様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

令和　６ 年　 ６ 月 ２６ 日現在

機関番号：１４３０１
研究種目：基盤研究(C)（一般）
研究期間：2021～2023
課題番号：２１Ｋ０５３４１

研究課題名（和文）Spatiotemporal mapping of gene expression to reveal mechanisms of cellular differentiation in E. coli biofilms

研究課題名（英文）Spatiotemporal mapping of gene expression to reveal mechanisms of cellular differentiation in E. coli biofilms

研究代表者
Ｒｏｂｅｒｔ　Ｍａｒｔｉｎ（ROBERT, Martin）

京都大学・薬学研究科・特定准教授

研究者番号：９０３６５４８７

交付決定額（研究期間全体）：（直接経費）　　3,100,000円

研究成果の概要（和文）：このプロジェクトは、大腸菌バイオフィルムの機能分化を、形態と遺伝子発現をモニターすることによって調べるものであった。我々は、グルコースがバイオフィルムに対して、代謝や酸ストレスに起因する機能的差異だけでなく、構造的差異を生じさせることを発見した。時空間的な遺伝子発現は、蛍光レポーターとプロテオーム解析の両方で観察され、形態学的な違いは、表面硬度の変化と局所的な栄養状態の変化から生じていた。加えて、運動性、マトリックス産生、タンパク質合成に関連する遺伝子発現とタンパク質レベルの変化も観察された。予定通り、バイオフィルムの時空間的差異から、細胞集団の共存と相互作用を示唆する新たな証拠を得た。

研究成果の学術的意義や社会的意義
この結果はバイオフィルムの成長と発達に対するグルコースの重要な影響を浮き彫りにした。 また、環境条件や薬剤などに対する耐性を試験するために使用できる単純な生物学的モデルにおける、多細胞の増殖と分化に関する基本的な洞察を提供する。バイオフィルムの生存と形成に重要な耐酸性、マトリックス産生、運動性遺伝子に関連した明らかな変化は、バイオフィルムの形成を制御するための新たなターゲットとなる可能性を示唆している。加えて、我々は関連分野の他の研究者にも有用な、オープンソースタイプの汎用的で安価なマルチモードイメージングシステムを開発した。

研究成果の概要（英文）：This project was about examining functional differentiation in E. coli biofilms by monitoring growth, morphology and gene expression during development.
We found that in complex media glucose affects biofilm formation resulting in structural differences as well as functional differences linked to metabolism and response to acid stress during growth on glucose. Differential spatio-temporal expression was observed both with fluorescent reporters and proteome analysis. Morphological differences arise from changes in surface hardness as well as local nutritional conditions. We observed changes in gene expression activity and protein levels related to motility, acid resistance, metabolism, biofilm formation and protein synthesis. As planned, we provided novel evidence for spatiotemporal differentiation in activity in different parts of the biofilm (top-bottom and center-edge axis) and over time (biofilm growth and aging) that suggest co-existence and interactions between these populations.

研究分野：microbiology 微生物学

キーワード：biofilm　E. coli　gene expression　imaging　differentation　bacterial growth　proteomics　stress resistance

※科研費による研究は、研究者の自覚と責任において実施するものです。そのため、研究の実施や研究成果の公表等については、国の要請等に基づくものではなく、その研究成果に関する見解や責任は、研究者個人に帰属します。

１．研究開始当初の背景

Bacterial biofilms represent interesting multicellular prokaryotic systems that are only starting to be characterized. They are the subject of interest both for fundamental reasons as well as for their links to resistance to various stress and antibiotics (1-2). There is some evidence that individual cells in biofilms may functionally differentiate but still little is known about the process. We therefore set out to use a model of *E. coli* biofilms on solid agar medium to explore how surface and nutritional conditions affect growth, morphology, and gene expression in biofilms.

２．研究の目的

The main objectives were to explore and quantify gene expression and metabolic activity in biofilms produced by the bacterium *Escherichia coli* (*E. coli*) using time-lapse fluorescence imaging of reporter gene expression, and spatio-temporal mapping of the cellular proteome using liquid chromatography-mass spectrometry. We also aimed to provide a characterization of molecular and structural changes occurring during biofilm formation on different surface and nutritional conditions. In order, to pursue these objectives, since some of the imaging requirements were not readily available, we also set out to develop our own imaging system inspired by open-source models (3).

３．研究の方法

We isolated single colonies of multiple strains of *E. coli*, pre-cultured overnight in LB medium and spotted a small aliquot of culture onto agar plates while manipulating surface hardness by varying agar concentration and changing nutritional conditions by using different concentrations of glucose or other carbon sources. Biofilm development and morphology were monitored using standard desktop scanners and a stereomicroscope during growth at 28C. According to the results、 we selected appropriate conditions for further exploration of gene expression as outlined below.

Spatiotemporal gene expression (promoter activity) was monitored using fluorescence imaging of strains expressing GFP under the control of various *E. coli* promoters (4). Image analysis was performed using Fiji/ImageJ.

Spatiotemporal protein expression was performed using LC-MS based proteomics to monitor expression in different locations (top-bottom layers and middle-center-edge sections) of the biofilms and at different time points. For proteome analysis biofilms were separated into top and bottom layers by shaking biofilms in PBS solution for several hours collecting the floating top part and agar-attached bottom part. The central, middle, and peripheral regions of a single biofilm were dissected by scraping the biofilm from the agar surface and collected different parts into separate tubes. The different samples were extracted using standard protocols and digested by trypsin. Resulting peptides were purified and analyzed by LC-MS (5). Data analysis was performed using MaxQuant (6).

We also developed a new imaging system to collect information about cell density, biofilm morphology, and promoter activity using different color LEDs and emission filters allowing a combination of transillumination, epi-illumination and fluorescence (Figure 1). Our system can collect time-lapse images of multiple biofilm samples (usually one image every 20 mins) over several days in an automated fashion.

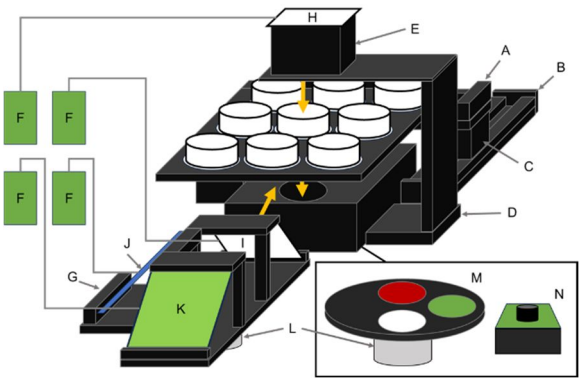

Figure 1. Diagram of our in-house designed and 3D-printed imaging system. Main components include the robotic sample platform and actuators (A-D), the transillumination unit (E, H), control systems (F), different LEDs sources (G, I, J, K), the spinning optical filter unit (M) and camera (N).

4．研究成果

During biofilm growth on agar in complex medium (LB), either supplemented or not with glucose, we observed diauxic growth on LB with glucose (Figure 2) resulting in increased biofilm structural complexity compared to LB alone. Growth paused for an extended period of time at high glucose concentration (> 0.5%) and eventually resumed after more than 10 days. Acid stress from acetate production is the most likely reason for this delayed growth phenotype.

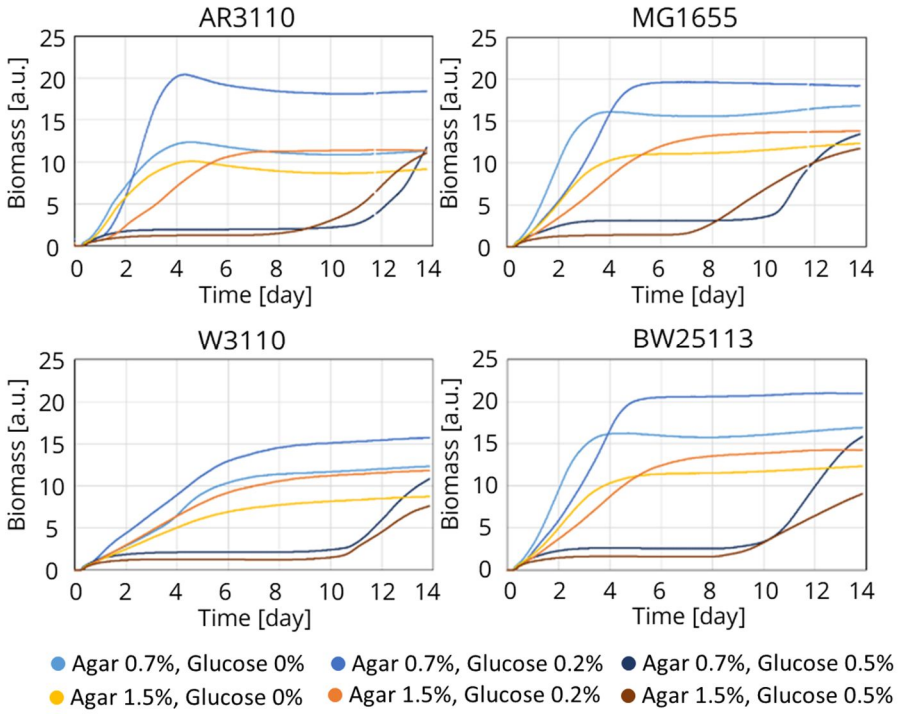

Figure 2. Growth curves for four different *E. coli* strains as quantified using transilluminated white light from a desktop scanner in the presence or absence of various concentrations of glucose. Biomass (arbitrary units) was quantified from time-lapse images using Fiji.

Other carbon sources were also tested. While glucose and similar glycolytic sources result in a reduction of growth at higher concentrations, gluconeogenic carbon sources showed growth kinetics and phenotypes similar to growth on LB. These highlight the importance of overflow metabolism and acid production in the process. Further experiments are ongoing to characterize this process in more details.

Using Congo Red which binds to curli fibers, we characterized curli expression in several strains *E. coli* K-12 including some producing curli but not cellulose (W3110), a strain producing neither (csgD knock-out) and a strain expressing both (AR3110) (Figure 3). The results show that the presence of either or both curli and cellulose result in different 3D structures that depend on surface hardness while strains lacking both produce more simple and flat and white biofilms in agreement with previous studies (1-2) although we observed an increase of red-staining components in the csgD knock-out after longer incubation. The reasons for this is not clear and will be further investigated.

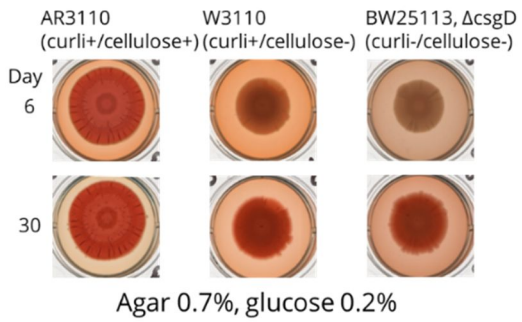

Figure 3. Images of biofilms grown for 6 or 30 days in the presence of Congo Red. The red color reveals the binding to curli and/or cellulose fibers produced by *E. coli*.

Using fluorescence imaging we observed changes in the cAMP synthesis enzyme (cyaA) and some specific ribosomal and motility related genes (rrnD, flgM, tar, etc.) during growth on various glucose concentrations (Figure 4). These results revealed variable spatio-temporal promoter activity during biofilm development. Using pH indicator dyes, we observed local pH changes in the agar medium that correlated with different growth phases (not shown). As shown above, when adding glucose, a first rapid growth phase using glucose as carbon source releases significant acid that affects growth at high concentration (> 0.5%). We are currently exploring this in further details.

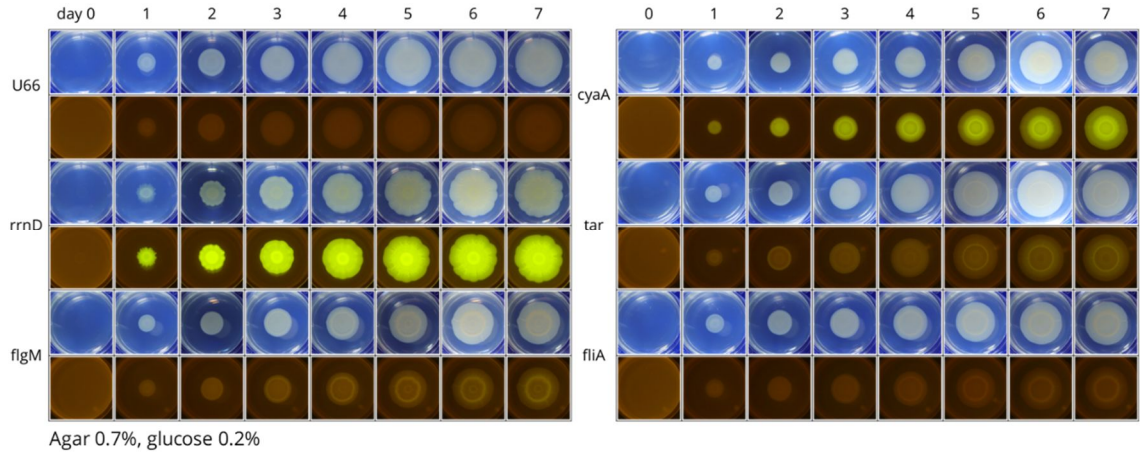

Figure 4. Bright-field and fluorescence time-lapse imaging showing growth (bright field) and promoter activity (GFP fluorescence) for the indicated genes. pU66 is a promoter less strain used as negative control. Images were taken over a seven-day period and one representative image is shown for each day.

While fluorescent signals are readily detectable, the 3D structure of biofilms requires to normalize the results to cell density/biomass. To facilitate this, we developed our own customized imaging system that can perform time-lapse imaging of nice samples over several days. This is achieved using two bright-field modes and fluorescence in two different colors using different LED sets for excitation and long-pass or bandpass filters for emission.

To complement the above experiments, proteome analysis of biofilms produced from the AR3110 strain was performed using LC-MS and allowed to identify over 2000 different proteins. Single biofilms were separated into three parts (center, middle and edge) as shown in Figure 5 (left panel) as well as in two vertical layers (top and bottom). The results revealed clear changes in spatio-temporal expression of proteins involved in biofilm production (curli), pyrimidine metabolism, acid stress response and motility as summarized in the model below, shown in Figure 5 (right panel). Specifically, proteins related to the flagellum were mainly expressed at the bottom of the biofilm while curli (matrix) were expressed in the top layer and edge. Expression of acid stress response proteins increased over time and in the upper layer while those related to protein synthesis decreased over time. Pyrimidine biosynthesis related proteins were mainly found in the middle of biofilms while chemotaxis-related proteins were more abundant at the center of the growing biofilm.

As originally proposed for this project, we succeeded in demonstrating spatiotemporal functional differentiation in *E. coli* biofilms. Our results suggest involvement of motility genes in attachment to surface and other cells, the importance of the stress response in specific parts of the biofilm and also localized curli expression in the top layer. These changes are induced by local carbon source consumption and resulting acidification and response. Later, growth can resume when acid is consumed and there is significant shift in carbon source use.

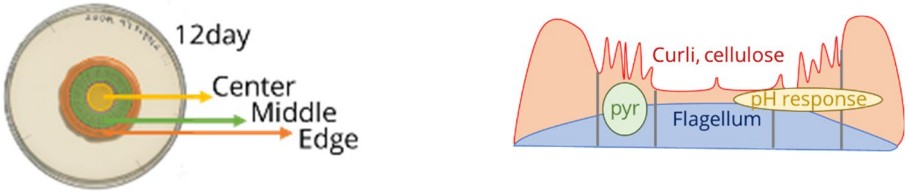

Figure 5. The left panel shows how a biofilm growing on top or an agar plate was processed into three different samples representing different radial positions (center, middle and edge). The right panel shows a biofilm lateral trans-section model summarizing the specific spatial localization of different functional categories of proteins as revealed in the proteomics experiments and following functional category enrichment analysis (text annotations). Vertical lines mark the separation between center, middle and edge regions while the top and bottom layers are shown in red and blue colors, respectively.

As planned, we have characterized biofilms with respect to their curli and cellulose expression, nucleotide (cAMP) biosynthesis and motility-related genes among others. We have not yet tested the effect of specific drugs on these processes, and this should be the focus of future studies.

Finally, we have also explored the role of an uncharacterized gene on biofilm development. We observed that its absence results in greater motility which is probably explains our observation that its overexpression affects biofilm morphology and increases apparent cell/biofilm density. Further investigations are ongoing to characterize this process further.

Overall, our results agree with some recently published studies (7) but provide a different perspective and a more in depth view and characterization of *E. coli* biofilms especially at the level of nutritional/metabolic and proteome analysis. Our study provides additional details from a less explored perspective about *E. coli* biofilm formation, its growth over longer periods (3-21 days) and functional and metabolic differentiation that will be useful for future studies and for better understanding of baterial biofilms.

Specific output

The results presented in this report highlight some of our important findings. We presented our results at several conferences in both domestic and international settings. Two publications are in preparation and will report on the above described results regarding biofilm development and gene expression findings as well as the development of the our original imaging system. A symposium on the topic of microbial multicellularity organized by the PI will be held in November 2024 in Fukuoka during the 47th annual meeting of the Molecular Biology Society of Japan (November 27-29, 2024).

Acknowledgements

The PI is very grateful to Prof. Hirotada Mori for providing research equipment for this project and for numerous discussions. We thank the National BioResource Project (NBRP) for providing most of the *E. coli* bacterial strains used in this project. We also wish to thank Prof. Regine Hengge (Humboldt U.) and Knut Drescher (Biozentrum, U. Basel) for providing the AR3110 strain and their derivatives.

References

1. Serra, D. O., Richter, A. M. & Hengge, R. Cellulose as an Architectural Element in Spatially Structured Escherichia coli Biofilms. Journal of Bacteriology 195, 5540–5554 (2013).
2. Serra, D. O., Richter, A. M., Klauck, G., Mika, F. & Hengge, R. Microanatomy at Cellular Resolution and Spatial Order of Physiological Differentiation in a Bacterial Biofilm. mBio 4, 10.1128/mbio.00103-13 (2013).
3. Nuñez, I. et al. Low cost and open source multi-fluorescence imaging system for teaching and research in biology and bioengineering. PLOS ONE 12, e0187163 (2017).
4. Zaslaver, A. et al. A comprehensive library of fluorescent transcriptional reporters for Escherichia coli. Nat Methods 3, 623–628 (2006).
5. Rappsilber, J., Mann, M. & Ishihama, Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. Nat Protoc 2, 1896–1906 (2007).
6. Tyanova, S. et al. The Perseus computational platform for comprehensive analysis of proteomics data. Nat Methods 13, 731–740 (2016).
7. Díaz-Pascual, F. et al. Spatial alanine metabolism determines local growth dynamics of Escherichia coli colonies. eLife 10, e70794 (2021).

５．主な発表論文等

〔雑誌論文〕　計0件

〔学会発表〕　計12件（うち招待講演　1件／うち国際学会　2件）

１．発表者名 Robert M.
２．発表標題 Opening and closing the box of bacterial metabolic function: from enzyme discovery to adaptive evolution and biofilms using E. coli.
３．学会等名 Annual Meeting of Japan Society for Environmental Biotechnology. Tokyo（招待講演）
４．発表年 2022年

１．発表者名 Fujino Y. Sakai, R. and Robert M.
２．発表標題 Exploring transitions in metabolic state during the development of bacterial biofilms.
３．学会等名 RIKEN BDR Symposium 2023 “Transitions in Biological Systems”. Kobe（国際学会）
４．発表年 2023年

１．発表者名 Fujino Y. and Robert M.
２．発表標題 Exploring developmental processes in growing E. coli biofilms
３．学会等名 ゲノム微生物学会
４．発表年 2021年

１．発表者名 Robert M.
２．発表標題 Exploring patterns of growth and differentiation during E. coli biofilm formation.
３．学会等名 日本農芸化学会関西支部 第525回講演会
４．発表年 2023年

１．発表者名
酒井 隆之介, Zhao Yifan, Robert M

２．発表標題
明視野および蛍光モードでバイオフィルムの動態を捉える タイムラプスイメージングシステムの開発

３．学会等名
第19回 21世紀大腸菌研究会

４．発表年
2023年

１．発表者名
Zhao Yifan, Sakai R., Fujino Y., Hoshiba Y., Robert M.

２．発表標題
Investigating the role of YccA on E. coli biofilm growth and development

３．学会等名
第19回 21世紀大腸菌研究会

４．発表年
2023年

１．発表者名
干場 悠介, 藤野 祐紀子, Zhao Yifan, 酒井 隆之介, Robert M.

２．発表標題
Effects of glucose on the growth of E. coli biofilms in rich medium

３．学会等名
第19回 21世紀大腸菌研究会

４．発表年
2023年

１．発表者名
Robert M.

２．発表標題
E. coli biofilms as a developmental model

３．学会等名
第19回 21世紀大腸菌研究会

４．発表年
2023年

１．発表者名
Robert, M.

２．発表標題
Biofilming beyond B. subtilis

３．学会等名
BACELL 2023（国際学会）

４．発表年
2023年

１．発表者名
Sakai. R, Zhao, Y, and Robert, M.

２．発表標題
Development of imaging system to track biofilm morphology and temporal gene expression in growing biofilms.

３．学会等名
The 46th Annual Meeting of the Molecular Biology Society of Japan

４．発表年
2023年

１．発表者名
Zhao, Y and Robert, M.

２．発表標題
Investigating the role of YccA on E. coli biofilm growth and development.

３．学会等名
The 46th Annual Meeting of the Molecular Biology Society of Japan

４．発表年
2023年

１．発表者名
Fujino. Y and Robert, M.

２．発表標題
Exploring culture conditions and fractionation methods for spatio-temporal analysis of E. coli biofilms.

３．学会等名
The 46th Annual Meeting of the Molecular Biology Society of Japan

４．発表年
2023年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

―

6．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考
研究協力者	藤野　祐紀子 (FUJINO Yukiko)		
研究協力者	酒井　隆之介 (SAKAI Ryunosuke)		
研究協力者	趙　一帆 (ZHAO Yifan)		
研究協力者	干場　悠介 (HOSHIBA Yusuke)		

7．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

8．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関