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研究課題名（英文）Visualization of metabolic dynamics during pattern formation in bacteria

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研究成果の概要（和文）：大規模コロニーと固体媒質上でのバイオフィルムパターン中で大腸菌が 成長する過程における動的遺伝子発現のパターン形成の研究を行った。蛍光たんぱく質を用いて、これらのコロニー構造の中で、遺伝子発現の時空間的分布を調べた。
その結果、大腸菌は栄養的な条件と寒天培地の硬さに応じて様々なタイプの構造を形成できるということが示された。これらの要因に依存して、折りたたみや環状構造がコロニーで形成されて遺伝子発現のパターンが観測された。我々の研究から、大腸菌コロニーの複雑な時空間的組成が明らかとなり、大規模な集団の中では個々では遺伝子的に同一な細胞が異なった生態的活動を見せることを示している。

研究成果の学術的意義や社会的意義
Understanding bacterial colonies and biofilms is important as they represent communities as they occur in nature and in host organisms and are a major challenge for drug resistance some industrial processes. Our results reveal the emergent complexity of gene expression within these structures.

研究成果の概要（英文）：We studied pattern formation and the dynamics of gene expression in the bacterium E. coli growing into large colony and biofilm structures on solid media. We tested various environmental conditions leading to different patterns and then investigated the spatio-temporal distribution of gene expression within those structures using fluorescent protein reporters.

Our results show that E. coli can form various types of structures depending on agar hardness and nutritional conditions. The resulting colonies develop complex wrinkle and ring structures in which the profiles of gene expression vary and can result in preferential expression in ring-like structures near the center or periphery of the colony or within wrinkles. Our work thus reveals the complexity of spatio-temporal organization in colonies and suggests that bacterial cells that are otherwise genetically identical can display division of labor leading to different physiological properties within large collectives.

研究分野：生化学、微生物学

キーワード：gene expression　bacterial colony　biofilm　division of labor　time-lapse fluorescence　differentiation　pattern formation　metabolic activity

１．研究開始当初の背景

Complex patterns are apparent everywhere in nature, from plant/flower shapes to the skin of animals. They are exquisitely demonstrated during the development of multicellular organisms from a single cell. However, such patterns can also be produced by bacteria growing on semi-solid media (agar) and during biofilm formation when these colonies produce extracellular polysaccharides and proteins that convey specific adhesive properties to the colony. The development of these structures is usually dependent on the hardness or the surface properties of the supporting medium and the nutritional conditions. Because the secretion of amino acids has been shown to be important for formation of these spatio-temporal structures there thus appears to be a functional link with metabolic activity (energy and biomass production). Moreover, we showed how in well-mixed cultures *Escherichia coli* (*E. coli*) cells secrete multiple metabolic products and that this can lead to temporal patterns in cell populations (Robert et al., 2012). This suggests that such interactions are likely to affect cell-cell interactions in space too and thus be relevant for pattern formation. More recently, oscillations have been observed to occur during biofilm growth and found to be dependent on the metabolic state of cells and local nutrient availability, thus also suggesting that exchange of metabolites are occurring within the colony (Liu et al., 2015). The formation of complex structures in colonies and biofilms has been previously shown to depend on environmental factors and cell motility. However, relatively little is known about how pattern formation and metabolic activity relate and affect each other and how gene expression varies during these events.

Insights into these processes can provide evidence of functional differentiation within large population in colonies and also help characterize and understand the properties of biofilms. Moreover, although this work is based on a simple bacterial system, the basic principles by which cell-populations organize are generally well conserved and it is expected that some of the results can be applicable to other more complex forms of multicellular and developmental processes including tissues and even cancer.

２．研究の目的

The main objective of this project was to explore the connections between metabolic function and pattern formation in bacterial colonies and biofilms. In addition, we aimed to obtain evidence for possible differentiation and division of labor with macro-colonies. To do this, we visualized metabolic gene expression dynamics in growing colonies of *E. coli*. The study mainly focused on producing a variety of colony and biofilm patterns by varying the nutritional and agar properties of the supporting growth medium and on visualizing patterns of gene expression in both space and time during the formation of large colonies and biofilms.

Processes occurring within large colonies and biofilms appear similar to differentiation principles that also occur in other complex organisms. Studying structural and functional differentiation in populations of interacting bacterial cells is thus an important goal and it is hoped that our project might also suggest ways in which a simpler model that can be easily manipulated could be useful in other fields. By collecting spatio-temporal patterns of gene expression we hoped to highlight some of the complexity and high level of organization in large communities of interacting individuals such as bacterial colonies and biofilms.

３．研究の方法

The main methods used for the project were based on bacterial liquid pre-cultures followed by spotting onto agar plates, of different composition, to produce various colony and biofilm patterns. Most conditions tested were based on modifications of existing ones (Serra et al., 2013.). We made use of various strains of *E. coli* including wild-type K12 strains, their derivatives, and strains where specific genes had been modified to produce fusions to yellow fluorescent protein (YFP), provided by the laboratory of H. Mori. We also used a large collection of *E. coli* strains, each carrying a single plasmid-based transcriptional promoter controlling the expression of green fluorescent protein (GFP) (Zaslaver et al., 2006, commercialized by Dharmacon Inc.). Both agar hardness and the composition of nutrients in the culture medium were manipulated. Colony growth dynamics and the associated changes in gene expression were monitored using time-lapse imaging in bright field and/or in fluorescence mode to capture and localize fluorescence signals within growing colonies. For this, we used macro imaging of colonies using either a flatbed desktop scanner or a digital SLR camera placed above a blue LED system on which cultures dishes were placed. We collected still images every 20 mins for several days (usually 3 to 7 days). When desirable, a fluorescence microscope was used offline to obtain finer localization details.

４．研究成果

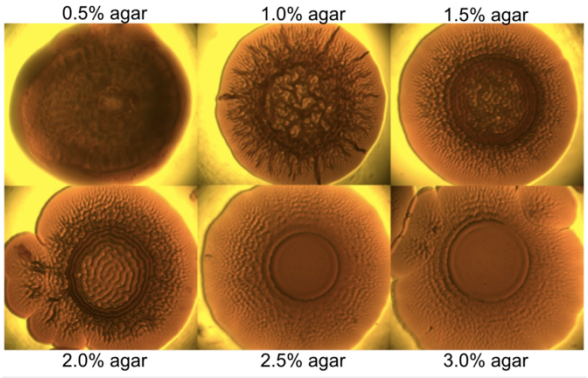
Overall, our results provide some of the first line of evidence, using multiple target reporters of gene expression, supporting complex dynamics of gene expression and functional differentiation within *E.*

coli colonies and biofilms. More details are provided below.

Colony and biofilm pattern formation under various environmental conditions

We first spent efforts to produce a variety of patterns on different agar compositions (hardness and nutrient). Most of the data were collected on Luria-Bertani (LB) medium (with or without additional carbon source) containing between 0.5 to 1.5% agar and incubated at 37°C. We used conditions similar to those described elsewhere (Tokita et al., 2009) to produce eden-like, ring type and DLA-like patterns at 37C. However, in most cases, these patterns were either too slow to develop or difficult to reproduce.

We therefore chose to test alternative conditions using salt-free LB agar on either 0.5 or 1.5% agar at 28°C, conditions known to promote the production of the protein curli important for biofilm formation (Serra et al., 2013). These structures had the advantage of being easier to produce, forming relatively rapidly over 3-5 days and showing the desired level of structural complexity. At first, we mainly used the W3110 wild-type strain and were able to obtain different structures (see the figure on the right) depending on the agar concentration.



Patterns of gene expression in growing colonies and biofilms

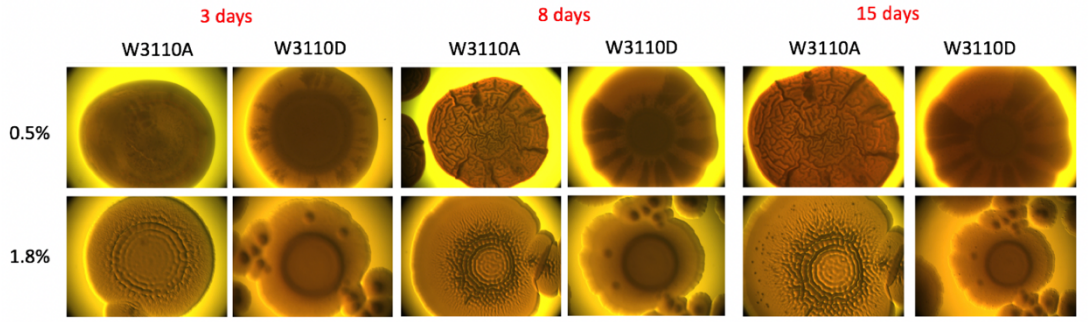
Once we successfully obtained different types of pattern, we started imaging gene expression within colonies using a set of yellow-fluorescent protein (YFP) fusions to metabolic genes representing the TCA cycle, catabolite repression and acid metabolism (crp, icd, ackA, and rpoS).

We detected and imaged expression for these and other metabolic genes and regulators on different agar concentrations (mainly 0.5 and 1.8%) as well as different nutritional conditions (LB with or without addition of glucose or glycerol). Both qualitative and quantitative differences were observed in both pattern shape and development as well as gene expression intensity (data not shown). Many genes patterns appeared to first be expressed in the region where cell suspensions are originally inoculated or plated and expression appeared to propagate radially, with different dynamics. In addition, we observed in some cases interesting waves of gene expression which reflect a specific space-time dependent induction of expression. This process is likely due to dynamically changing local availability of nutrients and other factors that induce wave-like patterns of gene expression and growth that propagate radially from the center of the colony. Although we are not able to clarify the process in details, it is also possible that such propagation is triggered by biochemical and/or bio-mechanical interactions between cells within the colony.

In some cases, genes appeared to be relatively uniformly expressed and the main variation in signal intensity within the colony appeared to be linked to cell density. As such these signals may reflect the higher density of cells rather than higher gene expression level. However, it has not been yet possible to assign clearly the respective contribution of cell density and the actual scale of gene expression to the overall fluorescence signals. For this some form of normalization of signals normalization would be required (using cell density measurement with other reporters, for example). However, we could also observe other more specific patterns that emerge and that can't be simply explained by variations in cell density.

One phenomenon we observed while performing the above experiments what that whereas the W3110 strains consistently produced the expected complex pattern structures, we struggled to reproduce such structures using the YFP fusion strains. We originally thought it was a reproducibility or technical issue, but multiple attempts consistently led to similar results. We eventually traced the strain details and found out that the host strain used to produce the fusion proteins (sub-strain W3110D) was a different variant from the sub-strain W3110A that we had been using to produce the colony patterns. The most significant difference is that the W3110D strain carries mutations in the rpoF gene that affect its expression, resulting in defects in flagellar protein biosynthesis and thus cell motility (Jishage and Ishihama, 1997). We confirmed the difference in colony phenotypes between the two strains on two different agar concentrations (see the top figure on the next page).

We observed important differences in colony structure. The more amorphous and smooth appearance of the W3110D colonies when compared to the presence of radial wrinkles and rings in the W3110A strain (on 0.5% and 1.8% agar, respectively) can therefore be most likely explained by lack of motility in this sub-strain.



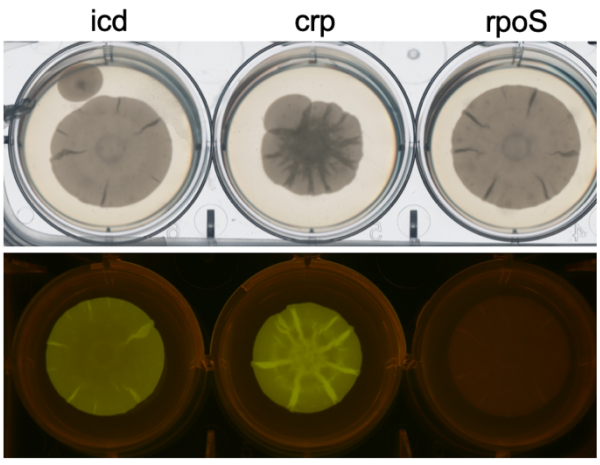
In spite of those difficulties, we nonetheless obtained interesting results using those strains that and observed a variety of gene expression pattern that were affected by the presence of glucose and glycerol (not shown). A decrease in the expression of crp, for example, was clearly observed within the colony in the presence of glucose as could be expected.

Effect of cyclic-dinucleotides on pattern formation

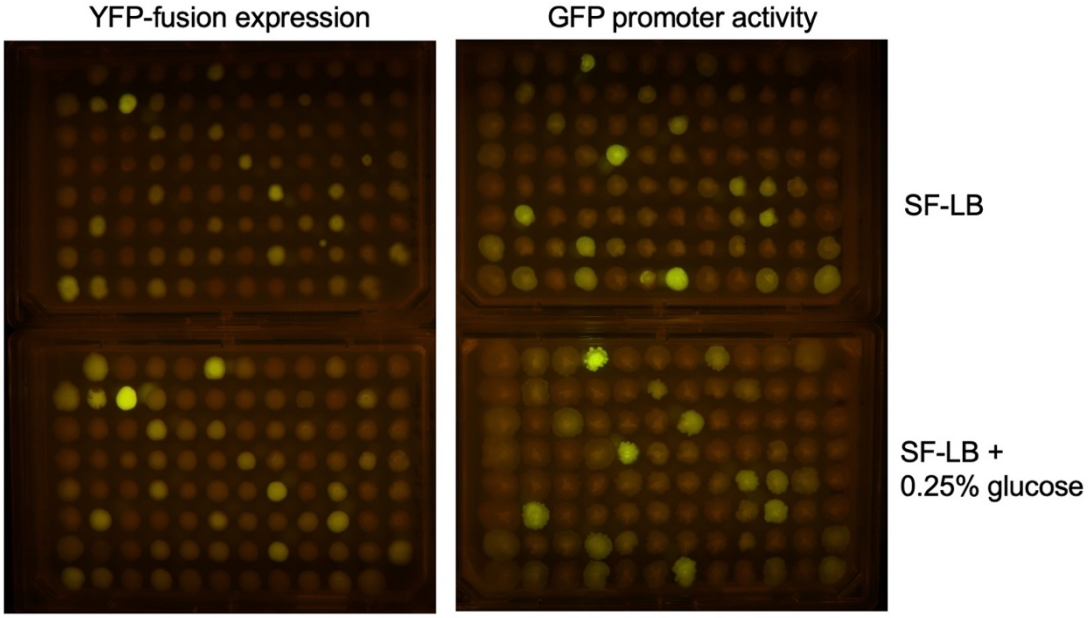
Because c-di-nucleotide signaling has been reported to play a role in bacterial motility and in the formation of biofilms, we were interested in testing whether analogs of c-di-GMP and c-di-AMP synthesized in the laboratory of H. Isobe (Fujino et al., 2014) would have an effect on colony or biofilm formation in our system. However, after several trials using different concentrations of the analogs (up to 10 uM) we could not detect any clear and reproducible effects on the pattern formation process. While we can't exclude the possibility that externally added c-di-nucleotides might have an effect, at least under the conditions tested here, (either added to the agar preparation, added to the bacterial suspension or directly spotted on top of agar) we did not observe a clear effect. If potentially active, such compounds were either not sufficiently soluble or abundant to reach their target on or within cells.

Imaging of gene expression using transcriptional promoter strains

Since there was no easy and quick solution to the problem for the several dozens of YFP fusion proteins, we considered possible alternative experimental resources. We decided to test a collection of transcriptional promoter strains that express a GFP proteins under the control of over 2000 native *E. coli* promoters (Zaslaver et al., 2006). The host strain is a K-12 derivative MG1655 that different but closely related to the W3110 we had used for previous experiments. Using this strain under similar conditions to those or the prior experiments we were able to reproducibly generate patterns displaying the expected type of complex structures similar to those obtained using sub-strain W3110A. At first, we analyzed the dynamics of expression for three selected genes (icd, crp and rpoS) on salt-free LB medium (0.5% agar) supplemented with 0.25% glucose. The 7-day end point patterns are shown on the figure on the right, in bright field (top) and fluorescence (bottom) mode. Note that while the two panels show images of the same strains, they represent different colonies grown in parallel, so that the exact shape varies a bit. Expression of both icd and crp, two metabolism-related genes, is readily detected over the whole colony with prominent signals in specific radial wrinkles and ring structures. On the other hand, rpoS gene expression was undetectable under the same conditions.

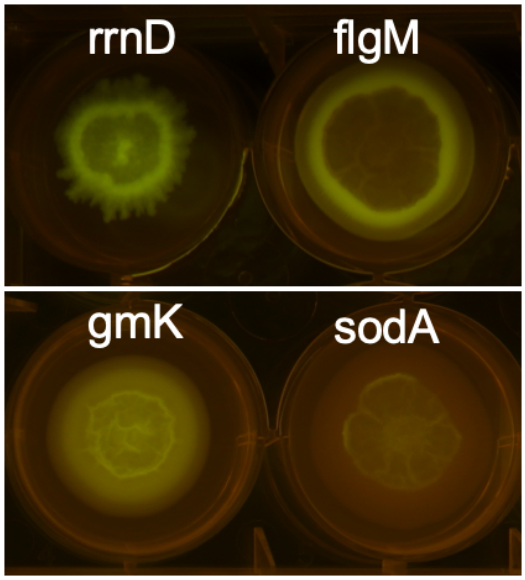


Using this larger promoter collection, we were able to screen a wider range of metabolism-related genes as well as other categories of genes (structural, membrane proteins, motility-related, etc.). We also compared some expression signals between both collections (YFP-fusion and GFP promoter) and at least for a few of the genes tested that were common to both collections the qualitative patterns of expression were similar, although actual colony structure varied due to the reasons described above.



As seen in the above figure, good fluorescence signals were obtained for many genes in both collections on salt-free LB (SF-LB) medium in the absence (top panels) or presence (bottom panels) of 0.25% glucose. Some examples of strains in which glucose either activates or represses expression are clearly visible. Note that the identity of the promoter strain is not shown, and that the left and right panels do not correspond to the same strains and therefore left-right panel comparisons are not possible. The above figure shows the results of an experiment using a high-density, 96-well array of colonies. Because this format produces much smaller colonies (5 to 8 mm in diameter), some complex pattern structures are less apparent than in the low-density format (figure below) where colonies reach diameters of 2 to 5 cm.

We therefore confirmed selected interesting findings in a secondary, low-density format to better evaluate localization differences. An example is shown in the figure on the right for four different genes, showing different patterns of gene expression. In such a way, we generated a series of spatio-temporal patterns of gene expression for several dozens of different promoters (not shown). Overall, the results show that gene expression varies in both intensity and localization within different parts of the colony and that cells in these different locations are likely interacting in complex ways, exchanging resources and information in ways that remain to be established. The results shown here are for only a small but representative subset of the data collected. We plan to publish more details of our findings elsewhere.



Finally, due to the unexpected challenges in producing suitable patterns for analysis of gene expression by fluorescence that were described above, as well as some other technical details, we were unable to pursue one aspect of our original proposal. Specifically, this refers to the mapping of metabolites in an around colonies using mass spectrometry methods. While we have not been able to analyze this directly yet, from our results, we can at least infer that the observed propagation of bacterial cells and gene expression within the growing colony that generated wave-like patterns likely reflects dynamical gradients of some metabolites. We hope that this additional and complementary component of the project can be addressed in future experiments.

Overall, during this project, we have been able achieve most of our original objectives and show that a simple bacterial model can reveal complex spatio-temporal patterns of gene expression that reflect the metabolic state of cells in different parts of the colony. Such findings provide information about the cellular diversity within large colonies and biofilms. Since basic principles of organization appear very similar to those in more complex organisms some of our results may also be relevant to developmental processes in multicellular organisms, including plants and animals.

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５．主な発表論文等

〔雑誌論文〕（計　　1 件）

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② Zhou X., Shoji W., Robert M. Gene expression patterns in growing *E. coli* biofilms and macro-colonies. E. coli systems biology workshop: Annual meeting in Awaji. Awaji Yumebutai, Hyogo, Japan, March 13-15, 2019
③ Robert M. Patterns of metabolic gene expression within *Escherichia coli* biofilms and macro-colonies. Advances in Physics of Emergent orders in Fluctuations (APEF2018), The University of Tokyo, Tokyo, Japan, November 12-15, 2018
④ Zhou, X, Shoji W., and Robert M. Metabolic activity within *Escherichia coli* macrocolonies and biofilms. The 19th International Conference on Systems Biology (ICSB 2018), Lyon, France, October 28-November 1, 2018
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６．研究組織

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