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Isolation and identification of a pathogenic bacterium, *Exiguobacterium oxidotolerans* XP-2, from the abnormal diseased mature sporophytes of a commercially cultivated brown seaweed *Saccharina japonica*

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Abstract

Diseases of the commercially cultivated brown seaweed *Saccharina japonica* have been noticed since the 1960s in China. However, the usually opportunistic nature of the pathogenic bacteria of seaweeds is still a main obstacle for isolating and identifying pathogenic bacteria responsible for causing diseases of the cultivated *S. japonica*. In this study, a Gram-positive bacterium XP-2 was isolated from the abnormal diseased mature sporophytes of *S. japonica.* Pathogenicity test indicated that XP-2 could cause green-rotten disease in healthy juvenile sporophytes and significant algal ultrastructural changes were observed after *S. japonica* was infected by XP-2. The cells shrank, and the thylakoids of the chloroplasts became blurred after 12 h of re-infection. At 24 h slight deformation was observed in the structure of the mitochondria, chloroplasts, and nucleus. There also were many vacuoles in the infected cells. After 31 h of re-infection, mitochondrial structure and thylakoids became more indistinct. SEM and TEM observations indicated that XP-2 was rod-shaped with no flagella. Analysis of the full length 16S rRNA gene sequence and physiological and chemical characteristics indicated that XP-2 was closely related to *Exiguobacterium oxidotolerans* 12280 ^{T}. Based on these results, XP-2 was designated *Exiguobacterium oxidotolerans* XP-2. The pathogenicity of *E. oxidotolerans* XP-2 was identified by Koch's Postulate under laboratory conditions. These results enrich the growing list of pathogenic bacteria in commercially cultivated seaweeds and also provide a foundation for investigating the virulence mechanisms of pathogenic bacteria of commercially cultivated seaweeds in the near future.

Keywords Abnormal disease · *Exiguobacterium oxidotolerans* XP-2 · Green-rotten disease · Pathogenic bacteria · *Saccharina japonica*

Introduction

Epibacterial species associated with seaweeds are diverse and may have beneficial or detrimental effects on their hosts. For example, certain epiphytic bacteria are beneficial for the morphology, growth, and development of many seaweed species (Egan et al. 2013; Singh 2013; Singh and Reddy 2014). However, in some cases, epibacteria associated with macroalgae can become pathogenic and harmful to their hosts and can cause disease outbreaks. Pathogenic bacteria for seaweeds are considered opportunistic pathogenic bacteria, because they are present on the surface of macroalgae and can become pathogenic when environmental conditions change, like increased temperature and the variation of light intensity (Egan et al. 2013; Wang et al. 2014). By using the

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traditional culture-dependent method and re-infection assay, many pathogenic bacteria have been isolated and identified in farmed seaweeds.

Several pathogenic bacterial strains have been reported for various diseases in the farmed seaweeds such as *Laminaria/Saccharina, Porphyra/Pyropia* and *Gracilaria* species in China, Japan, and Korea (Gachon et al. 2010; reviewed in Egan et al. 2014). Some of these pathogenic bacteria were identified by fulfilling Koch's postulates (reviewed in Egan et al. 2014). For example, *Alteromonas* sp., isolated from decaying *Laminaria japonica* (now *Saccharina japonica*) (Sawabe et al. 1992), was capable of inducing thallus bleaching lesion on *Laminaria religiosa* (Vairappan et al. 2001). *Pseudoalteromonas elyakovii* was considered to be associated with spot-wounded fronds of *S. japonica* (Sawabe et al. 2000). However, the virulence mechanism of pathogenic bacteria in farmed macroalgae is poorly understood. Firstly, it is difficult to identify the true pathogens from saprophytes or other secondary colonizers that benefit from the diseased macroalgae (Egan et al. 2013). Another reason is that the pathogenic bacteria lose their pathogenicity after being cultured for several generations in laboratory conditions. Thus, there is no stable experimental model for investigating the virulence mechanisms behind the diseases of the farmed macroalgae.

Saccharina japonica is a very important economic seaweed worldwide because of its high consumption as food and utilization in commercial products (FAO 2016). China contributes 60% of production and 90% alginate totally (FAO 2016). Since the successful cultivation of *S. japonica* in 1958, disease outbreaks have been observed at nursery and field cultivation stages (Tseng 1994). However, research work on isolating and identifying the pathogenic bacteria have been conducted only since 1979 (Chen et al. 1979). A large number of epiphytic alginic acid-decomposing bacteria from the diseased *S. japonica* have been isolated and screened in vitro for their ability to induce a disease similar to that observed in the field (e.g., Liu et al. 2002; Wang et al. 2003a). The alginic acid-decomposing bacteria were not the taxonomic terminology for pathogenic bacteria but were referred to all of those bacteria which could decompose the cell wall of the cultivated *S. japonica* (Chen et al. 1979; Wang et al. 2014). The pathogenic alginic acid-decomposing bacteria responsible for the most destructive green rot/falling off disease at nursery stage were identified by Koch's postulates (Chen et al. 1979, 1981; Wang et al. 2003b). Further, it was found that the number of alginic acid-decomposing bacteria on diseased juvenile sporophytes (4.88×10^7 cfu g^{-1}) was up to 100–500 times that of the healthy ones [$(0.8–4.3) \times 10^5$ cfu g^{-1}] (Lin et al. 2004). Other than alginic acid-decomposing bacteria, other bacterial genera were also reported to be pathogenic for the cultivated *S. japonica*. Bacteria of the genus *Pseudomonas* are known to secrete alginase that can decay the cell wall and cause rotten disease (Chen et al. 1986; Ding 1990) and have been considered to be the major bacterial pathogen for the rotten-diseased juveniles of cultivated *S. japonica*. *Alteromonas macleodii* is a pathogenic bacterium found to cause rotten disease in healthy *S. japonica* under laboratory conditions (Wang et al. 2004; 2005). *Alteromonas espejiana*, isolated from bleaching diseased sporophytes of *S. japonica*, was identified as pathogenic bacteria (Wang et al. 2006). *Pseudoalteromonas*, *Vibrio, Halomonas*, and *Bacillus* were dominant bacteria in hole-rotten-diseased sporophytes (Wang et al. 2008). However, whether these dominant bacterial species are pathogenic or not has not been confirmed by re-infection assay. Some pathogenic bacteria have been isolated and identified in cultivated *S. japonica*. However, these pathogenic bacteria are opportunistic, and due to the lack of an experimental model between the pathogenic bacteria and cultivated *S. japonica*, it is difficult to examine the virulence mechanisms.

Recently, disease outbreaks have become very complex in the field cultivation. Diseases may differ from year to year, and it is difficult to track the causative factors for such unidentified diseases. The bottleneck issue is to obtain the pathogenic bacteria that can maintain their pathogenicity in culture, which will help to establish a stable experimental model for studying the virulence mechanisms. The present study aimed to isolate pathogenic bacteria from the abnormal diseased mature sporophytes of commercially cultivated *S. japonica*. Pathogenicity of the isolated strain was identified by re-infection assay, and finally the strain was identified to the species level. The results of this study will help to establish a stable experimental model between the pathogenic bacterial strain and commercially cultivated *S. japonica.* Detailed knowledge of virulence mechanisms can significantly prevent and mitigate the disease outbreaks during both nursery and field cultivation stages.

Material and methods

Sample collection

The abnormal diseased mature sporophytes of *S. japonica* (Fig. 1) were collected on 9 July 2018 from the *S. japonica* cultivating farms, located in Xiapu, China (N 26° 30′ 06.87″ E 119° 53′ 34.11″). There were many abnormal diseased areas scattered on the edge of the mature sporophytes with white rotten holes and filamentous epiphytes, penetrating into the epidermal cells (see details in Fig. 1). Three replicate of samples from diseased mature sporophytes were cut into 10 cm^2 pieces with a sterile surgical blade and tweezers and were transferred to the laboratory in sterile zip lock plastic bags with ice packs within 20 h of collection.

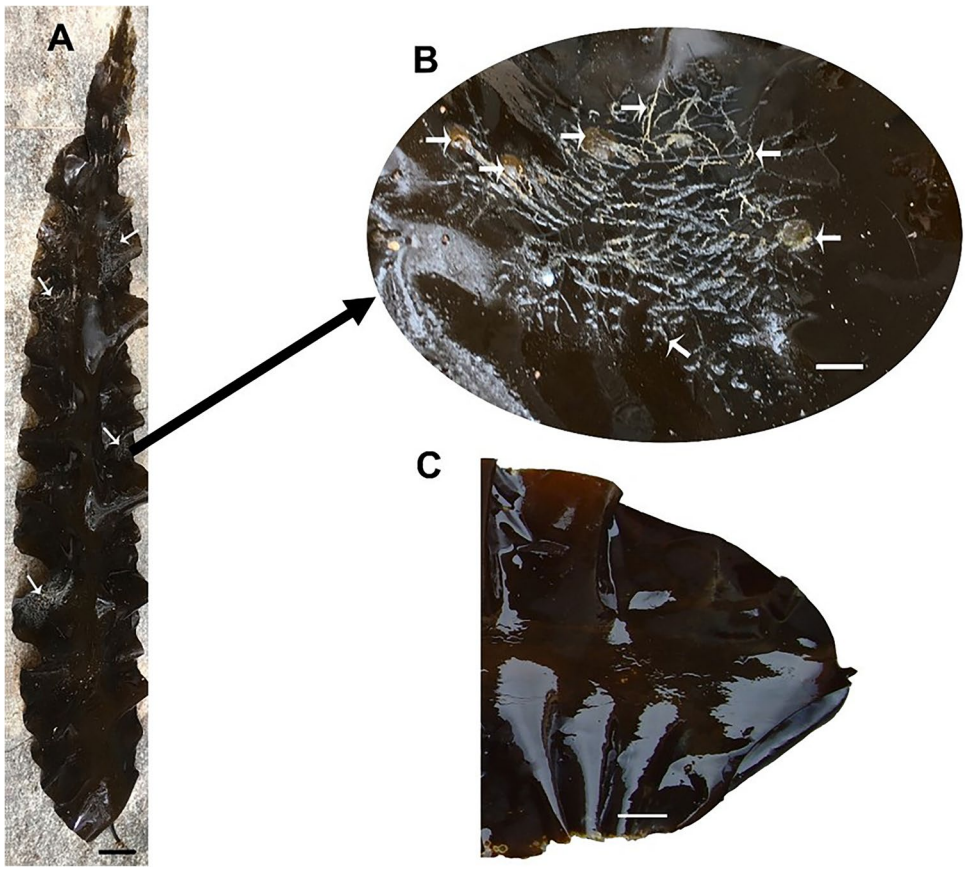

Fig. 1 Abnormal diseased mature sporophytes of cultivated *S. japonica.* **A** Abnormal diseased mature sporophytes (white arrows showed the disease areas). **B** The inset indicates the disease symptoms, including white dots (white arrows) and white filamentous epiphytes (white arrows) on the surface of the diseased mature sporophytes. **C** Healthy mature sporophyte. Bars: **A** 5 cm; **B** and **C** 0.5 cm

Bacterial isolation

The diseased tissues of 10 cm^2 area were rinsed at least three times with sterile seawater (the salinity of the seawater was about 30‰) to remove loosely attached free living organisms and other particles. The epiphytic bacteria were isolated from the tissues by using sterile cotton applicator and then were suspended in 5 mL sterile sea water. The suspension of epiphytic bacteria was diluted using tenfold dilution plate method and spread over Zobell 2216 E marine agar (Ying et al. 2019). A 100 µL of each dilution was spread in triplicate on Zobell 2216 E marine agar (Oppenheimer and Zobell 1952). The plates were then incubated at 25 °C for 24 h. The bacteria were selected on the basis of morphology, such as shape and pigment. The purified strains were obtained by streaking on the plates for three times. The purified bacterial isolates were then stored using 15% (v/v) glycerol at − 80 °C for further analysis.

Re-infection assay

Pathogenicity tests were carried out according to Wang et al. (2004) to determine whether the isolated strains may cause any disease in healthy *S. japonica*. Fresh healthy juvenile sporophytes (10 cm in length) of *S. japonica* were collected from Weihai Changqing Ocean Science & Technology Co., Ltd, located in Rongcheng, Shandong province, China, on 24 December 2018. After collection samples were processed within 4 h, healthy juvenile sporophytes were cut into small pieces (1.0 × 1.0 cm) with a sterile surgical blade. Each tissue was cut in the center and put into 24-well Coster cell culture plates (USA). The tissue sections were treated individually with the isolated strains (density was 1.0×10^7 cfu mL^{-1}). The same size of tissue sections of the healthy juvenile sporophytes were used as a control group and treated with autoclaved Zobell 2216E liquid medium. The plates were then incubated at 10 °C with 90 µmol photons m^2 s^{-1} (light: dark = 12:12 h) for 35 h. The infected tissues were observed under a light microscope (Nikon, Japan). Replication level was 6. Pathogenicity of the isolated bacterial strains was identified by re-infection assay.

Observations of ultrastructure of the infected S. japonica

The re-infection assay and the incubation conditions were the same as mentioned in the "Re-infection assay" section. Control and infected tissues were collected at 12, 24, and 31 h after infection by pathogenic bacteria. Samples were fixed with 2.5% glutaraldehyde in phosphate buffer for 4 h

and then rinsed three times with 0.1 M phosphate buffer saline (PBS) for 15 min and post-fixed with 1% osmium tetroxide for 1.5 h. Following that, the samples were rinsed with PBS and dehydrated with ethanol (50%, 70%, 90%, and 100%) for 15 min and kept at room temperature (Maurin et al. 1993). Following Kim et al. (2016), the samples were immersed in Spurr's epoxy resin and polymerized overnight at 72 °C okdkdven. The samples (thin sections) were stained with uranyl acetate and lead citrate for 15 min. Finally, samples were observed using a JEM-1200EX TEM microscope (JEOL, Japan).

Morphological observations of the pathogenic bacterial strain by scanning electron and transmission electron microscope

The cell morphology was examined by scanning electron microscope (SEM). The pathogenic bacterial strain was grown on Zobell 2216E Marine broth at 25 °C for 24 h. The cells were fixed with 2.5% (v/v) glutaraldehyde for 4 h and then rinsed with 0.1 M phosphate buffer. Then the cells were post-fixed in 1% OsO_4 for 2 h, washed with 0.1 M phosphate buffer, and were dehydrated with a graded series of ethanol/water (10% v/v to 100%) at 10-min intervals. The specimens were passed through 50% (once) and 100% isoamyl acetate twice. All samples were critical-point dried and gold-coated. The cells were observed under SEM by using JEM 1200-EX (JEOL, Japan).

The specimens of pathogenic bacteria for transmission electron microscopy (TEM) were referred to Yumoto et al. (2002). Pathogenic bacterial strain was grown on Zobell 2216E Marine broth and incubated at 25 °C for 24 h. The bacterial cells were suspended in physiological saline solution. A tiny drop of the suspension was mounted on a carbon coated copper grid, following which cells were negatively stained with 1% phosphotungstic acid. The specimens were observed under with JEM 1200EX TEM microscope (JEOL, Japan).

Molecular identification of the pathogenic bacterial strain

Molecular identification of pathogenic strain was achieved by blasting the full length of 16S rRNA gene sequence according to Fisher et al. (1998). Bacterial DNA was extracted by using plant DNA kit (Tiangen, China) following protocols of the manufacturer. The full length 16S rRNA gene sequence was amplified by PCR, using universal primers (27F: AGAGTTTGATCCTGGCTCAG; 1429R: GGTTACCTTGTTACGACTT) (Moreno et al. 2002). PCR amplification reaction was performed in triplicate of 20 µL mixture, containing 11 µL of double distilled water, 2.5 µL of 10 × KOD Buffer, 2.5 µL of template DNA, 2.5 µL of 2.5 mM dNTPs, 1 µL of each primer (5 µM), and 0.5 µL of KOD polymerase. Amplified DNA fragments were sequenced by Sangon Co., Ltd, Shanghai, China.

Full length 16S rRNA gene sequence of the pathogenic bacterial strain was compared with those other related bacterial species using NCBI data base (https://blast.ncbi.nlm.nih.gov). The 16S rRNA gene sequences of the related bacterial strain were retrieved from the NCBI database (www.ncbi.nlm.nih.gov) and aligned by using the Clustal X 1.81 program (Thompson et al. 1997). Phylogenetic trees were reconstructed based on the neighbor-joining (NJ) (Tamura and Nei 1993). Phylogenetic analysis was carried out by using MEGA X software (Felsenstein 1985; Kumar et al. 2018).

Physiological and chemical characterization by Biolog Gen III Microplate

Biolog microbial identification system (Biolog, Hayward, USA) was used to classify the physiological fingerprint of the pathogenic bacterial strain according to the manufacturer's protocol. The pathogenic bacterial strain was cultured in Zobell 2216E marine agar at 25 °C for 24 h, and bacterial colonies were re-suspended in inoculating fluid A (Biolog). The inoculum was prepared according to the user guidelines, and transmittance of bacterial cell suspension was measured by turbidity meter (Biolog TM).

The 100 µL of culture bacteria were inoculated into each well of Gen III microtest device and incubated at 25 °C for 24 h. The results of the Biolog were obtained at 600 nm automatically by the standardized Micro station system (Biolog Inc., USA). The results were analyzed on the basis of an extensive species library in the Biolog GEN III database.

Results

Isolation of bacterial strains from the abnormal diseased mature sporophytes of commercially cultivated S. japonica

The abnormal disease occurred in mature sporophytes in June 2018 during the nursery stage. The diseased areas (white arrows in Fig. 1A) scattered over the edge of the diseased sporophytes (Fig. 1A). The diseased symptoms included white spots, and more white filamentous epiphytes penetrated into the epidermal cells of the diseased mature sporophytes. (Fig. 1B). Using the conventional culture-dependent method, a total of 12 epiphytic bacterial strains were isolated from the abnormal diseased mature sporophytes. The pathogenicity of these strains was determined by re-infection assay.

Re-infection assay

The purified isolated bacterial strains (1.0×10^7 cfu mL^{-1}) were infected to the healthy juvenile sporophytes of *S. japonica*. Among the 12 isolated strains, only XP-2 could cause green-rotten disease, not abnormal disease. Compared to the control groups (Fig. 2A), no disease symptoms were observed at 12 h after XP-2 infection (Fig. 2B). The color of the infected tissue became white at 24 h (Fig. 2C), while at 30 h, the infected tissue turned green (Fig. 2D), and the green infected tissue became rotten at 31 h (black arrows, Fig. 2E).

Moreover, TEM observations of the infected cells indicated ultrastructure changes after infection by XP-2. In the control groups at 12, 24, and 31 h (Fig. 3A, B, and C, respectively), the structure of chloroplasts, mitochondria, and nuclei showed the normal morphology during the re-infection assay. After infection by XP-2 at 12 h, shrinkage of nucleus occurred. Chloroplast thylakoids became less distinct (Fig. 3D). At 24 h after infection by XP-2, the chloroplast thylakoids deformed, and some vacuoles were observed in the cytoplasm. More shrinkage of nucleus was observed (Fig. 3E). The structure of chloroplasts, mitochondria, and nucleus became indistinct at 31 h after infection by XP-2 (Fig. 3F).

Morphological characteristics of pathogenic bacterial strain of XP-2

XP-2 was Gram-positive and appeared as light orange color, forming 1–1.5-mm diameter circular colony with an entire edge (Fig. 4A). SEM (Fig. 4B) and TEM (Fig. 4C) showed that XP-2 strain was rod-shaped, 1.45–2.1 μm in length, and 0.7–0.9 μm in width with no flagella.

Molecular identification and phylogenetic analysis and verification of Koch's postulates

The 16S rRNA gene sequence of XP-2 consisted of 1445 bp. After blasting the 16S rRNA sequence in NCBI database, it showed 98.95% similarity with *Exiguobacterium oxidotolerans* JCM12280^T. The phylogenetic analysis was performed on the basis of 16S rRNA gene sequences of other related species (Fig. 5). In the phylogenetic tree, XP-2 clearly clustered with *Exiguobacterium oxidotolerans* JCM12280^T, and therefore we designated XP-2 as *Exiguobacterium oxidotolerans* XP-2. The 16S rRNA gene sequence of XP-2 has been deposited in NCBI database with the accession number of MW131367.

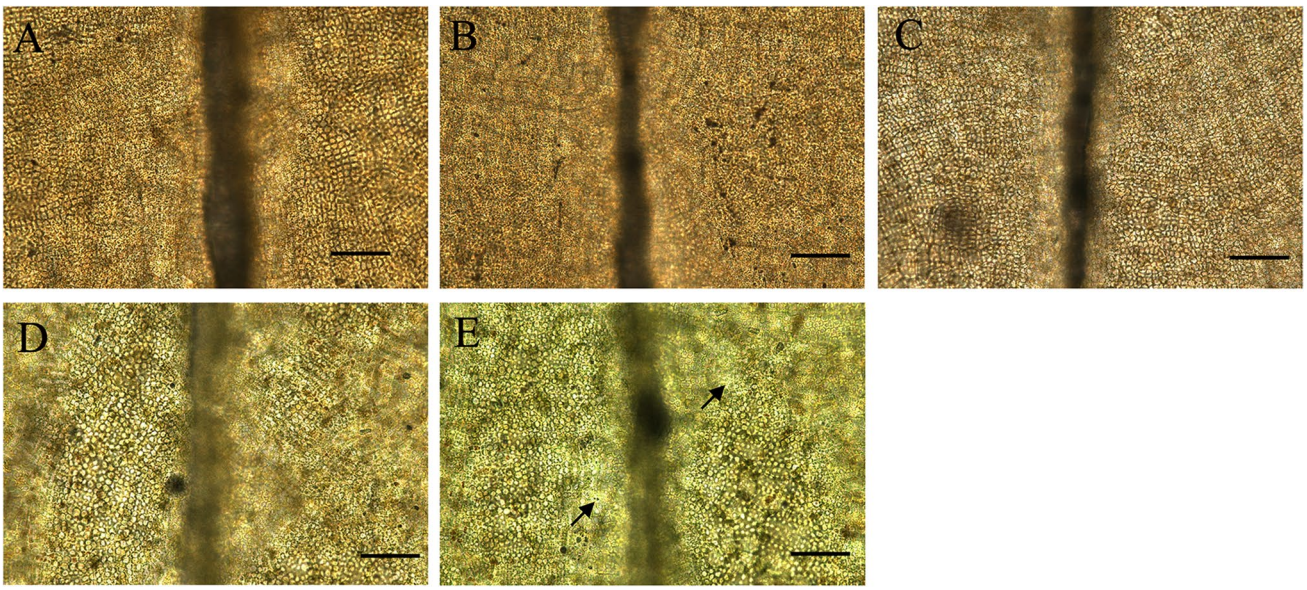

Fig. 2 Re-infected tissues of healthy juvenile sporophytes by XP-2. **A** control group; **B**: re-infected tissue of healthy juvenile sporophytes at 12 h; **C** infected tissue of healthy juvenile sporophytes at 24 h; **D** green-rotten disease symptoms were observed in the infected tissue at 30 h; **E** serious green-rotten disease symptoms (black arrows) examined in the infected tissue of healthy juvenile sporophytes at 31 h. Bar = 100 μm

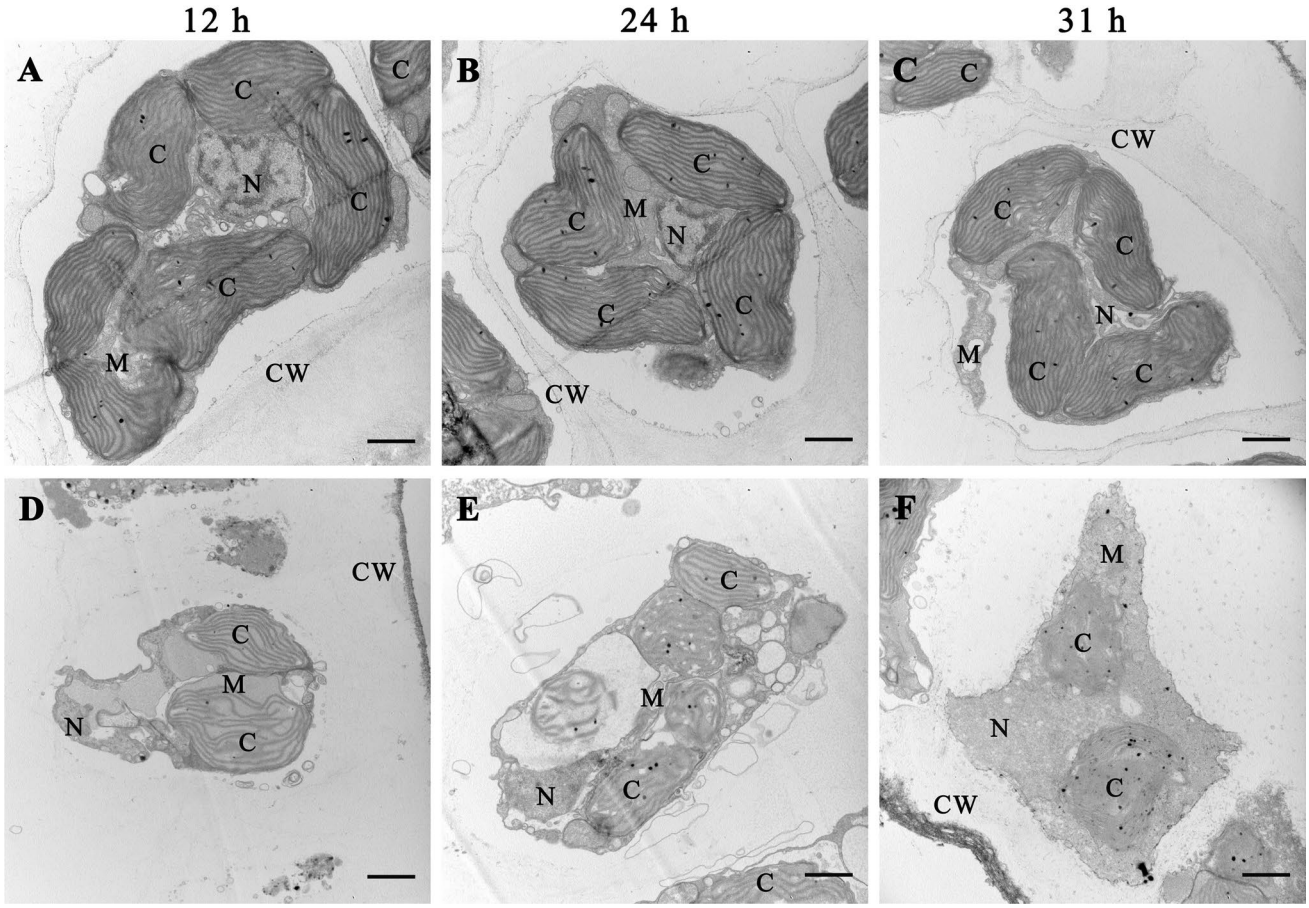

Fig. 3 Ultrastructural changes of the infected *S. japonica* cells by XP-2. **A** Control group at 12 h; **B** control group at 24 h; **C** control group at 31 h; **D** re -infected cell at 12 h; **E** re-infected cell at 24 h; **F** re-infected cell at 31 h. CW, cell wall; C, chloroplasts; N, nucleus; M, mitochondria. Bar = 2.0 μm

The bacterial strain re-isolated from green-rotten-diseased tissues of the healthy juvenile sporophytes induced by strain of XP-2 was proven to be the same according to 16S rDNA sequencing. The results verified that strain XP-2 was the pathogen which could cause the green-rotten disease under laboratory conditions.

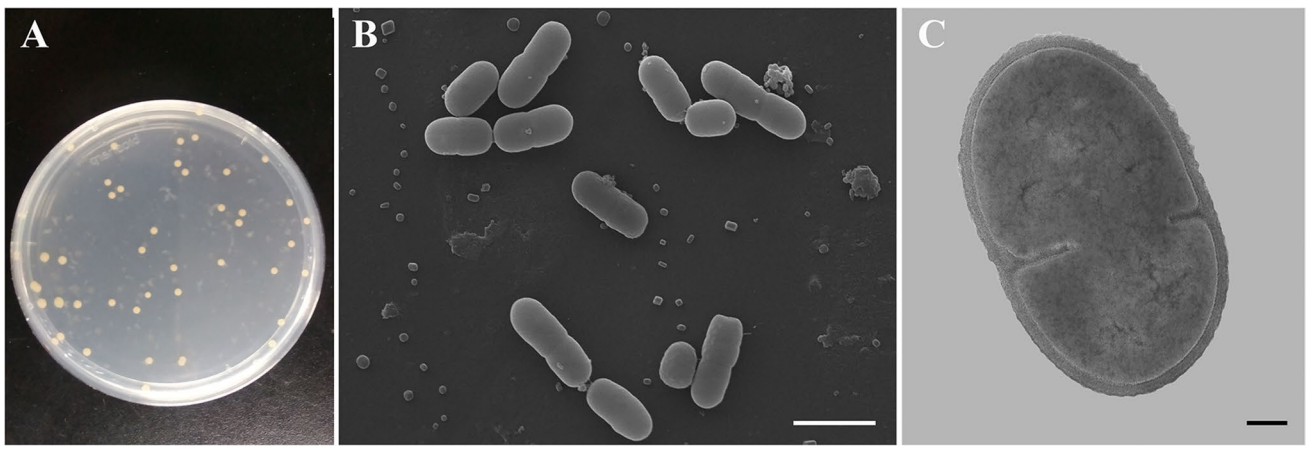

Fig. 4 Morphological characteristics of XP-2. **A** XP-2 colonies on Zobell 2216E culture medium; **B** morphology of XP-2 observed by SEM (bar = 2 μm); **C** morphology of XP-2 observed by TEM (bar = 500 nm)

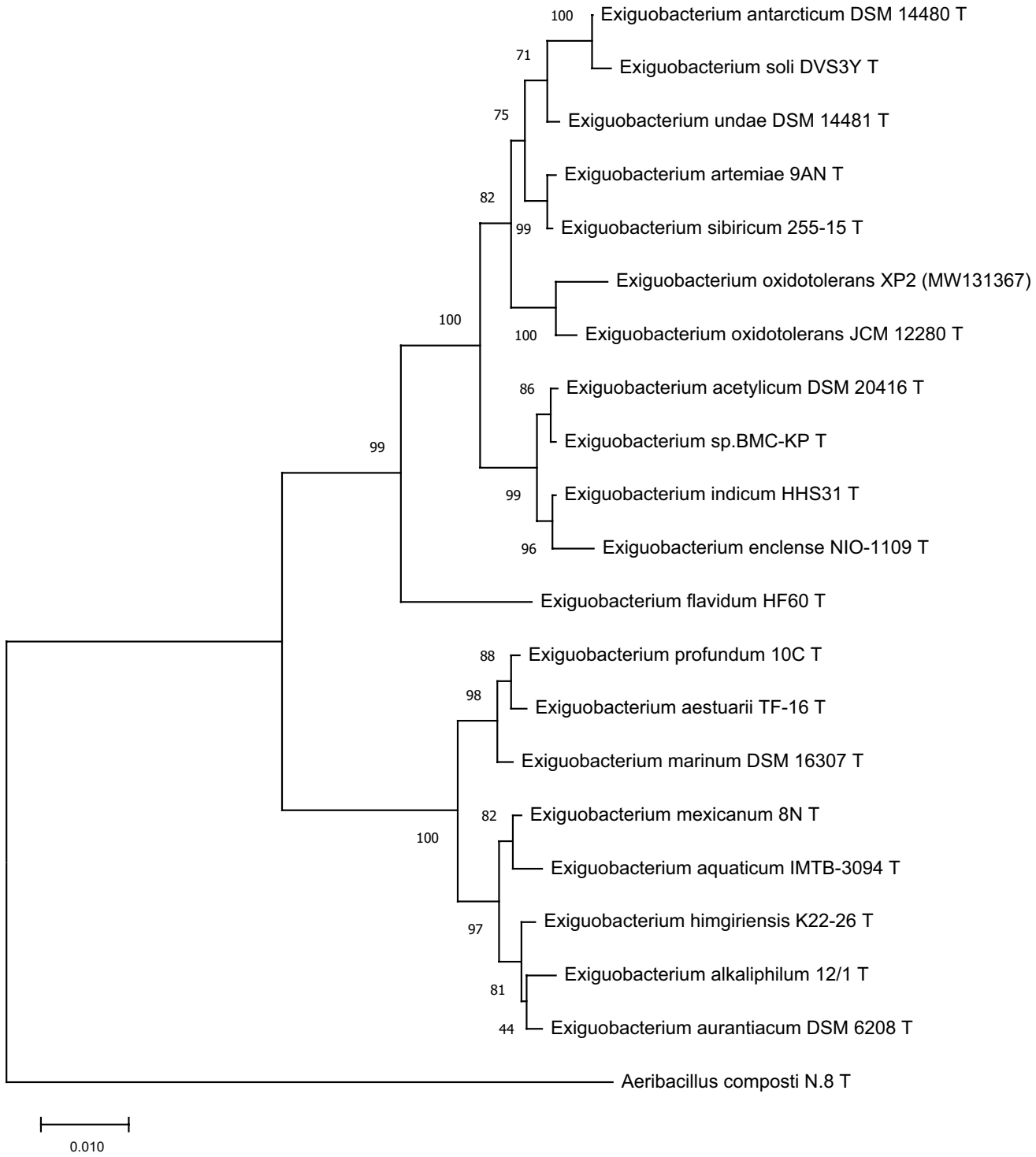

Fig. 5 Phylogenetic tree based on 16S rRNA gene sequences showing the position of XP-2 with other related species using neighbor-joining method. *Aeribacillus composti* was used as out group. Bootstrap values were shown for the nodes in percentage after 1000 re-samplings. Bar= 0.01.

Biochemical and physiological characterization of XP-2

The Biolog Gen III Microplate findings showed that XP-2 utilized carbohydrates, as well as amino acids and other chemical derivatives. The isolated strain XP-2 metabolized a number of carbohydrates, like D-fructose, αD-glucose, D-cellobiose, sucrose, D-trehalose, gentiobiose, propionic acid, pectin, dextrin, hexose PO_4 i.e. (D-glucose-6-PO_4, D-fructose-6-PO_4). The metabolized amino acids included

Table 1 Biochemical and physiological characterization of the XP-2 strain based on Biolog Gen III Microplate

Positive reactions	
Lithium chloride	Aceto acetic acid
D-cellobiose	PH 6
Dextrin	4% NaCl
D-gluconic acid	1% NaCl
D-fructose	8% NaCl
D-mannitol	L-serine
Acetic acid	Gentiobiose
D-glucose-6-PO_4	Propionic acid
D-fructose-6-PO_4	Guanidine HCl
Glycerol	Tween 40
N-acetyl D-glucosamine	Sodium butyrate
α D-glucose	$^{\beta}$Methyl D-glucoside
L-alanine	Gelatin
Sucrose	L-glutamic acid
Inosine	Potassium tellurite
Pectin	
D-trehalose	
Weak positive reactions	
D-maltose	D-turanose
D-galactose	γamino-butyric acid
Mucic acid	1% Sodium lactate
N-acetyl βD-manosamine	L-aspartic acid
$^{\beta}$Hydroxy-D,L-butyric acid	
Negative reactions	
L-lactic acid	L-malic acid
D-lactic acid methyl ester	$^{\alpha}$Keto-butyric acid
L-arginine	D-galacturonic acid
D-glucuronic acid	Citric acid
D-saccharic acid	L-histidine
L-pyroglutamic acid	D-sorbitol
3-Methyl glucose	D-melibiose
p-Hydroxy phenylacetic acid	D-malic acid
Glycyl-L-proline	Myo-inositol
L-galactonic acid lactone	Sodium bromate
Quinic acid	D-aspartic acid
αketo-Glutaric acid	D-fucose
D-serine	D-serine
Glucoronamide	N-acetyl D-glactosamine
Methyl pyruvate	Tetrazolium Blue
L-fucose	Fusidic acid
Tetrazolium violet	Troleandomycin
Bromo-succinic acid	Vancomycin
N-acetylneuraminic acid	D-mannose
αhydroxy butyric acid	D-Raffinose
Stachyose	Rifamycin SV
Formic acid	Minocycline
L-rhamnose	Lincomycin
Nalidixic acid	D-arabitol
$^{\alpha}$D-lactose	Niaproof 4
Aztreonam	D-salicin
PH 5	

L-alanine, L-glutamic acid, L-serine, gelatin some carboxylic acid, acetic acid and acetoacetic acid. It may grow well at pH 6 and can also tolerate 1–8% of NaCl. However, some of carbohydrates and amino acids were poorly metabolized by XP-2. The strain showed sensitivity to vancomycin, rifamycin SV, lincomycin, minocycline, Niaproof 4, tetrazolium violet, and troleandomycin. The metabolic profile of XP-2 based on high and low consumption of carbohydrates, amino acids, and other chemical derivatives were depicted in Table1. To maintain the vital activities for survival, *E. oxidotolerans* JCM12280 ^{T} consumes compounds such as D-fructose, D-maltose, and gelatin. Our isolated XP-2 also consumes D-fructose, D-maltose, and gelatin compounds. Metabolic characteristics of XP-2 slightly matched with *E. oxidotolerans* JCM12280 ^{T}. On the basis of the above 16 S rRNA sequence blasting, phylogenetic analysis, and biochemical and physiological characteristics, we designated XP-2 as *Exiguobacterium oxidotolerans* XP-2.

Discussion

In this study, we identified a pathogenic bacterial strain XP-2, isolated from the abnormal diseased mature sporophytes. XP-2 could cause green-rotten disease (not abnormal disease) in healthy juvenile sporophytes in laboratory conditions. Pathogenecity of XP-2 was tested by both re-infection assay and by Koch's postulate. Based on the sequence of 16S rRNA gene and physiological and chemical characterization of XP-2, we designated XP-2 as *Exiguobacterium oxidotolerans* XP-2. This bacterium was definitely an opportunistic pathogenic bacterium for the cultivated *S. japonica*.

The genus *Exiguobacterium* was first designated by Collin in 1983. So far, 20 species have been identified within this genus from diverse habitats, including marine water, permafrost to hot spring, food processing plants, and soil (see review by Pandey 2020). Various species of *Exiguobacterium* have been studied for industrial use, including enzyme production (Kasana and Pandey 2018), bioremediation of pesticide, and degradation of toxic compounds (Rizvi et al. 2016; Mohapatra et al. 2017), and some of them have the potential to promote plant growth (Chauhan et al. 2015; Kumar and Verma 2018). The agro-utility of *Exiguobacterium* has been confirmed by its ability

to suppress the fungal diseases of cereal crops and to inhibit the growth and development of plant pathogens (Selvakumar et al. 2009).

Exiguobacterium spp. have been isolated from green, red, and brown seaweeds (Del olmo et al. 2018). *Exiguobacterium marinum* was isolated from the green seaweed *Caulerpa cylindracea* (Rizzo et al. 2016) and *Exiguobacterium profundum* from the brown seaweed *Turbinaria ornata* (Karthick and Mohanraju 2018). Moreover, Singh et al. (2011) reported that *Exiguobacterium homiense* associated with the red seaweed *Gracilaria dura* had the ability to produce growth promoting hormone enhancing the number of buds and growth of *G. dura.* Till date, no pathogenic *Exiguobacterium* species has been documented in seaweeds. However, Anguiano-Beltrán et al. (2012) reported that *Exiguobacterium* sp. could cause mortality on abalone in commercial farms at the post larval stage.

Exiguobacterium oxidotolerans was first reported in 2004 (Yumoto et al. 2004). A number of *E. oxidotolerans* T-2-2 ^{T} strains, like STR36, AMBL-20 T, SGAA-2, A011, N4-1p, CNU020, Kopri 20,246, and isolate 2, have been isolated from variety of the habitats like rhizosphere, glaciers, corals, sea mud and sediments, dead brown algae, and shrimps. These strains played a major role in enzyme production, such as exopolysaccharide and alkaline lipase production, and showed antimicrobial activity and Bgl1C (β-glucosidase), catalase, and protease activity, and were useful for industrial applications (Lee et al. 2005; 2009; Chen et al. 2010; Bharti et al. 2013; Cai et al. 2014; Chellaram et al. 2015; Seki et al. 2017; Yasin et al. 2021).

So far, culture-dependent method and 16S rRNA gene amplicon sequencing have been applied for identifying the pathogenic bacteria of diseased cultivated *S. japonica.* By using conventional culture-dependent method, species of *Pseudoalteromonas* (Sawabe et al. 1998; 2000; Wang et al. 2008), *Alteromonas* (Wang et al. 2004, 2005; Peng and Li 2013), and *Vibrio* sp. (Wang et al. 2008) have been identified as the pathogenic bacteria for various diseased *S. japonica.* However, using 16S rRNA gene amplicon sequencing, *Granulosicoccus*, *Litorimonas*, *Tenacibaculum*, and *Blastopirellula* were found to be dominant in green rotten-diseased *S. japonica* sporelings as compared to the healthy ones at nursery stage (Li et al. 2020). It was implied that these genera were possibly associated with green-rotten disease of the sporelings of *S. japonica*. In the case of field cultivation during the harvest season (from April to June), *Halomonas* sp. was the most dominant bacterial species on green rotten-diseased sporophytes. OTUs of genera *Sulfitobacter* and *Loktanella* of the Roseobacter group were enriched in diseased tissues suggesting these taxa could be related to the opportunistic pathogens (Zhang et al. 2020). Different findings may have resulted from the limitation of the culture-dependent method and also the fact that different disease symptoms may be caused by different pathogenic bacteria. Moreover, the results from culture-dependent method and using 16S rRNA gene amplicon sequencing could not be compared with each other, as these results focused on different diseases. Therefore, in order to gain deeper and comprehensive understanding of the pathogenic bacteria of the diseased cultivated *S. japonica*, it is highly recommended to combine the conventional culture-dependent method and 16S rRNA gene high-throughput sequencing. Additionally, whether the potential pathogenic bacteria identified by the 16S rRNA gene high-throughput sequencing are the actual pathogenic bacteria or not still need to be tested by isolation, purification, and re-infection assay. The findings of the pathogenic bacterium *E. oxidotolerans* XP-2 in this study enriched the list of the pathogenic bacteria for cultivated *S. japonica*.

So far, research on the diseases of commercially cultivated *S. japonica* is still at its early stages. The main issue for bacterial diseases of cultivated *S. japonica* is the complexity of disease outbreaks. Rather than re-occurring year after year, the observed disease outbreaks are highly variable with different disease symptoms in different years at both nursery stage and cultivation stage, which may be caused by fluctuations of biotic and abiotic parameters during cultivation in *S. japonica* farms (Prof. Gaoge Wang, pers. communication). Abnormal disease was found on the mature sporophytes in July 2018. Although we have tried to isolate and identify the pathogenic bacteria for abnormal disease, we have not been fully successful yet. The reasons for difficultly in identifying the pathogenic bacteria for commercially cultivated *S. japonica* can be summarized as follows: (1) it has been identified that pathogenic bacteria of the cultivated *S. japonica* are opportunistic pathogenic bacteria, which means that they are present on the healthy *S. japonica* and only become pathogenic when the environmental factors deteriorate during cultivation, for example, increased temperatures and the variation of light intensity. (2) Only about 1% of the epiphytic bacteria of *S. japonica* are cultivable, the true pathogenic bacteria maybe not cultivable using the conventional culture-dependent method. (3) Polymicrobial pathogenic bacteria have been found for coral diseases (Sato et al. 2016) and the red alga *Delisea pulchra* (Kumar et al. 2016). Polymicrobial pathogenic microorganisms also occurred in the sporelings of *S. japonica* at nursery stage. When re-infecting the healthy sporelings by combining one isolated pathogenic bacterial strain and two other fungal isolated pathogenic strains, disease symptoms appeared 3–4 h earlier compared to 8–12 h when re-infected by each individual pathogenic strain (Prof. Gaoge Wang pers. communication). (4) It is hard to identify which is the first causative agent whenever there is a disease outbreak because of the polymicrobial pathogenic agents. Therefore, *E. oxidotolerans* XP-2 is also an opportunistic pathogenic bacterium for the cultivated *S. japonica.*

Conclusions

China is a world leader in the production of the brown seaweed *S. japonica*. With the successful cultivation of the *S. japonica* cultivation since the 1950s, numerous diseases of biotic and abiotic origin have been described at both nursery stage and field cultivation stage. Previous research were highly focused on observations and descriptions of the disease outbreaks. However, all strategies for disease management of commercially cultivated seaweeds are based on the fundamental understanding of the pathogenic bacteria. So far, very little is known about the virulence mechanisms induced by pathogenic bacteria and the virulence factors in the cultivated *S. japonica*. Elucidating the virulence mechanisms and how these processes are regulated by the biotic and abiotic environment is of urgent importance. Only once this has been achieved, rapid diagnosis can be applied for the effective management of *S. japonica* diseases. The identified pathogenic bacterium *E. oxidotolerans* XP-2 in this study will help to establish a stable experimental model of pathogenic bacteria and *S. japonica* to elucidate the virulence mechanisms. Diseases of *S. japonica* could be prevented and mitigated by the deeper understanding of the virulence mechanisms of the pathogenic bacteria in the near future.

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