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Membrane filter (pore size, 0.22–0.45 μm; thickness, 150 μm) passing-through activity of *Pseudomonas aeruginosa* and other bacterial species with indigenous infiltration ability

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Abstract

Bacteria growing on MF-Millipore filters (thickness, 150 μm) passed through the underlying membrane by their infiltration activity. *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Escherichia coli* passed through a 0.45-μm pore size filter within 48–96 h. *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Listeria monocytogenes* passed through a 0.3-μm pore size filter. *P. aeruginosa* passed through a 0.22-μm pore size filter. The membranes which allowed passing-through of bacteria showed normal bubble point values in the integrity test. Studies with isogenic *S. marcescens* mutants indicated that flagellum-dependent motility or surface-active exolipid were important in the passing-through. *P. aeruginosa* PAO1 C strain defective in twitching motility was unable to pass through the 0.22-μm filter. Scanning electron microscopy showed bacteria passing-through the 0.22-μm filter. Millipore membrane filters having well-defined reticulate structures will be useful in the study of infiltration activity of microbes.

Keywords: Millipore membrane filter; Reticulate structure; Infiltration activity; *Pseudomonas aeruginosa*; Bacterial motility

1. Introduction

During a study searching for signal molecules released from biofilms formed on one side of a membrane filter (Kurabo Intercell, pore size, 0.45 μm) that was used to separate two chambers, we found bacteria in the chamber fluid which should have been sterile because of the intervening filter. So, *Pseudomonas aeruginosa* ATCC 27853 used in the above experiment was suspended in saline and examined for filterability through a membrane filter (pore size, 0.45 μm) under conventional pressure filtration. The obtained filtrate was sterile, and all viable cells in the suspension were trapped by the 0.45-μm pore size membrane filter.

The above contradictory finding seemed to be a reflection of active and passive bacterial behaviors under different conditions. Bacterial cells passively streaming in a forced liquid flow will be trapped completely by the membrane filter with a pore size smaller than the bacterial size. However, given enough time to grow on the filter surface, bacteria will gradually infiltrate the reticulate structure of the filter (thickness, 150 μm) and pass through to the other side of the filter. Since reticulate structures are ubiquitous in the micro-scale world in nature, it seemed natural that bacteria have such an ability. In clinical practice, long-term usage of an in-line filter unit has been suspected as a possible source of catheter-related infection. *Escherichia coli* and *P. aeruginosa* were shown to be able to pass through 0.45-μm pore size disposable filter unit during 24-h flow of Ringer's solution in experiments simulating intravenous fluid administration [1].

For a direct examination of the filter infiltration activity of bacteria, cell suspensions of several species of bacteria were spotted on the central area of a Millipore membrane filter placed on an agar medium. After designated incubation times, the filter was removed and the agar plate was further incubated to examine the presence of passed-

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through bacteria at the small localized agar surface corresponding to the spotted location.

2. Materials and methods

2.1. Bacterial strain

The bacterial strains, their sources and relevant characteristics are described in Table 1. Most bacterial strains were grown in LB broth or on an LB agar (1.5%) medium [2]. *Listeria monocytogenes* was grown on blood agar medium.

2.2. Examination of membrane passing-through activity

An autoclaved MF-Millipore™ membrane filter (Millipore; diameter, 25 mm; pore size, 0.22, 0.3, or 0.45 μm) was placed and dried for 45 min on an LB agar or a blood agar plate. 10 μl of bacterial suspension (approximately 2×10^8 CFU ml^{-1} of saline) was spotted at the center of the filter on the agar medium without direct contact of a pipette tip. Following absorbance of the formed droplet (horizontal diameter, <4 mm) into the filter after about a 15-min incubation, the plate was inverted and incubated at 30°C. At 24-h time intervals (except the first 18-h incubation), the membrane filters were removed, the remaining agar plate was incubated for 24 h, and examined for the presence of colonies. When bacterial growth was positive at the agar surface corresponding strictly to the spotted locus of the filter, the bacterium was considered to be active in passing-through the filter within the designated time of the filter presence on the agar surface.

2.3. Examination of membrane integrity

The membrane filter fixed in a Swinnex filter holder (Millipore) was examined for bubble points using Integrity Tester (Millipore) as described by the manufacturer. The membrane filter through which bacteria passed was washed gently to remove bacterial mass on the filter surface, then examined for its bubble point. A conventional pressure filtration test with the used membrane filter (bacteria on the surface were washed out) fixed in a filter holder was carried out using 2 ml of suspension of *Serratia marcescens* pigmented strain 274. The whole volume of the filtrate was incubated for examination of the presence of viable bacteria.

2.4. Twitching motility

Twitching motility of *P. aeruginosa* strains was examined by observing the spreading halo between the Petri dish plastic surface and the LB agar (1%) medium which was stab-inoculated with bacteria to the bottom. For clear visualization of the formed bacterial halo, the agar me-

Table 1
Bacterial strains and relevant characteristics

Strain	Relevant characteristics	Source/reference
Gram-negative species		
P. aeruginosa		
ATCC 27853	wild-type, twitching motility-positive	Difco
GIFU 274	wild-type, twitching motility-positive	E. Yabuuchi
PAO1 T	wild-type, twitching motility-positive	J. Kato
PAO1 C	twitching motility-negative	J. Kato
S. marcescens		
274	wild-type	[2]
274 Smu-1a	serrawettin W1-negative mutant	[2]
274-AB1	Fla^- mutant	[2]
NS 38	wild-type	[8]
NS 38-09	serrawettin W1-negative mutant	[4]
NS 38-11	Fla^- mutant	[4]
NS 45	wild-type, non-pigmented	[4]
NS 45-11	Fla^- mutant	[4]
K. pneumoniae		
Fu1	wild-type	[9]
Fu1-m21	capsule-less mutant	[9]
E. coli		
ATCC 25922	wild-type	Difco
Gram-positive species		
Bacillus subtilis		
ATCC 21331	wild-type	ATCC
Listeria monocytogenes		
EGD	wild-type	[7]
Staphylococcus aureus		
ATCC 25923	wild-type	Difco

dium was removed en bloc and the remaining Petri dish was stained with 1% crystal violet solution [3].

2.5. Scanning electron microscopy

Bacterial mass growing on the inoculated membrane surface was scraped off using a hard paper edge. The membrane was fixed as described previously [4], and vacuum-dried. Ethanol and isoamyl acetate, which dissolve texture polymers of the membrane filter, were not used. The membrane filter was mechanically torn to see its inside and coated by an ion sputter (E-1030, Hitachi). Observation was made with an S-5000N microscope (Hitachi).

3. Results and discussion

3.1. Validation of the method disclosing bacterial passing-through activity

Without using complicated devices, it was easy to control experimental conditions and obtain distinctive results regarding time lapse-dependent filter passing-through activity of bacteria. As seen in Fig. 1, the difference between passing-through activity of an *S. marcescens* parent strain and a mutant is evident after 24 h. Since location of colony growth on the agar plate was restricted to the inoculated area on the membrane, and properties (e.g. production of characteristic pigments) of bacteria growing on the agar medium were identical to the inoculated bacteria, passing of the bacteria through the membrane was evident.

We examined the intactness of the filter, after observing bacterial colonies on the agar surface under the membrane filters. The bubble point values of the membrane filters which permitted passing-through of bacteria were the same or slightly higher than that of unused control Millipore membrane filters (2110, 2460, and 3520 hPa for 0.45-, 0.3-, and 0.22-μm pore size filter, respectively). In addition, the used membrane filters (pore size greater than or equal to 0.22 μm) which permitted *P. aeruginosa* passing-through showed complete intactness in producing an *S. marcescens*-free filtrate when a suspension of the bacteria was filtered by the conventional pressure filtration method (experiments were done in triplicate, data not shown). Thus, growing bacteria on the membrane filter did not spoil the trapping function of the Millipore filter against the passively streaming bacterial cells in a short time period (less than 1 min).

3.2. Bacterial passing-through of membrane filters differing in pore sizes

Four representative Gram-negative and three Gram-positive species passed through Millipore filter with pore size 0.45 μm, although they differed in time required (Table 2). When pore size was 0.3 μm or smaller, many species were unable to pass through the membrane within 96 h. In contrast, *P. aeruginosa*, *S. marcescens*, and *L. monocytogenes* were able to pass through these filters. It was noteworthy that times needed for passing-through

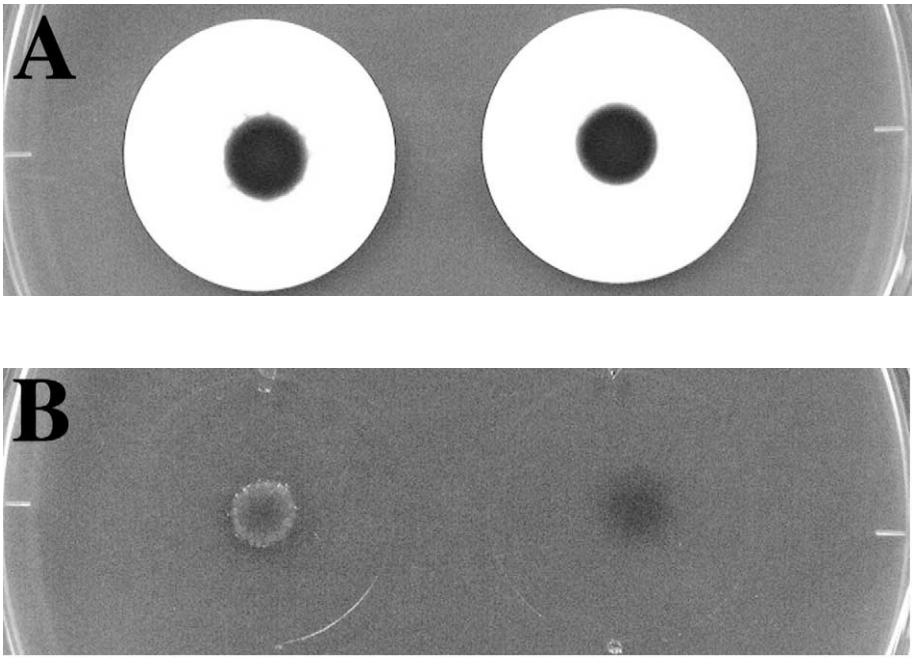

Fig. 1. Membrane filter passing-through activity of *S. marcescens*. A: Millipore membrane filters (diameter, 25 mm; pore size, 0.45 μm) point-inoculated with bacteria (ca. 2×10^6 CFU) at the center of the filters placed on an LB agar plate. The plate was incubated at 30°C for 24 h. B: Above filters carrying growing bacteria were removed aseptically and the plate was further incubated for 24 h. Left, wild-type NS 38; bacterial growth is evident on the agar surface. Right, flagellum-less mutant NS 38-11, bacterial growth was not observed on the agar surface.

Table 2
Membrane filter passing-through activity of bacteria

Strain	Time (h)[a] for passing-through filter with pore size (μm)		
	0.45	0.3	0.22
P. aeruginosa			
ATCC 27853	10	24	72
GIFU 274	10	24	96
PAO1 T	10	24	72
PAO1 C	12	48	>96
S. marcescens			
274	13	72	>96
274 SMu-1a	24	96	ND[b]
274-AB1	>96[c]	>96	ND
NS 38	14	96	>96
NS 38-09	24	96	ND
NS 38-11	72	>96	ND
NS 45	24	96	ND
NS 45-11	96	>96	ND
K. pneumoniae			
Fu1	>96	>96	ND
Fu1-m21	96	>96	ND
E. coli			
ATCC 25922	48	>96	ND
B. subtilis			
ATCC 21331	96	>96	ND
L. monocytogenes			
EGD	12	24	>96
S. aureus			
ATCC 25923	96	>96	ND

[a]Earliest designated time of passing-through, confirmed in triplicate.
[b]ND, not determined.
[c]>96 means absence of passing-through after 96 h.

increased when pore size became smaller, presumably reflecting the bacterial response to pore size smaller than ordinary bacterial size and the long distance in the Millipore membrane filter (100 times or more longer than ordinary bacterial length). A membrane filter from another manufacturer (Advantec, Tokyo; pore size, 0.30 μm; thickness, 140 μm) also permitted passing-through of *P. aeruginosa* (details will be published elsewhere).

Among wild-type strains, *Klebsiella pneumoniae* Fu1 (mucoid colony former) did not pass through the 0.45-μm pore size filter within 96 h, whereas its isogenic capsule-less mutant was able to pass through the same membrane filter. Encapsulation of a bacterial cell body seemed to affect the filter passing-through activity of this bacterium.

Some special bacterial abilities (e.g. indigenous translocating ability) seemed to contribute to this filter passing-through activity. Isogenic *S. marcescens* mutants defective in flagellum-dependent motility [2,5] or production of serrawettin W1 (surface-active exolipid) [2,5] demonstrated slower passing-through activity in comparison with the respective parent strains 274, NS 38, and NS 45 (Table 2). *L. monocytogenes* is a small Gram-positive rod with flagellum-dependent motility. The remarkable tissue invasiveness [6] and dendritic spreading behavior in solid agar substrate [7] of *L. monocytogenes* are consistent with the filter infiltration ability exhibited herein as prompt passing-through activity of the bacteria (Table 2).

Although it was very slow, flagellum-less species such as *S. aureus* and *K. pneumoniae* (capsule-less mutant) demonstrated passing-through activity. Bacterial mass expansion itself by cell multiplication seemed to contribute to pass-

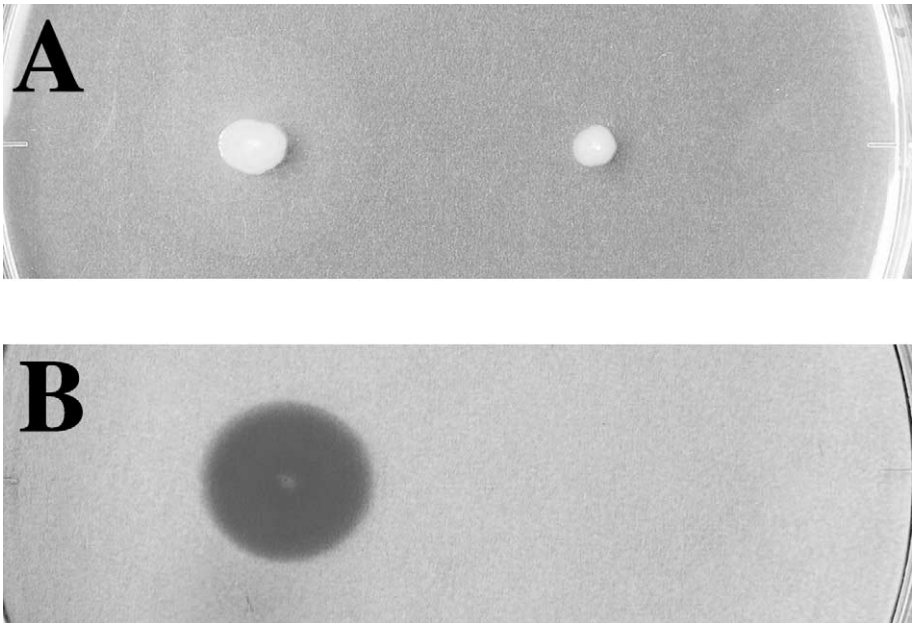

Fig. 2. Twitching motility visualized by crystal violet staining. A: *P. aeruginosa* growing on an LB agar (1%) plate after stab inoculations and 24-h cultivation. B: The stained plastic dish after removal of agar medium. Bacteria which spread by twitching motility are visible on the left side (PAO1 T strain). Such stained bacteria are absent on the right side (PAO1 C strain), indicating negative twitching activity.

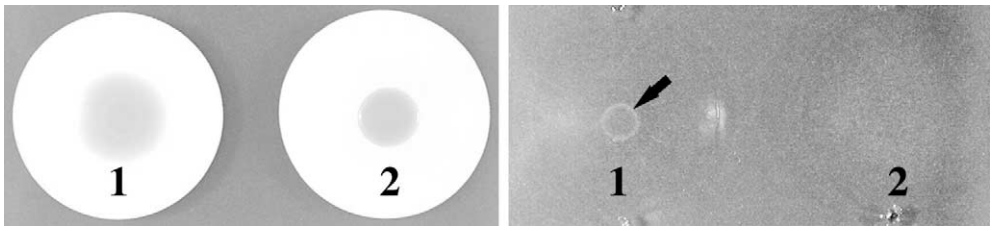

Fig. 3. Passing-through activity of 0.22-μm pore size filter by *P. aeruginosa*. Left panel: Millipore membrane filters (pore size, 0.22 μm) point-inoculated with bacteria (ca. 2×10^6 CFU) at the center of the filters placed on an LB agar plate. The plate was incubated at 30°C for 96 h. Right panel: Above filters carrying growing bacteria were removed aseptically and the plate was further incubated for 9 h. Strain PAO1 T (1) demonstrated passing-through activity and formed O-ring bacterial growth pattern (indicated by an arrow). Growth of strain PAO1 C (2) on the agar surface was not observed.

ing-through of the filter of 0.45 μm pore size. This result is similar to the 'grow-through phenomenon' reported with 0.45-μm pore size filters previously [1]. Filament-forming microbes such as filamentous fungi are known to invade animal and plant tissues by expansive extension of the filament tip. Local pressure generated by a multiplying bacterial population may have some role in infiltration to the reticulate tissues.

3.3. *Passing-through of 0.22-μm pore size filter by* P. aeruginosa

As shown in Table 2, *P. aeruginosa* strains demonstrated remarkable passing-through activity to Millipore membrane filters. *P. aeruginosa* strains used herein were positive in flagellum-dependent swimming motility, and type IV pilus-dependent twitching motility except the PAO1 C strain which is a spontaneous twitching motility mutant as shown in Fig. 2. *P. aeruginosa* strains, except the PAO1 C strain, demonstrated passing-through activity with the 0.22-μm pore size membrane filter (Table 2, and Fig. 3). Mutational studies on the role of twitching motility in passing-through of the membrane are now in progress. It was noteworthy that the growing bacteria on the agar surface formed an O-ring pattern (Fig. 3) suggesting favorable spatio-temporal conditions for the passing-through. Other bacterial species also showed this tendency of O-ring formation by depending on the time of filter removal (data not shown).

3.4. *Scanning electron microscopic examination*

Electron microscopic examination visualized passing-through bacteria at the exit side of the 0.22-μm pore size MF membrane filter inoculated with *P. aeruginosa* strain PAO1 T, and not with strain PAO1 C (Fig. 4). In contrast to the inoculated membrane surface, bacteria were recognized sparsely and singly, consistent with the O-ring forming sparse distribution of micro-colonies on the agar surface (data not shown). As seen in Fig. 4, passing-through

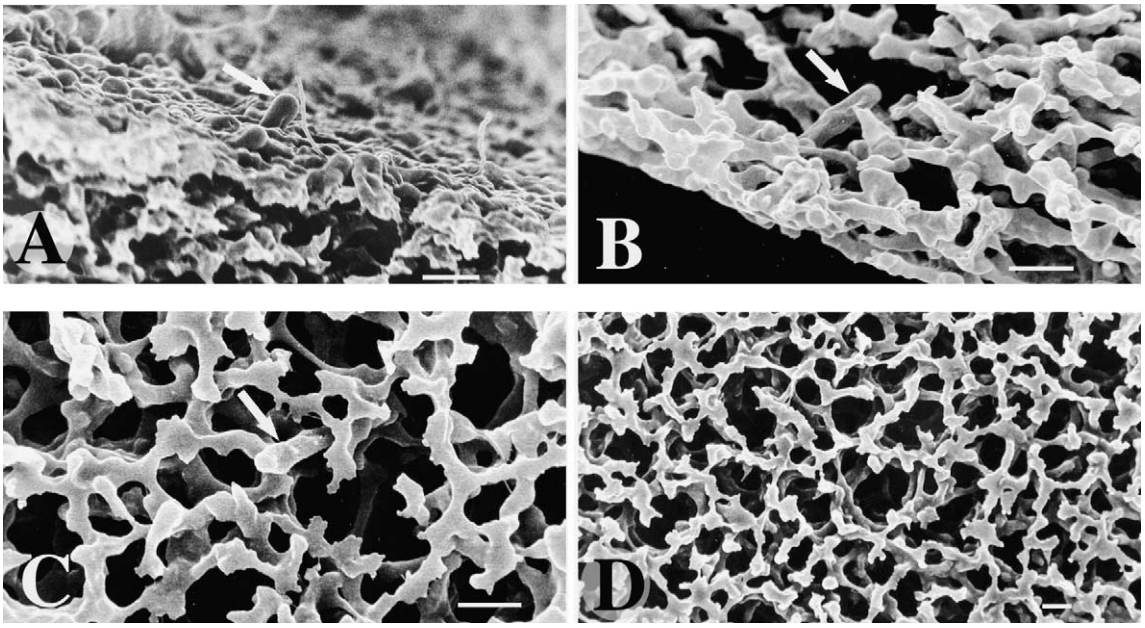

Fig. 4. Scanning electron micrographs of Millipore membrane filter (pore size, 0.22 μm) 120 h after inoculation with *P. aeruginosa* strain PAO1 T (A–C) and strain PAO1 C (D). A: Side view of the inoculated membrane surface. B: Side view of the membrane facing an agar medium. C,D: Surfaces of the membranes facing an agar medium. On the exit side of the membrane inoculated with strain PAO1 C, no bacteria were observed (D). Arrows indicate bacteria infiltrating the membrane. Each bar = 1 μm.

bacteria seemed to be not so small (although slender), and distances between polymer net fibers constructing the membrane are mostly longer than 0.22 μm. Examinations of un-inoculated MF membrane filters with and without fixation also showed wide distances between polymer net fibers (data not shown). Three-dimensional effects of the thick (150 μm) polymer net seemed to provide non-filterable conditions for bacteria in conservative pressure filtration.

3.5. Prospects

P. aeruginosa and *S. marcescens* are known for the production of various exo-enzymes such as proteinase. Destruction of the reticulate structures composed of biopolymers by these enzymes may open the way for bacterial infiltration in the body. However, complete lysis of tissues may not be necessary, the bacteria have an indigenous ability to squeeze through partially destroyed reticulate tissues. Membrane filters that are defined strictly in their chemical composition and graded filtration ability by manufacturers may be useful experimental substrates for the examination of the indigenous infiltration activity of tissue-intruding or reticulate-structure-inhabiting bacteria.

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