Fibrivirga Algicola Gen. Nov., Sp. Nov., An Algicidal Bacterium Isolated from a Freshwater River

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Abstract

An aerobic, gram-negative, pink-colored, non-motile, rod-shaped algicidal bacterium, designated JA-25^T was isolated from the freshwater of the Geumgang River, Republic of Korea. It grew at 15–30°C, 6.0–9.0 pH, and in the presence of 0–1% (w/v) NaCl. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain JA-25^T belongs to the Family ‘*Spirosomaceae*’ and is most closely related to *Fibrella aestuarina* BUZ 2^T (93.6%). The strain JA-25^T showed < 90% sequence similarity to other members of the Family ‘*Spirosomaceae*’. The average nucleotide identity(ANI), *in silico* DNA-DNA hybridization and the average amino acid identity(AAI) values based on the genomic sequences of JA-25^T and *F. aestuarina* BUZ 2^T were 74.4, 20.5 and 73.6 %, respectively. The genomic DNA G + C content was 52.5mol %. The major cellular fatty acids were Summed feature 3 (C16:1 ω6c/C16:1 ω7c), C16:1 ω5c, C16:0 (> 10%). The genomic DNA G + C content was 52.5 mol %. The major respiratory quinone was MK-7 and the polar lipids were phosphatidylethanolamine, two unidentified aminolipids, two phospholipids and five unidentified lipids. Considering the phylogenetic inference, phenotypic and chemotaxonomic data, strain JA-25^T should be classified as a novel species of the novel genus *Fibrivirga*, with the proposed name *Fibrivirga algicola* sp. nov. The type strain is JA-25^T (= KCCM 43334^T = NBRC 114259^T).

Introduction

The family ‘*Spirosomaceae*’ is a member of the order *Cytophagales* within the phylum *Bacteroidetes* (García-López et al., 2019). Currently, this family comprises 25 genera including the genus *Fibrella* in the List of Prokaryotic names with Standing in Nomenclature (<https://lpsn.dsmz.de/family/spirosomaceae>). Cells in this family are gram-negative, aerobic, or facultative anaerobic, and non-spore forming, with variable motility. Rods have various degrees of curvature, sometimes resulting in rings, coils, and undulating filaments. Colonies contain a pink or yellow, non-water-soluble pigment. The major menaquinone is MK-7 and the major polar lipid is phosphatidylethanolamine. The G + C content calculated from genome sequences is approximately 35.1–56.4%. The genus *Fibrella* was proposed by Filippini et al. (Filippini et al., 2011), and its antagonistic interactions against the cyanobacterium *Nostoc muscorum* have been reported (Svercel et al., 2011). In the present study, the novel algicidal strain JA-25^T, which was isolated from freshwater, was described based on genotypic, chemotaxonomic, and phenotypic approaches, as a representative of a novel species of a new genus *Fibrivirga*.

Materials And Methods

Isolation and culture conditions

Freshwater was collected from the Geumgang River in South Korea (35° 50' 38.1" N, 127° 24' 51.8" E). To obtain the novel species, the freshwater was diluted in 50 mM phosphate buffer and spread on Reasoner's 2A agar (R2A; MBcell, Seoul, Korea) plates. The plates were incubated at 25°C for 2 weeks, after which the colonies were streaked on R2A agar at least three times to obtain pure colonies. The

identity of the colonies was determined using 16S rRNA sequencing. Based on the 16S rRNA sequencing results, one colony designated as JA-25^T was selected for polyphasic characterization. Strain JA-25^T was routinely cultured on R2A agar at 25°C for 3 days for phenotypic, physiological, and chemotaxonomic tests, and maintained in glycerol suspensions (20%, v/v) at -80°C. For comparative analyses, *F. aestuarina* DSM 22563^T was purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany) and cultured under the same conditions as strain JA-25^T.

Harmful algae and algal culture

The strain of harmful algae used in this study was *Heterocapsa triquetra* (LIMS-PS-1783) and obtained from the Library of Marine Samples Korea (Korean Institute of Ocean Science and Technology, Busan, Korea). The algal strain was cultured in f/2-Si medium (Sigma-Aldrich, St. Louis, MO, USA), at 20°C with a light intensity of 40 µmol/m²/s, with a 14:10 h light:dark cycle.

16S rRNA gene phylogenetic analyses

Chromosomal DNA of strain JA-25^T was extracted using a DNA extraction kit (iNtRON Biotechnology, Gyeonggi-do, South Korea). The 16S rRNA gene was amplified using the AccuPower PCR PreMix (Bioneer, Daejeon, Korea) and the bacteria-specific primer sets 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-AAGGAGGTGATCCAGCCGC-3'). 16S rRNA gene sequencing was performed as previously described (Roh et al., 2008). The almost full-length 16S rRNA gene sequence (1,451 bp) was assembled and curated using SeqMan Pro 14 (DNASTAR, Madison, WI, USA). Phylogenetic neighbors were identified and pairwise sequence similarities were calculated using EzBioCloud (Yoon et al., 2017). Phylogenetic relationships between closely related species were determined using the MEGA6 program (Tamura et al., 2013). Phylogenetic trees were constructed using the neighbor-joining (Saitou and Nei, 1987), maximum-parsimony (Fitch, 1971), and maximum-likelihood methods (Felsenstein, 1981), and evolutionary distances were calculated using Kimura's two-parameter model. To evaluate the stability of the phylogenetic trees a bootstrap analysis was performed by obtaining a consensus tree based on 1,000 randomly generated trees.

Whole genome sequencing analyses

The genomic DNA of strain JA-25^T was extracted and purified using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The purified genomic DNA was quantified using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Inc, Waltham, MA, USA). A genome library was prepared using the Nexera DNA Flex Library Prep Kit (Illumina, San Diego, CA, USA). The average library size was 550 bp and a draft genome sequencing was performed using a Novaseq 6000 platform (Illumina) with 101 bp paired-end reads according to the manufacturer's protocols. Low-quality reads were trimmed using a quality threshold of Q20. The raw data were assembled using Unicycler 0.4.7 (Wick et al., 2017). The average nucleotide identity was calculated using JSpecies (Richter and Rosselló-Móra, 2009), and the distance (1-similarity) was visualized by plot of R environment ver. 4.0.4 (<https://www.r-project.org/>). The average amino acid identity (AAI) values were calculated with a 20% identity cut-off by using the AAI Calculator (<http://enve->

omics.ce.gatech.edu/aai/index). The digital DNA–DNA hybridization (dDDH) was calculated using Genome-to-Genome Distance Calculator 2.1 (<https://ggdc.dsmz.de/ggdc.php>) using formula 2. The genome BLAST distance phylogeny (GBDP) tree was inferred from the draft genome of strain JA-25^T (<https://ggdc.dsmz.de/ggdc.php>). The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and draft genome sequences of strain JA-25^T are MN559427 and WAEL01000000, respectively.

Phenotypic and chemotaxonomic characteristics

To investigate its morphological and physiological properties, strain JA-25^T was routinely cultivated on R2A agar at 25°C. Gram staining was performed using the non-staining method described by Buck (Buck, 1982), using a Gram staining kit (bioMérieux, Marcy-l'Etoile, France). Growth under anaerobic conditions was determined after incubation for 1 week in a GasPak (GasPak EZ Anaerobe Pouch System; BD, Franklin Lakes, NJ, USA) on R2A. Cell morphology was observed using a transmission electron microscope (H-7650; Hitachi, Tokyo, Japan). Gliding motility was investigated as described by Bowman *et al.* (Bernardet *et al.*) using a modified R2A medium. Growth assessment under various conditions, including temperature (10, 15, 20, 37, 40, and 45°C) and pH conditions (pH 5, 6, 7, 8, 9, 10, and 11) was performed on R2A agar. The pH values were adjusted by adding the following buffers as needed: 10 mM MES for pH 5.0 and 6.0; 10 mM bis-Tris propane for pH 7.0, 8.0, and 9.0; and 10 mM CAPS for pH 10.0 and 11.0. NaCl tolerance (0, 1, 2, 3, and 5%, w/v) was tested on R2A agar. Production of flexirubin-type pigments was determined according to the reversible color shift to red, purple, or brown when yellow or orange colonies were covered with aqueous 20% KOH solution (Siddiqi *et al.*, 2016). Hydrolysis of CM-cellulose and starch was tested by adding 0.5% of the respective polysaccharides to R2A agar medium. API ZYM, API 20NE and 50CH strips (bioMérieux) were used to determine the physiological and biochemical characteristics. API 20NE and 50CH results were recorded after 3 days, and those of API ZYM were recorded after 1 day at 25°C. Catalase and oxidase activities were measured using a 3% (v/v) hydrogen peroxide solution and 1% (w/v) tetramethyl-p-phenylenediamine (bioMérieux), respectively. The quinone of strain JA-25^T grown on R2A medium was analyzed by high-performance liquid chromatography (Waters, Milford, MA, USA) as described by Hirashi *et al.* (HIRAISHI *et al.*, 1996). Cellular fatty acid composition was analyzed according to the instructions provided in the Sherlock Microbial Identification System (Miller, 1982), using strain JA-25^T and *F. aestuarina* DSM 22563^T cultivated under the same conditions on R2A agar at 25°C for 3 days. Fatty acids were analyzed by gas chromatography (Hewlett Packard 6890; Agilent Technologies, Santa Clara, CA, USA) and identified using the Microbial Identification software package (Sasser, 1990) based on the TSBA6 database. Polar lipids of strain JA-25^T were extracted and separated by two-dimensional thin-layer chromatography on a silica gel glass plate (Merck, Darmstadt, Germany) as described by Xin *et al.* (Xin *et al.*, 2000). The separated polar lipid spots were detected by spraying the plate with 5 % ethanolic molybdophosphoric acid (for total polar lipids) and ninhydrin (aminolipids) according to the method described by Minnikin *et al.* (Minnikin *et al.*, 1984). Phospholipids were detected by spraying the plate with the Zinzadze reagent (Minnikin *et al.*, 1984).

Algicidal activity assay

The algicidal activity assay was based on a previous work by Cho et al (Cho and Kim, 2018) with slight modification. Strain JA-25^T was cultured in R2A broth medium for 3 days, and then bacterial cells were collected at ~ 3,000 × g. The bacterial cell pellets were washed thrice using algal medium, subsequently diluted with algal medium. Bacteria at concentrations J1(6mg/ mL), J2(3mg/mL), J3(1.5mg/ mL) and J4(0.75mg/ mL) were added into the 24-well plates with harmful algae in exponential growth phase. Equal amounts of algal medium were used as controls. The plates were cultivated under the culture conditions of harmful algae. The algicidal activity was calculated every day using the following equation. The assay was performed in triplicates.

$$\text{Algicidal rate (\%)} = \frac{N_c - N_t}{N_c} \times 100$$

Where N_c is the number of algal cells in the control group, and N_t is the number of algal cells in treatment group.

Results And Discussion

Phylogenetic and phylogenomic analyses

The strain JA-25^T showed the highest 16S rRNA gene sequence similarity to the type strain of *F. aestuarina* BUZ 2^T (93.6%) followed by *Spirosoma migulaei* 15J9-8^T (89.4%). The pairwise 16S rRNA gene sequence similarity values between strain JA-25^T and the other members of the family ‘*Spirosomaceae*’ were below 90.0%. Strain JA-25^T was found to cluster with *F. aestuarina* BUZ 2^T in the neighbor-joining tree (Fig. 1), and this cluster was independent from other related genera in the family ‘*Spirosomaceae*’. The maximum likelihood tree, neighbor-joining, and maximum-parsimony trees all showed the same topology. The draft genome assembly of strain JA-25^T contained 60 contigs (N50, 670,798 bp) with a 1377.57× coverage. The total estimated combined length of the genome was 6,718,605 bp with 52.5 mol% G + C content. A whole genome based phylogeny of strain JA-25^T using MIGA (Rodriguez-R et al., 2018) has been performed (Figure S1). The completeness of the genome was very high at 97.2%, and the probability of contamination was very low at 0.9%. Strain JA-25^T most likely belonged to the family ‘*Spirosomaceae*’. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession number WAEL01000000. The ANI, AAI, and dDDH values of strain JA-25^T against the most related *F. aestuarina* BUZ 2^T were 74.4%, 73.6%, and 20.5% respectively, and against closely related genera within the family were 70.7–74.4%, 61.8–73.6% and 19.5–20.5%, respectively (Table S1). These values are below the defined thresholds for species delineation, 95–96% for ANI and 70% for dDDH (Chun et al., 2018; Rosselló-Móra and Amann, 2015; Goris et al., 2007), and support the inclusion of strain JA-25^T within a novel genus in the family ‘*Spirosomaceae*’. In addition the AAI values based on protein sequences showed relatedness values in the range of 61.8–73.6% (Table

S1), which is satisfied the threshold range of 60–80% to separate different genera proposed by Luo et al. and Rodriguez and Konstantinidis (Luo et al., 2014; Rodriguez-R and Konstantinidis, 2014), also indicating a novel genus in the family ‘*Spirosomaceae*’. Genome annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). A total of 5,440 genes, including 5,393 CDSs, 66 pseudogenes, and 47 RNA genes were annotated in *Fibrella* sp. JA-25^T. The assembly contained one, three, and one of full-length 5S, 16S, and 23S rRNA genes, respectively, 40 tRNA genes, and two non-coding RNAs. OrthoVenn2 (<https://orthovenn2.bioinfotoolkits.net/>) was used to analyze shared or independent gene clusters between JA-25^T and the closest relative *F. aestuarina* BUZ 2^T. Both strains shared 3,840 gene clusters. Strain JA-25^T and BUZ 2^T had 66 and 77 independent gene clusters, respectively. Interestingly photo-driven protein as bacteriorhodopsin and Brp/Blh family beta-carotene dioxygenase gene were only detected JA-25^T.

Phenotypic and chemotaxonomic characteristics

Strain JA-25^T was found to be a gram-negative, catalase-positive, catalase- positive, and oxidase-negative. Colonies of strain JA-25^T grown on R2A were circular, smooth, and pink. The cells were rod-shaped (0.8–1.2 µm wide and 1.5–3.5 µm long) as shown in Figure S2. Gliding motility was absent, and the production of flexirubin pigments was negative. The optimal growth of strain JA-25^T was obtained at 25°C and pH 7.0, in a medium containing 0% (w/v) NaCl. Strain JA-25^T produced a pink pigment with absorbance peaks at 480 and 510 nm, with a major peak at 482 nm which was similar to *F. aestuarina* BUZ 2^T. Isolation source, colony color and growth range for temperature and pH characteristics could distinguish between closely related genera and strain JA-25^T. A detailed comparison of the characteristics of JA-25^T with those of its close relatives in the family ‘*Spirosomaceae*’ is provided in Table 1. The cellular fatty acid profiles of strain JA-25^T and *Fibrella aestuarina* BUZ 2^T are shown in Table S2. The dominant fatty acids of strain JA-25^T were summed feature 3 (comprising C16:1 ω6c and/or C16:1 ω6c; 40.8%), C16:1 ω5c (21.4%) and C16:0 (10%). Although the overall fatty acid composition of the strain JA-25^T was similar to that of strain BUZ 2^T, it could be distinguished from its closest phylogenetic neighbors due to a smaller proportion of summed feature 3 and a larger proportion of C16:0. Menaquinone 7 (MK-7) was the predominant respiratory quinone in strain JA-25^T. The major polar lipids of strain JA-25^T were phosphatidylethanolamine, two unidentified aminolipids, two phospholipids and five unidentified lipids (Figure. S3).

Algicidal activity

Strain JA-25^T showed algicidal activity after 4 days of co-culture. The concentrations of J1(6mg/mL) and J2(3mg/mL) showed the highest algicidal activity toward the harmful algae, *H. triquetra* (Fig. 2). Meaningful algicidal activity was not observed in bacterial culture supernatant and bacterial cell extracts against harmful algae (data not shown). Algicidal activity was more effective in bacterial co-culture. Therefore, more studies are needed to understand these results, such as changing the extraction methods and bacterial culture conditions. The amount of bacterial inoculum is an important factor for algicidal activity, as shown in the differences between treatment groups (Hu et al., 2019). Under a scanning

electron microscope, a large number of algal cells surrounded by microorganisms were observed, and the burst algal cells to which microorganisms were attached were also observed. Further study is needed, but it is suggested that the mechanisms of algicidal effects are based on direct interaction (Hu et al., 2019).

Conclusion

The algicidal strain JA-25^T was isolated from freshwater in Korea. The strain phylogenetically related to the genus *Fibrella* was characterized using a polyphasic approach. Based on the phylogenetic inference, phenotypic and chemotaxonomic data, strain JA-25^T should be classified as a novel species of the novel genus *Fibrivirga* within the family 'Spirosomaceae' for which the name *Fibrivirga algicola* sp. nov. is proposed. The type strain is JA-25^T (= KCCM 43334^T = NBRC 114259^T).

Declarations

Description of *Fibrivirga* gen nov

Fibrivirga (Fi.bri.vir'ga, L. fem. n. *fibra*, a fibre or filament; N.L. fem. n. *virga*, a rod; N.L. fem. n. *Fibivirga*, a filamentous rod).

Cells are pink-pigmented, rod-shaped, non-motile, gram-negative and obligately aerobic. The major respiratory quinone is MK-7. The predominant cellular fatty acids are summed feature 3 (comprising C16:1 ω 6c and/or C16:1 ω 6c; 40.8%), C16:1 ω 5c (21.4%) and C16:0 (10%). The type species is *Fibrivirga algicola*

Description of *Fibrivirga algicola* sp. nov.

(Al.gi.co'la. L. fem. n. *alga* (gen. *algae*), a seaweed; L. masc./fem. suff. -cola, dweller;; and L. masc./fem. n. *incola*, an inhabitant, dweller; N.L. fem. n. *Algicola*, inhabitant of algae)

Cells are Gram-stain-negative, aerobic, The cells are rod-shaped (0.8–1.2 μ m wide and 1.5–3.5 μ m long). Gliding motility is absent, and the production of flexirubin pigments is negative. Growth occurs at 15–30°C (optimum 25°C), and pH 6–9 (optimum 7.0) and does not require NaCl. The major polar lipids are phosphatidylethanolamine, unidentified aminolipids, phospholipids and five unidentified lipids. Catalase is positive but oxidase is negative. Nitrate reduction is negative. In API ZYM tests, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase and *N*-acetyl- β -glucosaminidase are positive. In API 50CH assays, acid is produced from d-xylose, d-galactose, d-glucose, d-fructose, mannose, methyl-d-mannoside, methyl-d-glucopyranoside, *N*-acetyl-glucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, sucrose, trehalose, inulin, melizitose, d-raffinose, turanose, gentibiose. The type strain is JA-25^T (= KCCM 43334^T = NBRC 114259^T). The DDBJ/ENA/GenBank accession numbers for the 16S rRNA gene and genome sequences of *Fibrivirga algicola* JA-25^T are MN559427 and WAEL010000000, respectively.

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Data Availability Statement: Due to confidentiality agreements, supporting data can only be made available to bona fide researchers subject to a non-disclosure agreement.

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Conflicts of Interest: The authors declare no conflict of interest.

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Tables

Table 1. Differential characteristics between strain JA-25^T and closely related genera in the family ‘*Spirosomaceae*’

Strains: 1, *Fibrivirga algicola* JA-25^T (this study); 2, *Fibrella aestuarina* BUZ 2^T (this study); 3, *Spirosoma migulaei* 15J9-8^T (Okiria et al., 2017); 4, *Rudanella lutea* 5715S-11^T (Weon et al., 2008) + Positive, negative; ND, not available.

Data of *F. algicola* JA-25^T and *F. aestuarina* BUZ 2^T were obtained in this study. In API ZYM strips, two strains were positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase and N-acetyl-b-glucosaminidase activities.

Characteristics	1	2	3	4
Isolation source	Fresh water	Costal mud	Soil	Air
Colony colour	Pink	Pink	Pale Yellow	Orange
Cell morphology	Rods/Filaments	Rods/ Filaments	Rods	Rods/ Filaments
Growth range for temperature (°C)	15-30	15-37	10-30	10–40
Growth range for pH	6-9	6-8	6.5-8.5	6-11
Oxidase	–	–	+	+
Catalase	+	+	–	+
Hydrolysis of gelatin	-	+	ND	–
CM-Cellulose	–	+	ND	–
Agar	–	+	ND	–
Enzyme activity(API zyme)				
Trypsine	+	+	–	–
α-chymotrypsin	+	–	+	–
α- Manocidase	–	+	+	+
α-Fucosidase	–	+	–	–
DNA G+C content (%)	52.5	56.5	47	55

Figures

Figure 1

Neighbor-joining tree showing strain JA-25^T among related taxa. Branch points and bootstrap values (>80%, NJ/ML/MP) calculated by the neighbor-joining(NJ), maximum likelihood(ML) and maximum parsimony(MP) methods. *Verrucomicrobium spinosum* DSM 4136^T was used as an outgroup. The scale bar represents 0.05 substitutions per nucleotide position.

Figure 2

Algicidal(Heterocapsa) activity of strain JA-25T. A: Algicidal activity of JA-25T treatment J1(6mg/mL), J2(3mg/mL), J3(1.5mg/mL), J4(0.75mg/mL). B Microscoph 1: Con, 2: Treat(JA-25/6mg), C SEM picture 1. Con(Hetrocapsa), 2, 3, 4 : Treat(JA-25/6mg).

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