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Restriction-Modification-Guided Plasmid Engineering for Burkholderia species

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Article

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Abstract

Genetic manipulation of non-model bacteria is frequently constrained by endogenous restriction-modification (RM) systems that recognize and degrade incoming DNA, limiting the development and application of genetic tools. This challenge is particularly evident in *Burkholderia*, a diverse genus encompassing clinically, environmentally, and biotechnologically important species. Here, we define the RM landscape of *Burkholderia* and use this information to establish a rational strategy for improving genetic accessibility.

Using the genetically recalcitrant *Burkholderia cepacia* as the primary experimental system and *Burkholderia thailandensis* as a comparative model, we combined methylome data analysis, and experimental DNA-transfer assays to identify RM-associated barriers to plasmid uptake. Analysis of 1,044 publicly available REBASE records, including 53 named *Burkholderia species*, together with their predicted RM recognition motifs, revealed extensive diversity in RM systems and their target sequences. Systematic elimination of individual and combinatorial RM recognition sites from plasmid DNA progressively enhanced transformation and conjugation efficiencies, demonstrating that multiple RM targets collectively restrict incoming DNA. Comparative analyses further revealed species-specific differences in genetic permissiveness between *B. cepacia* and *B. thailandensis*.

Leveraging these findings, we developed an RM-optimized pan-*Burkholderia gfp* containing vector that enabled stable fluorescence and live-cell confocal imaging during mammalian cell infection. Our study establishes RM-informed vector design as a generalizable framework for overcoming endogenous DNA-transfer barriers and expands the genetic toolkit available for functional genomics, microbial engineering, and infection biology in *Burkholderia*.

Introduction

Genetics with non-model bacteria remains a major challenge in microbiology due to limited genetic tools, intrinsic DNA restriction systems, and poor experimental tractability [1, 2]. The genus *Burkholderia* comprises a diverse group of Gram-negative bacteria that include major human, animal, and plant pathogens, as well as environmentally persistent opportunistic species [1]. Clinically important members such as *Burkholderia pseudomallei*, the causative agent of melioidosis, and species within the *Burkholderia cepacia* complex are associated with high mortality, intrinsic antimicrobial resistance, and severe outcomes in vulnerable patient populations [1, 3]. Despite their global health relevance, mechanistic studies of *Burkholderia* biology remain limited, because many species are exceptionally difficult to genetically manipulate [1, 2]. This lack of reliable genetic tools continues to impede functional studies of virulence, persistence, and environmental adaptation across the genus [1, 2].

Multiple approaches have been explored to introduce foreign DNA into *Burkholderia*, including chemical transformation, electroporation, and conjugation [2, 4, 5]. More recently, CRISPR-Cas and transposon-based tools have been applied for the genetic manipulation of these species; however, their implementation also relies on efficient delivery of foreign DNA [2, 6, 7], often requiring electroporation of

relatively large amounts of DNA (1–10 μg). These approaches frequently fail, even when diverse plasmid backbones, origins of replication, and selection markers are employed [2, 4]. While such failures are often attributed to protocol optimization or plasmid incompatibility, the underlying biological barriers responsible for this genetic inaccessibility remain poorly defined. Moreover, commonly used and readily available vectors are relatively large and often contain kanamycin, tetracycline, or spectinomycin-resistance cassettes as selection markers, which have been used at comparatively high concentrations for mutant selection [2, 7–10]. In particular, the contribution of host restriction-modification (RM) systems, which protect bacterial genomes by recognizing and degrading unmethylated and incorrectly methylated foreign DNA, has not been systematically evaluated as a primary obstacle to plasmid entry in *Burkholderia species* [11, 12].

Beyond improving genetic accessibility, the development of a stable GFP-expressing *Burkholderia* strain has important implications for host-pathogen interaction studies [13, 14]. Visualization of intracellular *Burkholderia* during infection remains technically challenging because wild-type bacteria lack an intrinsic fluorescent signal, whereas transient labeling approaches are often limited by signal loss, photobleaching, incomplete labeling, and incompatibility with prolonged live-cell imaging [13, 15]. These challenges become even more pronounced in complex biological systems, including three-dimensional tissues and organoid models, where background autofluorescence and limited penetration of exogenous dyes further compromise bacterial detection [15]. Consequently, stable plasmid-mediated GFP expression provides a valuable approach for reliable bacterial tracking during infection while avoiding repeated labeling procedures [13, 14]. In addition to stable reporter expression, incorporation of inducible regulatory elements can provide temporal control over reporter activity, enabling fluorescence to be modulated according to experimental requirements [8].

Here, we demonstrate that active RM systems constitute a major molecular barrier to genetic manipulation in *Burkholderia*. Using *B. cepacia* and *B. thailandensis* as experimental models, we combined methylome analysis, comparative RM profiling, and systematic plasmid engineering to identify and eliminate host RM recognition motifs from the plasmid. Comparative analysis of RM recognition motifs compiled from 1,044 publicly available records of *Burkholderia* (53 species) further enabled the development of a genus-informed plasmid design strategy. We show that synonymous sequence redesign markedly improves both transformation and conjugation efficiencies while preserving plasmid architecture and biological function. Finally, stable expression of a Superfolder green fluorescent protein (sfGFP) reporter during host-cell infection demonstrates the utility of this approach for downstream functional studies. Collectively, our findings establish RM systems as a principal determinant of genetic inaccessibility in *Burkholderia* and provide a rational framework for RM-guided plasmid engineering across the genus, with implications for future work on high-containment pathogens such as *B. pseudomallei*.

Results

Conventional DNA delivery approaches reveal a barrier to genetic accessibility in Burkholderia

To assess genetic accessibility in *Burkholderia*, we evaluated commonly used DNA delivery methods, chemical transformation, electroporation, and conjugation, in *B. cepacia* and *B. thailandensis*. Three plasmids (3–6 Kb) representing distinct vector backbones, and origins of replication, pBBR-based pRL814, pSUP-based pPK15534, and pRL27, were tested using these approaches (Fig. S1) [7, 8, 16]. Despite optimization, none of the plasmids yielded transformants or transconjugants in either *Burkholderia* species on selective plates, whereas the plasmids were readily propagated in *Escherichia coli* DH5 α and MG1655 (Fig. 1a-c).

To determine whether the failure to recover plasmid-containing *Burkholderia* resulted from inappropriate antibiotic selection, we evaluated antibiotic susceptibility and alternative selectable markers. Minimum inhibitory concentration (MIC) analysis identified suitable antibiotic concentrations for selection (Table S1), with chloramphenicol providing the most effective positive selection at 60 µg/mL. However, replacing the spectinomycin resistant cassette with a chloramphenicol resistance cassette and optimizing the selection conditions did not enable plasmid recovery in *B. cepacia* and *B. thailandensis* on selective plates (Fig. 1a-c). It indicated that selectable-marker incompatibility was unlikely to account for the observed failure of DNA transfer.

Together, the inability to recover plasmids across multiple DNA delivery methods, vector backbones, and selection conditions suggested that *B. cepacia* and *B. thailandensis* possess intrinsic barriers that limit the establishment of incoming DNA (Fig. 1d).

Database analysis reveals diverse RM systems and recognition motifs across Burkholderia

To investigate whether RM systems contribute to the poor genetic accessibility of *Burkholderia*, we first examined RM systems and their associated recognition motifs in *B. cepacia* and *B. thailandensis* using available methylome data from REBASE (Table S2) [17]. This analysis identified Type II and Type III RM systems and their recognition motifs in the two species (Table S2a and S2b) [11, 17]. Comparison with *B. pseudomallei* K96243 revealed that these motifs were also represented in this clinically important species, suggesting conservation of specific RM recognition sequences among *Burkhlderia* species.

We next expanded the analysis to determine the distribution and diversity of RM systems across the genus. RM systems and their associated recognition motifs were analysed across 1,044 publicly available records of *Burkholderia* to understand broader genus-level coverage. Of these, 931 entries were assigned to 53 named Burkholderia species. The remaining 113 entries lacked a named species-level classification and comprised 84 entries annotated as *Burkholderia species*, 13 *Burkholderia* phage entries, 12 *Burkholderiaceae* bacterium entries, and four *Burkholderiales* bacterium entries. These 113 entries were therefore not included in the count of named species. Type I, Type II, and Type III RM systems were widely distributed across *Burkholderia;* although their composition and recognition-motif profiles varied substantially among species (Fig. 2; Table S3) [11, 18]. Notably, several Type II and Type III recognition motifs identified in *B. cepacia* and *B. thailandensis* were also present across multiple *Burkholderia* species, indicating that these RM-associated sequences are broadly represented within the genus (Table S2 and S3).

Analysis of recognition-motif frequency further revealed marked differences in their occurrence across the *B. pseudomallei* K96243, *B. cepacia* and *B. thailandensis* (Table S2b). Type II motifs were particularly abundant across the species, and Type III recognition motif was highly represented, whereas the analysed Type I motifs were comparatively less frequent.

We subsequently examined commonly used plasmid backbones for the presence of these RM recognition sequences. Multiple Type I, Type II, and Type III target sites were identified within the plasmid sequences, providing a potential molecular basis for their inefficient establishment in *B. cepacia* and *B. thailandensis* (Fig. S1). Together, these analyses implicated RM Systems as candidate barriers to incoming plasmid DNA and provided a rationale for systematically removing RM recognition sites to determine their contribution to genetic accessibility in *Burkholderia*.

Systematic RM-site removal identifies major barriers to plasmid acquisition

To determine the contribution of individual RM recognition sites to plasmid restriction, we used the smallest conjugatable plasmid (pPK15534; 3.7kb) as the parental backbone for RM-guided engineering (Fig. 1e) [16]. The plasmid contains an origin of replication, a chloramphenicol resistance (*cat*) cassette, a mobilization element, and a sfGFP reporter gene [16]. Guided by the identified distribution of RM recognition motifs, the plasmid was systematically redesigned by selectively disrupting individual Type II and Type III RM recognition sites while preserving its overall size, backbone architecture, and essential functional elements (Fig. 1e). The resulting constructs were evaluated for transformation and conjugation efficiencies in *B. cepacia* and *B. thailandensis* (Figs. 3 and 4).

Type I recognition site removal did not show improvement in plasmid acquisition. Disruption of individual Type II RM recognition sites improved transformation efficiency to varying degrees compared with the parental plasmid, whereas modification of individual Type III recognition sites produced little or no improvement. Simultaneous disruption of all three Type II recognition sites (pPL540) resulted in a substantially greater increase in transformation efficiency than modification of individual Type II sites in both *B thailandensis* and *B. cepacia* (one-way ANOVA, $p < 0.0001$ for both species) (Fig. 3a and b). These results indicate that multiple Type II recognition sites collectively contribute to restriction of incoming plasmid DNA.

To further distinguish the contributions of Type II and Type III RM recognition sites, pPL540 was compared with pPL542, in which both identified Type III recognition sites were disrupted. pPL540 exhibited significantly higher transformation efficiency than pPL542 in both *B. thailandensis* ($p < 0.0001$) and *B. cepacia* ($p = 0.0001$), whereas removal of type III sites alone resulted in only marginal improvement (Fig. 3a and b). Type III RM-site removal similarly produced little improvement in conjugative plasmid transfer. Type II RM systems yielded relatively restriction impact on the transformation than conjugation of parental plasmid. Species level difference for the type II removal gave almost 1000 to 10000-fold in transformation and less than 5-fold in conjugation (Fig. 3a and b).

Thus, although both classes of recognition sites contribute to plasmid restriction, Type II recognition sites represent the major barrier to plasmid establishment under the conditions tested.

These finding demonstrate that RM recognition sites restrict plasmid acquisition in *Burkholderia* and that systematic removal of all Type II sites provides a substantially greater improvement in DNA delivery than modification of individual type II or Type III sites alone (Fig. 3c-f).

RM-site engineering reveals species-specific barriers to plasmid acquisition

RM-sites removal improved plasmid acquisition in both *B. thailandensis* and *B. cepacia*, although the magnitude of improvement differs between species and DNA delivery methods (Figs. 3 and 4). During transformation, progressive disruption of Type I, Type II, and type III RM recognition sites resulted in corresponding increases in plasmid acquisition in both species (Figs. 3 and 4). Constructs carrying individual or partial combinations of RM-site modifications exhibited intermediate efficiencies, whereas the fully engineered plasmid pPL545 consistently produced the highest transformation efficiency (Figs. 3 and 4a-d). Within each species, transformation efficiencies differed significantly among the engineered constructs (one-way ANOVA, $p < 0.0001$), demonstrating a strong influence of RM-site composition on plasmid establishment.

Despite the similar response to progressive RM-site removal, transformation efficiencies were consistently higher in *B. thailandensis* than in *B. cepacia* (Fig. 3a,c,d,g,h; 4a,b,e,f). In contrast, conjugation efficiencies were broadly comparable between the two species (Fig. 3b,e,f,i,j; 4c,d,g,h). Progressive RM-site removal nevertheless improved conjugative plasmid transfer, with pPL545 exhibiting the highest conjugation efficiency among the engineered constructs in both species (Fig. 4c,d,g,h). These contrasting patterns indicate that species-specific differences in genetic accessibility depend on the route of DNA delivery. In particular, the lower transformation efficiency of *B. cepacia*, despite conjugation efficiencies comparable to those for *B. thailandensis*, suggests the presence of additional barriers associated with transformation-mediated DNA uptake or early plasmid establishment.

The contribution of individual RM-site modifications to conjugative transfer also differed between species (Fig. 3a,b). In *B. cepacia*, simultaneous disruption of all three Type II recognition sites significantly enhanced conjugation compared with constructs carrying individual Type II RM-site modifications ($p < 0.001$) (Fig. 3e). In *B. thailandensis*, however, cumulative Type II RM-site disruption did not provide a significant additional improvement over the individually modified constructs (Fig. 5f). Two-way ANOVA further identified significant effects of bacterial species, plasmid construct, and their interaction (Fig. 3b,e,f,i,j; 4c,d,g,h,j), demonstrating that the effect of RM-site engineering depends on both plasmid design and recipient host.

These finding reveal host- and delivery-route-dependent barriers to plasmid acquisition in *Burkholderia*. RM-site engineering improved DNA transfer in both species. However, comparable conjugation

efficiencies suggest that conjugative transfer can reduce the species-specific disparity observed during transformation. Despite these differences, complete RM-site engineering consistently generated the most efficient plasmid backbone, supporting pPL545 as the lead construct for subsequent functional applications (Fig. 4i and j).

gfp containing pPL545 vector enables visualization during mammalian cell infection

Having established the RM motif-reduced plasmid as the most efficient construct for DNA delivery, plasmid maintenance was further evaluated through prolonged serial subculturing for 96 generations, under both chloramphenicol-selected and nonselective conditions. Colonies recovered under both conditions retained detectable GFP fluorescence, with no nonfluorescent colonies observed among the approximately 20–30 colonies examined per strain. These findings provide qualitative evidence of sustained reporter maintenance in both *Burkholderia species* in the absence of antibiotic selection. We next evaluated the utility of pPL545 as a stable fluorescent reporter for *Burkholderia*. The optimized plasmid carrying sfGFP was introduced into *B. cepacia* and *B. thailandensis*, and fluorescence was assessed before and after mammalian cell infection. In *B. cepacia* and *B. thailandensis*, the pPL545 produced high fluorescence intensity (two-tailed unpaired *t-test, p* < 0.0001) (Fig. S2), demonstrating that extensive RM-site engineering in vector backbone and *gfp* gene did not compromise reporter expression or plasmid functionality.

We next examined whether the pPL545 could facilitate visualization of *Burkholderia* during mammalian cell infection. MCF-7 cells were infected with GFP-expressing *B. cepacia* and *B. thailandensis* then readily detected in association with infected cells by fluorescence microscopy (Fig. 5a-g). High-resolution confocal imaging further revealed GFP-expressing bacterial within infected cells (Fig. 5h-m), demonstrating that reporter fluorescence was maintained during infection. Three-dimensional reconstruction of confocal z-stacks further supports the intracellular localization for fluorescent bacteria within the host-cell volume (Fig. 5n-p).

These results demonstrate that the RM motif-reduced *gfp* containing vector (pPL545) retains the functional reporter expression and enables fluorescence-based visualization of *Burkholderia* during mammalian cell infection. Thus RM-guided plasmid engineering not only improves genetic accessibility but also provides a functional platform for imaging-based studies of *Burkhlderia*-host interaction.

Discussion

Genetic manipulation remains a major bottleneck in *Burkholderia* research despite extensive efforts to optimize DNA delivery [1, 2, 4, 11, 19]. Our findings identify RM-mediated restriction as an important and experimentally tractable barrier to plasmid establishment [11, 18, 20]. Systematic elimination of RM recognition motifs progressively improved both transformation and conjugation, with the fully engineered pPL545 showing the highest DNA-delivery efficiency in *B. cepacia* and *B. thailandensis*. These findings indicate that the fate of incoming DNA depends not only on how efficiently it enters the cell, but also on its compatibility with the host's DNA-recognition machinery (Figs. 3 and 4) [11, 20, 21].

Previous approaches to *Burkholderia* genetic manipulation have largely emphasized physical DNA delivery, including optimization of electroporation, chemical transformation, and conjugation, as well as variation of plasmid backbones, replication origins, and selectable markers [1, 19, 22–24]. Our results provide a complementary explanation for why such optimization may remain insufficient, even when DNA delivery is achieved, incoming plasmids containing host-recognized RM motifs remain susceptible to restriction before successful establishment. Consistent with this interpretation, the failure of multiple plasmid backbones and delivery approaches in our initial experiments was not overcome by changing selectable markers, whereas sequence-level removal of RM recognition sites substantially improved plasmid acquisition (Fig. 1; Table S1; Fig. S1) [25]. Thus, physical delivery and plasmid architecture remain important determinants of genetic accessibility, but their effectiveness can be constrained by the sequence compatibility of the incoming DNA with host RM systems [11, 20].

The use of a chloramphenicol resistance cassette (*cat*) as a selectable marker in our engineered plasmids is well-suited for genetic studies in *Burkholderia* (Figs. 3 and 4). Chloramphenicol holds no therapeutic role in the clinical management of *Burkholderia* infections (such as melioidosis by *B. pseudomallei* or *B. cepacia complex* infections), which rely instead on frontline agents like ceftazidime, meropenem, or trimethoprim-sulfamethoxazole [26]. Because *Burkholderia species* possess intrinsic multi-drug resistance mechanisms against chloramphenicol that naturally limit susceptibility, employing a *cat* marker for plasmid selection poses no clinical risk of propagating or selecting for clinically resistant strains [27–29]. Furthermore, it provides a dedicated, reliable mechanism for plasmid maintenance in the laboratory without overlapping with critical last-line human therapeutics [26, 28].

The progressive response to RM-site removal provides mechanistic insight into this restriction barrier. Individual Type II RM-site modifications produced variable improvements, whereas simultaneous removal of multiple Type II sites resulted in a substantially greater increase in transformation efficiency [25]. Removal of Type III sites alone had a comparatively limited effect, but elimination of the remaining sites from an extensively engineered backbone further increased DNA acquisition (Figs. 3 and 4) [30, 31]. The superior performance of pPL545 therefore supports a cumulative model in which multiple recognition sites collectively determine the susceptibility of incoming plasmid DNA to restriction (Fig. 4) [11, 32]. At the same time, the differences between the two species indicate that RM recognition does not fully account for genetic accessibility. Transformation was consistently less efficient in *B. cepacia* than in *B. thailandensis*, whereas conjugation efficiencies were more comparable, suggesting that additional host- and delivery-route-dependent processes influence DNA uptake or early plasmid establishment (Figs. 3 and 4) [12, 33, 34].

A major advantage of the present strategy is that RM recognition sites were disrupted through synonymous sequence modifications rather than removal of functional genetic elements. This allowed the nucleotide sequence to be redesigned while preserving encoded proteins, plasmid architecture, reporter activity, and selectable functions (Fig. 4; Table S6). The findings therefore highlight the primary nucleotide sequence of a vector as an underappreciated determinant of genetic compatibility. Conventional strategies often address plasmid size, replication origin, or selection systems; RM-aware

sequence engineering adds host-specific DNA recognition to these considerations and provides a rational alternative to empirical vector optimization [17, 25, 35].

RM-site engineering also offers an alternative to methylation-based strategies used to protect incoming DNA from restriction. Pre-methylation approaches can require prior identification and functional reconstruction of host methyltransferases and may need to be adapted to individual strains with distinct RM repertoires [18, 25, 32]. In contrast, removing recognition motifs directly from the incoming DNA reduces dependence on host-matched methylation and specialized donor systems. As genome sequences, methylome datasets, and curated RM annotations become increasingly available, this approach could allow restriction barriers to be considered during vector design rather than addressed only after unsuccessful DNA delivery [17, 36].

The genus-wide analysis further extends this principle toward a pan-*Burkholderia* strategy. Comparative analysis of RM recognition motifs across 1,044 REBASE records of *Burkholderia* representing 53 *Burkholderia species*, revealed recognition sequences shared among multiple members of the genus (Fig. 2; Table S2; Table S3). This broader taxonomic representation provided a basis for identifying commonly occurring RM targets and incorporating their removal into the plasmid redesign strategy. pPL545 was therefore designed to reduce susceptibility to restriction across a broader RM landscape rather than a single experimentally characterized host (Fig. 2; Table S3). Although experimental validation was performed in *B. cepacia* and *B. thailandensis*, the genus-wide analysis provides a foundation for extending this approach to additional *Burkholderia species* with diverse RM repertoires (Fig. 2; Table S3). The resulting framework shifts vector development from strain-by-strain empirical optimization toward sequence-informed design based on the RM landscape of the target bacterial group. Importantly, this strategy is complementary to, rather than a replacement for, broad-host-range replication systems and established DNA-delivery methods [1, 2, 5].

The functional characterization of the optimized vector demonstrates that improved DNA accessibility can be achieved without compromising downstream biological utility. pPL545 retained robust GFP expression and enabled fluorescence and confocal visualization of *B. cepacia* and *B. thailandensis* during mammalian cell infection (Fig. S2; Fig. 5).

More broadly, efficient establishment of recombinant DNA is a prerequisite for many genetic approaches in *Burkholderia* [1, 6, 7]. The optimized backbone could therefore support applications including genetic complementation, transcriptional reporter assays, and other plasmid-dependent studies, while RM-compatible vectors may also improve delivery of constructs used for allelic exchange, transposon mutagenesis, and CRISPR-based manipulation. These applications were not directly tested here and should therefore be regarded as potential extensions rather than demonstrated capabilities [25]. The underlying sequence-design principle may likewise be applicable to other genetically recalcitrant bacteria in which endogenous RM systems limit horizontal DNA acquisition.

We propose that the RM-aware strategy and plasmid pPL545 facilitate genetic manipulation across *Burkholderia* genus based on our database analysis of motif data from 1,044 REBASE records including

53 *Burkholderia species* (Fig. 2; Table S3). Though, this hypothesis has been experimentally validated in only two species, our tool should be applicable to pan-*Burkholderia species*. Additionally, factors other than RM systems, such as CRISPR-Cas-mediated defense, may influence the entry or establishment of vectors and foreign DNA in *Burkholderia* species, which needs to be addressed.

Conclusion

This study demonstrates that restriction-modification (RM) systems constitute a major determinant of the poor genetic tractability of *Burkholderia* by restricting the establishment of incoming plasmid DNA. In this work, we developed genetic tools and RM-aware strategy for mobilization or transformation of codon optimized exogenous vector into *Burkholderia* strains to express exogenous genes.

Collectively, this study provides both mechanistic insight into the role of RM systems in limiting horizontal DNA acquisition and a practical strategy for improving genetic accessibility in *Burkholderia*. We anticipate that the principles described here will facilitate the development of more effective molecular tools for functional genetics in this important bacterial genus and provide a foundation for extending RM-guided plasmid engineering to other genetically recalcitrant bacteria.

Methods

Bacterial strains, plasmids and culture conditions

Burkholderia cepacia ATCC 25416 (UCB 717) and *Burkholderia thailandensis* ATCC 700388 (E264) were used as the experimental models throughout this study. Both *B. cepacia* and *B. thailandensis* are BSL-2 microbes. *Escherichia coli* DH5α was used for routine cloning and plasmid propagation, whereas meso-diaminopimelic acid (mDAP) auxotrophic *E. coli* WM6026 served as the donor strain for conjugation experiments [16]. The plasmids used included pRL814, pPK15534, pRL27, and the engineered derivatives of pRL814 and pPK15534 generated in this study [7, 8, 16]. Bacterial strains, plasmids, and primers used throughout the study are listed in Tables S4-6.

Bacterial cultures were routinely grown in Luria-Bertani (LB) broth under aerobic conditions at 37°C with shaking at 200–250 rpm. Tryptic soy broth (TSB) or Super Optimal Broth with Catabolite repression (SOC) was used as the recovery medium following DNA delivery experiments where indicated. Antibiotics were added as required for plasmid maintenance and selection at concentrations determined empirically for each strain. Detailed antibiotic concentrations and selection conditions used for individual experiments are described in the corresponding sections below.

Plasmid stability was evaluated in *B. cepacia* and *B. thailandensis* by serial subculturing for 96 generations. Parallel cultures were maintained in the presence and absence of chloramphenicol selection. Cultures were monitored at an OD_{600} of approximately 0.5, with sampling performed at defined generation intervals. Optical density and relative fluorescence units (RFU) were monitored at hourly intervals. At the final assessment, samples were plated under selective and nonselective conditions, and

recovered colonies were examined for GFP fluorescence under blue-light illumination to assess reporter retention.

All experimental work involving bacterial cultures and recombinant plasmids was performed in an institutional Biosafety Level 2 (BSL-2) laboratory at Manipal Academy of Higher Education, Manipal, India. Personnel involved in the study had received appropriate training in microbiological handling and biosafety practices. Laboratory work was conducted using appropriate personal protective equipment (PPE) and in accordance with institutional biosafety guidelines. Bacterial cultures and biological materials transported to shared research facilities were maintained in sealed, labelled secondary containers in accordance with institutional transport procedures. Laboratory surfaces, equipment and reusable materials were decontaminated using institutionally approved procedures. Bacterial cultures, contaminated consumables and other biological waste were subjected to autoclaving or other institutionally approved decontamination procedures before disposal.

Identification of restriction-modification systems and recognition motifs

Restriction-modification (RM) systems and their associated recognition motifs were identified using an integrated methylome and bioinformatic workflow. Genome assemblies of *Burkholderia cepacia* ATCC 25416 (GCF_000473485.1), *Burkholderia thailandensis* E264 (GCF_000012365.1), and *Burkholderia pseudomallei* K96243 (GCF_000959285.1) were obtained from the NCBI Assembly database [37] and used for genome-level analyses. Publicly available methylome information and RM annotations were used to identify predicted methylated DNA motifs, restriction endonucleases, methyltransferases, and their corresponding recognition sequences [36]. RM annotations were cross-referenced with the NEB REBASE database to assign RM system types and recognition motifs. Protein domain annotation of predicted RM-associated proteins was performed using HMMER (hmmscan) against the Pfam database [32, 38, 39].

RM recognition motifs reported for 1,044 publicly available *Burkholderia*-related entries listed in REBASE database (accessed on February 19, 2026) were manually compiled into a reference dataset for comparative analysis (Table S3) [36]. The compiled dataset was used to assess the diversity and distribution of RM recognition motifs across the genus (Table S3). Recognition motifs identified from this dataset were subsequently searched within the plasmid sequences used in this study to determine their occurrence and frequency (Fig. 1e; Table S2b) [40]. These analyses were used to prioritize RM recognition sites for subsequent plasmid redesign while preserving plasmid architecture and functional genetic elements (Fig. 1e).

RM-guided plasmid engineering

Plasmid engineering was performed using pPK15534 as the primary vector backbone for rational elimination of restriction-modification (RM) recognition motifs [16]. RM recognition sites identified from REBASE were selected for sequence modification. Individual Type II and Type III recognition sites were initially disrupted, followed by the construction of plasmids carrying cumulative Type II, cumulative Type III, and combinatorial Type I, Type II, and Type III RM-site modifications [25, 35]. All sequence modifications were designed to eliminate RM recognition motifs while preserving plasmid size, backbone organization, origin of replication, selectable marker, reporter gene, and other functional genetic elements. Plasmid redesign was performed iteratively, with each round of sequence modification re-evaluated to ensure elimination of the target RM recognition motif without introducing additional RM recognition sites.

RM recognition motifs were disrupted by introducing synonymous nucleotide substitutions based on the codon usage preferences of the *Burkholderia* genus [41]. Alternative synonymous codons encoding the same amino acid were selected using *Burkholderia* codon usage tables, thereby eliminating RM recognition motifs while preserving the amino acid sequence. Because codon usage is highly conserved between *B. cepacia* and *B. thailandensis*, a common *Burkholderia* codon usage scheme was used for plasmid redesign. Sequence modifications were additionally evaluated to avoid the introduction of new RM recognition motifs elsewhere in the plasmid sequence. All engineered plasmids were propagated in *Escherichia coli*, verified by PCR amplification of the modified regions, and confirmed by Sanger sequencing before use in downstream transformation and conjugation experiments. PCR amplification was performed using PrimeSTAR Max DNA Polymerase (Takara Bio), and plasmid assembly was carried out using the In-Fusion Snap Assembly Master Mix (Takara Bio) according to the manufacturer's instructions [42, 43]. Primers were synthesized by Eurofins Genomics or Integrated DNA Technologies (IDT). Plasmids generated in this study are listed in Table S6, and primer sequences used for plasmid construction and verification are provided in Table S5.

DNA delivery by transformation, electroporation and conjugation

Plasmid DNA was introduced into *B. cepacia* and *B. thailandensis* using chemical transformation, and conjugation [44, 45]. Electroporation with *B. cepacia* and *B. thailandensis*, and *E. coli* strains was performed using a Bio-Rad MicroPulser electroporator with pulse voltages ranging from 1.8 to 2.5 kV, followed by recovery in TSB or SOC medium at 37°C under aerobic shaking conditions (200–250 rpm) [46]. Conjugation was performed using the agar-plate mating method with *E. coli WM6026* as the donor strain. Donor and recipient mixtures were incubated at 30°C for 14 hours on LB plate with 100uM DAP, after which bacterial cells were harvested, and after two times washing using LB media, cells were incubated at shaking at 37°C for 2 hours in LB without DAP and antibiotic, followed by plating onto selective media for transconjugant enumeration.

Transformation and conjugation experiments were performed using both the parental plasmids and the RM-engineered plasmid derivatives generated in this study. The same optimized transformation,

electroporation, and conjugation protocols were applied to all plasmid constructs to enable direct assessment of the effect of RM recognition-site elimination on DNA delivery efficiency. Following DNA delivery, bacterial cultures were recovered under non-selective conditions before plating onto selective LB agar containing the appropriate antibiotics. Recipient-only and donor-only controls were included in each experiment to monitor background growth and selection specificity.

Transformation efficiency was calculated as the number of transformants recovered per microgram of plasmid DNA, whereas conjugation efficiency was calculated as the percentage of transconjugants recovered relative to the total recipient population in the mating mixture following conjugation. Each experiment was performed using three independent biological replicates under identical experimental conditions.

Antibiotic susceptibility testing

To establish appropriate selection conditions for plasmid maintenance and recovery of transformants, the antibiotic susceptibility profiles of *B. cepacia* and *B. thailandensis* were evaluated using serial dilution method [47]. Chloramphenicol, kanamycin, and spectinomycin were tested over a range (20–500 µg/mL) of concentrations to determine their suitability for selection in both *Escherichia coli* and *Burkholderia* strains (Table S1). The minimal inhibitory concentration (MIC) of appropriate antibiotics were used consistently throughout all subsequent transformation, conjugation, and plasmid maintenance experiments unless otherwise stated.

Cell culture, bacterial infection and fluorescence microscopy

Human breast adenocarcinoma MCF-7 cells were maintained in Dulbecco's Modified Eagle Medium/F-12 (DMEM/F-12K) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin-streptomycin, at 37°C in a humidified incubator with 5% CO_2 (New Brunswick™ S41i). Cells were routinely propagated in T-25 tissue culture flasks and seeded into 6-well tissue culture plates for fluorescence microscopy or glass-bottom imaging dishes for confocal microscopy. Cell numbers were determined using a hemocytometer prior to infection.

GFP-expressing *B. cepacia* and *B. thailandensis* carrying the RM-engineered *gfp* containing plasmid were used for fluorescence measurements and infection assays. Bacterial GFP fluorescence was quantified from 200 µL bacterial suspensions using a Varioskan™ LUX Multimode Microplate Reader and normalized to the corresponding optical density (OD_{600}). Bacterial inocula were estimated from OD_{600} measurements, adjusted by serial dilution, and used to infect MCF-7 cells at multiplicities of infection (MOIs) of 10 and 50, as calculated from the bacterial and host-cell counts. Infected cultures were incubated at 37°C in 5% CO_2, and GFP fluorescence was quantified prior to microscopic analysis.

Fluorescence microscopy was performed using an Olympus IX83 inverted fluorescence microscope equipped with a 40× objective. Images were acquired at 6 h and 18 h post-infection before and after washing infected monolayers with phosphate-buffered saline (PBS) to remove non-adherent bacteria.

Phase-contrast and FITC fluorescence images were collected from identical fields of view and merged using Olympus cellSens Dimension 4.4 software to visualize GFP-expressing bacteria relative to host-cell morphology.

High-resolution confocal imaging was performed using an Olympus IX85 laser-scanning confocal microscope equipped with a 100× oil-immersion objective. GFP was excited using a 488 nm laser, and fluorescence emission was collected between 500 and 550 nm. Optical sections (z-stacks) were acquired at 6 h and 18 h post-infection before and after PBS washing, and merged fluorescence and transmitted-light images were generated. Three-dimensional (3D) volume-rendered reconstructions were produced from the z-stack image series using cellSens software to visualize the spatial distribution of GFP-expressing bacteria within infected cells. Host cells were imaged without additional fluorescent staining, enabling direct visualization of GFP-expressing bacteria against the native cellular morphology.

Personnel at the shared microscopy facility were informed of the biological nature of the samples, which contained GFP-expressing *Burkholderia* strains. Sample handling and imaging procedures were conducted in accordance with the facility's approved BSL-2 biosafety practices.

Statistical analysis

Data collection was done using Microsoft Excel and statistical analyses were performed using Microsoft Excel, GraphPad Prism version 8.0.1 (GraphPad Software, San Diego, CA, USA), and R software. Bioinformatic analyses were performed on a Linux-based computing platform. Data are presented as mean ± standard deviation (SD) from three independent biological replicates, unless otherwise stated.

Comparisons among three or more groups within a single bacterial species were performed using ordinary one-way ANOVA to evaluate differences in mean values across groups. Dunnett's or Tukey's multiple-comparison tests were subsequently applied, as appropriate, for comparisons against a common control or between all possible pairs of groups, respectively. Comparisons involving two experimental factors (*Burkholderia species* and plasmid construct) were analyzed using ordinary two-way ANOVA to assess the effects of each factor and their interaction, followed by Sidak's multiple-comparison test for selected pairwise comparisons. Comparisons between two independent groups were performed using unpaired two-tailed Student's *t*-tests. These parametric tests were selected for comparing group means, assuming independent observations, approximately normally distributed residuals, and homogeneity of variance, as applicable. A P value of < 0.05 was considered statistically significant. Exact statistical tests, sample sizes (n), and significance levels are provided in the corresponding figure legends.

Declarations

Additional information

Ethical approval

Ethical clearance was granted by the Institutional Ethics Committee of Kasturba Hospital, Manipal, India (IEC number- 197/2024 and 308/2022). Experimental work involving *Burkholderia cepacia* ATCC 25416, *Burkholderia thailandensis* ATCC 700388, and *Escherichia coli* strains was conducted under the approval of the Institutional Biosafety Committee (IBSC), Manipal Academy of Higher Education, Manipal, India. The proposal was approved at the IBSC meeting held on 28 November 2023, with the approval documented in a certificate dated 30 November 2023. The biosafety review covered the bacterial strains used in the study, recombinant plasmid constructs, antibiotic-resistance markers employed for selection, and the potential for unintended genetic transfer. All experimental procedures were conducted in the institutional BSL-2 laboratory in accordance with the conditions specified by the IBSC.

Competing interests

The authors declare no competing interests.

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Author Contribution

PL conceived the overall study. SJ did all the bacterial, molecular biology and bioinformatic works guided by PL. RA and SJ did cell culture experiments and microscopy with help of BG. Data analysis was done by PL, and SJ. SJ, and PL drafted the first draft of the manuscript, and all authors (CM, BG, and RA) participated in the improvement of the draft to the final version of the manuscript.

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Data Availability

Most of the datasets generated and analyzed during this study are included in this published article and its Supplementary Information files. Additional data supporting the findings of this study, including plasmid sequence information and related materials not included in the manuscript, are available from the corresponding author upon reasonable request.

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Figures

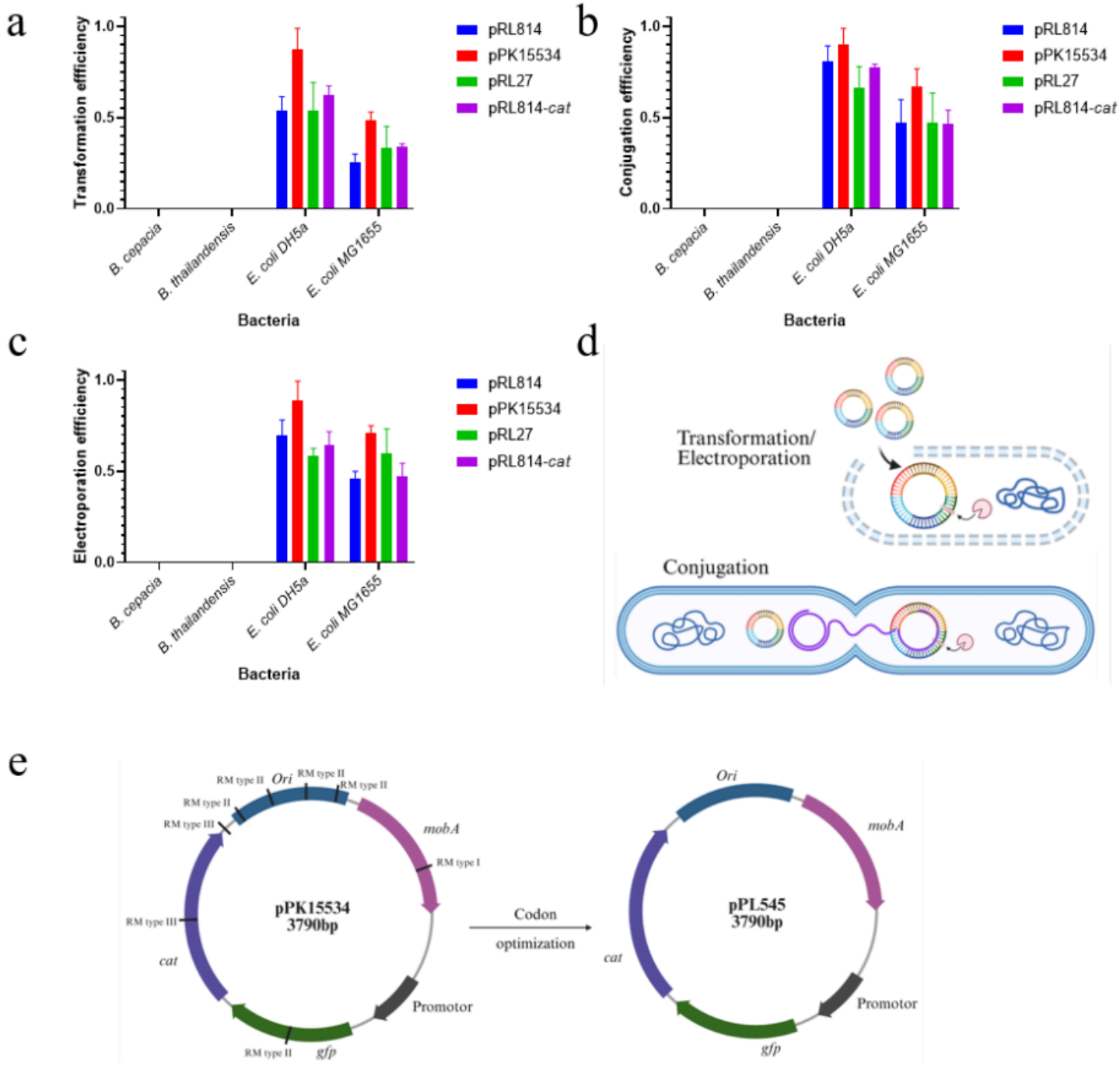


Figure 1

Conventional DNA delivery approaches and plasmid architecture in *Burkholderia*. aTransformation efficiency, **b** conjugation efficiency, and **c**electroporation efficiency of the indicated plasmids (*pRL814*, *pPK15534*, *pRL27,* and pRL814-*cat*) in *B. cepacia* and *B. thailandensis*. *E. coli* donor/cloning strains served as positive controls for plasmid delivery. No detectable transformants or transconjugants were recovered from either *Burkholderia* species using the tested DNA delivery approaches, whereas successful plasmid transfer was observed in the corresponding *E. coli* controls. Data are presented as mean ± SD from three independent biological experiments. Within each species, statistical significance was assessed using ordinary one-way ANOVA followed by Dunnett's multiple-comparision testk.

Comparisons between species were performed using ordinary two-way ANOVA followed by Sidak's multiple-comparison test. *P*-values are indicated in the figure. **d**Schematic representation of the principal DNA-delivery approaches, including transformation/electroporation and conjugation. **e** Schematic comparison of the parental plasmid pPK15534 and the engineered plasmid pPL545, illustrating their respective plasmid architectures and functional elements. Data in **(a-c)** are presented as mean ± SD from three independent biological experiments.

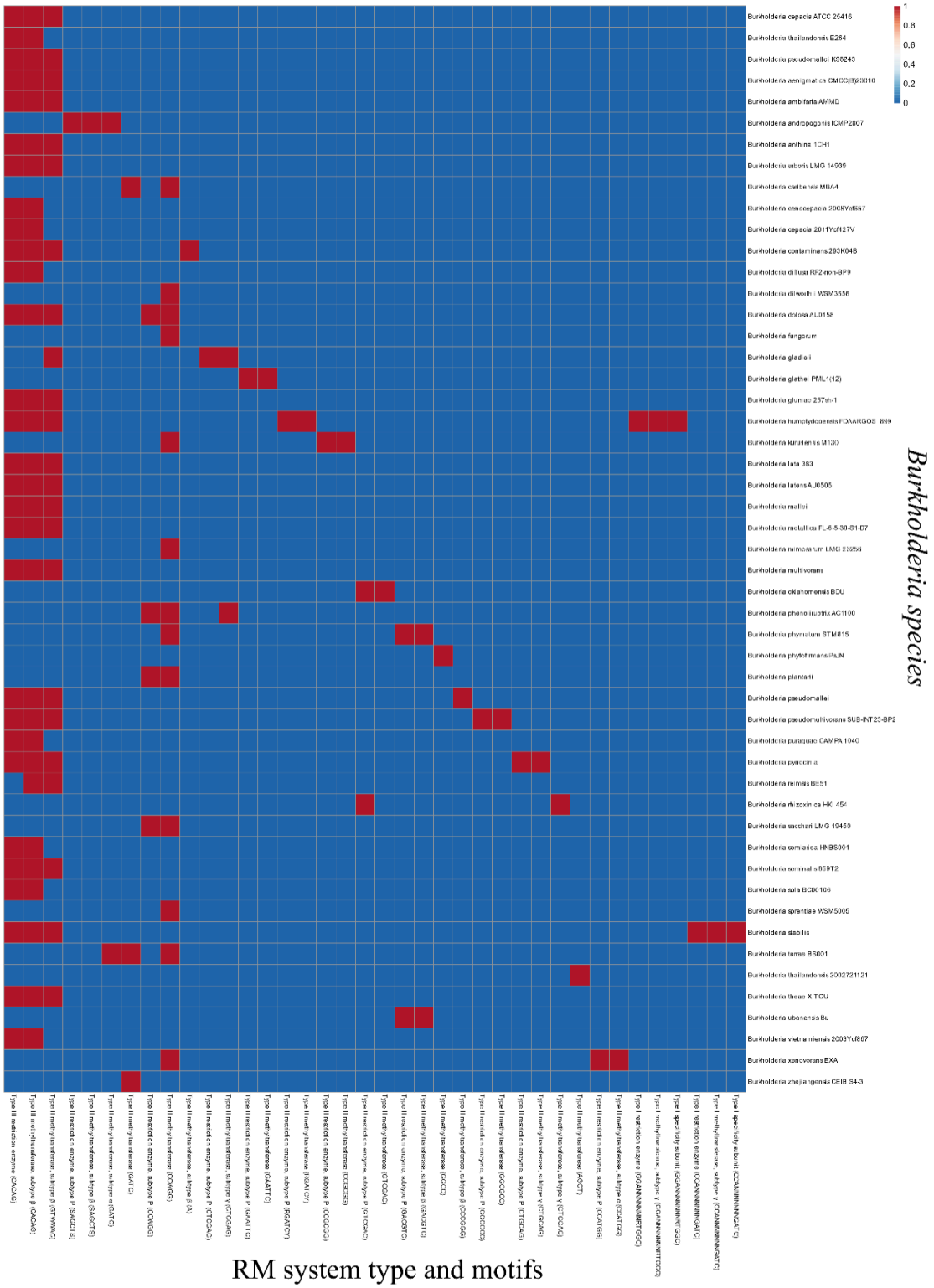


Figure 2

Comparative distribution of restriction-modification (RM) systems and recognition motifs across the *Burkholderia* genus. Heat map illustrating the presence and distribution of bioinformatically identified RM enzymes and their corresponding recognition motifs among representative *Burkholderia*entries retrieved from the REBASE database. The analysis was based on 1,044 publicly available *Burkholderia*-related records in REBASE (accessed February 19, 2026). For comparative visualization, 56 entries were selected, comprising the first REBASE entry for each of the 53 named *Burkholderia*species, along with three specifically included reference strains: *Burkholderia cepacia* ATCC 25416 and *Burkholderia thailandensis* E264, which were used as experimental models in this study, and *Burkholderia pseudomallei* K96243, which was included exclusively for comparative bioinformatic analysis. Five entries with no RM motifs were excluded, these were *Burkholderia cordobensis* LMG 27620, *B. perseverans*INN12, *B. sordidicola* S170, *B. stagnalis* MSMB735WGS, and *B. territorii* RF8-non-BP5. Consequently, 51 entries are displayed. Red and blue cells indicate the presence (1) and absence (0), respectively, of the corresponding RM enzyme or recognition motif. Organism entries are displayed in their original input order, whereas RM features are hierarchically clustered. The heat map was generated using the ClustVis web application (Metsalu and Vilo, 2015; https://biit.cs.ut.ee/clustvis/). The complete dataset of 1,044 REBASE records are provided in Supplementary Table S3 (Tables S3a-c).

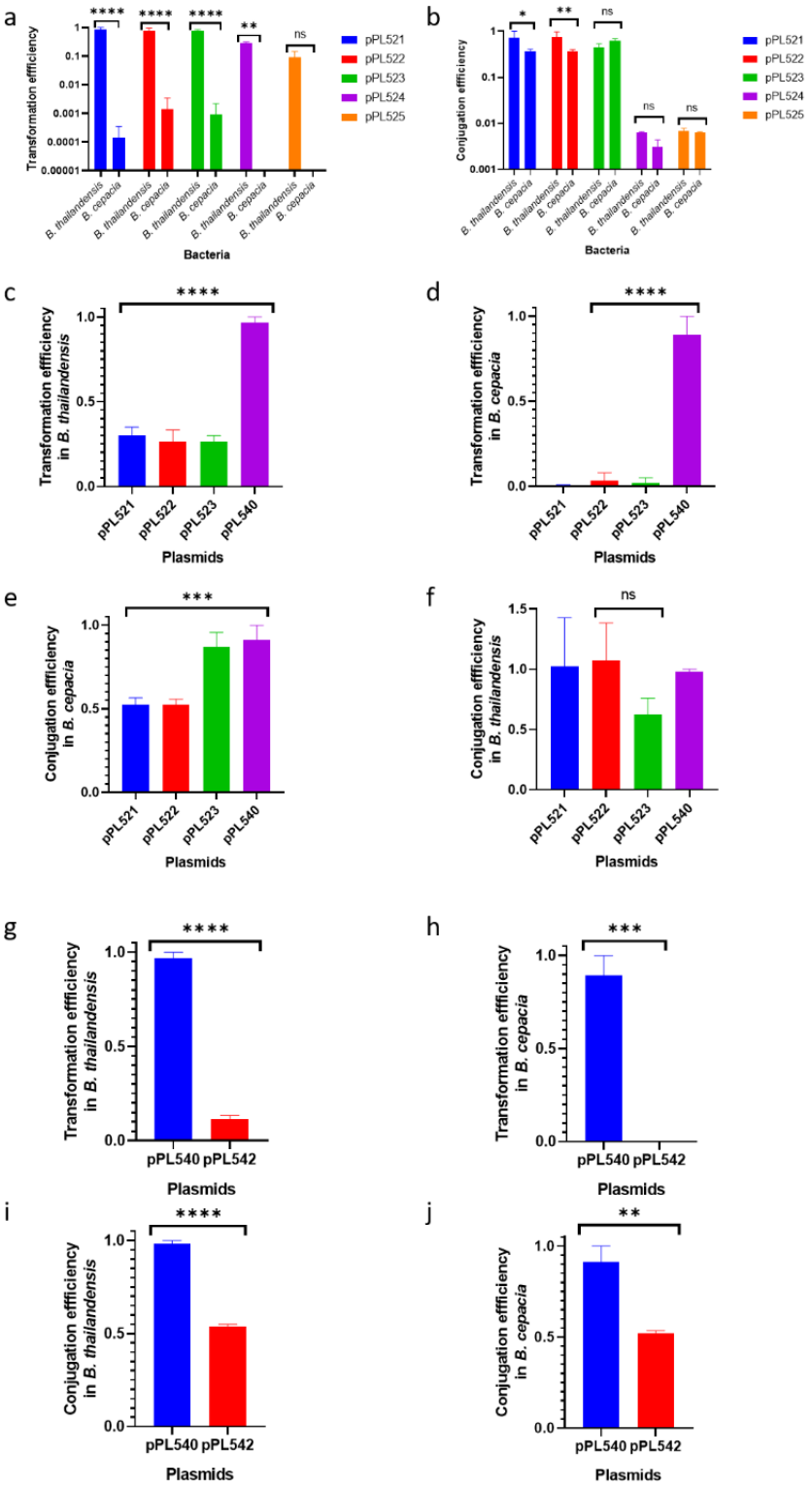


Figure 3

Systematic removal of restriction-modification (RM) recognition sites reveals their contribution to plasmid DNA delivery in *Burkholderia*. (a-b)Transformation efficiencies of plasmids carrying individual first, second, or third Type II RM recognition-site removals compared with the construct lacking all three Type II RM recognition sites (pPL540) in *B. thailandensis*(a) and *B. cepacia* (b). **(c-d)** Transformation efficiencies of pPL540 compared with pPL542, which lacks both identified Type III RM recognition sites,

in *B. thailandensis* (c) and *B. cepacia*(d). **(e-f)** Conjugation efficiencies of plasmids carrying individual Type II RM-site modifications compared with pPL540 in *B. thailandensis* (e) and *B. cepacia* (f). **(g-h)**Transformation efficiencies of pPL540 and pPL542 in *B. thailandensis*(g) and *B. cepacia* (h). **(i-j)** Conjugation efficiencies of pPL540 and pPL542 in *B. thailandensis* (i) and *B. cepacia* (j). Data are presented as mean ± SD from three independent biological replicates. Statistical analyses for **(a-b)** were performed using ordinary one-way ANOVA followed by Dunnett's multiple-comparison test; **(c-d)** and **(g-j)**were analyzed using two-tailed unpaired *t*-tests. For **(e-f)**, statistical significance within each species was assessed using ordinary one-way ANOVA followed by Dunnett's multiple-comparison test, while comparisons between species were performed using ordinary two-way ANOVA followed by Sidak's multiple-comparison test. *P* values are indicated in the figure; ns, not significant; $P < 0.05$, $P < 0.01$, $P < 0.001$, $P < 0.0001$.

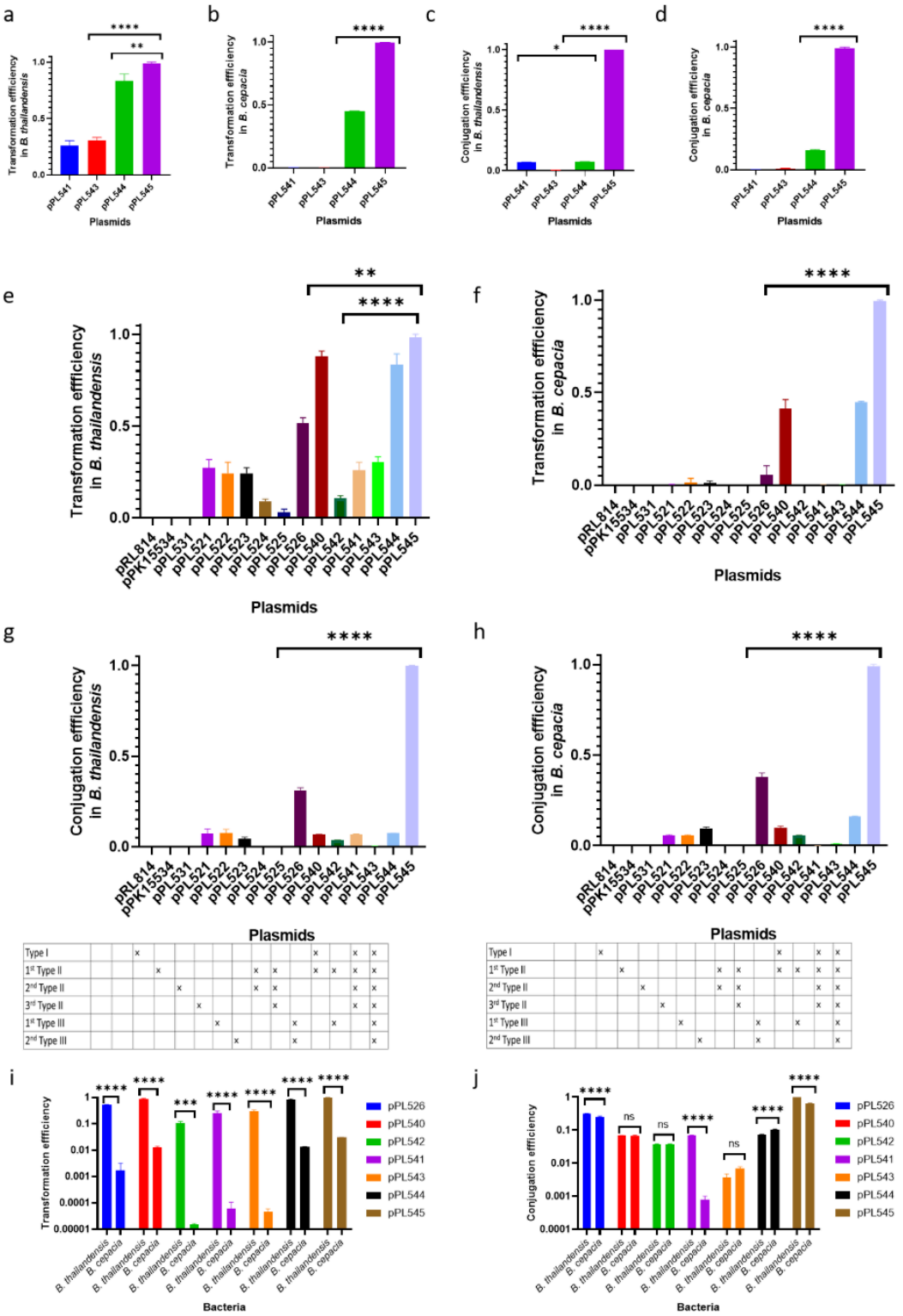


Figure 4

Progressive and combinatorial removal of RM recognition sites improves plasmid DNA delivery in *Burkholderia*.

(a-d) Transformation and conjugation efficiencies of selected combinatorial RM-site-engineered plasmids. Transformation efficiencies in *B. thailandensis*(a) and *B. cepacia* (b), and conjugation

efficiencies in *B. thailandensis* (c) and *B. cepacia* (d), were compared among plasmids carrying Type I, Type II, and Type III RM recognition-site modifications, including pPL541, pPL543, pPL544, and the fully engineered pPL545. **(e-h)**Comprehensive evaluation of plasmid acquisition following progressive and combinatorial RM-site removal. Transformation efficiencies in *B. thailandensis* (e) and *B. cepacia* (f), and conjugation efficiencies in *B. thailandensis* (g) and *B. cepacia* (h), were evaluated across the engineered plasmid series, including individual, cumulative, and combinatorial Type I, Type II, and Type III RM-site modifications. **(i-j)**Comparative evaluation of selected engineered plasmids in *B. thailandensis*and *B. cepacia*. Transformation (i) and conjugation (j) efficiencies were compared for selected constructs representing distinct RM-site engineering strategies. Data are presented as mean ± SD from three independent biological replicates (n = 3). Statistical analyses in panels (a-h) were performed using ordinary one-way ANOVA followed by Tukey's multiple-comparison test. Comparisons between *B. thailandensis* and *B. cepacia* in panels (i-j) were performed using ordinary two-way ANOVA followed by Sidak's multiple-comparison test. P values are indicated in the figure; ns, not significant; $P < 0.05$, *$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$.

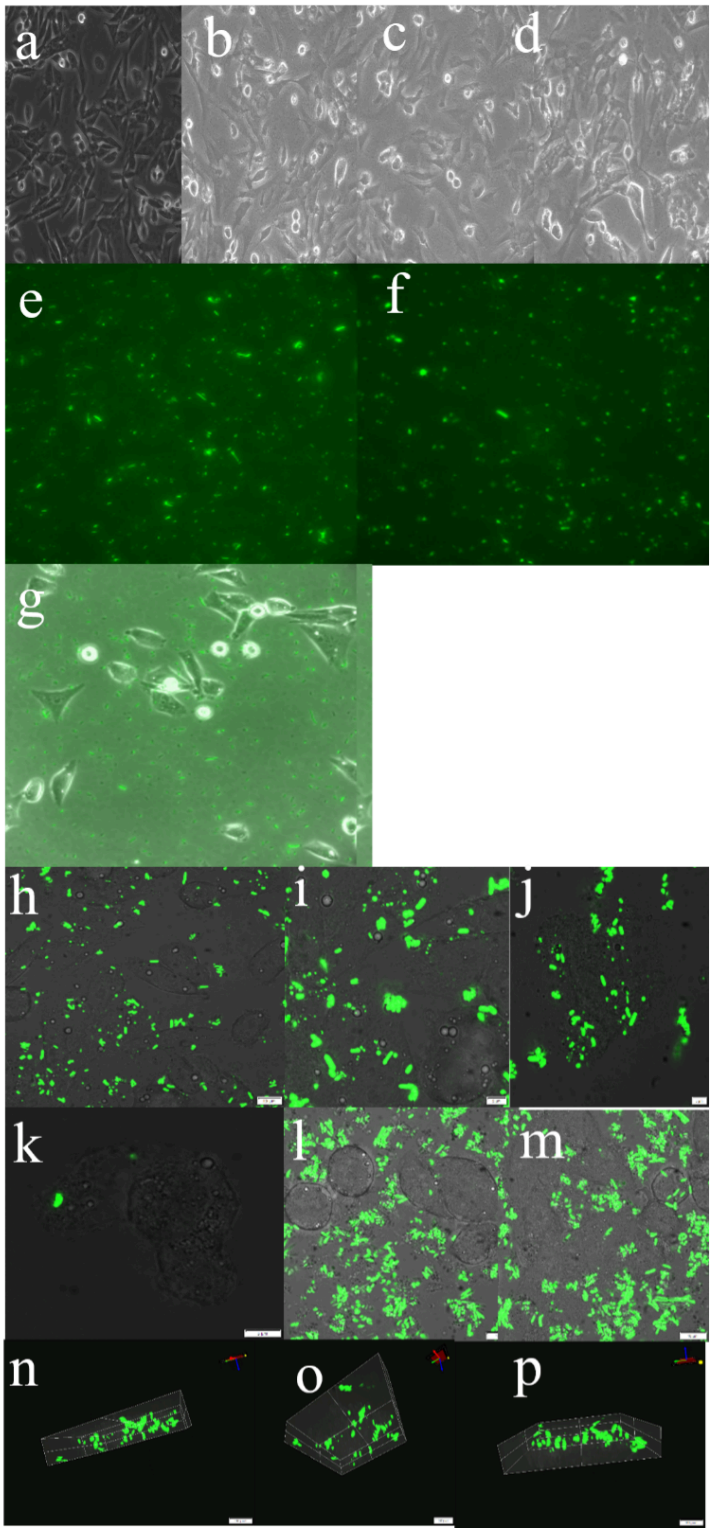


Figure 5

Stable intracellular expression of the engineered *gfp* containing plasmid during host-cell infection. Representative fluorescence and confocal microscopy images of MCF-7 cells infected with GFP-expressing *B. cepacia* and *B. thailandensis* carrying the RM-engineered plasmid. Phase-contrast images showing host-cell morphology are presented in **a-d**. Corresponding GFP fluorescence images demonstrating intracellular bacteria are shown in **e** and **f**, and the merged fluorescence and phase-

contrast image is shown in **g**. Representative confocal optical sections illustrating GFP-expressing bacteria associated with infected host cells are shown in **h-m**. Three-dimensional volume-rendered reconstructions generated from confocal z-stack image series further confirm the intracellular localization of GFP-expressing bacteria within host cells (**n-p**). Images were acquired using an Olympus IX83 inverted fluorescence microscope (**a-g**) and an Olympus IX85 confocal microscope equipped with a 100× oil-immersion objective (**h-p**). Images shown are representative of independent biological experiments. Scale bars are indicated in the respective panels.

Supplementary Files

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- TableS2.docx
- TableS3.xlsx
- TableS4.docx
- TableS5.docx
- TableS6.docx