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 various 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 conjugal transfer of plasmid pOLA52, which carries 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 non-methylated 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,M,S) is formed, whereas methyltransferase of the DNA requires formation of a complex of only the HsdM and HsdS proteins (M,S) (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 conjugal 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 conjugal 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 conjugal 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 Saul, 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 Saul system was not sufficient for producing strains that efficiently take up foreign DNA (6), again questioning the importance of RM systems as barriers to conjugal transfer.

In the current study, we aimed to clarify the impact of a restriction-modification system in the conjugal 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 AACN6GTGC, 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 possesses three different methylation-requiring type IV systems, EcoKMrA, 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.
TABLE 1 Bacterial strains, plasmids, and oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Bacterial strain, plasmid, or oligonucleotide</th>
<th>Description (relevant genotype and/or phenotype) or sequence (5' to 3')</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1655 (K-12 strain)</td>
<td>F' λ− ilvG rfb-50 rph-1</td>
<td>CGSC</td>
</tr>
<tr>
<td>MG1655-RN</td>
<td>Spontaneous Rif' and Nal' derivative of MG1655</td>
<td>This study</td>
</tr>
<tr>
<td>MG1655-A</td>
<td>Spontaneous NalN' derivative of MG1655</td>
<td>This study</td>
</tr>
<tr>
<td>MG1655-hsdR^INT</td>
<td>TargetTron insertion at nucleotide 1740</td>
<td>1741 of hsdR, Rif' Nal'</td>
</tr>
<tr>
<td>MG1655-hsdM^INT</td>
<td>TargetTron insertion at nucleotide 720</td>
<td>721 of hsdM, Kan'</td>
</tr>
<tr>
<td>MG1655-hsdR^COMPL</td>
<td>MG1655-hsdR^INT with phsR for complementation, Rif' Nal' Tem'</td>
<td>This study</td>
</tr>
<tr>
<td>MG1655-hsdR^CONTROL</td>
<td>MG1655-hsdR^INT with expression vector pMSC83, Rif' Nal' Tem'</td>
<td>This study</td>
</tr>
<tr>
<td>DH10B</td>
<td>F' endA1 recA1 galE15 galK16 supG rpsL ΔlacX74 φ80lacZΔM15 araD139 Δ(ara leu)7697 merA Δ(mrr-hsdRMS-mcrBC) λ −</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

**Plasmids**

- pACD4K-C-loxP: Linearized TargetTron plasmid with a T7 promoter; Cam' Kan-Δtd (Sigma)
- pACD4K-C-loxP (hsdR): pACD4K-C-loxP targeted for hsdR of MG1655 (LR1/LR2/LR3) (This study)
- pACD4K-C-loxP (hsdM): pACD4K-C-loxP targeted for hsdM of MG1655 (LR4/LR5/LR6) (This study)
- pAR1219: Expresses T7 DNA polymerase under the control of the IPTG-inducible lac UV5 promoter; Amp' (Sigma)
- 706-Cre: Expression plasmid for Cre recombinase driven by the thermosensitive promoter cI578; Tem' (Gene Bridges GmbH)
- pOLA52: Plasmid of 45.7 kb with two restriction sites for hsdR (EcoKI); Amp' (41)
- pHHA45: Plasmid of 51.6 kb without restriction sites for hsdR (EcoKI); Amp' (31)
- pMSC83: Cloning vector used for complementation; Tem' (This study)
- phsR: R.EcoKI from MG1655 cloned into pMSC83 (This study)

**Oligonucleotides**

- LR1 (hsdR IBS): AAAAAAGCTTATAATTATCCCTACATCGCGGCTATGTGCGCCCAGATAGGGTG (Sigma)
- LR2 (hsdR EBS1d): CAGATTGTCCAAAACTGGTGATAACAGATAAGGCGCTTATATTAAAATACCTACCTTTCTTTG (Sigma)
- LR3 (hsdR EBS2): TGAACGCCATTTCATATTACGGTGATCGATGAGGAAAGTGTCT (Sigma)
- LR4 (hsdR-V-R): TCCAGCTGGGCTGGGAACGTG (TAGC)
- LR5 (hsdM IBS): AAAAAAGCTTATAATTATCCCTACATCGCGGCTATGTGCGCCCAGATAGGGTG (Sigma)
- LR6 (hsdM EBS1d): CAGATTGTCCAAAACTGGTGATAACAGATAAGGCGCTTATATTAAAATACCTACCTTTCTTTG (Sigma)
- LR7 (hsdR EBS2): TGAACGCCATTTCATATTACGGTGATCGATGAGGAAAGTGTCT (Sigma)
- LR8 (hsdM-V-F): CCAATGTCTGAGAGCCTT (TAGC)
- LR9 (hsdR-C-F): GTCTAATGCGCGCGGAAGTGA (TAGC)
- LR10 (hsdR-C-R): GCGCAGCTGAAAGGATAGGTA (TAGC)

* For bacterial strains and plasmids, the relevant genotype, phenotype, and other characteristics are shown. Abbreviations: INT: interruption; COMPL: complementation.

**Materials and Methods**

*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. The conjugation experiments were performed from donors with (hsdM^+^) and without (ΔhsdM) 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 conjugal 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 TargetTron insertion mutants of E. coli MG1655.** Two TargetTron 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 DNA 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 TargetTron 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.
Complementation of hsdr with phsdr. 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 mutant, we amplified a 3,633-bp fragment encompassing the hsdr gene on the chromosome, MG1655-hsdR COMPL, with a complemented 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 inoculated 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 × 10^8, 1.04 × 10^8, and 0.67 × 10^8, 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 × 10^8, 2.79 × 10^8, and 0.17 × 10^8, 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 × 10^7, 7.73 × 10^7, and 3.58 × 10^7 from the donor MG1655-A or between the ratios of 9.47 × 10^7, 6.25 × 10^7, and 7.66 × 10^7 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 × 10^3 and 3.37 × 10^4 for MG1655-hsdR COMPL and MG1655-hsdR CONTROL, respectively.
CONJUGATIVE EFFECT OF hsdR FROM THE UNMETHYLATED DONOR STRAIN MG1655-hsdR<sup>INT</sup> INSERTED INTO THE COMPLEMENTED STRAIN MG1655-hsdR<sup>COMPL</sup>

**FIG 2** Conjugative effect of hsdR from the unmethylated donor strain MG1655-hsdR<sup>INT</sup> inserted into the complemented strain MG1655-hsdR<sup>COMPL</sup> 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 the wild-type strain; more surprisingly, the methylated plasmid into the complemented strain was significantly higher than the wild type. This does not preclude the possibility that such genes exist. The two parental *E. coli* strains used in their study may be missing regulatory genes responsible for the conjugational uptake in the recipient, 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 exist. 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, 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 plasmid, 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).
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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REFERENCES


