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Genome-wide screen for *Escherichia coli* genes involved in repressing cell-to-cell transfer of non-conjugative plasmids

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ABSTRACT

Acquiring new genetic traits by lateral gene transfer is a bacterial strategy for environment adaptation. We previously showed that *Escherichia coli* could laterally transmit non-conjugative plasmids in co-cultures containing strains with and without the plasmid. In this study, using the Keio collection, a comprehensive library of *E. coli* knock-out mutants for non-essential genes, we screened for genes responsible for repressing cell-to-cell plasmid transfer in recipient cells. By stepwise screening, we identified 55 'transfer-up' mutants that exhibited approximately 2- to 30-fold increased activities. We confirmed plasmid acquisition by these 'up' mutants and revealed that there were no significant changes in antibiotic resistance in the original Keio strains. The presumed functions of these gene products covered a wide range of activities, including metabolism and synthesis, transport, transcription or translation and others. Two competence-gene homologues (*ybaV* and *yhiR*) were identified from among these genes. The presumed localizations of these 55 gene products were estimated to be 34 cytoplasmic proteins, 20 in or around the cell surface and 1 unknown location. Our results suggest that these 55 genes may be involved in repressing plasmid uptake during cell-to-cell plasmid transfer.

1. Introduction

Lateral gene transfer between bacterial cells contributes to bacterial adaptations to varying environmental conditions and ultimately results in bacterial evolution [1–3]. However, in human environments, this results in the undesirable spread of pathogenic, antibiotic resistance or artificially engineered genes [2,4–8]. There are three known mechanisms for lateral gene transfer in bacteria: conjugation, transduction and transformation [2]. For DNA transfer from donor to recipient cells, conjugation and transduction involve specific structures, i.e. conjugative pili and phage capsids, respectively. However, transformation is primarily performed by recipient cells that express genetic competence for the uptake of extracellular free DNA [9,10]. Transformation competence can be induced both naturally and artificially, although not all bacterial species develop natural competence [1,9,10].

Escherichia coli is not assumed to be transformable under natural conditions, although it can develop high genetic competence under artificial conditions such as exposure to high Ca^{2+} concentrations [11,12]. Recent reports have shown that *E. coli* can express modest genetic competence under certain conditions that may arise in its environment [13–20]. Relevant to these findings, we found that spontaneous lateral transfer of non-conjugative plasmids occurred in an *E. coli* mixed-cell culture [21–23]. Based on subsequent analyses, we hypothesized that this cell-to-cell plasmid transfer resulted from the transformation of plasmid DNA released from co-cultured cells [23]. However, the detailed molecular mechanisms for this process, including the genes involved, remain uncertain.

In this study, we used the Keio collection established by Baba et al. [24] to explore for genes responsible for repressing cell-to-cell plasmid transfer in recipient cells. The Keio collection is a comprehensive library of *E. coli* knock-out mutants for 3985 non-essential genes, which constitute 90% of all the genes in the *E. coli* K-12 genome. This collection was previously used in several genome-wide screens for genes involved in various cell functions [25–27]. To apply the Keio collection to screen for genes involved in cell-to-cell plasmid transfer, we devised a 96-well microplate assay system for cell-to-cell plasmid transfer [28]. Then, using the Keio strains as plasmid recipients, we screened for 'transfer-up' mutants (hereafter referred to as 'up' mutants) that promoted

Abbreviations: cam, chloramphenicol; tet, tetracycline; kan, kanamycin; PEG, polyethylene glycol; TSB, Tryptic Soy Broth.

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plasmid acquisition. The knock-out genes among these 'up' mutants were considered candidates for repressing cell-to-cell plasmid transfer. Here we present our screen results and our analyses for 55 'up' mutants.

2. Materials and methods

2.1. E. coli strains, plasmids and materials

CAG18439 [29] (MG1655 derivative; F^-, λ^-, *lacZ118(Oc), lacI3042::Tn10(tet^r), rph-1*), BW25113 [24,30] (F^-, *rrnB, ΔlacZ4787, HsdR514, Δ(araBAD)567, Δ(rhaBAD)568, rph-1*), Keio strains [24] (BW25113 derivatives, Δ*(single gene)::kan^r*), pSY510 and pGBM1 were obtained from the National BioResource Project (NIG, Japan): *E. coli* (http://www.shigen.nig.ac.jp/ecoli/strain/top/top.jsp). Plasmids pHSG399-F6 (*cam^r*, pMB1 origin) and pUC19-tet (*tet^r*, pMB1 origin) were constructed as described previously [31]. Chloramphenicol (cam), tetracycline (tet), polyethylene glycol (PEG; molecular mass = 8000) and Luria–Bertani powder (LB, Lennox) were purchased from Sigma. Tryptic Soy Broth (TSB) was from Becton, Dickinson. Distilled water (DNase- and RNase-free, molecular biology grade) and kanamycin (kan) were from Invitrogen. Microplates (96-well) and pin replicators were purchased from Nippon Genetics. Nylon66-membrane filters (pore size: 0.45 μm, Biodyne A) were from Pall. Agar powder (guaranteed reagent grade) and other general reagents were from Wako.

2.2. Screen for 'up' mutants involved in cell-to-cell plasmid transfer

A screen for 'up' mutants from among the Keio strains as recipient cells used 96-well microplates according to a previously described protocol [28] with some modifications. The main modification was decreasing the number of cells inoculated into the first selection medium. Under this condition, most of the Keio mutants resulted in no positive wells in which transformed cells grew. Therefore, 'up' mutants were effectively screened by counting the number of positive wells. More screening details are provided below.

To screen for 'up' mutants in 96-well microplates, transformants were selected twice in liquid media containing two antibiotics. Plasmid-donor cells (CAG18439 harboring pHSG399-F6) were pre-cultured in 10 mL of LB broth (tet: 75 μg/mL; cam: 100 μg/mL) at 37 °C for 22 h. Cultured donor cells were recovered by centrifugation and suspended in 7 mL of LB broth. Recipient cells (each Keio strain) were pre-cultured at 37 °C for 22 h in 200 μL LB broth (kan: 75 μg/mL) in microplate wells, recovered by centrifugation and suspended in 50 μL of the above donor cell suspension. Five microliters of each mixture of Keio and donor cells was inoculated onto TSB agar (1.5%) prepared in microplate wells and cultured in quadruplicates at 25 °C (duplicates) and at 37 °C (duplicates) for 16 h. The cultured cells in wells were suspended in 100 μL of LB broth, small amounts (approximately 0.2 μL) of the suspensions were transferred with a 96-pin replicator to 100 μL of the first selection LB broth containing cam (100 μg/mL for plasmids) and kan (75 μg/mL for Keio strains) and then cultured at 37 °C for 16 h. The same manipulations were repeated for the second selection. The turbidities (OD_{600} values) of the resulting second selection cultures were determined using a microplate reader (Multiskan JX, Thermo Fisher Scientific), and the wells that showed apparent cell growth were counted. These quadruplicate screens were performed twice; thus, we obtained eight results per Keio strain. Under these conditions, most Keio mutants resulted in 0/8 positive wells. The mutants that produced 4/8–8/8 positive wells were regarded as 'up' mutants.

For a quantitative assay for the plasmid transfer in 'up' mutants, mixed culture was carried out with a colony biofilm on TSB agar (1.5%) prepared in a polystyrene plate (ø 90 mm). This was a culture system that was more sensitive for detecting cell-to-cell plasmid transfer, as described previously [21]. Transformants were then colonized and counted on LB agar plates containing two antibiotics (cam: 50 μg/mL; kan: 75 μg/mL). Two Keio mutants (*cysM* and *tyrA*) that showed plasmid transfer frequencies around the average value were used as control strains.

2.3. Plasmid isolation and colony-direct PCR analysis of the produced transformants

To confirm antibiotic resistance and exclude the possibility of contamination by the original strains, double-resistant colonies that appeared were re-streaked onto fresh LB agar containing cam and kan, cultured overnight at 37 °C and then used for plasmid preparation and PCR (Fig. 1).

Plasmids were isolated from the double-resistant transformants that were produced, digested with EcoRI and analyzed by 0.8% agarose-gel electrophoresis using conventional methods [32].

Colony-direct PCR for the double-resistant colonies produced used the primers (ATCGTTCCCACTGCGATGCT, CCAGCAGGCGAAAA TCCTGT) for *lacI*, which is present in the Keio strains' chromosomes but not in the CAG18439 chromosome. PCR reactions using KOD Dash as the PCR enzyme were carried out with an initial denaturation at 94 °C for 4 min, 35 cycles of 30 s at 98 °C, 40 s at 66 °C (−0.1 °C per cycle), 50 s at 72 °C and final extension at 72 °C for 5 min.

2.4. Data analyses for screened genes

Information on the screened genes was obtained from the following databases: PEC (http://www.shigen.nig.ac.jp/ecoli/pec/index.jsp), Uniprot (http://www.uniprot.org/), Echobase (http://www.york.ac.uk/res/thomas/index.cfm), GenoBase (http://ecoli.naist.jp), and Ecocyc (http://ecocyc.org/).

2.5. Natural and artificial transformation of 'up' mutants

Natural transformation of the screened Keio mutants with purified plasmids in liquid and colony-biofilm culture was done as described previously [33]. Artificial transformation of the screened Keio mutants with purified plasmids was performed using three typical methods: $CaCl_2$ method [12,32]; PEG method [32,33]; and electroporation [33]). BW25113 (the original strain of the Keio mutants) and two Keio mutants (*cysM* and *tyrA*) were used as the controls.

3. Results and discussion

3.1. Screen for 'up' mutants involved in cell-to-cell plasmid transfer

To screen for cell-to-cell plasmid transfer 'up' mutants from among 3985 strains of the Keio collection, we adopted a screening system using 96-well microplate [28]. In brief, plasmid-donor cells (harboring a plasmid containing a *cam^r* gene) and recipient cells (individual Keio strains containing a *kan^r* gene on their chromosomes) were cultured on agar media in microplate wells. Then transformants were selected twice by culturing portions of the co-cultured cells in liquid media containing two antibiotics (kan and cam). If Keio strains that had acquired plasmids appeared, then double-resistant cells appeared and selectively grew in these wells. The numbers of positive wells for eight experiments for each Keio strain were counted.