Growth of *Enterobacter amnigenus* and *Escherichia coli* spheroplasts in marine broth containing penicillin

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Spheroplasts of the intestinal bacteria *Enterobacter amnigenus* and *Escherichia coli* were generated and cultivated in marine broth. Their spheroplasts enlarged and formed giant cells in marine broth containing 300μg/mL penicillin, whereas they elongated and formed filamentous cells in marine broth containing 3μg/mL penicillin. During growth, larger spheroplasts were more frequently observed at 24°C than at 30°C and 37°C, indicating that the optimal culture temperature for the enlargement of spheroplasts differs from that required for cell division. In marine broth containing 30μg/mL penicillin, both enlarged and elongated cells were observed. Most of the filamentous cells were observed after appearance of the giant spheroplasts. This morphological change may be due to penicillin inefficiency.

Keywords: bacterial spheroplast, enlarged cell, filamentous cell, marine broth, penicillin concentration

1. Introduction

There are two methods for generating giant spheroplasts of bacterial cells. One method is by spheroplast cultivation in broth containing an inhibitor of peptidoglycan synthesis, such as penicillin, after lysozyme treatment of bacterial cells [1, 2]. Another method is by lysozyme treatment after elongation of bacterial cells using cephalexin [3]. Our method using marine broth is simpler than these two methods [4].

Incubation of spheroplasts formed by lysozymes with penicillin enlarges spheroplasts of the Gram-negative bacterium *Escherichia coli* [2] and the Gram-positive bacterium *Bacillus subtilis* [5]. Although DNA replication is generally related to cell division, spheroplasts replicate their DNA without undergoing cell division under incubation conditions [4]. Furthermore, spheroplasts of *Escherichia coli* can recover to their native cellular morphology via cell wall resynthesis [6].

Although *Escherichia coli* spheroplasts grew and enlarged in marine broth containing 600μg/mL penicillin, they elongated in the absence of penicillin [4], suggesting that penicillin concentration in marine broth influences cell morphology during the growth of bacterial spheroplasts.

In this study, we evaluated the cell morphology of spheroplasts of the intestinal bacteria *Enterobacter amnigenus* and *Escherichia coli* during growth in marine broth containing 3, 30, and 300μg/mL penicillin at 24°C, 30°C, and 37°C. These two species belong to the family Enterobacteriaceae of Gram-negative bacteria [7].

2. Materials and Methods

Giant spheroplasts were prepared using a modified version of the method previously described by Kusaka [1] and the spheroplast incubation method reported by Kuroda et al. [2]. *Enterobacter amnigenus* NBRC 105700 and *Escherichia coli* SCS1 with pHRP311 [4] were grown on marine broth agar (Difco). The harvested cells (0.003 g) were suspended in a buffer (1 mL) consisting of 0.1 M Tris-HCl (pH 7.6) and 0.3 M sucrose. Lysozyme (200μg/mL, Wako) was added to the cell suspension, and the suspension was incubated at 37°C for 15 min. After the suspension was divided into 3 aliquots of 330μL each, the cells were harvested by centrifugation for 5 min at 3000 rpm and resuspended in 330μL marine broth containing 3, 30, or 300μg/mL penicillin G (Serva). This suspension (2μL) was diluted in 1 mL marine broth containing 3, 30, or 300μg/mL penicillin G. The suspension was incubated at 24°C, 30°C, and 37°C. We used spheroplasts at various time points of growth: 3, 9, 24, 54, and 99 h. Phase contrast microscopy images were obtained using an Olympus CKX41.

3. Results

Spheroplasts of *Enterobacter amnigenus* and *Escherichia coli* enlarged in marine broth containing 300μg/mL penicillin (Figs. 1 and 2). The optimal temperature for the enlargement was 24°C in both *Enterobacter amnigenus* and *Escherichia coli* (Figs. 1 and 2). Most of the enlarged spheroplasts of *Enterobacter amnigenus* maintained inner and outer membranes, with the outer membrane more enlarged than the inner membrane (Fig. 3). The *Enterobacter amnigenus* spheroplasts were up to >30μm in diameter (Fig. 3).

In contrast, the spheroplasts elongated in marine broth containing 3μg/mL penicillin at 24°C, 30°C, and 37°C (Figs. 1 and 2). These filamentous cells of *Escherichia coli* were also observed in the absence of penicillin [4].

In marine broth containing 30μg/mL penicillin, both enlarged and elongated cells were observed (Figs. 1 and 2). Most of the filamentous cells were observed after the appearance of the giant spheroplasts (Figs. 1 and 2).

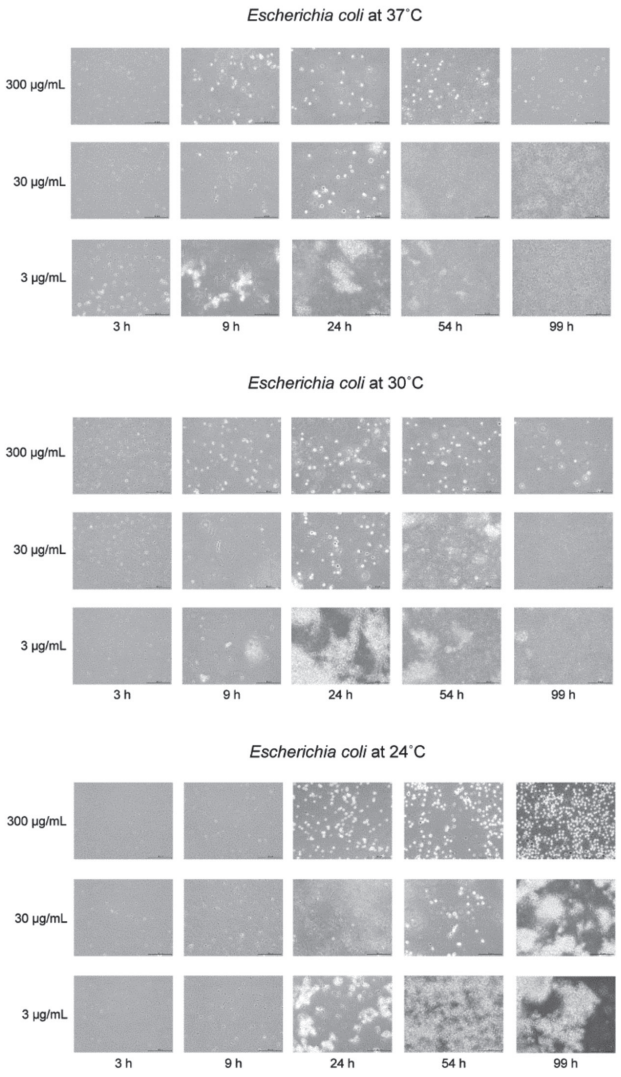

Figure 1. Phase contrast micrographs of *Escherichia coli* cells in marine broth containing 3, 30, and 300μg/mL penicillin.

Phase contrast microscopy images were obtained using an Olympus CKX41; bar = 100μm. The cells were incubated at 24°C, 30°C, and 37°C. We used cells at various time points of growth: 3, 9, 24, 54, and 99 h.

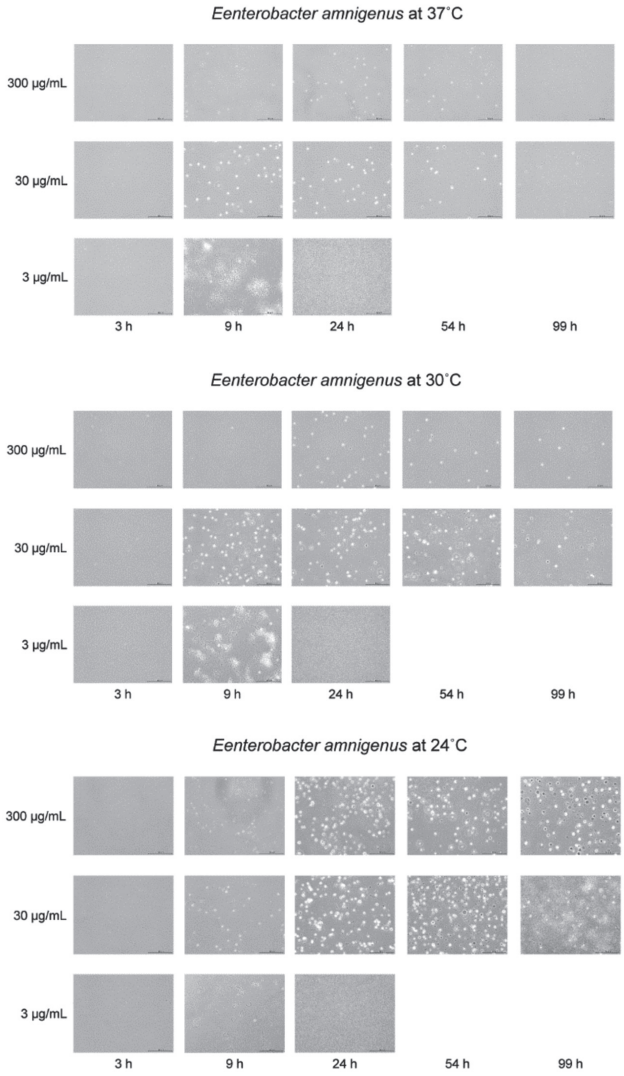

Figure 2. Phase contrast micrographs of *Enterobacter amnigenus* cells in marine broth containing 3, 30, and 300μg/mL penicillin.

Phase contrast microscopy images were obtained using an Olympus CKX41; bar = 100μm. The cells were incubated at 24°C, 30°C, and 37°C. We used cells at various time points of growth: 3, 9, 24, 54, and 99 h.

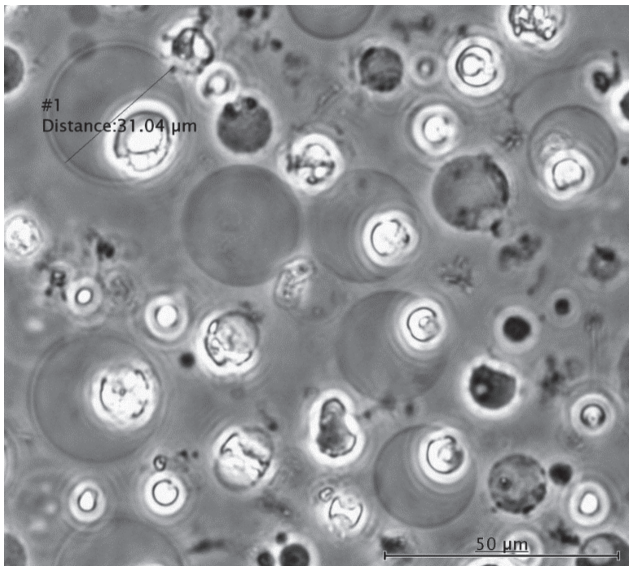

Figure 3. Giant spheroplasts of *Enterobacter amnigenus* in marine broth containing 300µg/mL penicillin.
Phase contrast microscopy images were obtained using an Olympus CKX41; bar = 50µm. The cells were incubated at 24°C. We used cells at 99 h of growth.

4. Discussion

Not only marine bacterial spheroplasts but also those of intestinal bacteria enlarged in marine broth containing penicillin [4, 8]. It suggests that high salt concentration in the broth may be suitable for enlargement of bacterial spheroplasts. The optimal temperature for the cell division and growth of *Escherichia coli* is 37°C, whereas the optimal temperature for the enlargement of the spheroplasts is 24°C (Figs. 1 and 2). In addition, the rate of DNA replication during the enlargement of *Escherichia coli* spheroplasts is slower in the presence of penicillin than in the absence of penicillin [4]. Thus, the activity of cells may have a lower level of growth during spheroplast enlargement compared with that of cell division.

Although the spheroplasts of *Enterobacter amnigenus* and *Escherichia coli* enlarged and formed giant spheroplasts in marine broth containing 300µg/mL penicillin, they elongated and formed filamentous cells in marine broth containing 3µg/mL penicillin (Figs. 1 and 2). This elongation of *Escherichia coli* was also observed in the absence of penicillin [4], suggesting that the penicillin concentration of 3µg/mL is ineffective. Thus, the intestinal bacterial spheroplasts elongated and formed filamentous cells under ineffective penicillin.

In marine broth containing 30µg/mL penicillin, the elongated (filamentous) cells were observed after the appearance of the enlarged cells giant spheroplasts (Figs. 1 and 2), suggesting that this morphological change may be due to the inefficiency of penicillin. In marine broth containing 30µg/mL penicillin, giant spheroplasts were observed more frequently in *Enterobacter amnigenus* than in *Escherichia coli* (Figs. 1 and 2), suggesting that *Enterobacter amnigenus* spheroplasts may have a higher sensitivity to penicillin than *Escherichia coli* spheroplasts.

The giant spheroplasts generated by the spheroplast cultivation method have been reported to have a single membrane, i.e., the inner membrane [2, 5]. However, most of the giant spheroplasts of *Enterobacter amnigenus* have both inner and outer membranes (Fig. 3), with the outer membrane being larger than the inner membrane (Fig. 3). The outer membrane had a maximum size of >30µm in diameter (Fig. 3). The rupture of the outer membrane was sometimes observed under the microscopy. Such a large outer membrane was not observed in the *Escherichia coli* giant spheroplasts under the microscopy. It is uncertain whether the outer membrane is completely separated from the inner membrane in the *Enterobacter amnigenus* spheroplasts.

An outer membrane which is larger than an inner membrane is observed in normal cells of other bacteria, for example, *Dictyoglomus thermophilum* [9] and *Thermotoga maritima* [10]. These thermophilic bacteria are evolutionarily closely related [11] and the earliest diverging bacteria from a common ancestor of organisms [12]. The bacteria belonging to Aquificae, Dictyoglomi, and Thermotogae are Gram-negative, which have inner and outer membranes. According to Errington's hypothesis [13], a common ancestor of organisms had a single membrane. If so, when and how the earliest diverging bacteria had two membranes? A common ancestor might have two (or more) membranes. During the evolution, cell wall might have been synthesized between the membranes.

5. Conclusion

Our findings demonstrate that the enlargement of bacterial spheroplasts can occur in marine broth containing penicillin for intestinal bacteria, and penicillin concentration in the broth plays an important role in the enlargement. At present, the giant spheroplasts are useful for cell membrane analysis using patch clamp technique.

In the future, they will be useful for cell engineering analysis using micromanipulation technique.

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References

[1] Kusaka I (1967) Growth and division of protoplasts of *Bacillus megaterium* and inhibition of division by penicillin. J Bacteriol, **94,** 884-888.

[2] Kuroda T, Okuda N, Saitoh N, Hiyama T, Terasaki Y, Anazawa H, Hirata A, Mogi T, Kusaka I, Tsuchiya T, Yabe I (1998) Patch clamp studies on ion pumps of the cytoplasmic membrane of *Escherichia coli*. J Biol Chem, **273,** 16897-16904.

[3] Martinac B, Rohde PR, Cranfield CG, Nomura T (2013) Patch clamp electrophysiology for the study of bacterial ion channels in giant spheroplasts of *E. coli*. Methods Mol Biol, **966,** 367-380.

[4] Takahashi S, Nishida H (2015) Quantitative analysis of chromosomal and plasmid DNA during the growth of spheroplasts of *Escherichia coli*. J Gen Appl Microbiol, **61,** 262-265.

[5] Nakamura K, Ikeda S, Matsuo T, Hirata A, Takehara M, Hiyama T, Kawamura F, Kusaka I, Tsuchiya T, Kuroda T, Yabe I (2011) Patch clamp analysis of the respiratory chain in *Bacillus subtilis*. Biochim Biophys Acta, **1808,** 1103-1107.

[6] Ranjit DK, Young KD (2013) The Rcs stress response and accessory envelope proteins are required for *de novo* generation of cell shape in *Escherichia coli*. J Bacteriol, **195,** 2452-2462.

[7] Paradis S, Boissinot M, Paquette N, Bélanger SD, Marte EA, Boudreau DK, Picard FJ, Ouellette M, Roy PH, Bergeron MG (2005) Phylogeny of the Enterobacteriaceae based on genes encoding elongation factor Tu and F-ATPase β-subunit. Int J Syst Evol Microbiol, **55,** 2013-2025.

[8] Takayanagi A, Takahashi S, Nishida H (2016) Requirement of dark culture condition for enlargement of spheroplasts of the aerobic anoxygenic photosynthetic marine bacterium *Erythrobacter litoralis*. J Gen Appl Microbiol, **62,** 14-17.

[9] Saiki T, Kobayashi Y, Kawagoe K, Beppu T (1985) *Dictyoglomus thermophilum* gen. nov., sp. nov., a chemoorganotrophic, anaerobic, thermophilic bacterium. Int J Syst Bacteriol, **35,** 253-259.

[10] Huber R, Langworthy TA, König H, Thomm M, Woese CR, Sleytr UB, Stetter KO (1986) *Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C. Arch Microbiol, **144,** 324-333.

[11] Nishida H, Beppu T, Ueda K (2011) Whole-genome comparison clarifies close phylogenetic relationships between the phyla Dictyoglomi and Thermotogae. Genomics, **98,** 370-375.

[12] Pace NR (1997) A molecular view of microbial diversity and the biosphere. Science, **276,** 734-740.

[13] Errington J (2013) L-form bacteria, cell walls and the origins of life. Open Biol, **3,** 120143.

ペニシリン含有マリン培地におけるエンテロバクターと大腸菌の培養

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腸内細菌であるエンテロバクターおよび大腸菌のスフェロプラストを作成し，マリン培地において培養した。300μg/mLのペニシリン含有マリン培地においてスフェロプラストは巨大化したのに対して，3μg/mLの濃度の場合には，細胞伸長し，繊維化細胞となった。培養において，30℃および37℃よりも24℃の場合に，より大きなスフェロプラストが観察できた。これは通常の２分裂と細胞巨大化における適温と異なっている。30μg/mLの濃度の場合には，巨大化細胞および伸長細胞の双方が観察された。多くの繊維化細胞は巨大化細胞が現れたのちに観察できた。この細胞形態の違いはペニシリン濃度の違いによって生じる。

キーワード：細菌スフェロプラスト, 巨大化細胞, 繊維化細胞, マリン培地, ペニシリン濃度