論文の内容の要旨

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論文題目

Elucidation of the plasmid effect on the host at single-cell level
(プラスミドが宿主に及ぼす影響の一細胞レベルでの解明)

Chapter 1. Introduction

Plasmids are circular or linear extrachromosomal replicons which are found in many microorganisms. Plasmids can provide functional benefits to the host such as antibiotic-resistance and novel metabolic capacity for xenobiotics. Plasmids are able to spread such functional genes horizontally among bacteria through conjugative transfer, and promote the rapid evolution and adaption of bacteria (Trevors, 1986). Conjugation is the process by which DNA is transferred from one bacterium to another via cell-cell contact or a bridge-like connection between two cells. Many studies have clarified the details of the plasmid effects on host cell physiology, suggesting that the host factors possibly affect the recipient range and conjugative transfer frequency of the plasmid (Shintani *et al.*, 2014) and that the factors expressed from transferred plasmids may affect the expression network of their host chromosome (Takahashi *et al.*, 2015). However, all of these studies were done at population level, and this may hide some variation among the individual cells. That is the averaged data cannot show the diversity of the responses in individual cells, and therefore, single cell level analysis will be necessary. Meanwhile, previous studies on crosstalk between plasmids and their host were performed with plasmid-harboring strains after long-term cultivation in which the host were presumably fully adapted to the plasmid, the interaction among the adaptation process to the conjugated plasmid is still unknown. The objective of my research is to uncover the plasmid effect on the host during plasmid adaptation process (Fig.1) at single-cell level. For this purpose, I employed two techniques, one is a combination of fluorescence-labelling system and flow cytometry, another is based on the label-free and non-invasive Raman spectroscopy.

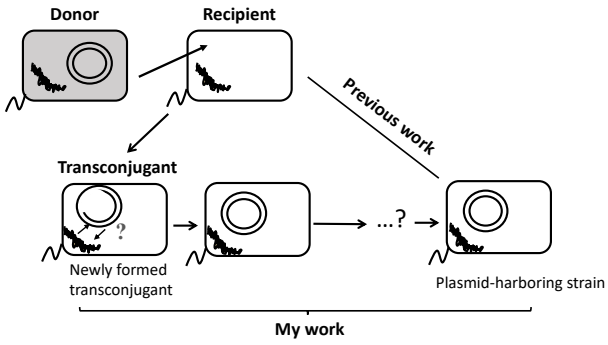

Fig. 1. Hypothetical diagram of plasmid adaptation process after conjugative transfer. Conjugation requires contact (mating process) between donor (plasmid-harboring) and recipient (plasmid-free) cells, leading to

formation of transconjugants (recipient cell acquired plasmid). Newly formed transconjugants were cultured, and then they will be “plasmid-harboring strain” after plasmid-host adaptation.

Chapter 2. Elucidation of the host adaptation process after plasmid conjugation using transcriptome analysis

In this chapter, 2-hour mating assay was performed with *P. putida* SM1443*(*pBP136*::gfp*) (donor) and *P. putida* KT2440RG (recipient). In this system, *gfp* gene conceding reporter protein GFP was encoded on incompatibility group (Inc) P-1β plasmid pBP136*::gfp* under the regulation of *lac* promoter. GFP expression was repressed by LacI in donor and was derepressed in transconjugant. Flow cytometer was introduced to detect and separate 10^7 of newly-formed transconjugant cells. Part of the collected cells (named “2-h”) were continually cultured for several days (transfer culture to fresh medium every day). Log phase of “1-day” and “3-day” cultures were collected as plasmid-adaptive-process samples. Transcriptome analysis was performed on the duplicated samples of recipient cells (KT), newly formed transconjugant cells (2-h), adaptive-process cells (1-day and 3-day) and 3-day transconjugant with undergoing cell sorting (3-day-sort) respectively. Compared to transcriptome of 3-day cells, there is a significant change in 3-day-sorted cells which indicated that flow cytometric cell sorting has effect on transcriptome analysis (Fig. 2). Initially, I want to explore host adaptation process start from 2-h transconjugant, however, due to effect of cell sorter, it was only used to compare with 3-day-sort transconjugant. A total number of 242 differentially expressed genes (DEGs) were discovered between the two samples, the mainly up-regulated genes are related to signal transduction mechanisms protein and mainly down-regulated genes are related to inorganic ion transport and metabolism (Table 1). The transferred plasmid has drastic effect on host indeed, compared to recipient, a total number of 1475 and 1404 DEGs were discovered from 1-day and 3-day transconjugants respectively. Function of differential expressed genes in the 2 combination are the similar. Meanwhile, the gene expression between 1-day to 3-day transconjugant are not changed too much, it may indicates plasmid adaptation process nearly complete, and the sampling points should be set more earlier to catch more intense period during plasmid host adaptation in the future work.

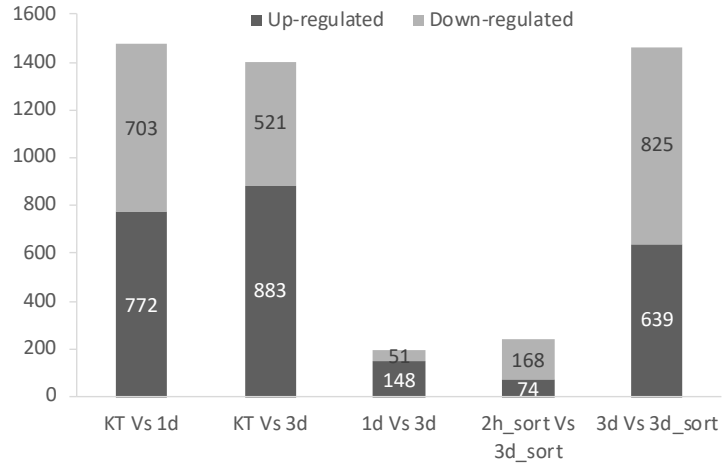

Fig. 2. Up-, down- regulated genes detected during plasmid-adaptation process samples. KT Vs 2h: gene expression of 1-day transconjugant was compared with it of KT, so do others.

Table 1. Distribution list of main differentially expressed genes based on COG classifier.

	COG	All	UP-regulated	All %	COG	Down-regulated	All %
KT Vs 1d	-	1104	91	8.2	-	172	15.6
	E	480	90	18.8	E	71	14.8
	C	274	75	27.4	S	69	16.4
KT Vs 3d	-	1104	129	11.7	-	113	10.2
	E	480	75	15.6	E	61	12.7
	C	274	67	24.5	S	55	13.1
1d Vs 3d	-	1104	63	5.7	E	14	2.9
	L	200	14	7.0	R	7	1.5
	S	420	14	3.3	-	6	0.5
2h_sort Vs 3d_sort	-	1104	22	2.0	-	33	3.0
	T	232	8	3.4	P	20	7.8
	E	480	7	1.5	C	14	5.1

[C] Energy production and conversion, [E] Amino acid transport and metabolism, [L] Replication, recombination and repair, [P] Inorganic ion transport and metabolism, [R] General function prediction only, [S] Function unknown. [T] Signal transduction mechanisms.

Chapter 3. Discrimination of plasmid-harboring and -free strains using single-cell Raman spectroscopy

Raman spectroscopy, label-free and non-invasive detection technique, has already been used in several studies on single cells(Li *et al.*, 2012). In this part, on the basis of Raman spectroscopy techniques, I developed a method to distinguish between plasmid-harboring and -free bacterial strains at the single-cell level without harming or modifying cells. *P. putida* KT2440 and *E. coli* W3110 were used as the hosts for the plasmid pB10 and plasmid RP4. Each strain with or without plasmid pB10 or RP4 was cultured in minimum medium supplied by glucose or succinate as the sole carbon source. Raman spectra of single cells from log and stationary phase were recorded, denoised using Singular Value Decomposition (SVD), and followed by classification of plasmid-harboring and -free strains using Random Forest (RF) algorithm (Lee *et al.*, 2013). For discriminating, half of spectra (30-50) of each strain were selected to constructing training dataset to building the classification model and the remaining half spectra were used to forming test dataset to validating the classification accuracy. Applicaiton of the combination of Raman spectroscopy technique and RF algorithm to *P. putida* KT2440 strains at both log and stationary phases successfully discriminated plasmid-free strain from pB10- or RP4-harboring strains (Fig. 3). On the other hand, I was also able to distinguish plasmid-free *E. coli* W3110 strain from pB10- or RP4-harboring strains with Raman spectra at log phase, but failed to distinquishing these strinas at stationary phase. I am still looking for the accurate reason.

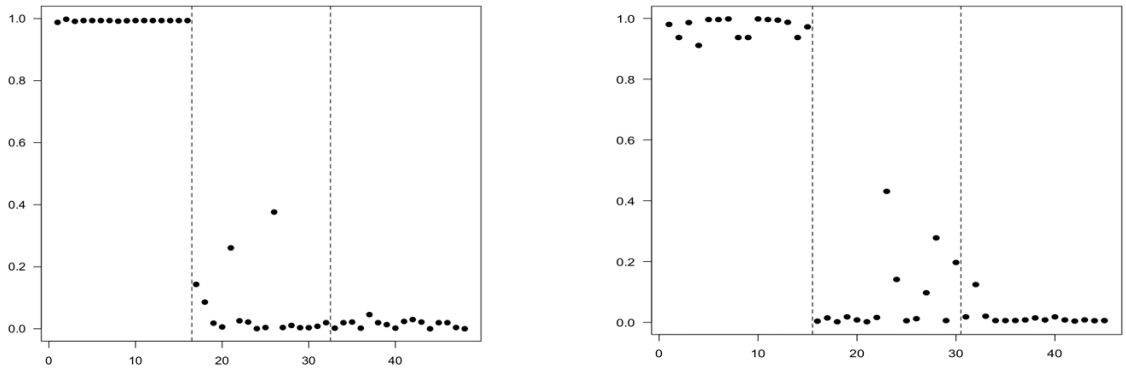

Fig. 3. Score plot of validation result from spectra of *P. putida* KT2440 strains of log-phase (left) and stationary phase (right) samples. X axis: 3 parts from left to right represent to *P. putida* KT2440, *P. putida* KT2440(RP4), *P. putida* KT2440(pB10) respectively. Y axis: reliability score of the plasmid-free, (1 means plasmid-free and 0 means plasmid-harboring). Each dot represents validation score of one Raman spectrum from a single cell.

Chapter 4. Summary and future prospect

Compared to previous study, my study is the first time to explore plasmid effect on host specially for plasmid adaptation process. I explored two techniques for biological analysis at single cell level. With the combination of flow cytometry and RNA-seq analysis, I found that flow cytometric cell sorting change transcriptome of cells with large extent. This result indicates that it can be solved by applying same treatment of each sample with cell sorter in the future. Furtherly, the earlier sampling point (lag phase before 1-day sampling) should be set to exploring host adaptation process. To this end, single (or a smaller number of) cell transcriptome technique should be combined, because current cell sorting cannot be adopted to gather enough cell numbers for RNA extraction. RNA extraction method should be modified to obtain RNA from less cells like 10^5 even 10^3 cells in the future work. On the other hand, I successfully distinguished the plasmid-harboring cells from plasmid-free cells with Raman spectra. It provides the possibility to detecting the plasmid transfer using Raman spectroscopy in future. If single cell-picking technologies, such as laser microdissection or optical tweezer, are combined, this new method can be applied to clarify the interaction between plasmid and host cell at the single cell level without any sampling preparation or modification of cells such as GFP-labeling or mutation construction.