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1 **Direct detection of mRNA expression in microbial cells by fluorescence *in situ***

2 **hybridization using RNase H-assisted rolling circle amplification**

3

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17 **ABSTRACT**

18 Meta-analyses using next generation sequencing is a powerful strategy for studying
19 microbiota; however, it cannot clarify the role of individual microbes within microbiota.
20 To know which cell expresses what gene is important for elucidation of the individual
21 cell's function in microbiota. In this report, we developed novel fluorescence *in situ*
22 hybridization (FISH) procedure using RNase-H-assisted rolling circle amplification to
23 visualize mRNA of interest in microbial cells without reverse transcription. Our results
24 show that this method is applicable to both gram-negative and gram-positive microbes
25 without any noise from DNA, and it is possible to visualize the target mRNA expression
26 directly at the single-cell level. Therefore, our procedure, when combined with data of
27 meta-analyses, can help to understand the role of individual microbes in the microbiota.

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30 **Introduction**

31 Microbiota consists of several bacterial species, including non-cultivable microbes, via
32 interactions such as nutrients and growth promoting substance. One of the goals of
33 microbiology is to understand the impact of microbiota on the environment, animals
34 including human to marine sponges, and plants including rhizosphere. Thus,
35 meta-analyses such as metagenomics and metatranscriptomics using next generation
36 sequencing (NGS) are powerful strategies for studying microbiota. In particular,
37 high-throughput transcriptome sequencing (RNA-seq) data that contain amount and
38 type of expressed mRNA are an important for understanding the behavior of the
39 microbiota [1].

40 Meanwhile, RNA-seq data cannot clarify the role of individual microbes because
41 complete reference genome of individual microbes is often not available even when

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42 metagenomic analysis is performed simultaneously. To clarify the role of individual
43 microbes, single cell genome sequencing of individual microbes in the microbiota has
44 been a trend in recent years [2,3], however high cost restrict many researchers from
45 performing these analysis.. Therefore, fluorescence *in situ* hybridization (FISH) of
46 mRNA is an important method for understanding 'who is doing what?' in the
47 microbiota with low cost.

48 However, compared with the oligo-FISH for 16S rRNA, which is commonly used
49 in microbiology [4], mRNA is difficult to visualize by FISH, because the mRNA of
50 interest in a cell has an extremely low copy number compared to the rRNA. In fact,
51 several high-sensitivity FISH methods for mRNA detection have been developed using
52 enzymatic signal amplification such as catalyzed reporter deposition-FISH
53 (CARD–FISH) [5] and its derivative methods (e.g. two pass tyramide signal amplification

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54 FISH [6] and double CARD–FISH [7]). However, these methods cannot compare mRNA
55 expression level because the signal for these methods diffuses over the entire cell.
56 Although single-molecule FISH (smFISH) can detect a single mRNA molecule [8]
57 even in microbial cells [9], this procedure is much expensive than the other FISH method
58 because it needs nearly 50 different detection probes carrying the same fluorophore
59 against one transcript of interest to increase detection sensitivity [10]. In addition,
60 RNA-seq data without a reference genome, especially from microbiota, are fragmented,
61 and the mRNA sequence of interest is often too short when designing nearly 50
62 detection probes.
63 A click-amplifying FISH method, termed ClampFISH, was recently developed to
64 amplify the fluorescence signal [11]. However, this method is time-consuming and
65 confounded by background noise presumably due to nonspecific hybridization of the

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66 probe. Most currently available FISH methods, including RNAscope [12] and RollFISH [13],
67 rely on simple hybridization of oligonucleotide probes to mRNA sequences. Therefore,
68 these methods cannot clearly distinguish between fluorescent signals originating from
69 mRNA and genomic DNA, especially in microbes. The prokaryotic mRNA is an
70 unspliced RNA; thus, the oligonucleotide probes cannot be set at the exon-exon junction
71 to distinguish between genomic DNA and mRNA, which commonly used in
72 reverse-transcription (RT)-PCR for detection of eukaryotic mRNA.

73 FISH using rolling circle amplification (RCA), which uses complementary DNA
74 (cDNA) generated from mRNA by RT, has a potential to detect a single mRNA
75 molecule in a eukaryotic cell [14]. This method makes it possible to distinguish between
76 mRNA and genomic DNA with high probability, because it is difficult to initiate RCA
77 reaction using the genomic DNA as a primer. The RCA product (RCP) contains a hyper

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78 tandem-repeat of a DNA sequence complementary to a padlock probe (PLP). The RCP
79 eventually becomes a platform on which a sufficient amount of the detection probe can
80 hybridize. Therefore, single fluorescent probe for detection allows this method to be
81 available at approximately 50-fold lower cost per gene as compared with the
82 conventional smFISH. However, for the detection of microbial mRNA, the exonuclease
83 treatment of cDNA to produce initiation point of RCA reaction will be difficult to
84 optimize for each individual mRNA considering the operon structure, especially using
85 the fragmented sequence data from RNA-seq. For these reasons, a cost-effective and
86 user-friendly FISH method that detects only the target mRNA in a microbial cell is
87 needed.

88 Recently, we developed RNase H-assisted RCA (RHa-RCA) [15]. The PLP used in
89 this procedure can be set without full-length sequence information of target RNA;

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90 therefore, PLP can be set even with a fragmented mRNA sequence such as RNA-seq
91 data. In addition, this procedure can detect only RNA. Here we demonstrate a novel
92 FISH method based on RHa-RCA for the visualization of mRNA expression level in
93 microbial cells at a single-cell level.

94 **Results and Discussion**

95 Figure 1 shows the scheme and workflow for our method of visualization of expressing
96 mRNAs of interest in microbial cells. After cell fixation and permeabilization,
97 RHa-RCA–FISH consists of five reaction steps (**Fig. 1A**): (i) PLP hybridization to a
98 sequence of interest in the target mRNA molecule; (ii) circularization of hybridized PLP
99 by SplintR ligase; (iii) producing nick site in the hybridized mRNA by RNase H; (iv)
100 RCA using phi29 DNA polymerase to create RCP from the nick site; and (v) finally,
101 detection probe hybridization to visualize RCP.

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102 Using this method, it is possible to complete all steps except fluorescence
103 microscopic observations in approximately 8 h (**Fig. 1B**). Typically, we perform up to
104 step (iv) on the first day and then store the samples at 4°C, as RCPs are stable at this
105 temperature. On the following day, step (v) and microscopy were performed.
106 At first, we have tested whether RHa-RCA–FISH detects mRNAs of green
107 fluorescent protein (GFP) expressed in *Escherichia coli* cell. *E. coli* BL21(DE3)
108 transformed with a plasmid carrying GFP gene (pET-AcGFP) [16] was cultured by
109 inducing GFP expression through the addition of isopropyl-β-d-thiogalactopyranoside
110 (IPTG), whereas the *E. coli* without the addition of IPTG were maintained as negative
111 control (non-induced cell). The non-induced cells were cultured with 2% glucose to
112 inhibit leaky expression of GFP mRNA. The GFP expression was confirmed by
113 fluorescence microscopy using the harvested cells 2 h after the addition of IPTG

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114 (**Supplementary Fig. 1**). Considering that mRNA expression was observed before
115 protein expression, the cells harvested 1 hour after addition of IPTG were used for
116 RHa-RCA–FISH.

117 No fluorescent signal was observed in non-induced cells, even though the cells
118 were also transformed by pET-AcGFP (**Fig. 2A**). In contrast, fluorescent signals were
119 clearly observed in GFP-induced cells (**Fig. 2B**). This result clearly shows that
120 RHa-RCA–FISH specifically detected GFP mRNA molecules and not the GFP DNA in
121 the vector. In addition, the fluorescence signals formed spot-like shapes (**Fig. 2C**), as
122 observed in microbial cells using smFISH [9,10]. Unfortunately, bacterial cells are too
123 small to distinguish each RCP, so it was impossible to determine the exact number of
124 spots in the constrained space of these cells. To count the exact number of spots in a

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125 microbial cell, the use of super-resolution microscopy such as Stochastic Optical
126 Reconstruction Microscopy (STORM) is required [17].

127 Because *E. coli* is a gram-negative bacteria, the utility of RHa-RCA–FISH in
128 microbiota will be limited if this method cannot be performed using the same protocol
129 for gram-positive bacteria, which have a thick peptide glycan coat. Therefore, next we
130 investigated whether RHa-RCA–FISH is applicable to *Brevibacillus choshinensis* as
131 control of gram-positive bacteria.

132 Using *B. choshinensis* harboring a plasmid carrying the DsRed gene under control
133 of the native P2 promoter, we observed fluorescence from the DsRed protein starting at
134 12 h after the start of the culture. The fluorescence of DsRed gradually increased,
135 reaching a maximum at 72 h (**Fig. 3, upper images**).

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136 On the other hand, the fluorescent signal from DsRed mRNA by RHa-RCA–FISH
137 was first observed 12 h after culture initiation, reaching a maximum at 24 h and then
138 decreasing (**Fig. 3, middle images**). These results indicate that RHa-RCA–FISH can be
139 performed following the same protocol even for gram-positive bacteria.

140 Interestingly, the signal in *B. choshinensis* varied greatly between individual cells
141 (**Fig. 3, middle images**), while the fluorescent signals from GFP mRNA in *E. coli*
142 showed minimal variation between cells (**Fig. 2B**). The reason the DsRed mRNA
143 expression level varied between individual cells may be that mRNA expression from the
144 P2 promoter occurs during cell wall synthesis [18], which varies with the cell cycle stage.
145 In addition, it was likely that the number of signals could be counted in cells with low
146 mRNA abundance (**Fig. 3, lower images**). These results indicate that RHa-RCA–FISH
147 have a potential to compare the amount of mRNA expression at the single-cell level.

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148 Finally, we performed our protocol for simultaneous detection of both GFP and
149 DsRed mRNA in suspension containing *E. coli* and *B. choshinensis* cells. As a result,
150 red fluorescence (Alexa -568) and green fluorescence (Alexa -488) were observed from
151 separate cells (**Fig. 4**). Although both the strains have similar cell shapes that cannot be
152 discriminated under the microscopy we were able to distinguish between the two strains
153 based on the species-specific fluorescence from labelled probes. This result indicates
154 that RHa-RCA–FISH can simultaneously detect multiple specific mRNAs, even in
155 microbiota containing both gram-positive and gram-negative bacteria.

156 Here we report that our method for *in situ* analysis of mRNA expression level in
157 both gram-positive and gram-negative bacteria has potential to detect differences in
158 amount of the mRNA between individual microbial cells without background noise
159 from genomic or plasmid DNA. In this report, we performed RHa-RCA–FISH using

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160 both gram-positive and gram-negative bacteria in liquid culture; thus, the samples used
161 for RHa-RCA–FISH contain almost no impurities other than bacterial cells. In contrast,
162 the samples that many researchers want to study, such as feces, soil, and symbiont,
163 contain impurities other than bacterial cells. However, we believe that RHa-RCA–FISH
164 can be perform for samples containing impurities because oligo-FISH for 16S rRNA has
165 been performed on many samples including feces [19], soil [20] and symbiont [21]. However, it
166 is important to examine the method of fixing and permeabilization according to the state
167 of the sample to be observed.

168 In this report, we have demonstrated that RHa-RCA–FISH will be able to
169 compare the amount of mRNA expression in rod-shaped bacterial cells. However,
170 whether the expression levels of mRNA can be compared greatly depends on the
171 physical size and shape of the microbial cells. It is difficult to compare the expression

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172 level in the cells of small cocci with a commonly used fluorescence microscope. The
173 FISH signal and the cells were almost the same size, therefore, the mRNA expression
174 level was not revealed in *Streptococcus thermophilus*, which used for fermentation of
175 yogurt (data not shown). Thus, we believe that super-resolution microscopes such as
176 STORM [17] will become essential in microbiology.

177 We used RNase H to digest RNA/DNA-hybrid but not DNA/DNA-hybrid
178 molecules, therefore, our method can clearly distinguish between mRNA and DNA
179 even prokaryotic mRNA remains unspliced. In addition, because the PLP used in this
180 method can be set anywhere in the mRNA sequence [15], the PLP can be set even in the
181 RNA-seq data which may contain short sequence. Thus, we believe that our method for
182 visualizing RNA molecules directly within cells could help understand the role of
183 individual microbes in the microbiota when combined with the data of meta-analyses.

184

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185 **Material and methods**

186 **Padlock probe and detection probe**

187 Each PLP position is shown in **Supplementary Figs. 3–4**. The PLP and detection probe
188 sequences are shown in **Supplementary Table 1**. All PLP and PCR primers were
189 purchased from Eurofins genomics (Ebersberg, Germany). Alexa labeled detection
190 probes were purchased from Japan Bio Services Co., LTD.（Asaka, Saitama, Japan).

191 **Expression of GFP mRNA in *E. coli***

192 The *E. coli* strain BL21 (DE3) (Novagen, Merck Millipore, Darmstadt, Germany)
193 carrying pET-AcGFP was grown in LB medium (1% tryptone, 0.5% yeast extract, 1.0%
194 NaCl, and 50 μg/mL ampicillin) for 14–16 h at 30°C with shaking. The cultures were
195 diluted 1:1000 in fresh LB medium and incubated at 30°C with shaking. Growth was
196 monitored by the measurement of the optical density at 660 nm (OD_{660}). When the

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197 OD_{660} was approximately 0.6, GFP expression was induced by the addition of IPTG
198 (final concentration, 0.5 mM). The GFP-induced cells for RHa-RCA–FISH were
199 harvested 1 h after IPTG addition. GFP-induced and non-induced cells were collected in
200 1.5 mL tubes by centrifugation at 10,000 × *g* for 2 min at 4°C. Cells were immediately
201 suspended in saturated ammonium sulfate (SAS) solution to inhibit RNase activity and
202 store at 4°C before use. This treatment would keep the total RNA in the cells stable
203 without degradation for at least 2 weeks (**Supplementary Fig. 2**).

204 **Construction of expression vector for *B. choshinensis* and *in vitro* transcription**

205 The coding sequence of DsRed was isolated from the pDsRed-monomer vector
206 (Clontech/TaKaRa Bio, Ohtsu, Shiga, Japan) by digestion with the appropriate
207 restriction enzymes. The resulting fragment was cell-free cloned into pNI-His (Takara
208 Bio) for expression in *B. choshinensis* cells as pNI-DsRed or pET-21d (Novagen) for *in*

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209 *vitro* transcription as pET-DsRed. Detailed procedures for cell-free cloning are

210 described in the **Supplementary Material and Methods**.

211 **Expression of DsRed in *B. choshinensis***

212 The *B. choshinensis* strain HPD31-SP3 (Takara Bio) carrying pNI-DsRed was grown in

213 2SYF medium (20.0 g/L fructose, 40.0 g/L Phytone Peptone (Becton, Dickinson, and

214 Co., Franklin Lakes, NJ), 5.0 g/L Ehrlich bonito extract (Kyokuto Pharmaceutical

215 Co.LTD, Tokyo, Japan), and 0.15 g/L $CaCl_2 \cdot 2H_2O$) containing 50 μg/mL neomycin for

216 12, 24, 48, or 72 h at 30°C with shaking. DsRed protein expression was confirmed by

217 fluorescence microscopy. The cells were harvested in 1.5 mL microcentrifuge tubes by

218 centrifugation at 10,000 × *g* for 2 min at 4°C. Cells were suspended in SAS and stored

219 at 4°C before use.

220 **Cell fixation and permeabilization**

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221 The stored cells in SAS solution (600 μL) were transferred to a 1.5 mL tube and
222 centrifuged at 20,000 × *g* for 1 min at 4°C to remove the SAS solution. The cell pellet
223 was resuspended in 300 μL of 4% paraformaldehyde and incubated at room temperature
224 for 15 min to fix the cells. After centrifugation, the cell pellet was resuspended with
225 300 μL of 70% ethanol and incubated at room temperature for 60 min to dehydrate the
226 cells. After centrifugation under the same conditions to remove 70% EtOH, the cell
227 pellet was resuspended with 262.5 μL of 1× TE buffer. Then, 37.5 μL of lysozyme
228 solution (200 μg/mL in 50% glycerol) was added to the cell suspension solution and
229 incubated at room temperature for 10 min to digest the cell wall. After centrifugation at
230 same conditions to remove the lysozyme solution, the cells were washed twice with
231 300 μL of 1× phosphate-buffered saline (PBS). After centrifugation, the cells were

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232 resuspended with 14 μL of μDW and transferred to 0.2 mL of PCR tube to prepare the

233 RCA reaction.

234 **FISH using RHa-RCA**

235 The cells were mixed with 20 pmol of PLP in a buffer containing 20 mM Tris-acetate

236 (pH 7.5), 10 mM magnesium acetate (MgAc), and 50 mM potassium glutamate (KGlu)

237 in a final volume of 20 μL. The PLP was hybridized by incubation at 95°C for 1 min,

238 slowly cooling to 30°C over 30 min, and incubation for 10 min at 30°C. To the cells

239 was added 10 μL of ligation mixture (20 mM Tris-acetate [pH 7.5], 10 mM MgAc,

240 1.2 mM ATP, 50 mM KGlu, 10 mM dithiothreitol, and 25 units of SplintR ligase (New

241 England BioLabs, Ipswich, MA)), followed by incubation at 37°C for 10 min to seal the

242 PLP. In our previous report [15], the ligase was inactivated by heating, but this step was

243 omitted in this study to shorten the reaction time and simplify the procedure. The

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244 detection sensitivity was not affected (data not shown). The RHa-RCA reaction was
245 started by mixing 30 μL of the ligated mixture with 20 μL of a reaction mixture
246 containing 20 mM Tris-acetate (pH 7.5), 10 mM MgAc, 80 mM ammonium sulfate,
247 10 mM KGlu, 2.0 mM deoxynucleoside triphosphate, 0.004 units of pyrophosphatase
248 (New England BioLabs), 0.06 units of RNase H (BioAcademia, Osaka, Japan), and
249 500 ng of DNA-free phi29 DNA polymerase (Kanto Chemical, Tokyo, Japan). The
250 mixture was incubated at 30°C for 2 h followed by enzyme inactivation at 65°C for
251 10 min.

252 The RCA reaction mixture containing the cells was transferred to a 1.5 mL tube.
253 After centrifugation at 20,000 × *g* for 1 min at 4°C, the cells were washed with 1× PBS
254 at room temperature, suspended in 2× saline sodium citrate, and transferred to a 1.5 mL
255 black tube. After addition of the Alexa-labeled oligonucleotide, the cell suspension was

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256 incubated at 37°C for 3 h to hybridize the oligonucleotide to RCP. After centrifugation
257 at 20,000 × *g* for 1 min at 4°C, the cells were washed twice with 1× PBS at 37°C for
258 15 min. The cells were suspended in 1× PBS and stored at 4°C in 1.5 mL black tubes
259 before observation by microscopy.

260 RCA and hybridization were performed in bench-top cleanroom [22] to prevent
261 contamination that could cause nonspecific hybridization..

262 **Imaging and analysis**

263 A drop of cell suspension hybridized with the detection probe was placed on 1%
264 agarose pads containing 1× PBS . Images of each sample were taken on a fluorescence
265 microscope (Nikon ECLIPSE E600 for *E. coli* and Nikon ECLIPSE Ti2-E for
266 *B. choshinensis*, Tokyo, Japan) equipped with a phase-contrast objective CFI PlanApo
267 DM 100×(Nikon) and an ORCA-Flash4.0 V3 camera (Hamamatsu Co., Shizuoka,

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268 Japan). Typically, the RHa-RCA–FISH, GFP, and DsRed fluorescence images were
269 taken using an exposure time of 100 msec. The images were analyzed using ImageJ
270 software (NIH).
271
272

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340 **Contributions**

341 H. T, T. K, Y. N, and Y. O conceived of this study and designed the experiments. K. H
342 performed nearly all of the experiments. K. H and S. K collected and analyzed the FISH
343 images. T. K and Y. O obtained the necessary financial support. H. T, K. H, and Y. O
344 prepared the initial draft of the manuscript. T. K, S. K, T. A, K. W, and Y. N revised the
345 manuscript. All authors contributed to and have approved the final manuscript.

346 **Competing interests**

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347 Hiroshima University has filed patent applications related to the technology described in
348 this work to the Japan Patent Office. H. Takahashi, T. Aki, Y. Nakashimada, and Y.
349 Okamura. are listed as inventors on the patents. No one received personal or
350 institutional revenue associated with the patent applications. The JST as the funder had
351 no role in the study design, data collection and analysis, decision to publish, or
352 preparation of the manuscript. K. Horio, S. Kato, K. Watanabe, and T. Kobori declare
353 no potential conflict of interests.
354

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355 **Figure legends**

356 **Figure 1.** Sheme and workflow for visualization of mRNA in microbial cells. by RNase

357 H-assisted RNA-primed rolling circle amplification (RHa-RCA–FISH). (A) Schematic

358 representation of RHa-RCA–FISH procedure. (B) Workflow of the procedure and

359 required time for *in situ* visualization of mRNA expression.

360

361 **Figure 2.** FISH detection of GFP mRNA in *E. coli* cells. (A) Detection of GFP mRNA
362 in non-induced *E. coli* cells carrying a GFP expression vector and (B) in GFP-induced
363 cells. Scale bar, 10 µm. (C) Magnified image of box in (B). Scale bar, 5 µm. An overlay
364 of the phase contrast (grayscale) and Alexa-568 labeled probes (red) targeting the RCP
365 from GFP mRNA is shown.
366

367 **Figure 3.** FISH detection of DsRed mRNA in *B. choshinensis* as a Gram-positive

368 bacterium.

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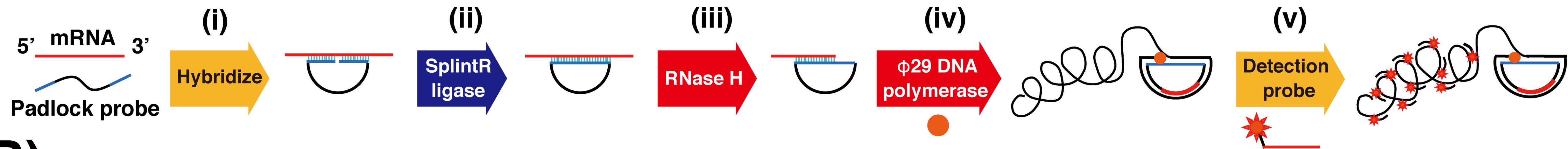
369 Upper images show fluorescence of DsRed protein expressed in *B. choshinensis*; middle
370 images show the signal of detection probes using FISH; lower images show the
371 magnified image of the square box in middle images. The cells in the upper and middle
372 images are not from the same sample because the protein is denatured by the FISH
373 procedure. Overlays of the phase contrast (grayscale), DeRed protein (red), and
374 Alexa-488 labeled probes (green) targeting the RCP from DsRed mRNA are shown.
375 Scale bar, 10 µm.
376
377 **Figure 4.** Simultaneous detection of GFP and DsRed mRNA in a mixture of *E. coli* and
378 *B. choshinensis* cells.
379 An overlay of the phase contrast (grayscale) and Alexa-568 labeled probes (red)
380 targeting the RCP from GFP mRNA and Alexa-488 labeled probes (green) targeting the
381 RCP from DsRed mRNA are shown. Scale bar, 10 µm.

(A)



(B)

Cell fixation
PFA
15 min
Cell permeabilization
Ethanol
60 min
Lysozyme
10 min
Wash
1 min
Wash
1 min
(89 min)

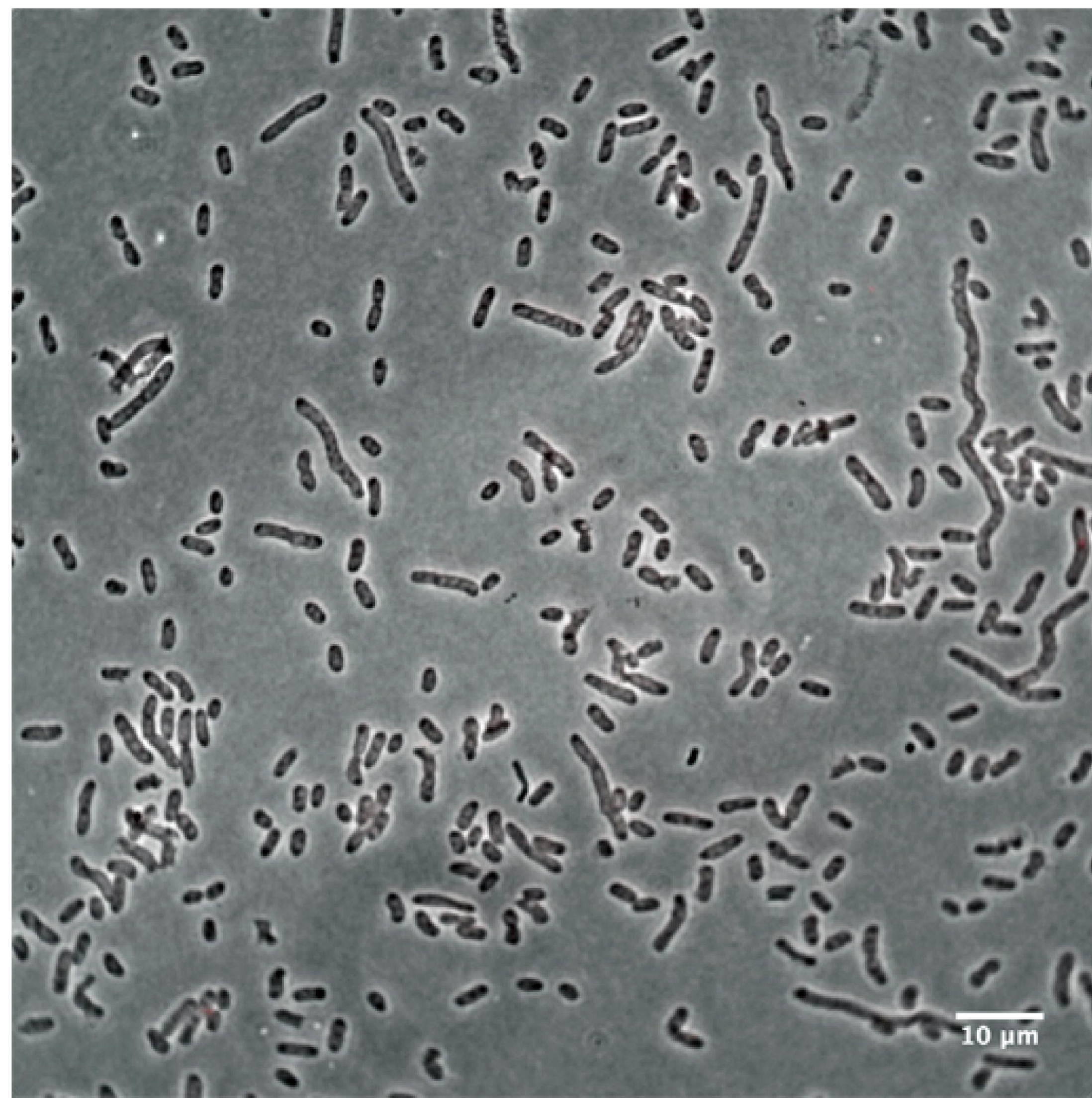
RNaseH-assisted rolling circle amplification (RHa-RCA)
Padlock probe hybridization
Step (i)
43.5 min
Ligation
Step (ii)
10 min
RHa-RCA reaction, Step (iii, iv)
130 min
Wash
1 min
(184.5 min)

Fluorescence *in situ* hybridization (FISH)
Detection probe hybridization, Step (v)
180 min
Wash
15 min
Wash
15 min
(210 min)

(total: 483.5 min)

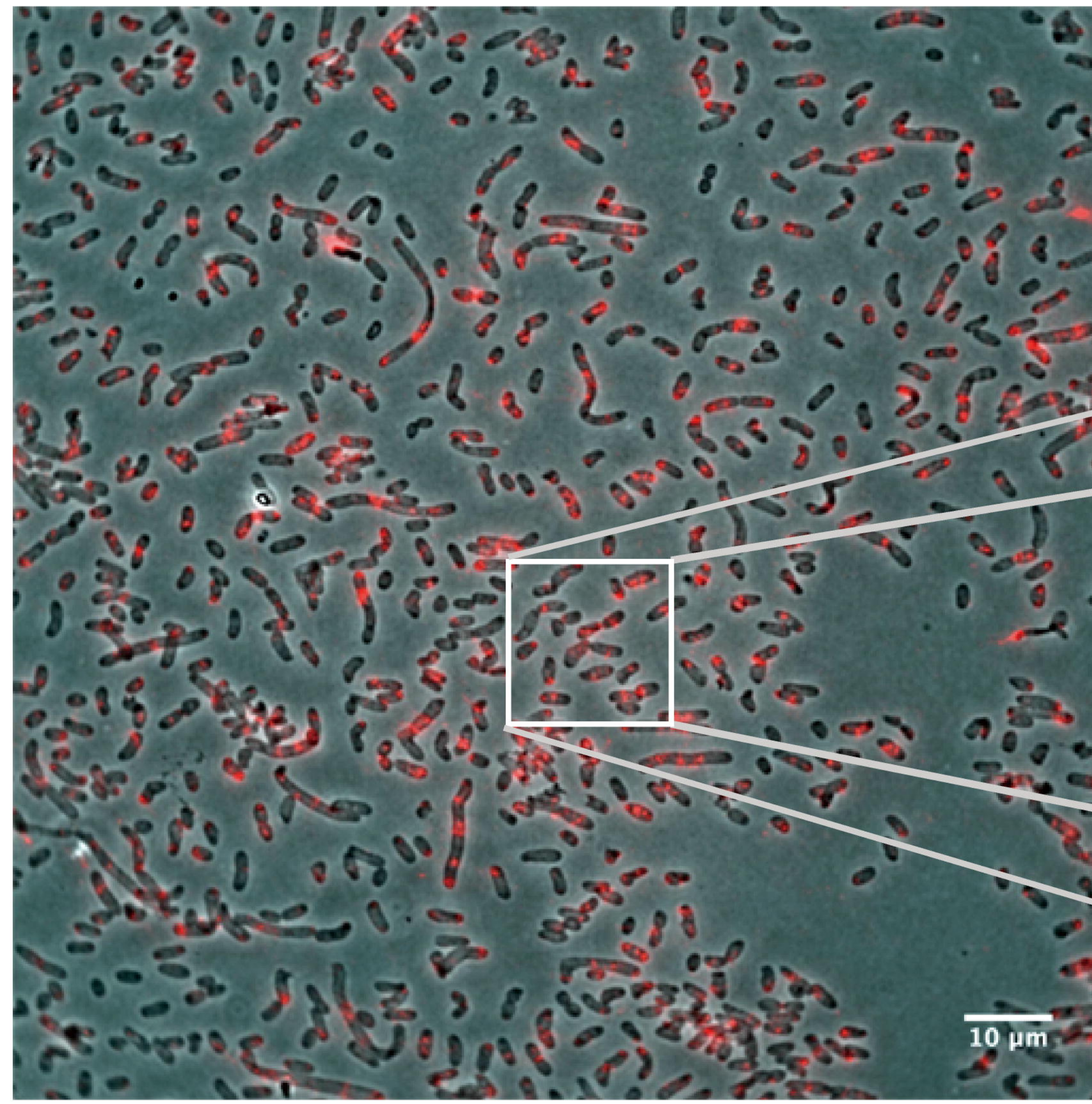
(A)

non-induced cells

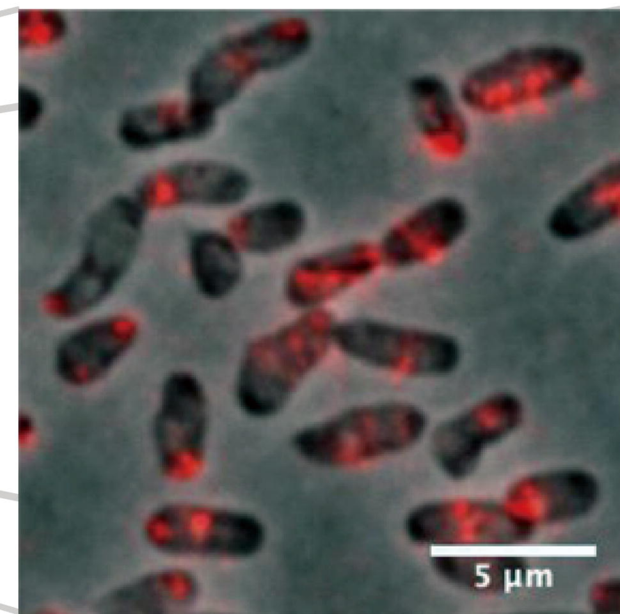


(B)

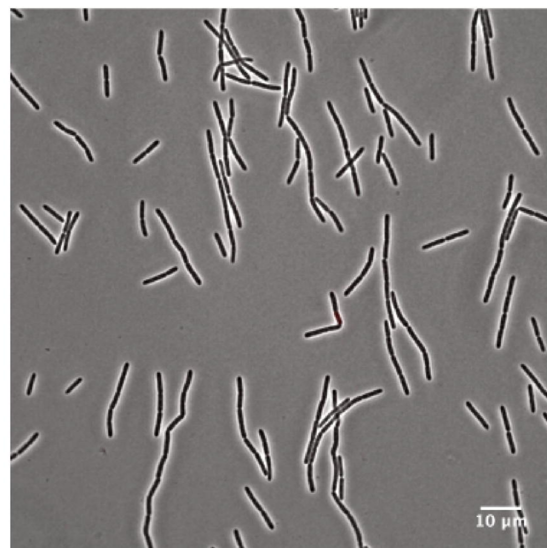
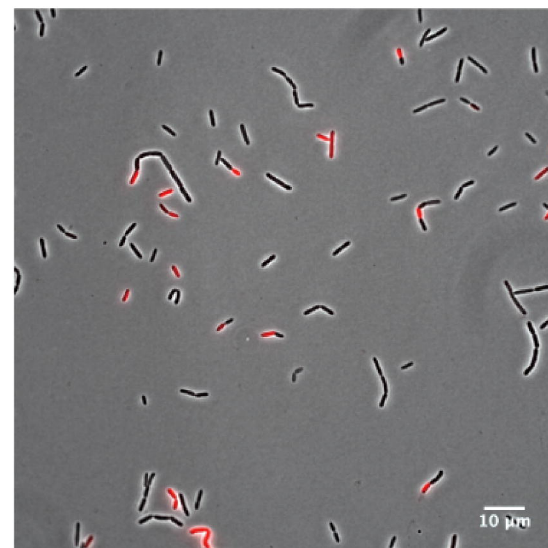
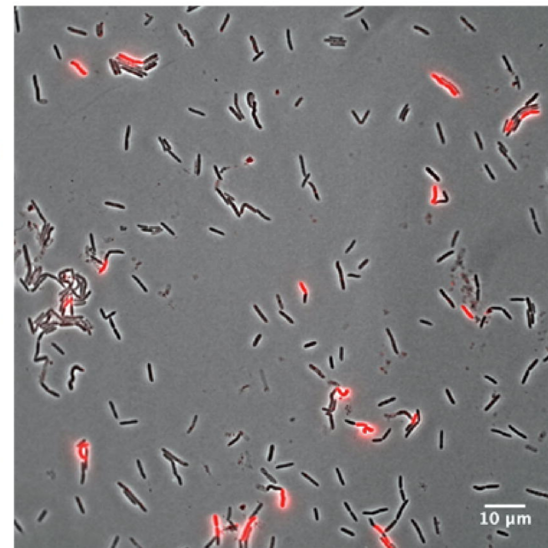
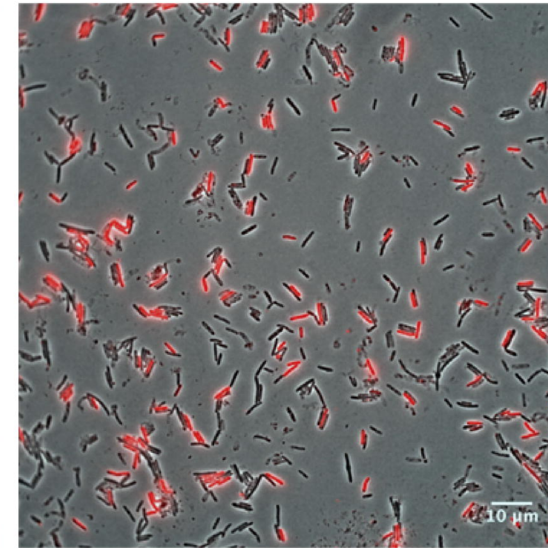
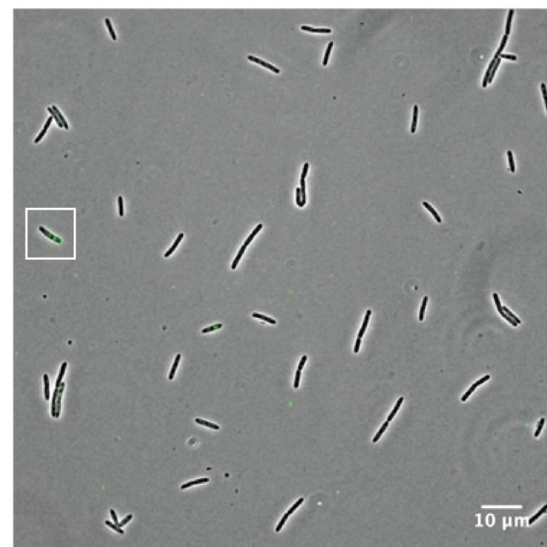
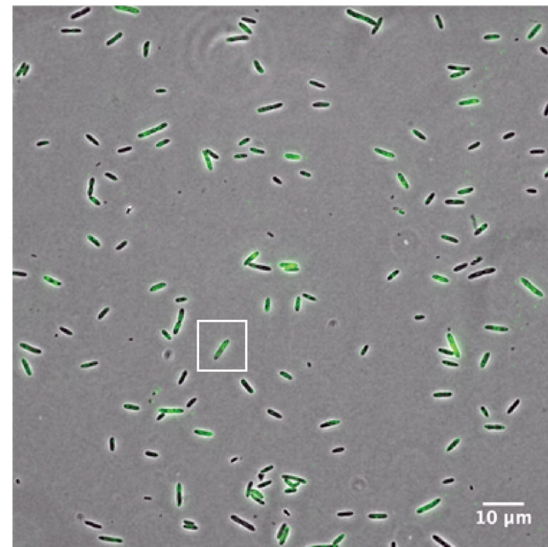
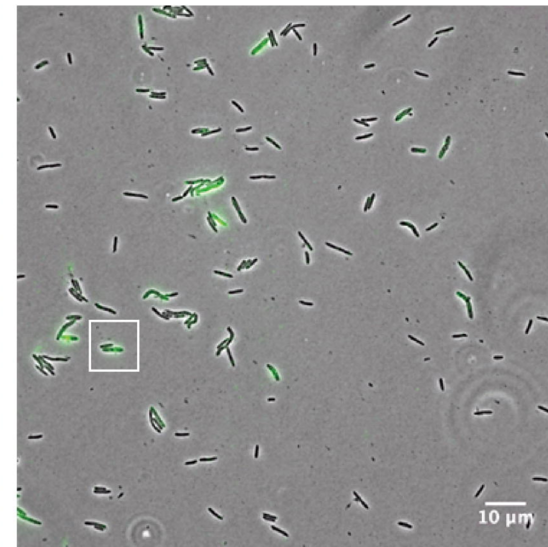
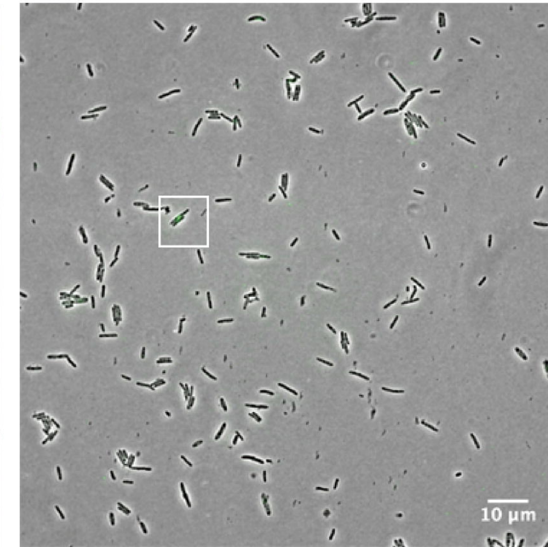
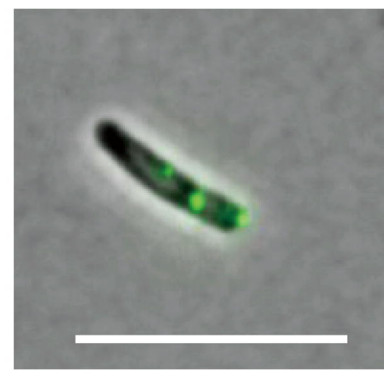
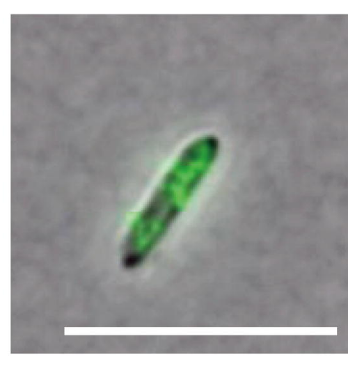
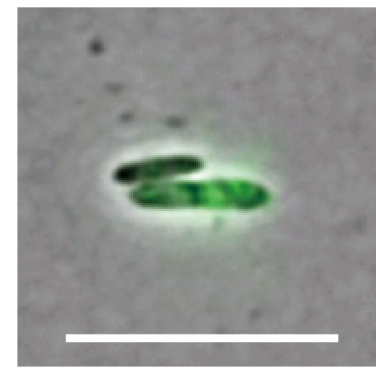
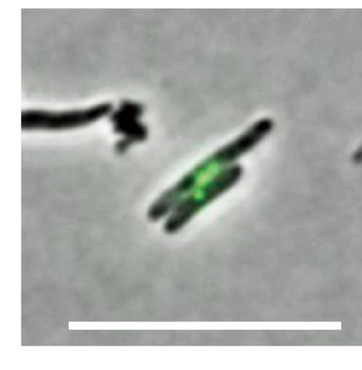
GFP induced cells

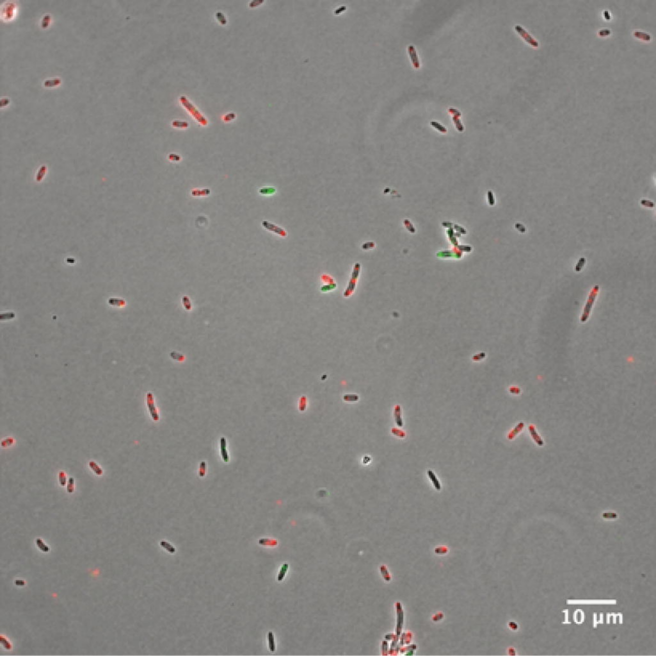


(C)



Cultivation time (hours)

	12	24	48	72
DsRed				
Alexa-488 (Wide angle)				
Alexa-488 (Magnified)				


10 µm