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研究課題名（和文）シングルセルトラッキングによる細菌バイオフィルム形成の解析

研究課題名（英文）Single cell tracking to analyze biofilm formation

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研究成果の概要（和文）：日和見病原菌である緑膿菌Pseudomonas aeruginosa は、バイオフィルムのモデル細菌である。そのムコイド株は、細胞外多糖のアルギン酸を大量産生することで、嚢胞性線維症患者の死亡原因となる。我々は、ムコイド株が高頻度に復帰変異を生じ、アルギン酸産生能を失うことを見出した。そこで、蛍光タンパク質の発現が変化するレポーターをムコイド株に導入し、この復帰変異株を区別可能な系を確立した。現在、これらの株をフローサイトメトリーや広視野共焦点顕微鏡を用いて解析している。また、ムコイド株コロニーにおける局所的な復帰変異率の定量化や復帰変異に関わる主要因子の同定に取り組んでいる。

研究成果の学術的意義や社会的意義
We have generated a reporter strain through this work that has the potential to enable the clarification of the biofilm forming properties of the mucoid mutant strain. This research has potential to clarify the ecological significance of polysaccharide overproduction.

研究成果の概要（英文）：　Pseudomonas aeruginosa (PA), an opportunistic human pathogen, is often used as a model organism to understand biofilms. The PA mucoid mutant produces copious amounts of the polysaccharide alginate and is a leading cause of mortality in cystic fibrosis patients.
We found that the mucoid strain rapidly acquires spontaneous mutations, resulting in a loss of the alginate overexpression phenotype; this new strain is called the “revertant”. To distinguish and track these different mutants, we generated a reporter strain in the mucoid mutant background to report the phenotype through expression of fluorescent proteins. We are characterizing the transition from mucoid (green) to revertant (red) using flow cytometry, wide-field and confocal microscopy. Moreover, we are beginning to quantify the frequency and location of appearance of the reversion mutant within mucoid colonies. We are still working to clarify the major factors that induce spontaneous mutations in the mucoid mutant.

研究分野：農学

キーワード：Pseudomonas aeruginosa　mucoid phenotype　spontaneous mutation　phenotypic switching

１．研究開始当初の背景

1. Biofilms: Microorganisms inhabit nearly every explored environment on earth, from the most mundane to the most extreme: from soil to high-temperature thermal vents in ocean. Typically, bacteria live not as independent entities, but rather is members of surface-associated social communities, called biofilms. Biofilms are made up of cells embedded within a sticky extracellular matrix that consists of exopolysaccharides (EPS), proteins, and extracellular DNA (eDNA) (see **Fig. 1**). The matrix is a complex physical structure that regulates the local environment by facilitating communication, enabling the sharing of resources, and providing protection from the environment and predators. Biofilm based infections frequently infect medical implants, the urinary tract, and gastro-intestinal tract. Remarkably, biofilms are 1000x more resistant to antibiotics compared to free swimming planktonic cells. In recent years biofilm related research has increased tremendously.

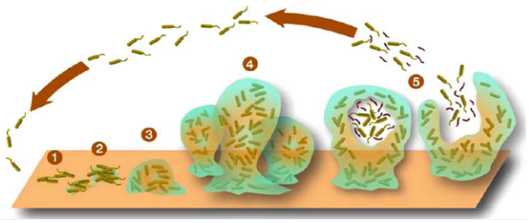

Fig. 1 The biofilm life cycle (Fig. from Musk, D. J., Jr. *et al. Curr. Med. Chem.* **13**, (2006).). (1) Cells make surface attachment. (2) EPS is produced (3 and 4): biofilm architecture develops and matures. (5) single cells are released.

2. *Pseudomonas aeruginosa (PA):* *PA* is an adaptable, ubiquitous environmental bacterium that is also an opportunistic pathogen that forms biofilms during infection. The World Health Organization recently named *PA* as a critical target for the development of novel antibiotics. *PA* causes up to 10% of nosocomical infections with high fatality rates, mainly affecting immunosuppressed individuals, severe burns patients, and leads to fatal lung failure in cystic fibrosis (CF) patients.

Active surface motility and EPS production are crucial for biofilm development for *PA*. *PA* utilize hair-like appendages, called type IV pili (TFP), to 'twitch' (translocate) across surfaces[8]. Psl, a component of EPS is crucial to biofilm development because it enables cell-surface adhesion and serves as a chemo-attractant. In fact, *PA* follow trails of Psl slime left by other cells, speeding microcolony formation. Furthermore, Psl production is linked to down regulation of motility via the 'motility master regulator' cyclic diguanylate (c-di-GMP) and alginate production.

However, for reasons not fully understood, wild type (WT) *PA* biofilm infections in CF patient lungs undergo a phenotypic switch to alginate overproducers (mucoid variant)**.** The mucoid variant has even greater antibiotic resistance and eventually displaces WT, becoming dominant. The observed biofilm architecture also changes massively: WT biofilms are relatively flat and uniform while mucoid variants tend to form taller microcolony structures with large gaps.

２．研究の目的

My long-term goal for this project was to clarify the foundational rules governing *PA* mucoid variant biofilm formation by quantifying changes to motility, localization of cell-cell communication, and heterogeneity of the chemical signals in the local environment. This strategy was to be quantitatively based on tracking and analyzing the motion of individual cells within bacterial communities and compiling these data into searchable libraries. This proposal is motivated by my previous work on cell tracking, surface motility, and microcolony formation with *Vibrio cholerae*.

３．研究の方法

Traditional bacteriological techniques such as genetic manipulation and knockout mutant strain comparisons have generated much of what is known about *PA* biology and biofilm development. However, in addition to the genetic factors, it is becoming clear that epigenetic factors modulated by the physical environment also strongly affect biofilm development. The intricate interplay of surface recognition, motility, cell-to-cell signaling within biofilms, and gene expression, all occurring in a dynamic physical environment is still poorly understood.

In this proposal, I planned to extend novel cell-tracking tools that can record and analyze an entire community of cells at the *single cell level* and merge it with the 3D capabilities of confocal laser scanning microscopy (CLSM). I aimed to combine these imaging and analysis tools with microfluidics to precisely control environmental conditions and with flow cytometry to analyze the spatio-temporal secretion of alginate.

Specific Aim 1: *refine and extend the novel high-throughput single-cell tracking technique by coupling it to observations from CLSM of mono- and co-cultures of WT and mucoid reporter strains, respectively*. Fundamentally characterizing the differences in biofilm development dynamics will shed light on where the mucoid variant begins to dominate, enabling us to access how it becomes dominant.

Specific Aim 2: *engineer confined and structured microfluidic environments to precisely control and modulate environmental topology, fluid flow, and chemical background, tightly coupling these observations to single-cell tracking and confocal microscopy to determine microscopic cues in the biofilm development that are not discernable through traditional imaging and analytical methods.*

４．研究成果

Through our research we found that the mucoid strain rapidly acquires spontaneous mutations, resulting in a new strain that lacks the alginate overexpression phenotype; this new strain is called the “revertant” to signify its reversion to a WT-like phenotype. Images of Indian ink inverse staining show that the mucoid strain produces a large amount of alginate surrounding the cells while WT do not show a similar ink exclusion (see **Fig. 2**). During typical culturing conditions on agar plates, we observed this transition in real-time, with the revertants appearing at the edges of the mucoid colonies over the course of 12-18 h (see **Fig. 3**).

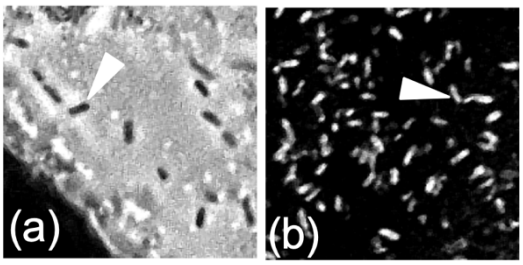

Fig. 2. (a) Mucoid cells (black) in alginate (light). (b) WT cells in Indian ink.

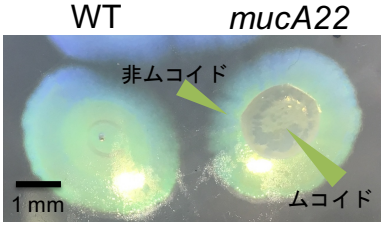

Fig. 3. (left) WT and (right) mucoid colonies. The clear region on the right is a mucoid colony surrounded by revertants.

These revertant mutants, while they did not produce copious amounts of alginate in the channels, are indistinguishable under wide-field imaging. Thus, although we had begun to record flow-cell movies and had started to track the motion and build databases of bacterial behavior, since we could not distinguish the different strains from each other, we instead began to focus on developing a “reporter” strain with the ability to indicate the state of the bacteria; if the bacteria were producing large amounts of alginate, they would simultaneously produce the red fluorescent protein mCherry indicating that it was still mucoid.

However, if a spontaneous mutation occurred where the mucoid became a revertant, the strain would then produce the green fluorescent protein SFGFP. We designed a reporter strain in the mucoid background such that when the P_{algD} promoter was working mCherry (or dsRed) would also be produced. On the other hand, if a mutation occurred in the promoter, the P_{lacZ} would instead turn on, thereby producing SFGFP (indicating transition to a revertant).

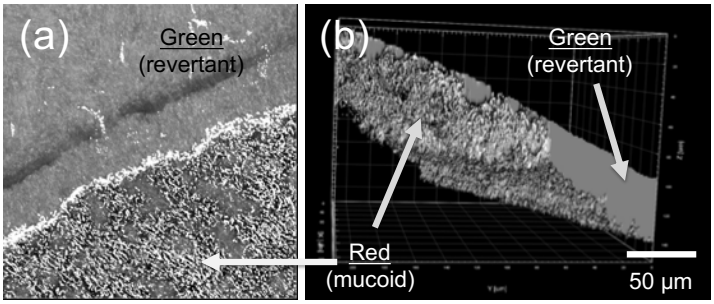

Fig. 4. (a) Fluorescent confocal image from the top at the edge of a colony on an agar plate. (b) Side view of the same slice. The reporter strain enables us to directly visualize the location of both strains within the original colony.

We are currently characterizing the transition from mucoid (green) to revertant (red) using confocal microscopy and flow cytometry. The reporter strain is useful for imaging and quantifying the location of appearance of the revertant mutants within mucoid colonies. This will enable us to isolate and identify the major causes of this spontaneous revertant. Confocal images of a colony that has the appearance of the revertant mutant at its edges is shown in **Fig. 4**. We find that the revertant tends to "escape" from within the larger mucoid colony, for reasons still unclear.

We are still working to clarify the major factors that induce spontaneous mutations in the mucoid mutant. Another hint comes from flow cytometry after culturing in for various amounts of time. For example, we can quantify using flow cytometry at particular points in time to determine the mutation rate. For example cells grown in liquid medium for 24 h show a particular signature: cells with high red or green fluorescence are overwhelmingly mucoid or revertants, respectively. Interestingly, a population of cells with very low fluorescence exists in the lower "no color" box (Fig. 5(b)); these cells are sampled randomly and appear to approximately 50/50 each of mucoid and revertant.

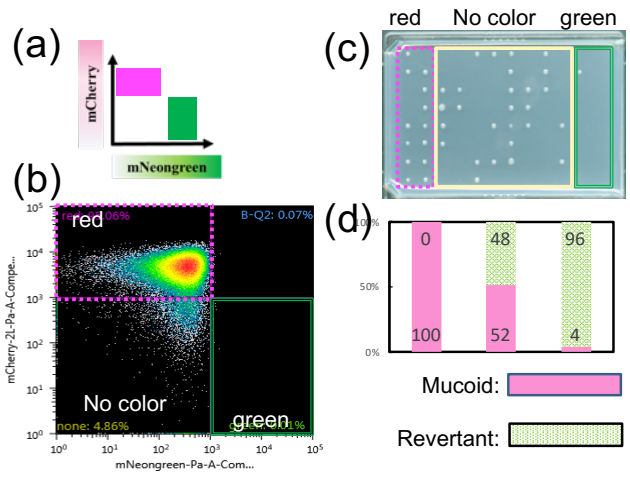

Fig. 5. (a-b) Axes for the flow cytometry of reporter strain cells grown in 24 hr LB culture. (c) Cells from each region are spotted onto a grid on agar and the phenotype is quantified in (d).

I am still working to clarify the causes and drivers of these random mutations and the reasons that there are significant numbers of cells that lack fluorescence altogether. I believe that clarifying the reasons for the spontaneous mutation leading to polysaccharide overexpression is relevant not only for *P. aeruginosa*, but for other bacteria that make similar transitions from "normal" to "EPS overexpression" phenotypes due to changing environmental conditions. In the case of *P. aeruginosa*, specifically, this organism actually appears to transition from WT to mucoid, and then to a WT-like revertant. I aim to summarize these results in the near term in an academic publication.

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[学会発表]（計　14　件）

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[図書]（計　　件）

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