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The EcoKI Type I Restriction-Modification System in *Escherichia coli* Affects but Is Not an Absolute Barrier for Conjugation

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The rapid evolution of bacteria is crucial to their survival and is caused by exchange, transfer, and uptake of DNA, among other things. Conjugation is one of the main mechanisms by which bacteria share their DNA, and it is thought to be controlled by varied bacterial immune systems. Contradictory results about restriction-modification systems based on phenotypic studies have been presented as reasons for a barrier to conjugation with and other means of uptake of exogenous DNA. In this study, we show that inactivation of the R.EcoKI restriction enzyme in strain *Escherichia coli* K-12 strain MG1655 increases the conjugational transfer of plasmid pOLA52, which carriers two EcoKI recognition sites. Interestingly, the results were not absolute, and uptake of unmethylated pOLA52 was still observed in the wild-type strain (with an intact *hsdR* gene) but at a reduction of 85% compared to the uptake of the mutant recipient with a disrupted *hsdR* gene. This leads to the conclusion that EcoKI restriction-modification affects the uptake of DNA by conjugation but is not a major barrier to plasmid transfer.

The exchange of chromosomal and/or extrachromosomal DNA, such as plasmids, viruses, and transposons, is crucial for the evolution of bacteria and their ability to adapt to new environments. Exchange of genetic material occurs among both related and unrelated species of bacteria and is driven by the three horizontal gene transfer (HGT) mechanisms: conjugation, transformation, and transduction (1–3). Restriction-modification (RM) systems are described as major barriers to HGT (4–6) and comprise restriction endonucleases with a cognate methyltransferase. These recognize and cleave DNA not modified by the methyltransferase, thereby making the bacterium able to distinguish between its own (methylated) DNA and incoming nonmethylated DNA.

Based on their protein-complex subunit composition and functionality, RM systems can be divided into four types; this study focuses on type I. Type I systems require products of the three genes *hsdR* (restriction), *hsdM* (methylation), and *hsdS* (sequence specificity) and cleave randomly at a remote distance from the recognition sequence. Restriction occurs only when a protein complex of all three gene products (R_2M_2S) is formed, whereas methylation of the DNA requires formation of a complex of only the HsdM and HsdS proteins (M_2S) (7).

Some studies have indicated that transfer by conjugation is unaffected by RM systems but that unmodified phage or free DNA in transformation is readily degraded (8–11). This has led to the view that the conjugational transfer of plasmids through a single-stranded DNA intermediate is immune to restriction by RM systems, as the great majority of these recognize only nonmethylated double-stranded DNA (12–17). Other studies have, however, contradicted this. In 1964, Arber and Morse (18) proposed that host specificity (RM systems) might play a role in the acceptance or rejection of DNA transferred by conjugation in *Escherichia coli*. In many studies from the 1960s, the transfer was measured with recombinants of Hfr strains (18–20), but Arber and Morse made a phenotypic study showing that the conjugational transfer of episomes (with the ability to express genes without integration into the bacterial chromosome) was affected in the same manner as in phages (18). Other experiments have shown reduced conjugational transfer between different bacterial species with diverse restriction-modification systems, indicating that they may be the cause of this reduction in transfer (21, 22), but none of these observations have been confirmed with isogenic strains by modern molecular techniques. Recent studies indicated that SauI, a type I RM system for *Staphylococcus aureus*, may be a barrier to transfer into and between *S. aureus* isolates (23), but Veiga and Phino showed that inactivation of the SauI system was not sufficient for producing strains that efficiently take up foreign DNA (6), again questioning the importance of RM systems as barriers to conjugational transfer.

In the current study, we aimed to clarify the impact of a restriction-modification system in the conjugational gene transfer of single-stranded plasmidic DNA (24, 25) at the genotypic level. We focused on the impact of the type I RM system EcoKI, with the recognition sequence $AACN_6GTGC$, in the transfer of conjugative plasmids between RM variants of the *E. coli* K-12 strain MG1655. In addition to possessing the type I RM system, which we examine in the current study, MG1655 possess three different methylation-requiring type IV systems, EcoKMcrA, EcoKMcrBC, and EcoKMrr. All three systems are sequence specific and will not interfere with plasmid transfer between isogenic strains used in the current study (26–29). We provide evidence that the RM system EcoKI has a significant and negative effect on conjugation but also that this is not a major barrier to conjugation.

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TABLE 1 Bacterial strains, plasmids, and oligonucleotides used in this study

Bacterial strain, plasmid, or oligonucleotide	Description (relevant genotype and/or phenotype) or sequence (5′ to 3′)[a]	Source or reference
Escherichia coli strains		
MG1655 (K-12 strain)	F$^-$ λ$^-$ *ilvG rfb-50 rph-1*	CGSC
MG1655-RN	Spontaneous Rif^r and Nal^r derivate of MG1655	This study
MG1655-A	Spontaneous $NaN_3{}^r$ derivate of MG1655	This study
MG1655-*hsdR*^{INT}	TargeTron insertion at nucleotide 1740|1741 of *hsdR*, Rif^r Nal^r	This study
MG1655-*hsdM*^{INT}	TargeTron insertion at nucleotide 720|721 of *hsdM*, Kan^r	This study
MG1655-*hsdR*^{COMPL}	MG1655-*hsdR*^{INT} with p*hsdR* for complementation, Rif^r Nal^r Tet^r	This study
MG1655-*hsdR*^{CONTROL}	MG1655-*hsdR*^{INT} with expression vector pMSC83, Rif^r Nal^r Tet^r	This study
DH10B	F$^-$ *endA1 recA1 galE15 galK16 nupG rpsL* Δ*lacX74* ϕ80*lacZ*ΔM15 *araD139* Δ(*ara leu*)*7697 mcrA* Δ(*mrr-hsdRMS-mcrBC*) λ$^-$	Invitrogen
Plasmids		
pACD4K-C-loxP	Linearized TargeTron plasmid with a T7 promoter; Cam^r Kan-Δ*td*	Sigma
pACD4K-C-loxP (*hsdR*)	pACD4K-C-loxP retargeted for *hsdR* of MG1655 (LR1/LR2/LR3)	This study
pACD4K-C-loxP (*hsdM*)	pACD4K-C-loxP retargeted for *hsdM* of MG1655 (LR4/LR5/LR6)	This study
pAR1219	Expresses T7 RNA polymerase under the control of the IPTG-inducible *lac* UV5 promoter; Amp^r	Sigma
706-Cre	Expression plasmid for Cre recombinase driven by the thermosensitive promoter *c*I578; Tet^r	Gene Bridges GmbH
pOLA52	Plasmid of 45.7 kb with two restriction sites for *hsdR* (EcoKI); Amp^r	41
pHHA45	Plasmid of 51.6 kb without restriction sites for *hsdR* (EcoKI); Amp^r	31
pMSC83	Cloning vector used for complementation; Tet^r	This study
p*hsdR*	R.EcoKI from MG1655 cloned into pMSC83	This study
Oligonucleotides[b]		
LR1 (*hsdR* IBS)	AAAAAAGCTTATAATTATCCTTACATCGCGGCTATGTGCGCCCAGATAGGGTG	Sigma
LR2 (*hsdR* EBS1d)	CAGATTGTACAAATGTGGTGATAACAGATAAGTCGGCTATATTAACTTACCTTTCTTTGT	Sigma
LR3 (*hsdR* EBS2)	TGAACGCAAGTTTCTAATTTCGGTTCGATGTCGATAGAGGAAAGTGTCT	Sigma
LR4 (*hsdR*-V-R)	TCCAGCTGGCTGCGGAACTGC	TAGC
LR5 (*hsdM* IBS)	AAAAAAGCTTATAATTATCCTTAGATTGCGCCGCCGTGCGCCCAGATAGGGTG	Sigma
LR6 (*hsdM* EBS1d)	CAGATTGTACAAATGTGGTGATAACAGATAAGTCGCCGCCGTTAACTTACCTTTCTTTGT	Sigma
LR7 (*hsdM* EBS2)	TGAACGCAAGTTTCTAATTTCGATTCAATCTCGATAGAGGAAAGTGTCT	Sigma
LR8 (*hsdM*-V-F)	CCAATGATCTGGACGACCTT	TAGC
LR9 (*hsdR*-C-F)	GGTCATTGCCCGGAAAGGTA	TAGC
LR10 (*hsdR*-C-R)	GGCAGCCTGAAGGATGAAGT	TAGC

[a] For bacterial strains and plasmids, the relevant genotype, phenotype, and other characteristics are shown. Abbreviations: INT, interruption; COMPL, complementation.
[b] The genes targeted by the primers used in the construction of knockout strains or complementary plasmids are shown in parentheses at the end of the entry.

MATERIALS AND METHODS

Media and reagents. *E. coli* cells were cultured in brain heart infusion (BHI) broth at 37°C. For growth on agar, Luria-Bertani (LB) or BHI agar plates were used. The following antibiotics and concentrations were used: ampicillin (Amp), 50 μg/ml for cloning or 100 μg/ml in HGT assays; chloramphenicol (Cam), 25 μg/ml; kanamycin (Kan), 25 μg/ml; tetracycline (Tet), 5 μg/ml or 10 μg/ml; rifampin (Rif), 25 μg/ml; and nalidixic acid (Nal), 25 μg/ml.

Strains and plasmids. Bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table 1. 32 The conjugation experiments were performed from donors with (*hsdM*$^+$) and without (Δ*hsdM*) the ability to methylate the DNA and with functional, disrupted, and complemented restriction abilities. Two plasmids with a published DNA sequence, pOLA52 and pHHA45 (GenBank accession numbers EU370913.1 and JX065630.1, respectively), were used to assess the influence of the RM systems on conjugational transfer. pOLA52, belonging to the IncXI incompatibility group, has two recognition sites for the EcoKI system, while pHHA45, belonging to the IncN incompatibility group, does not contain any sites. Both IncXI and IncN plasmids are known to transfer in the single-stranded form (24, 25, 30, 31).

Construction of TargeTron insertion mutants of *E. coli* MG1655. Two TargeTron mutants with interruptions, MG1655-*hsdR*^{INT} and MG1655-*hsdM*^{INT}, were created by following the guidelines from Sigma-Aldrich (32) for insertion mutations in *E. coli* strains, with the plasmid pAR1219 as the source of T7 RNA polymerase. Plasmid pACD4K-C-loxP was used as the donor for the group II intron, retargeted by PCR with primers designed for position 1740|1741 in *hsdR* (primers LR1, LR2, and LR3) and position 720|721 in *hsdM* (primers LR5, LR6, and LR7). Gene disruptions were induced by the addition of 20 μl of a 10 mM stock solution of isopropyl-β-D-thiogalactopyranoside (IPTG) to 2 ml of culture.

The plasmids (retargeted pACD4K-C-loxP and pAR1219) were cured by overnight growth in broth, followed by plating on BHI, and patched on BHI with 50 μg/ml Amp and BHI plus 25 μg/ml Cam to identify plasmid-free isolates. MG1655-*hsdR*^{INT} was made electrocompetent as described by D. O'Callaghan and A. Charbit (33), but the glycerol washing step was performed with the full-strength original volume (100 ml). MG1655-*hsdR*^{INT} was further transformed with the plasmid 706-Cre to remove the kanamycin resistance marker (Gene Bridges GmbH). Single colonies of both MG1655-*hsdM*^{INT} and MG1655-*hsdR*^{INT} were tested by PCR for the TargeTron insert and removal of the kanamycin gene (MG1655-*hsdR*^{INT} only) by using *Taq* polymerase (Fermentas) and internal and external primers LR3 and LR4 (*hsdR*) or LR7 and LR8 (*hsdM*). For further verification, the PCR products were purified using a GFX purification kit (GE Healthcare) and sequenced by Macrogen Korea.

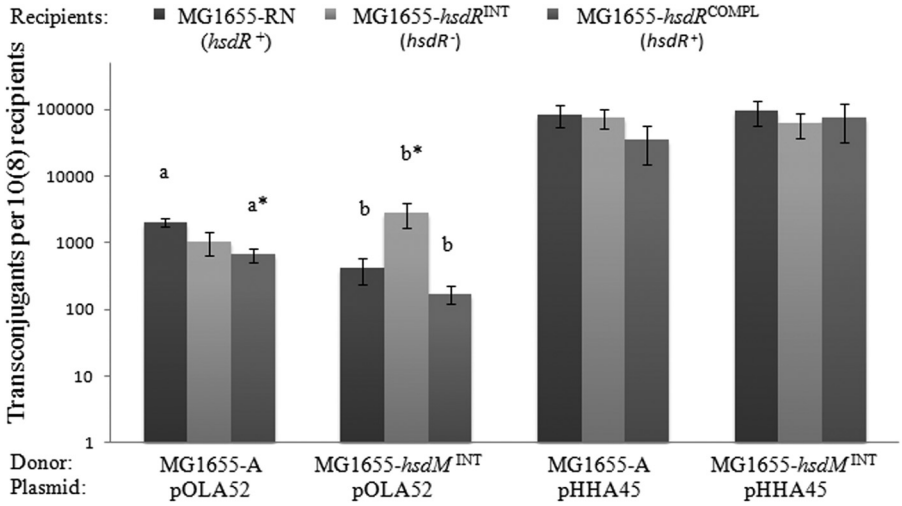

FIG 1 Single-stranded horizontal gene transfer with isogenic donor and recipient strains of *E. coli* K-12 MG1655. All experiments were performed at least in triplicate, and data are means of transconjugants per 10^8 recipients, with standard errors of the mean (SEM). The levels of transfer of the plasmids for the three recipients were compared individually for each donor (strain and plasmid). Statistically significant differences were observed between the recipients MG1655-*RN* and MG1655-*hsdR*^{COMPL} with the donor MG1655-*A* (a*, $P = 0.021$) and between MG1655-*hsdR*^{INT} and the remaining two recipients with the donor MG1655-*hsdM*^{INT} (b*, $P = 0.029$) with the plasmid pOLA52, which harbors two recognition sites for EcoKI. No statistically significant differences were observed with the two donors with the plasmid without recognition sites for EcoKI, pHHA45.

Complementation of *hsdR* with p*hsdR*. For complementation in the *hsdR* mutant, we amplified a 3,633-bp fragment encompassing the *hsdR* gene with the primers LR9 and LR10 by PCR and cloned the resulting fragment into the PvuII site of pMSC83. For a detailed description, see the supplemental material.

Horizontal gene transfer of single-stranded DNA by conjugation. The strains MG1655-*A* and MG1655-*hsdM*^{INT} were transformed with the plasmid pOLA52, containing two recognition sites for EcoKI, or pHHA45, lacking EcoKI recognition sites, to act as donors in the conjugation experiments. The strains MG1655-*RN*, with a functional *hsdR* gene on the chromosome, MG1655-*hsdR*^{INT}, with an interrupted restriction gene, and MG1655-*hsdR*^{COMPL}, with a complemented restriction gene, were used as recipients.

Two individual conjugation experiments were performed, one with transfer of pOLA52 and one with pHHA45. In both cases, overnight cultures of donor and recipients were reinoculated into fresh preheated BHI medium and grown to an optical density at 600 nm (OD_{600}) of 0.5. Then, 1-ml samples of each donor and recipient were mixed in 24-well microtiter plates and incubated at 37°C for 5 h. Conjugation mixtures were diluted and plated on selective plates for CFU counting.

RESULTS

Horizontal gene transfer of single-stranded DNA by conjugation. The results of the conjugational transfers are presented in Fig. 1.

All three recipients, MG1655-*RN*, MG1655-*hsdR*^{INT}, and MG1655-*hsdR*^{COMPL}, accepted the methylated plasmid pOLA52 from donor MG1655-*A* at high ratios (transconjugants per 10^8 recipients) of 2.05×10^3, 1.04×10^3, and 0.67×10^3, respectively. The level of conjugational transfer into the MG1655-*hsdR*^{COMPL} strain complemented with the *hsdR* gene in *trans* was significantly lower ($P = 0.021$) than in the MG1655-*RN* strain, with a wild-type functional *hsdR* gene on the chromosome, possibly due to the higher copy number, stronger promoter, or both.

For the transfer of the unmethylated plasmid pOLA52, with two recognition sites, from the donor MG1655-*hsdM*^{INT} to the three recipients, MG1655-*RN*, MG1655-*hsdR*^{INT}, and MG1655-*hsdR*^{COMPL}, the ratios were 0.42×10^3, 2.79×10^3, and 0.17×10^3, respectively. The recipient MG1655-*hsdR*^{INT} showed a statistically significantly ($P = 0.029$) higher level of transfer, which was more than 6.5 times higher than those of the two recipients with functional *hsdR* genes.

In the transfer of pHHA45, no significant difference was observed between the ratios of 8.45×10^4, 7.73×10^4, and 3.58×10^4 from the donor MG1655-*A* or between the ratios of 9.47×10^4, 6.25×10^4, and 7.66×10^4 from the donor MG1655-*hsdM*^{INT} to the recipients MG1655-*RN*, MG1655-*hsdR*^{INT}, and MG1655-*hsdR*^{COMPL}, respectively (see Table S1 in the supplemental material).

Complementation of *hsdR* restores restriction activity. The restriction gene *hsdR* was cloned into the expression vector pMSC83 under the control of the arabinose promoter pBAD, which is known to be leaky in rich media (34). The conjugation experiment was therefore performed without addition of arabinose to avoid overexpression of the *hsdR* gene, which could potentially be harmful to the cell. As a control for sufficient *hsdR* expression and to verify that the decrease in transfer observed in Fig. 1 was caused by expression of the *hsdR* gene alone and not the vector pMSC83, conjugation with the complemented strain and a control strain with the pMSC83 vector was performed. For the control experiment, the methylation-deficient donor MG1655-*hsdM*^{INT} was used with each plasmid (pHHA45 and pOLA52). The results of the conjugative control experiment are presented in Fig. 2.

In experiments with the unmethylated plasmids pHHA45 and pOLA52, the conjugational transfer of pOLA52 to MG1655-*hsdR*^{COMPL} was significantly decreased ($P = 0.00031$) compared with that to MG1655-*hsdR*^{CONTROL}, with transfer ratios of 0.21×10^3 and 5.89×10^3, respectively. With the transfer of pHHA45, no significant difference was observed between the transfer ratios of 5.70×10^4 and 3.37×10^4 for MG1655-*hsdR*^{COMPL} and MG1655-*hsdR*^{CONTROL}, respectively.

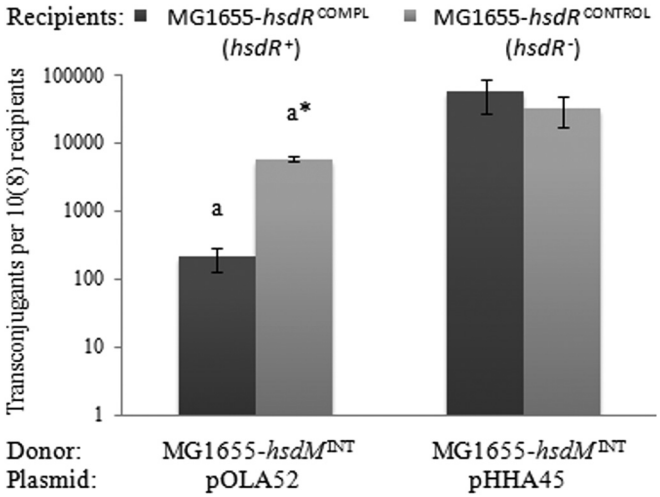

FIG 2 Conjugative effect of *hsdR* from the unmethylated donor strain MG1655-*hsdM*^{INT} inserted into the complemented strain MG1655-*hsdR*^{COMPL} compared to that in a control strain harboring the empty vector pMSC83. For conjugation with the unmethylated plasmid pHHA45, which lacks recognition sites for MG1655-*hsdR*, no significant difference was observed between the complementation and control strains. For conjugation with the plasmid pOLA52, which has two recognition sites, a significant difference was observed between the two recipients (a*, $P = 0.00031$).

DISCUSSION

Previous studies of the influence of RM systems in conjugation have generated conflicting conclusions in relation to the effect of RM systems on plasmid transfer (8–15). This has led to some controversy on how restriction-modification systems act on the uptake of single-stranded DNA, such as plasmids transferred by conjugation. An obvious driver of the conflicting conclusions from these studies is the fact that many of these studies were carried out in an era before the emergence of molecular techniques in microbiology and before the genetic determinants responsible for the RM phenotypes were identified.

The current study aimed to utilize isogenic strains and defined knockout genetic constructs to study how RM systems influence plasmidic transfer and showed that the type I restriction-modification system EcoKI in *E. coli* K-12 MG1655 affects conjugational transfer if the transferred DNA includes nonmethylated recognition sites.

Transfer of the methylated plasmid pOLA52 from the wild-type host to the three different recipients, with different restriction abilities, was not expected to have any significant influence on its uptake, as the plasmid was modified as "self" and in all three cases should have been immune from degradation. Transfer of the nonmethylated plasmid into the complemented strain was significantly lower than into the wild-type strain; more surprisingly, the same was true for the methylated plasmid. One explanation for this decrease might be incompatibility between pOLA52 and the vector carrying the complementation gene *hsdR*, but this was not expected, as the two replicons belong to IncXI and ColE10, respectively. This was also verified by the control experiment (Fig. 2), where the results clearly showed that the decrease in transfer was caused by the restriction gene alone and not the vector pMSC83. Therefore, a more likely explanation may be that overexpression of EcoKI, resulting in the formation of R_2M_2S complexes rather than M_2S complexes, leads to degradation of hemi-methylated DNA, as in the type II systems described by Nelson et al. (35).

The unmethylated plasmid pOLA52 was taken up significantly less in the wild-type strain than in strain MG1655-hsdR^{INT}, which has an interrupted restriction gene. The plasmid without recognition sites for EcoKI can transfer efficiently between donor and recipients independently of methylation and restriction abilities. This was shown by the transfer of the plasmid pHHA45 from both the methylation-deficient donor MG1655-*hsdM*^{INT} and the MG1655-*A* donor with the functional *hsdM* gene to three different recipients with functional, disrupted, and complemented restriction genes.

Even though the transfer efficiencies of the two plasmids, pOLA52 and pHHA45, differ by nearly 2 logs in the *hsdR*-disrupted recipient, we find pHHA45 an appropriate control plasmid, as both plasmids are narrow-host-range plasmids and about the same size. More plasmids could be investigated to confirm our observation that the conjugational transfer or uptake in *E. coli* K-12 strain MG1655 is in fact dependent not only on the donor's methylation and the recipient's restriction abilities but also on the presence of recognition sequences on the plasmid. The results of this genotypic study are in agreement with the study performed by Arber and Morse (18), where the RM-deficient strains were selected based on phenotypes by testing their ability to restrict different phages, but they were not further characterized.

From the present study, we have shown that a type I RM system can act as a barrier to the conjugational transfer of plasmids, but in many of the previous studies, the restriction is described only phenotypically (10, 18) or as a transfer between unrelated species (8, 11, 12) without our knowing the type of RM system involved. There is evidence that a type-III-like RM system may act as barrier to transformation in *S. aureus* strains (36) but not to conjugational transfer. Further, the present study focuses on only a single system, in a single isolate, with only two different plasmids. This leads to the questions of whether all type I systems influence conjugational transfer and whether the three remaining systems have the same ability to protect hosts from invading foreign DNA. Murray and colleagues suggest that the protection from foreign DNA might be altered by alleviation of chromosomal restriction genes, which might lead to uptake (37–39).

Pérez-Mendoza and de la Cruz (40) investigated two different knockout libraries, the Keio collection of single knockouts and a random insertion library, to determine how recipient cells contribute to bacterial conjugation (40). Their only finding was that the lipopolysaccharide (LPS) showed strong conjugation inhibition when conjugation was performed in liquid, but with filter mating, the reduction was restored. The Keio collection is based on a restriction-deficient K-12 strain variant (BW25113), which explains why they did not find EcoKI as a contributing factor in conjugation, as we did in this study.

Even though Pérez-Mendoza and de la Cruz did not find any genes responsible for the conjugational uptake in the recipient, this does not preclude the possibility that such genes exists. The two parental *E. coli* strains used in their study may be missing regulatory genes responsible for conjugational transfer. To identify possible barriers to conjugational uptake, as well as uptake by the other HGT mechanisms, good and poor recipients must be identified by phenotype and compared at the genetic level.

In summary, the EcoKI RM system found in *E. coli* K-12 strain MG1655 affects the conjugational transfer of plasmid pOLA52,

harboring two recognition sites, but the results imply that this effect is not absolute and that uptake is still possible, though at a lower level. The results showed 4.88-times-higher uptake of methylated pOLA52 than of the unmethylated plasmid in wild-type MG1655. This leads to the conclusion that plasmids with the same methylation pattern as the recipient can have a competitive advantage when entering a new host.

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