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An *in vitro* model for a packed bacterial mass formation with special attention to less interstitial spaces

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細菌間間隙の少ない，細菌密度の高い細胞集団の作成モデル

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Abstract：

Objective: This study aimed to establish a new model for bacterial mass formation with special attention to less interstitial spaces, in other words, a model for a well-packed bacterial mass.

Materials and methods: *Enterococcus faecalis* or *Propionibacterium acnes* microorganisms were centrifuged onto a cover glass, and allowed to stand for 60 min under 95% humidity. Then, the bacteria were cultured under restricted supply of media (25 μl at 60 min intervals) for 72 h for *E. faecalis* aerobically and for 5 d for *P. acnes* anaerobically. Excess media were immediately removed using sterilized absorbing papers, so the media stayed in the layer where bacteria were growing.

Results: After the 60 min-standing, the centrifuged bacteria were firmly adhered on the glass surface, whereas those without 60 min-standing were washed away. The bacterial density in the cultured bacterial mass was higher than in those formed by centrifugation or filtration, meaning that bacteria might multiply to throng and fill up the inter-bacterial spaces to make a packed monolayer, then, piled up to make the second layer, and continued one after another. When excess culture media were supplied, bacteria formed a less packed bacterial mass.

Conclusion: The well-packed bacterial mass seemed to be quite equivalent to the so called "biofilm" in regard to insufficient infiltration of molecules into the bacterial mass, although the constituting bacteria do not produce extracellular polymers.

抄録：

本研究は細菌間の間隙の少なさに注目した細菌塊形成の新しいモデル，言い換えれば，細菌同士が密着した細菌塊の形成モデル，を確立することを目的としている。*Enterococcus faecalis* あるいは *Propionibacterium acnes* の生菌をカバーグラス上に遠心によって付着させ，湿気を保って 60 分放置後，*E. faecalis* の場合 72 時間にわたって好気的に，*P. acnes* の場合 5 日間嫌気的に，1 時間毎に 25 μl の培地を供給した。余分な培地は直ぐに滅菌した吸収紙を用いて除去し，遠心付着させた 1 層の細菌層の高さに培地が供給され，その中で細菌が増殖するようにした。60 分間の放置中に細菌はカバーグラスに強く付着したと思われ，放置しなかった場合，容易に剥がれて洗い流された。この様にして形成された細菌塊は，遠心細菌塊や濾過細菌塊に比べ細菌密度が高く，細菌が密接して細菌間のスペースが無い状態で増殖し，その後，その上にまた密な細菌層を形成していると思われた。この様に密に形成された細菌塊は，細胞外に多糖体を形成しないにもかかわらず，いわゆる「バイフィルム」と同様，物質の浸透が極めて悪い状態にあると思われた。

1. Introduction

Descriptive observations have shown that infecting bacteria can form a biofilm, *i.e.* a thin bacterial layer, on solid surfaces[1] in certain cases. It has been recognized that the biofilm formation is one of the important virulent factors in infective diseases, including native valve endocarditis, osteomyelitis, middle ear infections, medical device-related infections, ocular implant infections, and chronic lung infections in cystic fibrosis patients[2,3] . The virulence that has been suggested to biofilm is the resistance against antibacterial drugs and immunological phagocytosis because of the encasement of biofilm within extracellular polymeric substances (EPS), physiological heterogeneity and the altered growth phenotypes[4-7] of bacterial cells as they form biofilm.

The *in vivo* formation of amorphous materials, as EPS, covering a bacterial mass observed *in situ* by scanning electron microscopy has been reported and it has been suggested that the bacterial mass is biofilm. However, it is rather difficult to analyze the biochemical composition of amorphous materials; especially it is probable that various molecules of host origins may- interact with the bacterial masses[8, 9] . Moreover, the resistant features against antibacterial drugs or the immunological removal *in situ* has not been clearly demonstrated, although artificial *in vitro* models revealed some approvals for those[10, 11] .

Even in the *in vitro* models, the barrier function of EPS does not seem to be perfect, because antibacterial drugs are effective to kill biofilm bacteria with time[12] , even if a higher concentration is needed. If so, the transportation of molecules into biofilm should relate to the biofilm resistances. Since the molecules are transported through interstitial spaces and the connected channels, the void volume of bacterial masses may be important for biofilm functions.

However, most *in vitro* models allows attached bacteria to grow in the excess bulk of culture media under uncontrolled conditions[13] , so that bacteria may grow three dimensionally making more wider interstitial spaces.

In this study, we aimed to form a bacterial mass as a new model in which limited amount of media was provided; such restricted nutrient mode caused to dip bacterial cells to grow within a monolayer, and then, on top of this layer, a new layer, and so forth. The bacterial structure and the bacterial density in the bacterial mass were analyzed with confocal laser scanning microscopy (CLSM).

2. Materials and Methods

2.1 Microorganisms

From the American Type Culture Collection (ATCC), *Enterococcus faecalis* ATCC 19433 and *Propionibacterium acnes* ATCC 11827 were obtained. They were cultured anaerobically for, overnight and 48h respectively, in Brain Heart Infusion (BHI; Difco Laboratories, Detroit, MI) broth media and harvested by centrifugation (Tomy Seiko Co., LTD. Tokyo, Japan). After washing with sterilized 40 mM phosphate buffer, pH 7.0, by centrifugation (10,000×g, 10 min, 4℃), the precipitate was resuspended and homogenized in sterilized BHI broth. Finally the suspension was adjusted to give the optical density value 1.00 at 660 nm with sterilized BHI broth. The fresh bacterial suspension was used for the preparation of bacterial mass formation.

2.2 Formation of cultured bacterial mass

An aliquot of 400 μl bacterial suspensions of *E. faecalis* and *P. acnes* were centrifuged, respectively, using CytoFuge (StatSpin Technologies, Norwood, MA, USA) onto a glass-surface. Thus, the bacterial organisms were spun down at 1,060 g for 30 min onto a cover glass (2.4cm x 5.5 cm: Matsunami, Osaka, Japan) as shown in Fig. 1. The centrifuged bacteria were allowed to stand for 60 min in a humidity cabinet (95% humidity) at 37℃ without providing any watery components including nutrients. During the 60 min-standing, most of the centrifuged bacteria seemed to attach firmly onto the cover glass, because they were resistant to washing procedure using sterilized 0.9% NaCl solution (3-5 drops 5 times each).

Under the restricted nutrient supply of 25 μl of BHI broth media (BHI supplemented with yeast extract, cysteine, hemin and vitamin K)[14] at 60 min intervals, the attached bacterial organisms were allowed to grow for 72 h aerobically for *E. faecalis* and for 5 d in an anaerobic glove box (model AZ-Hard; Hirasawa, Tokyo, Japan) containing 80% N_2, 10% H_2 and 10% CO_2 for *P. acnes*, respectively. Excess amount of media was immediately removed using sterilized absorbing

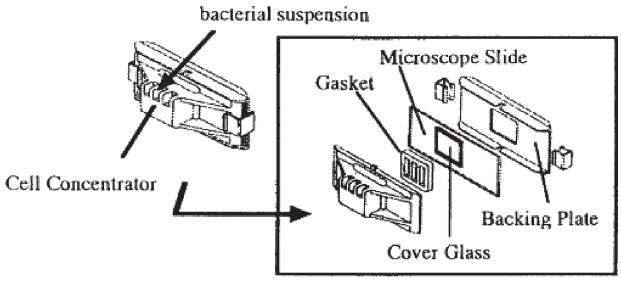

Fig 1. CytoFuge assembly

papers. In some cases, the attached bacteria were allowed to grow in a batch of 200 μl BHI broth surrounded by a sterilized gasket.

Cultured bacterial mass was observed and analyzed using confocal laser scanning microscopy (CLSM, Olympus FluoView 300, Tokyo, Japan).

When needed, a cultured bacterial mass was detached from the cover glass, and dispersed with 200 μl of sterile 40 mM phosphate buffer, pH 7.0 to determine the optical density values at 660 nm and other analyses.

To make standard curve showing the relationship between the numbers of bacterial cells and fluorescent intensity, various concentrations of bacterial suspensions were centrifuged onto cover glasses using the Cytofuge as mentioned earlier. After denaturation by 70 % isopropyl alcohol for an hour in a humidity cabinet (95 % humidity) at 37℃, bacterial cells were stained with propidium iodide (PI) for 15 min. A calibration curve between the total fluorescent intensity of PI and the number of isopropyl alcohol-killed organisms in a range from 10^6 to 10^9 was obtained. Thus, values of fluorescent intensity represented bacterial numbers.

2.3 Formation of centrifuged and filtrated bacterial mass

Bacterial cells of *E. faecalis* and *P. acnes* were allowed either to be centrifuged (10,000x g, 5 min, 4℃) or to be filtered using the Filter System with 0.45 μm cellulose Acetate membranes (Corning, New York, USA). The bacterial mass formed at the bottom of the centrifugation tube or on the filter were carefully removed and placed onto a sterilized cover glass for further image analysis using CLSM after denaturation by 70% isopropyl alcohol for an hour in a humidity cabinet (95% humidity) at 37℃. When needed, the bacteria mass was dispersed with 200 μl of sterile 40 mM phosphate buffer, pH 7.0 to determine the optical density values at 660 nm and other analyses.

2.4 Sample Preparation for CLSM observation

Before staining with PI, bacteria in cultured bacterial mass were denatured by 70 % isopropyl alcohol for an hour in a humidity cabinet (95 % humidity) at 37℃, followed by washing with sterilized 0.9% NaCl solution.

The layered-sectioning confocal images of 0.4 μm thickness were captured with CLSM for the observation of PI stained bacteria in bacterial mass. Fluorescent intensities were also measured using CLSM.

CLSM operation mode was as follows: For imaging in transmission mode, excitation from a 488-nm laser was used. For imaging denatured bacterial cells stained with PI, a 543-nm laser was used and the emission wavelength was set at 610 nm (red channel). When bacterial masses were stained with fluorescein isothiocyanate (FITC), a 488-nm laser was used with the emission at 510 to 530 nm (green channel). A dry objective lens (x40) was used for these experiments. Microscope images were analyzed using the FluoView FV 300 software. The biofilm thickness was measured as the distance from the substratum (cover glass) to the biofilm-air interface.

2.5 Determination of bacterial density of bacterial mass

The bacterial density was defined as bacterial numbers per unit area. Bacterial cell numbers were expressed in fluorescent intensity, which correlated with bacterial numbers as shown as the standard curve described earlier.

2.6 Immunoglobulin G (IgG) infiltrations through cultured bacterial mass

Rabbit IgG specific to *P. acnes* were from our stock, which was the rabbit serum immunized with whole cell of *P. acnes* strain ATCC 11827. FITC-conjugated goat IgG against rabbit IgG were purchased from the ICN Biomedicals Inc (Aurora, OH).

The cultured bacterial mass of *P. acnes* was incubated with the rabbit antiserum (1:1000) for 60 min at 37℃ and washed with phosphate buffer. They were further incubated with FITC-goat IgG (1:100) and washed with the same buffer. The infiltration of IgG was determined using CLSM by tracing FITC fluorescence.

2.7 Statistical analysis

The paired t-test was applied for statistical analysis using Microsoft Excel 2007 for Windows (Microsoft Asia Ltd, Tokyo, Japan). p values <0.025 were considered to be significant.

3. Results

3.1 Bacterial adherence on to a glass surface

Although the bacterial cells of *E. faecalis* or *P. acnes* were spun down onto glass surfaces of a histological cover glass, they were not adhered firmly to the surfaces, as all of them were easily washed out. However, when the centrifuged cells were allowed to stand for 60 min without excess watery supply in a 95% humidity cabinet at 37 ℃ , quite many of them adhered onto the surface firmly, and they were not washed out (Fig. 2).

When bacteria were collected by centrifugation or filtration, bacterial masses were easily formed. But, although they were placed onto a cover glass, they did not adhere onto the surface, and they were easily washed out.

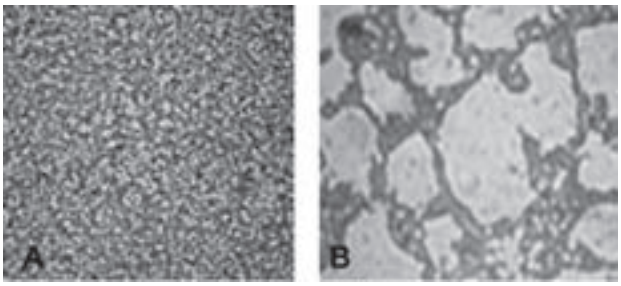

Fig 2. Differential interface microscope images of adhered bacterial cells after they were spun down over histological cover glass.
(A) Firmly attached cells, after standing for 60 min with no nutrient supply.
(B) Cells washed away when not allowed to adhere for 60 min.

3.2 Bacterial culture of the centrifuged cells on a cover glass

When *E. faecalis* or *P. acnes* cells were allowed to grow under limited nutritional supply (25 μl at 60 min intervals) for 3 and 5 d respectively, tight bacterial cell layers were formed. The media volume was the maximum to dip the bacterial layer without excess overlay. Care was taken to let bacterium grow sideward on the glass surface. After a bacterial monolayer was made, the second layer was formed on it, and layers were added on the layers as shown in Fig. 3, which was observed by CLMS. This was actually the bacterial mass adhering onto solid surface (cover glass) and making bacterial thin layers. *E. faecalis cells* produced bacterial layers of average 17.4 μm (n= 3, SD: 0.4) thickness for 3 d, while *P. acnes* produced bacterial layers of average 8.0 μm (n= 3, SD: 0.3) for 5 d. It should be noted that amorphous materials covering these cultured bacterial masses were not observed in all cases using *E. faecalis* and *P. acnes*.

However, a supply of excess amount of media (50 μl at 60 min intervals) caused bacterial detachment from the glass surfaces, probably because bacteria might moved up into media, grew in the excess media and flowed out. Even when 25 μl medium was supplied; the shorter frequencies of nutrient supply (10 min interval and 30 min interval) caused to fail the bacterial adherence.

3.3 Bacterial density in bacterial masses

The bacterial density, expressed as fluorescent intensity/unit area, was lower in case of centrifuged or filtrated bacterial masses of *E. faecalis* or *P. acnes* than the cultured bacterial masses (Table 1), indicating much more bacterial cells were packed in the cultured bacterial masses. Multiplied bacterial cells might throng to fill up the inter-bacterial spaces. When centrifuged and adhered bacterial cells of *E. faecalis* or *P. acnes* were cultured on the cover glass in 200 μl

Table 1 Cultured and non-cultured Biomasses

Distances from bottom to top (μm)	Cultured Biomass	Non-Cultured Biomass	
	With restricted media volume	Centrifuged	Filtered
3.2	3.34×10^8	1.36×10^8	2.60×10^8
2.4	4.81×10^8	1.42×10^8	2.51×10^8
1.6	5.61×10^8	1.48×10^8	2.31×10^8

Data expressed as total fluorescent intensity per unit area 0.5 cm^2.

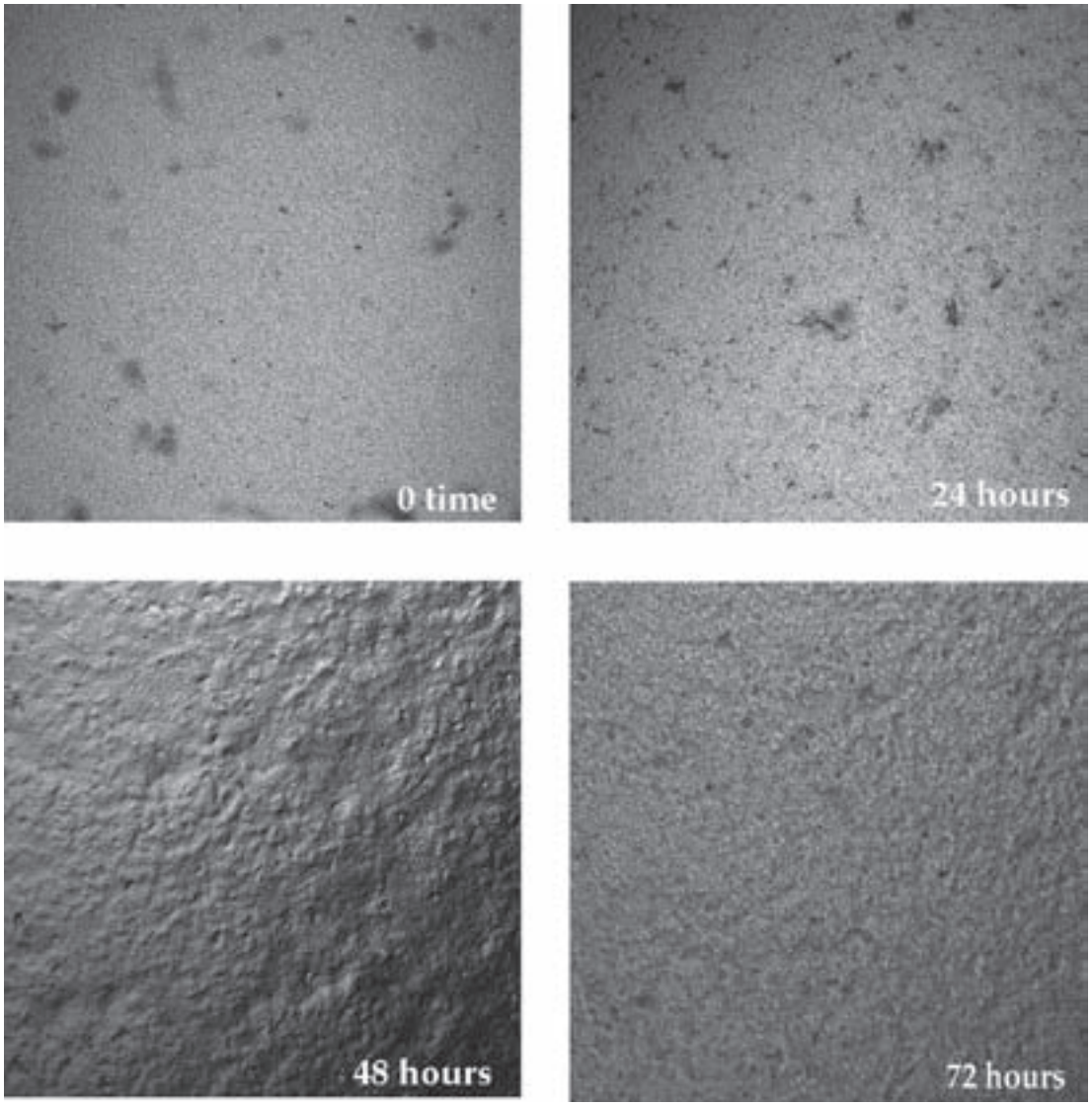

Fig 3. CLSM image of cultured bacterial mass using the transmitted mode (laser differential interference mode).

Images were captured for *E. faecalis* during its 72 h growth on cover glass. The bacteria numbers increased with time, making layers.

volume of culture media, bacterial density was significantly lower than those made with restricted media volume (Table 2 and Fig. 4), probably because the bacteria might grow upward within the culture media, making much more inter-bacterial spaces.

The bacterial density of the filtrated bacterial mass was lower than the centrifuged one, probably because the mechanical forces to pack bacteria were stronger in centrifugation than in filtration.

3.4 Immunoglobulin infiltration through the cultured bacterial mass

Excess amounts of rabbit polyclonal specific antibodies against *P. acnes* infiltrated into the cultured bacterial mass of *P. acnes*. Thus, the fluorescence-positive *P. acnes* cells that reacted with the specific IgG and then with FITC labeled anti-rabbit antibodies were detected using CLSM. This might indicate that inter-bacterial spaces were connected each other to make pathways for transport molecules, such as nutrients and immunoglobulin. However, when the well-packed bacterial masses, cultured with restricted media supply, were compared with loosely-packed bacterial masses cultured with simultaneous media supply (Fig. 5), the IgG infiltration was significantly less in packed cultured bacterial masses (n=9, 56.1 ±

Table 2 Bacterial Density of Cultured Biomasses

Bacterial species	Nutritional mode	Average fluorescent intensity	SD	P value
E. faecalis	Restricted	685.35	88.88	<0.001
	Excess	199.16	22.13	
P. acnes	Restricted	393.78	247.98	<0.05
	Excess	109.18	37.26	

Paired *t*-test; n=3

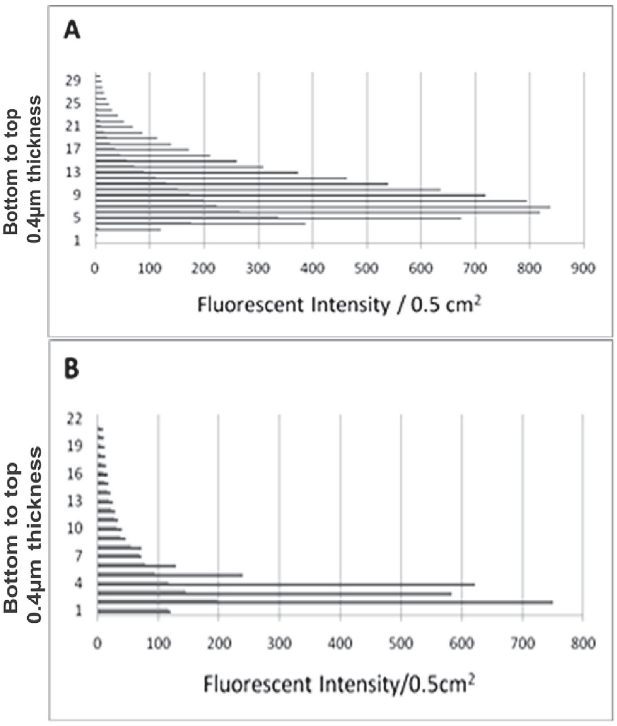

Fig 4. Quantitative analysis of horizontal optical sections within *E. faecalis* (A) and *P. acnes* (B) bacterial masses (n=3).

■ Cultured with restricted nutrient volume
■ Cultured with excess nutrient volume

84.34) than in loose cultured bacterial masses (n=9, 660 ±89). Since the bacterial density in packed cultured bacterial mass was higher than in loose cultured bacterial mass, the inter-bacterial spaces might be indicated to be narrower and the connected pathways were complicated.

Discussion

Bacterial cells of *E. faecalis* and *P. acnes* produced thin bacterial layers, which quite resemble to so called 'biofilm' on a solid glass surfaces, because a biofilm is defined as a thin film-like bacterial structure formed on a slid surfaces[15] . As the first step to produce thin bacterial structure, bacteria have to attach to the solid surfaces. It should be noted that both *E. faecalis* and *P. acnes* cells firmly adhered onto glass surfaces when they were centrifuged onto the surfaces and kept for 60 min without excess watery media. Since the centrifuged cells without 60 min-standing were easily peeled off by washing, the firm adherence was generated during the 60 min-standing. Both of *E. faecalis* and *P. acnes* do not produce considerable amounts of extracellular polysaccharide, but various polysaccharide, such as glycoprotein, are produced as cell wall components[16,17], which may be sticky and may have been used to adhere to glass surfaces.

After centrifugation, cells may contact with glass surfaces at less points as happened during the centrifugation at 1,060 g for 30 min, each bacterium may flexibly widen the adhering area by increasing chemical binding points, because glycoprotein is distributed widely throughout the cell wall. It is probable that, the more the chemical adhering points increase on each cell, the stronger adherence of the cell is generated. It may have happened only on the wet surfaces without excess watery media, probably because the bacterial cells can increase contacting area to the glass surface with time, whereas in the presence of excess watery media, the cells may swim out into the watery media.

The restricted watery amounts on the solid surfaces may be most likely probable and common inside host bodies, such as in lesions, or outside host bodies, such as on skins and mucous membranes, where only limited amounts of tissue fluid, exudates and/or secretions are provided. Excess amounts of those watery components may easily flow out from the surfaces after wetting the surfaces unless structural pools are formed there. In addition, those host watery components contain glycoprotein that may assist the bacterial attachment.

Such attached bacteria under the restricted bulk of watery components grow only within a watery bulk, and multiply most likely sideward. If so, bacteria absent areas may be fulfilled by such multiplying

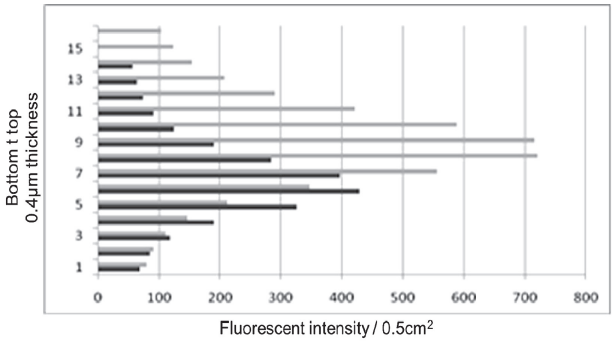

Fig 5. Relative fluorescent intensity of FITC in cultured bacterial masses.

Decreased fluorescent intensities within packed bacterial mass revealed the impaired infiltration of IgG macromolecules within such dense bacterial biomass (n=9).

■ Packed cultured biomass

■ Loose cultured biomass

bacteria. Between these bacteria, firm attachment could be similarly formed. Thus, these restricted watery amounts on the solid surfaces of human hosts may encourage the attached bacterial to produce bacterial monolayer first, and then, further the second layers. The multi-layered areas may function as the structural dikes to pool the watery media where bacteria may throng to full up the vacant rooms, resulting in higher bacterial density, as shown in cultured bacterial masses in the present study.

Although it is quite difficult, at present, to determine the actual shapes and volume of the spaces three dimensionally, inter-bacterial spaces in a bacterial mass should be small and narrow when bacterial density is high.

The IgG could infiltrate into the bacterial masses and combine with the bacterial cells, meaning that inter-bacterial spaces fused to each other and constituted channels. Thus, various molecules, including nutrients and anti-bacterial agents, can infiltrate into bacterial masses and react with bacteria within bacterial masses. If so, infiltration of molecules is affected by the structural narrowness of channels or chemical reactivity of fluid to the molecules in the channels, together with the reactivity of bacteria to molecules.

So-called 'biofilm' is characterized by its protective functions against antibacterial drugs or immunological attacks[10], together with its structural thin layer formation. This has been ascribed by the formation of extracellular polymeric substances (EPS)[2,4,6], which is functioning as a barrier of entire biofilm structure against antibacterial agents or immunological attacks. However, these protective functions are not perfect, because, for example, the anti-bacterial agents affects with time, meaning protection mode is 'delayed'[12]. If so, infiltration of associating molecules is insufficient or slow through 'biofilm'[18].

It is probable, when a bacterial mass is constituted with higher bacterial density meaning that narrower channels are made inside the bacterial mass even without formation of EPS, penetration of antibacterial drugs is restricted or delayed. Immunological removal of bacteria from bacterial masses may not be easy if immunological molecules infiltrate restrictedly and, phagocytes are too large to pass through the channels.

The present study suggests that micro colonies of bacteria, not only of specific so-called biofilm-forming-bacteria, which are often proven to produce EPS *in vitro*, but also more common bacteria *in situ* may constitute bacterial layers equivalent to a so called "biofilm".

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