ABSTRACT

Antibiotic-tolerant persister cells of *Pseudomonas aeruginosa* PAO1 is successfully detected in the water-in-oil (WO) microchamber array. The frequency of appearance of the persister cells enclosed in the WO chamber after treatment by carbenicillin was much higher than that by the conventional agar plate culture, suggesting that the quorum-sensing signal transduced in the chamber increases the frequency of persister appearance. Furthermore, persister cells could be easily recovered from WO chamber since each chamber was directly accessible from outside.

KEYWORDS: Microchamber, Persister Bacteria, Single Cell Measurement, Quorum-Sensing

INTRODUCTION

Opportunistic infection by bacteria resistant to multiple antibiotics is a serious problem in hospital. Antibiotic-resistance of bacteria is generally classified into the two categories, acquired resistance and natural resistance (tolerance) (Figure 1). The acquired resistance is caused by the change in the genotype of the cell. In contrast, very small fraction of bacteria shows the natural resistance without the genotypic change, and these bacteria are called as the “persister”. However, the nature of the persister is not known well because the systematic study of them is not easy due to the very low frequency of appearance in the population (less than 1%).

Here we developed a microdevice for the study of the persister bacteria. We aimed to efficiently detect the persister of *Pseudomonas aeruginosa* PAO1 and to monitor its properties for long periods under an optical microscope. To achieve this, we improved and applied the water-in-oil (WO), directly accessible microchamber array we previously developed (Figure 2) [1].

EXPERIMENTAL

Preparation of hydrophilic-in-hydrophobic micropatterned surface - A hydrophobic polymer of carbon-fluorine (CYTOP, Asahi-glass) was spin-coated on a cleaned coverglass (30 mm in diameter, Matsunami) at 2000 rpm for 30 s and then baked for 1 h at 180°C. Photolithography was carried out with a high-viscosity photoresist (AZP4903, AZ Electronic Materials) because the CYTOP-coated surface had very low friction and could not be fully covered with a low-viscosity photoresist. The resist-patterned substrate surface was dry-etched with O₂ plasma by a reactive ion etching system (RIE-10NR, Samco) to expose the hydrophilic SiO₂ glass surface. The thickness of the CYTOP layer was ~1 μm as measured by a laser microscope (VF-7500, KEYENCE).

Formation of WO microchamber array - The hydrophilic-in-hydrophobic micropatterned coverglass was attached to the bottom of a perforated Petri dish (35 mm in diameter) and covered with aqueous solution (bacterial suspension). Oil (Fluorinert FC40, Sigma), which has a higher density than water, was then flowed into the aqueous solution near the substrate surface. The hydrophilic SiO₂ glass surface retained the aqueous solution, while the hydrophobic surface was covered with oil. As a result, many WO chambers were formed simultaneously.

Figure 1: Acquired and natural resistances of bacteria to antibiotics.

Figure 2: Directly accessible WO chamber array.
**Antibiotic treatment and culture of bacteria in WO microchamber array** – An antibiotic, carbenicillin (final 5 mg/ml) was added to suspension of *Pseudomonas aeruginosa* PAO1 precultured to the stationary phase (O.D._(600nm)~1.0) in trypti-case soy broth, and further cultured for 3 hr at 37°C. Then the cells were collected, washed, resuspended into fresh medium (O.D._(600nm)~0.2), and enclosed in WO chamber as described above. After enclosure, whole device was put in the incubator at 37°C and cultured overnight. The fraction of the cell showing multiple divisions were counted and the frequency of persister appearance was calculated. For the conventional persister assay using agar plate, series of dilution of cell suspension were prepared after carbenicillin treatment and inoculated on agar plates, and number of colony was compared with that of control experiment without carbenicillin treatment.

**RESULTS AND DISCUSSION**

Our new device could prepare more than three hundred thousand WO chambers in one chip simultaneously just by exchanging cell culture medium on the hydrophilic-in-hydrophobic micropatterned surface with oil (Figure 2). In newly designed chamber array, each chamber could be distinguished by the numbering on the surface (Figure 3). Bacterial cells were enclosed during the chamber formation process. Enclosure of the cell was stochastic and many chambers were remained empty, but roughly 20-30% of chambers contained single cells (Figure 4, left).

In the control experiment without antibiotic treatment, most cells showed multiple cell divisions after overnight culture (Figure 4, right). The divided cells showed active flagellar motions, indicating the high metabolic activities. Next, the cells were pretreated with carbenicillin at 5 mg/ml (roughly 100-times higher than the minimal inhibitory concentration [MIC]) for 3 hr before the enclosure in the chamber. The persister could be easily found under an optical microscope after the overnight culture (Figure 5, right, indicated by a solid circle).

Frequency of persister appearance estimated by the culture in the WO chamber (1.5±0.72%, n=4) was unexpectedly much higher than that estimated by the conventional agar plate culture (0.1±0.03%, n=4) (Figure 6 and 7, Table 1). It has been reported very recently that the autoinducing “quorum-sensing” signal among PAO1 cells increased the frequency of persister appearance [2], and the quorum-sensing signal was transduced even in isolated single cells when PAO1 was enclosed in the small volume at picoliter level [3]. So, enclosure in the WO chamber would be an efficient way to detect and investigate the persister bacteria. Effect of quorum-sensing signal on the frequency of persister appearance in WO chamber can be confirmed more clearly by antibiotic treatment of the cells after enclosure in the chamber.

**Table 1. Comparison of frequencies of persister appearance in Pseudomonas aeruginosa PAO1 between two methods**

<table>
<thead>
<tr>
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<th>Frequency of persister appearance (%)</th>
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<tbody>
<tr>
<td>Agar plate</td>
<td>0.10 ± 0.03 (n = 4)</td>
</tr>
<tr>
<td>WO chamber</td>
<td>1.5 ± 0.72 (n = 4)</td>
</tr>
</tbody>
</table>

**Figure 4: Number of cells in WO chamber with different sizes just after enclosure (left) and number of cells after 24hr culture (right).**

**Figure 5: Detection of the persister cells of Pseudomonas aeruginosa PAO1 (indicated by solid circle) in WO chamber array.**

**Figure 6: Increase in the cell numbers in each WO chamber after overnight culture. (Left) Control. (Right) Carbenicillin treated.**

**Figure 7: Detection of persister of PAO1 by conventional agar plate.**

**Figure 8. Recovery of persister from WO chamber with micropipette.**
CONCLUSION

We have succeeded in detection of the persister of Pseudomonas aeruginosa PAO1 in theWO chamber. The mechanism of persister appearance and reversible switching dynamics between persister and sensitive cells will be revealed if cells are treated with antibiotics in the chamber and continuously monitored under an optical microscope. Furthermore, because in our device each chamber was isolated by oil and accessible from outside, we could recover the persister cells easily using micropipette (Figure 8). This result indicates the further application of WO chamber array as a container of the cell culture. The WO chamber array can be used for the screening of the bacteria that is resistant to the specific antibiotics and subsequent gene identification responsible for acquired resistance.

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REFERENCES


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