

Full Length Research Paper

Effects of urea on length distribution and morphology of *Escherichia coli* and *Salmonella enterica* subsp. *enterica* cells

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Our previous studies showed that urea in acidic broth induced swarmer morphotypes in *Proteus mirabilis*, but the effects to other bacterial species remain unsolved. Here we report effects of urea on morphology and size distribution of urease-negative *Escherichia coli* and *Salmonella enterica* serovar Abony grown in urea-containing broth. Statistical analysis indicated lognormal distribution of the cellular lengths below a threshold level, suggesting that the growth process of bacterial cells obeys some random multiplicative process below a threshold value, despite any other factors affecting the process above the threshold value, to produce elongated cells. In urea broth, the distribution shifted to larger size and enormously elongated cells appeared. Morphological studies in urea broth revealed that filamentous cells of *E. coli* and *S. Abony*, accompanied with incomplete chromosomal segregation without forming hyperflagellates were induced at logarithmic growth phase, unlike swarmer cells in *P. mirabilis*. Since cell division protein FtsZ (filamenting temperature-sensitive mutant Z) assembled round the chromosome segregated point and cells were divided into short rods after cell counts had reached to a threshold level, urea in broth was responsible for delay of chromosomal segregation.

Key words: Chromosomal segregation, enterobacteria, filamentous cells, urea.

INTRODUCTION

Bacterial shapes differ among various genera, and it is a characteristic feature of some bacteria to grow and multiply in filaments, clusters or chains. All these morphological properties are of value in the identification and classification of bacterial strains, though a particular species may show variations of shape and size according to environments including nutrient conditions. For example, bacterial cells grown in rich nutrient medium are

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Abbreviations: BSA, Bovine serum albumin; DAPI, 4’,6-diamidino-2-phenylindole; EDTA, ethylenediaminetetraacetic acid; FtsZ, filamenting temperature-sensitive mutant Z; P2, testing broth containing 2 g/L peptone; P2U, testing broth containing 2 g/L peptone and 25 g/L urea; P50, testing broth containing 50 g/L peptone; P50U, testing broth containing 50 g/L peptone and 25 g/L urea; PBS, phosphate buffered saline.
significantly larger than in nutrient-poor medium (Schaechter et al., 1958). The small cell sizes due to starvation conditions increase resistance to acid, oxidative stress and proteases (Clements and Foster, 1999; Watson et al., 1998). Although, other environmental factors such as temperature (Wiebe et al., 1992) and osmotic shock (Baldwin et al., 1988) have been reported to affect the size of bacteria, cell size reduction appears to be induced predominantly by nutrient limitation. On the other hand, harmful environments such as low water activity (Mattick et al., 2000), refrigerated temperature (Mattick et al., 2003), host innate immunity (Justice et al., 2006), high hydrostatic pressure (Ishii et al., 2004) and presence of chelating agents (Fujihara et al., 2009) inhibit bacterial cell division resulting in the production of filamentous cells by unknown mechanisms. Similarly, several antibacterial reagents blocking FtsZ, the tubulin homologue that forms cytokinetic Z ring (Löwe and Amos, 1998) are also known to yield filamentous cells (Beuria et al., 2005; Jaiswal et al., 2007; Wang et al., 2003).

We have previously demonstrated that urea, a denaturation agent for protein structure, induced swarmer morphotypes of Proteus mirabilis strains in acidic broth (Fujihara et al., 2011). Although, biological functions of urea remain largely undocumented, urea is known to delay cell cycles and induce apoptosis in eukaryotic cells without inhibition of DNA synthesis (Michea et al., 2000). To assess the biological function of urea to enterobacteria, in the present study, we examined effects of urea to morphology and cell length distribution of urease-negative Escherichia coli and Salmonella enterica subsp. enterica serovar Abony.

MATERIALS AND METHODS

Bacterial strains and growth condition

Escherichia coli FDA strain (same progenitor of ATCC 25922) and Salmonella enterica subsp. enterica serovar Abony K103 strain (same progenitor of NCTC 6017) were grown in Luria broth (SIGMA-ALDRICH, Tokyo, Japan) for 18 h at 37°C. Testing broth consisting of 5 g sodium chloride, 5 g dipotassium hydrogen-phosphate, 0.651 g magnesium chloride hexahydrate and 2 or 50 g of Bacto-Peptone (Becton, Dickison and Company, Sparks, MD, U.S.A.) was supplemented with 25 g urea (P2U or P50U) or without urea (P2 or P50) in 1 L of distilled water. The amount of urea used in this study was based on the level of human urine (Griffith et al., 1976). The broth medium was adjusted to pH 7.1 and filter-sterilized.

Small amount of bacterial cells approximately 10^5 colony forming unit obtained from the Luria broth cultures were inoculated into the testing broth and incubated at 37°C. After 6, 8, 10, 12, 14 h incubation, living cells were counted by plating on MacConkey agar plates (NIHON Pharmaceutical, Tokyo, Japan).

Measuring of bacterial cell length

Bacterial cells were harvested after 8, 10 and 12 h incubation, since countable cells were not apparent until 6 h incubation. They were collected by centrifugation at 1,000 g for 1 and suspended in broth to adjust to an appropriate numbers of bacterial cells for observation using an Eclipse TE2000-U fluorescence microscope (Nikon, Tokyo, Japan) equipped with a VB-7010 digital camera (KEYENCE, Osaka, Japan). After the microscopic images recorded as color scale data were converted into binary data, the area of each bacterial cell was measured by numerical analysis software, MATLAB (MathWorks, Natick, MA, U.S.A.). Then, we calculated the length and the width of more than 1,000 bacterial cells from an area as described previously (Wakita et al., 2010).

Immunofluorescence microscopy

Chromosome and FtsZ ring organization were observed as described by Den Blaauwen et al. (1999) with a modification. Cells grown for 8 h were fixed in 2.8% neutral-buffered formalin and 0.04% glutaraldehyde for 15 min at ambient temperature. Cells were washed twice in phosphate buffered saline (PBS), pH 7.2, and subsequently suspended in 0.1% Triton X-100 in PBS for 45 min at ambient temperature. The cells were washed three times in PBS and resuspended in PBS containing 100 μg of lysozyme per ml and 5 mM ethylenediaminetetraacetic acid (EDTA) for 45 min at ambient temperature and then the cells were washed three times in PBS. FtsZ was stained with a polyclonal anti-FtsZ rabbit antibody (1:200; Agrisera, Vännas, Sweden) diluted in PBS containing 1% bovine serum albumin (BSA) for 2 h at 37°C. The cells were washed three times with PBS containing 0.05% (v/v) polyoxyethylene sorbitan monolaurate. The cells were then treated with secondary antibodies, Cy3-conjugated goat anti-rabbit antibody (1:1000; Rockland Immunochemicals, Inc., Gilbertsville, PA, U.S.A.) diluted in PBS containing 1% BSA for 2 h at 37°C. The cells were further washed three times in PBS–0.05% polyoxyethylene sorbitan monolaurate.

In contrast, nucleoids were stained using 2.5 μg/ml of 4',6-diamidino-2-phenylindole (DAPI) (Dojindo, Kumamoto, Japan) and visualized with a fluorescence microscope Eclipse TE2000-U. Images were captured and submersed by using a VB-7010 digital camera.

RESULTS

Growth curve

Growth curve of both bacterial strains were shown in Figure 1. Although, no marked difference in growth speed was evident depending on the concentration of peptone, viable bacterial counts in the stationary phase increased under the conditions of high level of peptone. On the other hand, addition of urea delayed the proliferation. Higher cell counts were measured in P2 and P50 as compared to P2U and P50U after 8 h incubation, though after 12 h incubation higher counts were obtained in broth of higher concentration of peptone irrespective of addition of urea.

Statistical analysis of bacterial cell length distribution

Cell length distributions after 8, 10 and 12 h were shown in Table 1. Average cell length was longer in the logarithmic growth phase in all the broth media. In
Figure 1. Growth curves of *E. coli* and *S. Abony* in broth. About 100 bacterial cells were inoculated in each broth and after 6, 8, 10, 12, 14 h incubation at 37°C; living bacterial cells were counted by plating on MacConkey agar plates.

In addition, cells increased length in P50 rather than in P2. In urea-containing broth, the highest counts of cells over 5 m long were seen in the logarithmic phase and cells had divided into shorter rods along with growth. The microscopic observation revealed that elongated cells divided irregularly into short and long cells. Similarly with average of cell length, P50U produced highest counts of *E. coli* cells longer than 10 m, whereas P2U did in *S. Abony*. The probability densities of cell length about *E. coli* and *S. Abony* after 8 h incubation are shown in Figure 2a and b, respectively. It is noticed that the distributions with urea shift to longer direction of cell length from that without urea. Furthermore, all the distributions in Figure 2a and b have thick tails. It just reminds us of lognormal distribution.

Figure 2c and d show that the cumulative length distributions are well fitted except the tail part by the solid curves which are lognormal distributions in cumulative form given by:

\[
N(x) = \frac{1}{2} \left[ 1 - \text{erf} \left( \frac{\ln(x/L)}{\sqrt{2}\sigma} \right) \right]
\]

Where \( x \), \( \sigma \) and \( L \) are the length, the standard deviation, and the median, respectively. \( \text{erf}(z) \) is the error function defined by

\[
\text{erf}(z) = \left( \frac{2}{\sqrt{\pi}} \right) \int_{0}^{z} \exp(-y^2)dy
\]

After 10 and 12 h incubation, cell length distribution had shifted to shorter direction (data not shown).

Effects of urea on the chromosome and cytokinetic Z ring organization

We examined the effects of urea on Z ring assembly and karyomitosis by fluorescence microscopy (Figure 3). While the chromosome of short rods grown in urea-negative broth replicated by binary fission and Z rings located at the middle of cells, the multiple chromosomes distributed haphazardly in elongated cells grown in broth containing urea. The lateral length of nucleoid in elongated cells was not equal and chromosomal segregation was not seen in the middle of cells. FtsZ assembled round the point of karyomitosis as well as cells grown in broth without urea. No bacterial cells were stained in negative controls without anti-FtsZ antibody.

DISCUSSION

In the present study, broth medium containing urea induced elongated cells during logarithmic phase of growth, though optimum peptone concentration was different between *E. coli* and *S. Abony*. Most elongated cells had divided into short rods after stationary phase. In general, cell length and DNA content start to decrease transition point between the logarithmic phase and the stationary phase (Akerlund et al., 1995) and cells grown in nutrient-rich medium exhibit longer cell length than in nutrient-poor medium (Schachter et al., 1958). Our findings in broth medium without urea confirmed this, but P2U shifted to longer length in large majority of *S. Abony* cells than P50U. It remained unclear why optimal peptone concentration to produce elongated cells was different between *E. coli* and *S. Abony*. Polymerization of FtsZ, one of the central cell division proteins (Madabhushi and Marians, 2009) depends on multiple factors including pH, concentrations of magnesium, potassium, calcium, competing nucleotide triphosphates, macromolecular crowding and the presence of FtsZ interacting proteins (González et al., 2003; Mukherjee and Lutkenhaus, 1998, 1999; Mukherjee et al., 1998). Inhibitors such as antibiotics against Fts proteins were known to produce filamentous cells, though they did not affect karyomitosis (Beuria et al., 2005; Jaiswal et al., 2007). Additionally, induction of *sulA* (*sfiA*), one of the SOS genes, also mediates inhibition of FtsZ assembly without influence on karyomitosis in *E. coli* (Bernhardt...
Table 1. Cell length distributions after 8, 10, 12 h incubation. More than 1,000 cell lengths were measured and the abundance ratio of shorter than 5, 5 to 10, 10 to 20, 20 to 30, 30 to 40, 40 to 50 μm and longer than 50 μm was shown.

<table>
<thead>
<tr>
<th>Broth</th>
<th>Incubation period (h)</th>
<th>Measured cell number</th>
<th>Average±S.D. (μm)</th>
<th>Cell length (μm) distribution (%)</th>
<th>Max (μm)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&gt;5</td>
<td>5-10</td>
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<td>P2</td>
<td>8</td>
<td>1999</td>
<td>3.211±0.011</td>
<td>93.7</td>
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</tr>
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<td></td>
<td>10</td>
<td>1379</td>
<td>2.912±0.010</td>
<td>97.6</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1189</td>
<td>2.467±0.009</td>
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</tr>
<tr>
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<td>40.2</td>
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</tr>
<tr>
<td></td>
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<td>1383</td>
<td>4.049±0.014</td>
<td>74.5</td>
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<tr>
<td></td>
<td>12</td>
<td>1302</td>
<td>2.967±0.012</td>
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<td>6.1</td>
</tr>
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<td>E. coli</td>
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<td>4.131±0.010</td>
<td>73.6</td>
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<tr>
<td></td>
<td>10</td>
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<td>S. Abony</td>
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<td>70.9</td>
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<td>1168</td>
<td>3.015±0.010</td>
<td>95.7</td>
<td>4.2</td>
</tr>
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</table>

and de Boer, 2005; Mukherjee et al., 1998). Urea in broth did not inhibit assembly of FtsZ despite random segregation of chromosomes, though formation of matured Z ring was not explored in the present study. Therefore, it is most likely that appearance of filamentous cells was due to delay
of chromosomal segregation. Since normal segregation of chromosomes was evident in swarmer cells in \textit{P. mirabilis} (Fujihara et al., 2011), appearance of filamentous cells in urea-containing broth may be accounted by another mechanism distinct from differentiation of swarmer cells. In addition, statistic distribution of bacterial cell length showed a lognormal distribution under growing process that was consistent with those commonly observed in each body growth according to a random multiplicative stochastic process (Walita et al., 2010). Thus, our data demonstrated also quantity of the temporal cell length and the effects of urea on bacterial cell division, while to identify the statistical characteristics of bacterial growth process.

In conclusion, we demonstrated urea-containing broth medium induced elongated cells in logarithmic phase of growth of enterobacteria, though exact mechanism remained unsolved. FtsZ localized at the chromosome-segregated point, despite yielding short and long cells at random from the elongated cells. We indicated cumulative distribution of bacterial lengths for \textit{E. coli} in P50 and P50U, and for \textit{S. Abony} in P2 and P2U were lognormal after 8 h incubation except for the tail part of the distributions along with an evidence for a threshold value of cell lengths. This implies that the growth process of bacterial cells obeys some random multiplicative process below a threshold value, despite any other factors affecting the process above the threshold value, to produce elongated cells. In urea-containing broth, the distribution shifted to larger size and enormously elongated cells appeared.

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Figure 3. Fluorescence micrograph of *E. coli* grown in P50/P50U and *S. Abony* in P2/P2U grown for 8 h. Short cells in broth without urea are shown in upper left windows and these windows are same magnification as larger windows showing elongated cells in urea broth. Cells were immunostained with polyclonal anti-FtsZ rabbit antibody followed by Cy3-conjugated goat anti-rabbit secondary antibody. Nucleoids were visualized by treating the cells with DAPI. FtsZ is shown in red, and the DAPI-stained nucleoids are shown in blue. Bar = 10 μm.

REFERENCES


