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Analysis of cultivable aerobic bacterial community composition and screening for facultative sulfate-reducing bacteria in marine corrosive steel*

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Abstract Anaerobic, aerobic, and facultative bacteria are all present in corrosive environments. However, as previous studies to address corrosion in the marine environment have largely focused on anaerobic bacteria, limited attention has been paid to the composition and function of aerobic and facultative bacteria in this process. For analysis in this study, ten samples were collected from rust layers on steel plates that had been immersed in seawater for different periods (i.e., six months and eight years) at Sanya and Xiamen, China. The cultivable aerobic bacterial community structure as well as the number of sulfate-reducing bacteria (SRB) were analyzed in both cases, while the proportion of facultative SRB among the isolated aerobic bacteria in each sample was also evaluated using a novel approach. Bacterial abundance results show that the proportions are related to sea location and immersion time; abundances of culturable aerobic bacteria (CAB) and SRB from Sanya were greater in most corrosion samples than those from Xiamen, and abundances of both bacterial groups were greater in samples immersed for six months than for eight years. A total of 213 isolates were obtained from all samples in terms of CAB community composition, and a phylogenetic analysis revealed that the taxa comprised four phyla and 31 genera. Bacterial species composition is related to marine location; the results show that Firmicutes and Proteobacteria were the dominant phyla, accounting for 98.13% of the total, while *Bacillus* and *Vibrio* were the dominant genera, accounting for 53.06% of the total. An additional six facultative SRB strains were also screened from the isolates obtained and were found to encompass the genus *Vibrio* (four strains), *Staphylococcus* (one strain), and *Photobacterium* (one strain). It is noteworthy that mentions of *Photobacterium* species have so far been absent from the literature, both in terms of its membership of the SRB group and its relationship to corrosion.

Keyword: marine corrosive steel; cultivable aerobic bacteria; facultative sulfate-reducing bacteria; bacterial community composition; 16S rRNA gene sequencing

1 INTRODUCTION

Steel corrosion is becoming an increasingly serious problem worldwide as it has major social and economic impacts. Corrosion involves a variety of complex processes, including those that are physical, chemical, and biological (Kip and van Veen, 2015). In

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this context, microbially influenced corrosion (MIC), also known as biocorrosion, refers to the acceleration of this process because of the presence of biofilms on the surfaces of metals and alloys (Beech and Sunner, 2004). The earliest recorded example of MIC was reported in 1891, and this process was then described in detail almost two decades later (in 1910). Bacterial numbers and the nature of these communities in steel rust layers were initially analyzed using traditional cultivation methods (Guo et al., 2006; Bermont-Bouis et al., 2007). However, due to modern technological developments, bacterial communities in steel rust layers can now be identified using culture-independent techniques (Jones et al., 2007; Lee et al., 2008). Community structures can also be analyzed using molecular biological techniques, including, but not limited to, fluorescence in situ hybridization (FISH), terminal restriction fragment length polymorphism, and the use of high-throughput Illumina MiSeq sequencing (Jones et al., 2007; Lee et al., 2008). Microscopic techniques can also be used to count bacteria, and the combination of FISH and confocal microscopy presents an even more versatile technique that can be employed to directly evaluate the importance of different bacteria (Dang and Lovell, 2002a, b). Although previous research in this area has greatly enhanced our understanding of corrosive bacteria, numerous deficiencies nevertheless remain, including the interrelationship amongst taxa, the functions performed by bacteria during the corrosion process, and the underlying mechanism itself. Additional research on bacterial communities in corrosive environments is required, especially those that can be cultured.

Three categories of bacteria are found in corrosive environments, aerobic, anaerobic, and facultative, classified according to their demands for oxygen. The proportions and types within these three categories change as corrosion time progresses; aerobic and facultative bacteria are dominant during the beginning and middle stages of experiments, respectively (Yang et al., 2011), while aerobic, anaerobic, and facultative taxa tend coexist simultaneously during later stages (Bermont-Bouis et al., 2007; Yang et al., 2011). A great deal of research has been carried out on anaerobic forms, including sulfate-reducing (SRB), iron-reducing (IRB), and nitrate-reducing bacteria (NRB) (Li et al., 2013). The first of these groups, SRB, were thought to be the most influential and dominant corrosion-accelerating factor linked to metal MIC in marine environments (Angell and Urbanic, 2000). Research has shown that SRB mainly occur as biofilms on metal surfaces as a result of their growth and metabolism; it is the interaction between the metallic matrix and SRB metabolites that accelerates metal corrosion. A range of studies have been carried out on SRB abundance, species, and corrosion mechanisms, with particularly emphasis on *Desulfovibrio* species (Iverson, 1968; King and Miller, 1971; Mansfeld, 2007; Xu et al., 2016); numerous species within this genus have been shown to corrode metal, including *D. desulfuricans* (Lopes et al., 2006) and *D. vulgaris* (Zhang et al., 2015, 2016). At the same time, other SRB genera have also been shown to have a corrosive effect on metals, including *Desulfotomaculum* (Cetin et al., 2007). It is also the case that other kinds of anaerobic bacteria can also contribute greatly to corrosion depending on environment; Iverson (1966) initially showed that MIC-causing methanogens are generated by the cathodic depolarization mechanism, as these bacteria are capable of consuming hydrogen. As corrosive nitrate reducers, NRB have also been shown to corrode C1018 carbon steel under anaerobic conditions (Xu et al., 2013). However, although previous studies have shown that IRB can inhibit metal corrosion by generating physical barriers (biofilms) that protect surfaces (Du et al., 2013), few studies have so far been performed other than on SRB.

Compared to anaerobic taxa, research on aerobic species has been limited and mainly restricted to sulfur-oxidizing (SOB), iron-oxidizing (IOB), and manganese-oxidizing bacteria (MOB) (Ashassi-Sorkhabi et al., 2012; Wang et al., 2012; Sun et al., 2014). Related research has noted the fact that aerobic bacteria form consortia, often encompassing the genera *Thiobacillus*, *Pseudomonas*, *Bacillus*, *Halomonas*, and *Marinobacter*, amongst others (Zuo, 2007; Pillay and Lin, 2013). Besides, *Roseobacter*, *Erythrobacter*, *Citrobacter*, *Klebsiella*, and *Proteus* were the dominant culturable bacteria on carbon steel plates immersed in seawater for one month (Bermont-Bouis et al., 2007; Lanneluc et al., 2015). Aerobic bacteria are thought to perform two separate functions during the metal corrosion process. On one hand, the presence of certain aerobic taxa can stimulate the corrosion rate due to biocatalytic reduction of oxygen by a biofilm, as seen, for example, in *Pseudomonas* and *Pseudomonas*-like organisms (Yuan and Pehkonen, 2007; Pillay and Lin; 2013; Li et al., 2016). In contrast, it has also been suggested that some

aerobic bacteria protect against corrosion by forming a biofilm on the surface of steel which removes corrosion-promoting agents (such as oxygen) that are a component of bacterial metabolism, or by secreting antimicrobial agents that can inhibit the growth of corrosion-causing species (Zuo, 2007; Eduok et al., 2016). The role of aerobic bacteria in corrosion is therefore very complex, and detailed information regarding the corrosive or anti-corrosive behavior of these assemblages is lacking.

Facultative bacteria are also known to play a very important role as the corrosive environment changes from aerobic to anaerobic during the corrosion process. To date, just a few reports on the corrosive properties of facultative anaerobic bacteria have been published, mainly concerning the genera *Vibrio* and *Bacillus* (Benbouzid-Rollet et al., 1991; Nikolaev and Plakunov, 2007; Yin et al., 2009). As is the case with their aerobic counterparts, facultative bacteria also perform two different functions such that they either promote or inhibit corrosion. The presence of some facultative taxa can stimulate the corrosion rate because of electron transfer and/or the catalysis of nitrogenase, *Vibrio natriegens* (Yin et al., 2009) for example, while the addition of these bacteria can also promote metal corrosion by anaerobic species (Benbouzid-Rollet et al., 1991). It has also been shown that biofilms produced by the gramicidin-producing species *Bacillus brevis* can inhibit SRB metal corrosion (Nikolaev and Plakunov, 2007). In this context, *V*. *neocaledonicus* is also thought to be a strong corrosion inhibitor because it forms a protective layer and produces extracellular polymeric substances (Moradi et al., 2015a, b).

SRB comprise an important category of facultative bacteria in this area because they perform sulfate reduction functions and are considered to be closely related to corrosion. Additional species that are also thought to perform corrosion-related functions include *Vibrio* and *Bacillus* species, while some facultative anaerobes that have been successfully isolated from samples and that have confirmed sulfate-reducing behavior include *Vibrio* species, *Stenotrophomonas maltophilia*, *B*. *cereus*, and *B*. *licheniformis* (Marez et al., 1971; Selvaraj et al., 2017). Studies on isolated strains remain inadequate, however, compared to research on anaerobic SRB; facultative SRB have also been utilized for the reduction of sulfur in fossil fuels prior to combustion, in particular biodesulfurization (Rath et al., 2012), including species of *Bacillus* and *Enterobacter* (Rath et al., 2012; Babul et al., 2014). The discovery of new facultative SRB will therefore be beneficial and will enable a clearer understanding of biological desulfurization and its mechanisms.

Corroded samples subjected to immersion times of different lengths were collected from sites at Sanya and Xiamen, China. Numbers of culturable aerobic bacteria (CAB) and SRB were determined for these samples and CAB isolated from plates were identified using partial 16S rRNA gene sequencing. Strains of facultative SRB were also screened from these isolated strains. The results of this study are likely to further our understanding of the corrosive functions of strains obtained as well as the relationships between different bacteria.

2 MATERIAL AND METHOD

2.1 Sample collection

The steel plates used in this analysis had the following composition (wt %): C: 0.16, Si: 0.12, Mn: 0.45, S: 0.029, and P: 0.019. A total of 12 samples were collected in December 2014, of which eight (i.e., SE1, SE2, SE3, SE4, SE5, SE6, SOH, and SSW) were collected from the Hongtang Bay coastal zone situated in Sanya City, Hainan Province, China. Sample SOH was collected from the rust layer of steel plates that had been immersed in seawater for six months, while sample SSW comprised seawater surrounding steel plates from Sanya; all other samples had been immersed in seawater for eight years. Four samples (i.e., XE4, XE5, XE6, and XSW) were collected from the coastal zone around the island of Gulang, located in Xiamen City, Fujian Province. Samples XE4, XE5, and XE6 were collected from the rust layers of steel plates that had been immersed in seawater for eight years, while sample XSW comprised seawater surrounding steel plates from Xiamen. In all cases, seawater pH, temperature, and salinity were measured using a multiparameter water quality detector (CTD90M, Germany).

All corroded samples were collected and quickly transferred into 10 mL sterile plastic centrifuge tubes following preparation according to a standard procedure. Initially, whole corroded steel plates were removed from the sea and large fouling organisms were removed with sterile forceps under sterile conditions before being rinsed with similarly cleaned seawater to remove unattached bacteria. Corroded samples were then transported to the laboratory on

dry ice within 12 h (Païssé et al., 2013; Sun et al., 2014).

2.2 Bacterial analysis

Approximately 2 g of each corroded sample was suspended in 18 mL of sterile seawater with seven glass beads and Tween 80 (working fluid concentration, 1:2 000 v/v; final volume ratio, 1:200 000 v/v), and it was subjected to mechanical grinding in a shaker incubator at 100 r/min for 30 min.

2.3 SRB and CAB determination

The most-probable-number (MPN) approach based on national standards (GB/T 14643.5-93) was used in this study for SRB determination. This involved the production of triplicate tenfold serial dilutions (between 10^{-1} and 10^{-3}) by inoculating 1 mL into 9 mL of sterile seawater; 1 mL samples of these tenfold serial dilutions (between 10^{-1} and 10^{-3}) were then separately transferred into liquid media in triplicate. The presence of SRB was then recorded by observing sample color change after aerobic incubation at 25°C for 21 days. Bacterial numbers in corroded product layers were then calculated using the statistical tables in the national standards (GB/T 14643.5-93).

Isolates were obtained from corroded samples using a 100-μL inoculation of tenfold serial dilutions (between 10^{-1} and 10^{-3}) placed onto the solid media in triplicate. In order to obtain a relatively broad phylogenetic range of different bacteria, 2216E and PGC media were also used to inoculate cell suspensions. The 2216E medium used in this study contained peptone (5.0 g/L), yeast (1.0 g/L), $FeC_6H_5O_7$ (0.1 g/L), and agar (20.0 g/L) with a pH of 7.8. The PGC medium contained KH_2PO_4 (0.5 g/L), NH_4Cl (1.0 g/L), $CaCl_2·6H_2O$ (0.06 g/L) $MgSO_4·7H_2O$ (0.06 g/L), yeast (1.0 g/L), $C_3H_5O_3Na$ (70%) (6 mL/L), $FeSO_4·7H_2O$ (0.004 g/L), $Na_3C_6H_5O_7·2H_2O$ (0.3 g/L), and agar (20.0 g/L) with a pH between 7.1 and 7.3. These media were both sterilized at 121℃ for 15 min; after four days at 25°C, the number of colony-forming units (CFU) were counted to assess bacterial growth. Plates of between 30 and 300 colonies were selected to count the number of CAB and results were expressed in CFU/g.

2.4 Isolating CAB using heterotrophic plate counts (HPC)

Colonies from half or one-quarter plates (i.e., about 30 strains of bacteria per plate) were picked in each case and subcultured onto fresh plates with the same medium until each isolate had the same colony morphology. The pure cultures obtained were then preserved as glycerol (15% v/v) stocks at -20°C and -80°C (Liu et al., 2012).

2.5 Screening facultative SRB

Aerobically isolated bacteria were inoculated into a Postgate B modified liquid medium (an anaerobic medium for SRB) containing KH_2PO_4 (0.5 g/L), NH_4Cl (1.0 g/L), $CaCl_2·2H_2O$ (0.1 g/L), Na_2SO_4 (1.0 g/L), $MgSO_4·7H_2O$ (2.0 g/L), $C_3H_5O_3Na$ (80%) (3.5 mL/L), yeast (1.0 g/L), $FeSO_4·7H_2O$ (0.5 g/L), vitamin C (0.1 g/L), and H-Cys-OH·HCl (0.5 g/L) with a pH between 7.0 and 7.2. Following 72 h incubation, the presence of a black precipitate in the bottles indicated the presence of FeS/H_2S, and the strain (facultative anaerobic bacteria) was determined as the target.

2.6 Genomic DNA extraction and 16S rRNA gene amplification

The genomic DNA from each bacterial strain isolated from solid media was extracted using a TIANamp Bacteria DNA Kit, following the manufacturer's instructions. The integrity of these DNA isolates were then examined using 1% agarose gel electrophoresis and concentrations were recorded by reading absorbance at 260 nm.

The 16S rRNA genes of bacteria isolated from corrosion products were then amplified using the 16S universal primers 27F and 1492R. The reaction mixture used for PCR in this case contained 2.0 μL of 10×Ex Taq buffer, 1.6 μL of 2.5 mmol/L dNTP mix, 0.8 μL of each primer, 0.5 μL of template, 0.2 μL of 5u Ex Taq, and 14.1 μL of ddH_2O. The PCR protocol used comprised an initial denaturation step at 95°C for 5 min followed by 24 cycles at 95°C for 30 s, at 55°C for 30 s, at 72°C for 1 min 30 s, and a final extension step at 72°C for 10 min. All samples were sent to Shanghai Majorbio Bio-pharm Technology Co., Ltd., where the 16S rDNA gene was sequenced.

2.7 Phylogenetic analysis

Sequence analyses were performed with reference to the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/) for genus identification. The software ClustalW was used to align closely related strains of sequences, and the Kimura-2 parameter was used to calculate

Table 1 Numbers of culturable bacteria and SRB in corroded samples and seawater

Source	Site	Immersed time (year)	No. of CAB (CFU/g)		No. of SRB
			2216E	PGC	
Sanya	SE-1	8	7.93×10^5	9.37×10^4	4.5×10^4
	SE-2	8	9.22×10^4	3.06×10^4	1.4×10^4
	SE-3	8	1.78×10^6	1.68×10^5	1.4×10^4
	SE-4	8	1.04×10^5	3.10×10^4	1.4×10^4
	SE-5	8	2.63×10^5	1.05×10^5	1.4×10^4
	SE-6	8	1.30×10^4	3.18×10^3	9.5×10^2
	SOH	0.5	9.91×10^6	2.53×10^6	9.8×10^4
	SSW		9.67×10^2	2.82×10^3	9.5×10^1
Xiamen	XE-4	8	1.44×10^4	4.94×10^4	8.5×10^3
	XE-5	8	4.95×10^4	1.03×10^4	3.5×10^3
	XE-6	8	1.09×10^4	1.59×10^4	7.0×10^3
	XSW		5.27×10^2	1.83×10^2	4.5×10^1

The unit of No. of SRB of the corroded samples is cell/g; the unit of No. of SRB of the seawater is L/g.

Table 2 Culturable bacterial community compositions at phylum level

	Total taxonomy (%)	Sanya: 2216E (%)	Sanya: PGC (%)	Xiamen: 2216E (%)	Xiamen: PGC (%)
Proteobacteria	49.30	31.46	16.43	0.47	0.94
Firmicutes	48.83	13.15	15.96	8.92	10.80
Bacteroidetes	0.94	0.47	0	0.47	0
Actinobacteria	0.94	0.47	0.47	0	0

Table 3 Culturable bacterial community compositions at genus level

	Total taxonomy (%)	Sanya: 2216E (%)	Sanya: PGC (%)	Xiamen: 2216E (%)	Xiamen: PGC (%)
Bacillus	40.38	10.80	12.21	7.04	10.33
Vibrio	12.68	8.45	4.23	0	0
Microbulbifer	6.10	2.81	2.35	0.47	0.47
Pseudoalteromonas	6.10	4.69	1.41	0	0
Ruegeria	5.16	2.81	2.35	0	0
Halobacillus	3.76	1.88	1.88	0	0
Fictibacillus	2.35	0	0.47	1.41	0.47
Rhodobacteraceae	2.35	1.88	0.47	0	0
Alpha	1.88	1.88	0	0	0
Others	19.25	10.33	7.51	0.94	0.47

evolutionary distances. Phylogenetic tree construction was performed using the software MEGA 5.1 using the neighbor joining approach and the statistical support of hypotheses was assessed using 1 000 bootstrap replicates.

3 RESULT

3.1 CAB and SRB abundances

The colonies formed on plates exhibited a variety of morphological appearances, pigmentation levels, and growth patterns after four days of incubation. Plate count analysis revealed variations in the number of CAB from corroded samples in different media. Results show that the number of CAB on samples grown in the 2216E medium ranged between 1.30×10^4 and 1.78×10^6 CFU/g and between 1.09×10^4 and 4.95×10^4 CFU/g in the case of plates immersed for eight years from Sanya and Xiamen, respectively, while numbers on the plate immersed for just six months (SOH, Xiamen) were 9.91×10^6 CFU/g. In contrast, the numbers of CAB recorded on SSW and XSW samples were 9.67×10^2 CFU/mL and 5.27×10^2 CFU/mL, respectively, while the number of CAB ranged between 3.18×10^3 and 1.68×10^5 CFU/g and between 1.03×10^4 and 4.94×10^4 CFU/g in the case of samples grown in the PGC medium and that had been immersed at Sanya and Xiamen for eight years, respectively. The number of CAB of SOH (Xiamen) was 2.53×10^6 CFU/g, while the number of SSW and XSW were 2.82×10^3 CFU/mL and 1.83×10^2 CFU/mL, respectively.

The color of some tubes changed after aerobic incubation for 21 days at 25°C. The results of the MPN method show that the number of SRB ranged between 9.5×10^2 and 4.50×10^4 CFU/g and between 3.5×10^3 and 8.5×10^3 CFU/g in the case of samples immersed for eight years at Sanya and Xiamen, respectively, while the number of SRB in SOH was 9.8×10^4 CFU/g. Similarly, the numbers of SRB in SSW and XSW were 9.50×10^1 CFU/mL and 4.50×10^1 CFU/mL, respectively (Table 1).

3.2 Identification of isolated bacteria

Results show that all sequences matched at least one identified strain with a degree of similarity between 97% and 100%. This suggests that no novel bacterial species were identified; a representative of each operational taxonomic unit (OTU) was then selected for phylogenetic analysis using 16S rRNA gene sequences (Fig.1).

Identification results for isolated bacteria are shown in Figs.2, 3 (combined analysis of isolated strains from the two media), Tables 2, 3. Four prokaryotic phyla were identified within the nine corroded samples, Proteobacteria (49.30%),

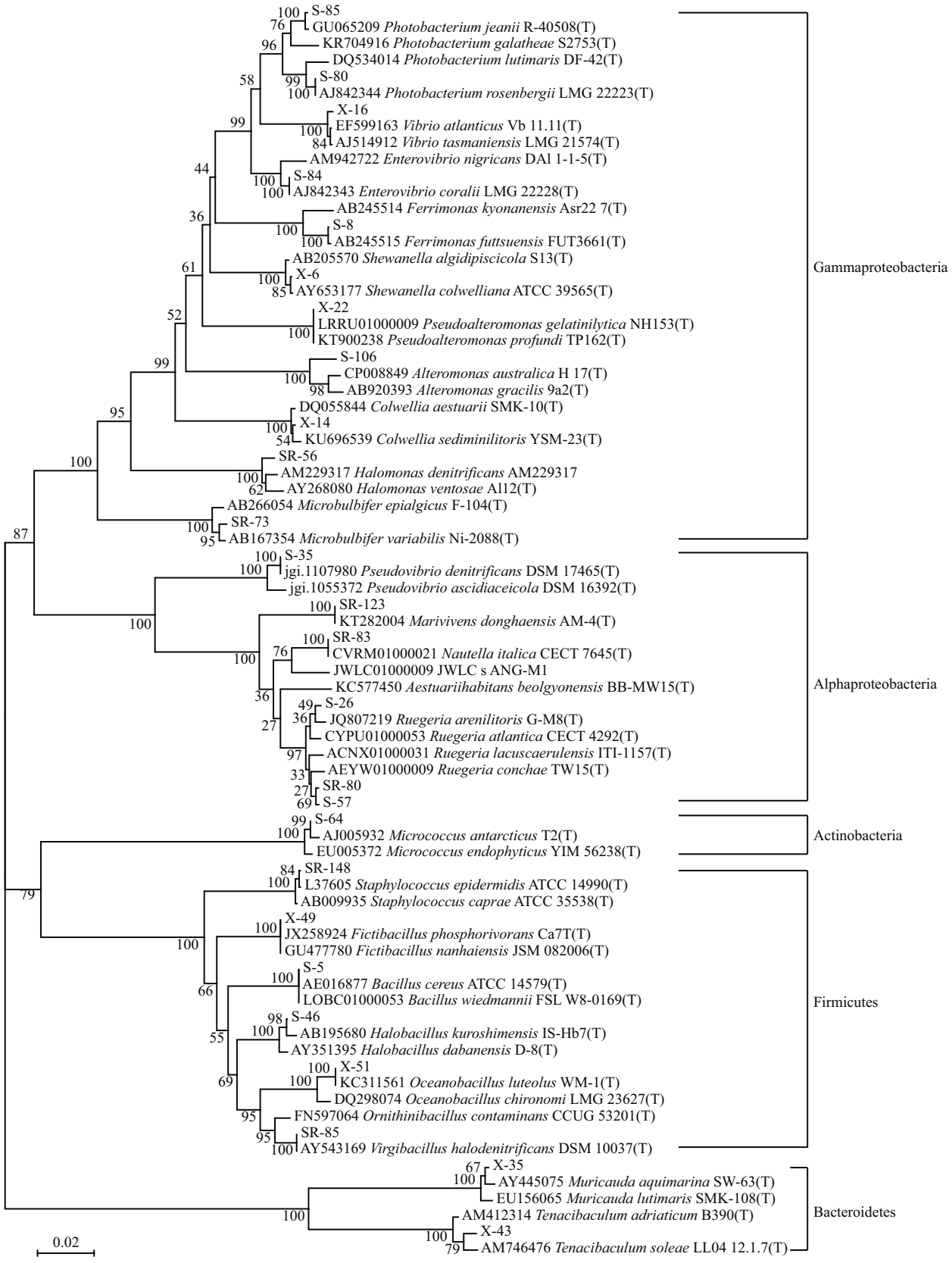

Fig.1 Neighbor joining phylogenetic tree based on 16S rDNA sequences for OTUs of bacteria isolated by solid media culture and related species from GenBank

Numbers are bootstrap percentages from 1 000 replicates.

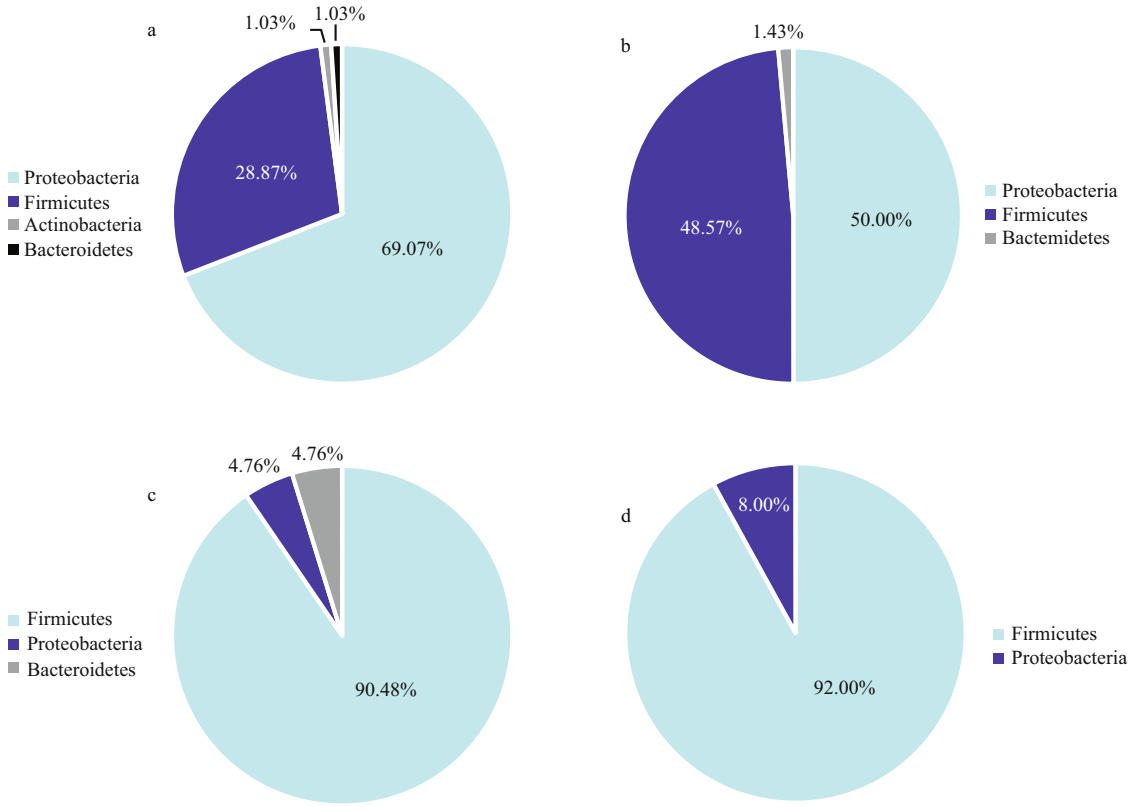

Fig.2 Relative abundances of CAB at the phylum level within corroded samples

Samples from Sanya isolated on 2216E (a) and PGC (b) media, respectively. Samples from Xiamen isolated on 2216E (c) and PGC (d) media, respectively.

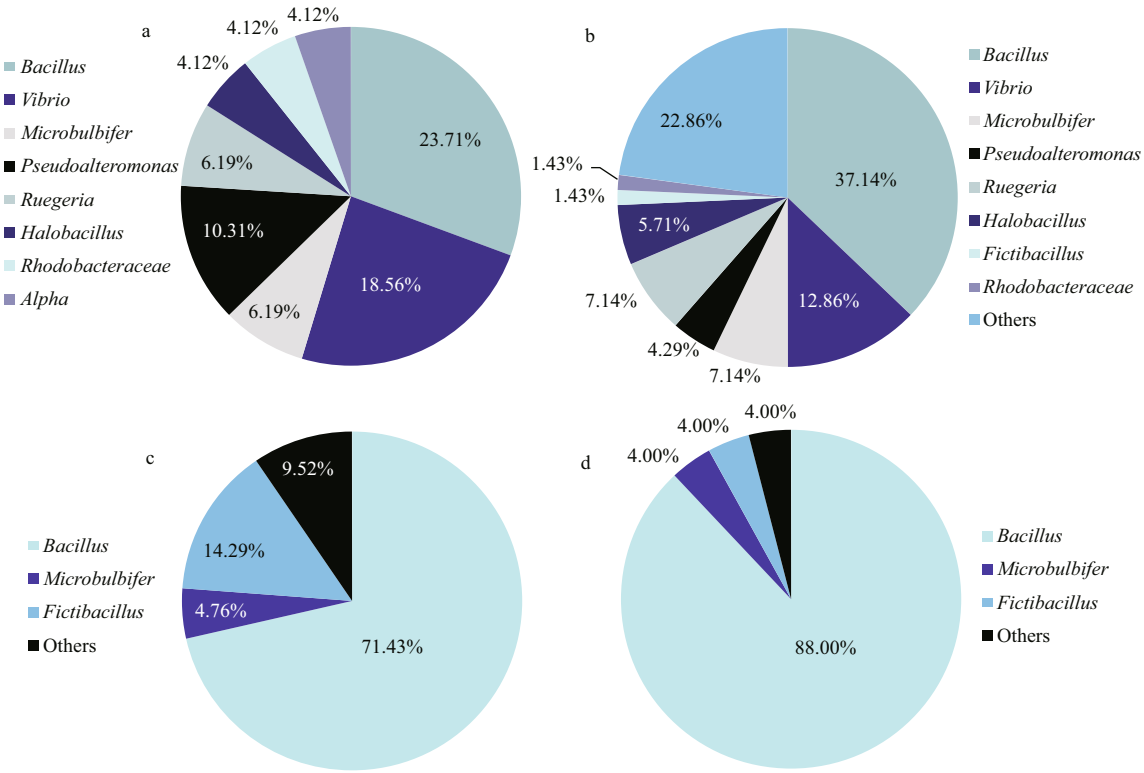

Fig.3 Relative abundances of CAB at the genus level within corroded samples

Samples from Sanya isolated on 2216E (a) and PGC (b) media, respectively. Samples from Xiamen isolated on 2216E (c) and PGC (d) media, respectively.

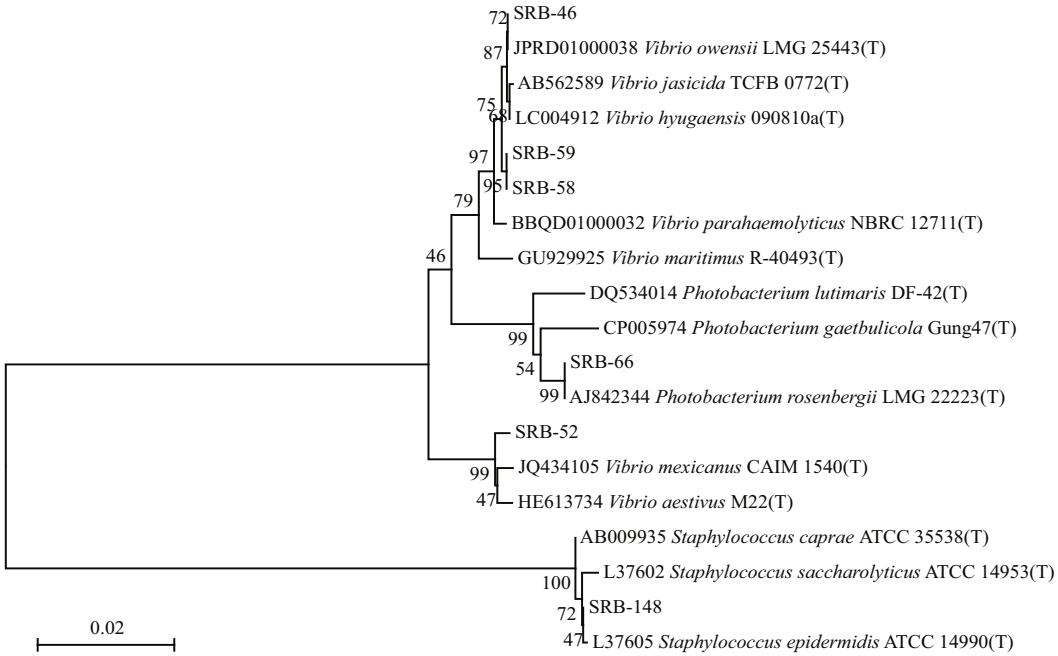

Fig.4 Neighbor joining phylogenetic tree based on 16S rDNA sequences for facultative SRB and related species from GenBank

Numbers are bootstrap percentages from 1 000 replicates.

Firmicutes (48.83%), Bacteroidetes (0.94%), and Actinobacteria (0.94%) (Fig.2 and Table 2). All four of these phyla were identified in the corroded samples from Sanya, with Proteobacteria dominant followed by Firmicutes. In contrast, just three (i.e., Firmicutes, Proteobacteria, and Bacteroidetes) were found in the corroded samples from Xiamen; Firmicutes was dominant in this case, followed by Proteobacteria.

A large number of sequences were classified at the genus level and 31 were identified in the nine corroded samples. Nine genera were most abundant, encompassing 80.75% of the total, *Bacillus* (40.38%), *Vibrio* (12.68%), *Microbulbifer* (6.10%), *Pseudoalteromonas* (6.10%), *Ruegeria* (5.16%), *Halobacillus* (3.76%), *Fictibacillus* (2.35%), *Rhodobacteraceae* (2.35%), and *Alpha* (1.88%) (Fig.3 and Table 3). A total of 28 and 19 genera were found in the samples from Sanya and Xiamen, respectively; *Bacillus* was dominant regardless of geographic location, ranging in abundance between 7.04% and 12.21%, while *Vibrio* and *Fictibacillus* were the second most dominant in samples from Sanya and Xiamen, respectively.

3.3 Facultative SRB

Six bacterial strains (i.e., SR-59, SR-46, SR-58, SR-52, SR-148, and SR-66) belonging to facultative SRB were obtained by screening isolates. Results show that four of these strains belonged to the genus *Vibrio* within the family Vibrionaceae (class Gammaproteobacteria), and were identified as distinct species. Data show that SR-59 is similar to *V. parahaemolyticus* NBRC 12711(T) BBQD01000032 (99.46% similarity with the type strain), while SR-46 and SR-58 are similar to *V. owensii* LMG 25443(T) JPRD01000038 (99.92% and 99.43% similarity with type strains, respectively), and SR-52 is similar to *V. mexicanus* strain CAIM 1540(T) JQ434105 (99.07% similarity with the type strain). In contrast, results show that SR-148 can be assigned to the genus *Staphylococcus* within the family Staphylococcaceae (class Bacilli) and is particularly similar to *Staphylococcus epidermidis* strain ATCC 14990(T) L37605 (99.7% similarity with the type strain). Phylogenetic analyses of the 16S rRNA gene sequence from strain SR-66 showed that this isolate belongs to the genus *Photobacterium* within the family Vibrionaceae (class Gammaproteobacteria) and that it is identical to *Photobacterium rosenbergii* strain LMG 22223(T) AJ842344 (100% similarity to the type strain). These phylogenetic results are presented in Fig.4.

4 DISCUSSION

4.1 SRB and CAB abundances on corroded samples

Bacterial abundances on corroded steel are

determined by numerous factors, including salinity, pH value, temperature, and immersion time (Guo et al., 2006). The results of this study show that the numbers of SRB and CAB on immersed steel are related to immersion time and sea location. Related research in this area has also noted that the maturity or age of biofilms is one of the most important factors governing bacterial communities (Neriagonzález et al., 2006) and it is well known that these populations develop and mature over the course of years, months, days, or even hours (Li et al., 2013). In this study, the numbers of SRB and CAB associated with corroded samples decreased with immersion time; the numbers recorded on samples immersed for just six months were greater than those seen on samples immersed for eight years. In similar work, Lanneluc et al. (2015) analyzed bacterial numbers in samples immersed for between one and eight weeks and demonstrated an increasing tendency over time. This result might be due to the exposure of steel to local acidification such that the redox potential decreases over time and the environment in the biofilm changes. As oxygen conditions change in the environment, the proportion of aerobic, facultative, and anaerobic bacteria will also vary (Yang et al., 2011). This study differs from the earlier work of Lanneluc et al. (2015) in terms of the respective corrosion stages investigated and this may explain the differences in results. Indeed, the variation reported here is corroborated by the study of Guo et al. (2006) who analyzed the numbers of corrosive bacteria in an internal carbon steel rust layer exposed to sea water for varying periods of time. The numbers of corrosive bacteria initially rose in this study and then subsequently dropped over the course of a period between one month and 120 months, while the proportion of culturable bacteria in corroded samples was found to be much higher than in seawater (Guo et al., 2006), a finding that is also consistent with our results. We also counted the numbers of CAB and SRB; our results show that samples immersed for six months had higher numbers of bacteria than their counterparts that had been immersed for eight years. The numbers of CAB and SRB in both samples were much higher than those recorded from seawater.

Data show that bacterial numbers are also related to sea location and likely change as a result of environmental variations. These conditions vary widely depending on location, and differences in temperature are particularly important for bacterial colonization. The results of this study show that the numbers of SRB and CAB in Sanya samples immersed for eight years were higher than those from Xiamen, a difference that may be because of seawater temperature. The water temperature at Sanya, a site in the tropics (25.14°C) was nearly 5°C higher than that at Xiamen which is in the subtropics (19.27°C), while there is little difference between the two in terms of either salinity (i.e., Sanya 33.97; Xiamen 31.96) or pH (i.e., Sanya pH 8.48; Xiamen pH 8.56). Previous research has shown that bacterial numbers in biofilms are associated with temperature (Bott, 1996), and the earlier work of Guo et al. (2006) also corroborates this conclusion; these workers also noted that the larger number of bacteria in Yulin station carbon steel rust layers compared to Qingdao is due to differences in temperature between the two locations. It is thought that temperature plays an important role in controlling bacterial numbers in carbon steel rust layers via biofilm metabolism; this variable also influences changes in most other biofilm parameters and their propagation in aquatic systems (Bott, 1996; Rao, 2010).

4.2 Isolated bacteria in corroded samples

Compared with seawater or ocean sediments, the bacterial community composition of corroded samples tends to be similar at the phylum level, but not at the genus level. Although previous work has shown that Proteobacteria is the dominant phylum in samples (Liu et al., 2014; Prasad et al., 2014), inconsistent results have been recovered regarding the genera; while *Flavobacterium* and *Shewanella* were the dominant genera in Kongsfjorden water and sediments, *Bacillus* species were shown to be dominant in marine sedimentary environments of the South China Sea (Liu et al., 2014; Prasad et al., 2014). The composition of bacteria in corrosive environments compared to other samples may therefore also be either similar or different.

The results of this study show that Proteobacteria and Firmicutes were the core phyla at both Sanya and Xiamen sites. Indeed, this result was also recovered when the same samples were studied using high throughput sequencing (Li et al., 2017). The conclusion that Proteobacteria is the dominant phylum in samples is also consistent with previous studies (Lee, et al., 2008; Dang et al., 2011; McBeth and David, 2016); Vigneron et al. (2016) also noted that the members of this phylum were dominant in a study of corroded samples from an offshore oil production facility. Proteobacteria are pioneer surface

colonizers, and can even sustain other species in this environment; this ecological preference explains their contribution to biofilm formation (Slightom and Buchan, 2009; Dang et al., 2011). Bacteria within this phylum therefore tend to have a close relationship with corrosion, and it is usual for them to be prevalent in these kinds of environments.

Members of the phylum Firmicutes were dominant in corroded samples from Xiamen, another unsurprising result. These bacteria have previously been recorded in large numbers in rust layer biofilms (Zhang and Fang, 2001; Luan et al., 2012) and earlier work has also shown that a number of species within this group are related to corrosion. Some SRB species produce hydrogen (e.g., *Tindallia texcoconensis*; Alazard et al., 2007) and, more importantly, some taxa within Firmicutes cause corrosion by generating organic acids and H_2S (e.g., *Acetobacterium carbinolicum*; Paarup et al., 2006). It is therefore unsurprising that large numbers of Firmicutes species were identified in this research.

The phyla Actinobacteria and Bacteroidetes were also identified in this study. This is noteworthy because species within these groups have been detected in previous research on corrosive bacterial communities using culture-independent techniques (Luan et al., 2012; Palaniappan and Toleti, 2016). Representatives of the first of these groups, Actinobacteria, have been shown to be dominant in regions characterized by high H_2S concentrations (Dong et al., 2017), while species within Bacteroidetes can contribute to the consumption of oxygen in biofilms (Kirchman, 2002) and therefore degrade complex biopolymers. These bacteria can also contribute to sustaining surface colonizers (Dang et al., 2011).

At the genus level, *Bacillus*, *Microbulbifer*, and *Vibrio* species all made up a significant proportion of the cultivable aerobic bacteria recorded at both Sanya and Xiamen. The first of these genera, *Bacillus*, is very important in steel corrosion and was unsurprisingly dominant in this study. This result agrees with previous studies that have shown *Bacillus* to be the dominant genus of bacteria present during the early stages of carbon steel corrosion, including on the corrosion surfaces of carbon steel plates extracted from a Brazilian oil platform (Bolton et al., 2010; Marques et al., 2012; Lanneluc et al., 2015). Interestingly, opposite corrosion-related functions might be seen between different *Bacillus* strains and metals; some strains of this bacteria accelerate the corrosion of steel, such as *B. subtilis* A1, *B. cereus* A4, and *B. subtilis* C2 (Qu et al., 2015; Parthipan et al., 2017), while others have been shown to inhibit this process (Wadood et al., 2015). As the corrosion mechanisms seen in the various species of *Bacillus* are very complex, one single mechanism cannot currently be proposed (Mansfeld, 2007).

The results of this study show that *Vibrio* was the second most dominant genus of bacteria in corroded samples from Sanya. These bacteria are also widespread in the corrosive environment; *Vibrio* species were detected with molecular methods in carbon steel after one month of immersion (Bermont-Bouis et al., 2007), and Chen (2014) also reported this genus to be dominant using culture methods. As is the case for *Bacillus*, *Vibrio* species play a very important role in steel corrosion and can also cause opposing effects. Several studies have shown that these bacteria can promote corrosion, for example; Cheng et al. (2010) observed a rapid increase in the corrosion of 303 SS and copper because of the N_2-fixation of *V. natriegens*, while the oxygen scavenger, *V. natriegens* can also promote the growth of SRB and enhance the corrosion of carbon steel and stainless steel (Dowling et al., 1988; Benbouzid-Rollet et al., 1991). In contrast, other studies have also shown that these species can inhibit corrosion; *V. neocaledonicus* has even been regarded as the most effective corrosion inhibitor because of its attachment, absorption of organic materials, and inhibitive layer formation (Moradi et al., 2015a, b). Few studies, however, have compared these varying characteristics in *Vibrio* species and current research has been insufficiently comprehensive to date. This remains an area for future research.

The fact that species within the genus *Pseudoalteromonas* were recorded in low numbers in this study is noteworthy because numerous previous investigations have discussed the characteristics of this bacteria. Species within this genus have chemoheterotrophic metabolic characteristics which have attracted attention because they can produce extracellular active compounds, including enzymes (Holmström and Kjelleberg, 1999; Sánchez-Porro et al., 2003). This ability enables these bacteria to compete for nutrition, settle on surfaces, live alongside other organisms, and absorb complex organic compounds (Holmström and Kjelleberg, 1999). Although related research has shown that *Pseudoalteromonas* species are dominant in fouling biofilms (Yu et al., 2014), few studies have addressed

the corrosive effects of these bacteria and results are variable. Moradi et al. (2014) reported that *Pseudoalteromonas* species can cause localized corrosion of 2205 duplex stainless steel (DSS) alloys via adhesion and the formation of a porous biofilm, while Zhang et al. (2016) reported that these bacterial can also inhibit corrosion via oxygen depletion and the formation of such a film. Research on this bacterial genus currently remains limited and few strains have so far been reported.

Although the remaining genera reported in this study were only present in small proportions, some of these strains have been previously shown to be related to corrosion. Some species of *Shewanella*, for example, noted in this study, have previously been linked with microbiologically influenced corrosion (Little et al., 1997). However, due to the lack of research concerning bacterial effects on corrosion performance, the degree to which this species can be understood to have an effect on corrosion remains difficult to assess. As both anaerobic and facultative species occur in corrosive environments, these habitats likely experience both the promotion and inhibition of bacterial growth and metabolism; further research in this area is necessary in light of these complex effects.

4.3 Comparative analysis of bacterial community composition

The bacterial community compositions of Sanya and Xiamen differed widely. Data show that more kinds of bacteria were present on the Sanya samples immersed for eight years (i.e., 28 genera in five phyla) than were present at Xiamen (i.e., 19 genera in three phyla), and we speculate that temperature was a major factor explaining this difference. Meng et al. (2016) also showed that the bacterial community compositions of crude oil-contaminated marine sediments and seawater are strongly linked to temperature; indeed, our data also show a slight difference in the dominant population of cultivable aerobic taxa between the corroded samples from Sanya and those from Xiamen. Species within Proteobacteria were dominant at Sanya while Firmicutes was the second most common phylum; in contrast, Firmicutes species were most common at Xiamen, followed by representatives from Proteobacteria. These results are consistent with those reported previously by Boudaud et al. (2010), who also found correlations between isolates and the geographical sampling zones. One other study has also demonstrated that a diverse phylogenetic range of bacteria are involved in the production of electricity due to changes in the environment (Holmes et al., 2004).

Because of the limited number of samples included in this study, differences between immersion times and bacterial community composition could not be clearly determined. Obvious differences were nevertheless found between the proportions of bacteria present in samples immersed for eight years (i.e., 29 genera within five phyla) and those that had been underwater for just six months (i.e., 12 genera within three phyla). This may, however, be due to the limited number of bacteria cultured in this study, as the number of strains isolated from the latter group of samples was considerable less than from those immersed for longer. The next step in our research will be to collect more samples to obtain more comprehensive and accurate test data regarding bacterial communities.

4.4 Six facultative SRB

The results showed that only six bacteria have a function of sulfate reduction. It seems that this reduction was carried out by producing of hydrogen sulfide which can blacken the media. SRB refers to two definitions; first one is the general designation of bacteria which can reduce sulfate (Gibson, 1990; Garrity et al., 2004) and the second is bacteria that reduce sulfate by dissimilation. In this study we referred to the former definition (Gall, 1975).

A limited number of previous reports have discussed facultative SRB (Rath et al., 2012; Babul et al., 2014). This study therefore represent significant progress as we screened six facultative SRB from our strains, and present the first report of *Photobacterium* in this context. No studies are presently available, however, that can be used for comparisons with our results.

Species of *Photobacterium* are common gram-negative coccobacilli that are distributed in marine habitats worldwide. Some of these species are capable of producing antibacterial compounds, including polyunsaturated fatty acids (Moi et al., 2017). Although these species have been widely utilized for environmental monitoring, no study to date has associated them with corrosion. This may be because the method used in this study differs from previous research in which facultative bacteria are screened from their anaerobic counterparts (Wang, 2014); for this reason alone, higher numbers of facultative SRB

were likely identified in this study. We are also the first to note the sulfate-reducing capabilities of this genus; future research will include corrosivity tests to verify our conclusions.

Numerous studies have discussed facultative SRB which use sulfates or other oxidized sulfides as electron acceptors to alienate organic matter, including research on the genera *Vibrio* and *Bacillus* (Benbouzid-Rollet et al., 1991; Moradi et al., 2015a, b). The corrosive impact of *Vibrio* in particular has been studied by many researchers, and members of this genus are also regarded as effective corrosion inhibitors (Benbouzid-Rollet et al., 1991; Moradi et al., 2015a, b). Few studies to date, however, have noted that *Staphylococcus* is a facultative SRB or described its corrosive effects; in one earlier study, Ponmariappan et al. (2004) noted a significant reduction in corrosion rate due to the formation of a metal surface biofilm.

5 CONCLUSION

The cultivable aerobic bacterial community compositions of corroded samples collected from steel plate rust layers immersed in seawater at Sanya and Xiamen were analyzed in this study. The results show that the number of bacteria present on immersed steel is related to both immersion time and sea location. In addition, the composition of CAB of corroded samples is also related to sea location. A total of 213 bacterial isolates were selected from different media in this study and were identified using 16S rRNA gene partial sequencing. Four bacterial phyla were identified, including Firmicutes and Proteobacteria which were most common. A total of 31 bacterial genera were identified within the nine corroded samples; data show that *Bacillus* species were most common, encompassing 40.38% of the total. Six facultative SRB were also screened from the strains isolated in this study.

6 DATA AVAILABILITY STATEMENT

All sequence data were submitted to GenBank under accession numbers between MF593988 and MF594194. The datasets analyzed in this study are available from the corresponding author on request.

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