 2216 plate (MA, Becton Dickinson) at 25 °C for 7 days. The isolates were routinely cultured on MA and stored in marine broth (MB, Becton Dickinson) supplemented with glycerol (20 %, w/v) at −80 °C. Reference strains F. jejuensis KACC 14158T and F. amylovorans KACC 14157T were purchased from the Korean Agricultural Culture Collection (KACC).

Bacterial DNA preparation, PCR amplification and sequencing of the 16S rRNA gene were carried out as described previously [2]. Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarities were achieved by the EzBioCloud server (www.ezbiocloud.net/ [3]) and the BLAST search program on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments were performed by using the CLUSTAL_W program [4] integrated in BioEdit version 7.2.5 software [5]. Phylogenetic analysis was performed by using the software package MEGA version 6 [6]. Phylogenetic trees were inferred using the maximum-likelihood [7], neighbour-joining [8] and maximum-parsimony [9] algorithms. Evolutionary distances of the maximum-likelihood and neighbour-joining methods were calculated using the Kimura two-parameter model [10] and following Jukes and Cantor [11], respectively. The robustness of the topology in the maximum-likelihood phylogenetic tree was evaluated by bootstrap analyses [12] based on 1000 resamplings.

The 16S rRNA gene sequences of strains ECD14T and EC2D5T were continuous stretches of 1425 and 1434 nt,
respectively. Preliminary sequence comparison with 16S rRNA gene sequences held in GenBank indicated that our isolates were closely related to members of the genus *Flavivirga*. The level of 16S rRNA gene sequence similarity between the two isolates was 95.5%. The closest relative of strain ECD14<sup>T</sup> was *F. amylovorans* JC2681<sup>T</sup> (96.5% 16S rRNA gene sequence similarity) followed by *Arenitalea lutea* P7-3-5<sup>T</sup> (95.0%) and *Algibacter aestuarii* KYW371<sup>T</sup> (95.0%). The most closely related species of strain EC2D5<sup>T</sup> was *F. amylovorans* JC2681<sup>T</sup> (96.8%) followed by *F. jejuensis* JC2682<sup>T</sup> (96.7%) and *Ar. lutea* P7-3-5<sup>T</sup> (96.5%). This relationship between our isolates and other members of the genus *Flavivirga* is also evident in the phylogenetic tree. Both the maximum-likelihood tree and the maximum-parsimony tree (Fig. 1) showed that strain ECD14<sup>T</sup> and strain EC2D5<sup>T</sup> formed distinct and separate branches with the clade comprising *F. amylovorans*. Although strain EC2D5<sup>T</sup> joined first with *F. jejuensis* in the neighbour-joining tree (Fig. S1, available in the online Supplementary Material), it is clear from 16S rRNA gene sequence similarity data and phylogenetic analysis that strain ECD14<sup>T</sup> and strain EC2D5<sup>T</sup> represent distinct and separate novel species in the genus *Flavivirga*.

Growth on various standard bacteriological media was tested by using MA, MacConkey agar (Becton Dickinson), nutrient

![Fig. 1. A maximum-likelihood tree based on 16S rRNA gene sequences showing the phylogenetic position of strains ECD14<sup>T</sup> and EC2D5<sup>T</sup>. Evolutionary distances, generated using the Kimura two-parameter model model, are based on 1291 unambiguously aligned nucleotides. Bootstrap values greater than 50% (1000 resamplings) for nodes conserved among maximum-likelihood analyses are shown. *Myroides odoratus* ATCC 4651<sup>T</sup> (M58777) was used as an outgroup. Closed circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-parsimony algorithm. Bar, 0.02 substitutions per nucleotide position.](https://www.microbiologyresearch.org/ijsem/2017/67/3089-3094/fig1.png)
agar (NA, Becton Dickinson), R2A agar (Becton Dickinson),
tryptic soy agar (TSA; Becton Dickinson) and ZoBell’s agar
[13] (5 g Bacto peptone, 1 g yeast extract, 0.1 g ferric citrate
and 15 g Bacto agar in 11 distilled water). Growth tempera-
ture [4, 7, 37 and 10–50 °C (at 5 °C intervals)] was tested on
MA. The pH range for growth was determined in MB
adjusted to pH 4–11 (at 1 pH intervals) by using 100 mM
acetate buffer (pH 4–5), 100 mM NaH₂PO₄/Na₂HPO₄ buffer
(pH 6–8) and 100 mM NaHCO₃/Na₂CO₃ buffer (pH 9–11)
[14]. Requirement and tolerance of sea salts (Sigma) or NaCl
(final concentration: 0–10 %, using increments of 1 %) for
growth was tested on ZoBell’s agar. Anaerobic growth was
tested on MA in a jar containing AnaeroPack-Anaero (Mit-
subishi Gas Chemical), which works as oxygen absorber and
CO₂ generator, for up to 14 days at 25 °C. Cells of strains
ECD14ᵀ and EC2D5ᵀ grown on MA at 25 °C for 3 days were
used for the physiological and biochemical tests. Cell mor-
phology was observed by transmission electron (CM-20, Phi-
lops) microscopy. Gliding motility was assessed by
examining wet mounts with a phase-contrast (IC50, Leica)
microscopy of a 48 h MB culture [15]. The Gram reaction
was determined by using the Gram staining method and the
KOH method [16]. Catalase and oxidase activities were
determined using 3 % (v/v) hydrogen peroxide and Kovacs’
reagent [17], respectively. Indole production was determined
with Kovacs’ indole reagent on SIM agar (Becton Dickinson)
supplemented with 3 % sea salts. H₂S production was deter-
mined on Kligler iron agar (Becton Dickinson) supple-
mented with 3 % sea salts according to Smibert and Krieg
[18]. The production of flexirubin-type pigments was inves-
tigated using the KOH test following the minimal standards
for the description of new taxa in the family Flavobacteria-
cae [15]. Carotenoid pigments were extracted with acetone and
the absorption spectrum (300–900 nm) was recorded
using a UV/VIS spectrophotometer (Ultrispec 2100pro, Bio-
chrom) [19]. Acid production from carbohydrates was tested
using phenol red broth base (Becton Dickinson) supple-
mented with 3 % sea salts. Hydrolysis of alginic acid (0.5 %,
w/v), carboxymethyl cellulose (CMC; 0.5 %, w/v), chitin
(0.5 %, w/v), egg yolk (5 %, w/v), starch (0.2 %, w/v), Tween
20 (1 %, w/v) and Tween 80 (1 %, w/v) was tested using MA
according to Smibert and Krieg [18]. Decomposition of
hypoxanthine (0.5 %, w/v), L-tyrosine (0.5 %, w/v) and xan-
thine (0.4 %, w/v) was tested using MA according to Gordon
et al. [20]. DNase activity was determined using DNase test
agar (Becton Dickinson) supplemented with 3 % sea salts in
distilled water. Other biochemical tests and enzyme activities
were performed using the API 20E, API 20NE and API ZYM
kits (bioMérieux). Antibiotic sensitivity was determined with
the disc diffusion method using commercial antibiotic-
impregnated discs (BBL Becton Dickson). After 5 days of
incubation at 25 °C on MA, the results were interpreted
according to the guidelines set by the Clinical and Laboratory
Standards Institute [21].

The isolates were rod-shaped (see Fig. S2) with yellow-
pigmented colonies. Flexirubin-type and carotenoid pig-
ments were present. Both two isolates could not grow on sea
salt-free ZoBell’s medium supplemented with NaCl and
required sea salts for growth. Strain ECD14ᵀ was sensitive to
(μg per disc unless otherwise stated) ampicillin (10) and
naldixic acid (30), but resistant to amikacin (30), chloram-
phenicol (30), erythromycin (15), gentamicin (10), kanamyc-
in (30), penicillin (10 IU), polymyxin B (300 IU), streptomycin
(10), tetracycline (30) and vancomycin (30). Strain EC2D5ᵀ
was sensitive to ampicillin, erythromycin and vancomycin,
but resistant to amikacin, chlorampheni-

col, gentamicin, kanamycin, naldixic acid, penicillin, poly-
myxin B, streptomycin and tetracycline. The detailed results
of physiological and biochemical analyses are given in
Table 1 and the species description. It is evident from
Table 1 that there are several phenotypic characters that
readily differentiate between strains ECD14ᵀ and EC2D5ᵀ
and the isolates from phylogenetically related species F.
amylovarans and F. jejuensis: absence or negative for motil-
ity, nitrate reduction, catalase activity and acid production
from rhamnose of strain ECD14ᵀ and positive for DNA
hydrolisis and negative for acid production from fructose
of strain EC2D5ᵀ.

For cellular fatty acid analysis, strains ECD14ᵀ and EC2D5ᵀ
and two reference strains were grown on MA for 3 days at
25 °C. Extraction of fatty acid methyl esters (FAMEs) and
separation by gas chromatography (GC) were performed by
using the Instant FAME method of the Microbial Identifica-
tion System (MIDI) version 6.1 and the TSBA6 database.
For polar lipid and isoprenoid quinone analyses, cells of strains
ECD14ᵀ and EC2D5ᵀ grown in MB for 3 days at 25 °C were
harvested and freeze-dried. Polar lipids were analysed by
using standard procedures [22]. Extracted lipids were sepa-
rated by two-dimensional thin-layer chromatography
(TLC) and identified by spraying with appropriate detection
reagents [23, 24]. Isoprenoid quinones were extracted and
purified according to the method of Minnikin et al. [22]
and analysed by TLC as described by Collins [25].

The cellular fatty acid profiles of strains ECD14ᵀ and
EC2D5ᵀ are described in Table 2. The common major fatty
acids (>10.0 % of total fatty acids) of the two isolates were
iso-C₁₅:₀, iso-C₁₅:₁ and unknown 13:65. This fatty acid
profile was similar to that of genus Flavivirga. However,
the proportions of iso-C₁₀:₀ 3-ΟH and summed feature 3
(C₁₆:₁ω７c/C₁₆:₁ω₆c) of strain ECD14ᵀ was higher than the
related strains. Strain EC2D5ᵀ contained more iso-C₁₅:₁ G,
but did not contain iso-C₁₅:₀ 3-ΟH. The common major
polar lipids of strains ECD14ᵀ and EC2D5ᵀ were phosphat-
dylethanolamine, an unknown amino lipid and an unknown
lipid. Another unknown lipid was detected as a major polar
lipid in strain EC2D5ᵀ. And an unknown phospholipid and
several unknown lipids were also detected in the two strains
(see Fig. S3). The polar lipid patterns of ECD14ᵀ and
EC2D5ᵀ were similar to the genus Flavivirga of which the
major polar lipids are phosphatidylethanolamine and varying number of unknown amino lipids, phospholipids and unknown lipids [1]. The predominant respiratory quinone detected was menaquinone-6 (MK-6), which is the characteristic respiratory quinone of the genus *Flavivirga* [1]. The DNA G+C contents of strains ECD14<sup>T</sup> and EC2D5<sup>T</sup> were 33 and 31 mol%, respectively, which are in the range of the genus *Flavivirga* [1].

Therefore, on the basis of the data presented here, strains ECD14<sup>T</sup> and EC2D5<sup>T</sup> represent novel species within the genus *Flavivirga*, for which the names *Flavivirga eckloniae* sp. nov. and *Flavivirga aquimarina* sp. nov. are proposed, respectively.

## DESCRIPTION OF FLAVIVIRGA ECKLONIAE SP. NOV.

*Flavivirga eckloniae* (eck.lō'-ni.ē). N.L. gen. n. *eckloniae* of *Ecklonia*, referring to the isolation of the type strain from *Ecklonia cava*).

Cells are Gram-stain-negative, non-motile, non-spore-forming, aerobic and rod-shaped (0.2–0.4×2.0–6.7 μm in size). Colonies are irregular, flat, smooth, yellow-pigmented and approximately 2–3 mm in diameter after 3 days at...
25 °C. Growth occurs on MA at pH 7–9 (optimally at pH 8) and at 10–35 °C (optimally at 25–30 °C). Requires a sea salts concentration of 2–5% (w/v) (optimum, 3%) for growth and growth does not occur on sea salt-free ZoBell’s medium supplemented with NaCl only. Catalase-negative and oxidase-positive. Flexirubin-type and carotenoid pigments are present. Nitrate is not reduced to nitrite. Indole and H₂S are not produced. Aesculin, agar, alginic acid, CMC, gelatin, starch, Tween 80 and L-tyrosine are hydrolyzed, but chitin, DNA, egg yolk, hypoxanthine, Tween 20, urea and xanthine are not. Positive for acetoac production, but negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase activities (API 20E).

Negative for assimilation of adipate, arabinose, caprate, citrate, gluconate, glucose, malate, maltose, mannitol, mannosé, N-acetyl-glucosamine and phenyl-acetate (API 20NE).

In the API ZYM gallery, acid phosphatase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase and naphthol-AS-BI-phosphohydrolase are present, but the other enzyme activities are absent. The major fatty acids are iso-C₁₅:₀, iso-C₁₅:₁ G and unknown 13:565. The major isoprenoid quinone is MK-6. The major polar lipids are phosphatidylethanolamine, one unknown amino lipid and one unknown lipid; smaller amounts of one unknown amino lipid, three unknown lipids and one unknown phospholipid are also detected.

The type strain is ECD14⁰T (=KCTC 52352T=JCM 31797T), isolated from seaweed Ecklonia cava obtained from the South Sea, Republic of Korea. The genomic DNA G+C content is 33 mol%.

**DESCRIPTION OF FLAVIVIRGA AQUIMARINA SP. NOV.**


Cells are Gram-stain-negative, non-motile, non-spore-forming, aerobic and rod-shaped (0.2–0.4×3.0–7.0 μm in size). Colonies are circular, convex, smooth, yellow-pigmented and approximately 1 mm in diameter after 3 days at 25 °C. Growth occurs on MA, at pH 7–9 (optimally at pH 8) and at 10–35 °C (optimally at 25–30 °C). Requires sea salts concentration of 2–5% (w/v) (optimum, 3%) for growth and growth does not occur on sea salts-free ZoBell’s medium supplemented with NaCl only. Catalase- and oxidase-positive. Flexirubin-type and carotenoid pigments are present. Nitrate is reduced. Indole and H₂S are not produced. Aesculin, DNA and L-tyrosine are hydrolyzed, but agar, alginic acid, CMC, chitin, egg yolk, gelatin, hypoxanthine, starch, Tween 20, Tween 80, urea and xanthine are not. Positive for acetoac production, but negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase activities (API 20E).

Negative for assimilation of adipate, arabinose, caprate, citrate, gluconate, glucose, malate, maltose, mannitol, mannosé, N-acetyl-glucosamine and phenyl-acetate (API 20NE).

In the API ZYM gallery, acid phosphatase, alkaline phosphatase, esterase (C4), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and valine arylamidase are present, but the other enzyme activities are absent. The major fatty acids are iso-C₁₅:₀, iso-C₁₅:₁ G and unknown 13:565. The major isoprenoid quinone is MK-6. The major polar lipids are phosphatidylethanolamine, one unknown amino lipid and two unknown lipids; smaller amounts of three unknown lipids and one unknown phospholipid are also detected.

The type strain is EC2D5⁰T (=KCTC 52353T=JCM 31796T), isolated from seaweed *Ecklonia cava* obtained from the South Sea, Republic of Korea. The genomic DNA G+C content is 31 mol%.

**Funding information**

This research was supported by the project on survey and excavation of Korean indigenous species of the National Institute of Biological Resources (NIBR) under the Ministry of Environment, Republic of Korea.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


