Agarose Gel Microcapsules Enable Easy-to-prepare, Picolitre-scale Single-cell Genomics, Yielding Near-complete Genome Sequences

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Agarose gel microcapsules enable easy-to-prepare, picolitre-scale single-cell
genomics, yielding near-complete genome sequences

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Abstract

A novel type of agarose gel microcapsule (AGM), consisting of an alginate picolitre sol core and an agarose gel shell, was developed to obtain high-quality single-cell amplified genomic DNA of bacteria. The AGM is easy to prepare in a stable emulsion with oil of water-equivalent density which prevents AGM aggregation, with only standard laboratory equipment. Single cells from a pure culture of *Escherichia coli*, a mock community comprising 15 strains of human gut bacteria, and a termite gut bacterial community were encapsulated within AGMs, and their genomic DNA samples were prepared with massively parallel amplifications in a tube. The genome sequencing did not need second-round amplification, and showed an average genome completeness that was much higher than that obtained using a conventional amplification method in microlitre scale, regardless of the genomic guanine-cytosine contents. Our novel method using the AGM allows many researchers to perform single-cell genomics easily and effectively, and can accelerate the genome analysis of yet-uncultured microorganisms.

40 Introduction

41 Microorganisms are ubiquitously distributed in diverse environments. They are often
42 associated with other organisms, and play important roles in ecosystems. However, the
43 majority of microbial species are difficult to culture with conventional methods and are
44 called yet-uncultured microorganisms¹. In the past two decades, they have been
45 extensively studied using culture-independent methods such as amplicon sequencing
46 analysis of small subunit ribosomal RNA (SSU rRNA) genes and shotgun sequencing
47 analysis of metagenomes. Metagenomics is a powerful tool for investigating the
48 ecological and physiological functions of microbiota based on their encoded genetic
49 repertoires. The recent development of the computational binning of metagenomic
50 fragments into respective microbial taxonomic assemblies has reinforced the utility of
51 metagenomics^{2,3}. However, computational binning based on the sequence composition,
52 homology to database sequences, and sequence read coverage of each fragment can
53 often fail to discriminate genomes of closely related (sub)species and to correctly sort
54 genomic regions exhibiting distinct features from others, such as rRNA genes,
55 prophages, and plasmids^{4,5}. Furthermore, a massive sequencing effort is required to
56 obtain the genome sequences of minor species in the microbial community³.

Single-cell genomics, in which whole genomic DNA is amplified from physically isolated single cells⁶, has been used as an alternative or complementary method to reveal the metabolic capacity of yet-uncultured microorganisms^{1,7}. Single-cell isolation can be achieved in many cases by using technologies such as fluorescence-activated cell sorting (FACS)¹, micromanipulators⁸, microfluidic devices^{9,10}, and encapsulation in water-in-oil droplets¹¹. Although various methods for whole genome amplification have been developed¹², multiple displacement amplification (MDA) using phi29 DNA polymerase with random primers is most widely used due to its relative simplicity, reliability, and applicability^{6,13}. However, MDA inherently accompanies extreme amplification bias among genome regions¹, which has been the major technical limitation in single-cell genomics. For example, the amplification bias caused lower average completeness of single-cell amplified genomes (SAGs) (47%) than that of metagenome-assembled genomes (MAGs) (93%) in a study of marine samples, although use of different coverage regions between SAG and MAG helped to obtain entire genome sequences¹⁴. Additionally, the high guanine-cytosine (GC) content of genomic DNA tends to increase the amplification bias^{1,15}. It has been reported that MDA in a small reaction volume, for example, when using a microchannel chamber (60 nL)¹⁰, nano-litre microwells (12 nL)¹⁶, virtual microfluidics (MDA in gel sheet, 60

nL)¹⁷, digital droplets generated by a microchannel (9.5–240 pL)^{18–20}, and gel bead²¹, can suppress amplification bias. Additionally, suppression of amplification bias using droplet MDA has been proved effective also for metagenomics: the droplet MDA more accurately presented microorganism composition than conventional microlitre scale bulk MDA²². However, these microfabrication techniques are not available for many microbiologists, and the amount of the amplification product is often too small for the direct genome sequencing; therefore, a second-round MDA is often required, which ultimately enhances the amplification bias²³.

To accelerate study using the single-cell genomics of yet-uncultured microorganisms, it is important to develop a simple method that prepares massive small reaction vessels using commercially available reagents, disposable plasticware, and equipment. For MDA reaction vessels, a microchannel can massively generate single-cell encapsulating water-in-oil droplets¹¹. However, MDA reagents are needed to be sequentially introduced into the droplets in the microchannel, which complexifies the microchannel structure. In contrast, single-cell encapsulating gel beads are more easily prepared by gelating cell-containing solated gel droplets^{21,23,24}. The MDA of the single-cell genome in the bead can be done only by exchanging reagents through gel.

However, the amount of MDA product in the gel matrix is generally insufficient for next generation DNA sequencing.

A hollow-core hydrogel microcapsule²⁵, which consists of a hydrogel shell and a sol core, is expected to be an efficient vessel for single-cell MDA. Many hollow-core hydrogel microcapsules have been reported to be useful for cell cultivations and cell transplantations: for example, pairs of a crosslinked polyethylene glycol (PEG) gel shell and a PEG sol core²⁶, an alginate and poly-L-lysine complex shell and an alginate sol core²⁷, and an agarose gel shell and an alginate sacrifice core²⁸. Recently, MDA in a hollow-core hydrogel microcapsule was used for discriminating single bacterial cells based on fluorescent in situ hybridization targeting a protein-coding gene²⁹. The microcapsule was prepared using simple emulsification gelation and consisted of a chemically-crosslinked soluble polyacrylamide gel core, an agarose gel middle layer, and a PCR primer-conjugated polyacrylamide gel shell. After genomic DNA from single *Escherichia coli* was amplified in the gel core using MDA, the target gene in the MDA product was further amplified in the microcapsule using emulsion PCR after solation of the core and the middle layer. However, the MDA product from single-cell genomic DNA, which was amplified in a picolitre-scale hollow core in a microcapsule, has not been fully evaluated for single-cell genomics.

Here, we introduce a novel method for single-cell genomics, using a newly developed AGM consisting of an agarose gel shell and an alginate sol hollow-core, which enables single-cell isolation and picolitre-scale MDA (Fig. 1a). The fabrication process of the AGM was optimised here for easier preparation using inexpensive equipment and reagents, such as a vortex mixer, centrifuges, and commercially available reagents without hazardous chemical crosslinker (Fig. 1b), although the size uniformity of AGM is sacrificed compared to the methods using microfabrication techniques^{29,30}. Thus, this novel type of AGM can be easily prepared and is scalable for many microbiologists. By using AGM, a single bacterial cell can be encapsulated in its picolitre-scale sol core and then subjected to MDA just by exchanging solutions (designated here 'MDA-in-AGM'). We demonstrate that MDA-in-AGM enables massively parallel single-cell genomics with much improved genome completeness, compared to a conventional method using FACS and microlitre-scale MDA.

Results

Preparation of AGMs containing single bacterial cells. Alginate gel cores containing bacterial cells were prepared using the emulsification/internal gelation method³⁰ (Fig. 1b). Briefly, a mixture of alginate solution, bacterial cells, and CaCO₃

nanoparticles was emulsified with isostearyl alcohol (ISA). The resulting microdroplets were gelated with calcium ions that were released from CaCO_3 nanoparticles by the addition of acetic acid to the water-in-oil emulsion. The concentration of bacterial cells was optimised using serial dilutions so that one alginate microdroplet contained one or no bacterial cell. The resulting alginate gel cores were washed sequentially with diethyl ether, 1-butanol, and Tris-HCl (pH 7.5) buffer.

Agarose solution (SeaPlaque, Lonza; final 2%) was added to the alginate gel cores suspended in Tris-HCl (pH 7.5) at 35°C, and the mixture was emulsified with 0.25% (v/v) Span 80 in polyglyceryl-6 octacaprylate (PGO) at 35°C (**Fig. 1b**). The use of PGO with a density of 0.997 g/mL, which is equivalent to that of water and AGMs (~1.0 g/mL), enabled the formation of a stable water-in-oil emulsion during the gradual gelation of agarose by cooling at 4°C. In addition, PGO improved the AGM preparation process by preventing the aggregation of AGMs and increasing their diameter and yield (Supplementary Fig. 1). After PGO was removed by mixing with diethyl ether and further with 1-butanol, the alginate gel cores were solated with a one-tenth volume of 0.5 M ethylenediaminetetraacetic acid (EDTA). The solation of alginate cores in AGMs was confirmed based on the core dissolution into the buffer after dissolving the agarose shells (Supplementary Fig. 2). The alginate sol core is expected to provide an

appropriate space for the MDA reaction, which was shown by the much greater yields of amplified DNA than those with an alginate gel core or agarose gel bead, as described below. The AGMs were washed with Tris-EDTA buffer (pH 7.4) to remove excess EDTA, and then suspended in a one-half volume of Tris-EDTA buffer and stored at 4°C.

Escherichia coli DH5α was used as a model microorganism. *E. coli* cells were added at a concentration of 3×10^8 cells mL⁻¹ alginate-CaCO₃ solution and were encapsulated in $5.64 \pm 1.72 \times 10^5$ AGMs (mean ± SD) in three independent experiments, which corresponded to $12.5\% \pm 5.4\%$ of the total AGMs. Of the AGMs containing *E. coli* cells, approximately 94% harboured a single cell in the core (Supplementary Table 1). In the three independent experiments, the diameters of the prepared AGMs were 39.6 ± 22.2 μm, 51.4 ± 13.3 μm and 49.3 ± 19.6 μm (Supplementary Fig. 3a). AGM diameters and core volumes showed normal distributions and log-normal distributions, respectively (Supplementary Figs. 3b and 4). The average of medians of AGM core volumes in the three experiments was 15.1 ± 6.3 pL (n = 3).

MDA of single-cell genomes within AGMs. AGMs (in 50 μ L suspension) were washed with sterile water and collected by centrifugation. The lysis of bacterial cells and denaturation of double-stranded DNA within the AGMs were carried out at room temperature for 30 min with 50 μ L of an alkaline solution, Buffer D2 of the REPLI-g UltraFast Mini Kit (Qiagen). Denaturation was stopped by neutralisation with the addition of the same volume of Stop solution from the kit, and the supernatant was then removed by centrifugation. Reaction Buffer (93.5 μ L) containing 11 μ L of REPLI-g UltraFast DNA Polymerase from the kit was added, and the resulting mixture was incubated at 30°C for 3 h. The reaction was stopped with one-tenth volume of 0.5 M EDTA, and the AGMs were washed three times with 200 μ L of Tris-EDTA buffer. All the reagents used in MDA and the AGM preparation were irradiated with ultraviolet light to degrade any contaminating DNA prior to use.

Among $4.6 \pm 2.9 \times 10^5$ AGMs prepared with *E. coli* cells, $8.9\% \pm 0.8\%$ exhibited DNA amplification, which corresponded to $77.0\% \pm 27.2\%$ of the AGMs containing *E. coli* cells ($n = 3$) (Supplementary Table 1). As shown in Fig. 2, amplified DNA filled the alginate sol core, whereas AGMs with alginate gel cores that were prepared without the solation step with EDTA or agarose gel beads containing no alginate core exhibited only limited or no DNA amplification by MDA.

181

182 Quality of single-cell genome sequences compared between MDA-in-AGM and
183 a conventional method using FACS-MDA. The quality of SAGs was evaluated and
184 compared with that obtained by a conventional method using a combination of FACS
185 for single-cell isolation and MDA (FACS-MDA) on a microlitre scale. AGMs
186 containing amplified DNA that was stained with SYBR Green I were individually
187 transferred to 0.2 mL PCR tubes with 29 μ L of sterile water under an epifluorescence
188 microscope equipped with a TransferMan NK2 micromanipulator (Eppendorf).
189 Amplified DNA was released to water by heating the tubes at 60°C for 5 min, and
190 directly used for preparation of sequencing libraries using the QIAseq FX DNA Library
191 Kit (Qiagen). No second round of MDA was necessary to obtain the libraries suitable
192 for genome sequencing. Paired-end sequence reads were generated on the Illumina
193 MiSeq platform with the Reagent Kit V3 (600 cycles), and a fixed number of read pairs
194 were randomly chosen and assembled *de novo* into contigs using SPAdes 3.13.0³¹ after
195 standard quality filtering. Contigs >1 kb were used for the subsequent analyses. Several
196 factors describing the genome sequence quality, including completeness and
197 contamination rate, were evaluated using CheckM³² and QUAST³³, and compared
198 between the SAGs obtained using MDA-in-AGM and FACS-MDA, respectively.

The genome completeness and the total sequence length of *E. coli* SAGs obtained by MDA-in-AGM rapidly increased during the sequencing effort, and the average genome completeness reached $93.0\% \pm 4.7\%$ ($n = 10$) for assemblies using 9×10^5 read pairs, while it was $33.6\% \pm 17.4\%$ ($n = 10$) in FACS-MDA (Student's *t*-test, $P < 0.01$) (Fig. 3a and Supplementary Table 2). The genome coverage calculated by mapping reads to the reference *E. coli* DH5 α genome sequence (BOCF01000000) was $98.0\% \pm 1.7\%$ in MDA-in-AGM ($n = 10$), which was much higher than $52.0\% \pm 13.8\%$ in FACS-MDA ($n = 10$) for 9×10^5 read pairs ($P < 0.01$) (Supplementary Table 2). In addition, the amplification bias among genome regions, which is inherent to MDA, was much improved in MDA-in-AGM compared to FACS-MDA (Fig. 3b). The steep decrease of number of contigs, i.e., smooth assembly of contigs, in MDA-in-AGM along the sequencing effort's depth was probably owing to the lower amplification bias (Fig. 3a). Other quality metrics of SAGs using all sequence reads are shown in Supplementary Table 3.

Single-cell genome analyses with a mock community and a natural sample. To evaluate the feasibility of MDA-in-AGM in a more realistic sample, we constructed a mock community comprising 15 cultured strains of human gut bacteria. The taxonomy

and compositions of each bacterial strain estimated using 16S rRNA amplicon analysis
 or metagenome analysis are shown in Supplementary Table 4. SAGs from the mock
 community were obtained using either MDA-in-AGM or FACS-MDA in the same way
 as above. Taxonomic compositions of SAGs were similar between MDA-in-AGM and
 FACS-MDA, both of which successfully recovered SAGs of most strains accounting for
 >5% of the mock community except *Faecalibacterium prausnitzii* (Supplementary
 Table 4). The overall genome completeness of SAGs with <5% contamination was
 significantly higher in MDA-in-AGM ($68.0\% \pm 23.3\%$, $n = 39$) than in FACS-MDA
 ($42.4\% \pm 20.5\%$, $n = 36$) for assemblies using 3×10^5 read pairs ($P < 0.01$)
 (Supplementary Tables 5 and 6). For example, SAGs of *Bacteroides thetaiotaomicron*
 and *Parabacteroides distasonis* exhibited clear differences between MDA-in-AGM and
 FACS-MDA in the genome completeness, total sequence length, number of contigs, and
 amplification bias, as seen in the *E. coli* SAGs (Fig. 3). The SAGs of most other strains
 also showed similar results (Supplementary Fig. 5).

Most sequence reads were mapped to their respective reference genomes: the
 mapping rates were 94.8% and 97.3% (median) for SAGs from MDA-in-AGM and
 FACS-MDA, respectively (Supplementary Table 7). Although the rates of cross
 contamination were slightly higher in MDA-in-AGM than in FACS-MDA, both were

lower than 5% (Supplementary Table 7). In *Ba. thetaiotaomicron* SAGs, sequence reads from a *Ba. thetaiotaomicron* plasmid were also obtained (Supplementary Table 7). Our results indicated that MDA-in-AGM is applicable to various bacterial species and improves genome completeness in both gram-positive and gram-negative bacteria, regardless of their GC content (Fig. 4). As for *Fa. prausnitzii*, it is possible that the cell lysis process using KOH was not effective and hence no SAGs were obtained in both MDA-in-AGM and FACS-MDA.

SAGs from MDA-in-AGM with >5% contamination, which would be caused either by contaminating extracellular DNA or by multiple cells being encapsulated in a single AGM, were binned into each bacterial species using metaWRAP³⁴. As a result, 30 additional SAGs with <5% contamination were obtained, and their genome completeness and contamination rates were 73.5% (median) and 0.9% (median), respectively (Supplementary Table 8).

DNA rearrangement during MDA, i.e., formation of chimeras, can complicate *de novo* assembly of single-cell genomes³⁵. In *E. coli* SAGs, the rates of chimeric reads were slightly higher in MDA-in-AGMs ($6.28\% \pm 1.09\%$) than in FACS-MDAs ($4.49\% \pm 1.32\%$) (Welch's *t*-test, $P < 0.01$; Supplementary Table 3). In SAGs from the mock community, the rates of chimeric reads were higher in MDA-in-AGM ($8.22 \pm 2.28\%$)

than in FACS-MDA ($3.55\% \pm 1.07\%$) (Welch's *t*-test, $P < 0.01$; Supplementary Tables 5 and 6). These rates of chimeric reads in MDA-in-AGM were similar to or slightly higher than the rate 6.19% in a previous study using a conventional MDA method for sequencing human haploid genomes³⁶.

Finally, we applied MDA-in-AGM to a natural environmental sample. We used the microbiota in the gut of the termite *Reticulitermes speratus*, which comprises several hundred bacterial species from diverse phyla³⁷. The entire guts of five worker termites were removed, and the gut contents were suspended. Then, bacterial cells were collected by centrifugation after digesting the extracellular DNA with DNase I, and the cells were washed with sterile water as described previously³⁸. The subsequent procedures were the same as those described above. We analysed 48 SAGs obtained by MDA-in-AGM and found high genome completeness ($78.6\% \pm 18.2\%$) and a low contamination rate ($1.0\% \pm 1.0\%$) (Table 1). The SAGs were taxonomically classified using the Genome Taxonomy Database Tool Kit (GTDB-Tk)³⁹ and were found to be affiliated with 13 bacterial classes, including known dominant groups in the termite gut, Spirochaetia, Bacteroidia, Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria, and Clostridia³⁷. In contrast, the genome completeness of SAGs obtained by FACS-MDA was only $37.7\% \pm 19.0\%$ ($n = 161$).

Although a different sequencing method, i.e., a combination of the Nextera XT DNA Library Prep Kit (Illumina) and the Illumina HiSeq 2500 platform (500 cycles), was used for the latter case, the number of reads (bases) used for assembly was much larger on average ($551 \text{ Mb} \pm 64 \text{ Mb}$, $n = 161$) than in the analysis of SAGs obtained by MDA-in-AGM ($256 \text{ Mb} \pm 49 \text{ Mb}$, $n = 48$) (Supplementary Table 9).

In addition to the 48 SAGs, we analysed 18 SAGs of the class Endomicrobia with small genome sizes ($\sim 1 \text{ Mb}$)⁴⁰ obtained by MDA-in-AGM, and $91.2\% \pm 9.8\%$ genome completeness was obtained. Furthermore, among them, eight SAGs of phylotype Rs-D17 were compared to the complete genome sequences obtained previously^{40,41}. The genome coverages calculated by mapping reads onto the two genomovars of Rs-D17 (Ri2008⁴⁰ and Ti2005⁴¹) reached $95.7\% \pm 1.3\%$ and $96.7\% \pm 0.9\%$, respectively (Supplementary Table 10). The composition of the termite gut community was estimated from SAGs using MDA-in-AGM or 16S rRNA amplicon analysis (Supplementary Table 11). The taxonomic composition of the SAGs showed a high positive correlation ($R = 0.834$, $P < 0.01$; Pearson's correlation coefficient) with that shown by the 16S rRNA amplicon analysis, whereas the frequencies of gram-positive bacteria were lower in MDA-in-AGM.

Discussion

MDA-in-AGM, the massively parallel MDAs in the picolitre-scale AGM cores, drastically improved the genome completeness of SAGs of *E. coli*, human gut bacteria in a mock community, and bacteria in the termite gut, compared to conventional microlitre scale FACS-MDA. Thus, the method is expected to contribute to revealing the metabolic capacity of yet-uncultured prokaryotes in environments such as animal intestinal tracts, soil, and hydrosphere.

It has been reported that the amplification bias in single-cell genomics can be suppressed by reducing the reaction volume of MDA using microfabrication techniques^{10,16,18,21,24}. The comparisons of MDA-in-AGM and other MDA methods are shown in Supplementary Table 12. Although the high genome completeness was also achieved in other miniaturised MDA methods, MDA-in-AGM does not require specialised equipment and provides scalable sample preparation.

The alginate sol core, employed in this study, successfully enhanced the yield of MDA compared to that obtained by MDA in gels (Fig. 2). This is probably attributable to the enhanced diffusivity of amplified DNA in the sol core⁴². MDA-in-AGM therefore does not require a second round of MDA, which is often necessary in other methods using microfabrication techniques (Supplementary Table 12)^{21,24}. The elimination of the

second-round MDA step resulted in the high genome completeness with suppressed amplification bias and enabled the simpler workflow.

The formation of chimeric sequences during MDA can affect the preciseness of the *de novo* assembly of SAGs⁴³. In the mock community, the rates of chimeric reads were comparable with those in droplet MDA²⁰ and slightly higher than conventional MDA methods (Supplementary Table 12)³⁶. Since the formation of chimeras is likely attributable to the branch migration of amplified DNA³⁵, a higher MDA reaction temperature by using thermostable phi29 DNA polymerase¹⁵ may decrease erroneous hybridization between replicons.

Our MDA-in-AGM is applicable to both gram-positive and gram-negative bacteria and also to genomes with high GC content (Fig. 4). Additional advantages of the AGM are its physical stability and permeability for small molecules, which have made buffer exchange and physical handling much easier. Although MDA-in-AGM can be performed without specialised equipment, the size distribution of AGM is difficult to control and the manual picking procedure using a micromanipulator suppresses throughput. To increase the uniformity of AGM and throughput of single-cell genomics using MDA-in-AGM, microchannels for preparation of uniform AGM, high-speed

AGM sorting using FACS, and molecular-barcoding⁴⁴ of SAGs may be adapted, although these approaches reduce the simplicity of the procedure.

Although the taxonomic composition of SAGs obtained by MDA-in-AGM was largely consistent with those shown by 16S rRNA amplicon and metagenomic analyses, a SAG of dominant *Fa. prausnitzii* was not recovered in either MDA-in-AGM or FACS-MDA (Supplementary Table 4). Thus, the failure was not specific to MDA-in-AGM but attributable to the MDA procedure. Since similar discrepancies in the taxonomic composition were found in gram-positive bacterial classes in the experiment using the termite gut microbiota (Supplementary Table 11), modification of cell lysis procedure, including an addition of lysozyme, may be required for certain gram-positive bacteria. To optimise such conditions, AGM is also superior because the exchange of solution is easier compared to other methods.

Lower average genome completeness of the mock community (68%) and the termite gut microbiome (79%) than that of *E. coli* (98%) (Supplementary Tables 2 and 5; Table 1) may also be attributable to the difference in easiness of cell lysis. Low genome completeness of SAGs in certain bacterial lineages and environmental samples, compared to SAGs of cultured *E. coli*, have been reported in previous studies, irrespective of the MDA methods^{1,45}. For example, in droplet single-cell MDA, the

genome completeness of *E. coli* and soil microbiota were $96.5\% \pm 2.2\%$ ($n = 16$) and $52.8\% \pm 24.3\%$ ($n = 17$), respectively²⁰. Cell lysis optimisation for different prokaryotic strains and environmental samples will be important also for increasing genome completeness.

Recently, when our experiments were on going, MDA using a hollow-core hydrogel microcapsule consisting of a crosslinked PEG gel shell and a dextran sol core, a similar capsule structure to AGM, was reported⁴². Although amplification bias has not been evaluated, single-cell MDA in the core increases the amount of MDA product when compared with MDA in the gel bead. In addition, the shell of a hollow-core hydrogel microcapsule prevents genomic DNA leaking from the core after alkaline denaturation prior to MDA. However, near-ultraviolet (UV) light (365 nm) used for photo chemical crosslinking of the PEG shell damaged cells and DNA due to photooxidation⁴⁶. We selected agarose as the AGM shell because it allows thermally mild reversible gelation, is commercially available, and is stable in MDA reagents, especially alkaline, neutralisation buffer, and EDTA.

AGMs or other reaction chambers containing single bacterial cells inevitably produces a proportion of chambers containing multiple bacterial cells²³, and it is difficult to completely eliminate contaminating DNA in our experimental procedures, as

in other previously developed methods¹. Nevertheless, the application of a computer program for binning metagenomic fragments, such as metaWRAP³⁴, enabled us to recover a considerable number of, at least, median-quality SAGs from mini-metagenome bins, which were resulted from multiple cells, by eliminating contaminating sequences (Supplementary Table 8).

In recent years, numerous studies analysing MAGs from environmental samples have been reported⁴⁷. Although single-cell genomics has different advantages and potentially increases the quality of research in combination with metagenomics, for example, by revealing strain-level heterogeneity⁴⁸ and by providing information on horizontally transferred genetic components²⁴, it is difficult for many microbiologists to perform. MDA-in-AGM is a much easier and less expensive method, and in addition, only a small amount of sample is needed. Thus, this method is also suitable for tiny specimens, such as the intestinal tracts of small insects and protist cells with endo- and ectosymbiotic bacterial communities.

Methods

Reagents. Ultrapure DNase/RNase-Free Distilled Water (Thermo Fisher Scientific, Waltham, MA) was used for all solutions prepared in-house. Solutions were autoclaved

378 at 121°C for 15 min and further decontaminated by UV radiation at 7.2 mW/cm²
379 overnight (12 h) in a laminar flow cabinet.

380 A suspension of uniform CaCO₃ nanoparticles (9.4% w/v, 100-nm diameter, pH
381 10) was provided by Shiraishi Central Laboratories (Hyogo, Japan). The conductivity
382 and pH of the CaCO₃ suspension were reduced by washing the particles using three
383 volumes of water. As well as Shiraishi's CaCO₃, fine calcium carbonate such as
384 precipitated calcium carbonate BioUltra (approximately 3-μm diameter; Sigma, St.
385 Louis, MO) is also used for alginate core gelation. Alginate solution (5%, w/v) was
386 prepared by dissolving sodium alginate (80–120 cP at 1% w/v, Wako, Osaka, Japan) in
387 water and stored at 4°C. Agarose solution was prepared by dissolving SeaPlaque (final
388 2%, w/v, Lonza, Basel, Switzerland) in water, and stored at 4°C. Polyglyceryl-6
389 octacaprylate (PGO; Nisshin Oillio, Tokyo, Japan), and Tris-EDTA buffer (TE;
390 BioUltra for molecular biology, pH 7.4, Sigma), and REPLI-g UltraFast Mini Kit
391 (Qiagen, Hilden, Germany) were used as described below.

392

393 Evaluation of PGO for agarose microdroplet gelation. After agarose solution (2
394 mL) was mixed with PGO or ISA, each containing 0.25% sorbitan monooleate (Span
395 80; Wako) (v/v) (20 mL, 35°C), by vortexing and gelated on ice, agarose beads were

recovered from PGO or ISA as described below. Large agarose aggregates were removed from the agarose beads using 300-µm cell strainers (pluriSelect, Leipzig, Germany). The yields and diameters measured with ImageJ (<https://imagej.nih.gov/ij/>) of the agarose beads were statically analysed with R (<https://www.r-project.org/>).

Preparation of *Escherichia coli* stock culture. After *E. coli* DH5α (TaKaRa Bio Inc) from a single colony on LB plate was cultured in two LB (200 mL) media, Cells of *E. coli* were harvested and washed twice in PBS (20 mL) by centrifugation. The cells were re-suspended in PBS (10 mL), and their density was determined with a cell-counting chamber. The *E. coli* cells (3.05×10^{10} cell/mL) were divided into aliquots in microtubes (0.5 mL each) and stored at –80°C.

Preparation of alginate cores containing *E. coli* cells. Cells of *E. coli* were encapsulated in alginate cores by the emulsification/internal gelation method³⁰ (Fig. 1b). *E. coli* cells from the stock culture (10 µL) were mixed with 990 µL of 2% sodium alginate solution containing 1% CaCO₃ and 50 mM acetate buffer (pH 7.0, Sigma-Aldrich, St. Louis, MO). The mixture was emulsified with 9 mL of isostearyl alcohol (ISA; Kokyu Alcohol Kogyo Co., Ltd., Chiba, Japan) containing 3% lecithin (Wako) in

414 a 50-mL tube by vortexing for 1 min. The emulsion was mixed with 2% acetic acid in
415 ISA (0.1 mL each) by vortexing for 1 min with 10 repetitions. During this procedure,
416 the pH of the mixture gradually decreased to 4.0 and the CaCO₃ nanoparticles were
417 dissolved. The released calcium ions from CaCO₃ gelated alginate microdroplets in the
418 emulsion. ISA was removed by mixing with 9 mL of diethyl ether and centrifugation at
419 4°C for 3 min using a swing rotor (3,000 × g, 5702R, Eppendorf, Hamburg, Germany),
420 and the residual ISA was further removed from the alginate cores by mixing with 50
421 mM Tris-HCl buffer (pH 7.5) containing 0.2% (v/v) Tween 20 (Tris–Tween 20) (5 mL),
422 followed by centrifugation, and then by repeating the procedure twice with 1-butanol (5
423 mL). The resulting alginate cores were suspended in one volume of 50 mM Tris-HCl
424 (pH 7.5) and filtrated through a 100-μm cell strainer (pluriSelect) to remove large
425 alginate aggregates. The filtrated alginate cores were further washed with Tris-HCl (pH
426 7.5) and collected in 2-mL microtubes. The volume of the alginate cores was calculated
427 from its weights and stored at 4°C after mixing with a one-half volume of Tris-HCl (pH
428 7.5). To adjust the number of *E. coli* cells in a single alginate core to less than two, 10
429 μL of 3.05×10^6 – 10^8 *E. coli* cells from the *E. coli* stock were mixed with 990 μL of the
430 alginate containing CaCO₃ suspension. After alginate cores containing *E. coli* were
431 prepared from the mixture, *E. coli* in the alginate cores were observed using SYBR

Green I (TaKaRa Bio Inc., Shiga, Japan) (Supplementary Fig. 6). At 3.05×10^6 cell/mL of the alginate mixture, 17.6% of the cores contained *E. coli* and their 78.8% carried single cells. Thus, 3.05×10^6 cell/mL of *E. coli* was used for alginate core preparation in the following experiments.

Preparation of AGMs containing *E. coli* cells. The supernatant of the alginate core suspension (600 μ L) was removed after centrifugation in a 50-mL tube. The residual alginate cores (400 μ L) were incubated at 35°C for 10 min and then mixed with the agarose solution as prepared above (2 mL, 35°C). The mixture was emulsified with 0.25% (v/v) Span 80 in PGO (20 mL, 35°C) by vortexing for 1 min (Fig. 1b). Then, the agarose was gelled by cooling on ice for 1 h. PGO was removed from the emulsion by washing as described above. The alginate gel core was solated by chelating calcium ions with the addition of a 1/10 volume of 0.5 M EDTA (Thermo Fisher Scientific). The AGMs were then suspended in one volume of TE buffer. Large debris in the suspension was removed through a 300 μ m cell strainer, and the AGMs were further washed with TE. The AGMs were diluted in 50 mM Tris-HCl (pH 7.5) containing 0.5% SeaPlaque agarose, 0.1% *p*-phenylene diamine (Wako), and 10,000-fold diluted SYBR Green I (TaKaRa Bio Inc., Shiga, Japan), and the morphology, density, diameter, and number of

encapsulated *E. coli* cells were observed under an inverted fluorescence microscope (IX71, Olympus, Tokyo, Japan) equipped with a CCD camera (BU-51LN, Bitran, Saitama, Japan). The AGMs were stored at 4°C. For long-term storage (more than a week), the AGMs were stored in 40% ethanol at –80°C and washed three times in 10 volumes of water before use.

Evaluation for alginate core solation in AGM. Alginate gel cores without *E. coli* were labelled with rhodamine 123 (Wako) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Tokyo Chemical Industry Co., Ltd., TCI, Tokyo, Japan) and *N*-hydroxysuccinimide (Wako)⁴⁹. AGMs containing rhodamine 123-labelled core were heated at 65°C for 5 min in the presence of 50 mM EDTA or 50 mM CaCl₂.

Residual cores were observed as mentioned above.

Effect of alginate core gelation and solation on MDA. Aliquots (50 µL) of the AGMs containing *E. coli* cells before solation of the alginate cores were mixed with one volume of cell lysis solutions containing 400 mM KOH with or without 10 mM EDTA and they were neutralised with 100 µL of Stop buffer from the REPLI-g UltraFast Mini Kit. The AGMs containing *E. coli*, AGMs without *E. coli* (negative control), and

468 agarose beads containing *E. coli* (control) were subjected to MDA as described below
469 and their amplified DNA were observed .

470

471 **MDA-in-AGM.** A 50 μ L aliquot of AGMs was centrifuged, and the collected AGMs
472 were washed with 200 μ L water. The cell lysis and DNA denaturation were carried out
473 with Buffer D2 (50 μ L) of the REPLI-g UltraFast Mini Kit at room temperature for 30
474 min. After denaturation was stopped with the Stop buffer (50 μ L), the supernatant was
475 removed by centrifugation. The Reaction buffer (93.5 μ L) containing 11 μ L phi29 DNA
476 polymerase from the kit was added and incubated at 30°C for 3 h (MDA-in-AGM). The
477 reaction was stopped with 1/10 volume of 0.5 M EDTA, and the AGMs were washed
478 with 200 μ L of TE buffer three times. The washed AGMs were stored at 4°C.

479

480 **FACS-MDA.** Single-cell MDA using a MoFlo XDP fluorescence-activated cell sorter
481 (Beckman Coulter Inc., Brea, CA) was performed as a control³⁸. After *E. coli*, a mock
482 bacterial community, and termite gut bacteria were stained with SYBR Green I or
483 CellTracker Green (Thermo Fisher Scientific), the stained bacteria were sorted into a
484 single cell per well of 96-well PCR plates. The cell lysis was carried out with Buffer D2
485 (1.5 μ L) of the REPLI-g UltraFast Mini Kit at room temperature for 30 min. After

denaturation was stopped with Stop buffer (1.5 µL), Reaction buffer (7.5 µL) containing 0.5 µL phi29 DNA polymerase from the kit was added and incubated at 30 °C for 3 h and 65 °C for 15 min. To check genome amplification and the degree of contamination, the MDA products were evaluated by direct Sanger sequencing of the 16S rRNA. PCR was performed with Bacteria-universal 16S rRNA gene primers: 27F (5'-AGAGTTTGATCMTGGCTCAG) and 1390R (5'-ACGGGCGGTGTGTACAA).

Mock community of human gut bacteria. A mock community of human gut bacteria was prepared using 15 isolates (Supplementary Table 4) obtained from Japan Collection of Microorganisms. A mixture of all glycerol stocks in equal amounts was used as the mock bacterial community. After the mock bacterial community was diluted 100-fold using water, the diluent (10 µL) was treated with DNase I (20 µL) at 30 °C for 30 min and washed with water (1 mL) three times. The washed bacterial pellet was suspended in water (10 µL). The MDA-in-AGM of the mock bacterial community was prepared from the suspension as mentioned above.

The total genomic DNA of the mock bacterial community was extracted using the DNeasy Ultra Clean Microbial Kit (Qiagen). The metagenome sequencing was performed by preparing a library of the purified total genomic DNA with the QIAseq

FX DNA Library Kit (Qiagen). Amplicon sequencing of 16S rRNA genes in the mock community was performed by preparing a library with the Nextera XT Index Kit 96 indexes (Illumina, Inc, San Diego, CA) and the MiSeq platform with Reagent Kit V3 (600 cycles). The bacterial composition of the mock community was estimated on the basis of the results analysed in QIIME2⁵⁰.

Termite gut bacteria. Specimens of the wood-feeding termite *Reticulitermes speratus* were collected at Tsukuba in Ibaraki Prefecture, Japan. The guts of five worker termites were removed, and the gut contents were suspended in solution U, which consisted of 9.2 mM NaHCO₃, 5.1 mM trisodium citrate dihydrate, 13 mM KH₂PO₄, 37 mM NaCl, 0.75 mM CaCl₂, and 0.4 mM MgSO₄. The extracellular DNA and protist DNA were digested using DNase I, and the bacterial cells were collected by centrifugation and washed with water³⁸. The washed termite gut bacteria were suspended in water (10 µL). The MDA-in-AGM of the termite gut bacteria was prepared using the suspension as mentioned above.

Genome sequencing and bioinformatics. AGMs containing amplified DNA, detected with SYBR Green I, were transferred individually to PCR tubes, under an

inverted fluorescence microscope equipped with a micromanipulator (TransferMan NK2, Eppendorf, Hamburg, Germany). Water (29 μ L) was added to each PCR tube with a single AGM and incubated at 60°C for 5 min, to release DNA by solating the agarose shell. To check the degree of contamination, we screened the MDA-in-AGM products by the direct Sanger sequencing of the 16S rRNA as mentioned above. After removing the contaminated samples, sequencing libraries were prepared using the QIAseq FX DNA Library Kit. When the sequencing libraries were constructed using adaptors with the concentration described in the manufacturer's instructions, a large number of concatenated adaptor sequences were formed. To suppress the generation of the artificial sequences, the concentration of adaptor sequences was adjusted to one-tenth of that in the manufacturer's instructions. Genome sequencing was performed on the MiSeq platform with Reagent Kit V3 (600 cycles). Sequence libraries for single cells isolated from the termite gut microbiota using FACS were prepared using the Nextera XT DNA Library Prep Kit (Illumina), and analysed on the Illumina HiSeq 2500 platform (500 cycles).

The generated short reads were trimmed using Cutadapt (<https://github.com/marcelm/cutadapt>), PRINSEQ (<http://prinseq.sourceforge.net/>), FASTX Trimmer, FASTQ Quality Trimmer

540 (http://hannonlab.cshl.edu/fastx_toolkit/download.html), and cmpfastq_pe
541 (http://compbio.brc.iop.kcl.ac.uk/software/download/cmpfastq_pe). The trimmed reads
542 were assembled using SPAdes ver. 3.13.0 (k-mer: 21, 33, 55, 77, 99, and 127, options: -
543 -sc, --careful)³¹ into contigs. Only contigs >1 kb were selected using SeqKit
544 (<https://bioinf.shenwei.me/seqkit/>) for the subsequent analyses. The fixed number of
545 read pairs was randomly chosen by SeqKit. Taxonomic classification of single-cell
546 genomes from termite gut samples was conducted using the Genome Taxonomy
547 Database Tool Kit (GTDB-Tk)³⁹. Genomic sequence data showing >5% contamination,
548 identified by using CheckM³², were sequentially subjected to binning, refinement, and
549 reassembly processes with the BINNING (including metaBAT2⁵¹, MaxBin2⁵², and
550 CONCOCT⁵³), BIN_REFINEMENT, and REASSEMBLE_BINS modules of
551 metaWRAP³⁴. For the single-cell genome analyses using *E. coli* DH5 α and the human
552 gut bacteria, trimmed reads were mapped onto known genome sequences using
553 Bowtie2⁵⁴, and the results were visualised using IGV ver. 2.3.26
554 (<http://software.broadinstitute.org/software/igv/>). The genome coverage obtained by
555 mapping reads without assembling them [total length (base) of mapped reads divided by
556 reference genome size] was calculated using BBtools ([http://jgi.doe.gov/data-and-](http://jgi.doe.gov/data-and-tools/bbtools/)
557 [tools/bbtools/](http://jgi.doe.gov/data-and-tools/bbtools/)). The chimeric read rate was calculated by mapping short reads to the

reference genome with the Barrows-Wheeler Aligner
(<https://sourceforge.net/projects/bio-bwa/files/>) and SAMtools (<http://www.htslib.org>).
The reference genomes are listed in Supplementary Table 4. Cross-contaminations of
genomic sequencing data with <5% contamination using CheckM³² (Supplementary
Table 7) were calculated by mapping sequencing reads to reference genomes in the
mock community¹⁷.

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Author contributions

Y. Y. and M. O. supervised the study. H. A., M. Y., Y. H., and M. O. designed the
study. H. A., M. Y., and Y. H. wrote the manuscript. H. A. elucidated the AGM
preparation protocol. M. Y. and M. S. performed next genome sequencings and their
data analyses. All authors contributed to and edited the manuscript.

576

577 Data availability

578 The raw fastq files (DRR252532–DRR253885) and assemblies for SAGs of termite gut
579 bacteria (BNTM01000000–BOCE01000000) were deposited into DDBJ under
580 BioProject accession no. PRJDB10679.

581

582 Competing interests statement

583 The authors have no conflicts of interest to declare.

584

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724

Figure legends

Fig. 1. Schematic view of an agarose gel microcapsule (AGM) and its preparation process.

a. A schematic diagram of the AGM developed in this study for the single-cell isolation and multiple displacement amplification (MDA). The AGM consists of an agarose hydrogel shell and an alginate sol core. The alginate core provides a picolitre-scale reaction chamber for MDA, while the agarose shell acts both as a permeable envelope for enzymes and small molecules and as a protective wall against larger particles and molecules such as bacterial cells and genomic/amplified DNA.

b. A preparation scheme for AGMs containing *Escherichia coli* cell(s). The alginate cores and agarose shells are gelated with Ca^{2+} from CaCO_3 and cooled in emulsion, respectively. PGO (polyglyceryl-6 octacaprylate) can suppress the aggregation of AGMs during agarose gelation by cooling. Finally, the alginate gel cores are solated with EDTA by chelating Ca^{2+} . ISA: isostearyl alcohol.

Fig. 2. Yield enhancement of multiple displacement amplification (MDA) by

alginate core solation.

The effect of alginate core solation on the productivity of MDA within agarose gel microcapsules (AGMs) was evaluated. The genomic DNA of *Escherichia coli* encapsulated within AGMs was amplified by MDA with or without alginate solation. A phase contrast image (red) and epifluorescent image (green, SYBR Green I) are overlaid. MDA in agarose gel beads (without an alginate core) was also conducted as a control experiment. Arrows indicate *E. coli* cells stained with SYBR Green I. Bar = 100 μm .

Fig. 3. Comparison of genome completeness and amplification bias of single-cell genomes obtained by MDA-in-AGM and FACS-MDA.

a. Completeness and number of contigs of SAGs during the sequencing process. Box-and-violin plots (blue: MDA-in-AGM; red: FACS-MDA) are shown for *Escherichia coli* and two bacterial species as examples from the mock community of human gut bacteria (Supplementary Tables 2, 5, and 6). Data points (blue circle) and their arithmetic means (rhombus) are shown on the plots. In the *E. coli* graphs, the results for DNA prepared from cultured *E. coli* cells without MDA are also shown (green circle).

In the left panels, significant differences are indicated with asterisks ($P < 0.05$) or double asterisks ($P < 0.01$).

b. The number of sequence reads mapped against genome regions is shown as an indicator of amplification bias caused during MDA. The number of reads ($\times 10^5$) used for *de novo* assembly (shown as 'Reads') and genome completeness (%) ('Compl.') estimated using CheckM are shown.

Fig. 4. Relationships between genome completeness and GC contents of single-cell genomes in the mock community of human gut bacteria.

Results were obtained by assembling randomly chosen 3×10^5 read pairs for each single-cell amplified genome. MS485-1-51 of MDA-in-AGM (2.6×10^5 read pairs) and CS1-94 of FACS-MDA (2.9×10^5 read pairs) were assembled using all read pairs. The bacterial species comprising the mock community and detailed results are shown in Supplementary Tables 4–6. Blue and red indicate the MDA-in-AGM and the FACS-MDA, respectively. Squares, circles, and rhombuses indicate gram-positive, gram-negative, and gram-variable bacteria, respectively.

Tables

Taxon of bacteria	MDA-in-AGM			FACS-MDA [†]		
	Number of SAGs	Genome completeness (%)	Contamination (%)	Number of SAGs	Genome completeness (%)	Contamination (%)
Spirochaetia	15	73.5 ± 20.6	0.9 ± 1.1	35	32.5 ± 19.0	0.3 ± 0.6
Bacteroidia	8	88.2 ± 8.7	1.3 ± 0.7	51	36.1 ± 17.7	0.6 ± 1.2
Alphaproteobacteria	2	87.0 ± 1.3	0.0 ± 0.0	8	52.9 ± 19.5	0.9 ± 1.7
Betaproteobacteria	7	87.5 ± 5.9	1.2 ± 0.3	9	35.3 ± 12.6	0.9 ± 0.8
Deltaproteobacteria	3	86.4 ± 6.7	0.5 ± 0.3	3	22.8 ± 16.7	1.1 ± 0.6
Epsilonproteobacteria	2	55.0 ± 48.5	2.2 ± 3.1	2	38.8 ± 20.7	1.2 ± 0.6
Clostridia	4	70.2 ± 23.6	1.3 ± 0.4	27	36.7 ± 16.2	0.7 ± 1.1
Actinobacteria	1	51.9	0.87	3	33.5 ± 15.9	1.9 ± 1.7
Planctomycetales	1	87.1	0.1	11	42.6 ± 21.0	0.6 ± 0.8
Synergistia	2	83.9 ± 22.7	1.2 ± 1.6	4	65.5 ± 21.9	0.2 ± 0.3
Fibrobacteria	1	72.8	0.0	n. d.	n. d.	n. d.
Deferribacteres	1	72.9	2.0	n. d.	n. d.	n. d.
Candidate division SR1	1	74.1	0.0	n. d.	n. d.	n. d.
Bacilli	n. d.	n. d.	n. d.	8	47.2 ± 23.2	1.4 ± 2.0
Endomicrobia*	18	91.2 ± 9.8	1.5 ± 1.0	n. t.	n. t.	n. t.
Total sequenced samples	48 (66)	78.6 ± 18.2 (82.0 ± 17.2)	1.0 ± 1.0 (1.1 ± 1.0)	161	37.7 ± 19.0	0.7 ± 1.1

Table 1 Summary of single-cell amplified genomes (SAGs) of symbiotic

bacteria in termite gut bacteria.

n. d.: not detected. n. t.: not tested.

[†] NGS libraries were made by Nextera XT and read by HiSeq.

Number of SAGs, genome completeness, and contamination including Endomicrobia

are shown in parentheses.

Figures

a

b

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Supplementary Files

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