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ORIGINAL ARTICLE

Non-exponential growth of *Mycobacterium leprae* Thai-53 strain cultured *in vitro*

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ABSTRACT

In this study, attempts were made to culture this bacterium in media supplemented with a variety of biological materials to determine why cultivation of *Mycobacterium leprae in vitro* has not this far been successful. A slight increase in the number of cells in medium supplemented with human blood plasma and an extract of nude mouse tissue as observed after more than 3 months of cultivation at 30 °C. To ascertain whether this increase was real growth, the growth was analyzed by droplet digital PCR, which showed a slow increase in the copy number of cell-associated DNA and the release of a large amount of DNA into the culture medium from bacterial cells during cultivation. These results were supported by electron microscopic examination of *M. leprae* in infected mouse tissues, which showed that most of the replicated bacteria had degenerated and only a few cells survived. Based on these results, it was postulated that many of the replicated cells degenerate during *M. leprae* growth and that only a few cells remain to participate in the next growth stage. This means that, unlike other cultivable bacteria, the growth of *M. leprae* is not exponential and the number of cells therefore increase extremely slowly. Thus, accurate judging of the success of *M. leprae* cultivation requires observation of growth over a long period of time and careful measurement of the increase in number of viable cells.

Key words cultivation, droplet digital PCR, growth *in vitro*.

Attempts to cultivate *Mycobacterium leprae in vitro* have not thus far been successful and the reasons for its failure to grow in artificial media are still unclear (1).

In a previous study, we used electron microscopy and the latest freeze-fixation technique to examine ML grown in nude mouse foot pads and found that only a few were alive (2). This observation suggested that, even in animals that are known to support the growth of this bacterium, its growth is unusual. The presence of so many degenerating cells suggested that the growth of ML is not logarithmic, which is in contrast to the growth of most other cultivable bacteria.

In this study, we attempted to determine the conditions suitable for cultivation of ML by trying to culture the ML Thai-53strain in liquid medium supplemented with a variety of materials, including animal serum, animal tissue extracts and human blood plasma. We found that human blood plasma, FBS and tissue extracts from nude mice could support some level of growth of this bacterium *in vitro*; however, the growth rate was extremely slow. We also found that, in the late stage of cultures, the cultured bacteria formed large bacterial aggregates similar to the globi commonly found in ML infected animal tissues. To confirm the growth of ML in these culture conditions, we analyzed growth using ddPCR and identified a slight increase in bacterial DNA in the cultures. Additionally, we found that plentiful ML-specific DNA was released from the cells

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List of Abbreviations: ML, *M. leprae*; ddPCR, droplet digital PCR.

during cultivation, which supports our electron microscopic findings when we studied ML-infected nude mouse tissues. Based on these experimental observations, we here discuss the growth mechanisms of ML.

MATERIALS AND METHODS

Bacterial strain

The Thai-53 strain, provided by the Leprosy Research Center of the National Institute of Infectious Diseases, Japan, was used throughout this study (3). For cultivation *in vitro*, ML cells were isolated from the foot pads of infected nude mice (8–9 months after infection) following the method described by Nakamura (4, 5). Briefly, the foot pads were cut into small fragments with scissors and then homogenized in sterile PBS (pH 7.0) with a glass homogenizer. Large tissue fragments were removed by low speed centrifugation, after which the supernatant containing ML cells was treated with trypsin (final concentration 0.05%) at 37°C for 60 min. The bacteria were then collected by centrifugation at 2380 *g* for 10 min and treated with 3% NaOH solution at 37°C for 15 min to kill contaminating bacteria. ML cells thus treated were collected by centrifugation and suspended in a small volume of culture medium. The final concentrations of bacteria used at the start of cultivation were approximately 10^6–10^7 cells/mL.

Culture medium and supplements

The basic culture medium used was the liquid NK260 medium designed by Nakamura and based on Kirchner's medium, which he used in trials of *in vitro* cultivation of ML (6). NK260 medium contains egg-yolk extract (10%), pyruvate (2 mg/mL) and transferrin (10 μg/mL) as additional nutrients. The pH of the medium is set from 7.0 to 7.2 (6). NK260 medium was originally only supplemented with 10% bovine serum. In this experiment, the following substances were substituted for bovine serum.

Human blood plasma
Human blood plasma that did not match the criteria for transfusion was donated by the Japan Red Cross Blood Center after government approval and used at a concentration of 10%–20% in the medium.

Normal mouse tissue extracts
After removing the internal organs, the body parts of one nude mouse were minced and ground in PBS with a glass homogenizer. Large tissue components were removed by centrifugation at 40 *g* for 10 min, and the tissue extracts in the supernatants used after passage through a 0.2 μm filter for sterilization.

Culture methods

Cultivation was performed in liquid media in small glass test tubes (10 mL volume) with screw caps. The isolated ML bacteria were suspended in culture media in the tubes and kept in an incubator at 30°–32°C for several months (6, 7). Media were replaced at 7–10 day intervals. The tubes were centrifuged at 2380 *g* for 15 min and fresh media added after the old media had been removed by pipetting.

Measurement of bacterial growth by cell count

The growth of ML was evaluated by counting the number of acid fast-stained (Ziehl–Neelsen stain) cells under 1000× magnification. The bacteria in the culture tube were collected by centrifugation at 2380 *g* for 10 min and resuspended in 1 mL of PBS. To make a homogeneous suspension, the suspension was repeatedly (more than 30 times) mixed using a 10 mL injection syringe with an 18-gauge injection needle. Ten microliters of the suspension were applied within a circle (1 cm in diameter) traced on a glass slide and stained using the Ziehl–Neelsen acid-fast staining method. The final cell counts were used to calculate the number of cells in 1 mL of suspension. These calculations were performed once a month over 5 to 6 months of cultivation. In the graphs showing bacterial growth, increases in the number of cells are expressed as fold changes relative to the number of cells at the starting point. This counting method is expected to have some error; however, test counting performed by several individuals revealed that the maximum error remained within twice the actual count number. Thus, it was decided that an increase of more than threefold could be considered significant.

Fluorescent staining to determine bacterial viability

To examine the proportion of live and dead bacteria in the culture, the bacteria were stained with a two-color fluorescence staining method (Live/Dead "BacLight" Bacterial Viability Kit; Molecular Probes, Eugene, OR, USA). The staining was performed in accordance with the manufacturer's instructions. Briefly, ML cells in culture medium were collected by centrifugation (2380 g for 10 min) and suspended in PBS. Next, 3 μL of the fluorescent dyes were added to 1 mL of the bacterial suspension and incubated in the dark for 15 min at room temperature, after which 3 μL of the stained suspension was placed on a glass slide, covered with a glass coverslip,

and observed using a fluorescence microscope (Keyence Ez9000; Keyence, Osaka Japan).

Extraction of DNA

DNA was extracted by a mechanical bacterial cell disruption method. In brief, 2 g of fine zirconia powder and 0.4 mL of bacterial suspension were mixed in small plastic tubes. The tubes were placed in a cell disruptor (Multi-beads shocker; Yasui Kikai, Tokyo Japan), which was operated at a rate of 3500 shaking cycles for 60 sec, repeated six times at intervals of 60 sec. Bacterial cell counts in acid-fast stained preparations of the suspensions before and after this procedure showed that more than 99% of the ML cells were disrupted by this method.

Droplet digital PCR

Droplet digital PCR was performed on a QX200 automated Droplet Digital PCR system (Bio-Rad, Hercules, CA, USA). Briefly, DNA templates were mixed with 2× QX200 ddPCR EvaGreen Supermix (Bio-Rad) and 2 pmol of an ML-specific primer pair (S13: 5′-CTCCACCTCCACCGGCGAT-3′ and S62: 5′-GACTAGCCTGCCAAGTCG-3′) (8, 9). The mixture was brought to 20 μL and subjected to oil droplet generation on an automated generator (Bio-Rad). The PCR cycling conditions were one cycle of 5 min at 95°C followed by 40 cycles consisting of 30 sec at 94°C with a 2°C/sec ramp rate and 1.5 min at 55°C with a 2°C/sec ramp rate, then 5 min at 90°C and a hold at 4°C. Droplets were read by a QX200 Droplet Reader (Bio-Rad) and analyzed with QuantaSoft software (Bio-Rad), which determines the concentration of a target gene as the copy number/mL based on the fraction of positive droplets.

To remove extracellular DNA before DNA extraction, the bacterial samples were treated with DNase (final concentration 50 ng/mL) in PBS containing 8 mM of MgCl2 for 30 min at 37°C and then heated at 85°C for 10 min in a steel heat block to inactivate the DNase.

RESULTS

Growth patterns of the *M. leprae* Thai-53 strain *in vitro* in NK260 media containing various supplements

Growth of the Thai-53 strain in NK260 media supplemented with several different biological materials is presented in Figure 1. As shown in Figure 1a, the strain cultured in PBS did not show any significant increase in

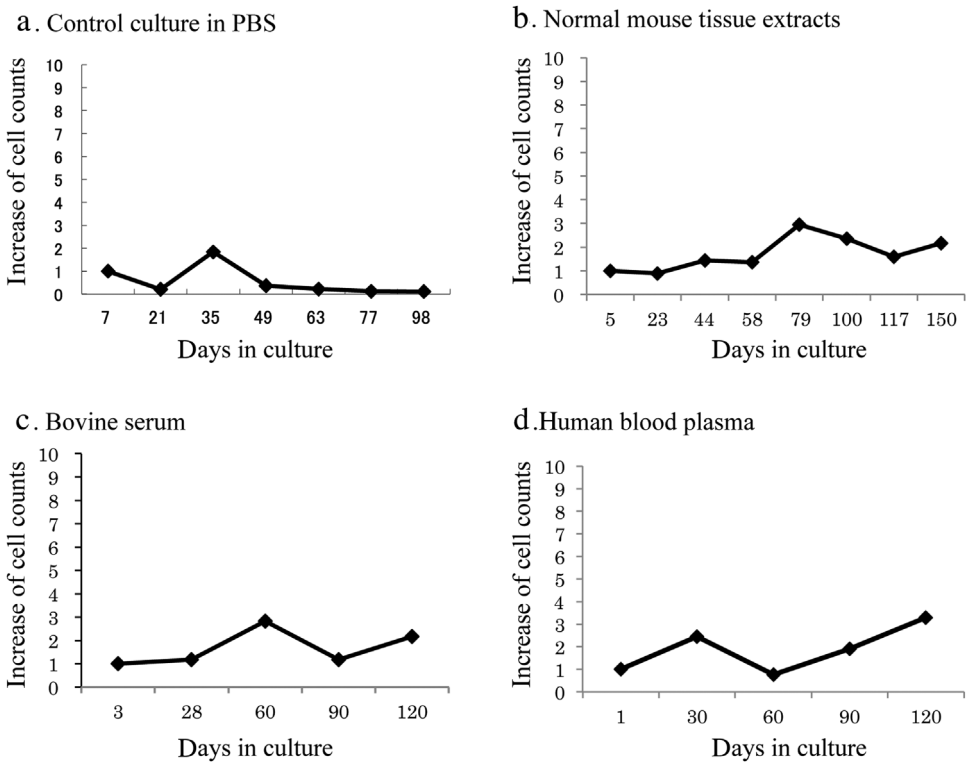

Fig. 1. Growth pattern of *M. leprae* strain Thai-53 in NK260 medium supplemented with various nutrients. Vertical scales show fold changes in the number of bacterial cells expressed as multiples of the counts on Day 1.

the number of cells. In contrast, some slight increases were observed in the supplemented media, as shown in Figures 1b–d. However, these increases were modest; the largest being fourfold; this was observed in the culture supplemented with human blood plasma (Fig. 1d). In all cases, growth was slow: it took 60 days or more to see a significant increase in cell counts.

Light microscopy of the cultured cells in the late stages of growth showed that many of the bacteria had aggregated into large bacterial masses similar to the globi observed in infected mouse or human tissue (10) (Fig. 2). Although similar bacterial aggregates were naturally found in the starting materials isolated from mouse foot pads, they were not as large as those found in the late stage of culture.

To visualize the dead cells in these aggregates, they were stained by the Live/Dead staining method. As shown in Figure 3, many of the cells in the cell aggregates found in the late stage cultures were stained green, indicating that they were alive. However, approximately 30% to 40%, were stained red or yellow, the colors of dead cells (Fig. 3). These results are largely consistent with those of our previous electron microscopic study of globi in infected nude mouse foot pads. Most of the bacterial cells observed in the electron micrographs were degenerate forms; less than half of them seemed to be alive (2).

These morphological observations suggest that not all ML cells participate in replication and that the growth of this bacterium is not exponential, unlike the growth observed in many other bacterial species. To confirm this phenomenon, the level of growth was analyzed by ddPCR.

In vitro analysis of growth by ddPCR

The ddPCR performed with DNA extracted from the culture on the first day of growth revealed the presence of much more ML DNA than expected (Fig. 4; untreated). Based on the assumption that the excess DNA probably resulted from contaminating bacterial DNA derived from the infected mouse foot pad tissues, the bacterial specimens were treated with DNase before DNA extraction to remove this contaminating DNA. As expected, after treatment there was much less bacterial DNA in the specimens from the initial stages (Fig. 4; DNase). Consequently, in the following ddPCR analyses, the specimens were tested under two conditions: DNase-treated and −untreated.

Figure 5 shows the results of ddPCR analysis of specimens cultured *in vitro* in NK260 medium supplemented with human blood plasma. The growth curve in this culture, as determined by cell counting, is shown in Figure 5c. As shown in this graph, the growth was very slow: by the late stage of the culture, the number of cells had increased to about twice the number present on the first day. Analysis with ddPCR showed that the total amount of DNA in the specimens that had not undergone DNase treatment gradually decreased, whereas the number of cells in specimens treated with DNase (DNase-treated) increased slowly to about twice that of the initial day (Fig. 5b). As had been demonstrated by electron microscopy and Live/Dead staining, some of the bacteria degenerated during cultivation. Thus it is possible that the DNA in some of these bacterial cells was released during cultivation. Nevertheless, the increase in cell numbers during cultivation after DNAase treatment suggests the possibility that this bacterium does grow *in vitro* (Fig. 5b).

The results from another culture grown in human plasma-supplemented medium are shown in Figure 6. In this case, the tissue from mouse foot pads was repeatedly treated with PBS to yield more bacteria for cultivation. It was anticipated that this process would reduce free DNA in the initial sample. However, ddPCR analysis of this culture showed an increase in free DNA during

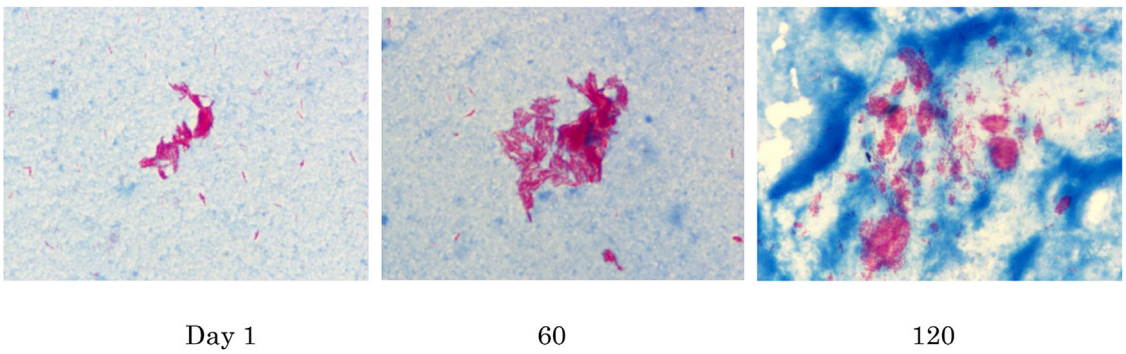

Fig. 2. Photomicrographs showing globi-like cell aggregates in cultured specimens on the first day of culture and Days 60 and 120 after inoculation. (Ziehl–Neelsen stain; magnification ×1000).

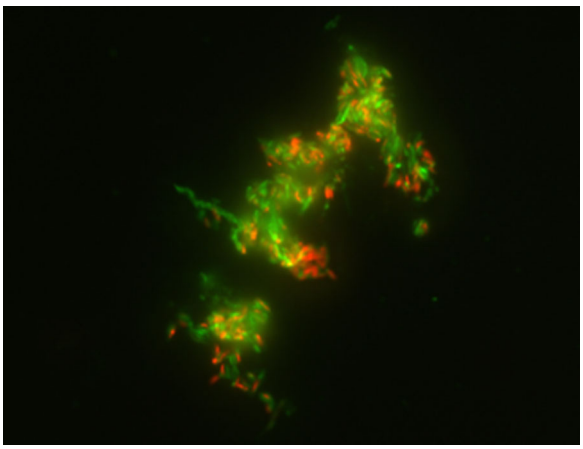

Fig. 3. Photomicrographs showing Live/Dead staining of bacterial aggregates in a cultured specimen on Day 130 after inoculation. (magnification ×1000).

cultivation and a slight increase in cell-associated DNA, suggesting the possibility of ML growth in this artificial medium (Fig. 6).

As a control experiment, ML was cultured in PBS at 30°C for approximately 100 days and its DNA analyzed by ddPCR. The results are presented in Figure 7. No increase in the bacterial cell count was observed (Fig. 7a) and, according to the ddPCR analysis, no increase in gene copy number was observed in either the DNase-treated or the untreated specimens (Fig. 7b). The gene copy numbers in the DNase untreated samples decreased drastically and disappeared after 90 days of culture.

These results of the copy number analysis suggest that, as observed in the Live/Dead staining and electron

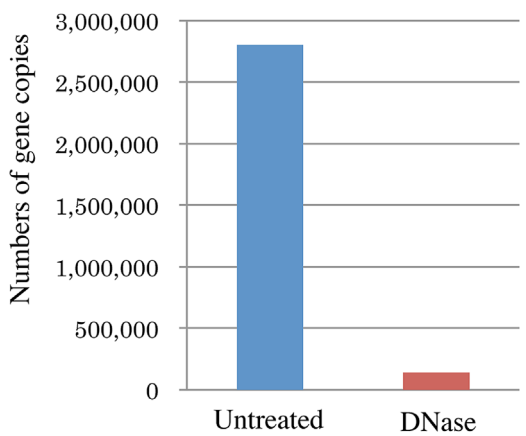

Fig. 4. Effects of DNase treatment on ML-specific DNA copy numbers in a specimen isolated on the first day of culture. Treatment of the cultured specimens with DNase before DNA extraction greatly reduces the numbers of ML DNA copies detected by ddPCR analysis.

microscopic examinations, some of the ML cells degenerated during culture and released their DNA whereas other cells proliferated.

In conclusion, the ddPCR analyses of the growth of ML in different growth media suggested that this bacterium grows *in vitro*, but that not all of the bacteria participate in replication. Thus, the exponential growth commonly observed in many bacteria was not observed for ML.

DISCUSSION

We have been attempting *in vitro* cultivation of ML for more than 10 years by testing the growth-promoting effects of adding a variety of nutrient supplements to the culture media. We observed a slight increase in the number of bacterial cells in media supplemented with human blood plasma. However, this increase was at most three- or four-fold higher than that on Day 1 of culture. Furthermore, the growth was so slow that it took more than 90 days to increase by this small amount. Therefore, the question of whether this was real growth remained. To determine whether growth did occur in this cultivation system, we carried out an analysis using ddPCR and found a slight increase in bacterial DNA during cultivation in media supplemented with human blood plasma. Additionally, we identified an unexpected new phenomenon in ML growth, that is, the release of bacterial DNA from the bacterial cells. This result is consistent with our electron microscopic observation of globi in mouse foot pads (2) and with the fact that Live/Dead staining of cultured specimens showed the presence of many dead cells. This phenomenon that not all of the replicated bacteria participate in the next step of growth and that as a consequence, the growth rate is not exponential. This pattern of growth differs completely from that of many cultivable bacteria. Thus, to confirm the *in vitro* growth of ML, it seems to be important to examine and analyze the cultures carefully for an extended period of time. The growth of ML had not been confirmed in many previous cultivation experiments, which is likely attributable to the unusual growth pattern of this bacterium.

Why this bacterium degenerates during growth is not clear. It is well known that ML forms large cell aggregates called globi. In this experiment, we identified globi-like large bacterial aggregates in the culture system. Therefore, it can be assumed that the formation of such bacterial aggregates is an important step in the growth of this bacterium. It can also be assumed that, in these aggregates, the degenerated bacteria may release some nutrients or growth factors and that these substances may support the growth of the remaining bacteria. Chemical and genetic analyses of the products

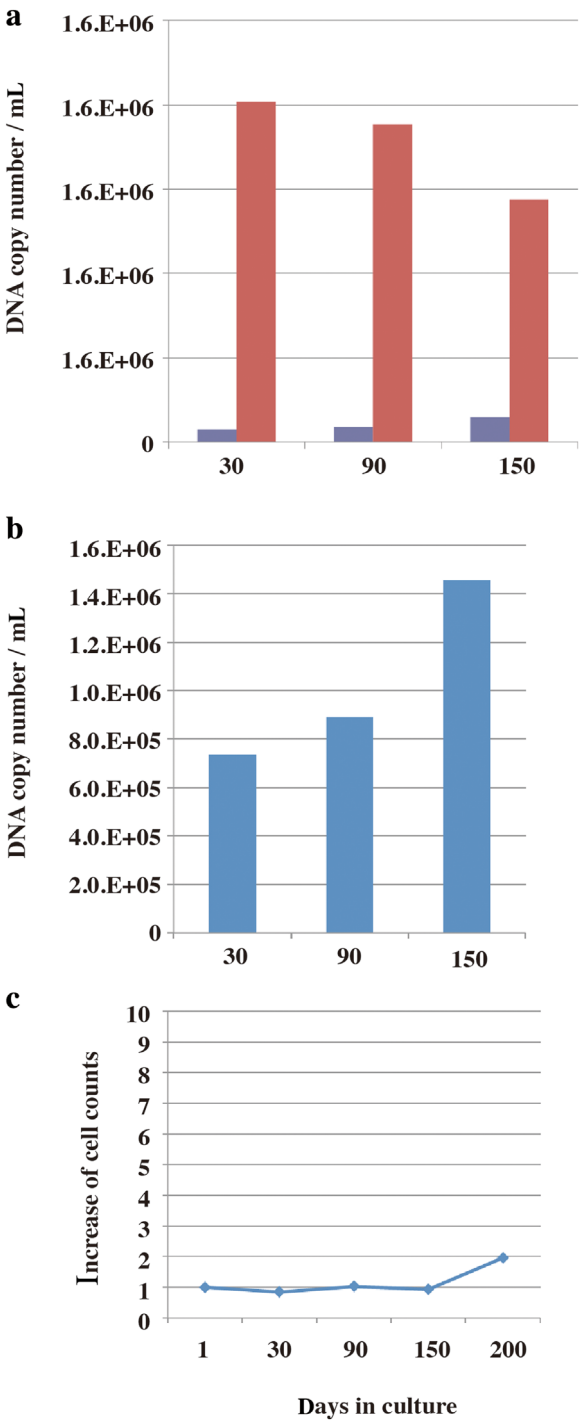

Fig. 5. (a) ddPCR analysis of the growth of ML cultured in NK250 supplemented with human blood plasma. Red, no DNase treatment; blue, DNase treated. (b) ddPCR analysis of DNase treated samples. The results of the DNase treated samples are shown on a different scale, showing an approximately two-fold increase in ML DNA copy numbers during 150 days of culture. (c) The growth pattern of ML expressed as fold changes in cell counts compared with the start of the experiment.

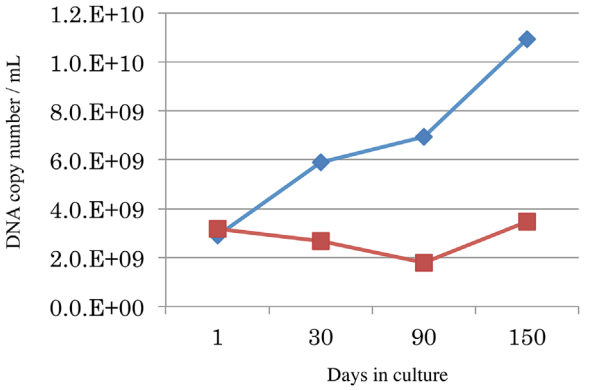

Fig. 6. ddPCR analysis of an ML culture in medium supplemented with human blood plasma. A culture different from that analyzed in Fig. 5 was analyzed. Blue line, no DNase treatment; red line, DNase treated.

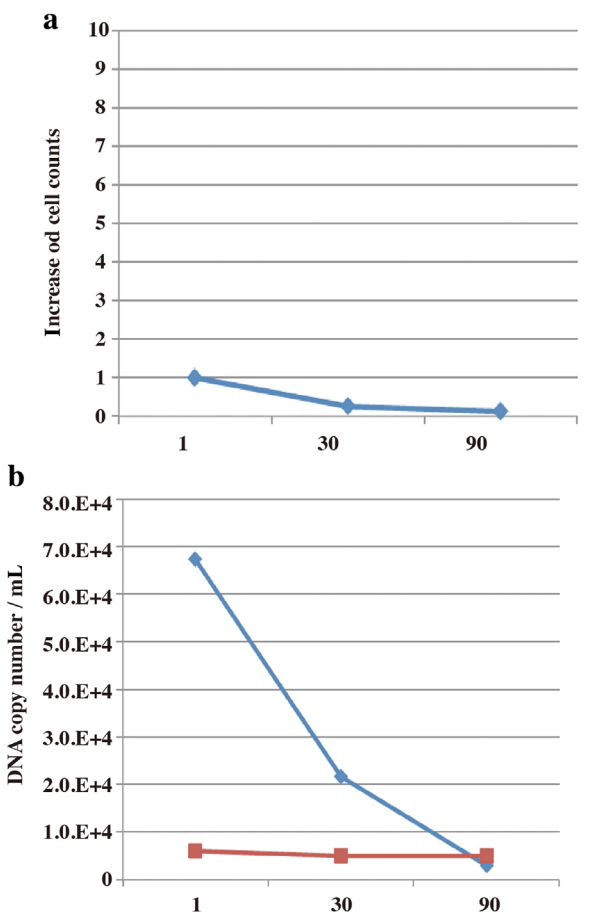

Fig. 7. Growth analysis of ML in PBS. (a) The growth pattern of ML in PBS without any supplements at 30°C and (b) the results of ddPCR analysis of the growth of ML in PBS. Red, treated with DNase; blue, without DNase treatment.

released from degenerated bacteria may be important in elucidating the growth mechanisms of ML.

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DISCLOSURE

The authors have no conflict of interest to disclose.

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