HOKKAIDO UNIVERSITY

Title	Identification and genetic characterization of microbes which are highly sensitive to hydrogen peroxide in the agar plate
Author(s)	渡邉, 統之
Citation	北海道大学. 博士(農学) 甲第15299号
Issue Date	2023-03-23
DOI	10.14943/doctoral.k15299
Doc URL	http://hdl.handle.net/2115/91545
Type	theses (doctoral)
File Information	Watanabe_Motoyuki.pdf

Instructions for use

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Identification and genetic characterization of microbes which are highly sensitive to hydrogen peroxide in the agar plate

(寒天培地中の過酸化水素に極めて高い感受性を示す微生物の同定および遺伝学的分析)

北海道大学　大学院農学院

生命フロンティアコース　博士後期課程

渡　邉　統　之

Table of contents

Chapter I

General Introduction

The cultivation of microbes is no doubt to be the most important procedure in the field of microbiology. Especially, the cultivation of microbes using solid growth medium allows researchers to obtain colonies, a mass of genetically identical cells multiplied from the single inoculated cell, which aids researchers to isolate single strain from the sample consists of multiple microbes (e.g. freshwater, soil and sludge samples). In the past, this cultivation approach was the only method to analyze the bacterial compositions within the sample, and thus it was the basis of the environmental microbiology. The limitation of this cultivation-based analysis was that the presence of the microbes which couldn't or hardly form colonies on the solid medium were not reflected. This limitation is often referred to the "great plate count anomaly" (1, 2), which is the phenomenon that when culturing microbes from the environmental sample, the number of colonies appears on the solid medium is always less than the number of cells observed under the microscope. This concept was further confirmed with the appearance of the next generation sequencing (NGS) technologies into this field of study. NGS allows researchers to use the total DNA/RNA extracted directly from the environmental sample and then PCR amplify the target sequences for the analysis (e.g. 16S rRNA sequence for bacterial identification), the whole process requires no cultivation and thus the accurate abundance of each taxaon in the sample could be reported without the cultivation bias. However, many microbes are known for their presences by NGS analyses but being classified as "Candidate phylum", which means their taxonomy are unconfident at the phylum level because no cultured representatives are present (3–5).

It is being said that only 1 % of the total microbes on this planet had been successfully isolated and cultivated to the date (1), and the microbes belong to the remaining 99% are always referred as the "uncultivated microbes" or "yet-to-be cultured microbes", which

are known to dominate various environments (6) and are believed to be rich of the novel genetic resources and metabolites. These unexplored microbial resources have high potentials to make contribution to the development of various fields which includes the field of industry, pharmacy, healthcare, genetic engineering etc. Although recent NGS technologies allow researchers to access genome information and transcriptome information without the need of cultivation (7), the DNA sequence information itself poorly explains the actual physiologies of the expressed products in the alive cells. To the date, many protein coding genes in the newly sequencing analyzed genomes are annotated as “hypothetical proteins”, which their functions are not predictable from the previously accumulated genetic information (8). In this era with developing NGS technologies, the successful cultivation of the viable microbes in the laboratory is still highly demanded in order to study the physiologies of the microbes and their gene products.

The limitation on the cultivation could be explained by the differences on the growth condition between the laboratory and the natural conditions which where microbes were isolated. Environmental microbes inhabit in various conditions ranging from the surface of the soil to the surface of the human gut, thus it is irrational to try culturing all these microbes in the single laboratory condition. Throughout the decades, researchers had developed various techniques to culture uncultured microbes from the nature; for examples: prolonging the incubation period for slow glowers (9, 10), co-culturing with other strain to fulfill the metabolic requirement (11–13), or by using the permeable membrane which allow the diffusion of natural nutrients and metabolites to mimic the natural environment (14, 15). For cultivation using solid growth medium, numerous attempts had focused on modifying the ingredients of solid growth medium and successfully improved the cultivability. For examples, researchers tried to improve the

cultivability by reducing the concentration of the nutritious substances in the medium (16–19), adding signal compounds or growth promotors (20, 21), or by using different gel solidifier instead of agar (22, 23). The use of gellan gum instead of the agar as the gel solidifier had revealed the presence of microbes which cannot form colonies on the agar growth medium (23). This study was followed by the study which detected the presence of the hydrogen peroxide (H_2O_2) in the solid medium which was solidified with agar (24). Furthermore, the generation of H_2O_2 was determined to occur during the autoclave sterilization step by the reaction between agar and phosphate (25). These findings surprised researchers since agar and phosphate are the common ingredients in many growth media and autoclave sterilization is also a common practice.

H_2O_2 is one of the reactive oxygen species (ROS) and it is known to damage microbial cell components, such as DNA and enzymes (26, 27). This non-polar compound diffuses through the microbial cell membranes and oxidizes the Fe(II) inside of the cell which cause the defection on the function of numerous enzymes which require ferrous iron (28); also, the hydroxyl radicals generated from H_2O_2 binds to the DNA molecule and causes damage (26). Researchers found that the H_2O_2 being generated in solid media could be the source of exogenous H_2O_2 stress, and it was believed to interfere the colony formation of many environmental microbes.

Previous studies indicated that the agar plates prepared by the separate autoclaving of the agar and phosphate have advantages on the yield and the novelties of the acquired colonies (24, 29, 30). However, since the generation of other growth-inhibiting substances during the autoclaving was also indicated (25), there was no study had successfully reported the independent effect of H_2O_2 on the colony formations. Thus, this study first focused on the effect of H_2O_2 in the agar plate on the colony formation of microbes.

Comparative cultivation of laboratory and environmental microbes under different H_2O_2 concentrations was performed to elucidate the critical effect of plate embedded H_2O_2 on the colony formation; the results of the experiment led to the isolation of microbes which their colony formation was highly inhibited by the presence of H_2O_2 in the medium. My study is the first study which selectively isolated environmental microbe which is vulnerable to the H_2O_2 in the agar medium. The genomic DNA sequence analysis on the isolated strains was consecutively performed to analyze their sensitivities genetically, which had revealed their potentials on H_2O_2 degradation by the presence of putative H_2O_2 degrading genes. Finally, the functions of the explored putative catalase genes were evaluated by self-cloning the gene to observe the resulted alteration on the physiological behaviors. The results of my studies had highlighted the importance of controlling the H_2O_2 concentrations in the solid medium during cultivation, and implied that the cultivation problems of the environmental microbes may be solved by using their own enzymatic potentials. This work would contribute to the isolation of uncultured microbes in the future.

Chapter II

Critical effect of H_2O_2 in the agar plate on the growth of laboratory and environmental strains

Abstract

Researchers previously showed that autoclaving in preparing agar media is one of the sources of hydrogen peroxide (H_2O_2) in the medium. This medium-embedded H_2O_2 was shown to lower the total colony count of environmental microorganisms. However, the critical concentrations of H_2O_2 detrimental to colony formation on the agar plate remain largely undetermined. Herein, I elucidated the specific effect of H_2O_2 on microbial colony formation on solid agar medium by external supplementation of varying amounts of H_2O_2. While common laboratory strains (often called domesticated microbes) formed colonies in the presence of high H_2O_2 concentrations (48.8 μM or higher), microbes from a freshwater sample demonstrated greatly decreased colony counts in the presence of 8.3 μM H_2O_2. This implies that environmental microbes are susceptible to much lower concentrations of H_2O_2 than laboratory strains. Among the emergent colonies on agar plates supplemented with different H_2O_2 concentrations, the relative abundance of betaproteobacterial colonies was found to be lower on plates containing higher amounts of H_2O_2. Further, the growth of the representative betaproteobacterial isolates was completely inhibited in the presence of 7.2 μM H_2O_2. This study thus clearly demonstrates that low micromolar levels of H_2O_2 in agar plates critically affect growth of environmental microbes, and large portions of those are far more susceptible to the same than laboratory strains.

Introduction

Hydrogen peroxide (H_2O_2), a reactive oxygen species is destructive to microorganisms (26, 27). Microbial susceptibility have long been investigated in a variety of laboratory cultures (31–42). These studies demonstrate the effect exerted by several

hundred micromolar to millimolar levels of supplementary H_2O_2 on bacterial survival. For instance, the *E. coli* K-12 strain W3110 survives after the 15 minutes treatment with 10 mM H_2O_2 (42), and the survival rate of *E. coli* K-12 strain AB1157 was below 5% after the 15 minutes treatment with 25 mM H_2O_2 (31). The majority of these studies exposed bacterial cells to H_2O_2 in liquid medium prior to spreading them on solid medium. This resulted in H_2O_2 stress that was exerted on bacterial cells in liquid medium, being evaluated on solid medium. Since the H_2O_2 content of the solid agar media was not determined, the H_2O_2 stress for microbial cells on solid (more specifically, agar) media are poorly assessed.

Agar has been commonly used as a growth media solidifier since the 1890s and modern microbiology would not exist without its extensive contributions to the field. Several factors that contribute to this effect in agar media have been established thus far, including the role played by H_2O_2 generation during agar media preparation as previously reported (24, 25).

A previous study demonstrated that autoclaving agar and phosphate in the same container during the media preparation (PT protocol, Phosphate-Together) resulted in the generation of H_2O_2 that remained entrenched within the medium (24). This retention of H_2O_2 in media is believed to be one of the factors responsible for the lowered colony counts of environmental microbes (24). H_2O_2 generation increases in a phosphate concentration-dependent manner, and is accelerated by high pH and ammonium concentrations (25). The generation of H_2O_2 can thus be reduced by autoclaving agar and phosphate separately (PS protocol, Phosphate-Separate) (24, 25). Comparative analyses of these two recipes using bacterial inoculums derived from several environmental samples revealed that the colony yield of PS plates was at least twice as high as that of

PT plates (24, 25, 29, 30). This was accompanied by a higher ratio of phylogenetically novel isolates on PS plates than that observed on PT plates (24, 29, 30). These results imply that the use of PT medium critically affects microbial colony formation, and the cultivability of hitherto-uncultivated microorganisms. The above-mentioned studies utilized PYG agar medium (containing peptone, yeast extract, and glucose as primary carbon and energy sources) as a model medium that potentially contains H_2O_2 at concentrations of ~ 15 μM or higher when plates are prepared using the PT protocol (24, 25).

While few studies previously reported that PT plates yield fewer colonies than that observed on PS plates, the effect might not be a consequence solely attributable to higher H_2O_2 content. This is because PT plates are discussed to contain other growth inhibiting substances that are generated during the autoclaving process (25). Thus, a simple comparison of growth between PT and PS plates does not accurately reflect the independent effects of H_2O_2.

The present study investigates the independent effect of H_2O_2 contained within agar medium on the growth of laboratory and environmental microbes. The modulation of H_2O_2 levels in media allowed investigation of the specific effects of H_2O_2 on microbial growth, independent of the effects exerted by other growth inhibiting substances that may have been generated during agar media preparation (25).

Materials and Methods

Environmental sample source and collection. Environmental samples were collected from the current beside Ono pond, a pond located at Hokkaido University, Sapporo, Japan (43˚07’N, 141˚34’E) in order to isolate microbes sensitive to low

micromolar levels of H_2O_2. The sediment surface was disturbed using an autoclave-sterilized ladle, and the water above containing floating sediment particles was subsequently collected into an autoclave-sterilized plastic laboratory bottle. The collected sample was immediately placed on ice and stored at 4 °C until further use in cultivation experiments.

Preparation of culture medium. PYG medium (containing peptone, yeast extract, and glucose) was prepared using the PW protocol (where W stands for Without phosphate buffer) for microbial cultivation from the environmental sample, as previously described by Tanaka et al. (24). Two solutions (solutions A and B) were prepared and autoclaved separately in different containers and subsequently mixed prior to solidification of the agar medium in a Petri dish. The pH of solution A containing 2.27 mM $(NH_4)_2SO_4$, 0.2 mM $MgSO_4$, and 45 µM $CaCl_2$, was adjusted to 8.1 before the addition of 16 g $liter^{-1}$ of Bacto agar. Solution B contained 0.1 g $liter^{-1}$ of Bacto peptone, Bacto yeast extract, and glucose (pH 6.7). Post mixing solutions A and B, H_2O_2 was added as required. Briefly, after cooling the autoclaved AB mixture to approximately 50 °C, commercially available H_2O_2 was added to the mixture at incremental concentrations ranging from 5–400 µM, before immediate solidification in a Petri dish. The final concentration of retained H_2O_2 in the agar plates was detected to be approximately 40% to 60% of that which was originally supplemented.

Cultivation and identification of microbes from environmental samples. Prior to initiation of the cultivation experiments, the environmental sample was inverse-mixed in a plastic bottle and left for one hour to allow large particles to settle. The liquid at the top was separated and serially diluted in a 10-fold series from 10^{-1} to 10^{-4} in sterile distilled water. From each dilution, 50 µl was spread on PW plates containing either 1.8 µM, 3.2

μM, 8.3 μM, or 17.3 μM H_2O_2. Each dilution was inoculated in quadruplicate for each H_2O_2 concentration, and the plates were subsequently incubated at 20 °C in dark for 10 days prior to estimation of CFUs.

At least 100 colonies from each H_2O_2 concentration were randomly chosen for identification (1.8 μM:384 colonies, 3.2 μM:288 colonies, 8.3 μM:192 colonies, 17.3 μM:384 colonies). Briefly, the partial region of 16S ribosomal RNA genes was PCR amplified using KOD FX Neo DNA polymerase (TOYOBO) and the primers set 10F' (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The PCR products were prepared for sequencing using the BigDye terminator cycle V3.1 sequencing kit (Thermo Fisher Scientific), and subsequently sequenced using the Applied Biosystems 3500XL genetic analyzer (Thermo Fisher Scientific) with the primer 341F (5′-CCTACGGGAGGCAGCAG-3′). Each colony was identified using the Ribosomal Database Project (RDB) classifier (43) with a confidence threshold of 80%. The full-length sequence of the amplified 16S rRNA gene obtained from the isolates OS-1 and OS-4 was determined and used for phylogenetic analysis.

Detection of H_2O_2 concentration in the medium. H_2O_2 concentration in the agar medium was estimated by freezing the medium overnight at -80 °C, followed by thawing in dark for three hours at room temperature. The liquid inside the agar medium was extracted onto the surface of the medium by syneresis, and was subsequently collected and diluted as liquid samples prior to detection of the H_2O_2 concentration.

The H_2O_2 concentration was analyzed by combining different aspects of the protocols previously published by Jiang et al. and Tanaka et al. (24, 44). In brief, freshly prepared 2X H_2O_2 assay reagent (200 mM sorbitol, 200 μM xylenol orange, 500 μM

$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, and 50 mM H_2SO_4) was added to the same volume of the liquid sample which was collected from the thawed frozen plate. Prior to this, an H_2O_2 eliminated blank for each liquid sample was prepared by adding bovine liver catalase to the liquid samples in order to eliminate H_2O_2, followed by incubation at room temperature for 40 min. The absorbance was subsequently read at 560 nm and compared to that of the H_2O_2 standard solution, the concentration of which was determined using the extinction coefficient of 43.6 $M^{-1} \cdot cm^{-1}$ at 240 nm. For each analysis, an average of triplicate measurements was made.

Evaluation of microbial sensitivity to H_2O_2. Eight bacterial strains were cultured on agar plates containing six different H_2O_2 concentrations. Five frequently utilized bacterial species included *Escherichia coli* K-12, *Pseudomonas putida* JCM 6157, *Sphingomonas paucimobilis* NBRC 13935^T, *Rhodococcus erythropolis* JCM 3201^T, and *Bacillus subtilis* subsp. subtilis str. 168. The present study resulted in the isolation of two strains that were highly sensitive to H_2O_2, namely OS-1 and OS-4. SO-S41 was a alphaproteobacterial strain that was isolated in the previous study (29). Each strain was cultured in PW liquid medium and harvested during the log phase. Cell suspensions were subsequently diluted, and 50 µl of the diluted suspensions were spread on PW plates containing either 0.6 µM, 2.9 µM, 7.2 µM, 13.3 µM, 48.8 µM, 85.3 µM, or 225 µM H_2O_2. Plates that were inoculated with *Escherichia coli* were incubated at 37 °C, those with *Pseudomonas, Sphingomonas*, *Rhodococcus,* and *Bacillus* strains at 28 °C, those with OS-1 and OS-4 at 20 °C, and those with SO-S41 at 25 °C.

Cultivation of OS-1 and OS-4 on various LB agar media. *Comamonadaceae* strain OS-1 and OS-4 were inoculated on standard and diluted Lysogeny Broth (LB) agar medium. The standard LB medium was prepared with 10g $liter^{-1}$ of Bacto Tryptone, 5 g

liter^{-1} of Bacto yeast extract and 5g liter^{-1} NaCl; if required, the solution was diluted with distilled water prior to the addition of 16g liter^{-1} Bacto agar. As result, LB agar plates with 1/1, 1/5, 1/10 and 1/20 amount of the nutrition were prepared. Additionally, a standard LB plates was prepared with amending catalase to eliminate the plate embedded H_2O_2; 1 ml (per liter) of filter-sterilized bovine liver catalase (2 mg/ml) was added to the autoclave-sterilized LB agar medium which was cooled down to approximately 40 °C before plating out. Strain OS-1 and OS-4 grown in the liquid PYG medium were inoculated on these LB agar media, followed by the 6 days of incubations at 20 °C.

Data availability. The 16S rRNA gene sequences of strain OS-1 and OS-4 has been deposited in the GenBank/EMBL/DDBJ databases under accession numbers LC710547 and LC710548. The partial 16S rRNA gene sequences behind Fig. 2 are deposited under accession number LC734098-LC734900.

Results

Evaluation of various microbial sensitivities to H_2O_2 in agar medium. PYG plates without supplemental phosphate (PW protocol, Without phosphate) that have been reported to yield less H_2O_2 than those prepared by the PS protocol (generated H_2O_2: PT>>PS>PW) were prepared (24). The concentration dependent effects of H_2O_2 on colony formation were assessed using PW plates supplemented with varying amounts of H_2O_2 before solidification.

The sensitivities of laboratory strains (see Materials and Methods) to H_2O_2 in agar plates was tested by spreading cell suspensions on PW plates supplemented with H_2O_2 (Table 1). The findings revealed that while *Sphingomonas* and *Pseudomonas* strains managed to grow on plates supplemented with 48.8 μM H_2O_2, *Escherichia* and

Rhodococcus strains showed growth at a much higher concentration of 85.3 μM. Further, *Bacillus* strain demonstrated growth even at 225 μM H_2O_2, the highest concentration tested. All of these commonly studied species were thus capable of growing on agar plates containing at least 48.8 μM H_2O_2, which is approximately twice the H_2O_2 concentration detected in conventional PT plates (24, 25).

TABLE 1 The growth of various bacterial strains on the PW plates with different H_2O_2 concentrations[a]

Bacterial type	Strain	H_2O_2 concentration of the PW plate (μM)						
		0.6	**2.9**	**7.2**	**13.3**	**48.8**	**85.3**	**225.0**
Common laboratory species	*Escherichia coli* K-12	+	+	+	NT	+	+	-
	Pseudomonas putida JCM 6157	+	+	+	NT	+	-	-
	Sphingomonas paucimobilis NBRC 13935^T	+	+	+	NT	+	-	-
	Rhodococcus erythropolis JCM 3201^T	+	+	+	NT	+	+	-
	Bacillus subtilis Subsp. Subtilis Str. 168	+	+	+	NT	+	+	+
Isolated in this study	OS-1	+	+	-	-	-	-	-
	OS-4	+	+	-	-	-	-	-
Isolated in the previous study	SO-S41	+	+	+	NT	-	-	-

[a] +: growth, - : no growth, NT = Not tested.

Comparison of colony diversity and frequency obtained from environmental sample at varying H_2O_2 concentrations. The freshwater microbial sample was inoculated on PW plates with four different H_2O_2 concentrations to elucidate its effect on microbial growth in the context of colony frequency and diversity. Supplemental H_2O_2 was added post autoclaving to ensure that plates only differed with respect to their final H_2O_2 concentrations. The highest H_2O_2 concentration of 17.3 µM was comparable to the H_2O_2 concentration present in PT plates, as previously reported (24). The CFUs were found to decrease with increasing H_2O_2 concentrations in PW plates (Fig. 1). This was evident from the finding that 20% of the CFUs that grew on the 1.8 µM H_2O_2 plates were lost on the 3.2 µM plates. Further, more than 60% were lost on the 8.3 µM plates, and more than 80% was lost on the 17.3 µM plates.

The H_2O_2 concentration also affected the taxonomic composition of the colonies, as depicted in Fig. 2 in terms of microbial diversity at the class level. While the relative abundance of the classes *Gammaproteobacteria*, *Flavobacteria,* and *Alphaproteobacteria* was much higher on plates that contained greater amounts of H_2O_2, a decrease in the relative abundance of the class *Betaproteobacteria* was evident when H_2O_2 concentrations were comparatively higher.

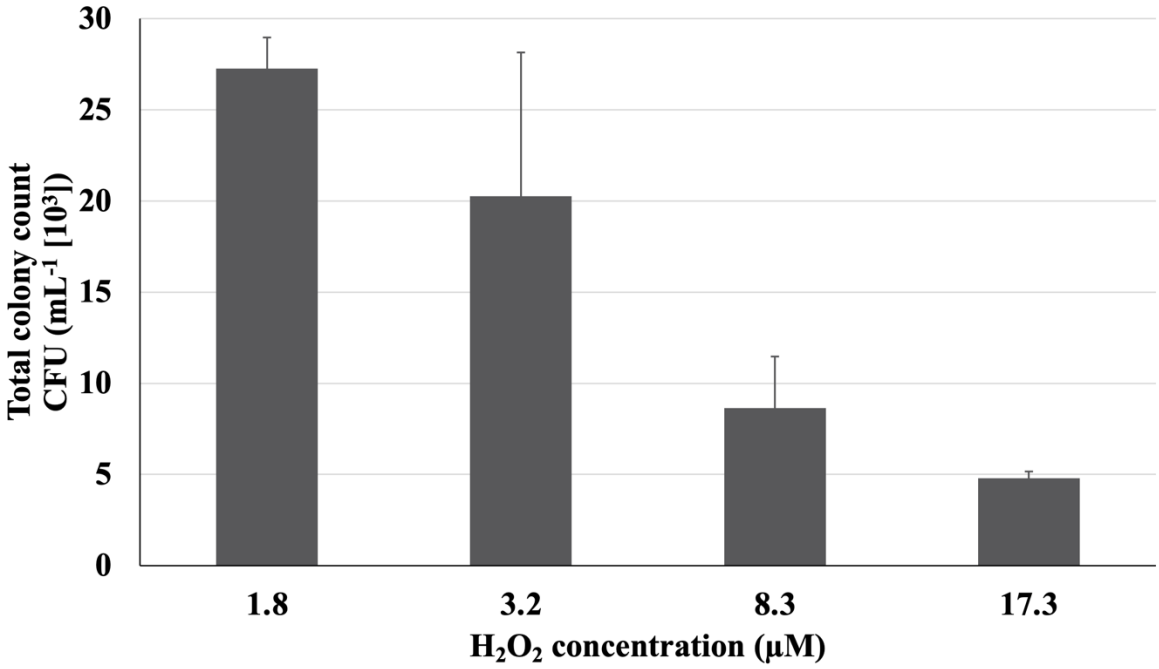


FIG 1 Total colony count of emergent colonies from the freshwater sample on PW plates with four different H_2O_2 concentrations. The number of colonies were counted after 10 days of incubation at 20˚C in dark. CFU counts are averages from four replicate agar plates and error bars represent standard deviations.

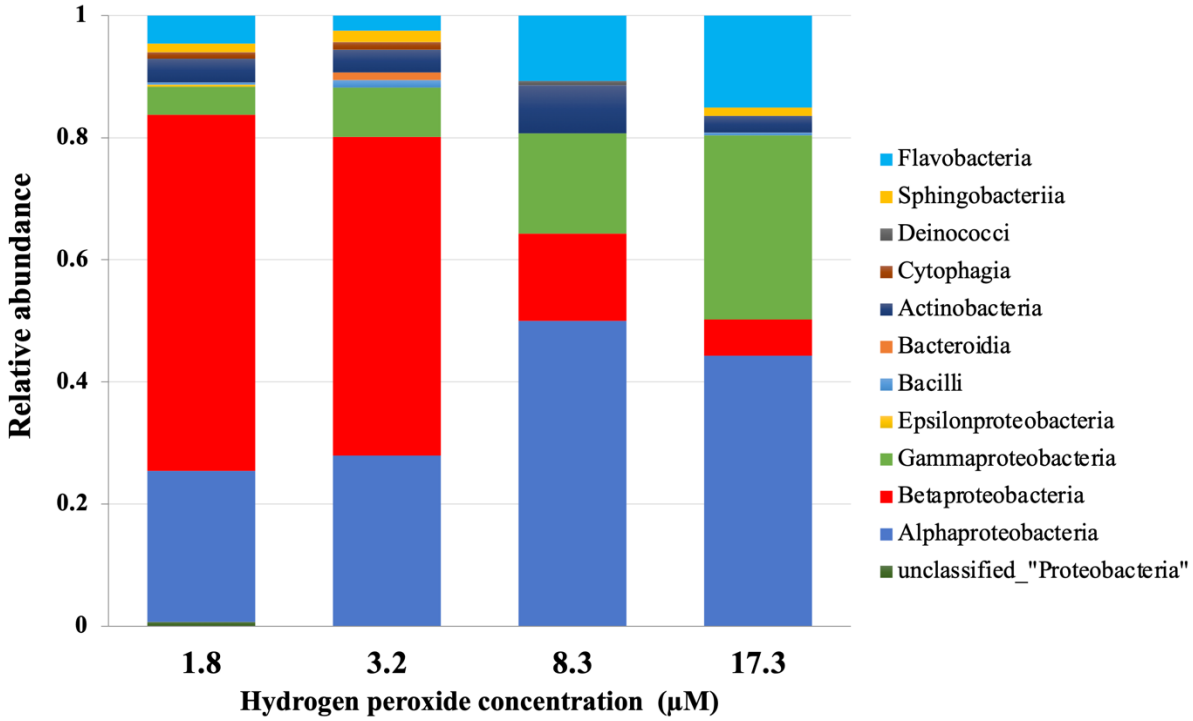


FIG 2 Comparison of the class level relative abundance of emergent colonies on PW plates with four different H_2O_2 concentrations. Colonies were randomly selected from the plates and identified by analyzing the amplified partial sequence of 16S rRNA gene using RDP classifier.

Isolation and characterization of microbes that are highly sensitive to H_2O_2. Based on the above-mentioned results, I attempted to obtain microbes that were highly sensitive to low micromolar levels of H_2O_2. More specifically, betaproteobacterial colonies were selectively collected among the identified colonies which depicted Fig. 2.

Of the various betaproteobacterial strains isolated from the 1.8 μM H_2O_2 plates, OS-1 and OS-4 were selected for further experimentation.

Analysis of the 16S ribosomal RNA gene sequence using the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that the strain OS-1 shared approximately 99% identity with certain *Rhodoferax* strains, and that the strain OS-4 was approximately 99% identical to certain *Curvibacter* strains. This implies that they may belong to these genera, both of which are classified under the family *Comamonadaceae*. Notably, the family *Comamonadaceae* also demonstrated the most obvious decrease in CFUs with increasing H_2O_2 concentrations (Fig. 3, Table 2).

The reproducibility of the results on H_2O_2 sensitivity of these two strains was confirmed after isolation and long-term preservation. Both strains were found to form colonies on PW plates supplemented with 0.9 μM H_2O_2, however, colony formation was completely abrogated on plates containing 13.3 μM H_2O_2, a concentration which is comparable to that present in conventional PT plates (Fig. 4).

Further, both OS-1 and OS-4 failed to form single colonies on plates supplemented with 7.2 μM H_2O_2 (Table 1). This indicates that their threshold of H_2O_2 sensitivity is substantially lower than the amount of H_2O_2 in conventional PT plates.

Additionally, the alphaproteobacterial strain SO-S41, previously isolated as the strain which grows on PS plates but not on PT plates, was also tested for its H_2O_2 sensitivity (29) (Table 1). The strain formed single colonies on the plates supplemented with 7.2 μM

H_2O_2, which is indicative of its lower sensitivity to H_2O_2 than that of OS-1 and OS-4.

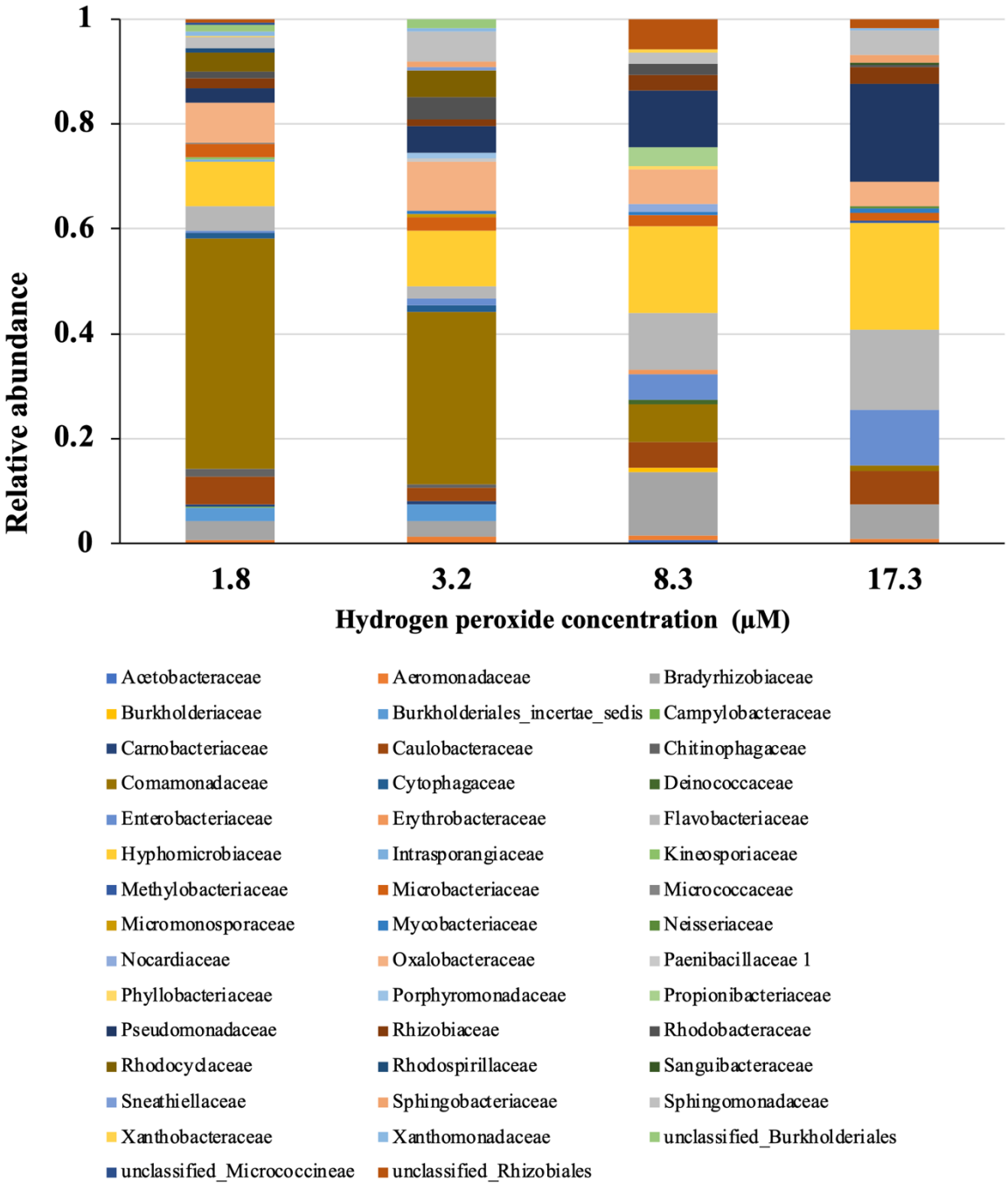


FIG 3 Comparison of the family level relative abundance of emergent colonies which was identified in Fig. 2.

TABLE 2 The count of identified colonies which was used to plot Fig. 3

Family	H_2O_2 concentration (μM)			
	1.8	**3.2**	**8.3**	**17.3**
Acetobacteraceae	0	0	1	0
Aeromonadaceae	2	2	1	2
Bradyrhizobiaceae	10	5	17	14
Burkholderiaceae	0	0	1	0
Burkholderiales_incertae_sedis	7	5	0	0
Campylobacteraceae	1	0	0	0
Carnobacteriaceae	1	1	0	0
Caulobacteraceae	15	4	7	14
Chitinophagaceae	4	1	0	0
Comamonadaceae	123	53	10	2
Cytophagaceae	3	2	0	0
Deinococcaceae	0	0	1	0
Enterobacteriaceae	1	2	7	23
Erythrobacteraceae	0	0	1	0
Flavobacteriaceae	13	4	15	33
Hyphomicrobiaceae	24	17	23	44
Intrasporangiaceae	1	0	0	0
Kineosporiaceae	1	0	0	0
Methylobacteriaceae	0	0	0	1
Microbacteriaceae	7	4	3	3
Micrococcaceae	1	0	0	0
Micromonosporaceae	0	1	0	0
Mycobacteriaceae	0	1	1	2
Neisseriaceae	0	0	0	1
Nocardiaceae	0	0	2	0
Oxalobacteraceae	21	15	9	10
Paenibacillaceae 1	0	1	0	0
Phyllobacteriaceae	0	0	1	0
Porphyromonadaceae	0	2	0	0
Propionibacteriaceae	0	0	5	0
Pseudomonadaceae	8	8	15	40
Rhizobiaceae	5	2	4	7
Rhodobacteraceae	4	7	3	1

Rhodocyclaceae	10	8	0	0
Rhodospirillaceae	2	0	0	0
Sanguibacteraceae	0	0	0	1
Sneathiellaceae	0	1	0	0
Sphingobacteriaceae	0	2	0	3
Sphingomonadaceae	6	9	3	10
Xanthobacteraceae	1	0	1	0
Xanthomonadaceae	2	1	0	1
unclassified_*Burkholderiales*	4	3	0	0
unclassified_*Micrococcineae*	1	0	0	0
unclassified_*Rhizobiales*	2	0	8	4

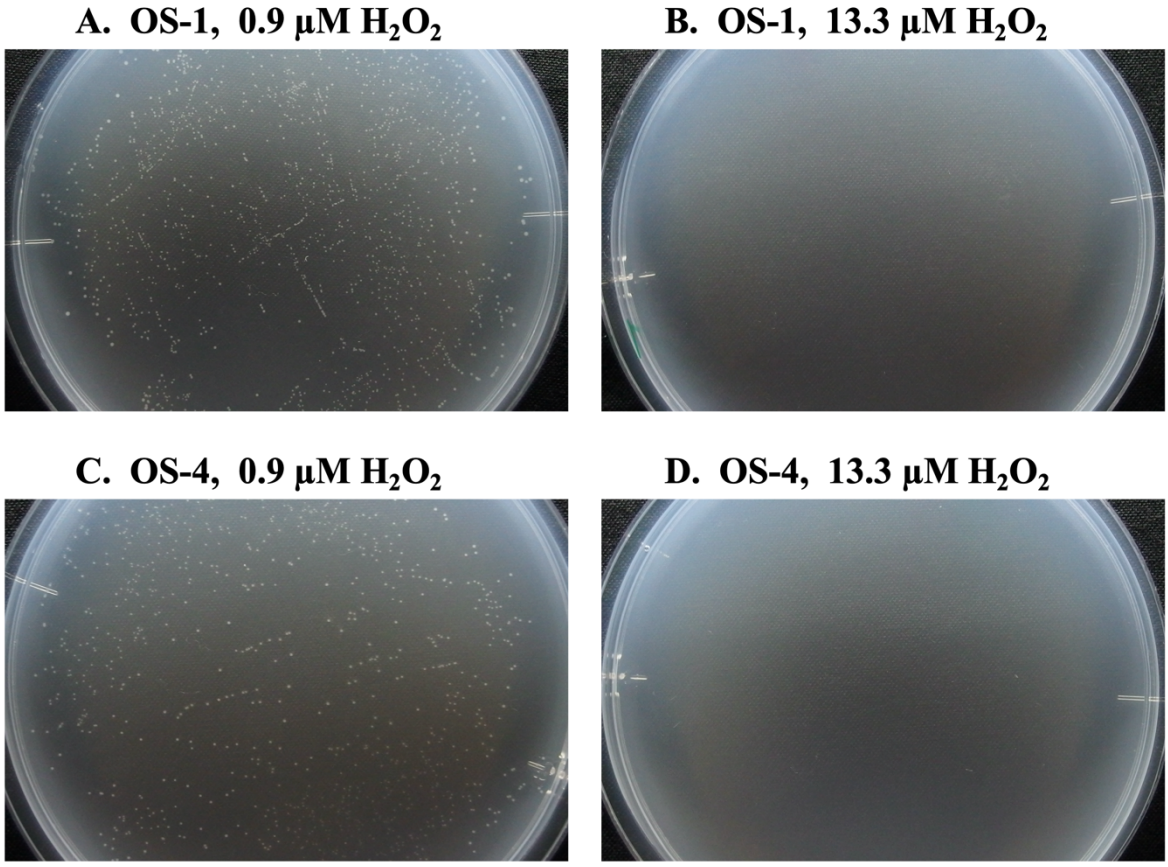


FIG 4 Colony formation of OS-1 and OS-4 on PW plates with 0.9 µM (A and C) and 13.3 µM (B and D) of H_2O_2. Pictures were taken after 5 days of incubation at 20°C. Cultivation experiment was performed in pentaplicates and the representative is shown.

Cultivation of strain OS-1 and OS-4 on LB medium. Strain OS-1 and OS-4 were cultivated on LB medium with varying concentration of the nutrient ingredients. Both strains showed no growth on the standard LB medium (Fig. 5 A, F), however, by amending the bovine liver catalase (final concentration of 2 ng/ml) to eliminate H_2O_2, OS-1 showed the formation of colonies (Fig. 5 B) while OS-4 did not (Fig. 5 G). The colony formation was observed for both strains as the concentration of the ingredients (Tryptone, yeast extract and NaCl) of the medium were diluted to 1/5, 1/10 and 1/20 (Fig. 5 C-E, H-J).

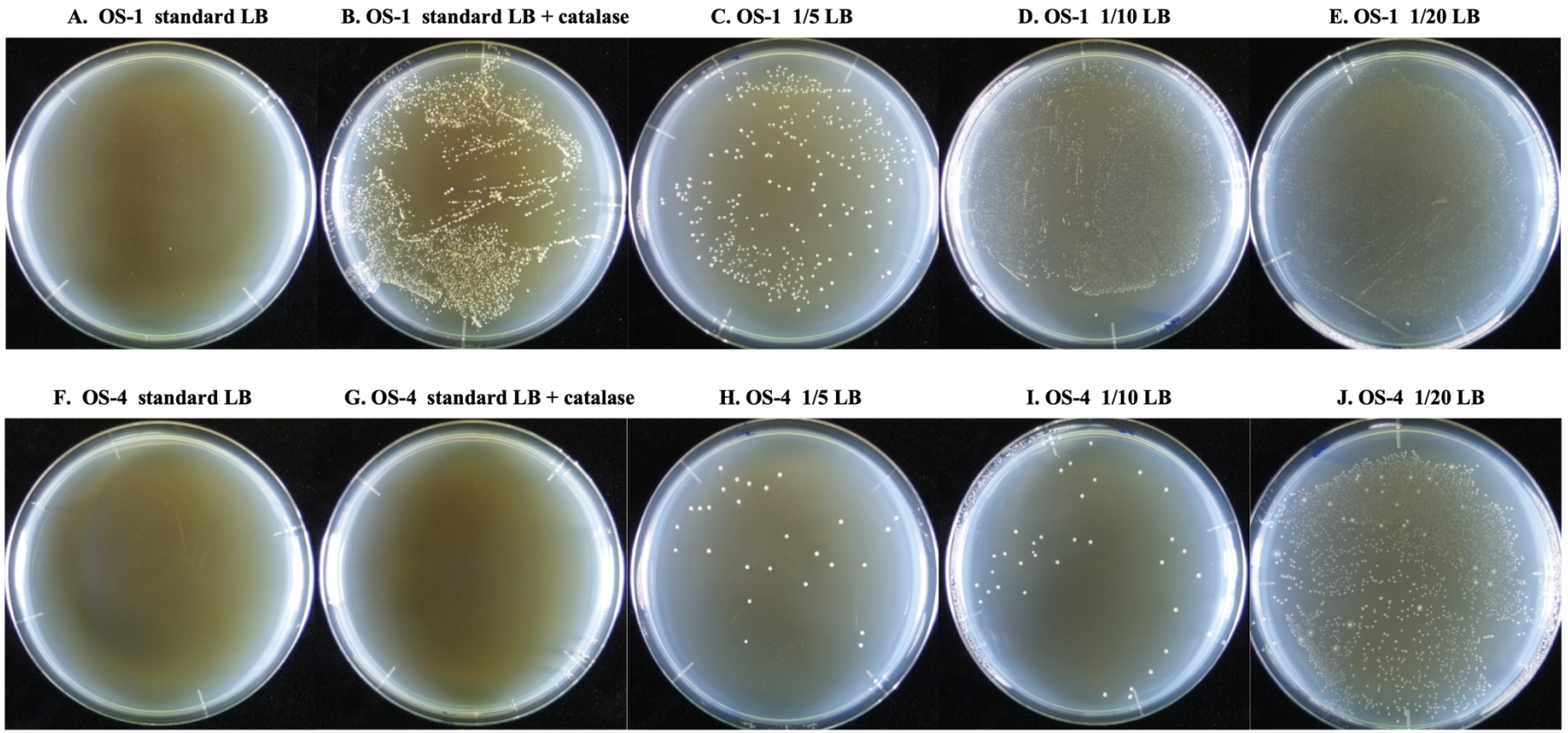


FIG 5 Colony formation of OS-1 (A-E) and OS-4 (F-J) on LB plates with varying dilutions. Standard (1/1) (A, B, F, G), 1/5 dilution (C, H), 1/10 dilution (D, I), 1/10 dilution (E, J). To 1/1 standard LB medium, bovine liver catalase was amended to the final concentration of 2 ng/ml (N, G). Pictures were taken after 6 days of incubation at 20°C. Cultivation experiment was performed in triplicates and the representative is shown.

DISCUSSION

The present study aimed to shed light on the specific effects of H_2O_2 on the cultivability (colony formation) of environmental isolates and laboratory strains. Since the previous studies that utilized PT and PS plates were limited by its inability to exclude the effects of other interfering substances that may have been detrimental to colony forming capacity, I used growth media that was prepared using an identical protocol to ensure that the PW plates only differed with respect to their H_2O_2 contents. These were prepared by supplementing agar medium post autoclaving with H_2O_2, thus allowing the manipulation of the H_2O_2 content, and were subsequently used to assess the H_2O_2 susceptibilities of various microbes (Table 1). All the tested laboratory strains successfully formed single colonies on PW plates containing 48.8 μM H_2O_2, thus indicating that their tolerance to H_2O_2 in agar plates was higher than the amount of H_2O_2 in conventional PT plates. The high tolerance of these strains may contribute to their growth on artificial media that contain a certain amount of H_2O_2, which possibly further led to their establishment as common laboratory strains.

The majority of studies that investigated microbial susceptibility to H_2O_2 exposed bacterial cells to millimolar levels of H_2O_2 in liquid medium prior to spreading on solid medium containing unknown amounts of H_2O_2 (31–42). In contrast, I analyzed the direct effect of H_2O_2 in agar plates on inoculated cells, so as to be able to determine the vulnerability of bacterial cells to the representative concentrations of ROS, when plated on solidified media. The results revealed that the well-known laboratory strains of *Escherichia*, *Pseudomonas*, *Rhodococcus* and *Sphingomonas* were able to form colonies at concentrations up to 48.8 – 85.3 μM H_2O_2 in agar plates, but were not able to form colonies in the presence of 225 μM H_2O_2 (Table 1), which is in sharp contrast to previous

findings that report resistance even to millimolar amounts of H_2O_2 in liquid medium. This significant difference between previous reports and my study probably exists on account of the previous studies have reported survivability in the presence of H_2O_2 in liquid medium, while my study demonstrated the effect of H_2O_2 on colony formation. This study therefore is the first study that demonstrates microbial susceptibility of pure strains, including *E. coli* strains, to potentially lethal concentrations of H_2O_2 on agar media.

I further hypothesized that the colony forming ability of many environmental microbes would potentially be significantly affected by H_2O_2 in agar plates. Given that 15 μM or higher H_2O_2 in agar plates, a concentration previously detected in PT media by us had an inhibitory effect on the growth of environmental microbes (24, 25), the effect of lower H_2O_2 concentrations was investigated in the present study. PW plates with four different H_2O_2 concentrations, namely 1.8 μM, 3.2 μM, 8.3 μM, and 17.3 μM were prepared and inoculated with a freshwater sample that served as a bacterial source. The number of emergent colonies were subsequently counted, which clearly revealed that H_2O_2 concentrations in agar plates were inversely related to colony numbers (Fig. 1). As shown in Fig.1, a remarkable decrease in CFUs was observed on the 8.3 μM plate, a H_2O_2 concentration which was obviously lower than the concentration previously detected in PT plates (~15 μM) (24). These results demonstrate the levels of H_2O_2 to which environmental microbes are sensitive for the first time.

The class level abundance of the colonies was directly influenced by H_2O_2 content. The identification of colonies on different plates revealed that colony variety differed between different H_2O_2 concentrations. The class *Betaproteobacteria* demonstrated a continuous decline as H_2O_2 concentrations increased (Fig. 2), which is indicative of the existence of H_2O_2 sensitive strains among *Betaproteobacteria*. This assisted us

substantially in the isolation of the betaproteobacterial strains OS-1 and OS-4 from plates supplemented with 1.8 μM H_2O_2. Both these strains failed to grow in the presence of 7.2 μM of H_2O_2, a concentration lower than that present in PT plates (Table 1), even after repetitive subculture in the laboratory, implying that they lack the ability to colonize conventional PT plates. Notably, in sharp contrast to the laboratory strains, both OS-1 and OS-4 were observed to have higher susceptibilities to H_2O_2 in agar plates.

During the routine cultivation of strain OS-1 and OS-4, I found that both strains do not form colonies on the LB agar medium. The standard recipe of the LB medium contains yeast extract, and it contains phosphates. Since yeast extract was autoclave-sterilized with agar in the same container, the generation of H_2O_2 was predicted (25). Both strains showed no formation of colonies on the standard LB medium (Fig. 5 A, F); but by eliminating the H_2O_2 with bovine liver catalase, OS-1 showed the formation of colonies (Fig. 5 B). Which indicated that for OS-1, the H_2O_2 in the LB agar medium was the main cause of the cultivation failure; however, OS-4 did not grow on the LB plated amended with catalase (Fig. 5 G), which indicates that the H_2O_2 in the LB plate was not the only obstacle of the colony formation of OS-4.

By diluting the ingredients of standard LB plates, both strains showed colony formation (Fig. 5 B-D, G-I). For OS-1, reduced amount H_2O_2 in the medium is one of the causes of the improved colony formation, by diluting the ingredients, the total amount of phosphate was reduced as the amount of yeast-extract was reduced. For OS-4, the improvement of the colony formation is possibly to be the result of the reduced amount nutrients itself, as growth medium with less amount of nutrients are reported to be favored for the cultivation of environmental microbes (17–19). The results indicated that the lower amount of H_2O_2 being generated in the diluted growth medium could be one of the

reasons why low nutrition medium yields more colonies of environmental microbes.

In order to further evaluate the effect of plate embedded H_2O_2 on the growth of environmental microbes, more samples from different locations and time period should be collected and examined. However, I successfully discovered microbes which are sensitive to low micromolar of H_2O_2 from my freshwater sample. Moreover, environmental microbes that grow on PS plates but not on PT plates have been sourced from water, sludge, sediment, and soil (24, 25, 29, 30), the majority (though not all) of which are in all likelihood sensitive to low micromolar levels of H_2O_2 in agar plates.

In the present study, I prepared agar media using 1.8 μM H_2O_2 as the lowest concentration, which yielded the highest CFUs from freshwater sample inoculums. However, given that the H_2O_2 content of freshwater samples has been reported to be in the nanomolar order (45) and in view of my evidence on the sensitivity of environmental microbes to H_2O_2, one may reasonably expect the existence of a number of microbes in these samples that are incapable of growing even on plates supplemented with 1.8 μM H_2O_2. The analysis of microbes with far more sensitivity to H_2O_2 therefore requires the preparation of plates with nanomolar levels of H_2O_2. While this may be achieved by the addition of catalase or pyruvate that reduce H_2O_2 to extremely low levels (25, 46–49), the technique cannot be applied to control H_2O_2 concentrations. The development of novel techniques that can successfully regulate H_2O_2 content in the nanomolar order are therefore essential for the analysis of more sensitive microbes.

My results demonstrated that the low micromolar levels of H_2O_2 in agar plates critically affected the growth of environmental microbes, which further implied that the micromolar levels of H_2O_2 generated during media preparation may be one of the causes of the “great plate count anomaly” (2).

Further studies on H_2O_2 sensitive strains, including a detailed analysis of the extent of their sensitivities to different H_2O_2 levels, as well as of the activities of catalase and other hydrogen peroxide-degrading enzymes encoded by their genomes, might aid elucidation of the mechanisms involved in H_2O_2 susceptibility. This may prove to be useful in the development of technological advancements that will permit the isolation of novel environmental microbes in the future.

Chapter III

Whole-genome sequencing analysis of the environmental isolates which are highly sensitive to H_2O_2 in the agar plate

Abstract

Aerobic microbes are known for their continuous generation of H_2O_2 in their cells, thus the genes of the H_2O_2 degrading enzymes are widely conserved among their genomes. Two *Comamonadaceae* strains OS-1 and OS-4 showed high sensitivity against the H_2O_2 in agar medium, however, they are highly possible to be possessing the H_2O_2 scavenging enzymatic genes since they both grow aerobically. To verify the genetic background of the high H_2O_2 sensitivity of these strains, their genomes had to be analyzed. In the present study, I investigated if strain OS-1 and OS-4 possess H_2O_2 degrading enzymatic genes or not by performing the whole genome sequencing analysis. The result revealed that both strains possess the DNA sequences being annotated as commonly conserved H_2O_2 degrading enzymes and the H_2O_2 sensory protein.

Furthermore, I focused on the putative genes of catalases since catalases are the most efficient H_2O_2 degrading enzymes and widely conserved. The putative catalase genes were found in both genomes, and each putative catalase gene was found to have intact ORF and thus they are less likely to be structural pseudogenes. The predict amino acid sequences of the putative catalase genes were compared against the reported catalases in the NCBI database, and the results indicated that the putative catalase genes of OS-1 and OS-4 show high similarities with the previously reported catalases. The results indicated the presence of the intact catalase genes in the genomes of microbial strains which are highly sensitive to H_2O_2.

Introduction

Through the isolation of *Comamonadaceae* bacterium strain OS-1 and OS-4, I proved the presence of microbes which their colony formation was largely affected by the

presence of low micromolar order of H_2O_2 in the agar plate (50). As in chapter 1, the colony formation of strain OS-1 and OS-4 were completely inhibited with the presence of 7.2 μM of H_2O_2 in the agar plates while common laboratory strains were resistant to at least 48.8 μM (Table 1). All strains in the Table 1 were cultivated under the aerobic condition, which indicates that those strains are always exposure to the oxygen while they were growing into visible colonies. Such exposure to approximately 20% of oxygen in the atmospheric air is an unusual hyperoxic condition for many environmental microbes and thus microbes on the solid medium could be receiving extraordinal oxidative stresses. However, aerobic microbes are known for their continuous generation of the ROS in their cells; which occurs as the result of the partial reduction of molecular oxygen during the aerobic respiration (51). To reduce the oxidative stress caused by these endogenous ROS, various enzymes are conserved among aerobic microbes to sense and degrade ROS to the safe level (27, 28, 52). As OS-1 and OS-4 both form colonies aerobically, they are assumed to possess these anti-ROS enzymes; and these enzymes should have potentials to degrade exogenous H_2O_2 coming from the agar plates. To verify if these two strains have anti-ROS enzymatic genes or not, their genomes were analyzed in this study.

Present study was performed mainly to check the presence of two major H_2O_2 degrading enzymes being conserved among aerobic microbes: Catalase and Alkyl hydroperoxide reductase (Ahp). These two enzymes are known to work together to degrade both endogenous and exogenous H_2O_2 (27). The presence of *oxyR* gene, which encodes the hydrogen peroxide-inducible gene activator, was also checked as it is known to upregulates the expression of Ahp genes and catalase genes with the presence of H_2O_2 influx (53). Along with strain OS-1 and OS-4, the genome sequence of the alphaproteobacterial strain SO-S41 was also analyzed in the same purpose since this

strain was argued to have similar H_2O_2 sensitivity (29).

Materials and Methods

Genomic DNA extraction. All three strains (OS-1, OS-4 and SO-S41) were grown in PYG liquid medium with shaking in 120 rpm for appropriate duration at 20 ˚C, and the genomic DNA was extracted using the phenol-chloroform method (54). The extracted genomic DNA was resuspended in the distilled water and preserved in 4 ˚C.

Sequencing library preparation and whole genome sequencing using iSeq 100. For iSeq 100 sequencing, the sequencing libraries for strain OS-1, OS-4 and SO-S41 were prepared using Nextera DNA Flex library prep kit (Illumina) according to the manufacturer's manual. The whole genome sequencing was performed using an iSeq 100 platform with paired-end 150-bp end, using iSeq 100 i1 Cartridge and iseq 100 i1 Flow cell (Illumina) according to the manufacturer's manual. Before each run, the i1 cartridge was thawed overnight on the bench top at room temperature. The library preparation and sequencing of strain SO-S41 was performed under the aid of Dr. Igarashi, a researcher of the Natural Institute of Advanced industrial Science and Technology.

Sequencing library preparation and whole genome sequencing using MinION Mk1B. For each strain, MinION Mk1B sequencing was performed twice. For strain OS-1 and OS-4, the sequencing libraries were prepared with Ligation sequencing kit SQK-LSK109 kit (Oxford Nanopore Technologies). For strain SO-S41, the sequencing library was prepared with Rapid sequencing kit SQR-RAD004 kit (Oxford Nanopore Technologies), with the extracted genomic DNA and the same DNA sample treated with Short Read Eliminator (Circulomics) to remove short DNA fragments. For all libraries being prepared, the whole genome sequencing was performed with MinION Mk1B

platform with FLO-MIN106 Flow cell (Oxford Nanopore Technologies).

Base-calling and quality-trimming the sequencing reads from iSeq 100 and MinION Mk1B. The iSeq 100 reads for OS-1 and OS-4 were quality trimmed with fastp v.0.12.4 (-q 15, -n 10, -t 1, -T 1, -l 20, -w 16, -g, and other parameters at the defaults) (55) and the iSeq 100 reads for SO-S41 was quality-trimmed with Trimmomatic v0.39 (SLIDINGWINDOW:6:30 MINLEN:78, and other parameters at the defaults) (56). The raw reads from MinION Mk1B for all strains were first base-called using the high-accuracy mode of the MinKNOW v4.1.22, followed by the quality-trimming with GNU Parallel v20181022 (57) and Nanofilt v2.7.1 (--quality 10, --length 500, --headcrop 75, and other parameters at the defaults) (58).

Hybrid Assembly of iSeq and MinION reads and gene annotation. For each strain, the sequence reads from iSeq 100 sequencing and MinION Mk1B sequencing were hybrid assembled with SPAdes v3.14.1 (-k 21,33,41,55,77 –nanopore, --careful, and other parameters at the defaults) (59, 60). For the assembly of OS-1 and OS-4 genomes, only paired-end reads were utilized for the assembly, while both paired-end reads and single reads were utilized for the assembly of SO-S41 genome.

For SO-S41 genome, 3 contigs which are longer than 200 nucleotides were generated, and outward-directing primers were designed from the ends of each contigs. By using SO-S41 genomic DNA as the template, PCR amplifications were performed using every possible combination of the designed outward-directing primers to elucidate the orientation of three contigs. As the orientation of the contigs were confirmed to be the parts of single chromosome, the regions between contigs were determined by sequencing the gap-bridging amplicons using an Applied Biosystems 3500xL genetic analyzer with Big Dye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific).

The assembled sequences were annotated using Prokka v1.14.6 (61) with default parameters, and all assembled circular chromosomal sequences were rotated manually to the start of the *dnaA* gene.

Analysis of the putative catalase genes and the construction of the phylogenetic tree. Based on the putative catalase gene sequences of OS-1 and OS-4, their theoretical translated amino acid sequences were predicted. The protein sequences were analyzed using the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with its protein BLAST (BLASTP) program. The putative catalase sequences of OS-1 and OS-4, the best hit catalase sequences from the BLASTP, and the sequences of two other top hits which their hosts are identified to the species level, the sequences of the catalase of common laboratory strains including the ones with confirmed catalase activities were acquired from the NCBI database to be included in the phylogenetic tree (Fig. 6). The list of the catalases is shown in Table 3. The predict amino acid sequences were aligned using the online Multiple Alignment using Fast Fourier Transform (MAFFT) on the EMBL-EBI website (62) and the phylogenetic tree was constructed. The constructed tree was visualized and edited using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

TABLE 3 Catalases included in the phylogenetic tree

Source organism	Catalase name	Protein ID	Description
OS-1	KatG	BDT69548	Environmental isolate from this study
	KatE	BDT66594	Environmental isolate from this study
OS-4	KatG	BDT71288	Environmental isolate from this study
Rhodoferax sp. OV413	KatG	WP_092757517	BLASTP top hit of the OS-1 KatG
	Catalase	SDP46826	BLASTP top hit of the OS-1 KatE
Rhodoferax bucti	KatG	WP_138514465	BLASTP top hit of the OS-4 KatG
Pseudomonas ullengensis	KatG	WP_183088820	BLASP hit of the OS-1 KatG, identified to the species level
Colimonas arenae	KatG	WP_061536890	BLASP hit of the OS-1 KatG, identified to the species level
Hydrogenophaga taeniospiralis	Catalase	WP_245638015	BLASP hit of the OS-1 KatE, identified to the species level
Variovorax paradoxus	KatE	CAA2101169	BLASP hit of the OS-1 KatE, identified to the species level
Rhodoferax aquaticus	KatG	WP_142809019	BLASP hit of the OS-4 KatG, identified to the species level
Undibacterium baiyunense	KatG	WP_212685166	BLASP hit of the OS-4 KatG, identified to the species level
Escherichia coli	KatG	NP_418377	Catalase of the common laboratory strain with reported activity
	KatE	YP_025308	Catalase of the common laboratory strain with reported activity
Mycobacterium tuberculosis	KatG	CCP44675	Catalase of the common laboratory strain with reported activity
Pseudomonas putida	KatG	WP_016499211	Catalase of the common laboratory strain with reported activity
	KatE	AAB40866	Catalase of the common laboratory strain with reported activity
Bacillus subtilis	KatX	NP_391742	Catalase of the common laboratory strain with reported activity
	KatA	NP_388762	Catalase of the common laboratory strain with reported activity
	KatE	NP_391784	Catalase of the common laboratory strain
Sphingomonas paucimobilis	KatG1	WP_007405955	Catalase of the common laboratory strain
	KatG2	WP_037568498	Catalase of the common laboratory strain
Rhodococcus erythropolis	KatG	WP_029254650	Catalase of the common laboratory strain

Results

Nearly complete genome sequence of *Comamonadaceae* bacterium strain OS-1. After quality-trimming, 4,535,428 paired-end reads were obtained from the iSeq 100 sequencing, and 610,015 reads (N_{50} read length of 2,113 bases, total read length of 1,069,535,023 bases) were obtained from the MinION Mk1B sequencing. The assembly resulted in one contig which was longer than 200 nucleotides (length of 5,121,829 nt; coverage of 64×) as a single chromosome. However, the presence of tandemly connected long repeating sequences was found between the ends of the contig and thus the circularity of the chromosome was not confirmed. This repeating sequence (~5.7 kb in length) was annotated as a ribosomal RNA repeat which contains three ribosomal RNA genes (5S, 23S and 16S) and two tRNA sequences (tRNA-Ala, tRNA-Ile). This sequencing result was consistent with the result of whole genome sequencing with PacBio Sequel lle system (PacBio) performed at Bioengineering lab, Kanagawa. The assembled single contig was with 5,121,829 bp in lengths with a 63.5% G+C content, which contains a total of 4,696 genes, 4,627 protein-coding genes, 56 tRNA genes, 12 rRNA genes, and one transfer-messenger RNA (tmRNA) gene. Two genes were annotated as putative catalase genes, one set of genes was annotated as putative alkyl hydroperoxide reductase, and three putative genes was annotated as *oxyR*.

Complete genome sequence of *Comamonadaceae* bacterium strain OS-4. After quality-trimming, 4,052,006 paired-end reads were obtained from the iSeq 100 sequencing, and 393,661 reads (N_{50} read length of 3,489 bases, total read length of 1,130,220,753 bases) were obtained from the MinION Mk1B sequencing. The assembly resulted in two contigs which were longer than 200 nucleotides (lengths of 3,763,098 nt, 318,875 nt; coverages of 68×,109×), the size of the contig and the difference on the

coverages indicate that the longer contig is a single chromosome and the other contig is a single plasmid. The ends of each contig were overlapping, thus the circularities were confirmed by PCR amplifying those regions. The assembled chromosome was 3,763,024 bp in lengths with a 60.2% G+C content, which contains a total of 3,547 genes, 3,501 protein-coding genes, 42 tRNA genes, three rRNA genes, and one transfer-messenger RNA (tmRNA) gene. The assembled plasmid was 305,179 bp in lengths with a 56.3% G+C content, which contains a total of 342 genes which are all protein-coding genes. One putative catalase gene, one set of gene was annotated as putative alkyl hydroperoxide reductase genes and one putative *oxyR* gene were found in the chromosomal DNA.

Complete genome sequence of *Alphaproteobacteria* bacterium strain SO-S41. After quality-trimming, 923,927 paired-end and 167,116 single-end reads were obtained from the iSeq 100 sequencing, and 20,906 reads (N_{50} read length of 1,350 bases, total read length of 25,855,567 bases) were obtained from the MinION Mk1B sequencing. The hybrid assembly resulted in three contigs which are longer than 200 nucleotides (lengths of 3,781,019 nt, 600,517 nt, and 55,596 nt; coverages of 30X, 29X, and 29X) and outward-directing primers were designed from the ends of each contigs. By using genomic DNA as the template, the orientation of these three contigs were confirmed by testing all possible combination of the primers. Amplification confirmed that three contigs are parts of the single circular chromosome. The gap regions between contigs were determined by manual sequencing and as result, a complete circular chromosomal DNA was determined. The chromosome was 4,443,179 bp in lengths with a 66.2% G+C content, which contains a total of 4,362 genes, 4,304 protein-coding genes, 53 tRNA genes, four rRNA genes, and one transfer-messenger RNA (tmRNA) gene. Two genes were annotated as putative catalase genes, one set of genes were found as putative alkyl

hydroperoxide reductase genes, and one gene was annotated as putative *oxyR* gene.

Analyzing the characteristics of the putative catalase genes in OS-1 and OS-4. The list of the putative catalase genes being found by the whole-genome sequencing analyses are in Table 4. The catalases of my own isolates (OS-1 and OS-4) were further analyzed**.** First, the putative catalase genes were re-sequenced and analyzed for its adequateness. Based on the genome sequencing analysis (63), strain OS-1 was found to harbor two putative catalase genes (locus tags of os1_07570, and os1_37390), and strain OS-4 was found to harbor one putative catalase gene (locus tag of os4_08060). All putative catalase genes of OS-1 and OS-4 were found to be an intact ORF with proper start and stop codon, and no frameshift was observed. The predict amino acid sequence of these enzymes showed considerable similarities against previously reported catalases throughout the sequences; based on these observations, these genes are less likely to be structural pseudogenes. Phylogenetic tree of catalase enzyme was illustrated in Figure 6. Each catalase from two strains showed closest relation with those identified in the genome of *Rhodoferax* spp., also a member of *Comamonadaceae*, though these enzymatic activities were not experimentally tested. In the tree, one OS-1 catalase (os1_37390) and OS-4 catalase were clustered with KatG enzymes which encode bifunctional catalase-peroxidase, and another OS-1 catalase (os1_07570) was clustered with KatE enzymes which encode monofunctional catalases, as well-studied *E. coli* catalase KatG and KatE with previously detected activities were clustered, respectively. Therefore, I designated these catalase gene as $katG_{os1}$, $katE_{os1}$ for strain OS-1 catalases, and $katG_{os4}$ for strain OS-4 catalase.

TABLE 4 Putative catalase genes found in the genome of microbes show high sensitivity against the H_2O_2 in agar plates

Source strain	Gene name	Annotated Product name	product size (aa)	Locus_tag	The highest hit on the BLASTP Protein name	% Identity	source organism
OS-1	*katG*	Catalase-peroxidase	716	os1_37390	Catalase/ peroxidase HPI	91.55%	*Rhodoferax* sp.
	katE	Catalase HPII	729	os1_07570	Catalase	83.72%	*Rhodoferax* sp.
OS-4	*katG*	Catalase-peroxidase	737	os4_08060	Catalase/ peroxidase HPI	96.33%	*Rhodoferax bucti*
SO-S41	*katG*	Catalase-peroxidase	739	sos41_36890	Catalase/ peroxidase HPI	74.19%	*Ketobacter alkanivorans*
	katE	Catalase C	703	sos41_09270	Catalase	82.62%	*Bosea* sp.

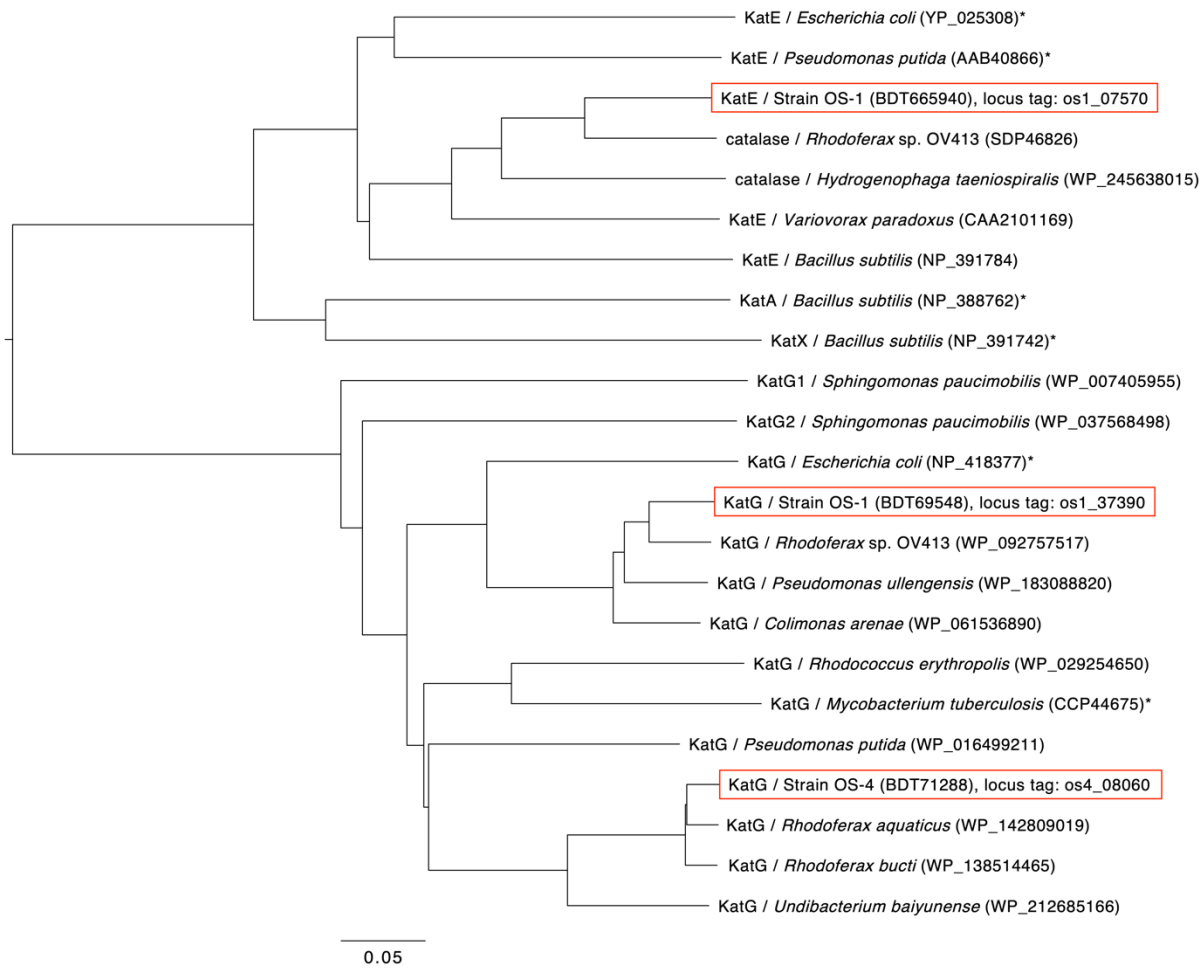


FIG 6 Phylogenetic tree of the catalases based on their predict amino acid sequences. The catalases of OS-1, OS-4 were shown in red frame. The catalases that have been experimentally tested for the activity were marked with an asterisk "*". Scale bar indicates 0.05 substitutions per amino acid position. Protein ID numbers are shown in the parenthesis after the host name.

Discussion

Present study had focused on the genomic background of the H_2O_2 sensitivities of environmental isolates OS-1 and OS-4. Thus, the whole-genome sequencing analyses were performed on the environmental isolates which showed high sensitivities against the plate embedded H_2O_2.

As the result of hybrid assembly of the whole genome, the chromosomal DNA sequences of strain OS-4 and SO-S41 were reported to be in complete circular form, while for the chromosomal DNA of strain OS-1, its circularity was not confirmed. The presence of the repeating sequences of ribosomal RNA operon was found in the chromosome of OS-1, and the presence of such tandem repeats was previously reported to be an obstacle for acquiring complete genome sequences (64, 65). The number of repeating operons were predicted to be more than three by analyzing the single long raw Nanopore read, however, it was not enough to confirm the numbers of repeats. Though the completeness of the genome sequence did not affect my investigation of the H_2O_2-related genes in the genome of OS-1.

Aerobic microbes are known to have H_2O_2 being generated inside of their cells due to the aerobic respiration; thus, many enzymes and proteins are conserved among aerobic microbes to degrade this endogenous H_2O_2 (27). The genome sequencing analysis of H_2O_2 highly sensitive strains revealed that all three sequenced genomes contained at least one putative gene of alkyl hydroperoxide reductases (Ahp) and one putative catalase, which are the two most common H_2O_2 degrading enzymes in aerobic bacteria; and at least one putative gene of *oxyR*, a gene activator which senses the accumulation of the exogenous H_2O_2 in the cell and upregulates the expression level of various genes including Ahp and catalase. The finding of these genes in the genome confirmed that the

high H_2O_2 sensitivities of strains OS-1, OS-4 and SO-S41are not caused by the lack of these common H_2O_2 degrading enzymatic genes.

Since all three strains grow aerobically, the presence of the putative Ahp genes in their genomes were as expected. Because Ahp is known as the primary degrader of the endogenous H_2O_2 in the model organism, *E. coli* (66); Ahp is believed to be an essential enzyme for microbes growing aerobically. However, the presence of putative catalase genes was out of my expectation as it is known to be the most essential H_2O_2 degrader against the exogenous H_2O_2 and catalases are known to degrade H_2O_2 in millimolar order.

The putative catalase genes of OS-1 and OS-4 were further analyzed. Two genes in OS-1 genome and one gene in OS-4 genome were annotated as catalase, however the annotation itself does not confirm that the gene is an intact protein coding gene. All three putative catalase genes started with the proper start codon and ended with the proper stop codon, which indicated that these gene are free of the frameshift mutation and thus these genes are less likely to be the pseudogenes. The size of the theoretical products of these genes were around 700-750 residues (Table 4), it matches the reported average size (700-850 residues) of the catalases found in proteobacterial genomes (67), this result further supported that these genes are complete catalase genes. As indicated by the phylogenetic tree (Fig. 6), the putative catalases of OS-1 and OS-4 were closely related to either KatG or KatE catalases in the NCBI database. Taken together, the putative catalase genes being found in both strains are highly possible to produce catalases.

As in Table 4, the catalase genes of strain SO-S41 showed similarities to the previously reported catalases. However, because strain SO-S41 could be phylogenetically novel in the order level and itself is a slow grower, the putative catalase genes of this strain were not further analyzed.

Although the presence of the catalase genes was indicated through the whole genome sequencing analysis, the sequence information is not sufficient to explain the expression level and the physiology of the product of the gene. Further study on the phenotypes of the gene products based on this genomic analysis should be performed to elucidate the cause of the H_2O_2 sensitivities of OS-1 and OS-4, and to develop a solution to enhance the colony formation of these strains.

Chapter IV

Self-cloning of catalase gene in environmental isolates improves the colony formation abilities under H_2O_2 pressure

Abstract

I previously demonstrated that strain OS-1 and OS-4, two H_2O_2 highly sensitive bacterial strains possess intact putative catalase genes in their genomes. As catalases are known to be the effective H_2O_2 degrading enzymes, their existences in the genome of OS-1 and OS-4 doesn't correlate with their high H_2O_2 sensitivities being observed; however, the characteristics of the product of these genes couldn't be evaluated sorely by the genomic sequence information itself. Herein, I elucidated the characteristics of the products of these putative catalase genes by self-cloning technique to increase the expression level in OS-1 and OS-4. The products of the cloned putative genes showed the H_2O_2 degrading ability during the catalase test, which indicated the gene products have activities as functional catalases. Furthermore, with the cloning of catalase genes, the colony forming ability of the OS-1 and OS-4 had drastically improved under H_2O_2 pressure. My study is the first study which self-cloned the catalase genes from the environmental H_2O_2-highly sensitive isolates and evaluated its effect on the colony forming ability. The results of this study clearly demonstrated that the high H_2O_2 sensitivity on the agar plate could occur even if microbes possess functional catalase genes in their genomes.

Introduction

In my previous study, *Comamonadaceae* bacterium strain OS-1 and OS-4 were isolated from the freshwater sample as microbes which are highly sensitive to agar medium-embedded H_2O_2 (50). Their colony formation was found to be completely inhibited in the presence of 7.2 μM H_2O_2 in the agar plate (50). The common agar growth medium was reported to contain ~15 μM of H_2O_2 (24), a concentration which does not

affect the growth of common laboratory strains (*E. coli*, *Pseudomonas putida, Bacillus subtilis* etc.) while it largely affects the growths of environmental microbes including OS-1 and OS-4 (50).

In aerobic microbes, H_2O_2 is endogenously generated during the aerobic respiration and its generation rate was determined to be 10-15 micromolar/s in *E. coli* (51). This intracellular H_2O_2 is immediately degraded by various enzymes including catalase (27, 28, 51), because the accumulation of 0.5 μM of intracellular H_2O_2 is considered to retards the growth (27). Strain OS-1 and OS-4 both grow aerobically and genome sequencing analysis of these strains demonstrated that they also possesses at least one putative catalase gene(s), despite showing high-sensitivity against H_2O_2 (63). I hypothesized that if their putative catalase genes in OS-1 and OS-4 were properly expressed and the translated products were functional, the colony formation of both strains would be much better on agar media. However, the function of the genes and their products remain uninvestigated. The high H_2O_2 sensitivity could occur if the functional catalase was not expressed from the gene, or the expression level was insufficient to detoxify the H_2O_2 from the agar medium. Although those putative catalase genes were not likely to be structural pseudogenes, even if those genes are intact, the translated products might have no function as catalase. The importance of the intracellular H_2O_2 degrading enzymes on the colony formation was already indicated, for example, the mutant strains of *Escherichia*, *Pseudomonas*, *Salmonella*, *Shewanella* and *Yersinia* species lacking genes for H_2O_2 degrading machinery are known to show decreased colony forming abilities (68–73).

In this study, catalase genes which were identified from the H_2O_2-sensitive environmental strains, were cloned and investigated to confirm whether the genes were

functional, and also to elucidate the relationship between the catalase activity and the colony forming ability on artificial agar media.

Materials and Methods

Construction of the plasmid vectors for self-cloning. The putative catalase genes of OS-1 and OS-4 were amplified from their extracted genomic DNA using the primers listed in Table 5. The amplified gene fragments were separately cloned to the plasmid vector by using In-Fusion cloning kit (Takara). The broad host range plasmid vector pHRP308 (RSF1010 replicon) (74) with following modification was used as the cloning vector. The gentamycin selective marker gene of pHRP308 was replaced with kanamycin gene and *lacZ* gene was removed, yielded a vector, pCPKM. Constructed plasmids were named as pOS1katG (harboring $katG_{os1}$), pOS1katE (harboring $katE_{os1}$) and pOS4katG (harboring $katG_{os4}$). As a positive control, the functional catalase genes from *E. coli* were also used since these catalases were previously detected with their activities (75, 76). *E. coli* catalase genes, *katG* and *katE* were PCR amplified from the extracted genomic DNA of *E. coli* k-12 strain with the primers in Table 5. The amplified *katG* and *katE* gene fragments were tandemly connected and cloned to the vector pCPKM, the plasmid was named as pECkatGE. To enhance the gene expression probability, the bacterial consensus promoter (TTGACA-17 nt-TATAAT) was located to the upstream of the cloned catalase genes on each plasmid.

TABLE 5 Primer sets being used for the cloning of catalase genes

Source strain	Gene name	Direction	Primer sequence
OS-1	*katG*	forward	5′-AGGAGACATTACATATGACCACCGAAGCCAAATGC-3′
		reverse	5′-CCTTGGATCCCTCGAGTTACGCCAGATCGAACCG-3′
	katE	forward	5′-AGGAGACATTACATATGACCCCCACCGCTACCCAA -3′
		reverse	5′-CCTTGGATCCCTCGAGCTATGCAGGTACAGTGGC-3′
OS-4	*katG*	forward	5′-AGGAGACATTACATATGACTACTGAAGCCAAATGCC -3′
		reverse	5′- CCTTGGATCCCTCGAGTCACACGAGGTCGAAGCG-3′
E. coli k-12	*katG*	forward	5′-GCTAAGGAAGCTAAAATGAGCACGTCAGACGATATC-3′
		reverse	5′-TATGTTGCGACATTACAGCAGGTCGAAACGG-3′
	katE	forward	5′-CGACCTGCTGTAATGTCGCAACATAACGAAAAGAAC-3′
		reverse	5′-ACTGCCTTAAAAAAATCAGGCAGGAATTTTGTCAATC-3′

Preparation of competent cells and the plasmid vector introduction. Cells of Wild type OS-1 and OS-4 grown in the liquid PW medium (Peptone-Yeast-Glucose medium prepared without phosphate buffer) (24) were collected by centrifugation, and the cell pellets were washed twice with sterilized 10% glycerol and cells were finally resuspended in 10% glycerol. With electroporation method using Gene Pulser Xcell Electroporation System (setting parameters: Exponential protocol, voltage: 1-1.6 kV, resistance: 400 Ω, capacitance 25 μF) (Bio-Rad), plasmid pOS1katG and pOS1katE were introduced to OS-1 cell, pOS4katG was introduced to OS-4 cell, pECkatGE and a blank vector pCPKM (as negative control) were introduced to both OS-1 and OS-4 cells.

Catalase test. Wild type OS-1, OS-4 and their transformants were grown on the PW medium until the visible colonies were observed, and a single colony was smeared on to the slide glass. 20 μl of 30% commercially available H_2O_2 was dropped on the top of the smeared colonies and the catalase activity was evaluated by the formation of oxygen bubbles as the result of H_2O_2 degradation.

Quantification of the cellular level H_2O_2 degrading activities. To quantify the H_2O_2 degrading ability caused by the introduction of catalase genes, the whole cell H_2O_2 degradation ability of OS-1, OS-4 and their transformants were detected. Wild type OS-1, OS-4 and their transformants were grown in the liquid PW medium (24) and diluted to $OD_{600} = 0.05$. To 5 ml of this cell suspension, H_2O_2 was added to the final concentration of 5 μM. After one minute of incubation at room temperature, cells were separated by the centrifugation (15,000 rpm, 3 minutes) and the supernatant was carefully collected. The supernatant was immediately mixed with the H_2O_2 assay reagent for H_2O_2 concentration detection as described by Watanabe et al. (50). As negative controls, 5 μM of H_2O_2 was also incubated with PW medium or the dead cells of OS-1 and OS-4. The dead cells were

prepared by autoclaving the grown cells at 121˚C for 15 minutes. The experiment using alive cells were performed in triplicates per strain.

Spotting growth assay. The colony forming abilities of the transformants were evaluated by the spotting growth assay on the PW agar medium. Wild type OS-1, OS-4 and their transformants were first cultured in PYG liquid medium and diluted to OD_{600} =0.1, followed by the 10-fold serial dilution to 10^{-7} in sterile distilled water. From each dilution, 10 µl was spotted on the surface of the PW agar medium with different H2O2 concentrations (0.9 µM, 13.3µM). The growth was evaluated after six days of incubation at 20˚C, spot with more than three colonies are defined as growth.

Results

Evaluation of the function of the self-cloned putative catalases. To confirm cellular catalase activity, wild type and transformant strains of OS-1 and OS-4 were used for conventional catalase test. As the result of the catalase test, wild type OS-1 and OS-4 and their blank vector transformants showed no formation of bubbles (Fig. 7 A, B, F, G), while the transformants with cloned catalases genes showed rapid formation of bubbles after the application of H_2O_2 droplet, which is likely to be the oxygen bubble resulted from the degradation of H_2O_2 (Fig.7 C-E, H, I).

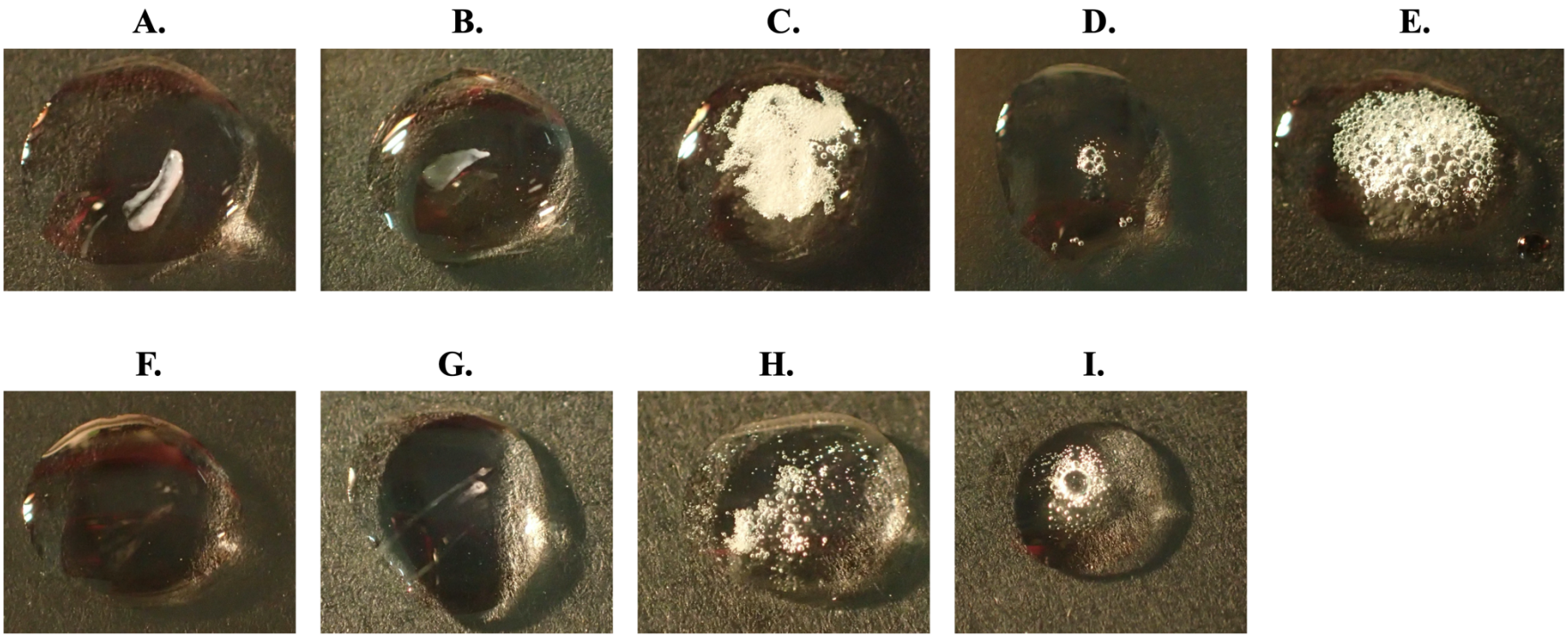
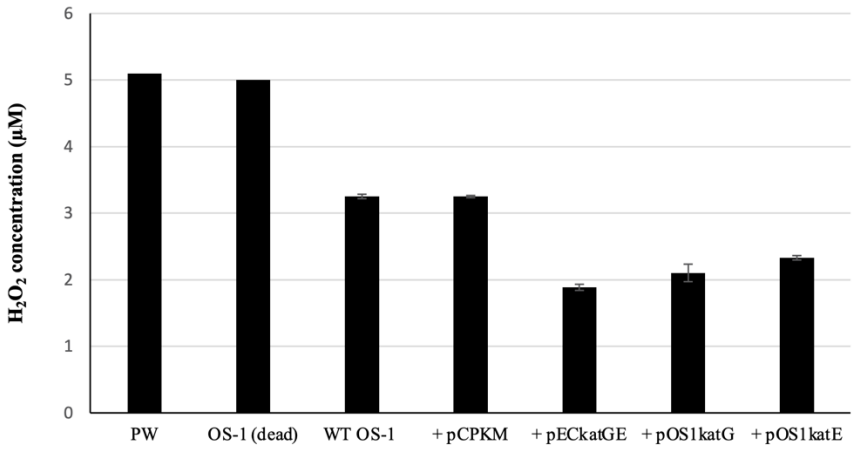


FIG 7 Detection of cellular catalase activity of OS-1, OS-4 and their transformants. Strains used in the panels; Wild type OS-1 (A), OS-1+ pCPKM (B), OS1 + pECkatGE (C), OS-1+ pOS1katG (D), OS-1 + pOS1katE (E), Wild type OS-4 (F), OS-4 + pCPKM (G), OS-4 + pECkatGE (H), and OS-4+ pOS4katG (I). Pictures were taken right after the 20 µl of 30% H_2O_2 was dropped on the top of smeared colonies.

Detection of the whole cell H_2O_2 degrading abilities in the liquid medium. To quantify the differences on the H_2O_2 degrading ability caused by the introduction of catalase genes, the whole cell H_2O_2 degradation ability of OS-1, OS-4 and their transformants were detected in the liquid medium (Fig. 8). In both strains, the wild type strain and the blank vector transformant showed similar degree of H_2O_2 degradation (Fig. 8 A, B). Catalase gene transformants showed improved degradation abilities in both strains; among OS-1 transformants, the *E. coli* catalase transformant showed more degradation over self-cloned transformants (Fig. 8 A), and this result was vice-versa in the OS-4 transformants (Fig. 8 B).



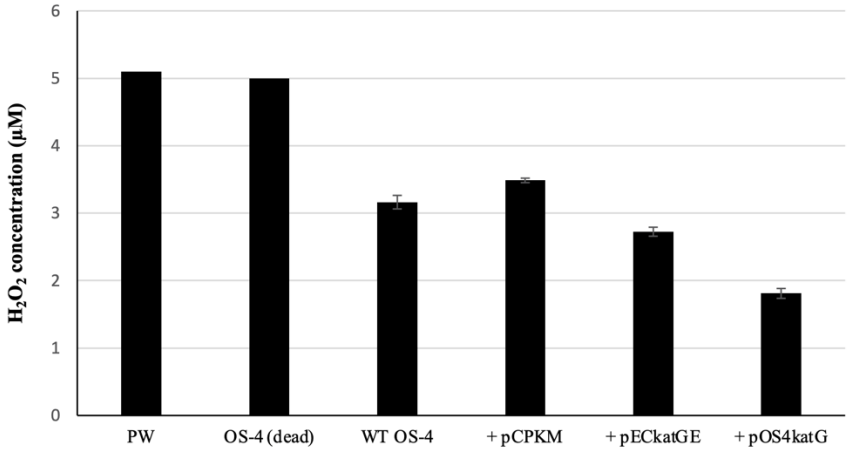


Fig 8 Whole cell H_2O_2 degradation ability assessment of OS-1, OS-4 and their transformants. Strains were diluted to OD_{600} = 0.05 in the liquid PW medium and extra H_2O_2 was added the final concentration of 5 μM. After one minute, the cell was removed with centrifugation and the remaining H_2O_2 concentration was analyzed. OS-1 and its transformants (A), OS-4 and its transformants (B). Dead cell samples were prepared by autoclaving the cells. Blank vector indicates the transformant with pCPKM vector. For sample which contains alive cells, the experiments were performed in triplicates and the error bars are shown.

The effect of the self-cloned catalase genes on the growth on the colony formation. The colony forming ability of wild type and transformants were evaluated by performing spotting growth assay. On the 0.9-μM H_2O_2 plate, both OS-1 and OS-4 strains showed similar growths among wild type, blank vector and catalase gene transformants, up to the 10^{-6} or 10^{-5} dilution (Fig. 9 A). On the plate with 13.3 μM H_2O_2, for both strains OS-1 and OS-4, the growth of wild type and blank vector transformants were only observed on the first spot, while the *E. coli* catalase transformants showed the growth up to 10^{-3} dilution spot, and self-cloning transformants showed the growth up to 10^{-5} dilution spot (Fig. 9 B).

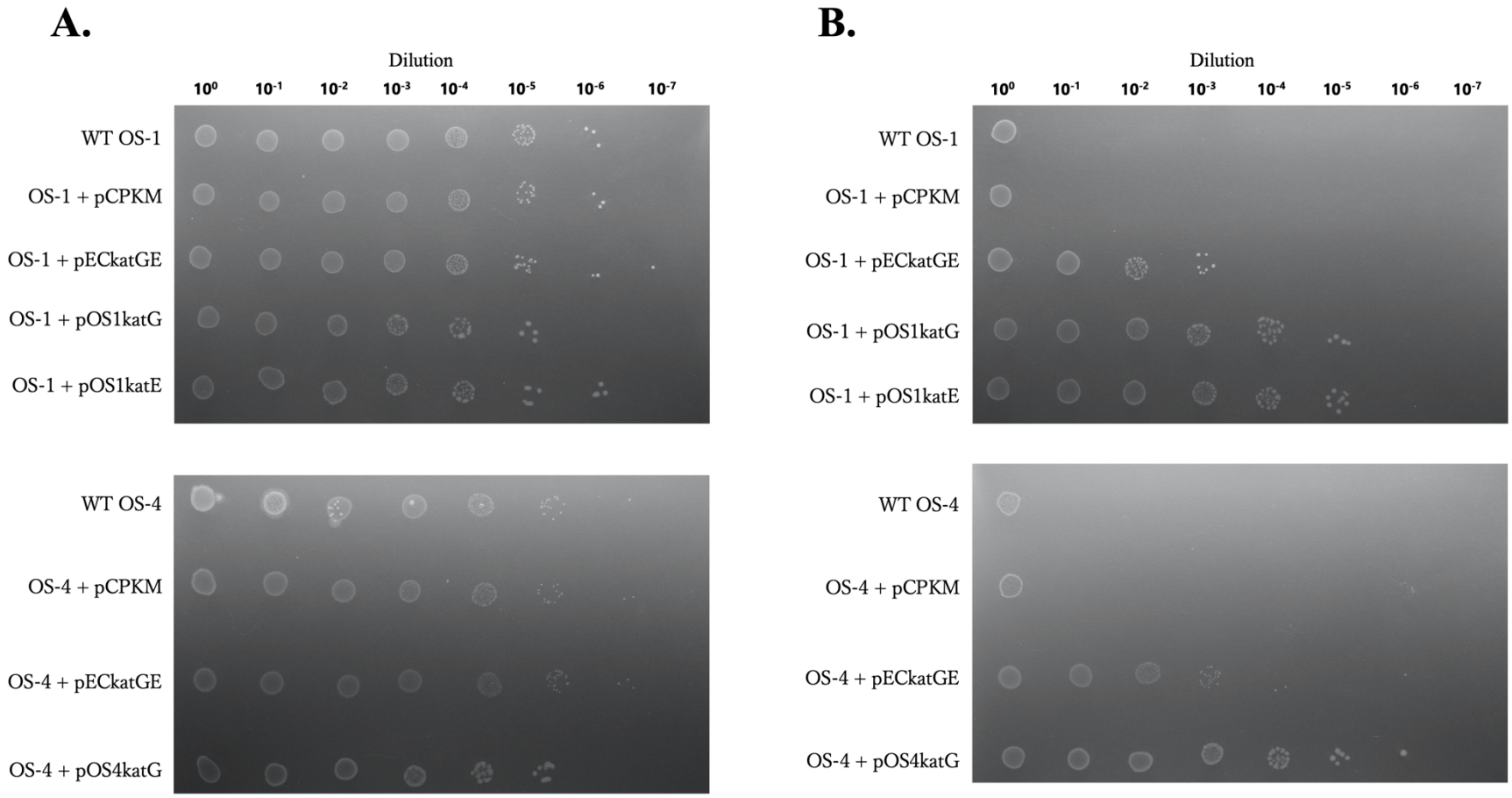


Fig 9 Growth of OS-1, OS-4 and their transformants on PYG plates with 0.9 μM (A) or 13.3 μM (B) H_2O_2. Strains grown aerobically in PYG liquid medium were collected and re-suspended to $OD_{600} = 0.1$ (dilution 10^0), then the cell suspension was serially diluted and 10 μl of each dilution was spotted. Pictures were taken after six days of incubation in 20˚C.

Discussion

This study aimed to evaluate the products of the putative catalase genes possessed by OS-1 and OS-4, two bacterial strains which showed high sensitivity against the H_2O_2 in the agar plate. As catalase is known to be the powerful H_2O_2 degrading enzyme which works intracellularly, its contribution against the invading exogenous H_2O_2 is not hard to imagine. To evaluate the effect of these putative catalase genes on the colony forming ability of their hosts; their catalase genes were self-cloned into their host for increasing the expression level of these genes. Unlike those laboratory strains which are frequently utilized in the gene recombination experiments, the environmental isolate OS-1 and OS-4 had no previously established host-vector system, which meant there was no confirmed information about which vector would be successfully maintained in the cells of these strains.

Plasmid vector was chosen to self-clone catalase genes. To increase the opportunities of the introduced vector to be retained in OS-1 or OS-4 cells, a broad host range plasmid vectors were chosen. My first attempt was made by using pBHR1 (funakoshi), a plasmid vector with pBBR122 replicon and which was known to be applicable to *E. coli* and a wide variety of Gram-negative bacteria. However, pBHR1 was poorly retained in the cells of OS-1 and OS-4, and I had to choose alternative vector for cloning. Plasmid vector pCPKM was chosen for the alternative vector. This vector was modified from pHRP308 with rsf1010 amplicon (74), which was known to be applicable to both Gram-positive and Gram-negative bacteria. On this plasmid vector, catalase genes were cloned to the downstream of the bacterial consensus promotor to increase the opportunities of the introduced genes to be expressed in the host cell.

The introduction of foreign genes to the environmental isolates are known to be

challenging because those isolates always lack information about the applicable gene vectors and the gene expression. The closest related strains of OS-1 and OS-4 were not reported with any transgenic experiments in the past, thus the self-cloning of their catalase genes was revealed to be difficult. First, the conjugative DNA transfer using *E. coli* was considered because of its high efficiency; however, this technique requires the screening of the transformants from the donor *E. coli* cells using antibiotics. Strain OS-1 and OS-4 were highly vulnerable to all available antibiotics and which made it impossible to perform screening after the conjugation. Alternatively, the electroporation method was chosen because it has more options of the antibiotics to be used. However, since no information was available on the parameters of the electric pulse to be applied, the successful introduction of the self-cloning vectors required numerous attempts.

The successful introduction of the plasmid vectors was confirmed by performing the traditional catalase test. The result of the catalase test with pECkatGE transformants had indicated that the broad host range vector used in this study was successfully maintained, and *E. coli* catalases were functionally expressed in these strains (Fig. 7 C, H). Furthermore, the formation of oxygen bubbles was also observed with the self-cloned transformants (Fig. 7 D, E, I), which was not observed with wild type OS-1 and OS-4 (Fig. 7 A, F). The evident difference on the result of catalase tests had clearly demonstrated that the self-cloned catalases of OS-1 and OS-4 were functionally active enzymes with H_2O_2 degrading abilities.

To quantify the H_2O_2 degrading abilities of wild type OS-1, OS-4 and their transformants, their H_2O_2 degradation was quantified in the liquid medium. As shown in Figure 8, wild type OS-1 and OS-4 both have ability to degrade certain amount of exogenous H_2O_2, and the degree of the degradation was not significantly different from

the catalase gene transformants. However, these little differences being observed had affected a lot on the colony formation as shown in Figure 9.

As shown in Figure 9, the result of the spotting growth assay indicated that the colony forming unit (CFU) of wild type strains in 13.3 μM H_2O_2 agar media was depressed to around 10^{-5} order of the magnitude compared to 0.9 μM, and also indicated that the self-cloning transformants keep the original CFU even in 13.3 μM of H_2O_2. Furthermore, the original CFU of the self-cloning transformants was also kept in 47.9 μM (data not shown). On top, endogenous catalase expression/activity was demonstrated to play a very important role on the colony formation.

Although the putative catalase genes were found in the genomes of OS-1 andOS-4, their expression at the mRNA level was not detected. In order to elucidate the function of the gene products, I performed the self-cloning of these catalase genes. My study is the first study that successfully self-cloned the catalase gene of the environmental H_2O_2-highly sensitive isolates by using broad host range plasmid vector, and I have proved that the expressed products of the putative genes were functional catalases. Since the enhanced expression of the self-cloned catalase genes had drastically increased the colony forming abilities of two strains (Fig. 9), The H_2O_2 in the agar plate was determined to be the main component which inhibited the colony formation of strain OS-1 and OS-4, and I proved for the first time ever that the insufficient expression of the catalase genes is one of the causes of the high sensitivities against plate embedded H_2O_2. My results imply the presence of similar environmental microbes, which are highly sensitive to the H_2O_2 in the agar plate regardless of harboring functional catalase genes in their genomes. Such microbes might be able tolerate the H_2O_2 pressures being found in the natural environment, but they could be vulnerable to the amount of H_2O_2 in the agar medium.

Indeed, the H_2O_2 concentration being detected form the nature environments are always in nanomolar orders (45, 77, 78), which might not be a threatening concentration as an exogenous H_2O_2; thus for many microbes, the expression of catalase genes might not be required in the ordinary time while they are inhabiting in the nature environment. Such environmental microbes possessing the catalase enzyme which could degrade millimolar order of H_2O_2 in is an intriguing fact; however, the expression of these genes might not be induced effectively on the agar plate. These microbes may not be able to cope with the micromolar order H_2O_2 in the agar plate by rapidly increasing the expression level of catalase genes.

The application of the catalase to the medium was previously performed to degrade H_2O_2 and to increase the colony yield of the environmental microbes (25, 79, 80); however, the presence of other growth inhibitor in the agar plate was indicated (25) and the degradation of these inhibiters might requires more than the simple supplement of the inexpensive enzyme. In such situation, ones may rely on the potential detoxifying abilities found in the genomes of environmental microbes themselves to enhance their colony formations.

Although the catalase gene cloning technique being performed in this study had significantly improved the colony forming abilities, this technique is not always an easily applicable method for variety of environmental microbes since host-vector system and gene introduction method etc. greatly depends on/differs between strains. During my self-cloning experiment, I also tried using EZ-Tn5 Transposase kit (Epicentre) along with the introduction of plasmid vectors. EZ-Tn5 kit inserts the foreign gene to the host genome by using transposase, which was believed to evade the restriction modification system of the host cell. However, this technique succeeded during the pre-experiment using

Pseudomonas putida, but not with my own isolates. Taken together, the introduction of the foreign gene to the random environmental microbe could be time and cost consuming.

Thus, by innovating the novel pre-treatment or the modified-cultivation technique which could effectively induce the expression of catalase genes (or other functional genes which degrade other growth inhibitors) in each microbe, the colony forming ability would possibly be improved and which might increase the opportunities of isolating novel environmental microbes.

Chapter V

General Discussion

This study attempted to elucidate the effect of H_2O_2 in the agar plate on the colony formation of microbes, including environmental microbes and common laboratory microbes. The generation of H_2O_2 during the preparation of agar medium was discovered previously (24), however, the independent effect of the plate embedded H_2O_2 on the colony formation was not evaluated in the related studies (24, 25, 29, 30). As the generation of H_2O_2 would possibly happen in various growth agar media and the cultivation using agar medium is still an important procedure for the isolation of novel microbes, the effect of plate embedded H_2O_2 on the colony formation was required to be evaluated in detail.

In chapter 2, I demonstrated the independent effect of H_2O_2 in the agar plate on the colony formation; H_2O_2 in agar plate was found to affect the colony forming abilities of microbes. This analysis was achieved by comparing the colony formation on agar plates prepared in the identical protocol but with different amount of amended H_2O_2. My study using such agar plate had successfully excluded the effects of other interfering substances, which was not performed previously.

My results also demonstrated that the colony formation of common laboratory microbes (e.g. *E. coli*, *Pseudomonas putida*, *Bacillus subtilis* etc.) would be affected by the micromolar level of H_2O_2 in the agar plate (Table 1), while their H_2O_2 tolerance in the liquid medium was reported to be in mM level (31, 36, 38). When microbes are cultured in the liquid medium, they are always cultured to the certain density thus the collective H_2O_2 degrading ability of the cells could lower the overall H_2O_2 concentration in the system. Comparatively, microbes are always diluted prior the inoculation on the solid medium to obtain isolate colonies, thus individual cell on the solid medium has to cope with the regional H_2O_2 (71). This difference on the H_2O_2 stress might explain the

difference on the H_2O_2 susceptibilities reported by past studies and my study. My study is the first study which showed that common laboratory strains are vulnerable to the micromolar level of H_2O_2 in the agar plate, with the quantification of the H_2O_2 concentrations of the agar plate.

With using environmental sample as the bacterial source, I confirmed that H_2O_2 level in the conventional growth medium lowers the colony yields and biases the variety of the colonies. I successfully isolated two betaproteobacterial strains which showed high H_2O_2 sensitivities to the plate embedded H_2O_2, OS-1 and OS-4. Their growth was completely inhibited by the presence of 13.3 μM of H_2O_2, which resembled the H_2O_2 level in the PT plate (approximately 15 μM) (Fig. 4). This isolation evidenced the presence of microbes which their colony formation would be inhibited by the H_2O_2 being generated in the conventional agar growth medium.

In chapter 3, I discovered the presence of putative genes which are related to the H_2O_2 degradation in the genome of OS-1 and OS-4. The presence of the alkyl hydroperoxide reductase (Ahp) was as expected since both strains grow aerobically, and Ahp is known as the primary degrader of the endogenous H_2O_2 which is generated by the aerobic respiration. However, it was out of my expectation that both strains were found to be harboring putative catalase gene, which is the well-studied H_2O_2 degrader. Although the presence of the gene being annotated as catalases would not confirm that these genes encode functional catalases, this result inspired me to perform further analysis on these genes for understanding the high H_2O_2 sensitivities of OS-1 and OS-4. The possession of the putative catalase genes was also confirmed with strain SO-S41, a strain which does not form colony on PT plates (29). This indicated that similar microbes (which are sensitive to plate embedded H_2O_2 while having catalase genes) could be found in the other

classes as well.

The catalase genes of OS-1 an OS-4 were evaluated in chapter 4. My results indicated that the gene products of the putative catalase genes are functional catalases with proper H_2O_2 degrading abilities. The expression level of these genes was increased by self-cloning technique and this operation resulted in the improvement of the colony forming abilities. OS-1 and OS-4 were not able to form colonies with 7.2 μM of H_2O_2 (Table 1), however the self-cloned transformants formed colonies on the plate with 13.3 μM of H_2O_2. Based on my finding that CFU from the freshwater sample was largely decreased with 8.3 μM of H_2O_2 in the agar plate (Fig. 1), microbes which have similar H_2O_2 sensitivities as OS-1 and OS-4 could be abundant in the freshwater sample and they could also possess functional catalase genes. The results of chapter 4 had also revealed that microbes could be sensitive to a growth inhibitor (H_2O_2) while having genes of functional enzymes which could degrade these inhibitors (catalase); which indicates that microbes may have potentials to overcome the inhibitors to form colonies in the laboratory condition.

My study had shed light on the effect of plate embedded H_2O_2 on the growth of various microbes. I reported for the first time that both environmental and laboratory strains could be sensitive to the micromolar order of H_2O_2 in the agar plate, and their sensitivities were found to be lower on the agar medium compared to the liquid medium. Through my results, I confirmed that H_2O_2 in the agar plate is one of the factors which inhibits the colony formation of the environmental microbes, which implies that the removal of H_2O_2 from the agar growth medium would be an effective approach to increase the cultivation efficiency on the solid medium. H_2O_2 in the agar plate could be easily degraded by the supplement of catalase or the pyruvate in to the medium, and fortunately these compounds are inexpensive and easily accessible on the market. However, in the further study on the

solid medium cultivation, researchers may find other growth inhibitors which require complicated procedures or expensive compounds to be removed from the medium. In such situation, researchers maybe able to count on the potentials of enzymes within the microbes themselves. If the expression of specific functional enzymatic genes could be effectively induced prior to the cultivation, it might aid microbes to be prepared to form colonies on the top of the solid medium without the complicated gene introductions.

Further studies will focus on the stimulation of such functional genes prior to the cultivation, which possibly improves the colony forming abilities of “yet-to-be cultured” microbes in various environmental samples.

References

1. Amann RI, Ludwig W, Schleifer K-H. 1995. Phylogenetic Identification and In Situ Detection of Individual Microbial Cells without Cultivation. MICROBIOL REV 59:33.
2. Staley JT, Konopka A. 1985. MEASUREMENT OF IN SITU ACTIVITIES OF NONPHOTOSYNTHETIC MICROORGANISMS IN AQUATIC AND TERRESTRIAL HABITATS. Annu Rev Microbiol 39:321–346.
3. Brown CT, Hug LA, Thomas BC, Sharon I, Castelle CJ, Singh A, Wilkins MJ, Wrighton KC, Williams KH, Banfield JF. 2015. Unusual biology across a group comprising more than 15% of domain Bacteria. Nature 523:208–211.
4. Hug LA, Baker BJ, Anantharaman K, Brown CT, Probst AJ, Castelle CJ, Butterfield CN, Hernsdorf AW, Amano Y, Ise K, Suzuki Y, Dudek N, Relman DA, Finstad KM, Amundson R, Thomas BC, Banfield JF. 2016. A new view of the tree of life. Nat Microbiol 1:16048.
5. Stackebrandt E, Frederiksen W, Garrity GM, Grimont PAD, Kämpfer P, Maiden MCJ, Nesme X, Rosselló-Mora R, Swings J, Trüper HG, Vauterin L, Ward AC, Whitman WB. 2002. Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int J Syst Evol Microbiol 52:1043–1047.
6. Lloyd KG, Steen AD, Ladau J, Yin J, Crosby L. 2018. Phylogenetically Novel Uncultured Microbial Cells Dominate Earth Microbiomes 3:12.
7. Dance A. 2020. The search for microbial dark matter. Nature 582:301–303.
8. Naveed M, Chaudhry Z, Ali Z, Amjad M, Zulfiqar F, Numan A. 2018. Annotation and curation of hypothetical proteins: prioritizing targets for experimental study 5:15.
9. Davis KER, Sangwan P, Janssen PH. 2011. Acidobacteria, Rubrobacteridae and

Chloroflexi are abundant among very slow-growing and mini-colony-forming soil bacteria: Slow-growing and mini-colony-forming soil bacteria. Environ Microbiol 13:798–805.

10. Song J, Oh H-M, Cho J-C. 2009. Improved culturability of SAR11 strains in dilution-to-extinction culturing from the East Sea, West Pacific Ocean. FEMS Microbiol Lett 295:141–147.
11. D'Onofrio A, Crawford JM, Stewart EJ, Witt K, Gavrish E, Epstein S, Clardy J, Lewis K. 2010. Siderophores from Neighboring Organisms Promote the Growth of Uncultured Bacteria. Chem Biol 17:254–264.
12. Tanaka Y, Benno Y. 2015. Application of a single-colony coculture technique to the isolation of hitherto unculturable gut bacteria: Isolation of uncultured gut bacteria. Microbiol Immunol 59:63–70.
13. Murugkar PP, Collins AJ, Chen T, Dewhirst FE. 2020. Isolation and cultivation of candidate phyla radiation *Saccharibacteria* (TM7) bacteria in coculture with bacterial hosts. J Oral Microbiol 12:1814666.
14. Chaudhary DK, Khulan A, Kim J. 2019. Development of a novel cultivation technique for uncultured soil bacteria. Sci Rep 9:6666.
15. Nichols D, Cahoon N, Trakhtenberg EM, Pham L, Mehta A, Belanger A, Kanigan T, Lewis K, Epstein SS. 2010. Use of Ichip for High-Throughput *In Situ* Cultivation of "Uncultivable" Microbial Species. Appl Environ Microbiol 76:2445–2450.
16. Ohta H, Hattori T. 1980. Bacteria sensitive to nutrient broth medium in terrestrial environments. Soil Sci Plant Nutr 26:99–107.
17. Janssen PH, Yates PS, Grinton BE, Taylor PM, Sait M. 2002. Improved Culturability of Soil Bacteria and Isolation in Pure Culture of Novel Members of the

Divisions *Acidobacteria* , *Actinobacteria* , *Proteobacteria* , and *Verrucomicrobia*. Appl Environ Microbiol 68:2391–2396.

18. Bartelme RP, Custer JM, Dupont CL, Espinoza JL, Torralba M, Khalili B, Carini P. 2020. Influence of Substrate Concentration on the Culturability of Heterotrophic Soil Microbes Isolated by High-Throughput Dilution-to-Extinction Cultivation. mSphere 5:e00024-20.
19. Gutleben J, Loureiro C, Ramírez Romero LA, Shetty S, Wijffels RH, Smidt H, Sipkema D. 2020. Cultivation of Bacteria From Aplysina aerophoba: Effects of Oxygen and Nutrient Gradients. Front Microbiol 11:175.
20. Bruns A, Cypionka H, Overmann J. 2002. Cyclic AMP and Acyl Homoserine Lactones Increase the Cultivation Efficiency of Heterotrophic Bacteria from the Central Baltic Sea. Appl Environ Microbiol 68:3978–3987.
21. Selvin J, Gandhimathi R, Kiran GS, Priya SS, Ravji TR, Hema TA. 2009. Culturable heterotrophic bacteria from the marine sponge Dendrilla nigra: isolation and phylogenetic diversity of actinobacteria. Helgol Mar Res 63:239–247.
22. Tamaki H, Sekiguchi Y, Hanada S, Nakamura K, Nomura N, Matsumura M, Kamagata Y. 2005. Comparative Analysis of Bacterial Diversity in Freshwater Sediment of a Shallow Eutrophic Lake by Molecular and Improved Cultivation-Based Techniques. Appl Environ Microbiol 71:2162–2169.
23. Tamaki H, Hanada S, Sekiguchi Y, Tanaka Y, Kamagata Y. 2009. Effect of gelling agent on colony formation in solid cultivation of microbial community in lake sediment. Environ Microbiol 11:1827–1834.
24. Tanaka T, Kawasaki K, Daimon S, Kitagawa W, Yamamoto K, Tamaki H, Tanaka M, Nakatsu CH, Kamagata Y. 2014. A Hidden Pitfall in the Preparation of Agar

Media Undermines Microorganism Cultivability. Appl Environ Microbiol 80:7659–7666.

25. Kawasaki K, Kamagata Y. 2017. Phosphate-Catalyzed Hydrogen Peroxide Formation from Agar, Gellan, and κ-Carrageenan and Recovery of Microbial Cultivability via Catalase and Pyruvate. Appl Environ Microbiol 83:e01366-17.
26. Park S, You X, Imlay JA. 2005. Substantial DNA damage from submicromolar intracellular hydrogen peroxide detected in Hpx - mutants of *Escherichia coli*. Proc Natl Acad Sci 102:9317–9322.
27. Sen A, Imlay JA. 2021. How Microbes Defend Themselves From Incoming Hydrogen Peroxide. Front Immunol 12:667343.
28. Mishra S, Imlay J. 2012. Why do bacteria use so many enzymes to scavenge hydrogen peroxide? Arch Biochem Biophys 525:145–160.
29. Kato S, Yamagishi A, Daimon S, Kawasaki K, Tamaki H, Kitagawa W, Abe A, Tanaka M, Sone T, Asano K, Kamagata Y. 2018. Isolation of Previously Uncultured Slow-Growing Bacteria by Using a Simple Modification in the Preparation of Agar Media. Appl Environ Microbiol 84:e00807-18.
30. Kato S, Terashima M, Yama A, Sato M, Kitagawa W, Kawasaki K, Kamagata Y. 2020. Improved Isolation of Uncultured Anaerobic Bacteria using Medium Prepared with Separate Sterilization of Agar and Phosphate. Microbes Environ 35:n/a.
31. Brandi G, Sestili P, Pedrini MA, Salvaggio L, Cattabeni F, Cantoni O. 1987. The effect of temperature or anoxia on Escherichia coli killing induced by hydrogen peroxide. Mutat Res Lett 190:237–240.
32. Panek HR, O'Brian MR. 2004. KatG Is the Primary Detoxifier of Hydrogen Peroxide Produced by Aerobic Metabolism in *Bradyrhizobium japonicum*. J Bacteriol

186:7874–7880.

33. Bakshi CS, Malik M, Regan K, Melendez JA, Metzger DW, Pavlov VM, Sellati TJ. 2006. Superoxide Dismutase B Gene (*sodB*)-Deficient Mutants of *Francisella tularensis* Demonstrate Hypersensitivity to Oxidative Stress and Attenuated Virulence. J Bacteriol 188:6443–6448.

34. Verneuil N, Rincé A, Sanguinetti M, Posteraro B, Fadda G, Auffray Y, Hartke A, Giard J-C. 2005. Contribution of a PerR-like regulator to the oxidative-stress response and virulence of Enterococcus faecalis. Microbiology 151:3997–4004.

35. Park B, Nizet V, Liu GY. 2008. Role of *Staphylococcus aureus* Catalase in Niche Competition against *Streptococcus pneumoniae*. J Bacteriol 190:2275–2278.

36. Saumaa S, Tover A, Tark M, Tegova R, Kivisaar M. 2007. Oxidative DNA Damage Defense Systems in Avoidance of Stationary-Phase Mutagenesis in *Pseudomonas putida*. J Bacteriol 189:5504–5514.

37. Xue X, Tomasch J, Sztajer H, Wagner-Döbler I. 2010. The Delta Subunit of RNA Polymerase, RpoE, Is a Global Modulator of *Streptococcus mutans* Environmental Adaptation. J Bacteriol 192:5081–5092.

38. Reder A, Höper D, Gerth U, Hecker M. 2012. Contributions of Individual σ^B -Dependent General Stress Genes to Oxidative Stress Resistance of Bacillus subtilis. J Bacteriol 194:3601–3610.

39. Jiang Y, Dong Y, Luo Q, Li N, Wu G, Gao H. 2014. Protection from Oxidative Stress Relies Mainly on Derepression of OxyR-Dependent KatB and Dps in Shewanella oneidensis. J Bacteriol 196:445–458.

40. Farizano JV, Torres MA, Pescaretti M de las M, Delgado MA. 2014. The RcsCDB regulatory system plays a crucial role in the protection of Salmonella enterica serovar

Typhimurium against oxidative stress. Microbiology 160:2190–2199.

41. Zhang L, Alfano JR, Becker DF. 2015. Proline Metabolism Increases *katG* Expression and Oxidative Stress Resistance in Escherichia coli. J Bacteriol 197:431–440.

42. Karas VO, Westerlaken I, Meyer AS. 2015. The DNA-Binding Protein from Starved Cells (Dps) Utilizes Dual Functions To Defend Cells against Multiple Stresses. J Bacteriol 197:3206–3215.

43. Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007. Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. Appl Environ Microbiol 73:5261–5267.

44. Jiang Z-Y, Woollard ACS, Wolff SP. 1990. Hydrogen peroxide production during experimental protein glycation. FEBS Lett 268:69–71.

45. Wilson CL, Hinman NW, Cooper WJ, Brown CF. 2000. Hydrogen Peroxide Cycling in Surface Geothermal Waters of Yellowstone National Park. Environ Sci Technol 34:2655–2662.

46. Flowers RS, Martin SE, Brewer DG, Ordal ZJ. 1977. Catalase and enumeration of stressed Staphylococcus aureus cells. Appl Environ Microbiol 33:1112–1117.

47. Harmon SM, Kautter DA. 1976. Beneficial effect of catalase treatment on growth of Clostridium perfringens. Appl Environ Microbiol 32:409–416.

48. Imazaki I, Kobori Y. 2010. Improving the culturability of freshwater bacteria using FW70, a low-nutrient solid medium amended with sodium pyruvate. Can J Microbiol 56:333–341.

49. Norrod EP, Morse SA. 1982. Presence of hydrogen peroxide in media used for cultivation of Neisseria gonorrhoeae. J Clin Microbiol 15:103–108.

50. Watanabe M, Igarashi K, Kato S, Kamagata Y, Kitagawa W. 2022. Critical Effect of H_2O_2 in the Agar Plate on the Growth of Laboratory and Environmental Strains. Microbiol Spectr 10:e03336-22.

51. Seaver LC, Imlay JA. 2001. Hydrogen Peroxide Fluxes and Compartmentalization inside Growing *Escherichia coli*. J Bacteriol 183:7182–7189.

52. Imlay JA. 2019. Where in the world do bacteria experience oxidative stress?: Oxidative stress in natural environments. Environ Microbiol 21:521–530.

53. Zheng M, Wang X, Templeton LJ, Smulski DR, LaRossa RA, Storz G. 2001. DNA Microarray-Mediated Transcriptional Profiling of the *Escherichia coli* Response to Hydrogen Peroxide. J Bacteriol 183:4562–4570.

54. Sambrook J, Russell D. 2001. Molecular cloning: a laboratory manual, 3rd ed, vol 1. Cold Spring Harbor Laboratory Press, NY.

55. Chen S, Zhou Y, Chen Y, Gu J. 2018. fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics 34:i884–i890.

56. Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–2120.

57. Tange O. GNU Parallel 20210622 ('Protasevich').

58. De Coster W, D'Hert S, Schultz DT, Cruts M, Van Broeckhoven C. 2018. NanoPack: visualizing and processing long-read sequencing data. Bioinformatics 34:2666–2669.

59. Antipov D, Korobeynikov A, McLean JS, Pevzner PA. 2016. hybridSPAdes: an algorithm for hybrid assembly of short and long reads. Bioinformatics 32:1009–1015.

60. Prjibelski A, Antipov D, Meleshko D, Lapidus A, Korobeynikov A. 2020. Using SPAdes De Novo Assembler. Curr Protoc Bioinforma 70:e102.

61. Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068–2069.

62. Madeira F, Pearce M, Tivey ARN, Basutkar P, Lee J, Edbali O, Madhusoodanan N, Kolesnikov A, Lopez R. 2022. Search and sequence analysis tools services from EMBL-EBI in 2022. Nucleic Acids Res 50:W276–W279.

63. Watanabe M, Igarashi K, Kato S, Kamagata Y, Kitagawa W. Genome sequences of Comamonadaceae bacterium OS-1 and OS-4, two H2O2 highly sensitive strains isolated from pond water. Microbiol Resour Annoucement. [under peer review]

64. Schmid M, Frei D, Patrignani A, Schlapbach R, Frey JE, Remus-Emsermann MNP, Ahrens CH. 2018. Pushing the limits of de novo genome assembly for complex prokaryotic genomes harboring very long, near identical repeats. Nucleic Acids Res 46:8953–8965.

65. Tørresen OK, Star B, Mier P, Andrade-Navarro MA, Bateman A, Jarnot P, Gruca A, Grynberg M, Kajava AV, Promponas VJ, Anisimova M, Jakobsen KS, Linke D. 2019. Tandem repeats lead to sequence assembly errors and impose multi-level challenges for genome and protein databases. Nucleic Acids Res 47:10994–11006.

66. Seaver LC, Imlay JA. 2001. Alkyl Hydroperoxide Reductase Is the Primary Scavenger of Endogenous Hydrogen Peroxide in *Escherichia coli*. J Bacteriol 183:7173–7181.

67. Yuan F, Yin S, Xu Y, Xiang L, Wang H, Li Z, Fan K, Pan G. 2021. The Richness and Diversity of Catalases in Bacteria. Front Microbiol 12:645477.

68. Ezraty B, Henry C, Hérisse M, Denamur E, Barras F. 2014. Commercial Lysogeny Broth culture media and oxidative stress: A cautious tale. Free Radic Biol Med 74:245–251.

69. Shi M, Wan F, Mao Y, Gao H. 2015. Unraveling the Mechanism for the Viability Deficiency of Shewanella oneidensis oxyR Null Mutant. J Bacteriol 197:2179–2189.

70. Wan F, Shi M, Gao H. 2017. Loss of OxyR reduces efficacy of oxygen respiration in Shewanella oneidensis. Sci Rep 7:42609.

71. Li X, Imlay JA. 2018. Improved measurements of scant hydrogen peroxide enable experiments that define its threshold of toxicity for Escherichia coli. Free Radic Biol Med 120:217–227.

72. Wan F, Feng X, Yin J, Gao H. 2021. Distinct H2O2-Scavenging System in Yersinia pseudotuberculosis: KatG and AhpC Act Together to Scavenge Endogenous Hydrogen Peroxide. Front Microbiol 12:626874.

73. Lee J-S, Heo Y-J, Lee JK, Cho Y-H. 2005. KatA, the Major Catalase, Is Critical for Osmoprotection and Virulence in *Pseudomonas aeruginosa* PA14. Infect Immun 73:4399–4403.

74. Parales RE, Harwood CS. 1993. Construction and use of a new broad-host-range lacZ transcriptional fusion vector, pHRP309, for gram- bacteria. Gene 133:23–30.

75. Loewen PC, Switala J, von Ossowski I, Hillar A, Christie A, Tattrie B, Nicholls P. 1993. Catalase HPII of Escherichia coli catalyzes the conversion of protoheme to cis-heme d. Biochemistry 32:10159–10164.

76. Singh R, Wiseman B, Deemagarn T, Jha V, Switala J, Loewen PC. 2008. Comparative study of catalase-peroxidases (KatGs). Arch Biochem Biophys 471:207–214.

77. Takeda K, Nojima H, Kuwahara K, Chidya RC, Adesina AO, Sakugawa H. 2018. Nanomolar Determination of Hydrogen Peroxide in Coastal Seawater Based on the Fenton Reaction with Terephthalate. Anal Sci 34:459–464.

78. Garcia PE, Gerea M, Diéguez MC. 2020. Natural levels of hydrogen peroxide (H2O2) in deep clear South temperate lakes: Field and laboratory evidence of photo- and biotic production. Sci Total Environ 727:138641.

79. Olson JB, Lord CC, McCarthy PJ. 2000. Improved Recoverability of Microbial Colonies from Marine Sponge Samples. Microb Ecol 40:139–147.

80. Adam D, Maciejewska M, Naômé A, Martinet L, Coppieters W, Karim L, Baurain D, Rigali S. 2018. Isolation, Characterization, and Antibacterial Activity of Hard-to-Culture Actinobacteria from Cave Moonmilk Deposits. Antibiotics 7:28.

Acknowledgements

I would like to express my deep gratitude to my supervisor Dr. Wataru Kitagawa, without his support, my research would have not proceeded to this point. Whose expertise, patience and generous support made everything possible during my study in the graduate school. I learned a lot through these five years with him, not only the experimental techniques, but also the techniques on logical thinking, critical thinking, and “scientific” presentation. His taught will surely be my treasure for the rest of my life, no matter what kind of career I would choose in the future. I can’t thank him enough for all his efforts and hard works which made my academic career to be exiting and meaningful.

I appreciate Dr. Yoichi Kamagata for his supports. He was the motivation for me to study in the graduate school of Agriculture in Hokkaido university and to work on the cultivation of uncultured microbes. For these years, no matter how busy he was, he always cared about me and my experiments, which was the strongest mental support. It was my pleasure to be the student of the greatest microbiologist, and I would be pleased if my works would be his aid in the future.

Dr. Yoshitomo Kikuchi and Dr. Souichiro Kato had helped me a lot on my studies. Although our researching themes were slightly different, they always treated my problems as theirs and gave me valuable suggestions. They also kindly welcomed me to their fields, and they always gave me opportunities to talk with their co-workers. Which had widened my perspectives and helped me to gain wide variety of knowledges.

The days I spent in the Hokkaido center of National Institute of Advanced Industrial Science and Technology (AIST Hokkaido) were always exiting because of the people I met there. I was always surrounded by the talented scientists with different expertise,

which was the biggest advantage that I got. They were always friendly and patient, wishing my success on my study in the graduate school. I really appreciate every staff who supported my career, especially, I really appreciate Dr. Kensuke Igarashi, Dr. Ryosuke Nakai, and Dr. Shusei Kanie for their supports. I also feel many thanks to Ms. Miyako Hata, who supported me a lot in the laboratory. She was the most patient teacher on the operation of the laboratory equipment and without her, I wasn't able to complete the identification of more than 1,200 bacterial colonies, which was one of the biggest experiments in my life.

I appreciate Prof. Teruo Sone for his warm supports. Although I had no opportunity to work on his topics, he always kindly welcomed me to his field of study, letting me to participate in his students' seminars and journal clubs. With him and his students, my days were always brilliant. Current and former students in the laboratory of Applied Molecular Microbiology (also known as oukin laboratory), were always supportive and the days I spent with them were the precious experiences.

My appreciation also extends to Dr. Seonghan Jang, who was the most respectful "senpai" that I had met in my life. We discussed a lot on our experiments inside and outside of the laboratory, and his attitudes toward his career had always motivated me to do better. I will never forget the days we spent with our common friend Mr. Kota Ishigami.

Last but not the least, my deep appreciation goes to my family members. My life wouldn't have been this complete without their understanding and supports. My gratitude extends to my deceased grandfather Akiyoshi Sano, who gave me opportunities for education and to be like him is my lifelong goal.