

# Genome-wide screen for *Escherichia coli* genes involved in repressing cell-to-cell transfer of a nonconjugative pSC101-derived plasmid

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**Abstract:** Acquiring new genetic traits by lateral gene transfer is a bacterial strategy for environmental adaptations. We previously showed that *Escherichia coli* laterally transmits nonconjugative plasmids in cocultures that contain strains with or without the plasmid. Using a pMB1-derived plasmid and the Keio collection, a comprehensive library of *E. coli* knockout mutants for nonessential genes, we recently screened for genes responsible for promoting or repressing cell-to-cell plasmid transfer in recipient cells. In this study, we used a pSC101-derived plasmid, instead of a pMB1-derived plasmid, to screen for repressing genes and identified 29 “transfer-up” mutants. Among these, four mutants are common to those previously screened using a pMB1-derived plasmid. Although the roles of the 29 gene products in plasmid transfer mechanism remain uncertain, it is interesting that 28 of the 29 screened genes map to two limited regions on the *E. coli* chromosome: 18 genes at 34.25–35.31 min and 10 genes at 12.62–13.35 min. Because these two regions commonly contain termination (Ter) sites for DNA replication (TerC: 34.64 min and TerH: 12.91 min), it is possible that chromosomal mutations around specific Ter sites may affect plasmid acquisition in the recipient cells.

**Keywords:** Lateral Gene Transfer, Keio Collection, pSC101-Derived Plasmid, Ter Site, *Escherichia coli*

## 1. Introduction

Bacteria adapt to varying environmental conditions through the application of lateral gene transfer between bacterial cells, ultimately resulting in bacterial evolution [1–3]. However, within human environments this results in the undesirable spread of pathogenic, antibiotic-resistant or artificially engineered genes [1, 4–8]. Bacteria use the following three mechanisms for lateral gene transfer: conjugation, transduction, and transformation [1]. For DNA transfer from donor to recipient cells, conjugation and transduction involve specific structures such as conjugative pili and phage capsids, respectively. However, transformation primarily involves recipient cells that express genetic competence for the uptake of free extracellular DNA [9, 10]. Transformation competence can be induced both naturally and artificially, although not all bacterial species develop natural competence [2, 9, 10].

Under natural conditions, *Escherichia coli* is not assumed to be transformable; however, it can develop high genetic competence under artificial conditions such as exposure to high Ca<sup>2+</sup> concentrations [11, 12]. Recent reports showed that *E. coli* could express modest genetic competence under certain conditions that may arise within its environment [13–20]. Relevant to these findings, we demonstrated that spontaneous lateral transfer of non-conjugative plasmids occurred in an *E. coli* mixed cell culture [21, 22]. On the basis of subsequent analyses, it was hypothesised that this cell-to-cell plasmid transfer resulted from a kind of transformation of plasmid DNA released from co-cultured cells [23]. However, the detailed molecular mechanisms underlying this process, including the genes involved, remain unknown.

In recent studies [24, 25], we used the Keio collection, established by Baba et al. [26], to explore the genes

responsible for regulating cell-to-cell plasmid transfer in recipient cells. The Keio collection is a comprehensive library of *E. coli* knockout mutants for 3,985 nonessential genes, which constitute 90% of all genes in the *E. coli* K-12 genome. This collection was used in several genome-wide screens for genes involved in various cellular functions [27–29]. We devised a 96-well microplate assay system for cell-to-cell plasmid transfer and screened eight “transfer-down” mutants [24] and 55 “transfer-up” mutants [25] (hereafter referred to as “down” mutants and “up” mutants, respectively) using the Keio strains as plasmid recipients.

In this study, we screened for “up” mutants using the pSC101-derived nonconjugative plasmid pGBM1 [23, 30], instead of the pMB1-derived plasmid pHSG399-F6, which was used in our previous study [25]. pHSG399-F6 exhibited a high transfer frequency, whereas pGBM1 exhibited a low transfer frequency [23, 31]; therefore, we considered pGBM1 more suitable for screening “up” mutants. In this study, we present our screening results and analyses of 29 new “up” mutants.

## 2. Materials and Methods

### 2.1. *E. coli* Strains, Plasmids and Materials

CAG18439 [32] [MG1655 derivative;  $F^-$ ,  $\lambda^-$ , *lacZ118(Oc)*, *lacI3042::Tn10(tet')*, *rph-I*], Keio strains [26] [ $F^-$ , *rrnB*,  $\Delta$ *lacZ4787*, *HsdR514*,  $\Delta$ (*araBAD*)567,  $\Delta$ (*rhaBAD*)568, *rph-I*,  $\Delta$ (*single gene*)::kan'] and pGBM1 [30] were obtained from the National BioResource Project (NIG, Japan): *E. coli* (<http://www.shigen.nig.ac.jp/ecoli/strain/top/top.jsp>). Tetracycline (tet), streptomycin (str), polyethylene glycol (PEG; molecular mass = 8000) and Luria–Bertani powder (LB, Lennox) were purchased from Sigma. Tryptic soy broth (TSB) was purchased from Becton, Dickinson. Distilled water (DNase- and RNase-free, molecular biology grade) and kanamycin (kan) purchased were from Invitrogen. Microplates (96-well) and pin replicators were purchased from Nippon Genetics. Nylon 66-membrane filters (pore size: 0.45  $\mu$ m, Biotodyne A) were purchased from Pall. Agar powder (guaranteed reagent grade) and other general reagents were purchased from Wako.

### 2.2. Screen for “up” Mutants Involved in Cell-to-Cell Plasmid Transfer

We screened for “up” mutants using the Keio strains as recipient cells using 96-well microplates, according to a previously described protocol [25] with some modifications. 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 pGBM1) were pre-cultured in 10 ml of LB broth (tet: 75  $\mu$ g/ml; str: 100  $\mu$ g/ml) at 37°C for 22 h. Cultured donor cells were recovered by centrifugation and re-suspended in 7 ml of LB broth. Recipient cells (each Keio strain) were pre-cultured at 37°C for 22 h in 200  $\mu$ l of LB broth (kan: 75  $\mu$ g/ml) in

microplate wells, recovered by centrifugation and re-suspended in 50  $\mu$ l of the above donor cell suspension. Each mixture (5  $\mu$ l) of Keio and donor cells was inoculated onto TSB agar (1.5%) prepared in microplate wells and cultured in quadruplicate at 25°C (duplicate) and 37°C (duplicate) for 16 h.

The cultured cells in wells were re-suspended in 100  $\mu$ l of LB broth. Small amounts (approximately 0.2  $\mu$ l) of this suspensions were transferred with a 96-pin replicator to 100  $\mu$ l of the first selection LB broth, which contained str (75  $\mu$ g/ml for plasmids) and kan (75  $\mu$ g/ml for Keio strains), and subsequently cultured at 37°C for 16 h. The same manipulations were repeated for the second selection. The turbidities (optical density 600 nm) of the resulting second selection cultures were determined using a microplate reader (Multiskan JX, Thermo Fisher Scientific). Wells that showed apparent cell growth were counted.

These quadruplicate screens showed in four results per Keio strain. Under these conditions, most Keio mutants resulted in 0/4 positive wells. The mutants that produced 2/4–4/4 positive wells were regarded as “up” mutants.

For a plating assay for plasmid transfer in “up” mutants, mixed cultures in each microplate well were plated onto LB agar plates that contained two antibiotics (str: 75  $\mu$ g/ml and kan: 75  $\mu$ g/ml) to confirm the production of double-resistant cells. To estimate plasmid transfer frequency in each “up” mutant, a plating assay was also used with a colony biofilm on TSB agar (1.5%) prepared in a polystyrene plate ( $\phi$  90 mm), as previously described [25].

### 2.3. Plasmid Isolation from the Produced Transformants

Double-resistant colonies that appeared in a plating assay were cultured in fresh LB broth that contained str and kan overnight at 37°C and were subsequently used for plasmid preparation. Plasmids were isolated, digested with EcoRI and analyzed by 0.8% agarose-gel electrophoresis using conventional methods [33].

### 2.4. Data Analyses for Screened Genes

Information for 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/>).

## 3. Results and Discussion

### 3.1. Screen for “up” Mutants and Confirmation of Transformants

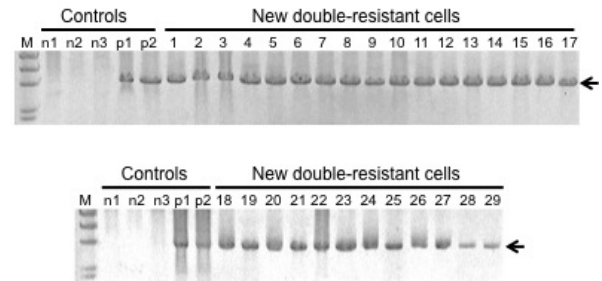
We adopted a screening system using 96-well microplates to identify cell-to-cell plasmid transfer “up” mutants among the 3,985 strains in the Keio collection [24, 25]. In brief, we cultured plasmid-donor cells (harboring pGBM1 that contained a *str<sup>r</sup>* gene) and recipient cells (individual Keio strains that contained a *kan<sup>r</sup>* gene on their chromosomes) on

agar medium in microplate wells. The transformants were subsequently selected twice by culturing portions of these cocultured cells in liquid media that contained two antibiotics (kan and str). If Keio strains had acquired plasmids, double-resistant cells appeared and selectively grew in these wells. The numbers of positive wells for four experiments for each Keio strain were counted.

Under these conditions, most samples resulted in 0/4 positive wells. However, 133 strains produced 1/4 or more positive wells and 36 strains produced 2/4–4/4 positive wells. We analyzed these 36 strains to confirm the occurrence of double-resistant cells using a plating assay (transformant selection on agar plates). Ultimately, we obtained 29 positive strains.

To ensure that no pseudo-positive mutants may result from unexpected str resistance arising in the original Keio mutants, we examined the str sensitivities of the original 29 plasmid-free Keio strains. None of the 29 original strains exhibited str resistance. In addition, the presence of plasmids in the screened cells was determined by plasmid isolation from new colonies that appeared in a plating assay. This confirmed that all 29 strains had full-length plasmids (Fig. 1). Because these 29 strains also exhibited obvious kan resistance, which is characteristic of the Keio strains, we concluded that the double-resistant cells that appeared after the cocultures were indeed transformants of the Keio strains that had

acquired plasmids from cocultured donor cells. Therefore, we selected these 29 mutants as “up” mutants (Table 1). The plasmid transfer frequencies of these “up” mutants were in the range of approximately  $10^{-9}$ – $10^{-8}$  using a plating assay, whereas those of most other Keio strains were below the limit of detection (approximately  $< 10^{-10}$ ).



**Figure 1.** Agarose gel electrophoresis results for *EcoRI*-digested plasmids isolated from the double-resistant colonies obtained by a plating assay used for plasmid-transfer experiments. The arrows indicate the bands for pGBM1 (4028 bp). Lane M: size markers ( $\lambda$  Hind III); lanes n1–n3: negative controls [DNA prepared from CAG18439 (n1) and original Keio strains (n2: *ybdG*, n3: *yneJ*)]; lanes p1–p2: positive controls (pGBM1); and lanes 1–29: plasmids prepared from new double-resistant colonies of Keio strains (1: *ybdZ*, 2: *ybdG*, 3: *yneJ*, 4: *ydeK*, 5: *marB*, 6: *marC*, 7: *eamA*, 8: *yneF*, 9: *yneH*, 10: *ydeW*, 11: *nfrA*, 12: *hipA*, 13: *tam*, 14: *nfrB*, 15: *lsrG*, 16: *envY*, 17: *cusA*, 18: *cusC*, 19: *ydeU*, 20: *ybdJ*, 21: *yneK*, 22: *ydeE*, 23: *dnaQ*, 24: *pheP*, 25: *ynfN*, 26: *yneI*, 27: *ydeH*, 28: *ydfR* and 29: *fepG*).

**Table 1.** Repressive genes that were screened in this study and their features

Gene name	Count	Location	Product function & feature	Gene location on chromosome (min)
<i>ybdZ</i>	4/4	C	MbtH-like protein that plays a role in amino acid activation by EntF	13.22
<i>ybdG</i>	4/4	IM	Predicted mechanosensitive channel	12.99
<i>yneJ</i>	4/4	C	LysR-family transcriptional regulator, lamB regulator	34.77
<i>ydeK</i>	4/4	IM	Predicted lipoprotein, Putative OM autotransporter adhesin	34.32
<i>marB</i>	4/4	P	Multiple drug resistance protein	34.87
<i>marC</i>	4/4	IM	Multiple drug resistance protein	34.84
<i>eamA</i>	4/4	IM	O-acetylserine/cysteine export protein	34.88
<i>yneF</i>	4/4	IM	Predicted diguanylate cyclase	34.68
<i>yneH</i>	3/4	C	Glutaminase selective for L-glutamine	34.71
<i>ydeW (lsrR)</i>	3/4	C	Lsr operon transcriptional repressor in the absence of phospho-autoinducer-2	34.45
<i>nfrA</i>	3/4	OM	Bacteriophage N4 receptor	12.66
<i>hipA</i>	3/4	C	Serine protein kinase required for persister formation; toxin of HipAB TA pair	34.25
<i>tam</i>	3/4	C	Trans-aconitate methyltransferase, SAM-dependent	34.60
<i>nfrB</i>	3/4	IM	Bacteriophage N4 receptor	12.72
<i>lsrG</i>	3/4	C	Autoinducer-2 degrading protein	34.59
<i>envY</i>	2/4	C	Thermoregulatory transcription activator of porin expression, AraC family	12.62
<i>cusA</i>	2/4	IM	Subunit of the cusCFBA copper efflux system	12.89
<i>cusC</i>	2/4	OM	Subunit of the cusCFBA copper efflux system	12.82
<i>ydeU</i>	2/4	OM	Putative ATP-binding component of transport system and adhesin protein	34.28
<i>ybdJ</i>	2/4	IM	Predicted inner membrane protein	13.04
<i>yneK</i>	2/4	U	Predicted protein	34.78
<i>ydeE</i>	2/4	IM	Major facilitator superfamily (MFS) transporter	34.90
<i>dnaQ</i>	2/4	C	$\epsilon$ subunit of DNA polymerase III, 3' to 5' proofreading exonuclease activity	5.09
<i>pheP</i>	2/4	IM	Phenylalanine amino acid-polyamine-organocation (APC) transporter	12.96
<i>ynfN</i>	2/4	U	Qin prophage; predicted protein	35.26
<i>yneI (sad)</i>	2/4	C	NAD <sup>+</sup> -dependent succinate semialdehyde dehydrogenase	34.73
<i>ydeH (dgcZ)</i>	2/4	C	Diguanylate cyclase that regulates motility and biofilm formation	34.94
<i>ydfR</i>	2/4	U	Qin prophage; predicted protein	35.31
<i>fepG</i>	2/4	IM	Subunit of ferric enterobactin ABC transporter and Ferric Enterobactin Transport System	13.35

+ Count: Count of positive wells in plasmid-transfer assay for 4 experiments. Location: C, cytoplasm; IM, inner membrane; OM, outer membrane; P, periplasmic space; U, unknown.

### 3.2. Analyses of Data of the Screened Genes

Table 1 summarizes the known characteristics of the 29 screened genes. Although we could not find direct linkages between the functions of the screened genes and plasmid transfer, we analyzed the following.

Half of these genes (15 of 29) were associated with cell-surface proteins, such as those in the inner membrane, outer membrane, and periplasmic space, which is consistent with the transmembrane phenomenon. Four genes were estimated to be involved in intra- and intercellular signaling with cyclic-di-GMP (*ydeH* and *yneF*) [34, 35] and autoinducer-2 (*ydeW* and *lsrG*) [36, 37], suggesting the involvement of various known signal transduction pathways. Another four genes (*ybdG*, *yneJ*, *marC* and *envY*) were the same as those that were screened in our previous study [25] using a pMB1-derived plasmid; however, the remaining screened genes (25 of 29) were different. No obvious functional connections were observed between the genes screened in this study and the eight genes for the “down” mutants previously screened [24] using a pMB1-derived plasmid. These results suggest that the above four common genes (*ybdG*, *yneJ*, *marC* and *envY*) may share plasmid-transfer mechanisms with different plasmids, although there may be some dissimilarities in these mechanisms. None of the 29 genes in Table 1 have been reported to be involved in DNA transfer or DNA uptake in *E. coli* or other bacteria, which suggests the uniqueness of this phenomenon, as also previously suggested [23–25, 31].

Moreover, by carefully surveying our results, interestingly, 28 of the 29 screened genes mapped to two limited regions on the *E. coli* chromosome; 18 genes at 34.25–35.31 min and 10 genes at 12.62–13.35 min (Table 1). The well positions of the 29 screened strains in the storage and culture microplates were distributed into separate plates and wells; therefore the concentration in gene loci is not an artifact resulting from the accidental contamination of one or a few specific plates. As additional supporting evidence, among the genes for the 97 strains that showed 1/4 positive wells in our initial screening, another 16 genes also mapped to these two chromosomal regions. Moreover, among the 55 “up” mutants screened in our previous study [25], 10 genes [6 genes (*ydeW*, *dcp*, *ydbK*, *ydfT*, *ybcS* and *fimZ*) in addition to the above 4 common genes (*ybdG*, *yneJ*, *marC* and *envY*)] belong to these two regions.

We observed that these two chromosomal regions commonly contained termination (Ter) sites for DNA replication (TerC: 34.64 min and TerH: 12.91 min) [38–40, [http://www.ecogene.org/old/topic.php?topic\\_id=228](http://www.ecogene.org/old/topic.php?topic_id=228)]. TerC is considered the predominant Ter site utilized *in vivo*. TerH is a weak but functional site, and TerI (13.46 min) is also in close proximity to TerH. Therefore, chromosomal mutations around specific Ter sites may induce structural or physiological disturbance in replication of chromosomal DNA and affect plasmid acquisition in the recipient cells. The product of *dnaQ*, the other gene screened in this study, is the  $\epsilon$  subunit of DNA polymerase III [41] (Table 1). This appears to be consistent with the above idea that some form of DNA synthesis disorder

may affect plasmid acquisition.

It is currently uncertain which is more important: individual functions of screened genes, DNA synthesis disorder or both. There may be rather complex mechanisms that vary according to the types of plasmid replicons. However, the results of this study include several interesting directions for investigating the molecular mechanisms of cell-to-cell plasmid transfer. Investigations that are more detailed will be necessary to unravel the overall mechanism of cell-to-cell plasmid transfer.

## 4. Conclusion

In this study, we have screened for genes responsible for repressing cell-to-cell plasmid transfer in *E. coli* using a pSC101-derived plasmid and the Keio collection. We identified 29 “transfer-up” mutants. Among these, four mutants are common to those previously screened using a pMB1-derived plasmid. It is interesting that 28 of the 29 screened genes map to two limited regions on the *E. coli* chromosome: 18 genes at 34.25–35.31 min and 10 genes at 12.62–13.35 min. Because these two regions commonly contain termination (Ter) sites for DNA replication (TerC: 34.64 min and TerH: 12.91 min), it is possible that chromosomal mutations around specific Ter sites may affect plasmid acquisition in the recipient cells.

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