

# Microscopic Appearance of Objects (Crown *E. coli*-glu-glu cells nema) from Agar Cultures of Antibiotic Crown *E. coli*-glu-glu Cells Stimulated with Glu-Glu

Shoshi Inooka\*

Japan Association of Science Specialists, Japan.

## \*Correspondence:

Shoshi Inooka, Japan Association of Science Specialists, Japan.

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## ABSTRACT

Synthetic DNA crown cells can be produced using sphingosine (Sph)-DNA-adenosine-monolaurin compounds and egg white. Previous experiments have demonstrated that both antibiotic compounds and antibiotic-producing cells could be produced using various combinations of DNA crown cells with partners such as microorganisms, other cells, extracts and organic compounds. However, whether these antibiotic-producing cells could be cultured has remained unclear. Previous research showed that both cell proliferation and various objects (hereafter referred to as Crown Asci-glu-glu cells nema) were observed in cultures of antibiotic-producing cells (Antibiotic crown Asci-glu-glu cells) produced using egg white powder enclosing DNA (Ascidian, Ascidian sea squirt) crown cells with Glu-Glu as a partner. The present study examined whether such cell proliferation and objects (Crown *E. coli*-glu-glu cells nema) were observed in cultures of egg white powder enclosing DNA (*E. coli*) crown cells with GluGlu and stimulated with GluGlu as a partner. The present findings showed that Crown *E. coli*-glu-glu cells nema were observed in these combinations.

## Keywords

DNA (*E. coli*) crown cells, Sphingosine-DNA, Antibiotic crown *E. coli*-glu-glu cells, Peptide (Glu-Glu), Crown cells nema.

## Introduction

Self-replicating artificial cells were first reported in 2012 [1] and the principal methods for preparing these artificial cells were reported in 2016 [2]. As the exterior of these cells consists of DNA, the cells were referred to as “DNA crown cells” in 2016 by the present author [3]. Synthetic DNA crown cells were produced using four common, commercially available compounds: sphingosine (Sph), DNA, adenosine, and monolaurin. These cells developed into fully self-replicating DNA crown cells when incubated in egg white.

Previous studies demonstrated that antibiotic and antibiotic-producing cells were produced by combining various DNA crown cells with different partners, such as microorganisms, other cells, extracts, and organic compounds [4-10]. However, the growth characteristics of these antibiotic-producing cells were unclear.

In previous reports, to clarify the characteristics of these cells, antibiotic-producing cells (antibiotic crown Asci glu-glu cells) were cultured on agar plates and L-glutamyl-L-glutamic acid (Glu-Glu) was added. As a result, cell proliferation and objects that varied in shape and size were observed, with the resulting cells referred to as “Crown Asci glu-glu cells nema”.

Crown cells nema can be multicellular or filamentous, and the objects that grow from antibiotic crown *E. coli*-glu-glu cells in response to the partner (Glu-Glu) were named “Crown *E. coli* glu-glu cells nema”. The present experiments therefore examined whether antibiotic-producing cells produced from DNA (*Escherichia coli*) crown cells formed Crown *E. coli* glu-glu cells nema with the addition of Glu-Glu.

## Materials and Methods

Antibiotic crown *E. coli*-glu-glu cells were prepared using the following three steps, as described previously [11,12]. The methods are provided here again for reference.

- Step 1 Preparation of DNA crown cells
- Step 2 Preparation of powder
- Step 3 Culture of powder

### Materials for these experiments

Sph (Tokyo Kasei, Tokyo, Japan), DNA (*E. coli*; Sigma-Aldrich, USA), adenosine (Wako, Tokyo, Japan), monolaurin (Tokyo Kasei), and adenosine-monolaurin (A-M) compound [12]. Monolaurin solutions were prepared to a final concentration of 0.1 M in distilled water. Agar plates were prepared using standard agar medium (SMA; AS ONE, Japan) and Glu-Glu (PEPTIDE; Institute of Peptides, Japan) was used as a partner and prepared to a concentration of 1.00 mg/mL.

### Step 1

#### Preparation of DNA (*E. coli*) crown cells [11,12]

Briefly, 180  $\mu\text{L}$  of Sph (10 mM) and 90  $\mu\text{L}$  of DNA (1.7  $\mu\text{g}/\mu\text{L}$ ) were combined, and the mixture was heated and cooled twice. A-M solution was then added and the mixture was incubated for 15 min at 37°C. Following the addition of monolaurin solution, the mixture was incubated for 5 min at 37°C to produce synthetic DNA crown cells.

These cells were then added to egg white and incubated for 7 days at 37°C. The egg white was recovered and used as a DNA (*E. coli*) crown cells.

### Step 2

#### Preparation of powder-enclosed DNA crown cells with Glu-Glu

- 1) First, 3 mL of Glu-Glu solution (300  $\mu\text{L}$  Glu-Glu in distilled water) was mixed with 3 mL of egg white;
- 2) The mixture was then incubated for 5 h at 37°C;
- 3) Approximately 20 mL of fresh egg white was then added to the mixture;
- 4) The mixture was then plated onto two Petri dishes and dried for 1–2 days at 37°C;
- 5) The dried material was then collected and ground to a powder using a mortar and pestle;

The powder, “Crown *E. coli* glu-glu-P”, was stored at room temperature and used as necessary (Figure 1).

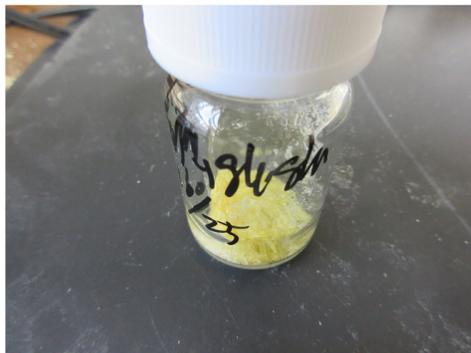


Figure 1: Powder used.

### Step 3

#### Cultivation of powder (preparation of antibiotic-producing cells)

Approximately 50 mg of powder (Crown *E. coli* glu-glu-P) was added to an agar plate and incubated for 2 days at 37°C. Approximately 1.5 mL of 0.1 M monolaurin solution was then poured onto each plate, which was then incubated for 2 days at 37°C. About 6.0 mL of distilled water was added to plates, dispersed on the plate surface. Objects on the plate were then recovered. Suspended objects were used as antibiotic-producing cells.

#### Cultures of antibiotic-producing cells

A total of 200  $\mu\text{L}$  of sample, which had been stored at 4°C for 3 days, was placed onto an agar plate and incubated for 1 day at 37°C.

#### Preparation of Crown *E. coli*-glu-glu cells nema

##### Experiment 1

After 7 days of culture of antibiotic-producing cells, approximately 1.5 mL of distilled water containing 100  $\mu\text{L}$  of Glu-Glu solution (1.0 mg/mL) was added to an agar plate and incubated for 5 h, 1 day, 2 days or 3 days at 37°C.

##### Experiment 2

After 3-day culture following addition of Glu-Glu in Experiment 1, objects that grew on the plate (Figure 17, within the frame) were collected, transplanted onto a new plate, and incubated for 1 day at 37°C. The objects that then grew on the plate were observed with the naked eye.

### Results

Figure 2 shows an agar plate at the beginning of culture using the powder (i.e., Crown *E. coli*-glu-glu-P). Powder particles of various sizes were observed throughout the Petri dish.

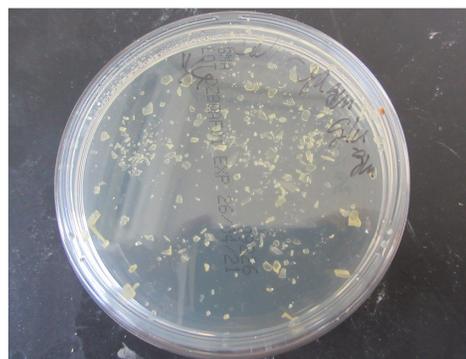


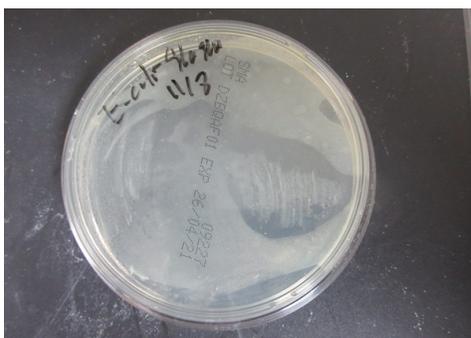
Figure 2: Agar plate at the beginning of culture using the powder (i.e., Crown *E. coli*-glu-glu-P). Powder particles of various sizes are observed throughout the Petri dish.

Figure 3 shows an agar plate at 2 days after monolaurin addition. Large, round, brown objects were observed on the plate. In addition, dot-like objects were also observed. Objects on the plate were collected and used as antibiotic-producing cells.



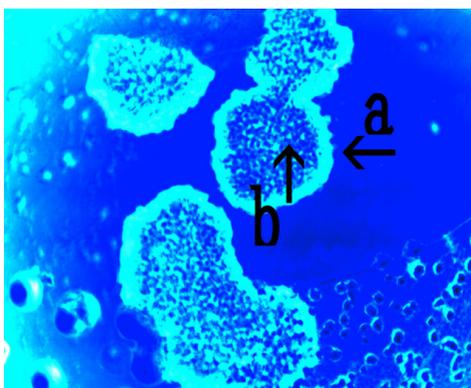
**Figure 3:** Agar plate 2 days after adding monolaurin. Large, round, brown objects are observed on the plate. In addition, dot-like objects are also observed. Objects on the plate were collected and used as antibiotic-producing cells.

Figure 4 shows an agar plate at 1 day of culture with the objects shown in Figure 3. Objects similar to microorganisms were observable with the naked eye across the entire plate.



**Figure 4:** Agar plate at 1 day of culture with the objects shown in Figure: 3. Objects similar to microorganisms are observable by the naked eye across the entire plate.

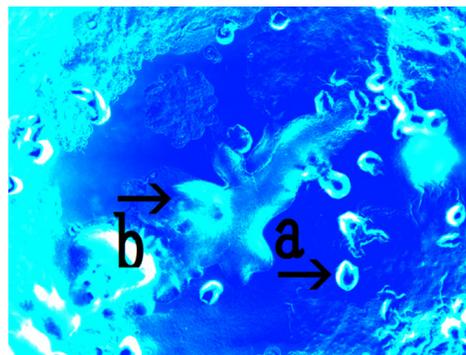
Figure 5 shows the microscopic appearance of objects (Example [Ex.]1) (before addition of GluGlu) grown for 1 day on the agar plate shown in Figure 4. Objects containing many small objects (Figure 5b) were observed (Figure 5a). Approximate size (Figure 5b) was 20 m.



**Figure 5:** Microscopic appearance of objects (Example [Ex.]1) (before addition of GluGlu) grown for 1 day on the agar plate as shown in Figure 4.

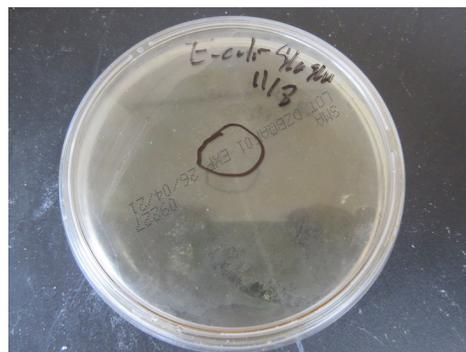
Objects containing many small objects (b) are observed (a). Approximate size (b), 20  $\mu$ m.

Figure 6 shows the microscopic appearance of objects (Ex.2) before addition of Glu-Glu to the agar plate shown in Figure 4. Several round objects were observed (Figure 6a), Cloud-like objects are also observed (Figure 6b). Approximate size (Figure 6a) was 100  $\mu$ m.



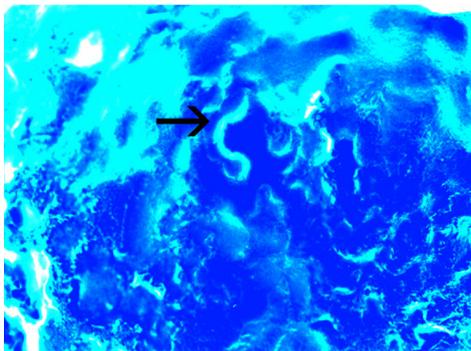
**Figure 6:** Microscopic appearance of objects (Ex.2) before addition of Glu-Glu to the agar plate shown in Figure 4. Several round objects are observed (a). Cloud-like objects are also observed (b). Approximate size (a), 100  $\mu$ m.

Figure 7 shows the microscopic appearance of objects (Ex.1) at 5 h after adding Glu-Glu to the plate shown in Figure 4. Broadly round like objects containing round objects were observed (Figure 7a). Also, an object that may have been constructed of several objects (Figure 7a) is observed (Figure 7b). Approximate size of the object shown in Figure 7a was 150  $\mu$ m.



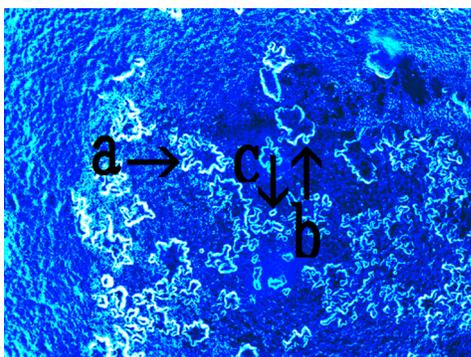
**Figure 7:** Microscopic appearance of objects (Ex.1) at 5 h after adding Glu-Glu to the plate shown in Figure 4. Round-like objects containing round objects are observed (a). Also, an object that may be constructed of several objects (a) is observed (b). Approximate size of objects (a): 150  $\mu$ m.

Figure 8 shows the microscopic appearance of objects (Ex.2) at 5 h after addition of Glu-Glu. Wiggly like objects ( $\rightarrow$ ) in various sizes are observed. The size of objects shown in Fig. 8 ( $\leftarrow$ ) is approximately 600  $\mu$ m in length.



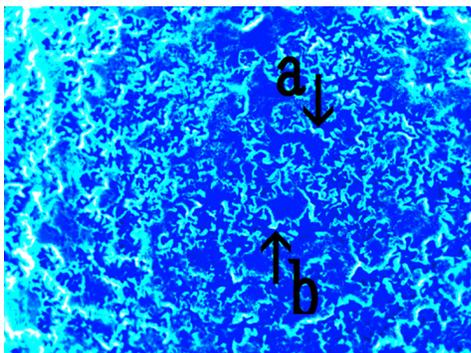
**Figure 8:** Microscopic appearance of objects (Ex.2) at 5 h after Glu-Glu addition. Wiggly objects (→) of various sizes are observed. The size of objects shown in Figure: 8 (←) is approximately 600  $\mu\text{m}$  in length.

Figure 9 shows the microscopic appearance of objects at 1 day after Glu-Glu addition. Most areas of membranes showed inclusion of jagged like objects (Figure 9a). Also, some parts of membranes also showed jagged like objects (Figure 9b). The size of the object (Figure 9c) is approximately 20  $\mu\text{m}$ .



**Figure 9:** Microscopic appearance of objects at 1 day after Glu-Glu addition. Jagged objects are seen in most of the membrane (a). Objects that are jagged in part of the membrane are also observed (b). The size of the object (c) is approximately 20  $\mu\text{m}$ .

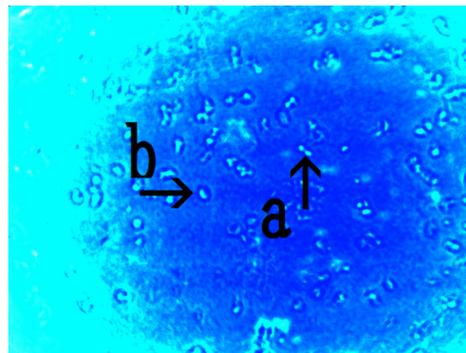
Figure 10 shows the microscopic appearance (Ex.1) of objects at 2 days after Glu-Glu addition. Many jagged-like objects are observed (Figure 10a). These objects may show some branching (Figure 10b). The approximate size of branch in Figure 10b is 30  $\mu\text{m}$ .



**Figure 10:** Microscopic appearance (Ex.1) of objects at 2 days after Glu-

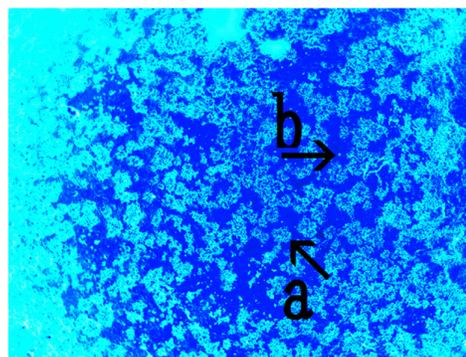
Glu addition. Many jagged-like objects are observed (a). These objects may show some branching (b). The approximate size of a branch in b is 30  $\mu\text{m}$ .

Figure 11 shows the microscopic appearance (Ex.2) at 2 days after Glu-Glu addition. Rod-shaped chains of objects are seen (Figure 11a), along with a single rod-shaped object (Figure 11b). The approximate width of the single object in Figure 11b is 40  $\mu\text{m}$ .



**Figure 11:** Microscopic appearance (Ex.2) at 2 days after Glu-Glu addition. Rod-shaped chains of objects are seen (a), along with a single rod-shaped object (b). The approximate width of the single object shown in b is 40  $\mu\text{m}$ .

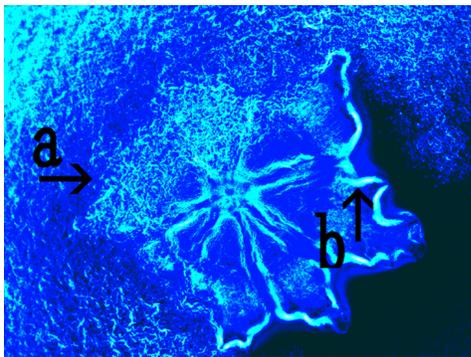
Figure 12 shows the microscopic appearance (Ex.3) of objects at 2 days after Glu-Glu addition. Chains of amorphous cloud-like objects (Figure 12a) are observed. The size of objects (Figure 12b) is approximately 5  $\mu\text{m}$ .



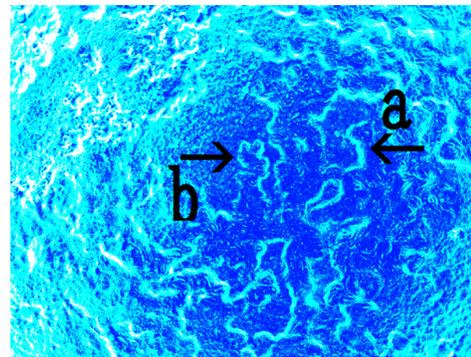
**Figure 12:** Microscopic appearance (Ex.3) of objects at 2 days after Glu-Glu addition. Chains of amorphous cloud-like objects (a) are observed. The size of object shown in b is approximately 5  $\mu\text{m}$ .

Figure 13 shows the microscopic appearance (Ex.4) of objects at 2 days after adding Glu-Glu. A large object comprising objects (Figure 13b) is observed (Figure 13a). Approximate size (Figure 13b) is 250  $\mu\text{m}$ .

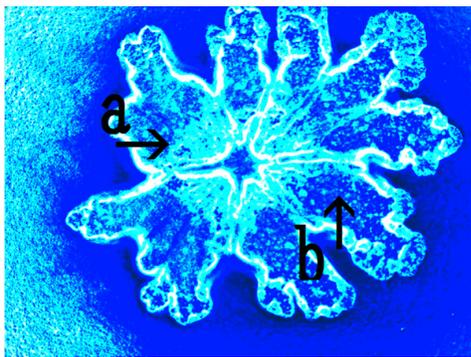
Figure 14 shows the microscopic appearance (Ex1) of objects at 3 days after Glu-Glu addition. Object like star anise are observed (Figure 14a), Approximate size is 10  $\mu\text{m}$  (Figure 14b).



**Figure 13:** Microscopic appearance (Ex.4) of objects at 2 days after Glu-Glu addition. A large object comprising objects (b) is observed (a). Approximate size (b), 250  $\mu\text{m}$ .

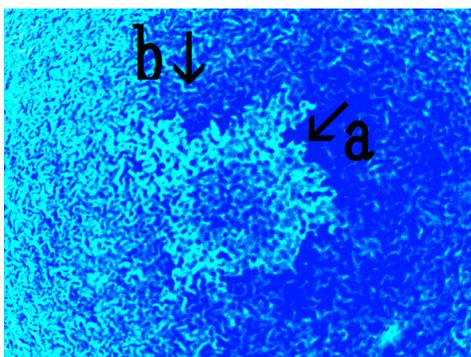


**Figure 16:** Macroscopic appearance of objects (Ex.3) at 3 days after Glu-Glu addition. Jagged-like objects are observed (a, b). Approximate length (b), 150  $\mu\text{m}$ .



**Figure 14:** Microscopic appearance (Ex.1) of objects at 3 days after Glu-Glu addition. Objects like star anise are observed (a). Approximate size, 10  $\mu\text{m}$  (b).

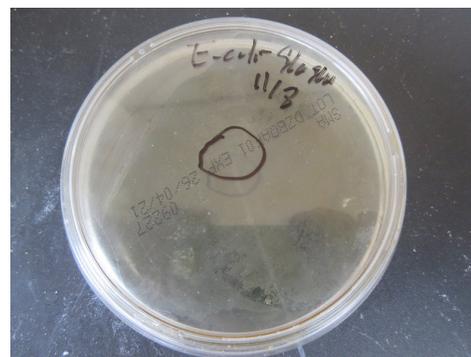
Figure 15 shows the microscopic appearance (Ex.2) of objects at 3 days after Glu-Glu addition. Objects like an assembly of rod-shaped objects (Figure 15b) are observed (Figure 15a), approximate length is 150  $\mu\text{m}$  (Figure 15b).



**Figure 15:** Microscopic appearance (Ex.2) of objects at 3 days after Glu-Glu addition. Objects like an assembly of rod-shaped objects (b) are observed (a). Approximate length, 150  $\mu\text{m}$  (b).

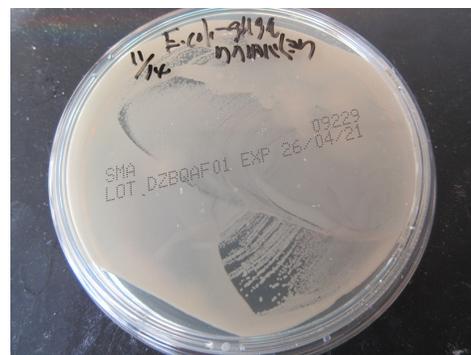
Figure 16 shows the macroscopic appearance of objects (Ex.3) at 3 days after Glu-Glu addition. Jagged-like objects are observed (Figure 16a, b), with an approximate size (Figure 16b) of 150  $\mu\text{m}$  in length.

Figure 17 shows an agar plate at 3 days after addition of Glu-Glu. Objects within the frame are collected and cultured.



**Figure 17:** Agar plate at 3 days after Glu-Glu addition. Objects within the frame are collected and cultured.

Figure 18 shows the culture of objects (i.e., within the frame in Figure 17) grown on an agar plate at 3 days after Glu-Glu addition. Objects with an appearance like microorganisms are observed throughout the whole plate.



**Figure 18:** Culture of objects contained within the frame in Figure 17. Microorganism-like objects are observed throughout the whole plate.

In previous studies, antibiotic-producing cells (antibiotic crown cells) were produced in combination with various DNA crown

cells and partners (e.g., microorganisms such as yeast, *Bacillus subtilis*, cells such as salmon roe, extracts such those as from bovine meat, and chemical compounds such as peptides [4-10]. Moreover, previous experiments [13] demonstrated that various unique objects appeared when antibiotic crown cells produced with Ascidian DNA and Glu-Glu as a partner were cultivated on agar plates and Glu-Glu was added to agar plates.

These objects were therefore named “Crown Asci glu-glu cells nema”, with “Crown Asci” referring to the Ascidian DNA used to prepare the DNA crown cells, “glu-glu” referring to the peptides added as a partner for the crown antibiotic cells; and “cells nema” as described in the Introduction. The purpose of the present study was to clarify whether antibiotic crown (*E. coli* glu-glu) cells would form crown cells nema with the addition of Glu-Glu.

Already, several unique objects were observed before the addition of Glu-Glu (Figures 5, 6). After the addition of Glu-Glu, objects that had not been present before the addition of Glu-Glu were observed. Most objects observed after the addition of Glu-Glu were jagged or wiggly in appearance (Figures 8-10, 16). Objects like star anise or like the assembly of star were also observed (Figure 13, 14).

These findings showed new objects born from the combination of antibiotic crown cells (*E. coli* glu-glu cells) with the addition of Glu-Glu. The presence of these objects led to the antibiotic crown *E. coli* glu-glu cells being termed “Crown *E. coli* glu-glu cells nema.” As described previously [13], crown cells nema consist of regenerated DNA crown cells.

Large objects were observed 3 days after the addition of Glu-Glu (Figure 14). Such objects were not observed before the addition of Glu-Glu and represented a kind of cells nema.

The present findings thus demonstrated that antibiotic crown cells form crown cells nema, and formed from DNA crown cells prepared with Sph, DNA (*E. coli*), adenosine, and monolaurin. These findings also demonstrated that antibiotic crown cells precede the formation of crown cells nema. Thus, the origin of crown cells nema is DNA crown cells and antibiotic crown cells have the ability to behave as proto-cells for crown cells in addition to showing anti-Bacillus characteristics.

In a previous report [13], most crown Asci glu-glu cells nema were described as forming a complex structure, so grouping of cells nema may be difficult based on size and shape alone.

On the other hand, in case of crown *E. coli* glu-glu cells nema, most of these may be formed with simple, round or rod shapes (Figures 7-11).

Although objects that may consist of complex structures were observed (Figures 13-15), those objects may comprise a line of assembly for simple objects (Figures 13b, 15b). On the other hand, such phenomena may be based on differences in the source

of DNA (derived biologically from single cells of *E. coli* or from multicellular Ascidians). Crown cells nema may consist of several crown cells and comprise a mass of crown cells that may form from regenerated DNA crown cells.

Using such materials (DNA from *E. coli* and Ascidians), crown cells nema may include components appearing round, rod-shaped or other complex shapes. Clarifying whether the source of the DNA used played a role in the formation of the objects was important. The present findings suggest that simple objects may form with the use of DNA from single-celled organisms (*E. coli*), while complex objects may form with the use of DNA from multicellular organisms (Ascidians). However, to clarify the significance of crown cells nema, further experiments on the formation of crown cells nema using various types of DNA crown cells are necessary.

The present findings on the proliferation of crown cells nema may have implications for the appearance of a single cell. Namely, as is well known, around  $415\sim 615 \times 10^{28}$  microorganisms exist in diverse and useful habitats all over the earth, of which over 99.9% remain unidentified. Because cells nema are living (Figures 17, 18), the existence of unidentified micro-organisms may be associated with the proliferation of crown cells nema. However, proving that such subjects (crown cells nema or crown cells) represent a kind of unidentified microorganism appears very difficult and it seems unlikely to search for the evidence from the natural environment. On the other hand, further study into the proliferation of crown cells nema may furnish researchers with useful hints toward resolving such issues.

The present study designated and identified antibiotic crown *E. coli* glu-glu cells. In addition, objects derived from antibiotic crown *E. coli* glu-glu cells after stimulation with a partner (Glu-Glu) were named Crown *E. coli* glu-glu cells nema. The objects consisting of crown cells nema are named crown cells.

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