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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.

Most samples resulted in 0/8 positive wells under these conditions. However, a total of 81 strains that produced 4/8–8/8 positive wells were picked up and used in a quantitative plating assay to count the transformants that were produced (colonies resistant to two antibiotics). As shown in Tables 1 and 2, 55 mutants were selected as 'up' mutants that showed approximately 2- to 30-fold higher activities than the controls. We categorized these into two groups: Group 1 (Table 1: 25 mutants with approximately 5- to 30-fold higher activities) and Group 2 (Table 2: 30 mutants with approximately 2- to 5-fold higher activities).

3.2. Confirmation of transformants

We used three different experiments to exclude pseudo-positive mutants. First, the cam sensitivities of the original 55 plasmid-free Keio strains in Group 1 and 2 were tested. None of the 55 original strains showed cam resistance (data not shown), indicating that cam resistance among the screened cells did not result from unexpected cam resistance arising in the original Keio mutants.

Second, the presence of plasmids in the screened cells were determined for Group 1 mutants by plasmid isolation. This confirmed that all of the cells tested had full-length plasmids (Fig. 1A).

Third, we determined whether the screened cells were Keio strains by PCR using primers for *lacI,* which occurs in the Keio strains but not in the plasmid-donor CAG18439. As shown in Fig. 1B, most of the screened cells in Group 1 were confirmed to be Keio strains. Although we did not obtain PCR products for four mutants (*dcp*, *ydeW*, *ykgF*, and *yneI*), we determined that they were probable Keio mutants because they exhibited obvious kan resistance, which is a characteristic of the Keio strains.

The results from these three experiments indicated that the double-resistant cells that appeared in the co-culture for screening were transformants of the Keio strains that had acquired plasmids from co-cultured donor cells.

3.3. Natural and artificial transformation of the 'up' mutants

To further characterize these 'up' mutants, we examined their transformability by natural and artificial transformation. We used the same plasmid (pHSG399-F6) that was used for screening and some other plasmids (pUC19-tet, pGBM1, and pSY110) that had different replication origins (pMB1 origin, pSC101 origin, and p15A origin). Then, as described in the Materials and Methods section, natural transformation (simple addition of purified plasmids to culture) and artificial transformation ($CaCl_2$ method [12,32], PEG method [32,33], and electroporation) were performed for eleven strains of the 'up' mutants in Group 1 (*mdtF, sgbH, ddpD, dcp, ydbK, rpsT, mrr, visC, ggt, afuB*, and *ybaV*) and one strain in Group 2 (*yhiR*). However, no obvious promotion was found for any of the mutants tested using both natural and artificial transformations (data not shown). This suggested that the mechanism of cell-to-cell plasmid transfer may be different from the mechanisms involved in simple natural transformation and artificial transformation.

3.4. Data summary for the screened genes and their presumed roles in cell-to-cell plasmid transfer

Table 1 summarizes the known characteristics of the Group 1 and 2 genes. Their gene products were localized in the cytoplasm (34 genes), in or around the cell surface (20 genes) and an unknown location (1 gene). The presumed functions of these gene products were: metabolism and synthesis (19 genes); transport (15 genes); transcription or translation (4 genes); genetic competence (2 genes); stress response (2 genes), envelope formation (2 genes); and unknown or others (7 genes) (The genes with multiple

Table 1
Repressive genes (Group 1) that were screened in this study and their features.

Gene name	Fold	Location	Category of product function	Product function & feature
mdtF (yhiV)	32	IM	Transport	MdtEF-TolC multidrug resistance efflux RND-type transporter, antibiotic resistance
sgbH(yiaQ)	26	C	Metabolism, synthesis	3-Keto-L-gulonate-6-phosphate decarboxylase; required for the aerobic utilization of L-ascorbate, metal-ion binding
ddpD	25	C	Transport	D,D-dipeptide permease system, ATP-binding component
dcp	20	C	Others	Dipeptidyl carboxypeptidase II, proteolysis, metal-ion binding
ydbK	19	C	Transport metabolism, synthesis	Probable pyruvate-flavodoxin oxidoreductase,4Fe-4S cluster binding
rpsT	19	C	Translation	30S ribosomal subunit protein S20, Response to stress, RNA binding
mrr	19	C	Others	Restriction of DNA with certain methylated adenine or cytosine residues, endonuclease activity
visC	18	C	Metabolism, synthesis	Putative FAD-dependent oxidoreductase, FAD/NAD(P)-binding
ggt	17	P	Metabolism, Synthesis	γ-Glutamyltranspeptidase, glutathione metabolism
afuB	14	IM	Pseudo gene	Ferric ABC transporter permease, CP4–6 putative prophage remnant
ycjO	14	IM	Transport	Probable ABC transporter subunit, sugar transport
yjeM	13	IM	Transport	Putative amino-acid transporter
aceB	13	C	Metabolism, synthesis	Malate synthase A, TCA & Glyoxylate cycle
yhjV	12	IM	Transport	Putative amino acid:H^+ symport permease
ycjF	11	IM	Unknown	Hypothetical protein, UPF0283 family, heat shock
pitA	10	IM	Transport	Low-affinity Phosphate transporter
yifK	9.2	IM	Transport	Predicted amino-acid transporter
lsrR(ydeW)	9.0	C	Transcription	*lsrACDBFGE* operon repressor for autoinducer-2 (AI-2) uptake, DNA binding
ybaV	7.9	P	Genetic competence DNA replication, recombination & repair	*B. subtilis* competence protein ComEA homolog
ybiS	7.0	P	Metabolism, synthesis envelope formation	L,D-transpeptidase linking Lpp to murein
ylbF	6.9	C	Unknown	Putative carboxylase
ykgF	6.8	C	Metabolism, synthesis transport	Putative electron transport protein, predicted amino acid dehydrogenase with NAD(P)-binding domain and ferridoxin-like domain
yhfA	6.7	C	Response to stress	Function unknown, OsmC family
damX(yhfB)	6.7	M	Envelope formation	Cell division protein, binds septal ring
yneJ	5.6	C	Transcription	LysR-family transcriptional regulator, lamB regulator, DNA binding

Fold: fold of plasmid-transfer promotion in each Keio strain compared to control strains. Location: C, cytoplasm; IM, inner membrane; M, membrane anchored; P, periplasmic space.

Table 2
Repressive genes (Group 2) that were screened in this study and their features.

Gene name	Fold	Location	Category of product function	Product function & feature
mraZ	4.9	C	Envelope formation	Cell division protein, Inhibitor of RsmH methytransferase activity
ybcS	4.9	P	Metabolism, synthesis	Phage lambda lysozyme R protein homolog, DLP12 prophage protein
yihO	4.8	IM	Transport	Putative transporter, function unknown
ymfL	4.6	C	Others	e14 prophage protein, predicted DNA-binding transcriptional regulator
ybeX	4.5	C	Transport	Heat shock protein, putative Co^{2+} and Mg^{2+} efflux protein, possible hemolysin
yeaU	4.5	C	Metabolism, synthesis	D-malate oxidase, NAD(+)-dependent
talA	4.2	C	Metabolism, synthesis	Transaldolase A, carbohydrate metabolism
fdoG	4.2	P	Metabolism, synthesis	Formate dehydrogenase-O, selenopeptide, cellular respiration
ycjV	4.1	C	Transport	Putative ATP-binding component of ABC transporter, malK paralog
fklB	4.0	C	Transport	FKBP-type peptidyl-prolyl cis–trans isomerase
frc	3.8	C	Metabolism, synthesis	Formyl CoA transferase, NAD(P)-binding
yahF	3.8	C	Metabolism, synthesis	Predicted acyl-CoA synthetase, NAD(P)-binding, succinyl-CoA synthetase domain
xapA	3.8	C	Metabolism, synthesis	Xanthosine phosphorylase, purine nucleoside phosphorylase II
avtA	3.8	C	Metabolism, synthesis	Valine-pyruvate aminotransferase
yjhX	3.7	C	Unknown	Function unknown
ydcF	3.7	C	Unknown	Hypothetical SAM-binding protein, DUF218 superfamily
marC	3.7	IM	Antibiotic resistance	Multiple drug resistance protein
ydfT	3.5	C	Transcriptionresponse to stress	Lambda Q antitermination protein homolog, Qin prophage
yeiQ	3.5	C	Metabolism, synthesis	Predicted NAD-dependent dehydrogenase, function unknown
ybhI	3.5	IM	Transport	Putative transporter, function unknown
fimZ	3.1	C	Transcription	Predicted fimbriae protein synthesis regulator, two-component response regulator, DNA binding
fumB	3.0	C	Metabolism, synthesis	Fumarase B, anaerobic, biofilm formation
ybdG	2.9	IM	Transport	Predicted mechanosensitive channel
ykiB	2.8	U	Unknown	Function unknown
yliE	2.7	IM	Unknown	Predicted cyclic-di-GMP phosphodiesterase
allB	2.7	C	Metabolism, synthesis	Allantoinase, allantoin for anaerobic nitrogen
ybbO	2.6	C	Metabolism, synthesis	Predicted oxidoreductase with NAD(P)-binding Rossmann-fold domain
envY	2.6	C	Transcription	Thermoregulatory transcription activator of porin expression, AraC family, DNA binding
degQ	2.4	P	Others	Periplasmic serine endoprotease, proteolysis
yhiR	2.3	C	Genetic competence	*H. influenzae* competence protein ComJ homolog, required for the utilization of DNA as a carbon source

Fold: fold of plasmid-transfer promotion in each Keio strain compared to control strains. Location: C, cytoplasm; IM, inner membrane; P, periplasmic space; U, unknown.

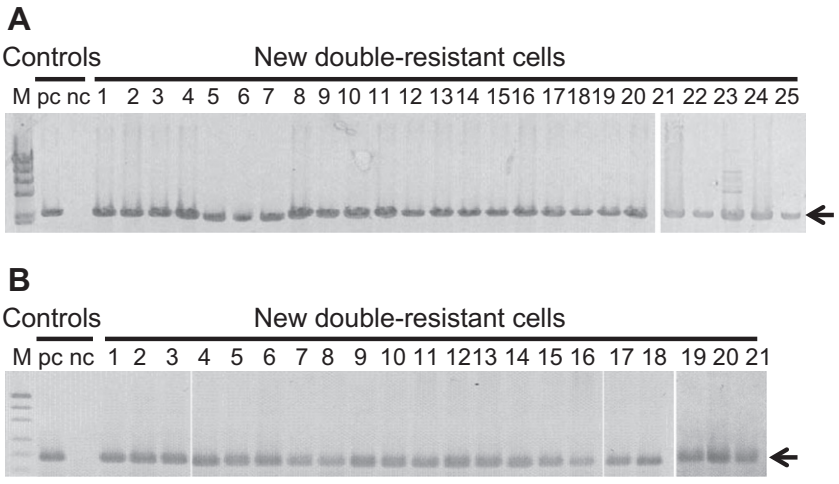

Fig. 1. Plasmid analysis (A) and PCR analysis (B) of colonies obtained by plasmid transfer experiments using Keio strains as recipient. Panel A: Agarose gel electrophoresis of EcoRI-digested plasmids isolated from the double-resistant colonies. The arrow indicates the band for pHSG399-F6 (2411 bp). Lane M: size marker; lane pc: positive control (pHSG399-F6); lane nc: negative control (DNA prepared from a Keio strain). Panel B: Agarose gel electrophoresis of colony-direct PCR products using the colonies as template. The arrow indicates the band (181 bp) specific for the Keio strains. Lane M: size marker; lane pc: positive control (a Keio strain); lane nc: negative control (donor strain: CAG18439 harboring pHSG399-F6).

functions were counted more than once). After carefully surveying these results, we could not deduce any simple explanations for the molecular mechanisms involved in this phenomenon. However, we did observe some interesting results.

The most interesting result was that the strong (approximately 5- to 30-fold) promotion of plasmid acquisition was observed by the 25 mutants in Group 1. This suggests that there may be strong repressive mechanism(s) in which many factors could be involved, either directly or indirectly. Alternatively, various types of non-lethal disturbance in cell functions by certain single-gene knock-outs may redundantly but strongly trigger this specific means of plasmid acquisition. However, after reviewing the known functions of those screened genes, we cannot deduce a feasible hypothesis for the mechanism(s) involved.

Another interesting result was that our screen identified two competence gene homologues: *ybaV* and *yhiR*. *ybaV* [34,35] of *E. coli* is a homologue of *comEA* [36] of *Bacillus subtilis*. The ComEA protein spans the cytoplasmic membrane and is a receptor for extracellular DNA in its periplasmic domain that assists in DNA translocation through the cytoplasmic membrane. Sequence analysis of the YbaV protein revealed that it probably has an N-terminal signal peptide for secretion and would be localized in the periplasmic space. The YbaV protein also has two helix-hairpin-helix motifs for DNA binding. Although there are no experimental data for the presumed functions and localization of the YbaV protein, it may be a DNA-binding (or DNA-sequestering) protein in the periplasmic space. Based on this assumption and the result obtained in this study, it is possible that the YbaV protein may sequester DNA in the periplasmic space and result in repressing DNA translocation through the cytoplasmic membrane. Although this putative function for YbaV is opposite to that of ComEA of *B. subtilis*, the lack of a membrane-spanning region in the YbaV protein compared with the ComEA protein may explain this functional difference.*yhiR* [34,35] of *E. coli* is a homologue of *comJ* [37] of *Haemophilus influenzae*. YhiR of *E. coli* has been predicted to be a cytoplasmic protein that contains a motif found in *N*-6-adenine-specific DNA methyltransferases. Finkel and Kolter [34] reported that YhiR was required for exogenous DNA processing (or digestion) in the cytoplasm to use DNA as a nutrient. This presumed function is reasonable for our results in this study, as inhibiting DNA digestion would promote the survival of foreign DNA that enters the cytoplasm.

Previously, using a similar screening system, we selected 'down' mutants from the same Keio collection [28]. Those knocked-out genes were considered as candidate essential or promoting genes for cell-to-cell plasmid transfer. We compared these essential and promoting genes with the repressive genes selected in this study. However, we found no apparent connections, although several genes in the former and latter groups belonged to the same operon (data not shown), which suggested that there was some relationship between them.

As described above, while two competence genes were screened in this study, the results of natural and artificial transformation for the 'up' mutants suggested that the mechanism of cell-to-cell plasmid transfer may differ from the mechanisms involved in simple natural and artificial transformations. This was also consistent with the results of similar transformation experiments using the 'down' mutants in our previous study [28]. Thus, the mechanism of cell-to-cell plasmid transfer may be rather complex, and more careful, detailed investigations will be necessary to unravel the overall mechanism of cell-to-cell plasmid transfer.

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