Detection Time Distribution of Microcolonies Formed by Individual Heat-Injured Cells of *Escherichia coli*

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The microcolony formation at 30°C on an enriched minimal salts agar plates by individual *Escherichia coli* cells heated at 50°C was monitored with a time-lapse shadow image analysis system, MicroBio µ3D™ AutoScanner. While the time course of microcolony count detected every half an hour for the unheated cells seemingly demonstrated a normal distribution, that for the heated cell population demonstrated totally the growth delay probably resulting from cell injury and also interestingly distributed in its rather deformed pattern with a tailing. Those patterns of the cumulative counts of appearing microcolonies during the post-heating cultivation period were expressed in three different mathematical models. This approach may be proposed as a rapid cultivation method predictable for enumeration of viable and repairable injured cells in practical use.

Key words : Heat injury / *E. coli* / Growth delay / Microcolony formation / Time-lapse image analysis system.

Different stress treatments of a microbial cell population have often been known to generate injured cells. How such injured cells can be successfully enumerated is a crucial problem in both the viability assay to rescue those and in practical pasteurization and sterilization processes to suppress those. Although cell staining methods using various kinds of fluorescent dyes have recently been employed to enumerate viable, injured and dead cells as rapid detection techniques (Schottroff et al., 2018), the cultivation methods still have been used universally as well. A rapid method to detect microcolonies formed on an agar plate has also been developed (London et al., 2010; Ogawa et al., 2012). In this method, an apparatus named MicroBio µ3D™ AutoScanner (Nippon Becton Dickinson Co., Ltd., Tokyo) equipped with the time-lapse shadow image analysis system is used (Ogawa et al., 2015).

The injured cells have mostly been detected and enumerated by using the double agar plating method, using both non-selective and selective media, the latter of which contains a growth-inhibiting substance selective to injured cells, e.g., sodium chloride or sodium deoxycholate. Therefore, a difference in colony counts obtained between these two types of media is generally regarded as sublethally injured cells (Wu, 2008). In our laboratory a novel double cultivation method without any selective principles has also been proposed previously for enumeration of the injured cell population (Tsuchido, 2017). Furthermore, Saito et al. (2021) have recently reported the effect of osmotic stress on
the colony formation rate of *E. coli* cells using the same apparatus as in this study.

In this study, we used a MicroBio µ3D™ AutoScanner to monitor and then analyze the detection time of microcolony formation by individual cells of heat-injured *E. coli* cells during the post-heating cultivation on an enriched minimal salts agar. Based on the data obtained, the growth dynamics of the heat-injured cell population were expressed in three mathematical models. And further, the distribution and degree of heat injury of the cell population reflected by delay in microcolony formation were evaluated and the quantitative growth delay indices derived from the above models were proposed for the prediction of the colony appearance process for the whole population.

*Escherichia coli* strain NBRC106482 (OW6), a proline-requiring mutant derived from the W3110 train, was used in this study. The bacterial cells were cultivated at 30°C in an enriched M9 (EM9) medium (7 g Na2HPO4, 3 g KH2PO4, 0.5 g NaCl, 1 g NH4Cl, and 0.25 g MgSO4·7H2O per liter) supplemented with 0.1% (w/v) vitamin-free Casamino acids (Difco) and 0.2% (w/v) glucose per liter (Kitagawa et al., 2000). For gelling the medium, agar was added at 1.5% (w/v) before autoclaving at 121°C for 20 min. The pre-culture was started by inoculation of a loopful colony into a test tube containing 5 ml of EM9 broth and then the tube was incubated overnight at 30°C with shaking. Afterward, a 100 ml flask containing 20 ml of fresh EM9 broth was inoculated with an aliquot of the pre-culture and the resultant culture was grown to mid-exponential phase (about 3×10^8 cells per ml) at the same temperature with shaking at 120 rpm. Cells were harvested and washed twice by centrifugation at 4°C, 5,000×g for 5 min with 50 mM potassium phosphate buffer (KPB) at pH 7.0. After that, the 10-times concentrated cell suspension in fresh buffer was kept at 4°C for 30 min before heat treatment (Katsui et al., 1981; Tsuchido et al., 1982).

The cell suspension was added to a sterile 100 ml flask containing 19.8 ml of KPB pre-heated to 50°C for rapid heating (Katsui et al., 1981) and then kept at this temperature for 20 min. Samples were withdrawn and diluted 10 times for instant cooling into a test tube containing KPB and kept at 30°C for 1 min to prevent cold shock before transferred to an ice water bath.

Heated cell samples were serially diluted with KPB and then an aliquot (0.1 ml each) of the diluted samples was plated on EM9 agar. The plates were then transferred into the MicroBio µ3D™ AutoScanner (Nippon Becton Dickinson Co., Ltd, Tokyo) for incubation at 30°C for 5 d (Ogawa et al., 2012). This apparatus is a time-lapse shadow image analysis system equipped with a temperature-controlled incubator and an automated microcolony growth analyzer. The monitoring system can detect microcolonies formed by bacterial cells on each agar medium of a maximum of 100 plates and the threshold size of one microcolony is about 65μm in diameter, although varying with colony shape characteristic for the kinds of bacterial strain and the medium used. The apparatus also outputs the final total viable counts (Ogawa et al., 2012 and 2015).

The pattern of the time course of microcolony formation by individual cells was analyzed by three mathematical models, Gompertz model, Logistic model (Tjørve and Tjørve, 2017) and FOR (the first order reaction) model (Hattori, 1981) as expressed in the following equations, Eqs. (1), (2) and (3), respectively.

\[
N = Ae^{-k(t-t_i)} 
\]  
\[
N = \frac{A}{1+e^{-(t-t_{1/2})}} 
\]  
\[
N = A[1-e^{-k(t-t_i)}] 
\]

where \(N\) and \(A\) are the relative microcolony number (%) at time \(t\) and at 5 d, the latter when the maximum value was taken as 100%. \(k\) is a shape parameter in Eqs. (1) and (2) or the specific colony formation rate (h⁻¹) in Eq. (3); \(t_i\) is the time (h) at the inflection point for Gompertz function, \(t_{1/2}\) is the time (h) of the midpoint (50%) of the final maximum number for logistic function and also \(t_i\) is the lag time (h) in FOR model.

From the analyzed data, the extent of cell injury of the cell population was evaluated by the following three growth delay time indices, as estimated by the difference in each time between unheated and heated cell populations. The first index is \(\Delta t_m\), which means the time difference at the peak of microcolony appearance occupied by the major population of heated cells, the second is \(\Delta t_{1/2}\), which is the difference in the time reaching half of the final microcolony number, and third is \(\Delta t_{av}\), which is the time difference of the weighted average as expressed in the following equation.

\[
\bar{t}_w = \frac{\Sigma pt}{\Sigma p} 
\]

where \(p\) (%) is the number of microcolony newly appearing at every 30 min interval after the lag time in this model.

The experiments were performed in triplicate and the number of microcolony counts was calculated to be relative values of the final total count. The data obtained were statistically analyzed in fitting to mathematical models with Microsoft Excel® for Office 365 software (Microsoft Corp., USA) for the distribution of colony number appearing at every 30 min of cultivation and with
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Based on these analyzed results, we calculated the values of three indices for the estimation of growth delay time described above, $\Delta t_p$, $\Delta t_{1/2}$ and $\Delta t_w$. The results of those analyses were summarized in Table 2. The results of Fig. 1 indicate that almost all of the surviving cells are injured after the heat treatment at 50°C for 10 min, as revealed by the growth delay in the appearance of each microcolony. The values of these indices were 4.67 h as $\Delta t_p$, and 19.82 h as $\Delta t_w$. And there were no significant differences in $\Delta t_{1/2}$ values obtained with between Gompertz, logistics and FOR models, being values of between 13 to 17 h (Table 2).

### TABLE 1. The values of parameters in mathematical models used to analyze the cumulative patterns of microcolonies formed on EM9 agar plates in heated cell population of E. coli.

| Model | Parameter | Cell population |
|-------|-----------|-----------------|
|       |           | Unheated | Heated |
| Gompertz | $t_i$ | 21.28±0.57 | 33.37±2.25 |
|         | $k$ | 1.42±0.17 | 0.10±0.01 |
|         | $R^2$ | 1.00 | 0.99 |
| Logistic | $t_{1/2}$ | 21.60±0.61 | 38.08±2.83 |
|         | $k$ | 2.09±0.23 | 0.14±0.01 |
|         | $R^2$ | 1.00 | 0.98 |
| FOR | $t_r$ | 20.88±0.65 | 25.35±1.46 |
|       | $k$ | 0.63±0.01 | 0.07±0.01 |
|       | $R^2$ | 0.97 | 0.99 |

See the text for the parameters of each model and $R$ is the correlation coefficient.

Data indicate the average in triplicate with SD.
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### TABLE 2

The index times of growth delay estimated directly or by using the mathematical models from the pattern of microcolony formation on EM9 agar in heat-injured cell population of *E. coli*.

| Index time of cell injury | Model     | Growth delay time (h) |
|--------------------------|-----------|-----------------------|
| ∆t₁/₂                    | Gompertz  | 15.61±2.27            |
| ∆t₁/₂                    | logistic  | 16.48±2.51            |
| ∆tᵣ                      | FOR       | 13.98±2.23            |

See the text for the index times and the data present the average±SD.

In particular, the ∆tᵣ indicates a time delay in the maximal appearance of detected microcolonies in the heat-treated population from that of the untreated population. According to our results, such a peaked population was largely occupied by less injured subpopulation and thus ∆tᵣ is an easy-to-evaluate but biased index. On the other hand, ∆t₁/₂ means a time delay in microcolony formation by half of the total cell population in the heat-treated population. Therefore, ∆t₁/₂ can be used as an all-inclusive indicator of evaluating the recovery rate of the injured population irrespective of the mathematical model used here (Fig. 1 and Table 1). The result of no significant differences in ∆t₁/₂ value among the three mathematical models indicate that any of these models can be available for practical use. Finally, ∆tᵣ is the difference in delay time of the weighted average calculated from the number and time of appearance of microcolony in every half an hour and can be broadly applied for practical use.

In this study we proposed a novel method to evaluate the distribution and the degree of cellular injury, which is reflected by the delay in microcolony formation of individual cells, in a heat-treated cell population of *E. coli* by using a time-lapse shadow image analysis system. The proposed growth delay indices may be available for the prediction and analysis of the growth pattern of surviving, but injured cell population generated by other lethal or sublethal stresses.

In conclusion, a novel method to analyze the delay time of microcolony formation detection with a MicroBio μ3D™ AutoScanner is proposed for the evaluation of an injured cell population. This approach is expected to be applied for practical use in the food industry, such as the preservation of thermally processed foods and the microbiological enumeration test. This system has a fascinating merit of high performance in a combination of automatic and simultaneous cultivation, measurement and assay for 100 agar plates.
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