Communication

Analysis of the Bacterial Community of Metal Scrap Using an Enrichment Culture Approach

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Abstract: Microbiologically influenced corrosion (MIC) of metal alloys is promoted by biofilms formed on metal surfaces. In the marine environment, MIC causes serious metal infrastructure problems, which lead to significant economic losses. In this study, we used an enrichment culture approach to examine the bacterial community that grows on metal surface at levels below the detection limit as a preliminary study for developing guidelines to prevent biofilm formation. An enrichment culture approach was employed to analyze the bacterial community on metal surface without biofilms and corrosion. Genomic DNA was extracted from culture sample after incubation in the enrichment culture with a metal piece, and then the V3–V4 variable regions of the bacterial 16S rRNA gene were amplified using the extracted genomic DNA as the template. Subsequently, using a next-generation sequencing approach, the amplified V3–V4 regions were sequenced, and the bacterial community was analyzed using the QIIME 2 microbiome bioinformatics platform. Using this enrichment culture approach, more than 80 bacterial genera were detected with *Sphingomonas* bacteria exhibiting the highest relative abundance (44%). These results demonstrated that this method could be useful for bacterial community analysis for bacteria below detection limits, and will serve as a basis for the development of the guidelines.

Keywords: 16S rRNA gene; bacterial community analysis; microbiologically influenced corrosion; metal piece; enrichment culture approach

1. Introduction

Microorganisms grow by adapting to environmental conditions. Biofilms are biological membranes enclosed in a matrix composed of polymeric substances produced by multiple species of microorganisms [1]. When microorganisms adhere to the metal surfaces of infrastructure in the marine environment such as bridges, offshore wind-power equipment and ocean-going vessels, biofilms form on the metal surfaces. The polymeric substance matrix of the biofilm protects the microorganisms within the biofilm from stress associated with chemicals and environmental changes [1]. Thus, a favorable environment for the growth of microorganisms is created within the biofilm [1]. When biofilms form over time on metal surfaces of infrastructure, the relative abundance of corrosion-causing microorganisms increases, ultimately leading to microbiologically influenced corrosion (MIC). Marine infrastructure components affected by MIC exhibit progressive metal degradation on the metal surfaces of the infrastructure, which reduces its useful lifetime. It is estimated that MIC is responsible for approximately 20% of metal corrosion affecting marine infrastructure components [2]. For example, in 2013, it was estimated that MIC causes economic losses of $2.5 trillion, which correspond to approximately 3.4% of the gross world product. MIC of the marine infrastructure is therefore a global problem.

High-throughput amplicon sequencing is an effective method for identifying the bacterial community that causes MIC. Sulfate-reducing and sulfur-oxidizing bacteria are

the major causative organisms of corrosion for several metal alloys including aluminum alloy [3], carbon steel [4], stainless steel [5] and other engineering steel [6–8]. In some cases, iron-oxidizing, nitrate-reducing and manganese-oxidizing bacteria have been associated with MIC [9]. Those previous characterizations of the bacterial community were carried out on samples with corrosion. On the other hand, a bacterial community is changed significantly during the early stages of biofilm formation to MIC development. In other words, major bacterial species that act on biofilm formation and development as well as MIC are different. Thus, we hypothesized that guidelines to prevent biofilm formation on the metal surfaces of marine infrastructure could be established if the structure of the bacterial community on the metal surfaces could be characterized before biofilm formation. Moreover, the lifetime of marine infrastructure might be extended if the effective utilization of such guidelines could reduce the occurrence of MIC. However, no studies have examined the bacterial community before biofilm formation on metal surfaces of marine infrastructure because the number of bacteria in the community is too low, so it is difficult to prepare the sufficient genomic DNA required for high-throughput amplicon sequencing.

Enrichment culture methods are used to increase the abundance of specific bacterial species within the total bacterial population of an environmental sample, and they are different from environmental enrichment, which is used to elicit normal behavior in captive animals [10–12]. Although the culture condition needs to be set according to the purpose of the research, this method can be used to grow bacteria below the detection limit to a detectable level. Using an enrichment culture approach, previous studies have reported metal corrosion-causing bacteria or bacterial communities growing on steel [13] and stainless steel [14,15]. However, the nutritional conditions used in previous studies differed from the natural environment because organic acids, sugars or yeast extract were added as carbon sources to the enrichment culture media. Those media contained high concentrations of carbon sources compared to the natural environment, and many kinds of environmental bacteria cannot grow in those media due to their growths are inhibited by high carbon concentrations [16]. Thus, the results of previous studies may not accurately reflect the communities in the natural environment, as poor-nutrient media should be used for enrichment culture media to obtain results closer to the actual target environment.

Here we report a preliminary study for developing guidelines to prevent biofilm formation. Using a synthetic medium with low concentrations of carbonate as the single carbon source, an enrichment culture was performed with a metal piece without biofilm or corrosion and genomic DNA was extracted from the culture sample. Subsequently, we amplified the V3–V4 variable regions of the bacterial 16S rRNA gene (amplicon) using the extracted genomic DNA as a template and then analyzed the bacterial community based on high-throughput sequencing of the V3–V4 variable regions. As a result, more than 80 bacterial genera were detected by applying an enrichment culture approach, which demonstrated that this method could be useful in developing the guidelines.

2. Materials and Methods

2.1. Sampling and Enrichment Culture

Several kinds of metal pieces (approximately 2.0 cm^2) consisting of stainless steel SUS304 and free of biofilm or corrosion were collected from a non-corroded area at the Akitsu Port, Hiroshima Prefecture, Japan. The temperature on the sampling day was approximately 20 °C, and there had been no precipitation for 1 week before sample collection. The collected metal pieces were each separated into a sterile tube, immediately placed in a cool box at 4 °C, and transported to our laboratory within 2 h.

To prevent contamination, flasks were autoclaved at 121 °C for 20 min and cooled to room temperature. Subsequently, each of the metal pieces were subjected to an enrichment culture for 24 h at 25 °C in a flask containing a synthetic medium (pH 7.0) consisting of 3.0 g/L NaCl, 1.7 g/L Na$_2$HPO$_4$·12H$_2$O, 1.0 g/L K$_2$CO$_3$, 0.3 g/L KH$_2$PO$_4$, 0.1 g/L NH$_4$Cl, 0.024 g/L MgSO$_4$·7H$_2$O and 0.0011 g/L CaCl$_2$·2H$_2$O. The growth of bacteria was assessed

by monitoring the OD_{600} value in comparison with cell-free control samples using an Eppendorf BioSpectrometer (Eppendorf, Hamburg, Germany).

2.2. Genomic DNA Extraction and PCR Conditions

After genomic DNA was extracted from the culture sample using an illustra^{TM} bacteria genomicPrep Mini Spin kit (GE Healthcare, Chicago, IL, USA), the concentration and purity were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The concentration of genomic DNA extracted from the enrichment culture sample with the highest OD_{600} value was 143 ng/μL. Using genomic DNA as the template, the V3–V4 variable regions were amplified by KOD -Plus- Neo (TOYOBO) with the bacterial domain–specific primers 341F (5′-TCGTCGGCAGCGTCAGATGTGTA TAAGAGACAGCCTACGGGNGGCWGCAG-3′) [17] and 805R (5′-GTCTCGTGGGCTC GGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′) [18]. The PCR protocol for amplification of the V3–V4 variable regions entailed initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s, and extension at 68 °C for 30 s, with a final extension at 68 °C for 5 min.

2.3. Sequencing Library Preparation, Sequencing, and Bioinformatics Analysis

Using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), the PCR products (approximately 450 bp) were purified. After libraries for high-throughput amplicon sequencing were prepared using a Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA), the concentration of the sequencing libraries was determined using a Quanti Fluor™ dsDNA System (Promega). The sequencing of libraries was performed using a MiSeq sequencer (Illumina) with MiSeq Reagent Kit v3 (Illumina). The sequence data have been deposited in the DDBJ database under BioProject number PRJDB13529, BioSample number SAMD00469933 and DRA number DRA014101.
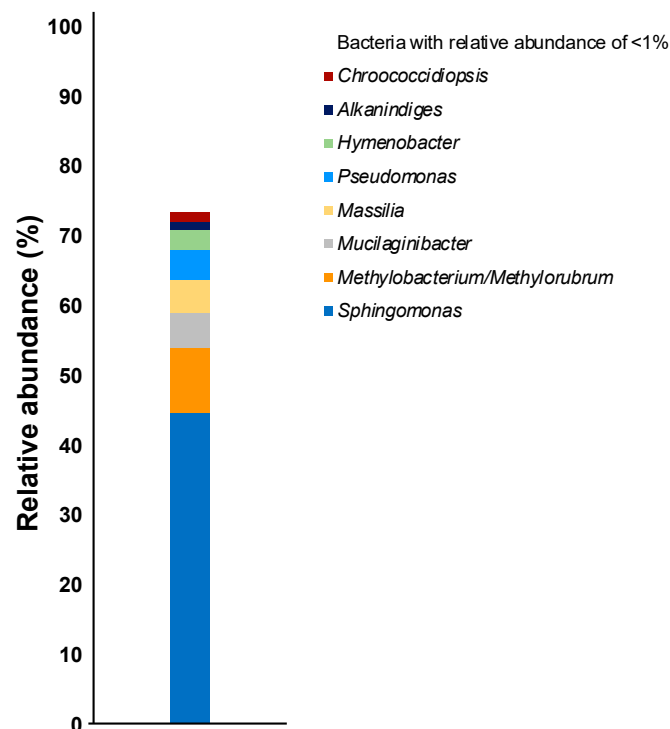
The analysis of the bacterial community was performed using the QIIME 2 microbiome bioinformatics platform [19]. After the quality of sequence data was confirmed using FastQC ver. 0.11.9 [20], the filtered sequence data were yielded. Subsequently, using the DADA2 algorithm [21], amplicon sequence variants (ASVs) were prepared based on the filtered sequence data. Using the Sliva taxonomic database ver. 138 [22,23], operation taxonomic units (OTUs) at 99% sequence identity were grouped based on the ASVs. The neighbor-joining tree based on a dataset consisting of more than 1% of the OTUs was constructed using MEGA 11 software [24].

3. Results and Discussion

When bacteria on metal surfaces are targeted for the analysis, the number of bacteria is too low for preparation of genomic DNA since high-throughput amplicon sequencing cannot be performed. Indeed, we could not extract genomic DNA from a non-cultured sample. Subsequently, to obtain a sufficient amount of bacteria for the preparation of genomic DNA while preventing deviation from the actual target environment, we used filter-sterilized seawater or artificial seawater as the enrichment culture media, but bacterial growth could not be confirmed. To increase the number of bacteria for preparation of genomic DNA, therefore, a synthetic medium containing low concentrations of nutrients was used for the enrichment culture. When this enrichment culture approach using the synthetic medium was performed for each of the metal pieces, the OD_{600} values increased from 0.08–0.14. As a control test, an enrichment culture was also performed without adding metal pieces, but no growth of the bacteria could be confirmed. Moreover, genomic DNA could be extracted from each of the culture samples, and the V3–V4 variable regions could be amplified using the genomic DNA as the template. Thus, genomic DNA extracted from the culture sample with the highest OD_{600} value was used for bacterial community analysis.

OTUs based on the analysis of genomic DNA from the cultured samples indicated the presence of more than 80 bacterial genera with the following genera present at >1% relative abundance: *Sphingomonas* (44%), *Methylobacterium/Methylorubrum* (9.4%), *Mu-*

cilaginibacter (4.9%), *Massilia* (4.8%), *Pseudomonas* (4.1%), *Hymenobacter* (2.9%), *Alkanindiges* (1.3%), and *Chroococcidiopsis* (1.2%) (Figure 1). To confirm the phylogenetic relationship between these bacterial genera, a neighbor-joining tree based on the V3–V4 variable regions was constructed (Figure 2). The neighbor-joining tree included α-Proteobacteria such as *Sphingomonas* sp. and *Methylobacterium* sp., β-Proteobacteria such as *Massilia* sp., γ-Proteobacteria such as *Pseudomonas* sp. and *Alkanindiges* sp., and Sphingobacteria such as *Mucilaginibacter* sp. In particular, *Sphingomonas* sp. 1–10 formed a major cluster with *S. kyungheensis* THG-B283[T] and *S. hankookensis* ODN7[T] and exhibited a 97.6–99.1% sequence similarity to *S. kyungheensis* THG-B283[T] (Table 1). These results suggested that a variety of bacteria existed on the metal surface before biofilm formation, and biofilm formation may be promoted when the bacterial community changes in environmental conditions and the relative abundance of bacteria with biofilm-forming ability increases. In fact, our previous study suggested that the bacterial community changes significantly in biofilms before and after the occurrence of metal corrosion [25]. On the other hand, the proportion of bacteria with a relative abundance of <1% was about 15.4% (Supplementary Table S1), and the decrease in this proportion is considered to be one of the improvements for future study. The proportion could be improved by changing the bacterial domain-specific primer set. According to Bukin et al. [26], the V2–V3 variable regions of the 16S rRNA gene are also useful for the identification of bacterial genera and species in bacterial community analysis. The V4–V5 variable regions of the 16S rRNA gene have a higher resolution for the identification of an archaeal community [27]. Thus, to decrease the proportion of unclassified bacteria, we are going to use those primer sets for bacterial community analysis in our next study.

Figure 1. Relative abundances of the bacterial communities in the culture sample at the genus level.
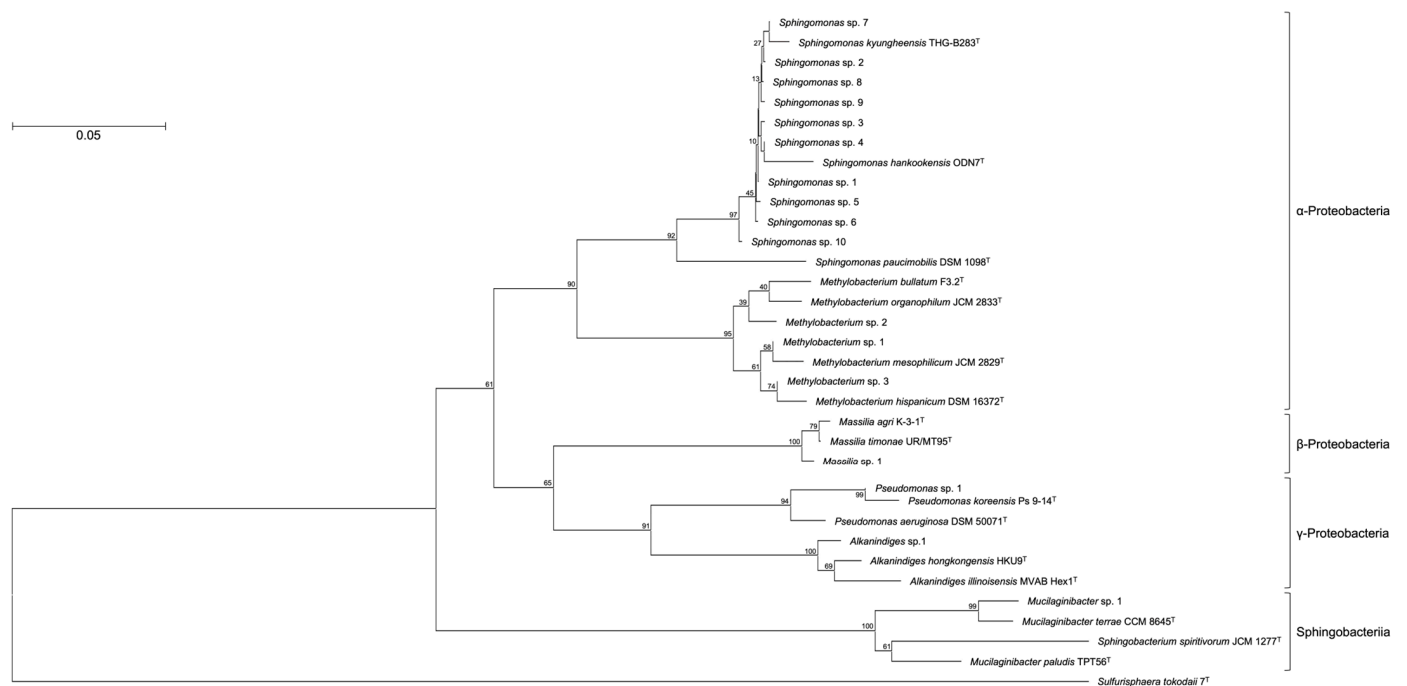
Figure 2. Consensus bootstrap phylogenetic tree constructed from analysis of the 16S rRNA gene sequences showing the relationships between OTUs comprising more than 1% and the related type strains. Each OTU is indicated as its representative type strain. The tree was rooted using *Sulfurisphaera tokodaii* 7^T as the outgroup. The bar indicates a 0.05% nucleotide substitution rate.

Table 1. Bacteria with more than 1% of OTUs observed in the culture sample.

OTU	Closest Relative		Accession No.	Identity (%)	Relative Abundance (%)
	Class	Species			
Sphingomonas sp. 1	α-Proteobacteria	*Sphingomonas kyungheensis* THG-B283^T	NR_118263	99.1	7.1
Sphingomonas sp. 2	α-Proteobacteria	*Sphingomonas kyungheensis* THG-B283^T	NR_118263	99.1	5.6
Sphingomonas sp. 3	α-Proteobacteria	*Sphingomonas kyungheensis* THG-B283^T	NR_118263	99.1	4.8
Sphingomonas sp. 4	α-Proteobacteria	*Sphingomonas kyungheensis* THG-B283^T	NR_118263	99.3	4.6
Mucilaginibacter sp. 1	Sphingobacteria	*Mucilaginibacter terrae* CCM 8645^T	NR_158094	99.7	4.3
Sphingomonas sp. 5	α-Proteobacteria	*Sphingomonas kyungheensis* THG-B283^T	NR_118263	97.6	4.2
Sphingomonas sp. 6	α-Proteobacteria	*Sphingomonas kyungheensis* THG-B283^T	NR_118263	99.1	4.0
Methylobacterium sp. 1	α-Proteobacteria	*Methylobacterium mesophilicum* JCM 2829^T	NR_115550	99.1	3.7
Pseudomonas sp. 1	γ-Proteobacteria	*Pseudomonas koreensis* Ps 9-14^T	NR_025228	99.1	3.5
Sphingomonas sp. 7	α-Proteobacteria	*Sphingomonas kyungheensis* THG-B283^T	NR_118263	99.3	3.1
Sphingomonas sp. 8	α-Proteobacteria	*Sphingomonas kyungheensis* THG-B283^T	NR_118263	99.3	2.4
Sphingomonas sp. 9	α-Proteobacteria	*Sphingomonas kyungheensis* THG-B283^T	NR_118263	99.1	2.0
Massilia sp. 1	β-Proteobacteria	*Massilia agri* K-3-1^T	NR_157781	98.5	1.9
Methylobacterium sp. 2	α-Proteobacteria	*Methylobacterium bullatum* F3.2^T	NR_116548	98.2	1.4
Sphingomonas sp. 10	α-Proteobacteria	*Sphingomonas hankookensis* ODN7^T	NR_116570	98.6	1.3
Alkanindiges sp. 1	γ-Proteobacteria	*Alkanindiges hongkongensis* HKU9^T	NR_115178	97.4	1.2
Methylobacterium sp. 3	α-Proteobacteria	*Methylobacterium hispanicum* DSM 16372^T	NR_112613	99.1	1.1

Sphingomonas bacteria are one species of the most abundant microorganisms in a variety of environments including freshwater, seawater and soils, due to the capacity of these bacteria to utilize a wide variety of organic compounds [28,29]. When *Sphingomonas* bacteria adhere to metal surfaces, microcolonies form and then biofilms develop over time [30]. Within developed biofilms in which *Sphingomonas* bacteria are the dominant species, an oxygen-free environment is created and the growth of other corrosive bacteria is accelerated, leading to MIC of the metal substrate [31]. In addition to *Sphingomonas* bacteria, other MIC-causing bacteria were observed such as *Massilia* and *Pseudomonas* bacteria (Figure 1). These bacteria reportedly accelerate metal corrosion via oxidation of

the metal surfaces to generate energy [32] and accumulate acidic metabolites [33]. These results suggest that *Sphingomonas* bacteria initiate biofilm formation when their growth is not inhibited by environmental stresses or nutrient limitations. The resulting growths of *Massilia* and *Pseudomonas* bacteria inside the biofilm encourage corrosion of the metal scrap surfaces.

In this study, bacterial community analysis was performed on metal pieces without biofilm or corrosion using a synthetic medium with carbonate as the single carbon source as a preliminary study for developing guidelines to prevent biofilm formation. The bacterial community on the metal pieces could not be analyzed without an enrichment culture approach. More than 80 bacterial genera were detected by applying an enrichment culture approach. In addition, many kinds of bacteria in the community were different from the commonly known MIC-causing bacteria, suggesting that the bacteria that act on biofilm formation are different from the MIC-causing bacteria. These results demonstrated that our idea of using low concentrations of carbonate for the enrichment culture media is useful for bacterial community analysis for bacteria below the detection limits. Moreover, the results of this study will serve as a basis for the development of guidelines for the prevention of biofilm formation on metal surfaces of infrastructure in the marine environment. To facilitate the development of the guidelines, we are planning to confirm the correlation between culture conditions and the type of sample that affects the bacterial community, which may be useful in improving the accuracy of the guidelines. These results will be presented in a future report.

4. Conclusions

The bacterial community before biofilm formation on metal surfaces has not been reported, but the analysis is necessary to develop guidelines for the prevention of biofilm formation. In this study, we demonstrated the effectiveness of bacterial community analysis using a low-nutrient media for an enrichment culture approach to identify the bacteria below detection limits. To develop accurate guidelines, it is necessary to clarify how enrichment culture conditions affect the bacterial community.

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Data Availability Statement: The 16S rRNA gene sequence of *A. hongkongensis* HKU9[T], *A. illinoisensis* MVAB Hex1[T], *M. agri* K-3-1[T], *M. timonae* UR/MT95[T], *M. bullatum* F3.2[T], *M. hispanicum* DSM 16372[T], *M. mesophilicum* JCM 2829[T], *M. organophilum* JCM 2833[T], *M. paludis* TPT56[T], *M. terrae* CCM 8645[T], *P. aeruginosa* DSM 50071[T], *P. koreensis* Ps 9-14[T], *S. hankookensis* ODN7[T], *S. kyungheensis* THG-B283[T], *S. paucimobilis* DSM 1098[T], *S. spiritivorum* JCM 1277[T], and *Sulfurisphaera tokodaii* 7[T] are available in the GenBank/EMBL/DDBJ databases under accession numbers NR_114676, NR_025254, KX672812, U54470, NR_116548, NR_112613, NR_115550, AJ400920, AM490402, NR_158094, NR_026078, NR_025228, NR_116570, NR_118263, X72722, D14026, and NR_028609, respectively.

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