博士論文

Development of mRNA visualization method specific to microbes within consortium

微生物コンソーシアム解析に向けた mRNA 特異的微生物可視化技術の開発

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主論文

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参考論文（学位要件論文）

1. Hirokazu Takahashi*, **Kyohei Horio***, Setsu Kato, Toshiro Kobori, Kenshi Watanabe, Tsunehiro Aki, Yutaka Nakashimada, Yoshiko Okamura, Direct detection of mRNA expression in microbial cells by fluorescence *in situ* hybridization using RNase H-assisted rolling circle amplification, *Scientific Reports*, **10**, 9588 (2020). DOI:10.1038/s41598-020-65864-7
   *: equally contributed
2. **Kyohei Horio**, Hirokazu Takahashi, Toshiro Kobori, Kenshi Watanabe, Tsunehiro Aki, Yutaka Nakashimada, Yoshiko Okamura, Visualization of gene reciprocity among lactic acid bacteria in yogurt by RNase H-assisted rolling circle amplification-fluorescence *in situ* hybridization, *Microorganisms*, **9**, 1208 (2021). DOI:10.3390/microorganisms9061208

関連論文

Hirokazu Takahashi, Masahiko Ohkawachi, **Kyohei Horio**, Toshiro Kobori, Tsunehiro Aki, Yukihiko Matsumura, Yutaka Nakashimada, Yoshiko Okamura, RNase H-assisted RNA-primed rolling circle amplification for targeted RNA sequence detection, *Scientific Reports*, **8**, 7770 (2018). DOI:10.1038/s41598-018-26132-x

主論文

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Contents

Chapter 1. Introduction

1.1. Background

Biotechnology, particularly in its application to the production of useful substances using microbes, has significantly increased living standards by aiding in the production of biopharmaceuticals, biochemical materials, and fermented foods. In the future, bitechnology is also expected to contribute to the achievement of the Sustainable Development Goals. Conventional microbial research has evolved based on the use of isolation and cultivation as a gold standard, therefore unculturable microbes that cannot be captured by this method were excluded from research. However, less than 1% of microbes in most environmental samples are culturable, and the remaining percentage of unculturable microbes are expected to possess several unknown activities [Amann et al. 1995, Rinke et al. 2013]. Therefore, expanding the available biological and genetic resources by utilizing isolation-independent research methods that can analyze a wider range of microbial species, can contribute to the development of further strategies for application in biotechnology. In addition, it could also help build sustainable society [Handelsman 2004].

To date, studies on the phylogenetic diversity of microbes with culture-independent methods have revealed the coexistence of known and novel microbes in environments, and the relationships among them have been inferred. In recent years, methods that focus on the "function" of microbes have been used, and it is desired to analyze microbial consortia in greater detail by clarifying not only "who is there" but also "what they are doing" [Handelsman 2004].

Recent developments in meta-analysis (metagenomics, metatranscriptomics,

metaproteomics, and metabolomics) have significantly advanced the functional analysis of microbial consortia. In particular, results of metatranscriptomic analysis using next-generation sequencing (NGS) well reflects the rapid changes in gene expression of microbes by sensing stimuli such as changes in environmental conditions [Moran 2009, Carvalhais et al. 2012, Franzosa et al. 2014, Goodwin et al. 2016]. However, the metatranscriptome data lacks the information of the "owner". To investigate "who is doing what", in other words, the function of individual microbes, it is necessary to identify which microbe is the “owner” of the mRNA, based on the data from analysis for performing further functional analysis. Therefore, there is a need for a technology that can connect mRNA and microbes for the analysis of microbial consortia.

1.2. Statement of the problem

In metatranscriptomic analysis, mRNA extraction is ordinarily performed from various microbial cells before sequencing. Thus, the host of the extracted mRNA is unknown. Therefore, the functions of individual microbes in the consortium cannot be revealed by metatranscriptomic analysis alone. Moreover, when the composition of the microbiota is unknown, metagenomic analysis is often performed to obtain reference sequences, but it is difficult to reconstruct whole genomes of all microbes, and to assemble the reads to large contigs [Qin et al. 2010]. To utilize functional microbes and their functional genes for biotechnological applications, it is important to understand how they function in their natural environment. To reveal how individual microbes function

and interact within a microbial consortium, transcriptome data needs to be combined with imaging techniques that can provide spatial information [Imdahl and Saliba 2020].

Fluorescence *in situ* hybridization (FISH) is a commonly used microbial imaging techniques, mainly to study the phylogenetic diversity of consortia by visualizing ribosomal RNA (rRNA) in microbial cells. Since the amount of mRNA in microbial cells is much lower than that of rRNA, mRNA cannot be visualized using the same method. Although FISH methods combined with signal enhancement techniques have been developed for eukaryotic cells and tissues [Larsson et al. 2010, Wang et al. 2012, Battich et al. 2013, Wu et al. 2018, Rouhanifard et al. 2019], these methods are not suitable for prokaryotic consortium analysis because of their specificity, high cost, and inability to detect short mRNA molecules. Table 1-1 shows a comparison of conventional mRNA-targeted imaging methods. All the methods in Table 1-1 targeted the specific sequences of not only mRNA but also genomic DNA since the probe recognized both DNA and RNA. Therefore, to detect mRNAs alone, a technique combined with RNA molecule-specific recognition should be developed.

The present study was undertaken to address these issues in imaging techniques of prokaryotic mRNA. Previous findings relevant to this study are summarized below.

1.3. Literature review

1.3.1. Recent studies analyzing microbial consortia using metatranscriptomics

Metatranscriptomic analysis has been applied to a variety of microbial

communities in marine [Amin et al. 2015, Moitinho-Silva et al. 2017], soil [Johnston et al. 2019], subsurface [Lau et al. 2016], and artificial environments [Luo et al. 2014, Nobu et al. 2015, Hao et al. 2020], and also within animals [McFall-Ngai et al. 2013, Bashiardes et al. 2016, Heintz-Buschart et al. 2018, Yang et al. 2020]. Metabolic pathways have been predicted by metagenome and genome sequences in databases, and the respective gene expression has been examined from metatranscriptome data to estimate microbial functions and interactions in complicated microbial communities. This approach revealed the following microbial interactions: interactions through the circulation of carbon, nitrogen, and sulfur [Lau et al. 2016, Moitinho-Silva et al. 2017, Hao et al. 2020], interspecies donation of signaling molecules that promote cell division [Amin et al. 2015], metabolic cooperation to degrade substrates that cannot be degraded by a single species [Luo et al. 2014], symbiotic relationships formed through the decomposition of inhibitor, such as hydrogen [Nobu et al. 2015], and symbionts that are interdependent with the host in terms of nutrition and energy [Bashiardes et al. 2016, Heintz-Buschart et al. 2018, Yang et al. 2020].

By analyzing the expression of metatranscriptomes targeting genes in metabolic pathways related to the observed functions, it is possible to estimate the microbes that play important functions in the consortium and the metabolic cooperation among multiple microbes. In relatively dominant microbial species, it is easy to obtain relatively long contigs even from metagenomes, and transcripts are relatively abundant. Therefore, they are likely to be of interest for gene expression analysis using expression level as a priority. On the contrary, the sequence data of minority microbial species tend

to be hidden by those of the dominant species. However, the results from recent studies reveal that the function of minority microbial species can have a significant impact on populations [Pester et al. 2010, Bodelier et al. 2013, Lawson et al. 2015, Jousset et al. 2017, Sato et al. 2019]. It was recently reported that *de novo* RNA-seq is useful for capturing the function of minority microbial species that are important in the consortium [Sato et al. 2019].

In addition, the following analytical techniques have been used to validate the estimated consortium functions and for more detailed analysis: targeted metabolite analysis [Amin et al. 2015], tracking of metabolites using stable isotopes [Lau et al. 2016], proteomics [Schmidt et al. 2013, Lau et al. 2016, Yang et al. 2020], single-cell genomics [Embree et al. 2014], and FISH targeting 16S rRNA to understand the location of microbial species [Moitinho-Silva et al. 2017]. By characterizing the metabolism based on functional estimation, it is possible to design a suitable medium, which may lead to the successful cultivation of unculturable microbes [Bomar et al. 2011].

1.3.2. Imaging technologies for microbes

1.3.2.1. 16S rRNA-targeted FISH

For microbial imaging, FISH has been widely employed to visualize cells by hybridizing fluorescently labeled oligonucleotide probes to rRNA, which is present in large amounts in cells [Sekiguchi et al. 1999, Dige et al. 2007, Dekas et al. 2014, Wada et al. 2016, Cardinale et al. 2018, Lukumbuzya et al. 2019]. For the detection of bacteria, universal probes designed for conserved regions of 16S rRNA [Amann et al. 1990, Daims

et al. 1999] and order-, family-, and genus-specific probes [Devereux et al. 1992, Raskin et al. 1994] have been developed to visualize the abundance and location of targeted bacterial species.

1.3.2.2. mRNA-targeted signal amplifying FISH

To detect mRNAs, which are much less abundant in cells than rRNA, many FISH methods combined with signal amplification techniques have been developed, mainly for eukaryotic cells and tissues [Larsson et al. 2010, Wang et al. 2012, Battich et al. 2013, Wu et al. 2018, Rouhanifard et al. 2019]. However, these techniques, which amplify signals from cDNA by reverse transcription [Larsson et al. 2010] or from scaffold probes directly hybridized to mRNA [Wang et al. 2012, Battich et al. 2013, Wu et al. 2018, Rouhanifard et al. 2019], cannot be directly used for prokaryotic mRNA detection. This is because prokaryotic mRNA is not spliced and contains the same sequence as the gene sequence of genomic DNA (gDNA), which may result in noise from nonspecifically hybridized scaffold probes. It has also been reported that the low reaction efficiency of intracellular reverse transcription reduces the detection rate in methods to detect cDNA [Lee et al. 2020].

CARD-FISH, two-pass TSA-FISH [Kubota et al. 2006, 2013] and 2C-FISH [Neuenschwander et al. 2015], which recognize mRNA with horseradish peroxidase (HRP)-labeled probes and amplify the signal by depositing large amounts of fluorescently labeled tyramide in the cells, have been reported to detect bacterial mRNA. However, endogenous HRP activity and excessively high sensitivity may affect specificity, limiting

its versatility [Kubota et al. 2006].

1.3.2.3. Single-molecule FISH

Recently, single-molecule FISH (smFISH) has been developed, in which multiple oligonucleotide probes labeled with the same fluorescence are directly hybridized to the target sequence to enhance the signal for detection [Raj et al. 2006, Raj et al. 2008, So et al. 2011, Skinner et al. 2013, Torre et al. 2018]. Badstöber et al. visualized the spatiotemporal interactions between pathogens and plants and algae using smFISH and demonstrated the possibility of using this technique to analyze interactions between organisms [Badstöber et al. 2020]. However, at least 32 to 48 probes with different sequences are required to detect an mRNA, so the cost per target mRNA becomes high [Badstöber et al. 2020]. Recently, Dar et al. developed a new mRNA detection method that solves this problem of smFISH and reported mRNA detection in bacteria [Dar et al. 2021]. However, in these techniques, which hybridize many probes to mRNA, the probe design is very complicated, and the noise originating from gDNA-hybridized probes cannot be controlled. Furthermore, due to the limitation of requiring a series of long sequences for probe recognition, it cannot be applied to short sequences obtained by metatranscriptomics, and mRNAs of gene family members with similar sequences are also difficult to identify [Larsson et al. 2010].

1.3.2.4. Rolling circle amplification-FISH

Rolling circle amplification (RCA) is an amplification method for single-strand

DNA, that utilizes the recognition of the target sequence by a circular probe and the highly accurate and continuous DNA synthesis by φ29 DNA polymerase [Li et al. 2009, Paez et al. 2004]. It enables highly sensitive detection of targets by detecting the reaction product, long single-strand DNA (RCA product, RCP) [Zhong et al. 2001, Lizardi et al. 1998]. This method has also been applied to RNA detection [Li et al. 2009, Takahashi et al. 2010]. However, the detection target of the circular probe was limited to the 3′-end [Takahashi et al. 2010], and the use of an additional primer to initiate the RCA reaction to detect the internal sequence produced noise. It has been reported that the 3′→5′ exonuclease activity of φ29 DNA polymerase eliminates the need for additional primers [Lagunavicius et al. 2008, 2009], but this activity has not been confirmed [Larsson et al. 2010]. RCA was combined with the padlock probe (PLP) system, which is made of single-stranded DNA with 10−20 mer recognition sequences at each end, with both ends are ligated to form a circular probe after hybridization to the target sequence [Larsson et al. 2004, Nilsson et al. 1994], *in situ* mRNA detection methods with high sequence specificity were developed [Larsson et al. 2010, Ke et al. 2013]. Since the total recognition sequence of PLP is 20−40 mer, short mRNA can be detected. In addition, since RCP is a tandem repeat of the complementary strand of PLP, it can be easily detected by *in situ* hybridization of fluorescent oligonucleotide probes with partial sequences of PLP. Therefore, only one fluorescent probe per target mRNA needs to be designed. The visualization of miRNAs in eukaryotic cells using the RCA method has been reported [Wu et al. 2016, Krzywkowski and Nilsson 2017, Lin et al. 2020].

Unlike short RNAs such as miRNAs, for which the entire sequence can be

hybridized to PLP and used as a primer, the method of hybridizing an additional primer to PLP to initiate the amplification reaction has been used for mRNA detection [Deng et al. 2017, Schneider and Meier 2017, Sountoulidis et al. 2020]. However, nonspecific signals may be produced due to nonspecific hybridization of the additional primers to intracellular molecules [Cao et al. 2020].

Table 1-1 Comparison of existing mRNA-targeted imaging methods.

Method	Detection principle	Fluorescently-labeled probes/mRNA	Length of the RNA sequence needed to recognize (mer)	Targeted organisms	Bacterial mRNA specific detection	References
CARD-FISH, Two-pass TSA-FISH	Hybridization of complementary probe and enzymatic reaction of HRP and tyramide.	1 (Tyramide-fluorophore)	20	bacteria, archaea	× (intrinsic HRP activity can cause non-specific signals)	Kubota et al. 2006, Kubota 2013
RNAscope, bDNA-smFISH	Recognize the target mRNA directly with "z"-shaped probes and making it a scaffold of signal amplification.	1~2	1 k	mammal	× (gDNA-hybridized target probes can cause non-specific signal)	Wang et al. 2012, Battich et al. 2013
RollFISH	Direct hybridization of recognition probes and RCA	1	1 k~2 k	mammal	× (gDNA-hybridized target probe can cause non-specific signal)	Wu et al. 2018
ClampFISH	Repeating hybridization of “c” form probes and ligation	2 (ClampFISH probes)	300	mammal	× (gDNA-hybridized target probe can cause non-specific signal)	Rouhanifard et al. 2019
smFISH	Direct hybridization of fluorescently-labelled probes	32~	640~	bacteria, archaea, eukaryotes	△ (gDNA-hybridized target probes can cause non-specific signal)	Raj et al. 2006, 2008, So et al. 2011, Skinner et al. 2013, Torre et al. 2018
par-seqFISH	Recognize the target mRNA with primary probes, and hybridize the fluorescently labeled probe to both ends of primary probes.	1	360~600	bacteria	△ (gDNA-hybridized target probes can cause non-specific signal)	Dar et al. 2021
cDNA-targeted RCA-FISH	RT and RCA	1	20~40	mammal	× (cannot distinguish cDNA and gDNA)	Larsson et al. 2010
mRNA-targeted RCA-FISH	RCA with additional RCA primer	1	20~40	mammal	× (non-specific hybridization of additional primer can cause non-specific signal)	Deng et al. 2017, Schneider et al. 2017, Sountoulidis et al. 2020

1.3.3. microbial single-cell RNA-seq - A technique expected to be developed further for better analysis of microbial consortia

Even in a population consisting of a single microbial species, gene expression is not the same and differs from cell to cell due to factors such as environmental changes, stress, and other stimuli [Ackermann 2015, Engl et al. 2019, Kuchina et al. 2021]. In environmental microbial consortia, where diverse microbes are heterogeneously mixed and can be altered by trivial stimuli, gene expression differs significantly in each microspace [Davis and Isberg 2016]. Data from transcriptome analysis averaging the expression levels of individual cells cannot capture such changes in gene expression in individual cells.

For eukaryotic cells and tissues, single-cell RNA-seq (scRNA-seq), which isolates individual cells and comprehensively analyzes gene expression in each cell, is now available [Picelli et al. 2013]. Methods for microbial scRNA-seq have also been developed in recent years [Blattman et al. 2020, Imdahl et al. 2020, Kuchina et al. 2021], but a technique that can be used to analyze a wide variety of microbes in the environment has not been fully developed [Imdahl and Saliba 2020-a]. If such a technique can be advanced further to reveal the genes expressed in environmental microbial consortia with greater accuracy, the estimation of interactions will be more precise. The accurate estimation will help to design efficient probes for imaging. This will also help imaging techniques access the hidden gene expression of important minority microbial species, greatly advancing the analysis of environmental microbial consortia.

1.3.4. RNA direct detection method

So far, we have developed new detection method using PLP combined with RCA, and this is a direct RNA detection method [Takahashi et al. 2018]. This method, called RNase H-assisted RCA (RHa-RCA), uses the target RNA directly as a primer for the RCA reaction. The conversion of RNA into primer is controlled by the substrate specificity of the nuclease, and DNA cannot be converted into a primer. Therefore, amplification is initiated when the PLP hybridizes to the RNA. On the other hand, indirect detection using padlock probe combined with RCA employed additional probe recognizing PLP sequence. Therefore, once PLP was circularized, even if it was hybridized with gDNA, the additional probe could start amplification. An indirect probe to recognize DNA or RNA.

1.4. Objectives & significance of the study

As mentioned above, we attempted the RHa-RCA would be applicable if the RCA reaction was able to proceed within a bacterial cell. The objectives of this study were, therefore: the development of a novel method to visualize mRNA specifically in bacterial cells, and the verification of the applicability of the method developed to visualize functional gene expression using a known microbial consortium.

Although conventional analysis using NGS has increased the accessibility of information on gene expression in microbial populations, the inability to pinpoint the origins of the transcripts and the use of population-averaged data limited the consortium

analysis to taxonomy-based estimation. Such estimation was insufficient to clarify "who is doing what?" Techniques for analyzing gene expression in microbial consortia by imaging, make it possible to perform a detailed analysis of individual microbes under original conditions (growth stage, environmental conditions, etc.) where they function. Such techniques would allow us to investigate how the functions of microbial consortia are achieved at the single-cell level, to analyze the mechanisms of unknown functions within it, and to efficiently search for conditions enabling fermentative production of new useful substances using microbial consortia. Furthermore, such techniques will contribute to the discovery of environmental conditions, nutrients, and microbial interactions necessary for the cultivation and isolation of unculturable microbes.

Consequently, this study will contribute to the expansion of available microbial and genetic resources by providing a technique for the detailed analysis of gene expression in microbial consortia and will also potentially lead to the construction and control of complex microbial systems. Therefore, this study is of great academic and social significance.

Chapter 2. Development of RHa-RCA-FISH for direct detection of mRNA in microbial cells

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2.1. Introduction

Imaging technology is a powerful tool to spatiotemporally link NGS data and single cells and can potentially reveal gene expression in individual cells within a microbial consortium. However, the currently available techniques for mRNA detection in bacterial cells do not meet the requirements for convenience and specificity.

RNase H-assisted RCA (RHa-RCA, Figure 2-1) has been developed by applying the RCA method using PLP to specifically detect RNA [Takahashi et al. 2018]. In this method, a 70-mer PLP harboring 15-mer recognition sequences in both ends were hybridized to the target mRNA, following the circularization with SplintR® ligase. Then, RNase H specifically recognized mRNA-PLP hybrid sequence and digested hybridized mRNA, and φ29 DNA polymerase synthesized a long ssDNA (RCA product, RCP) from the 3′-end of the mRNA. Binding fluorophores (e.g., SYBR Green II) to RCPs, the signal from mRNA was specifically amplified and made detectable [Takahashi et al. 2018].

When PLP is hybridized to the complementary RNA strand, RNase H specifically digests the RNA hybridized by PLP and makes it a primer of the RCA reaction. Therefore, noise derived from gDNA-hybridized PLP does not occur (Figure 2-1) [Takahashi et al. 2018]. In addition, a PLP has a total of 30 mer in its recognition sequence, enabling recognition of short RNA molecules. Also, this method is relatively inexpensive because it requires only one PLP and one FISH probe for mRNA detection. Therefore, RHa-RCA was combined with FISH to develop RHa-RCA-FISH, a method for *in situ* bacterial mRNA detection.

The detection targets of RHa-RCA-FISH were green fluorescent protein (GFP)

mRNA (for *Escherichia coli*) and red fluorescent protein from *Discosoma* sp. (DsRed) mRNA (for *Brevibacillus choshinensis*) because gene expressions are easily confirmed by fluorescence.

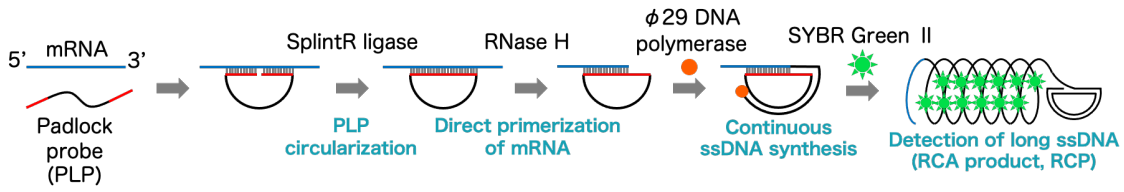

Figure 2-1 Schematic overview of the detection principle of RHa-RCA.

2.2. Materials and Methods

2.2.1. PLPs and FISH probes

Sequences of PLPs and FISH probes are shown in Table 2-1 and Table 2-2. PLPs were purchased from Eurofins Genomics, Inc. (Ebersberg, Germany). Fluorescently-labeled probes (FISH probes) were purchased from Japan Bio Services Co., LTD. (Saitama, Japan).

Table 2-1 Sequences of PLPs for *gfp* mRNA and *dsred* mRNA.

Name	**Sequence (5′→3′)**
PLP-GFP	AGCCCTCAGGCATGGttccttttacga*CCTCAATGCTGCTG* *CTGTACTAC*tcttcTGCGCTCCTGGATGT
PLP-DsRed	AGTCGCAGGTGTAGTGtttcttttactc*CCTCAATGCACATG*

	*TTTGGCTCC*tctttGTACACGGTCTTGA

The underlined letters are mRNA recognition sequences, and italic letters are FISH probe sequences.

Table 2-2 Sequences of FISH probes for *gfp* mRNA and *dsred* mRNA.

Name	**Sequence (5′→3′)**
Alexa568-FISH probe	Alexa568-CCTCAATGCTGCTGCTGTACTAC
Alexa488-FISH probe	Alexa488-CCTCAATGCACATGTTTGGCTCC

Alexa568-FISH probe and Alexa488-FISH probe are specific to RCPs of PLP-GFP and PLP-DsRed, respectively. Sequences were from Larsson et al. [Larsson et al. 2004, 2010].

2.2.2. Plasmids and bacterial strains

Bacterial strains and plasmids are shown in Table 2-3. *E. coli* BL21 (DE3) and pET21d were purchased from Novagen, Merck Millipore (Darmstadt, Germany). *B. choshinensis* HPD31-SP3, pNI-His, and pDsRed-Monomer vector were purchased from Takara Bio (Shiga, Japan). All plasmids were constructed using the cell-free cloning method [Takahashi et al. 2009].

Table 2-3 Bacterial strains and plasmids.

Bacterial strains	**Description**	**Manufacturers**
Plasmids		**References**

E. coli BL21 (DE3)	F^- *ompT hsdS*(r_B^- m_B^-) *gal dcm* (DE3)	Novagen
B. choshinensis HPD31-SP3	Spore-related gene *imp* and protease gene *emp* are disrupted in *B. choshinensis* HPD31.	[Mizukami et al. 2010] Takara Bio
pET-21d	Used as an expression vector of *gfp* in *E. coli* and for *in vitro* transcription of *dsred,* Amp^r	Novagen
pNI-His	An expression vector for *Brevibacillus*, His-tag fusioned, Nm^r	[Adachi et al. 1989] Takara Bio
pDsRed-Monomer vector	Holding *dsred* cassette	Takara Bio
pET-AcGFP	*gfp* was inserted between the *Nco*I and *Not*I sites of pET21d.	[Takahashi et al. 2010]
pET-DsRed	*dsred* was inserted between the *Nco*I and *Not*I sites of pET21d.	This study
pNI-DsRed	*dsred* was inserted between the *Nco*I and *Not*I sites of pNI-His.	This study

2.2.3. Media and cultivation of bacteria

2.2.3.1. *E. coli*

E. coli BL21 (DE3) was transformed with pET-AcGFP and grown in LB/amp medium (1% tryptone, 0.5% yeast extract, 1.0% NaCl, and 50 μg/mL ampicillin) in an L-formed test tube for 14–16 h at 30°C with shaking at 140 rpm. The preculture (3 μL) was added to 3 mL of LB/amp medium and cultivated at 30°C with shaking at 140 rpm. The expression of *gfp* was induced by adding 15 μL of 100 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) when the OD_{660} was approximately 0.6. One hour after the start of induction, cells were collected in 1.5 mL tubes by centrifugation at 10,000 × *g* for 2 min at 4°C. Non-induced cells were cultivated in LB/amp medium containing 2% glucose. Harvested cells were immediately resuspended in saturated ammonium sulfate (SAS) solution to inhibit degradation of RNA and stored at 4°C before use.

2.2.3.2. *B. choshinensis*

B. choshinensis HPD31-SP3 was transformed with pNI-DsRed and grown in 2SYF/Nm medium (20.0 g/L fructose, 40.0 g/L Phytone Peptone (Becton, Dickinson, and Co., Franklin Lakes, NJ), 5.0 g/L Ehrlich bonito extract (Kyokuto Pharmaceutical Co.LTD, Tokyo, Japan), and 0.15 g/L $CaCl_2 \cdot 2H_2O$, 50 μg/mL neomycin) in L-formed test tube for 12, 24, 48, or 72 h at 30°C with shaking at 220 rpm. The cells were harvested in 1.5 mL microcentrifuge tubes by centrifugation at 10,000 × *g* for 2 min at 4°C. Harvested cells were resuspended in SAS and stored at 4°C before use.

2.2.4. in vitro transcription

A plasmid was linearized by restriction enzyme digestion. *in vitro* transcription was performed using T7 RiboMAX™ Express Large Scale RNA Production System (Promega Madison, WI). Template DNAs were digested by RNase-free DNase I (New England BioLabs) treatment. The *in vitro* transcribed mRNA (IVT) was purified using a NucleoSpin® RNA Clean-up XS kit (Macherey-Nagel). The concentration of IVT was measured by Qubit® fluorometer and Qubit® RNA BR Assay Kit. The transcribed mRNA was stored at −80°C until use.

2.2.5. Real-time RHa-RCA

To check the detection sensitivity of PLP, real-time RHa-RCA was performed with IVT as a detection target according to Takahashi et al. [Takahashi et al. 2018]. The IVT was mixed with 250 fmol of PLP in 10 μL of hybridization mixture (1 μL of oligonucleotide annealing buffer (200 mM Tris-acetate (Tris-Ac) [pH 7.5], 500 mM potassium glutamate (KGlu), 5 mM ethylenediaminetetraacetic acid (EDTA)) and an appropriate amount of IVT), followed by incubation for 1 min at 95°C, 3 min at 45°C and 10 min at 30°C. After hybridization, 10 μL of the ligation mixture (1 μL of Ligation buffer (200 mM Tris-Ac [pH 7.5], 500 mM KGlu, 200 mM Mg-acetate (MgAc)), 0.8 mM ATP, 2 mM dithiothreitol (DTT), 25 units of SplintR® ligase (New England BioLabs, Ipswich, MA)) was added, and incubated for 10 min at 37°C to circularize the PLP. Finally, 20 μL of an RCA mixture (1.8 μL of buffer for SpRCA-1T (300 mM Tris-Ac [pH 7.5], 500 mM KGlu, 300 mM MgAc, 800 mM ammonium sulfate), 2 mM deoxynucleoside triphosphate

(dNTPs), 0.001 units of pyrophosphatase (New England BioLabs), 0.03 units of RNase H (BioAcademia, Osaka, Japan), 2× concentration of SYBR Green II (Invitrogen, Thermo Fischer Scientific, Waltham, MA, USA), and 200 ng of DNA-free φ29 DNA polymerase (Kanto Chemical, Tokyo, Japan)) were added, followed by incubation for 2 h at 30°C to synthesize RCPs. For real-time detection, RHa-RCA reactions were performed in a 96-well PCR plate, and fluorescence signals were measured every 10 min for 2 h with the FAM filter (excitation wavelength: 482 nm, fluorescence wavelength: 536 nm) of the Thermal Cycler Dice Real-Time System II (TP900, Takara Bio, Otsu, Shiga, Japan). All real-time RHa-RCA experiments were performed in triplicate, and solutions and mixtures were prepared in a bench-top clean room (KOACH 500F, Koken Ltd., Tokyo, Japan) [Takahashi et al. 2016] to prevent contamination.

2.2.6. Cell fixation and permeabilization

The cell density was adjusted to approximately OD_{660}=1 by SAS, and cells were centrifuged at 20,000× g for 1 min at 4°C to remove SAS. Then, cells were fixed by resuspending with 300 μL of 4% paraformaldehyde (PFA) in phosphate buffer (Nacalai Tesque Inc., Kyoto, Japan) and incubating at room temperature for 15 min. Cells were centrifuged again to remove 4% PFA, resuspended with 70% ethanol, and incubated for 1 h at room temperature for dehydration. After removing ethanol solution by centrifugation, cells were resuspended with lysozyme in 1× TE buffer to permeabilize the cell wall. Finally, lysozyme solution was removed by centrifugation and cells were washed twice with 1× phosphate-buffered saline (PBS; 137 mM NaCl, 8.1 mM Na_2HPO_4,

2.68 mM KCl, 1.47 mM KH_2PO_4, pH 7.4).

2.2.7. RHa-RCA-FISH

Pretreated cells were resuspended with an appropriate amount of dDW and transferred to a 0.2 mL tube. Next, 2 μL of oligonucleotide annealing buffer and 20 pmol PLP were added to the tube (final volume, 20 μL), and incubated for 1 min at 95°C, slowly cooled to 30°C, and incubated for 10 min at 30°C to hybridize the PLP. Then, 10 μL of ligation mixture (1.25 μL of Ligation buffer, 1.2 mM ATP, 3 mM dithiothreitol (DTT), 25 units of SplintR® ligase (New England BioLabs, Ipswich, MA)) was added to the tube and incubated for 10 min at 37°C to circularize the PLP. In this study, the inactivation step of the ligase was omitted to shorten and simplify the procedure. This small change would not adversely affect the reaction because the sensitivity of detection was equal or rather high. Finally, 20 μL of RCA mixture (2.25 μL of buffer for SpRCA-1T, 2 mM dNTPs, 0.004 units of pyrophosphatase (New England BioLabs), 0.06 units of RNase H (BioAcademia), and 500 ng of φ29 DNA polymerase (Kanto Chemical, Tokyo, Japan)) was added to the tube and incubated for 2 h at 30°C to synthesize RCPs. The RCA reaction was stopped by incubating for 10 min at 65°C.

After the reaction, cells were transferred to a 1.5 mL tube, centrifuged for 1 min at 20,000 × *g* at 4°C to remove the reaction solution, and washed with 1× PBS. Cells were transferred again to a 1.5 mL black tube, and FISH was carried out by incubating for 3 h at 37°C in 50 μL of FISH solution (100 pmol FISH probe-containing 2 × saline sodium citrate (SSC; 300 mM NaCl, 30 mM trisodium citrate, pH 7.0)). The cells were

centrifuged to remove the FISH solution, washed twice by incubating for 15 min at 37°C in 100 μL of 1×PBS.

2.2.8. Imaging and analysis

Samples were placed on 1% agarose pads containing 1× PBS. Images of each sample were taken on a fluorescence microscope (Nikon ECLIPSE E600 for *E. coli* and Nikon ECLIPSE Ti2-E for *B. choshinensis*, Nikon Corp., Tokyo, Japan) equipped with a phase-contrast objective CFI PlanApo DM 100× (Nikon Corp.) and an ORCA-Flash4.0 V3 camera (Hamamatsu Co., Shizuoka, Japan). All phase contrast and fluorescence images were captured using an exposure time of 100 ms. The images were analyzed using ImageJ software (v 1.52a, NIH, Bethesda, MD).

2.3. Results and discussion

2.3.1. Design of RHa-RCA-FISH

The schematic overview and workflow of RHa-RCA-FISH are shown in Figure 2-2 and Figure 2-3. The cell pretreatment method which enabled *in situ* RHa-RCA reaction and FISH was developed to be suitable for liquid reaction with reference to previous FISH methods [Maruyama et al. 2005, Hoshino and Schramm 2010, Haroon et al. 2013, Schneider et al. 2017]. In this procedure, lysozyme was adopted for permeabilizing the bacterial cell wall. Although treatment with Triton X-100, which is also used to permeabilize the cell membrane in mammalian cells was tried, almost no cell

harboring fluorescent signal was detected [Schneider et al. 2017]. Therefore, it was important to permeabilize the cell wall of bacterial cells to enable the *in situ* reaction.

After performing *in situ* RHa-RCA, RCPs are synthesized in the cells. Since RCPs are constructed with the tandem repeats of a complemental sequence of PLP, they can specifically be detected by hybridizing FISH probes having the same sequence to PLP (Figure 2-2). Furthermore, the maximum number of repeats of the complemental sequence of PLP is 5,143 to 20,571 in the 2 h of RHa-RCA reaction, therefore, signals from the FISH probe are sufficiently detectable (at least 48 probes are required in smFISH to detect bacterial mRNA [Skinner et al. 2013]).

In this method, all the steps from cell fixation to FISH can be completed in approximately 8 h (Figure 2-3). Since RCPs formed in the cells are stable at 4°C, the cells were stored at that temperature after the RHa-RCA reaction until the FISH step was performed the next day.

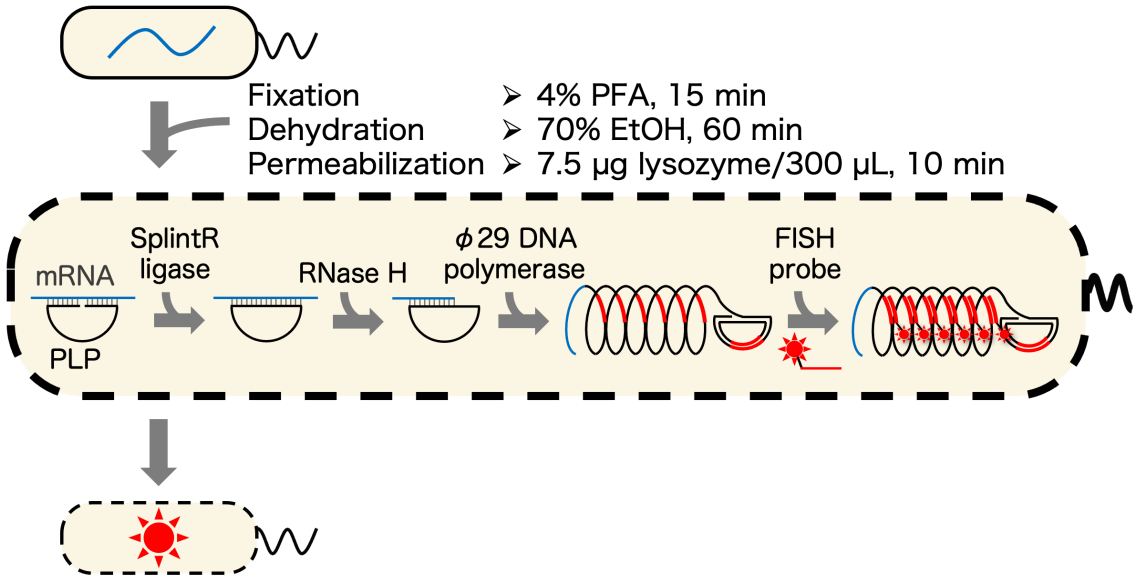

Figure 2-2 Schematic overview of RHa-RCA-FISH.

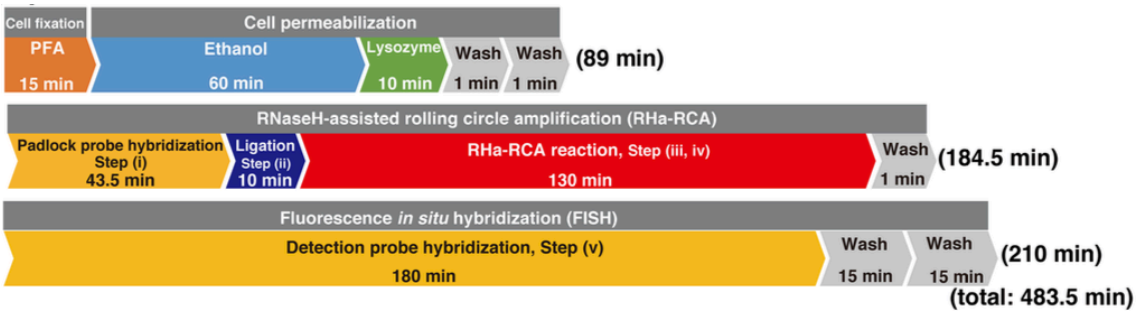

Figure 2-3 Workflow of the RHa-RCA-FISH procedure and required time for mRNA detection.

2.3.2. Developing RHa-RCA-FISH to Gram-negative model bacteria

gfp-expressing *E. coli* was used as a target for the detection of mRNA by RHa-RCA-FISH. pET-AcGFP was introduced into *E. coli* BL21(DE3) and cultured under conditions of induction (with IPTG) or non-induction (with glucose) of *gfp* expression. GFP fluorescence was obtained in the cells collected 2 h after the start of induction, while

there was no fluorescence in the non-induced cells.

After cell fixation, dehydration, and permeabilization with 7.5 μg of lysozyme/300 μL-reaction volume, RHa-RCA-FISH was applied to the *E. coli* cells. As a result, fluorescent signals from the RCP-hybridized Alexa568-labeled probe were detected from more than 80% of whole *gfp*-induced cells (Figure 2-4 B). On the other hand, no signal was obtained from non-induced cells (Figure 2-4 A). These results suggest that RHa-RCA-FISH was successfully applied to *E. coli* and specifically detected *gfp* mRNA in their cells. In addition, no non-specific signal was generated from cells having the *gfp* gene on the plasmid, indicating that the mRNA could be specifically detected without noise derived from DNA.

Also, as observed by smFISH [So et al. 2011, Skinner et al. 2013], the fluorescent signal formed a spot-like shape (Figure 2-4 C). Unfortunately, bacterial cells were too small to identify each RCP in the cell and determine the exact number of spots. Counting the number of spots in a microbial cell requires the use of super-resolution microscopy such as Stochastic Optical Reconstruction Microscopy (STORM) [Moffitt et al. 2016].

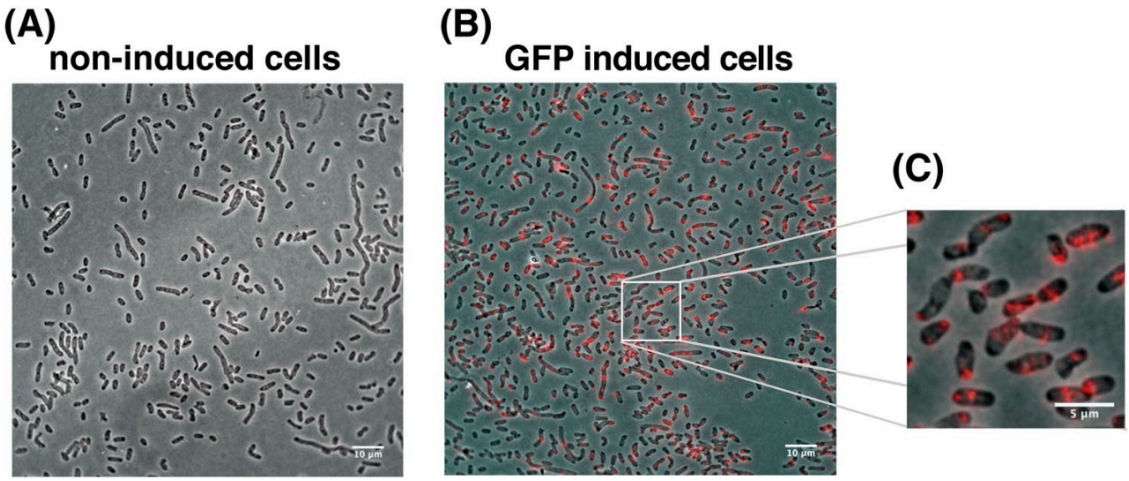

Figure 2-4 *in situ* detection of *gfp* mRNA in *E. coli* cells by RHa-RCA-FISH. (A) Detection of *gfp* mRNA in non-induced cells and (B) in induced cells. Scale bar, 10 μm. (C) Magnified image of the box. Scale bar, 5 μm. Overlays of the phase contrast (grayscale) and fluorescent signals from Alexa568-labeled probes (red) are shown.

2.3.3. Application of RHa-RCA-FISH to Gram-positive model bacteria

To apply RHa-RCA-FISH to Gram-positive bacteria, DsRed was expressed in *B. choshinensis* HPD31-SP3 and targeted *dsred* mRNA for *in situ* detection. In this experiment, *dsred* is driven by the native P2 promoter and expressed during the cell wall synthesis. When cells were cultured for 12 to 72 h and observed by fluorescence microscopy, the number of cells emitting fluorescence of DsRed protein gradually increased with the cultivation time (Figure 2-5, upper images).

By performing RHa-RCA-FISH using the same protocol as that built-in *E. coli*, the signal of the Alexa488-labeled probe was obtained from the *B. choshinensis* cells in all samples (Figure 2-5, lower images). It indicated that the same experimental conditions

can also be applied to *B. choshinensis*, a Gram-positive bacterium, as to *E. coli*, a Gram-negative bacterium. On the other hand, similar signals were detected in all cells in the detection of *gfp* mRNA in *E. coli*, but in *B. choshinensis*, the number of cells with signals varied between time and even among individual cells (Figure 2-5, magnified images). This may be because the timing of expression differs among cells due to relatively slow *dsred* induction. Since there is no significant difference in signal intensity among *gfp*-induced *E. coli* cells, whose gene expressions were expected to occur almost at the same time, the difference in signal intensity among cells observed in the *B. choshinensis* is not considered to have occurred because of using this technique. Therefore, the results obtained by RHa-RCA-FISH can reflect the differences in gene expression levels among the cells in the sample. However, some risks remained; prominent among them being that leakage of mRNA from the cell may affect the quantitative value of the gene expression levels, due to the improved cell permeability. Therefore, the quantitative value of bacterial mRNA detection by RHa-RCA-FISH would need to be evaluated future studies using cells large enough to count RCPs in the cell.

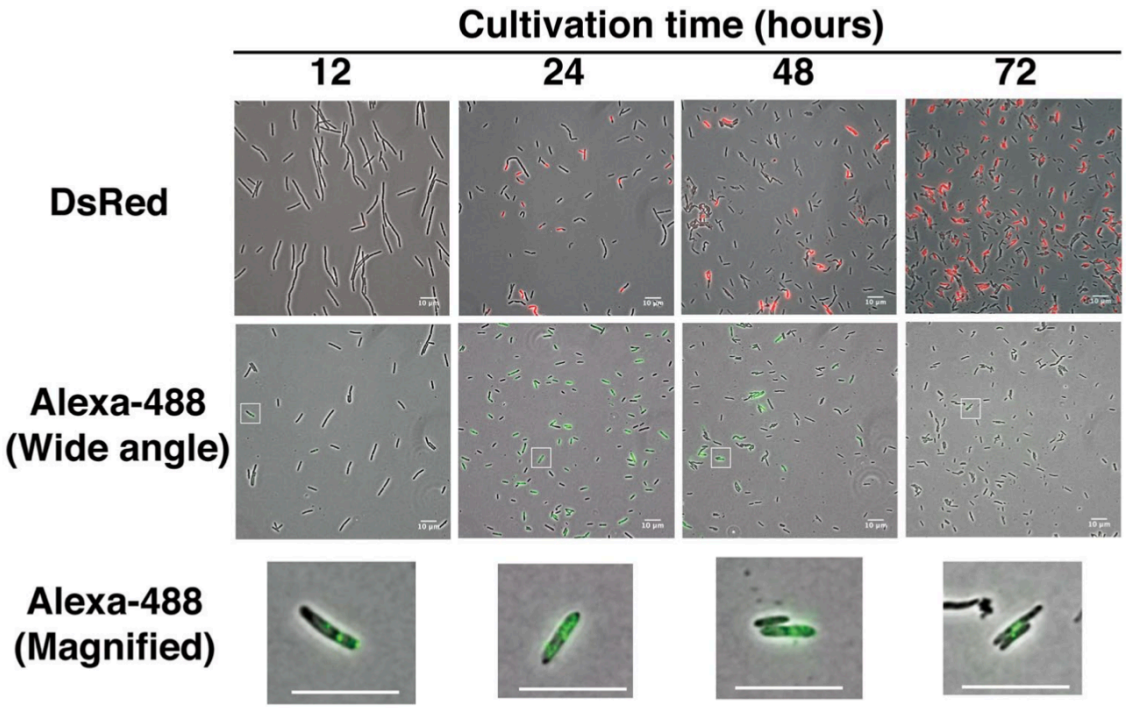

Figure 2-5 *in situ* detection of *dsred* mRNA in *B. choshinensis* cells by RHa-RCA-FISH. Upper images show fluorescence of DsRed protein expressed in *B. choshinensis*; middle images show the signal of detection probes using FISH; lower images show the magnified images of boxes in middle images. The cells in the upper and middle images are from different samples because the DsRed protein is denatured by the heating step of the PLP hybridization. Overlays of the phase contrast (grayscale), DsRed protein (red), and fluorescent signals of Alexa488-labeled probes (green) are shown. Scale bar, 10 μm.

2.3.4. *Simultaneous detection of mRNA within two bacterial species expressing different genes*

To investigate whether RHa-RCA-FISH can specifically detect gene expression

in mixed samples of different bacteria, *gfp*-expressing *E. coli* and *dsred*-expressing *B. choshinensis* were mixed and RHa-RCA-FISH was performed with both PLPs in one reaction. As a result, signals from Alexa568- and Alexa488-labeled probes were obtained from different cells （Figure 2-6）. This result indicates that RHa-RCA-FISH can specifically and simultaneously detect gene expression in different bacteria.

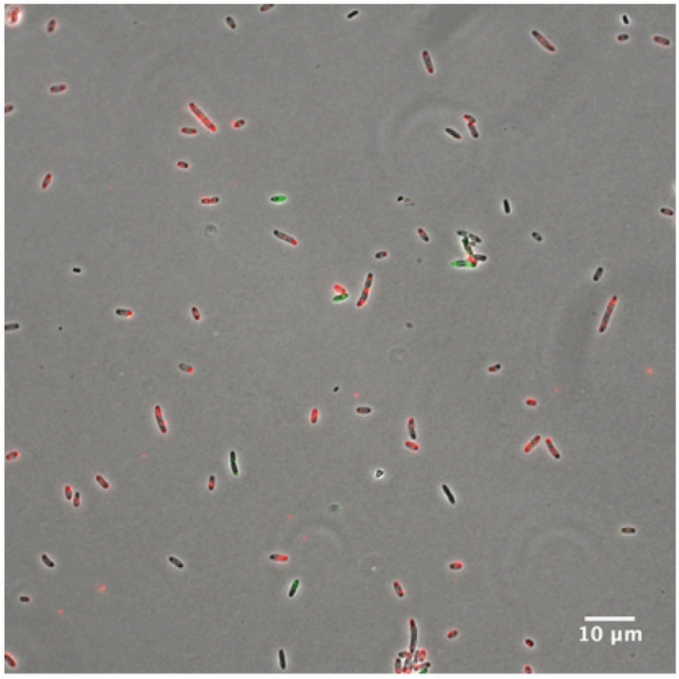

Figure 2-6 Simultaneous detection of *gfp* mRNA and *dsred* mRNA in a mixture of *E. coli* and *B. choshinensis* cells. An overlay of the phase contrast (grayscale) and fluorescent signals of Alexa568-labeled probes (red) from *gfp* mRNA and Alexa488-labeled probes (green) from *dsred* mRNA are shown. Scale bar, 10 µm.

Simultaneous visualization of the expression of a large number of genes has been done by *in situ* sequencing, in which multiple mRNA species are detected by

repeated stripping and hybridization of fluorescent probes. For example, par-seq FISH (a method based on direct hybridization of probe) has been applied to bacteria [Dar et al. 2021], and HybRISS (a method based on RCA) has been applied to mammalian cells [Lee et al. 2020]. Therefore, it would be also possible to visualize the expression of multiple genes in a single cell in the same way in RHa-RCA-FISH. In this case, the size of RCPs formed in the cells by the 2 h-reaction may affect the resolution of fluorescence microscopy. By adjusting the reaction time and observing with a high-resolution microscope and high-resolution camera, it would be possible to identify even if a large number of RCPs are formed in the cell. The use of such instruments that allow detailed analysis and the modification of the reaction will expand the possibilities of understanding microbial functions using this method.

2.4. Conclusion

In this chapter, the successful development of a direct detection method of bacterial mRNA, RHa-RCA-FISH by applying RHa-RCA to bacterial cells using model bacteria, was described (Figure 2-2). Using this method, target mRNA can specifically be recognized and detected in bacterial cells which contain genomic DNA, plasmid DNA, and cytoplasmic components, and do not generate non-specific signals from DNA.

In addition to being able to apply this method to both Gram-negative and Gram-positive bacteria, it was also possible to discriminate the expression of different genes in different bacteria within one reaction. Therefore, RHa-RCA-FISH may be applicable to

the analysis of samples of a mixture of microbes like a microbial consortium. However, since such samples contain impurities other than bacterial cells, unlike the liquid culture of model bacteria, optimization of the cell recovery method and pretreatment conditions before the reaction will be important.

RHa-RCA-FISH will help in more accurate prokaryotic consortium analysis by combining metatranscriptomic data with imaging technology. By using this technique for microbial consortium analysis, metatranscriptomic data will be magnified to the single-cell level, enabling higher resolution analysis of microbial functions and their interactions.

Chapter 3. Validation of applicability of RHa-RCA-FISH to microbial consortium analysis using lactic acid bacteria in yogurt

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Published as **Kyohei Horio**, Hirokazu Takahashi, Toshiro Kobori, Kenshi Watanabe, Tsunehiro Aki, Yutaka Nakashimada, Yoshiko Okamura, Visualization of gene reciprocity among lactic acid bacteria in yogurt by RNase H-assisted rolling circle amplification-fluorescence *in situ* hybridization, *Microorganisms*, **9**(6), 1208 (2021).

3.1. Introduction

In Chapter 2, a description of the development of *in situ* bacterial mRNA detection method RHa-RCA-FISH using model bacteria was provided, and it was mentioned that the method can be applied for the detection of, Gram-negative *E. coli* and Gram-positive *B. choshinensis*. However, unlike mRNA artificially expressed in model bacteria, the native gene expression of bacteria is expected to occur heterogeneously in microbes in the microbial consortium and the amount of mRNA in a cell is expected to be low. Therefore, we aimed to apply RHa-RCA-FISH to lactic acid bacteria (LAB) in yogurt to verify whether the native gene expression of bacteria can be visualized.

Yogurt is produced by the fermentation of milk by two species of lactic acid bacteria *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) [Sieuwerts et al. 2010]. During the process of lactic acid fermentation, *S. thermophilus* and *L. bulgaricus* form a simple consortium. Because of the different shapes of these LAB species, they can be easily identified by microscopic observation, and transcriptome analysis has already been conducted. Furthermore, since they were grown in milk containing various impurities rather than in artificial media (e.g., LB medium), they were ideal models for validating the applicability of RHa-RCA-FISH to microbial consortium analysis.

The addition of glass beads [Sauliner et al. 2007, Sieuwerts et al. 2010] and hard vortex [Sauliner et al. 2007, Sieuwerts et al. 2010, Zheng et al. 2016] were performed for extraction of RNA from lactic acid bacteria. Therefore, the cell wall of lactic acid bacteria may be tougher than that of *B. choshinensis*, which was used as a model of Gram-

positive bacteria in the process described in Chapter 2. Therefore, in this chapter, we reinvestigate the appropriate amount of lysozyme for cell permeabilization of each species of LAB.

3.2. Materials and Methods

3.2.1. PLPs and FISH probes

The sequences of PLP and FISH probe are shown in Table 3-1 and Table 3-2, respectively. Since the genome sequence of *S. thermophilus* strain 1131 was not available, the sequence of pyruvate-formate lyase activating enzyme gene (*pflA*) of five *S. thermophilus* species were aligned and PLP-*pflA* were designed to the sequence without single nucleotide polymorphism (SNP).

Table 3-1 Sequences of PLPs for *pflA* mRNA and *ldhD1* mRNA.

Name	**Sequence (5′→3′)**
PLP-*pflA*	TGTTGTTCGGGGTCAttccttttacag*CCTCAATGCTGCTGCTGT* *ACTAC*tcttcCGGGTCACAAACTTG
PLP-*ldhD1*#1	TCAAGTGAGTCTACGttcattttacag*CCTCAATGCACATGTTTG* *GCTCC*tcttaTGCTTGTACAGGTCG
PLP-*ldhD1*#2	CCATGATTTGCATGAttccttttacag*CCTCAATGCACATGTTTG* *GCTCC*tcttcTAGCGCCGAAGCCTT
PLP-*ldhD1*#3	CAACTTGGTCGCGAAttcattttacag*CCTCAATGCACATGTTTG*

	*GCTCC*tcttcTACCTATAACACCAA

The underlined letters are mRNA recognition sequences, and italic letters are FISH probe sequences.

Table 3-2 Sequence of FISH probe for *ldhD1* mRNA.

Name	**Sequence (5′→3′)**
Alexa568-FISH probe-2	Alexa568-CCTCAATGCACATGTTTGGCTCC

The fluorophore of Alexa488-FISH probe mentioned in Chapter 2 was changed to Alexa568 in the Alexa568-FISH probe-2. Alexa568-FISH probe (see Chapter 2) was used for PLP-*pflA* and Alexa568-FISH probe-2 was used for PLP-*ldhD1*.

3.2.2. Plasmids

The PCR products of *pflA* and *ldhD1* were obtained by PCR performed using primer sets shown in Table 3-3. pET-*pflA* and pET-*ldhD1* were constructed by inserting *pflA* and *ldhD1* into the *Nco*I and *Not*I sites of pET32a (Novagen).

Table 3-3 Sequences of PCR primers for *pflA* and *ldhD1*.

Name	**Sequence (5′→3′)**
pflA_F_NcoI	GAGAGAccatggCAGAAATTGATTACAGTCAG
pflA_R_NotI	GAGAgcggccgcTTAATCCAAGGTTTTAACNCCGATA
ldhD1_F_NcoI	GAGAGccatggCTAAAATTTTTGCTTACGCA

ldhD1_R_NotI	GAGAgcggccgcTTAGCCAACCTTAACTGGAGTTTCA

Lowercase letters are recognition sequences of *Nco*I and *Not*I, underlined letters are homologous sequences to *ldhD1*.

3.2.3. Fermentation of yogurt

LAB cells were fermented in milk and collected at three time points (3.5, 5.5, and 8.0 h), which were the first exponential phase, transition phase, and second exponential phase of LAB growth, respectively, according to Sieuwerts et al. and chosen to compare our results with transcriptome data [Sieuwerts et al. 2010]. The schematic representation of the LAB fermentation and collection is shown in Figure 3-1. A starter of a mixed culture of *L. bulgaricus* 2038 and *S. thermophilus* 1131 was purchased as commercial drinkable yogurt (Meiji Bulgaria yogurt, Meiji Holdings Co., Ltd., Tokyo, Japan) and fermented in sterilized non-fat milk (Takanashi Milk Products Co., Ltd., Kanagawa, Japan). First, 10 mL of milk was prewarmed at 42°C in a 50-mL conical tube before fermentation, followed by inoculation of 100 μL of yogurt and incubated for 3.5 h, 5.5 h, and 8.0 h at 42°C without shaking. Then, 20 mL of RNAprotect™ Bacteria Reagent (RBR) (Qiagen, Hilden, Germany) was added to cultures and mixed by vortex to disperse aggregated proteins in the yogurt and incubated for 5 min at room temperature to stabilize the RNA. Finally, the LAB cells were collected by centrifugation at 10,000 × *g* for 10 min at 25°C, and the cell pellets were resuspended in SAS and stored at 4°C until use.

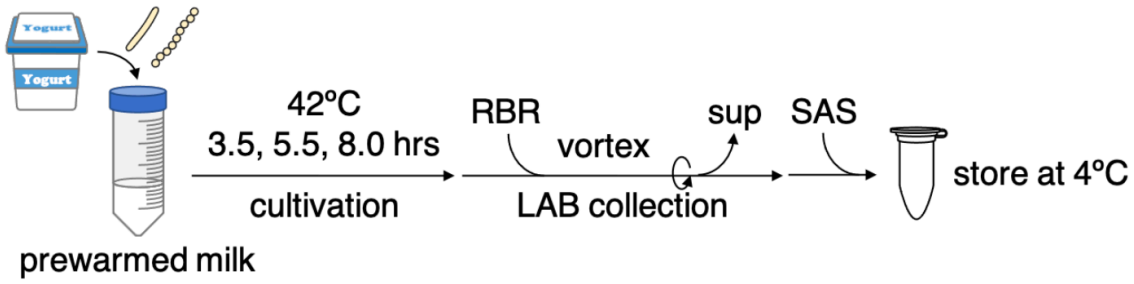

Figure 3-1 Schematic overview of fermentation and collection procedure of LAB cells.

3.2.4. Extraction of genomic DNA from LAB cells

First, 8.0-h fermented LAB cells were resuspended in 500 µL of cell resuspension buffer (10 mM Tris-Cl [pH 7.5], 0.15 M NaCl, 0.1 M EDTA), and 10 µL of 20 mg/mL lysozyme was added to the solution, followed by incubation at 37°C for 20 min. Next, 67.5 µL of cell lysis buffer (0.5 M Tris-Cl [pH 7.5], 5% sodium dodecyl sulfate) was added to the solution, and 100 µg proteinase K was added to the solution. The genomic DNA was extracted from the solution by the phenol-chloroform method and purified by treatment with RNase A and ethanol precipitation. The concentration of the genomic DNA was determined using a Qubit® fluorometer with Qubit® dsDNA BR Assay Kit (Invitrogen/Thermo Fischer Scientific, Waltham, MA), and DNA degradation was checked by agarose gel electrophoresis (1%, TAE buffer).

3.2.5. Pretreatment procedure

The pretreatment procedure was almost the same as that described in Chapter 2. In the cell permeabilization step, the amount of lysozyme added per reaction was

changed to 7.5, 100, and 1000 μg.

3.2.6. RHa-RCA-FISH

in situ mRNA detection of LAB using RHa-RCA-FISH was performed as described in Chapter 2.

3.2.7. Imaging and analysis

The sample preparation, observation, and analysis were performed as described in Chapter 2. All microscopic images were captured using a fluorescence microscope Nikon ECLIPSE Ti2-E (Nikon).

3.3. Results and discussion

3.3.1. Detection sensitivity checks of designed PLPs

Before the application of RHa-RCA-FISH to LAB, the detection sensitivities of the target mRNAs of the designed PLPs were evaluated by performing real-time RHa-RCA on IVT and comparing the amplification plots. The results showed that all PLPs detected the target IVTs and did not emit non-specific signals from non-target RNAs (Figure 3-2). PLP-*pflA* and PLP-*ldhD1*#1 showed comparable amplification plots and final fluorescence values, while that of PLP-*ldhD1*#2 and #3 were relatively low. Therefore, PLP-*pflA* and PLP-*ldhD1*#1 were used for RHa-RCA-FISH.

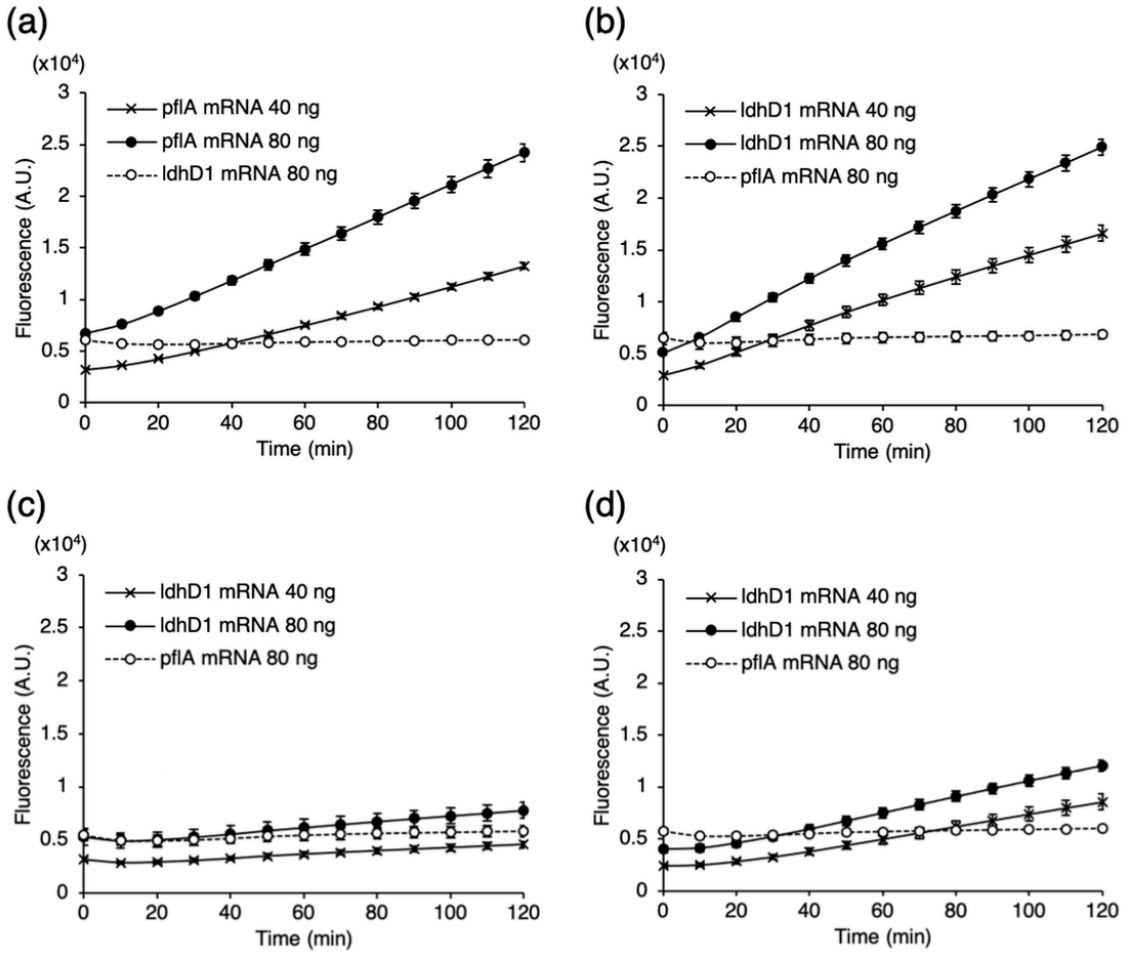

Figure 3-2 PLP detection sensitivity check. (a) Amplification plots of real-time RHa-RCA using IVT *pflA* and PLP-*pflA*. (b-d) Amplification plots of real-time RHa-RCA using IVT *ldhD1* and PLP-*ldhD1*#1 to #3, respectively. As a negative control, 80 ng of non-target IVT (IVT ldhD1 for PLP-*pflA* and IVT *pflA* for PLP-*ldhD1*) was used for each PLP.

In the RHa-RCA procedure, mRNA is primerized with RNase H, therefore, PLPs can be designed for all mRNA sequences, and not be limited to the terminal sequence. However, it has been found that there is a difference in their detection

sensitivity (Figure 3-2), which may be caused by the difference in hybridization and ligation efficiency of PLPs [Takahashi et al. 2018]. Schneider et al. suggested that the nucleotide pairing of the 5′- and 3′-ends of PLP, the secondary structure of mRNA and mRNA binding protein can affect the ligation efficiency [Schneider et al. 2017]. If the full-length sequence data of the target mRNA is obtained, the secondary structure can be calculated *in silico* [Rouskin et al. 2014]. However, since the RNA-seq data is fragmented, and genomic data lack the information of 5′- and 3′-untranslated regions of mRNA, it is difficult to accurately predict the secondary structure. Therefore, the accumulation of such information will make the design of optimal PLPs for detecting target mRNAs more accurate and enhance the applicability of this method to various samples.

3.3.2. Efficient collection of LAB cells from yogurt

Collecting LAB cells from yogurt containing milk-derived proteins, casein, and other solid impurities was an important step in applying RHa-RCA-FISH to LAB. Indeed, inadequate separation of LAB cells from impurities prevented mRNA detection by RHa-RCA-FISH. Under the culture conditions used in this study, the state of the culture medium (yogurt) was liquid at 3.5 h, semi-liquid at 5.5 h, and solid at 8.0 h. At 3.5 h, the culture medium was almost liquid, therefore, LAB cells could be separated from impurities to some extent by centrifugation alone. However, at later incubation times (5.5 h and 8.0 h), solid impurities increased, and LAB cells could not be separated.

When RNAprotect® Bacteria Reagent (RBR) was added to the yogurt, the solid impurities in 8.0 h-fermented yogurt were almost completely dispersed, and LAB cells

were easily obtained by subsequent centrifugation (Figure 3-3). This could be because the surfactant contained in RBR successfully dispersed the proteins.

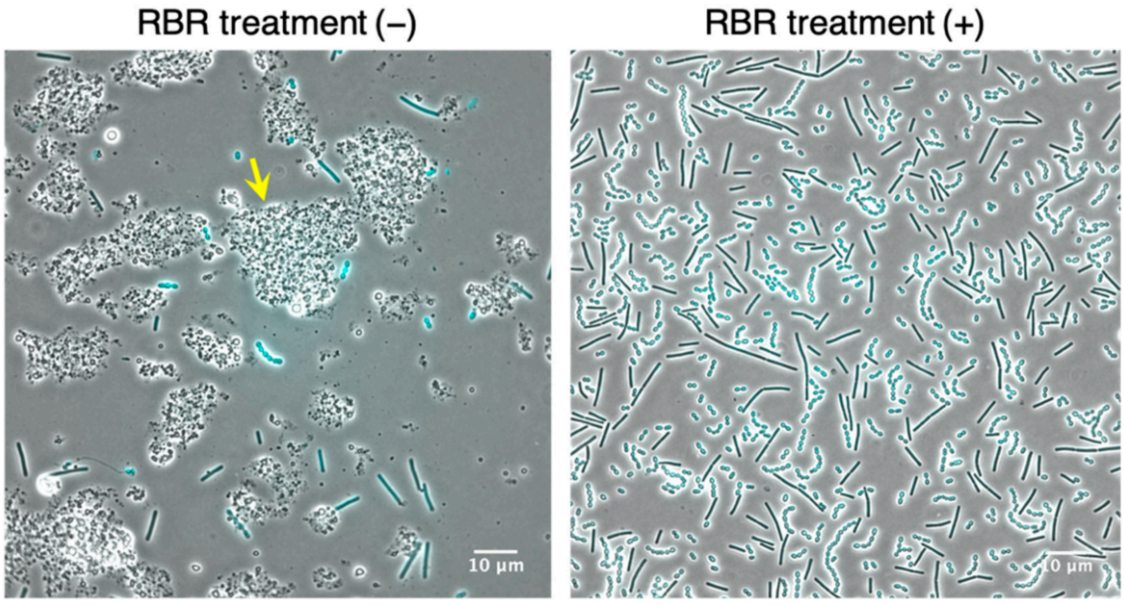

Figure 3-3 LAB cell collection by RBR treatment. Microscopic images of collected LAB cells from 8.0 h-fermented yogurt with (left) and without (right) RBR treatment are shown. LAB cells were stained by DAPI. Coccoid cells are *S. thermophilus* and rod-shaped cells are *L. bulgaricus*. The yellow arrow in the image of RBR treatment (-) indicates aggregated impurities. Overlays of the phase contrast (grayscale) and fluorescent signal of DAPI (cyan) are shown. Scale Bar, 10 µm.

Even though many methods have been developed to harvest LAB cells from yogurt, they mostly involve removing impurities. Such methods include detergent treatment [Gunasekera et al. 2000, 2002], enzymatic treatment [Gunasekera et al. 2000, 2002], pH adjustment [Sieuwerts et al. 2010], etc., which are labor- and time-consuming.

Here, we used a simple method using RBR to recover LAB cells much more efficiently. Several previous reports of transcriptomic analysis of LAB combined the use of RBR to prevent RNA degradation [Smeianov et al. 2007, Macklaim et al. 2013, Bisanz et al. 2014]. In this study, it was found that the use of RBR facilitates the collection of LAB cells from well-fermented yogurt. This method will simplify the procedure of LAB cell collection and facilitate the advancement of the morphological study and transcriptomic analysis of LAB.

3.3.3. Investigation of cell permeabilization conditions in S. thermophilus

Cell permeabilization was performed by adding 7.5, 100, and 1000 μg of lysozyme to 3.5 h-fermented LAB cells. RHa-RCA-FISH was performed targeting *pflA* mRNA. In the experiment conducted using 1000 μg-lysozyme, the signal from Alexa568-labeled probes was specifically detected from inside *S. thermophilus* cells (Figure 3-4, right and magnified images a-c). On the other hand, no signal was obtained from cells treated with 7.5 and 100 μg lysozyme (Figure 3-4, left and middle images). Therefore, 1000 μg of lysozyme per reaction was used for cell permeabilization of *S. thermophilus* for the subsequent experiments.

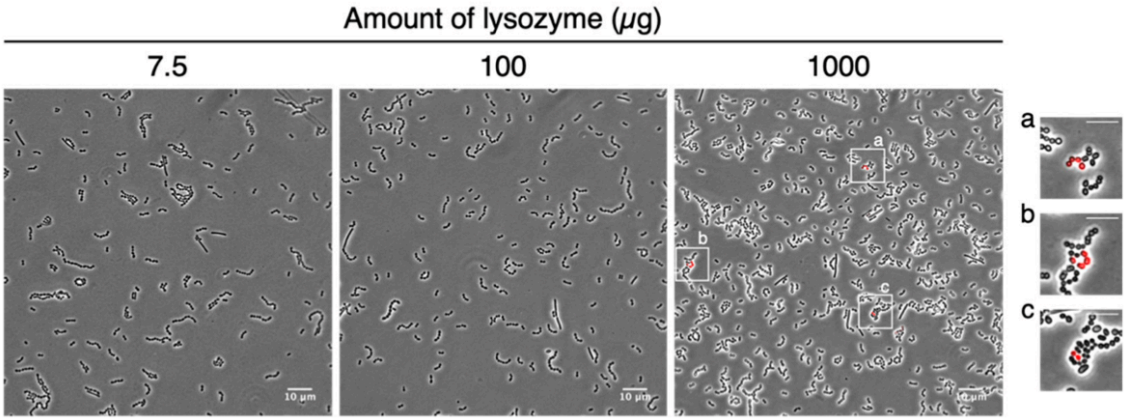

Figure 3-4 Investigation of the amount of lysozyme for permeabilizing *S. thermophilus* cells. *pflA* mRNA-targeted RHa-RCA-FISH was performed to 3.5 h-fermented LAB cells treated with 7.5, 100 and 1000 μg-lysozyme. "a" to "c" are magnified images of box in the image of cells treated with 1000 μg-lysozyme. Overlays of the phase contrast (grayscale) and fluorescent signals from Alexa568-labeled probes (red) are shown. Scale bars, 10 μm in large images and 5 μm in magnified images.

3.3.4. Visualization of pflA expression in S. thermophilus

The change in expression of *pflA* over the culture period was examined by microarray; highly expressed in the early stage of culture and then decreased under coculture conditions of two LAB species [Sieuwerts et al. 2010].

RHa-RCA-FISH was performed under the determined cell permeabilization conditions and visualized the temporal transition of expression of *pflA* in *S. thermophilus*. LAB cells were obtained from yogurt fermented for 3.5, 5.5 and 8.0 h, and RCPs formed from *pflA* mRNA were detected by Alexa568-labeled probe.

As a result, the number of *S. thermophilus* cells for which the Alexa568 fluorescence signal was detected differed between the three points of culture (Figure 3-5). Coccoid *S. thermophilus* cells with specific fluorescence signals were observed in LAB cells cultured for 3.5 and 5.5 h (Figure 3-5, left and middle images), but not in 8.0 h-cultured cells (Figure 3-5, right image). Sieuwerts et al. used mixed-culture of pure cultures of two LAB species as a starter of fermentation [Sieuwerts et al. 2010], which is different from the culture conditions of this study that used commercial yogurt. However, the pattern of variation in the number of *S. thermophilus* cells in which *pflA* expression was detected over time was roughly consistent with the previous report [Sieuerts et al. 2010]. These results indicate that RHa-RCA-FISH can be used to visualize gene expression in *S. thermophilus*.

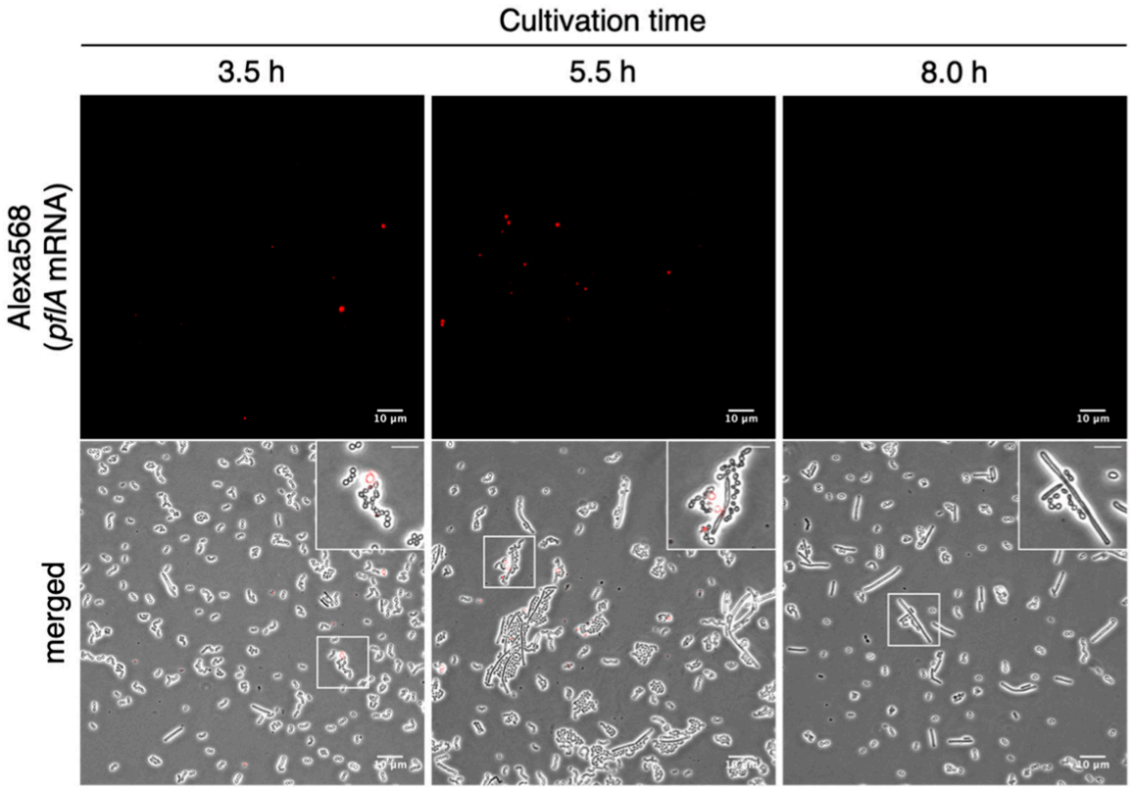

Figure 3-5 Time-course visualization of *pflA* expression in *S. thermophilus*. Overlays of the phase contrast (grayscale) and fluorescent signals from Alexa568-labeled probes (red) are shown in "merged", and pre-merged images are shown in "Alexa568". Magnified images of boxes are inserted in upper right of merged images. Scale bars, 10 μm in large images and 5 μm in magnified images.

3.3.5. Investigation of cell permeabilization conditions in L. bulgaricus

Using LAB cultured for 5.5 h, RHa-RCA-FISH was performed after cell permeabilization treatment with 7.5, 100, and 1000 μg of lysozyme. RHa-RCA-FISH was performed targeting *ldhD1* mRNA.

When treated with 100 μg of lysozyme, the signal of Alexa568 was specifically detected from within the cells of *L. bulgaricus* (Figure 3-6, middle image). On the other

hand, the signal was weak or undetectable in cells treated with 7.5 or 1000 µg of lysozyme (Figure 3-6, left and right images). Therefore, 100 µg of lysozyme per reaction was used for cell permeabilization of *L. bulgaricus*.

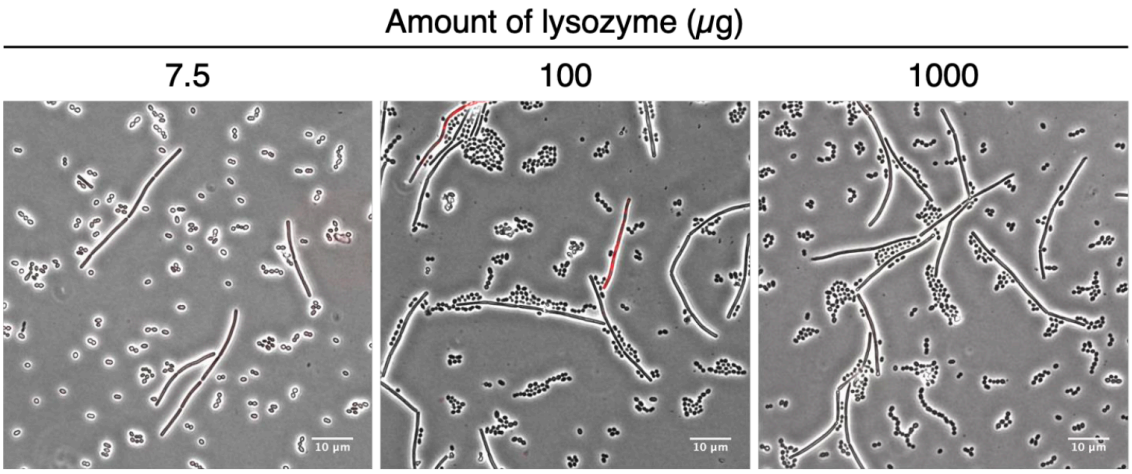

Figure 3-6 Investigation of the amount of lysozyme for permeabilizing *L. bulgaricus* cells. *ldhD1* mRNA-targeted RHa-RCA-FISH was performed on 5.5 h-fermented LAB cells treated with 7.5, 100 and 1000 µg-lysozyme. Overlays of the phase contrast (grayscale) and fluorescent signals from Alexa568-labeled probes (red) are shown. Scale bar, 10 µm.

3.3.6. Visualization of ldhD1 expression in L. bulgaricus

As in the *pflA* mRNA detection, RHa-RCA-FISH was performed to form RCPs from *L. bulgaricus ldhD1* mRNA. Since green autofluorescence was observed from small particles of residual impurities in collected LAB samples in preliminary tests, a new Alexa568-labeled probe was used to detect RCPs specifically (Table 3-2). *ldhD1*, which was selected as a target for detection, was found to be constantly expressed in

monoculture of *L. bulgaricus* by RNA-seq [Zheng et al 2016].

As a result, the fluorescence signal of Alexa568 was detected from the *L. bulgaricus* cells in all three points of the fermentation (Figure 3-7). The signal was detected from almost all *L. bulgaricus* cells, and some of them had multiple strong signals. These results indicate that RHa-RCA-FISH can be used to visualize gene expression in *L. bulgaricus*.

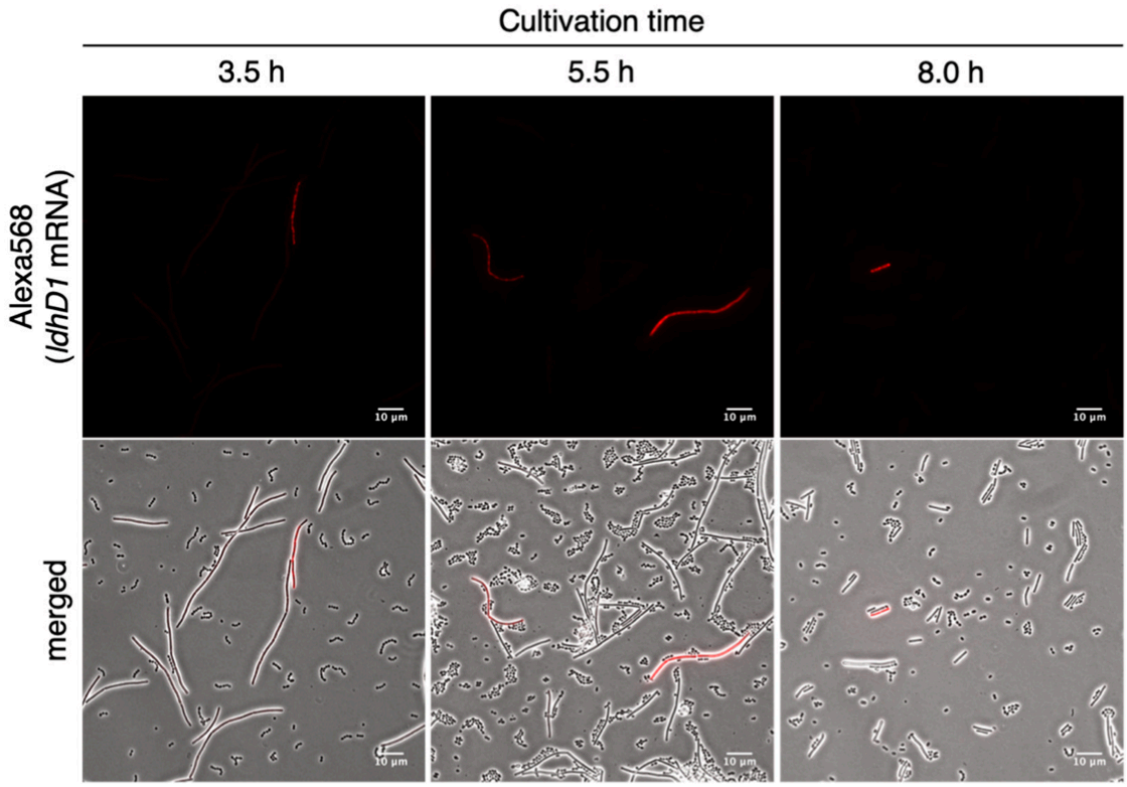

Figure 3-7 Time-course visualization of *ldhD1* expression in *L. bulgaricus*. Overlays of the phase contrast (grayscale) and fluorescent signals from Alexa568-labeled probes (red) are shown in “merged”, and pre-merged images are shown in “Alexa568”. Scale bar, 10 μm.

Unlike the spot-like signals observed in experiments involving *E. coli* and *B. choshinensis*, the signals obtained from *L. bulgaricus* cells were spread over the entire cell (Figure 3-7), which may be caused by the different recovery methods of LAB cells. *E. coli* and *B. choshinensis* were recovered by centrifugation alone, while RBR was used to remove impurities before the recovery of LAB (Figure 3-1). It is likely that the surfactant in the RBR increases the fluidity of the bacterial cell membrane, thereby aiding the penetration of the reagent into the cell [Glover et al. 1999]. The change in the state of the RNA or cytoplasmic components caused by this may have affected the packaging of the RCPs and caused them to take a relaxed form, resulting in non-convergence of the fluorescence signal.

3.4. Conclusion

This chapter describes the successful detection of known temporal gene expression of LAB cells collected from yogurt by performing RHa-RCA-FISH, demonstrating that this method can be applied to visualize the native gene expression of bacteria. Furthermore, gene expression was specifically detected in cells of each bacterial species (*S. thermophilus* and *L. bulgaricus*), even in mixed cultures. Also, the cells with the signals were not necessarily all cells, and the intensity of the signals varied from cell to cell even in the same sample. Therefore, this method can visualize gene expression in microbial consortia and can be used to study bacterial functions at the single-cell level.

In Chapter 2, Gram-negative bacteria (*E. coli)* and Gram-positive bacteria (*B.*

choshinensis) were treated with the same cell permeabilization conditions (7.5 μg-lysozyme/react) and were able to perform RHa-RCA-FISH. However, the same conditions could not be applied to LAB, which are also Gram-positive bacteria, and it was found that the cell permeabilization conditions described in Chapter 2 were not universal. Since previous reports have used relatively severe conditions for cell lysis in RNA extraction from LAB, the amount of lysozyme added was modified to permeabilize the tougher cell wall. Then, the conditions for successful detection were different even among the two species of LAB. Therefore, when this method is applied to more complex microbial consortia, it is hypothesized that searching for the optimal cell permeabilization conditions according to each sample or targeted bacterial species is key to detection.

If the taxonomic information of the constituent organisms is predictable, it may be possible to obtain information to predict the conditions necessary for the optimization of the pretreatment method, but it may be difficult for samples containing unknown or unculturable microbes. In such a case, a positive control may be necessary to examine the conditions for cell permeabilization. The universal probe for bacterial FISH that is commonly used in 16S rRNA-targeted oligo-FISH is designed for the conserved region of rRNA [Amann et al. 1990, Daims et al. 1999]. Therefore, by designing a universal PLP for the conserved region of 16S rRNA, it will be relatively easy to search for the optimal pretreatment method conditions.

Furthermore, to separate LAB from yogurt, it was necessary to break the original state in which they were growing. Therefore, the positions of individual bacterial cells became unclear, and the symbiotic relationship between the two species could not

be visualized by RHa-RCA-FISH. Reports of visualization of bacterial 16S rRNA with oligo-FISH in a variety of environmental samples have allowed conserving positional relationships between organisms [Badstöber et al. 2020, Dou et al. 2017, Prudent et al. 2018, Lukumbuzya et al. 2019, Sekiguchi et al. 1999, Dige et al. 2007, Cardinale et al. 2018, Wada et al. 2016]. Using such pretreatment methods before RHa-RCA-FISH will allow us to visualize bacterial interactions gene expression specifically. These findings are important for the application of RHa-RCA-FISH to samples containing several bacterial species and will be useful for the functional analysis of environmental microbial consortia.

Chapter 4. Conclusions

Metatranscriptomic analysis can provide evidence to understand the function of complex microbial consortia at the taxonomic group level, however this bulk analysis technique hides the origins of each mRNA molecule. Therefore, an imaging technique is helpful to learn the role of each microbial cell and is required for the manipulation at the single-cell level. On the other hand, signal amplification is required to visualize mRNA due to its low intracellular abundance, however previous imaging techniques still have problems with specificity and simplicity. Therefore, in this study, a novel imaging technique specific to mRNA molecule within a bacterial cell was developed in order to label the cells that play a specific function within a microbial consortium at the single-cell level using metatranscriptomic data.

In Chapter 2, RNase H-assisted RCA which is an mRNA-direct detection reaction was employed to discriminate mRNA molecule from DNA and combined with FISH, thereby establishing a novel imaging technique specific to mRNA molecule within a bacterial cell, named RHa-RCA-FISH. As in the model experiments, both inducible genes and native expressing genes were integrated into the expression vector, and the target mRNAs were specifically visualized depending on the induction or native growth phase within almost all cells. From the results, the author concluded that a new microbial mRNA-specific visualization technique was successfully developed.

In Chapter 3, RHa-RCA-FISH was applied to simple syntrophism in yogurt to check whether the known gene expression could be visualized in both lactic acid bacteria to verify the functions in the consortium. From the results, bacterial cells expressing the target mRNA were specifically visualized, and the imaging of gene expression reflected

the previous transcriptome data. Therefore, it was verified that this method can be applied to the detection of bacterial native gene expression and that specific detection is possible even in mixed cultures.

This study allows us to analyze microbial function at the single-cell level and to take a snapshot of the true players in a functioning microbial consortium. This made it possible to compensate for the lack of metatranscriptomic analysis and to reveal microbial interactions clearly on an individual cell. Accumulation of experimentally supported knowledge of microbial interactions will lead to more accurate estimates of the functions of complex microbial consortia than previously possible. This provides important information not only for the analysis of environmental microbial consortia but also for the isolation and cultivation of unculturable bacteria. Therefore, this study will facilitate the detailed analysis of microbial interactions in complex microbial systems, supporting the research of complex microbial communities and advancing the research of environmental microbial consortia, which are a treasure trove of unknown functions.

Based on the results and significance, this study is expected to contribute to the advancement of the study of unknown or unculturable microbes and genetic resources. Thus, this study has both academic and industrial significances.

4.1. Broader application and prospects

Recently, synthetic gut microbial ecosystems have been used as a good model to understand human gut microbiota [Vrancken et al. 2019]. Identifying the exact players

in the function of the original microbiota will lead to the construction of a more authentic model flora and progress in research. Spatial analysis of the gut microbiota using various imaging techniques has also been performed to analyze the relationship between biogeography and health [Tropini et al. 2017]. The analysis of the production of bioactive substances and the response of various compounds of the gut microbiome using gene-expression specific imaging methods will provide new insights into the regulation of gut microbiota that cannot be obtained by conventional taxonomic or genomic level analysis and may contribute to the development of tailor-made functional foods and drug discovery in the future.

Complex microbial systems are also expected to be used for the production of useful substances from wastes. The mixed culture containing alginate hydrolytic bacteria and methanogens degraded alginate and produced methane, but the percentage of alginate-degrading bacteria was expected to be low, and multiple analyses did not identify the responsible bacteria [Zhang et al. 2019]. RHa-RCA-FISH, in such a case, may provide strategies for controlling the population in a consortium as well as identification of mainly functioning alginate-degrading bacteria.

Furthermore, fluorescently labeled cells can be manipulated by fluorescent activating cell sorting (FACS). In fact, several studies have already reported the collection of specific bacterial cells from mixed samples using FACS combined with FISH-based methods [Batani et al. 2019, Neuenschwander et al. 2015]. When exploring novel antibiotics to address the recent antimicrobial resistance crisis, unculturable bacteria would be investigated. In such a case, metagenomic analysis will provide the partial

sequences of the antibiotic biosynthesis gene cluster. Using the sequence data for the PLP of RHa-RCA-FISH, it may be possible to obtain the specific bacterium by FACS and the whole genome sequence by whole genome amplification (WGA) and sequencing [Detter et al. 2002].

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関連論文

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