Modeling Surface Growth of *Escherichia coli* on Agar Plates

Hiroshi Fujikawa* and Satoshi Morozumi

Tokyo Metropolitan Institute of Public Health, Department of Microbiology, 3-24-1, Hyakunin-cho, Shinjuku, Tokyo 169-0073, Japan

Received 10 April 2005/Accepted 24 August 2005

Surface growth of *Escherichia coli* cells on a membrane filter placed on a nutrient agar plate under various conditions was studied with a mathematical model. The surface growth of bacterial cells showed a sigmoidal curve with time on a semilogarithmic plot. To describe it, a new logistic model that we presented earlier (H. Fujikawa et al., Food Microbiol. 21:501–509, 2004) was modified. Growth curves at various constant temperatures (10 to 34°C) were successfully described with the modified model (model III). Model III gave better predictions of the rate constant of growth and the lag period than a modified Gompertz model and the Baranyi model. Using the parameter values of model III at the constant temperatures, surface growth at various temperatures was successfully predicted. Surface growth curves at various initial cell numbers were also sigmoidal and converged to the same maximum cell numbers at the stationary phase. Surface growth curves at various nutrient levels were also sigmoidal. The maximum cell number and the rate of growth were lower as the nutrient level decreased. The surface growth curve was the same as that in a liquid, except for the large curvature at the deceleration period. These curves were also well described with model III. The pattern of increase in the ATP content of cells grown on a surface was sigmoidal, similar to that for cell growth. We discovered several characteristics of the surface growth of bacterial cells under various growth conditions and examined the applicability of our model to describe these growth curves.

Generally, microbial growth in liquid is planktonic, whereas the structure of a microbial colony grown on a surface is considerably complex (11, 23). The surfaces of solids, such as would exist on solid foods, are susceptible to attachment by and subsequent growth of microorganisms, where they might exist as a biofilm, a unique microbial community (22). Studies on microbial surface growth, therefore, are thought to be considerably important in many microbiological fields.

Microbial surface growth studies on kinetic parameters such as the rate constant of growth and the lag period have been done by many investigators (8, 18, 21, 27, 32, 33). Some investigators selected the colony size parameters such as the diameter and height as the measures of growth (27, 33). These measures of microbial growth are easy to measure and are for one dimension, but microbial colonies are three-dimensional objects by nature. Other investigators have studied the increase in the cell number of one colony with time (8). However, when we quantitatively evaluate microbial growth on a surface, the total cell numbers of colonies would be more appropriate as the measure of growth than those of a single colony. Thus, some of the investigators measured the increase in the total cell number of microbes inoculated on the surface of a specific food (2, 18, 20, 28). However, few investigators have studied microbial growth curves on a surface (2, 20, 28). Also, the basic characteristics of the population dynamics of microbial cells grown on a surface have not fully been elucidated.

For the last two decades, a number of mathematical models have been developed to quantitatively describe microbial growth in culture media and food (4, 9, 16, 25). Recently, we developed a new logistic model, which is a logistic model extended for the description of microbial growth (12, 13). Our model has the potential to predict bacterial growth at constant and dynamic temperatures in broth and liquid foods. More recently, we slightly modified the model to more precisely describe bacterial growth at the acceleration phase of growth (14, 15).

In the present study, therefore, we studied surface growth patterns of *Escherichia coli* cells under various conditions and modeled them using our model to evaluate the possibility that the model could describe and predict the surface growth of cells. We have already extensively studied the growth kinetics of this microorganism in liquid culture (12–14).

**MATERIALS AND METHODS**

Cell preparation. Cell suspensions of *E. coli* 5125, which was previously studied for growth in liquid media, were prepared by the method of Fujikawa et al. (12–14). Briefly, bacterial cells were activated on a nutrient agar plate (Nissui Pharmaceuticals, Tokyo, Japan) at 35°C for 24 h. Cells of several well-grown colonies on the plate were incubated in a nutrient broth (Nissui Pharmaceuticals) with shaking at 35°C for 24 h. Cultured cells were washed twice with phosphate buffer (pH 7.0) by centrifugation. Cells were then resuspended in the buffer. This gave a cell suspension of ca. 10^9 to 10^10 CFU/ml. The cell suspension was then diluted to 1:10^6 (in most of the experiments) with the nutrient broth and stored at about 5°C before use.

Incubation. Bacterial cells were surface-grown by using the membrane filter method (3, 19). Portions (1 ml) of the cell suspension prepared above were placed on a sterile filtration membrane with a 0.22-μm pore size and 25 mm in diameter (GSWP0250; Millipore, Bedford, MA) that was already set in a sampling manifold with 12 wells (no. 1225; Millipore). Cells in the suspension were collected on the membrane by vacuum filtration. These procedures were done in a sterile clean bench. The membrane was then placed at the center on the surface of a nutrient agar plate (Nissui Pharmaceuticals) that was already cooled to about 5°C.

Cells on the membranes were incubated in a humidified, programmed incubator (PR-3G; Tatsusi Espe Co., Osaka, Japan) at a constant or varying temperature (13). Immediately after each incubation period, the membrane in triplicate was taken from the incubator and cooled to about 5°C.
The temperature of the membrane on a plate was monitored with a digital thermometer (AM-7002; Anritsu Meter Co., Tokyo, Japan). The come-up time of the sample to reach a designated temperature was measured for the constant temperature experiment. This period, which was about 24 min for most of the constant temperatures, was taken into consideration (13). For a varying-temperature experiment, sample temperature was measured every 30 s throughout the experiment.

Agar plates with various nutrient levels. Agar plates with 1/5 and 1/25 nutrient levels of the original nutrient agar plates (15 g of peptone/liter and 5 g of meat extract/liter as nutrients; Nissui Pharmaceuticals) were prepared with agar (Bacto agar; Becton Dickinson, Sparks, MD) and nutrient broth (powder) containing peptone and meat extract (Nissui Pharmaceuticals). Agar plates without any nutrient broth were also prepared. All agar plates contained a constant amount of salt (5 g/liter). Their pHs were not adjusted because they were maintained at about 7 with the buffering action of the agar and the nutrient broth used. In the experiments with the low-nutrient-level agar plates, washed cells were suspended in the phosphate buffer and then collected on a membrane by vacuuming.

Cell counts. Cells grown on a membrane filter were suspended with 40 ml of buffered peptone water (pH 7.0; Nissui Pharmaceuticals), with Tween 80 in a sterile plastic bag using a stomacher for 1 min. The number of cells in the suspension was counted in duplicate using nutrient agar plates with the surface plating method. After each average (three per datum point) was transformed into the logarithm with base 10, the average and standard deviation of each point were then calculated. The counts of cells on a membrane without incubation were the initial value for each experiment.

**ATP measurement.** The ATP content of cells grown on a membrane filter was measured in triplicate by using the firefly luciferin-luciferase method with an ATP analyzer (AF100; TOA Electronics, Tokyo, Japan). Briefly, cells incubated on the filter membrane were placed with the membrane in a sample well of the analyzer. The ATP content of the cells was then measured with a reagent kit (AF-2L1; TOA Electronics) according to the manufacturer's technical manual. For samples whose ATP contents were over the measurement range of the analyzer, the cells on the membrane filter were suspended in 9 ml of deionized, cool water in a sterile plastic tube by sonicating them for 1 min. The ATP content of the cell suspension was then measured again with the analyzer according to the technical manual. The average of each datum point was then calculated.

**Model development.** Recently, we have developed a new logistic model of microbial growth (12, 13). The rate of growth of the model is expressed as follows: \( \frac{dN}{dt} = rN \left( 1 - \frac{N}{N_{\text{max}}} \right) \) (equation 1). Here, \( N \) is the population (arithmetic number) of the organism at time \( t \), \( r \) is the rate constant of growth, \( N_{\text{max}} \) is the maximum population, and \( N_{\text{lag}} \) is the inoculum size. \( n \) is an adjustment factor, which was called \( c \) in our previous study (12, 13).

To more precisely describe a linear growth during the acceleration period in a growth curve, we slightly improved equation 1 for the position of the adjustment factor \( n \) (14). In the improved model, the factor worked on the fraction \( N - N_{\text{lag}} \)/\( N_{\text{max}} \), instead of \( N/N_{\text{max}} \). The improved model can thus be expressed as follows: \( \frac{dN}{dt} = rN \left( 1 - \frac{N - N_{\text{lag}}}{N_{\text{max}}} \right) \left( 1 - \frac{N_{\text{lag}}}{N_{\text{max}}} \right) \) (equation 2). We will denote equations 1 and 2 as models I and II, respectively.

In the present study, we modified model II for the description of the curve of microbial surface growth. As seen later in the results section, surface growth curves were sigmoidal, whereas the curvature during the late exponential phase, or the deceleration phase, was larger than that in liquid medium (13). To represent this, model II was modified in the present study; the Richards model, which is an empirical one, was introduced in the term of \( 1/N_{\text{max}} \). In the Richards model, the term of \( 1/N_{\text{max}} \) of the original logistic model is changed to \( 1 - \left( N/N_{\text{max}} \right)^{m} \) by introducing a curvature parameter \( m \) (>0) (4, 30). The new growth model, therefore, can be described as follows: \( \frac{dN}{dt} = rN \left( 1 - N/N_{\text{max}} \right)^{m} \left( 1 - N_{\text{lag}}/N_{\text{max}} \right) \) (equation 3). Here, we will denote equation 3 as model III. Model III can describe sigmoidal curves with various curvatures during the deceleration phase compared to model II. With a larger \( m \), the curvature of the deceleration phase with model III becomes smaller.

**Numerical solution of the models.** Equation 3 was solved numerically with the four-order Runge-Kutta method using spreadsheet software (Microsoft Excel), similar to equations 1 and 2 (12-14). Parameter \( r \) was set to be a measured rate constant of growth during the exponential phase in an experimental growth curve (12-14). \( N_{\text{max}} \) and \( N_{\text{lag}} \) in the model, which are both the asymptotes, were estimated from experimental data (12-14). Parameters \( m \) and \( n \) were estimated by using the least-squares method that minimizes the means of the square error, MSE, between log-transformed cell concentrations predicted with the model and those observed at the observation points. Namely, parameter \( n \) was first estimated by using the experimental data from the initial time to that at the expo-

**RESULTS AND DISCUSSION**

**Growth at constant temperatures.** Growth curves of *E. coli* cells on a membrane surface placed on a nutrient agar plate were sigmoidal at various constant temperatures (10 to 34°C). One of the examples is shown in Fig. 1. The growth curves were successfully described with the new logistic model III. Model III could describe the curvature of the deceleration phase better than model II. That is, in Fig. 1, the difference between the measured and estimated cell numbers using model III with
a 13-h incubation (point P) was smaller (0.24 log unit) than that with model II (0.47 log unit). The value of MSE for cell numbers measured and estimated with model III during the growth period (0.021) was also smaller than that with model II (0.034). Similar results for both models were obtained at the other constant temperatures studied (data not shown). These results showed the superiority of model III to model II in the description of growth curves, and therefore model III was used thereafter in the present study.

Parameter values of model III for the growth curves are listed in Table 1. The values of MSE were very small. The values of \( m \) were almost constant at the temperatures studied with the average of 0.58, except for the value at 22°C. The values of \( n \) were also almost constant with the average of 3.0 without including the value at 10°C. The values of \( N_{\text{max}} \) for the curves were constant (~10.1 log units).

The Arrhenius analysis for \( r \) to the temperature was very linear with \( R^2 = 0.986 \) (Fig. 2). The linear regression line in the figure was expressed as follows: \( \ln r = -8.640 \times 1/T + 28.9 \) (equation 4). Here, \( \ln \) is the natural logarithm, and \( T \) is the temperature (K).

The temperature dependency of \( r \) was also analyzed with the square-root model, which is widely used (29). The relationship between \( r \) and \( T \) was expressed as \( \sqrt{r} = 0.0426 \times T - 0.0349 \). Here, the unit of \( T \) is in degrees Celsius (°C). The linearity was very good (\( R^2 = 0.977 \)) but slightly lower than that of the Arrhenius plot.

The duration of lag was longer at the lower temperature (Fig. 3). The relationship between lag and \( T \) (°C) could be experimentally expressed by using an exponential equation of \( \text{lag} = 15.800 \times T^{-2.59} \) with a high \( R^2 \) value of 0.980.

**Model comparison.** Model III was compared to the modified Gompertz and Baranyi models (4, 16), which have been well studied by many investigators, for the growth curves at the constant temperatures. All of the models described the growth curves well (Fig. 4). A curve predicted with one model crossed over the others several times during the growth period. More precisely, model III and the Gompertz model gave slightly better descriptions (less MSE) of the growth curves than the Baranyi model; the average MSE values between predicted and observed cell numbers at temperatures of 10 to 34°C were 0.030, 0.029, and 0.036 (log unit) for model III, the Gompertz model, and the Baranyi model, respectively. There were no significant differences in MSE between two of the three models at a confidence level of 0.95 by the \( t \) test.

$$\text{FIG. 2. Arrhenius plot for } r \text{ to the temperature. Circles are experimental. The straight line is the linear regression line.}$$

$$\text{FIG. 3. Temperature dependency of the duration of lag. Circles are experimental. The line is the regression line with an exponential function.}$$

$$\text{FIG. 4. Comparison of growth prediction by model III to those obtained by the Baranyi and Gompertz models. } E. \text{ coli growth curve at 26°C was analyzed with the models. Closed circles are experimental. Abbreviations: NLM, new logistic model III (solid line); Bar, Baranyi model (gray line); Gom, Gompertz model (dotted line). Bars show the standard deviations of the average viable cell counts.}$$
were more variable throughout growth (Fig. 4), similar to the growth curves in a liquid (12–14).

For \( r \) and \( \text{lag} \), which are the key parameters to characterize a growth curve, model III gave the best estimates among the models (Fig. 5). Model III gave the least \( \text{MSE} \) and high \( R^2 \) values of linearity for \( r \) and \( \text{lag} \) (Table 2). These results showed that model III was more suitable to describe the surface growth curves than the other models. The Gompertz model had a tendency to overestimate the rate constant (Fig. 5A), similar to growth curves in liquid (13, 26).

In the present study, we could not analyze the surface growth curves with the three-phase linear model developed by Buchanan et al. (9) because a curve-fitting program for the model, ABACUS, was unavailable to us. However, from the nature of the model where the rate constant of growth and the lag period are directly incorporated into the model, it could be expected that if our experimental data were analyzed with the model, the estimated values of \( r \) and \( \text{lag} \) would be equal to the measured ones in Fig. 5.

**Growth at varying temperatures.** With the parameter values of model III at the constant temperatures studied above, surface growths at varying temperatures were predicted with the model. For \( r \), the Arrhenius equation (i.e., equation 4) was used because of the better linearity than that of the square-root model as described above. For \( m, n \), and \( N_{\text{max}} \), the averages of the observed values that were described above were used, being 0.58, 3.0, and \( 10^{10.1} \), respectively. As shown in Fig. 6, the model successfully predicted growth with small values of \( \text{MSE} \) (Fig. 6A, 0.010, and Fig. 6B, 0.14 in log units). These results showed that the kinetics of bacterial surface growth was governed by temperature and that the model had the potential to predict the growth curves at various temperature profiles. There was a tendency that predictions at lower temperature ranges were slightly greater than actual measured cell counts (Fig. 6B), but the reason for this was not understood.

Predictions at various temperatures were also done with the square-root model for \( r \) in a preliminary study. As a result, predictions with the Arrhenius model were slightly better than those with the root square model (data not shown).

Predictions with the Baranyi model were studied at various temperatures in comparison with model III. Several investigators applied the Baranyi model to microbial growth at various temperatures (1, 6, 7, 20). The model was found to considerably overestimate bacterial growth in our study (Fig. 6A and B). This overestimation had already taken place during the first half of the observation period. Here, parameter \( \alpha_0 \) was experimentally estimated to be 0.0187. The overestimation with the Baranyi model was also observed by other investigators (1, 6, 7, 20). They also reported that growth curves described with the model strongly change with the value of \( \alpha_0 \) (1, 6, 7, 20). The same phenomena were observed in the present study. There were great differences in curve fittings with the model between constant and varying temperatures in our study. Baranyi and Roberts (4) reported that the value of the parameter is related to the physiological state of inoculated cells, but we cannot know in advance the optimal value of the parameter that gives the best prediction, especially at a varying temperature.

**Growth at various initial cell numbers and nutrient levels.** When *E. coli* cells grew on a surface at various initial cell numbers, the growth curves were also sigmoidal and well described with model III (Fig. 7). Regardless of the variety of the initial cell numbers, the rate constant of growth was constant, and the maximum cell population converged at a given value.

---

**TABLE 2. Comparison of models for parameters in Fig. 5**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Index</th>
<th>Value obtained with various models</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Model III</td>
</tr>
<tr>
<td>( r )</td>
<td>( \text{MSE} )</td>
<td>0.00090</td>
</tr>
<tr>
<td></td>
<td>( R^2 )</td>
<td>0.999</td>
</tr>
<tr>
<td>( \text{lag} )</td>
<td>( \text{MSE} )</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>( R^2 )</td>
<td>0.996</td>
</tr>
</tbody>
</table>

* The indices were calculated from the predicted and observed values of the parameters.

---

**FIG. 5.** Comparison of the rate constant (A) and the lag period (B) by model III with those by the Baranyi and Gompertz models. *E. coli* growth curves at 10 to 34°C were analyzed with the models. Symbols: ●, model III; ▲, Baranyi model; ■, Gompertz model. The straight line is the line of equivalence.

---

**FIG. 6.** Growth at varying temperatures. Predictions with the Arrhenius model (A) and the root square model (B) for the rate constant of growth. The observed values are shown by the symbols. /
This was also observed with *E. coli* growth in a liquid at the optimal and suboptimal temperatures (10, 13), but similar studies on surface growth have not been carried out to our knowledge. The values of *m* and *n* for the curves in Fig. 7 were also independent of the initial cell load (Table 3).

The nutrient level of a solid surface where microorganisms attach differs with place. Surface growth curves at various nutrient levels were then studied. The growth curves were sigmoidal and well described with model III (Fig. 8). Parameter values analyzed with the model are listed in Table 4. With the lower nutrient level agar plates, the rate of growth became slower and the value of *N* _max_ was lower. On the other hand, the duration of _lag_ was almost constant.

On agar plates without any (organic) nutrients added, bacterial cells did grow to a certain level (Fig. 8). This meant that the agar itself (Bacto agar) had a certain amount of nutrients to allow bacterial growth.

**Comparison with growth in a liquid.** The surrounding environment on a surface for microorganisms was considered to be very different from that in a liquid. Also, the freedom of microbial growth on the surface would be strictly limited compared to that in a liquid. Growth curves on a surface were thus compared to that in a liquid. Here, the experiments in a liquid (nutrient broth) were carried out by using methods we reported previously (13).

The growth curves on a surface at constant temperatures were very similar to those in a liquid. One example is shown in Fig. 9. In this figure, the values of *r* and _lag_ of the growth curve on the surface (2.05 1/h and 1.65 h) were very similar to those in the liquid (2.09 1/h and 1.24 h). The maximum cell numbers at the stationary phase were 10^10.2 (CFU per membrane) on the surface and 10^8.9 (CFU/ml) in the liquid in Fig. 9. The Arrhenius plot for *r* in the liquid at constant temperatures was also similar to that on the surface (data not shown). The only kinetic difference between them was the curvature in the deceleration phase of the growth curve, as observed in Fig. 1. In Fig. 9, *m* = 0.48 in model III for the surface, whereas *m* = 1 for the liquid (equal to model II).

It is interesting that while nutrients come to the bacterial cells from one vertical direction (for the agar plate) on a surface and from every direction in a liquid, the growth curve on the surface was almost the same as that in the liquid. Generally, the diffusion rate and the amount of nutrients are the limiting factors on microbial growth (9), as seen in Fig. 8. However, this might not be the case here, because the media for both growth analyses, which were broth and nutrient agar plate, contained a lot of nutrients for microbial growth.

**TABLE 3. Parameter values of model III for surface growth curves at various initial cell numbers in Fig. 7**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value obtained with an initial cell number (CFU)* of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^2</td>
</tr>
<tr>
<td><em>r</em> (1/h)</td>
<td>1.1</td>
</tr>
<tr>
<td><em>m</em></td>
<td>0.40</td>
</tr>
<tr>
<td><em>n</em></td>
<td>4.1</td>
</tr>
<tr>
<td><em>N</em> <em>max</em> (CFU)*</td>
<td>10^10.1</td>
</tr>
<tr>
<td>MSE (log unit)</td>
<td>0.020</td>
</tr>
</tbody>
</table>

*CFU per membrane.*
Comparison with increase in ATP. Growth in cell number on a surface was compared to the increase in the ATP content of cells grown under the same conditions at constant temperatures. The increases in the ATP contents of cells during the incubation showed sigmoidal curves, similar to the growth curves as shown in Fig. 1. One example is shown in Fig. 10. The ATP curves were also successfully described with model III (Fig. 10). The ATP curves were coincident with the growth curves at the temperatures studied; the values of $r$ and lag in the two curves in this figure were 0.70 1/h and 4.5 h for cell growth and 0.67 1/h and 4.7 h for the ATP increase, respectively. At some of the temperatures studied, the rate of ATP increase slightly preceded the cell growth; the ATP curve had a slightly shorter lag period. These results showed that the kinetics of the increase in the ATP content of cells was generally similar to that of the cell population.

These results led to the speculation that the ATP content of cells on a surface could be a measure of cell growth. The ATP measurement for microbial cells with the firefly luciferin-luciferase method is rapid and simple (17, 31). It takes less than 1 min to measure a sample with the ATP analyzer that we used. Thus, it can be a useful tool for the evaluation of microbial growth on a surface. The relationship between the cell number and ATP content for surface growth at various temperature patterns needs to be studied.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value obtained at nutrient level:</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$r$ (1/h)</td>
<td>1.7</td>
<td>1.5</td>
<td>1.5</td>
<td>0.98</td>
</tr>
<tr>
<td>lag (h)</td>
<td>3.0</td>
<td>2.9</td>
<td>3.0</td>
<td>3.4</td>
</tr>
<tr>
<td>$m$</td>
<td>0.45</td>
<td>0.49</td>
<td>0.44</td>
<td>0.55</td>
</tr>
<tr>
<td>$n$</td>
<td>2.5</td>
<td>3.0</td>
<td>2.9</td>
<td>3.8</td>
</tr>
<tr>
<td>$N_{max}$ (CFU)$^a$</td>
<td>$10^{10.1}$</td>
<td>$10^{9.65}$</td>
<td>$10^{9.9}$</td>
<td>$10^{9.25}$</td>
</tr>
<tr>
<td>MSE (log unit)</td>
<td>0.021</td>
<td>0.010</td>
<td>0.019</td>
<td>0.0047</td>
</tr>
</tbody>
</table>

$^a$ CFU per membrane.
The new logistic models II and III that we have proposed thus far are models extended from the original logistic model in structure. From our previous and present studies (12–15), we think that we can present a generic form of our models for the description of microbial growth curves. That would be model III. That is, when \( m = 1 \), it is equal to model II, as shown in Fig. 9. A sigmoidal curve described with model III changes with the values of \( m \) and \( n \). Namely, parameter \( m \) is related to the curvature during the deceleration period and \( n \) is related to \( lag \); with a smaller \( n \), the model describes a growth curve with a longer lag period, and vice versa (13, 14).

Our studies showed that regardless of the complexity of the microbial colony structure on a surface (11, 23), the growth curves inside the solid were well described with model III. We would like to further study microbial growth patterns inside a solid. We think that a systematic understanding is needed for microbial population dynamics in various fields where microorganisms grow.

REFERENCES

AUTHOR’S CORRECTION

Modeling Surface Growth of *Escherichia coli* on Agar Plates

Hiroshi Fujikawa and Satoshi Morozumi

*Tokyo Metropolitan Institute of Public Health, Department of Microbiology, 3-24-1, Hyakunin-cho, Shinjuku, Tokyo 169-0073, Japan*

Volume 71, no. 12, p. 7920–7926, 2005. Page 7924, Figure 6: There was an error in the calculation of the Baranyi model, resulting in an invalid Fig. 6. The corrected figure panels (below) show that the Baranyi model produced almost the same growth curves at the dynamic temperatures as the new logistic model.

![Figure A](image1.png)

![Figure B](image2.png)