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Bacterial Community Analysis of Biofilm Formed on Metal Joint

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Abstract: Microbiologically influenced corrosion (MIC) is caused by biofilms formed on metal surfaces, and MIC of metal alloys on marine infrastructure leads to severe accidents and great economic losses. Although bacterial community analyses of the biofilms collected from corroded metal have been studied, the analyses of biofilms collected from uncorroded metal are rarely reported. In this study, a biofilm formed on an uncorroded metal joint attached to a metal dock mooring at Akitsu Port was used as a model for bacterial community analysis. The bacterial community was analyzed by high-throughput sequencing of the V3–V4 variable regions of the 16S rRNA gene. Bacterial species contained in the biofilms were identified at the genus level, and *Alkanindiges* bacteria were the dominant species, which have been not reported as the dominant species in previous research on MIC. The genome sequences of known *Alkanindiges* bacteria do not have conserved gene clusters required to cause metal corrosion, which suggests that *Alkanindiges* bacteria do not corrode metals but act on the formation of biofilms. Those findings indicated that the bacterial community may change significantly during the process from biofilm formation to the occurrence of metal corrosion.

Keywords: 16S rRNA gene; bacterial community analysis; microbiologically influenced corrosion; biofilm; *Alkanindiges*

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1. Introduction

Metal alloys are widely used as raw materials for the construction of vessels, bridges, and factories using established molding technology to create products intended for long-term use in marine environments. In marine environments, biofilms are formed when microorganisms adhere to metal surfaces. Biofilms are complexes of microorganisms with a three-dimensional structure, which is enclosed in an extracellular polymeric substance (EPS) matrix [1]. Inside the biofilm, extracellular matrix proteins and exogenous genes are transported through the EPS matrix, which encourages microbial growth [1]. Moreover, a well-developed biofilm tightly adheres to metal surfaces by extracellular polysaccharides of the EPS matrix, which is difficult to remove completely. The microorganisms inside the biofilm are protected from stresses caused by environmental changes and chemical substances. As such, a favorable environment for microbial growth is formed inside the biofilm, and the microbial community develops to a high density over time. In biofilms formed on metal surfaces, microbiologically influenced corrosion (MIC) is caused by various microbial factors, such as metal oxidation by electron transfer from the metal surface to bacteria [2] and accumulation of corrosive metabolites [3]. MIC causes serious problems for metal infrastructure in the marine environment, and economic losses from MIC were estimated to be $2.5 trillion in 2013, which is equivalent to about 3.4% of gross world product [4]. MIC assessments in the marine environment have been reported for carbon steel [5], stainless steel [6], and low-alloy steel [7]. In those reports, *Bacillus*, *Desulfovibrio*, and *Desulfobacter* have been detected as the dominant bacteria, and the developmental mechanisms of those

bacteria are discussed. By contrast, bacterial community analyses of biofilms collected from uncorroded metal have rarely been reported, and our understanding of the community structure is limited. Thus, changes in bacterial community from development of biofilm to occurrence of metal corrosion have not been elucidated in detail. We consider that microbial community analyses of biofilms collected from uncorroded metal in the marine environment are important to understand the mechanism of MIC, and elucidation of the mechanism may lead to the establishment of measures to prevent the occurrence of metal corrosion [8].

High-throughput amplicon sequencing is a powerful method for the identification of target genes in large amounts of unexamined genetic material from the environmental microbiome [9]. In this method, microbial DNA is extracted from the environmental sample, and target genes are then amplified by polymerase chain reaction (PCR) using the extracted DNA as a template and primers designed to bind to the template. Subsequently, the amplified regions (amplicons) are sequenced by a next-generation sequencer. Since hundreds to thousands of different amplicons can be sequenced together by a next-generation sequencer, this method can efficiently identify target genes. In bacterial genomic DNA, several housekeeping genes are conserved to ensure cell maintenance and growth. In particular, the 16S rRNA gene encodes the 30S ribosomal subunit that is essential for the translation of mRNA and protein synthesis, but it has genus-specific variable regions, such as V1–V9 [10]. Almost all bacterial taxa can theoretically be identified based on the 16S rRNA gene sequence. Thus, bacterial community analysis based on high-throughput sequencing of 16S rRNA gene fragments can characterize the bacterial microbiome [10].

In this study, to investigate the bacterial community that contributes to MIC, a biofilm formed on an uncorroded metal joint attached to a metal dock mooring was used as a model. Using bacterial genomic DNA prepared from the biofilm, the variable region V3–V4 of the 16S rRNA gene was amplified by PCR, and high-throughput amplicon sequencing was carried out. The bacterial community was identified at the genus level based on the operation taxonomic units (OTUs).

2. Materials and Methods

2.1. Sampling

A biofilm that formed on an uncorroded metal joint attached to a metal dock mooring was collected in Akitsu Port in Hiroshima prefecture, Japan (Figure 1). After collection, the samples were put into sterile tubes and immediately placed in a cool box at 4 °C, then transported to the lab within a few hours.

Figure 1. Image of the sampling point. The sampling point for the collection of the biofilm is indicated by a black arrow.

2.2. Genomic DNA Extraction and PCR Conditions

Genomic DNA was extracted from the biofilm using an illustra™ bacteria genomicPrep Mini Spin Kit (GE Healthcare, Chicago, IL, USA) according to the manufacturer's instructions, and the concentration and purity were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Using the genomic DNA as a template, the V3–V4 variable regions of the 16S rRNA genes were amplified by KOD -Plus- (TOYOBO, Osaka, Japan) using bacterial domain-specific primers 341F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′) [11] and 805R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′) [12]. For amplification of the 16S rRNA genes, samples were subjected to 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, and the final extension at 68 °C for 5 min.

2.3. Sequencing Library Preparation, Sequencing, and Bioinformatics Analysis

The length of the PCR products was confirmed using 1.0% agarose gel electrophoresis, and the samples (approximately 450 bp) were then purified using a Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI, USA).

The microbiome sequence libraries were prepared using a Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. The concentrations of the sequence libraries were measured using a Quanti Fluor™ dsDNASystem (Promega). The libraries were sequenced using a MiSeq sequencer (Illumina) with a MiSeq Reagent Kit v3 (Illumina).

The bacterial community was analyzed using the QIIME 2 microbiome bioinformatics platform [13]. After the quality of the sequence data was confirmed using a FastQC ver.0.11.9 [14], the amplicon sequence variants (ASVs) were prepared based on the filtered data using the DADA2 algorithm [15]. The ASVs were grouped into OTUs at 99% sequence identity using the taxonomic databases of Sliva ver. 138 [16,17]. To construct a phylogenetic tree based on the variable regions of 16S rRNA gene sequences, the OTU dataset was also subjected to phylogenetic analysis with MEGA 11 software [18]. The consensus bootstrap phylogenetic tree was constructed using the neighbor-joining method.

3. Results and Discussion

Among the OTUs, a total of 18 genera of bacteria were observed, and the proportion of unclassified bacteria was less than 0.1% (Figure 2). *Alkanindiges* bacteria were observed as the dominant species, and their relative abundance was more than 95%. Apart from *Alkanindiges*, several bacteria such as *Sphingomonas* and *Pseudomonas* were also observed as OTUs, but the relative abundances of those bacteria were less than 3%. To confirm the phylogenetic relationship of the bacteria comprising more than 1% of the OTUs, a neighbor-joining tree based on the V3–V4 variable regions of the 16S rRNA gene was constructed. In the neighbor-joining tree, nine kinds of *Alkanindiges* bacteria formed a cluster with *A. hongkongensis* HKU9^{T}, *A. illinoisensis* MVAB Hex1^{T}, and *A. hydrocarboniclasticus* H1^{T} (Figure 3). *Alkanindiges* bacteria belong to a class of Gammaproteobacteria and can grow using several kinds of hydrocarbons degraded from petroleum [19], so *Alkanindiges* bacteria are a major contributor to the bioremediation of petroleum-contaminated soil [20–22]. To our knowledge, there is a report of Gammaproteobacteria being observed as more than 90% of OTUs in a biofilm collected from a metal surface [6], but there are no reports of *Alkanindiges* bacteria accounting for more than 90% of the total.

Lichen is a complex organism of algae, fungi, and bacteria that grows on a wide range of substrates, from natural surfaces to artificial materials such as metal, glass, rubber, and plastic [23]. For example, *Stereocaulon japonicum* and *Cladonia humilis* grow in Cu-polluted sites around Cu-roofed temple buildings by their Cu-accumulation capacity [24]. Fernández-Brime et al. reported the bacterial communities associated with a lichen symbiosis, and more than 20 orders of bacteria were observed in several lichenized samples [25].

Moreover, 11 genera of bacteria observed in this study, such as *Alkanindiges*, *Sphingomonas*, *Pseudomonas*, *Roseateles*, *Allorhizobium/Neorhizobium/Pararhizobium/Rhizobium*, *Brevundimonas*, *Reyranella*, *Massilia*, *Pantoea*, *Mucilaginibacter*, *Asinibacterium*, *Roseomonas*, *Hymenobacter*, *Paracoccus*, and *Bradyrhizobium* were also observed in the report by Fernández-Brime et al. [25]. Thus, the occurrence of metal corrosion may be induced by bacteria adhered to metal surfaces via lichen. To improve the accuracy of the measures to prevent the occurrence of metal corrosion, it is necessary to consider bacterial community analysis including lichen in the future.

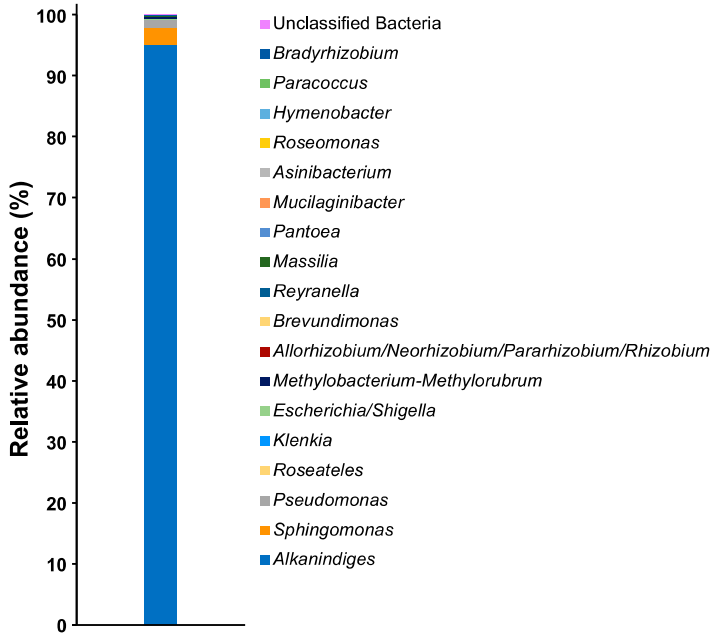

Figure 2. Relative abundances of the bacterial community in the biofilm at the genus level.

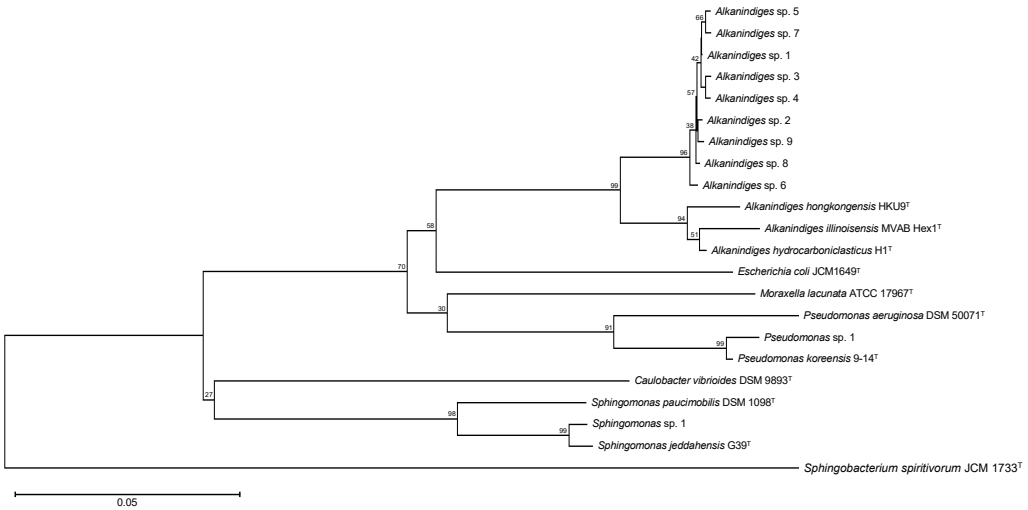

Figure 3. Consensus bootstrap phylogenetic tree of OTUs comprising more than 1% of the total 16S rRNA gene sequences. Each OTU is denoted by its representative strain type. The numbers at the nodes are percentages indicating the levels of bootstrap support based on neighbor-joining analysis of 1000 resampled datasets. The tree was rooted using *Sphingobacterium spiritivorum* JCM 1733^{T} as the outgroup. The bar indicates a 0.05% nucleotide substitution rate.

The detailed molecular mechanisms of MIC are still unclear, but electron transfer from the metal surface to MIC-causing bacteria has been demonstrated in a few reports. For example, *Pseudomonas aeruginosa* MCCC 1A00099 produces water-soluble electron transfer mediators, such as phenazine-1-carboxamide and pyocyanin, by phenazine-modifying enzyme, methyl transferase, and flavin-containing monooxygenase, which are encoded by *phzH, phzM*, and *phzS* genes. Those mediators activate the extracellular electron transfer pathway [26,27]. In the genome of *Methanococcus maripaludis* OS7, the genes that encode the small and large subunits of a [NiFe] hydrogenase (MMOS7_11590 and MMOS7_11600), the membrane proteins for secretion of the [NiFe] hydrogenase (MMOS7_11620 and MMOS7_11630), and a hydrogenase maturation protease (MMOS7_11610) are clustered [28]. Although *M. maripaludis* OS7 promotes MIC of iron, a deletion mutant strain lacking the gene cluster does not corrode iron. In *Alkanindiges* bacteria, the genome has been sequenced for two species, including *A. hydrocarboniclasticus* H1^{T} and *A. illinoisensis* DSM 15370^{T}. In those genome sequences, with the exception of MMOS7_11620 and MMOS7_11630, genes related to MIC are not present. This suggests that *Alkanindiges* bacteria do not cause MIC but act in the formation of biofilms before MIC occurrence.

In the KEGG PATHWAY Database [29], the biofilm formation pathway is registered for three species of Gammaproteobacteria, including *Escherichia coli*, *P. aeruginosa*, and *Vibrio cholerae*. To investigate the biofilm-forming capacity of *Alkanindiges* bacteria, the biofilm formation pathways of *A. hydrocarboniclasticus* H1^{T} and *A. illinoisensis* DSM 15370^{T} were compared using that of *P. aeruginosa*, which is the closest of the three species of Gammaproteobacteria to *Alkanindiges* bacteria (Figure 4). In *P. aeruginosa*, cAMP/Vfr signaling, Quorum sensing, and Gac/Rsm and c-di-GMP signaling pathways have been identified, and those pathways except the Quorum sensing pathway seem to be conserved in both strains. In particular, the function of c-di-GMP signaling pathway is enhanced by Psl, which is a neutral exopolysaccharide typically comprising D-glucose, D-mannose, and L-rhamnose. This exopolysaccharide is a crucial adhesive component of the biofilm matrix that promotes both cell-to-cell interactions and cell-to-metal surface attachment [30]. Thus, *Alkanindiges* bacteria may adhere to a metal surface by the function of the c-di-GMP signaling pathway, resulting in the formation of a biofilm. In addition, the development of biofilms can migrate to bacterial communities composed of MIC-causing bacteria and eventually cause MIC on the surface of metal joints. *Pseudomonas* species have been isolated and identified from various sources, such as soil, fresh water, and metal surfaces, and several species are suggested to be MIC-causing strains because those species can obtain energy by transferring electrons to extracellular solid substances [31]. In fact, we also observed *Pseudomonas* sp. 1, which shows high identity with the MIC-causing strain *P. koreensis* Ps 9-14^{T} [32]. Thus, the relative abundance of *Pseudomonas* sp. 1 in the biofilm may increase as time proceeds, which then encourages MIC on the surface of the metal joint.

Pathway/Gene/Product	1	2
cAMP/Vfr signaling pathway		
pilJ/twitching motility protein PilJ		
pilI/ twitching motility protein PilI		
chpA/chemosensory pili system protein ChpA		
chpC/chemosensory pili system protein ChpC		
pilH/ twitching motility two-component system response regulator PilH		
pilG/ twitching motility two-component system response regulator PilG		
cyaB/adenylate cyclase		
cpdA/3′,5′-cyclic-AMP phosphodiesterase		
crp/cyclic AMP receptor protein		
flrA, *fleQ*, *flaK*/sigma-54 dependent transcriptional regulator		
exsA/exoenzyme S synthesis regulatory protein ExsA		
Quorum sensing pathway		
trpE/anthranilate synthase component I		
trpG/anthranilate synthase component II		
pqsC/2-heptyl-4(1H)-quinolone synthase subunit PqsC		
pqsD/anthraniloyl-CoA anthraniloyltransferase		
pqsE/2-aminobenzoylacetyl-CoA thioesterase		
pqsH/2-heptyl-3-hydroxy-4(1H)-quinolone synthase		
mvfR, *pqsR*/LysR family transcriptional regulator, quorum-sensing system regulator MvfR		
rhlI, *phzI*, *solI*, *cepI*, *tofI, lasI*/acyl homoserine lactone synthase		
rhlR, *phzR*/LysR family transcriptional regulator, quorum-sensing system regulator MvfR		
rhlA/rhamnosyltransferase subunit A		
rhlB/rhamnosyltransferase subunit B		
rfbF, *rhlC*/rhamnosyltransferase		
lecA/PA-I galactophilic lectin		
lecB/PA-IIL fucose-binding lectin		
lasR/quorum-sensing system regulator LasR		
sagS/sensor histidine kinase SagS		
pa1611/sensor histidine kinase		
pa1976/sensor histidine kinase		
hptB/histidine phosphotransfer protein HptB		
hsbR/HptB-dependent secretion and biofilm response regulator		
hsbA/HptB-dependent secretion and biofilm anti anti-sigma factor		
flgM/negative regulator of flagellin synthesis FlgM		
fliA, *whiG*/RNA polymerase sigma factor FliA		
Gac/Rsm pathway		
ladS/sensor histidine kinase LadS		
barA, *gacS*, *varS*/sensor histidine kinase BarA		
retS/sensor histidine kinase RetS		
uvrY, *gacA*, *varA*/two-component system, NarL family, invasion response regulator UvrY		
rsmY/small regulatory RNA RsmY		
rsmZ/small regulatory RNA RsmZ		
csrA/ carbon storage regulator		
ppkA/serine/threonine-protein kinase PpkA		
c-di-GMP signaling pathway		
wspA/methyl-accepting chemotaxis protein WspA		
wspB/chemotaxis-related protein WspB		
wspE/sensor histidine kinase and response regulator WspE		
wspF/response regulator WspF		
wspR/response regulator WspR		
sadCD, tpbB, roeA, mucR/diguanylate cyclase		
bifA/c-di-GMP phosphodiesterase		
pslA/polysaccharide biosynthesis protein PslA		
pelB/polysaccharide biosynthesis protein PelB		
pelC/polysaccharide biosynthesis protein PelC		
pelD/polysaccharide biosynthesis protein PelD		
pelE/polysaccharide biosynthesis protein PelE		
pelF/polysaccharide biosynthesis protein PelF		
pelG/polysaccharide biosynthesis protein PelG		
alg44/mannuronan synthase		
fimX/multidomain signaling protein FimX		
fimW/cyclic-di-GMP-binding protein		

Figure 4. Presence/absence matrix of biofilm formation pathway genes in *A. hydrocarboniclasticus* H1^T and *A. illinoisensis* DSM 15370^T. Strains: 1, *A. hydrocarboniclasticus* H1^T; 2, *A. illinoisensis* DSM 15370^T. Green, orange, and gray squares indicate cases where the genes are conserved, similar genes are conserved, and genes are not conserved, respectively.

4. Conclusions

Bacterial community analyses of metal corrosion-free biofilms have rarely been reported, though analyses within different environments are required to understand the mechanism of MIC. Unlike previous studies on MIC, *Alkanindiges* bacteria were observed as the dominant species in this study. Moreover, the gene clusters required to cause metal corrosion were not conserved in the genome sequences of known *Alkanindiges* bacteria. Those findings indicated that the bacterial community changes significantly during the process from development of biofilm to occurrence of metal corrosion. For more detailed investigations, it will be necessary to analyze changes in the bacterial community over time.

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Data Availability Statement: The 16S rRNA gene sequences of *A. hongkongensis* HKU9^T, *A. hydrocarboniclasticus* H1^T, *A. illinoisensis* MVAB Hex1^T, *C. vibrioides* DSM 9893^T, *E. coli* JCM 1649^T, *M. lacunata* ATCC 17967^T, *P. aeruginosa* DSM 50071^T, *P. koreensis* 9-14^T, *S. jeddahensis* G39^T, *S. paucimobilis* DSM 1098^T, and *S. spiritivorum* JCM 1733^T are available in the GenBank/EMBL/DDBJ databases under accession numbers NR_114676, LT827059, NR_025254, NR_037099, NR_024570, NR_036825, AF094713, NR_025228, NR_158139, LN681566, and LC060921, respectively. Draft genome sequences of *A. hydrocarboniclasticus* H1^T and *A. illinoisensis* DSM 15370^T were deposited in DDBJ/EMBL/GenBank databases under accession numbers MLCN01000001–MLCN01000092 and JHUX01000001–JHUX01000012, respectively.

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