



Enhanced *Fusobacterium nucleatum* Genetics Using Host DNA Methyltransferases To Bypass Restriction-Modification Systems

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ABSTRACT Bacterial restriction-modification (R-M) systems are a first-line immune defense against foreign DNA from viruses and other bacteria. While R-M systems are critical in maintaining genome integrity, R-M nucleases unfortunately present significant barriers to targeted genetic modification. Bacteria of the genus *Fusobacterium* are oral, Gram-negative, anaerobic, opportunistic pathogens that are implicated in the progression and severity of multiple cancers and tissue infections, yet our understanding of their direct roles in disease have been severely hindered by their genetic recalcitrance. Here, we demonstrate a path to overcome these barriers in *Fusobacterium* by using native DNA methylation as a host mimicry strategy to bypass R-M system cleavage of transformed plasmid DNA. We report the identification, characterization, and successful use of *Fusobacterium nucleatum* type II and III DNA methyltransferase (MTase) enzymes to produce a multifold increase in gene knockout efficiency in the strain *Fusobacterium nucleatum* subsp. *nucleatum* 23726, as well as the first system for efficient gene knockouts and complementations in *F. nucleatum* subsp. *nucleatum* 25586. We show plasmid protection can be accomplished *in vitro* with purified enzymes, as well as *in vivo* in an *Escherichia coli* host that constitutively expresses *F. nucleatum* subsp. *nucleatum* MTase enzymes. In summary, this proof-of-concept study characterizes specific MTases that are critical for bypassing R-M systems and has enhanced our understanding of enzyme combinations that could be used to genetically modify clinical isolates of *Fusobacterium* that have thus far been inaccessible to molecular characterization.

IMPORTANCE *Fusobacterium nucleatum* is an oral opportunistic pathogen associated with diseases that include cancer and preterm birth. Our understanding of how this bacterium modulates human disease has been hindered by a lack of genetic systems. Here, we show that *F. nucleatum* DNA methyltransferase-modified plasmid DNA overcomes the transformation barrier and has allowed the development of a genetic system in a previously inaccessible strain. We present a strategy that could potentially be expanded to enable the genetic modification of highly recalcitrant strains, thereby fostering investigational studies to uncover novel host-pathogen interactions in *Fusobacterium*.

KEYWORDS *Fusobacterium*, *Fusobacterium nucleatum*, DNA methyltransferase, methylase, methylome, restriction-modification, R-M systems, transformation, bacterial genetics, bacteriophage genetics, cancer, virulence

Bacteria have multiple mechanisms to keep out foreign DNA elements, including physical barriers in the form of membranes and innate and adaptive nucleotide recognition systems to degrade foreign DNA before costly genome integration (1–3). This ability to recognize self versus nonself DNA is critical for productive genetic exchanges through horizontal gene transfers between close species to receive adaptive advantages (4–6). The two main nucleic acid surveillance systems bacteria deploy are restriction-modification (R-M) systems and CRISPR-Cas (clustered, regularly interspaced palindromic repeat-CRISPR-associated proteins) systems. In addition, a new system known as DISARM has joined the bacterial arsenal of DNA defense systems (7).

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CRISPR-Cas systems are considered adaptive immune components because of their ability to chromosomally integrate foreign (i.e., viral) DNA to create memory for subsequent encounters (8–11). In addition, the rather newly characterized bacteriophage exclusion (BREX) systems exist in 10% of the sequenced bacterial genomes and block phage DNA replication and lysogeny in infected cells (12, 13). BREX differentiates itself from R-M systems in that phage DNA is not cleaved or digested, which suggests a unique bacterial defense system. While R-M systems serve bacteria well for their survival and adaptation, they present significant challenges for researchers aiming to understand these organisms through genetic manipulation in the form of gene knock-outs. This genetic recalcitrance is widespread throughout the bacterial kingdom, and in many cases it has led researchers to gravitate toward using strains that have weak R-M systems that can be genetically modified, instead of using target strains that have desired phenotypes.

R-M systems consist of restriction endonucleases (REases) and DNA methyltransferases (MTase), which can either exist as a paired REase/MTase operon that can also contain additional specificity genes or as singular MTase genes (14–16). The system works when REases cleave DNA that does not have the proper MTase-induced methylation sequences, thereby signaling to the bacteria that the detected DNA is foreign and unwanted. R-M systems are classified as type I, II, III, or IV, according to their molecular structure, subunit composition, cleavage position, restriction site, and cofactor specifications (see Fig. S1 in the supplemental material). Type I systems (*hsdRMS* genes) cut exogenous DNA by forming protein complexes, and random cleavage usually happens at substantial distances from an asymmetric recognition sequence (400 to 7,000 bp) (17), while type II systems consist of an individual restriction endonuclease and methyltransferase that cleave DNA at symmetrical recognition sites (18). In a similar way to type I, type III forms a protein complex necessary for the restriction enzyme activity; however, the methyltransferase can function independently. DNA cleavage for type III R-M systems takes place 25 to 27 bp 3' to an asymmetrical recognition sequence that is 5 to 6 bp in length (19, 20). Furthermore, type IV systems asymmetrically recognize DNA sequences and cleavage by REases at a defined distance from the recognition sites. In addition, some of these systems contain multiple MTases that can be adenine or cytosine specific, as well as the REase showing methyltransferase activity (17, 21–23).

Fusobacterium, especially the species *Fusobacterium nucleatum*, has garnered significant attention since this bacterium was reported to be overrepresented in colorectal cancer tumors more than a decade ago (24–26). Classical studies have mainly focused on the role of *F. nucleatum* in oral infections and diseases, including periodontitis (27, 28), severe organ infections (29–32), and preterm birth (33–35). The majority of recent studies have shifted the focus to a potential direct causal role in adverse cancer phenotypes, including heightened inflammation (36–38), production of carcinogenic metabolites (39), induced metastasis (40–42), DNA damage (43–45), increased resistance to frontline chemotherapy drugs (46, 47), and overall worse patient prognosis (36, 48, 49). Despite an increasing interest in understanding how this bacterium contributes to cancer, there have been very few mechanistic studies of specific bacterial effector proteins, due to the genetic recalcitrance in most strains. Because of this, our current molecular studies have been limited to a few *Fusobacterium* strains that are able to acquire “naked,” unmethylated DNA and incorporate it into their genome by recombination with homologous sequences or, in the case of multicopy plasmids, by establishing a new episome. Of these strains, *F. nucleatum* subsp. *nucleatum* 23726 (transformation by electroporation) (50–52), *F. nucleatum* subsp. *polymorphum* 10953 (transformation by electroporation) (53), *F. nucleatum* subsp. *polymorphum* 12230 (transformation by sonoporation) (54), and a recent paper highlighting the first gene interruption in *Fusobacterium necrophorum* using DNA conjugation from *Escherichia coli* (55, 56). Needless to say, these four strains do not encompass the *Fusobacterium* subspecies landscape and their respective infections and diseases that researchers desire to study, and they highlight the need for molecular biology and biochemical studies to achieve universal genetics.

Seminal studies have successfully used MTases to modify and protect plasmid DNA to facilitate molecular genetics in several other bacterial species (57–59). This technique has been used successfully in many studies, but it was coined plasmid artificial modification (PAM) when it was used to enhance transformation in *Bifidobacterium adolescentis* (60). What we currently know about the R-M systems of *Fusobacterium* largely exist as bioinformatic predictions based on MTase classification in the REBASE database (61). However, this bioinformatic classification in most cases did not come with experimental DNA methylation analyses to match enzymes with their target sequences. Additionally, even when an MTase is matched with its recognition and methylation sequence, this does not guarantee that these modifications will be important for effective protection of plasmid DNA to increase transformation efficiency. Therefore, the goal of this study was to biochemically characterize and utilize a broad range of *F. nucleatum* MTases for host mimicry by methylation to accelerate bacterial genetics in previously inaccessible strains. We successfully report the use of *F. nucleatum* MTase enzymes produced in *E. coli* to protect plasmid DNA, facilitating a significant increase in chromosomal incorporation of plasmid and transposons in multiple *F. nucleatum* strains, as well as the development of the first gene deletion system in *F. nucleatum* subsp. *nucleatum* 25586 after only one previous study reporting a single-crossover gene interruption of the FomA porin (62). Our study is not exhaustive, because of the number of strains (>100 *F. nucleatum* strains in the NCBI genome database) and R-M systems (average of seven systems per genome [Fig. 1]) that could have been tested. However, we believe our strategies will provide a flexible roadmap for the scientific community to adopt MTase-based methods for genetic manipulation in *Fusobacterium*.

RESULTS

Bioinformatic identification and classification of R-M systems in *Fusobacterium*.

As shown in Fig. 1A, bacterial R-M systems act by blocking exogenous DNA from entering and being incorporated into the genome by digesting foreign, improperly methylated DNA. Scientists have exploited this defense mechanism by using strain-specific MTase enzymes to pretreat DNA before electroporation or natural competence to improve transformation efficiency (59). In this study, to identify potential *Fusobacterium* MTases to increase the efficiency of transformation and DNA recombination, we queried the online databases REBASE (61), FusoPortal (63), and NCBI (64) to characterize R-M systems. We analyzed 25 strains of *Fusobacterium nucleatum* in REBASE, covering the subspecies *F. nucleatum* subsp. *nucleatum*, *F. nucleatum* subsp. *animalis*, *F. nucleatum* subsp. *vincentii*, and *F. nucleatum* subsp. *polymorphum*, and cataloged the number and classification of their R-M systems, as shown in Fig. 1B (see also Table S1 in the supplemental material). There was an overall propensity for *F. nucleatum* strains to have a higher number of type II MTase genes, yet there was not a strong overall pattern for the number or class of R-M systems that differentiated the subspecies. As shown in Fig. 1C, we highlighted three strains of *F. nucleatum* covering subspecies *F. nucleatum* subsp. *nucleatum* and *F. nucleatum* subsp. *animalis*. The genetically tractable strain *F. nucleatum* subsp. *nucleatum* 23726 encodes 4 R-M systems, as shown in Fig. 1C: one type I, two type II, and one BREX system. *F. nucleatum* subsp. *nucleatum* 25586 lacks type I R-M systems but has three type II and one type III system containing two MTases that proved critical for enabling molecular genetics in this strain. Finally, 11 R-M systems (two type I and nine type II) were identified in *F. nucleatum* subsp. *animalis* 7_1; this was the highest quantity of R-M systems in our analyzed strains.

An orthodox type II R-M system includes two independent genes in an operon: an MTase and a REase. However, as shown in Fig. 1C, several lone type II MTases were discovered in multiple *F. nucleatum* subsp. *nucleatum* strains, and we later showed these are crucial for DNA methylation and molecular genetics. Our bioinformatic studies also confirmed the presence of the type II BREX system in several *F. nucleatum* subsp. *nucleatum* strains. The BREX system is generally composed of a 4- to 8-gene cluster (12), and in *Fusobacterium* it is predicted to methylate adenine residues in a mechanism similar to that in *E. coli* (65). However, since the restriction site for this enzyme is yet to be characterized and these systems have not been shown to be important for efficient

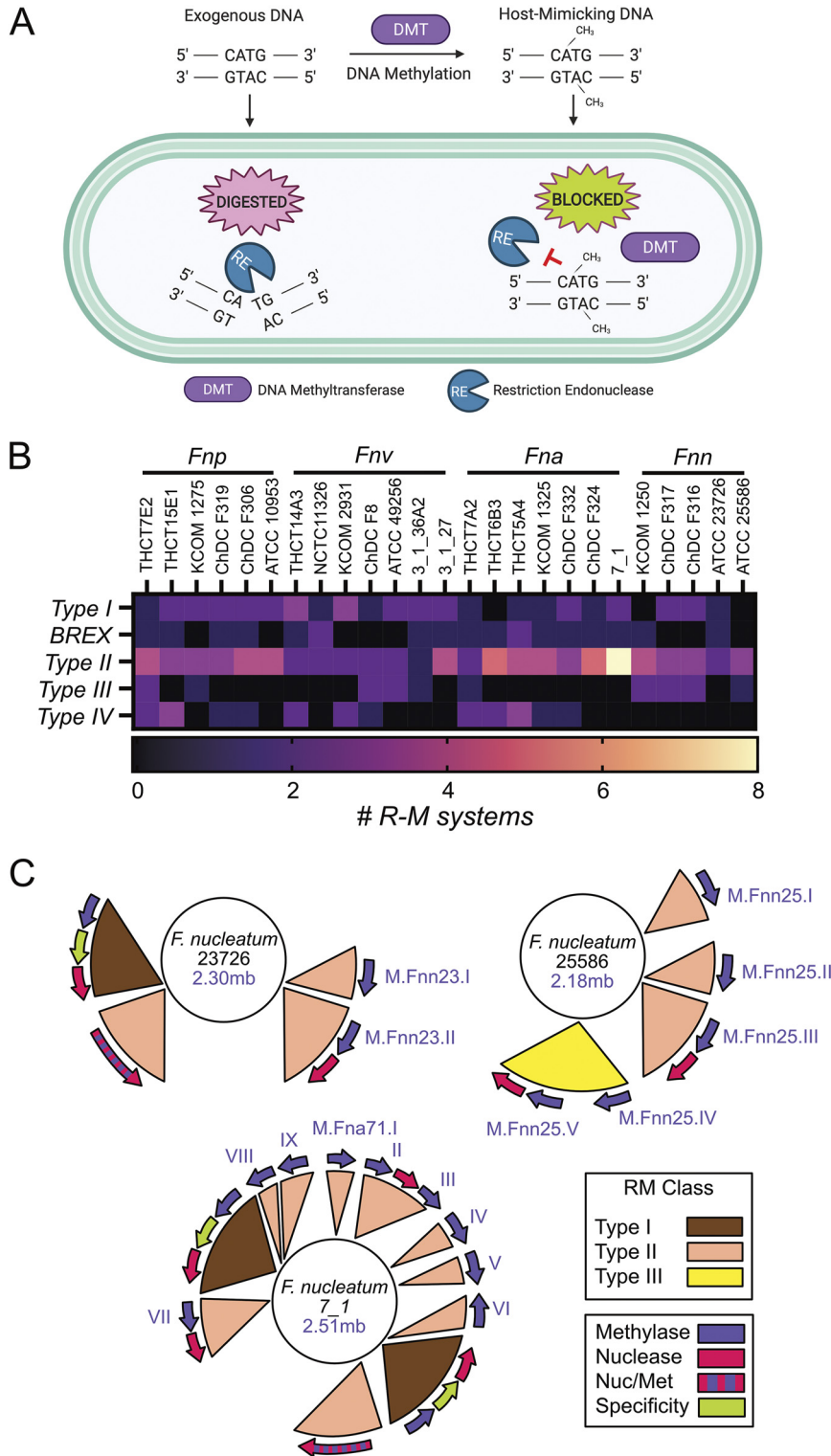


FIG 1 Restriction-modification system classification in *Fusobacterium*. (A) Overview of how R-M systems utilize bacteria-specific DNA methylation to mark the chromosome as self DNA, thereby restriction digesting invading DNA that does not contain the proper methylation patterns. Adapted from Hirokazu Suzuki (80). (B) Classification and quantitation of R-M systems in 25 strains of *F. nucleatum* covering the four subspecies: *F. nucleatum* subsp. *polymorphum* (*Fnp*), *F. nucleatum* subsp. *vincentii* (*Fnv*), *F. nucleatum* subsp. *animalis* (*Fna*), and *F. nucleatum* subsp. *nucleatum* (*Fnn*). (C) Genome location and renaming of type II and type III MTases in three strains of *F. nucleatum* subsp. *nucleatum* used in this study. R-M systems mapped on the genomes were recreated from those on the REBASE website.

molecular microbiology efforts, we did not focus on using these enzymes for plasmid protection. Finally, multiple type IV R-M systems were discovered in the *F. nucleatum* strains analyzed in this study, with type IV systems consisting of REases that will only cleave DNA that is methylated or glycosylated at either adenine or cytosine residues (21).

Recombinant production and characterization of *F. nucleatum* MTases. To focus our study, we chose to utilize and characterize all type II and type III MTase enzymes in the strains *F. nucleatum* subsp. *nucleatum* 23726 and *F. nucleatum* subsp. *nucleatum* 25586. We chose to focus on these MTase classes because the strain *F. nucleatum* subsp. *nucleatum* 25586 lacks type I systems and is still highly recalcitrant to genetic manipulation. However, we do realize that type I R-M systems could play a role in other strains and could be analyzed in the future. As shown in Fig. 2, we cloned (Fig. 2A), expressed, and purified (Fig. 2B) five enzymes (M.Fnn23.I, M.Fnn23.II, M.Fnn25.I, M.Fnn25.IV, and M.Fnn25.V). M.Fnn23.I and M.Fnn23.II were used to treat the plasmid pDJSVT13 as described below, which we previously used to knock out the *galkT* genes in *F. nucleatum* subsp. *nucleatum* 23726 (66).

Recombinant *F. nucleatum* MTases protect plasmid DNA from REase digestion.

To show that our recombinant enzymes from *F. nucleatum* subsp. *nucleatum* 23726 were active, we identified commercially available REases that matched the REBASE-predicted methylation sequences of M.Fnn23.I and M.Fnn23.II. By methylating the plasmid pDJSVT13 with M.Fnn23.I, we showed that adenine methylation in the sequence CATG blocked cleavage by the endonuclease NlaIII, which recognized the same sequence and cleaved 3' to the guanine (Fig. 2C). Next, we methylated pDJSVT13 with M.Fnn23.II, and we determined that methylation of the adenine in GATGC protected DNA from cleavage by SfiI, which recognizes GCATC(N₅) and cleaves 3' to the N₅ sequence (Fig. 2D). This protection of DNA from cleavage by methylation indicates that use of these enzymes in tandem would allow more efficient homologous recombination in *F. nucleatum* subsp. *nucleatum* 23726 postelectroporation.

Plasmid DNA methylated with recombinant MTases increases chromosomal integration for the *galkT* gene knockout plasmid pDJSVT13 in *F. nucleatum* subsp. *nucleatum* 23726. As shown in Fig. 2E, methylation of pDJSVT13 with M.Fnn23.I resulted in significantly more colonies after transformation, indicating that protected DNA was not degraded before homologous recombination with the *galkT* operon in *F. nucleatum* subsp. *nucleatum* 23726. M.Fnn23.II alone did not have a drastic effect but did increase efficiency. Finally, the combination of M.Fnn23.I and M.Fnn23.II resulted in an even further increase in transformation and chromosomal incorporation, thereby greatly enhancing the efficiency of creating gene knockouts.

As M.Fnn23.I appeared to be the dominant enzyme for protecting plasmid DNA in pDJSVT13, we made a pDJSVT13 Δ CATG plasmid, now called pDJSVT21, in which the four CATG sites were eliminated with silent single-nucleotide mutations to CACG. Figure 2F shows that pDJSVT21 transformed significantly better than pDJSVT13. The addition of M.Fnn23.I or M.Fnn23.II individually did not increase transformation efficiency over that of pDJSVT21. However, the addition of both enzymes did, which could mean that these enzymes are methylating at more than their bioinformatically predicted sites.

***In vivo* methylation of plasmids increased transformation of gene knockout and transposon plasmids.** We next developed plasmids that placed the M.Fnn23.I and M.Fnn23.II MTase genes downstream of a strong constitutive Anderson promoter (iGEM part BBa_J23101) and before a short terminator (iGEM part BBa_00014). Plasmid pDJSVT24 contains M.Fnn23.I, pDJSVT25 contains M.Fnn23.II, and pDJSVT26 contains both M.Fnn23.I and M.Fnn23.II (Fig. 3A). TOP10 *E. coli* containing one of the aforementioned plasmids expressing *F. nucleatum* subsp. *nucleatum* 23726 MTases was transformed with the *galkT* gene knockout plasmid pDJSVT13, followed by plasmid purification from overnight cultures. Upon transformation of this mixed pool of plasmids into *F. nucleatum* subsp. *nucleatum* 23726 and selection on thiamphenicol-containing medium to select for chromosomal incorporation of pDJSVT13, we showed that this simple method of plasmid methylation was effective at significantly increasing transformation rate. We noted that since this was a

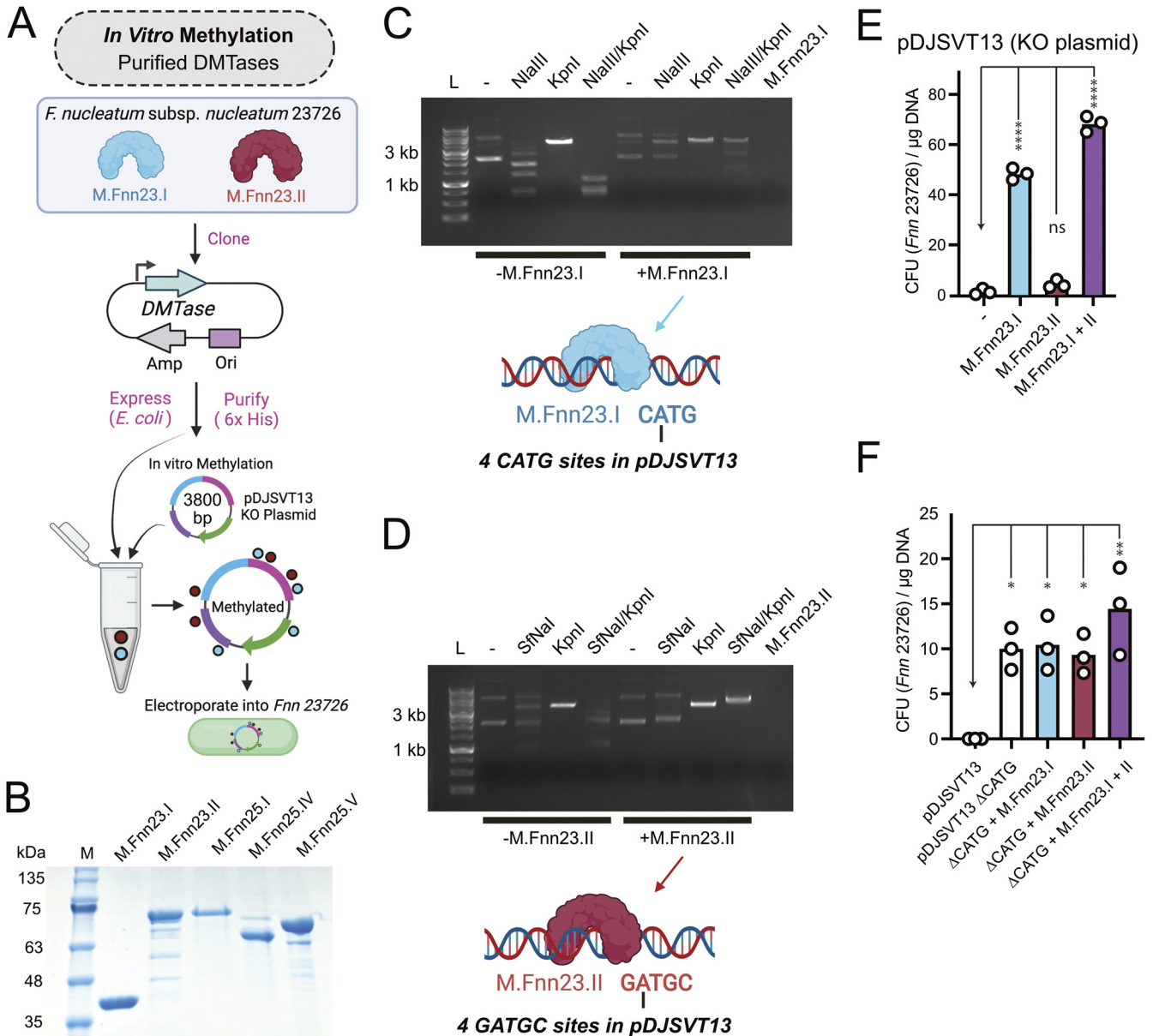


FIG 2 *F. nucleatum* MTases protect plasmid DNA and allow for more efficient chromosomal plasmid incorporation in *F. nucleatum* subsp. *nucleatum* 23726. (A) Schematic of our process to produce recombinant MTases that were next used to treat plasmid DNA *in vitro* prior to electroporation into *F. nucleatum* subsp. *nucleatum* 23726. (B) SDS-PAGE gel of five purified MTases from *F. nucleatum* subsp. *nucleatum* 23726 and *F. nucleatum* subsp. *nucleatum* 25586. (C) Methylation of pDJSVT13 with M.Fnn23.I protects against DNA cleavage by the REase NlaIII (NEB), which cuts at CATG sites. KpnI was used as a unique single-cut enzyme in pDJSVT13. The M.Fnn23.I lane is enzyme alone and shows no contaminating DNA brought in with the pure protein. (D) Methylation of pDJSVT13 with M.Fnn23.II protected against DNA cleavage by the REase SfiNI (NEB), which cut at GATGC sites. (E) Methylation of pDJSVT13 resulted in an increased number of transformants (in CFU per microgram of DNA). (F) By changing the four CATG sequences to CACG (Δ CATG), which are the target for the MTase M.Fnn23.I, transformation efficiency was significantly increased, even in the absence of methylation. Statistical values are as follows: ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. For panels E and F data, we used a two-way ANOVA.

mixed pool of plasmids, the efficiency of our transformations was likely higher than that shown in Fig. 3. M.Fnn23.I alone resulted in a marginal increase in efficiency, but methylation by both enzymes significantly increased transformation rates by more than 50-fold (Fig. 3B). As Top10 *E. coli* does possess Dam⁺ and Dcm⁺ methylation systems, we also used methylation-free *E. coli* ER2796 (67) and showed that plasmids purified from both strains transformed at the same rate into *F. nucleatum* subsp. *nucleatum* 23726 when pDJSVT26 was present and expressing M.Fnn23.I and M.Fnn23.II (Fig. 3C).

We next showed that the mini-Tn5 transposon harboring plasmid pBAMD1-4 (68) could be transformed into *F. nucleatum* subsp. *nucleatum* 23726, *F. nucleatum* subsp.

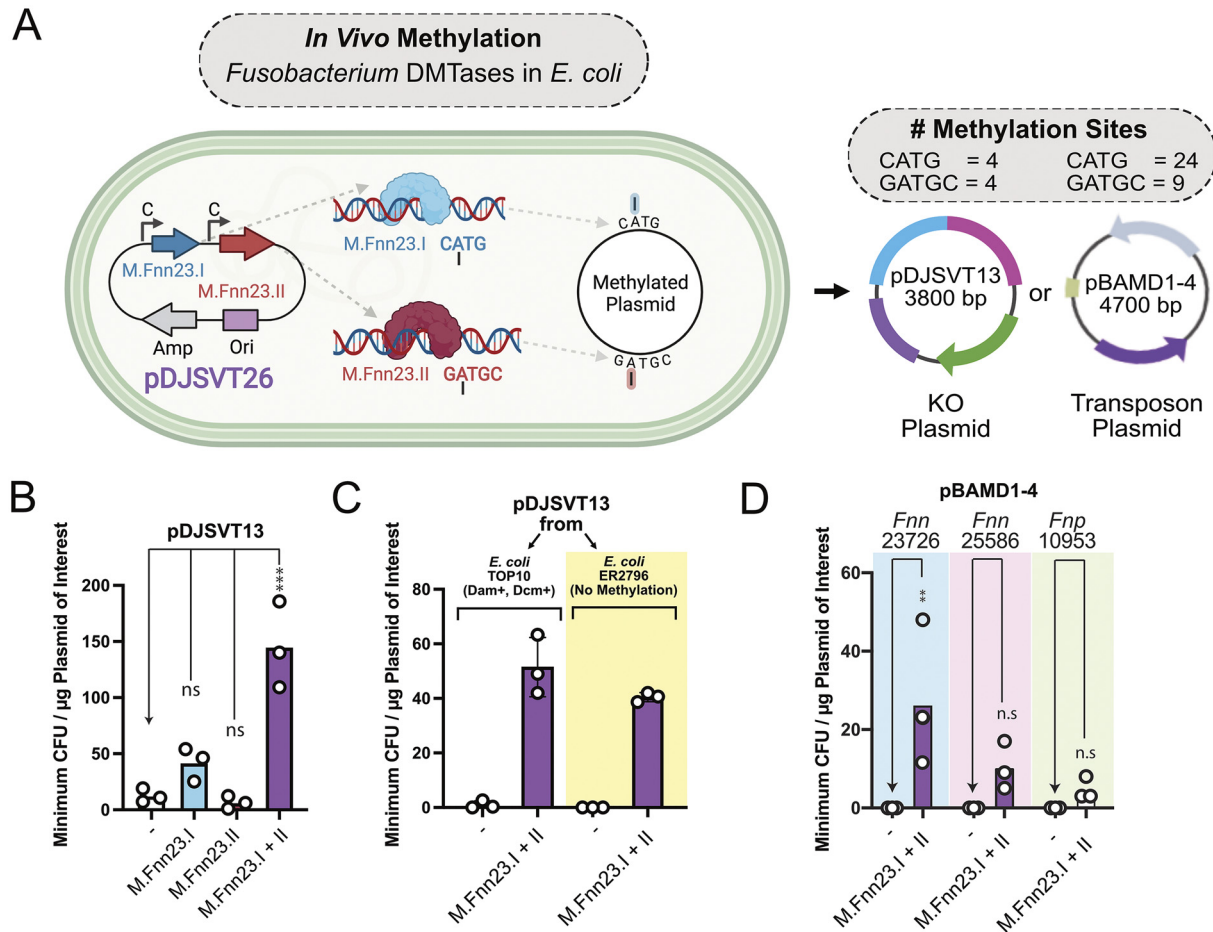


FIG 3 *In vivo* methylation in *E. coli* expressing M.Fnn23.I and M.Fnn23.II enhanced plasmid transformation and chromosomal incorporation of plasmids and transposons. (A) Schematic of *in vivo* methylation of plasmids with *F. nucleatum* subsp. *nucleatum* 23726 MTases. The C above the arrow indicates a promoter that is constitutively active for constitutive expression in *E. coli*. (B) Transformation of pDJSVT13 was significantly increased by coexpressing M.Fnn23.I and M.Fnn23.II. (C) Comparison of methylation-positive (TOP10) and methylation-negative (ER2796) *E. coli* strains revealed that native *E. coli* methylation did not inhibit the transformation of pDJSVT13 when *F. nucleatum* subsp. *nucleatum* 23726 MTases were concurrently expressed. (D) *In vivo* methylation of the pBAMD1-4 transposon plasmid allowed for transformation and chromosomal transposon insertion into multiple strains of *F. nucleatum*. Statistical values are as follows: ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. For panel B data we used a two-way ANOVA; for panel D data we used Student's *t* test analysis.

nucleatum 25586, and *F. nucleatum* subsp. *polymorphum* 10953 after methylation with M.Fnn23.I and M.Fnn23.II. Importantly, unmethylated plasmid was unsuccessful at producing transposon insertions in these three strains (Fig. 3D). We did note that this system is not highly efficient and would benefit from use of a more complete repertoire of MTases from the respective strains. Overall, compared with *in vitro* plasmid treatment with recombinant MTases, creation of an *E. coli* strain expressing *F. nucleatum* MTases worked just as well and required less effort than purifying multiple proteins. However, the difficulty of creating plasmids with a significant number of MTase genes makes this method increasingly challenging.

Passaging of a plasmid in *F. nucleatum* allowed for transformation into additional *F. nucleatum* strains. A common method of permitting a plasmid to be transformed into a genetically recalcitrant strain of interest is to first transform into a similar yet genetically competent strain, followed by re purification of the plasmid containing species-specific methylation patterns (Fig. 4A) (58). This plasmid frequently can then be transformed into the strain of interest. Here, we tested this classic method and showed that passage of the episomal, multicopy *Fusobacterium* plasmid pHS30 (50) in *F. nucleatum* subsp. *nucleatum* 23726 could be purified and then transformed into

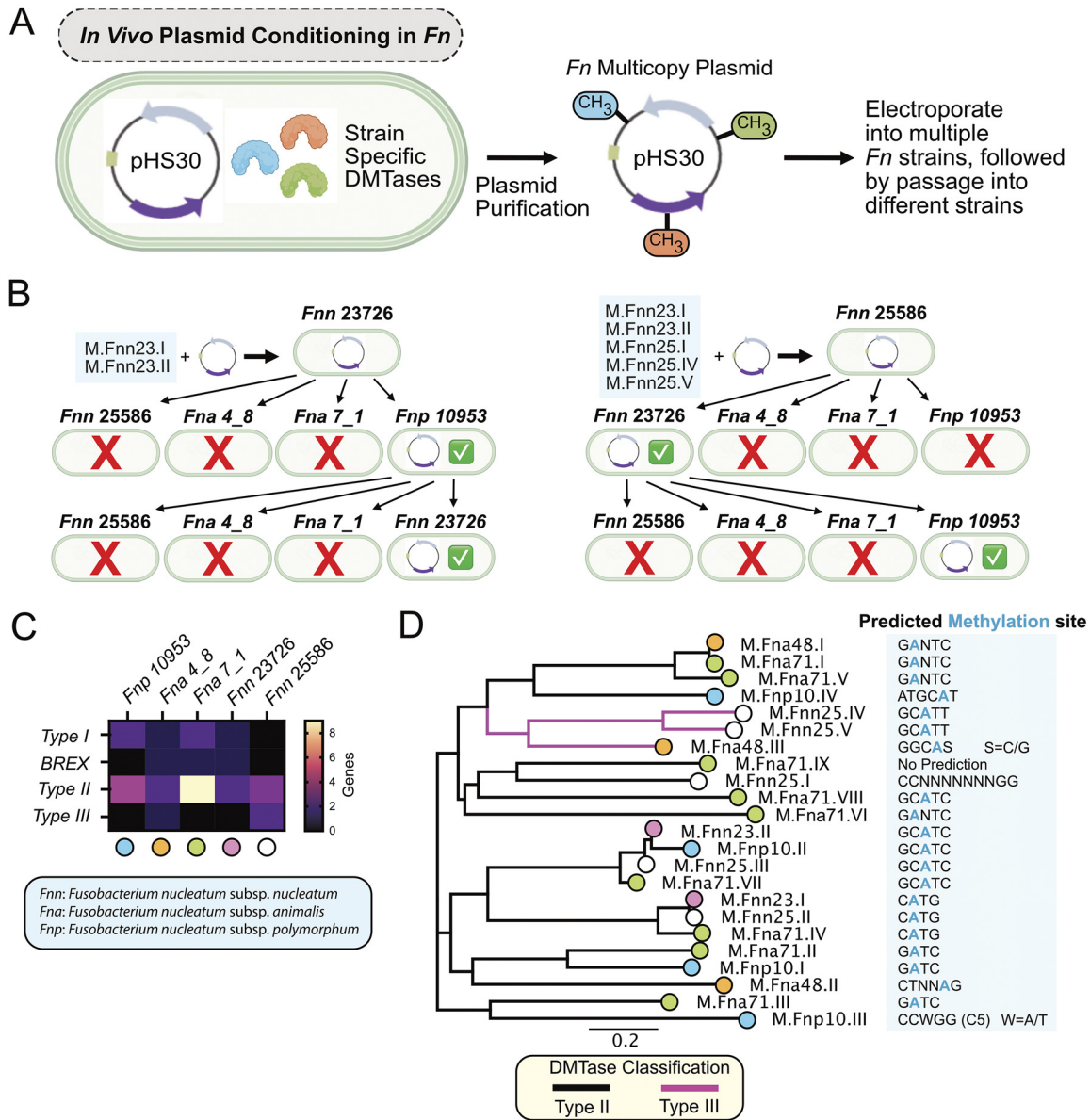


FIG 4 Passaging of a multicopy plasmid in *F. nucleatum* allowed passage to additional strains. (A) Schematic of our passaging method for the *Fusobacterium* multicopy plasmid pHS30 and purification of this plasmid for retransformation into different *F. nucleatum* strains. (B) pHS30 from *F. nucleatum* subsp. *nucleatum* 23726 can be transformed into *F. nucleatum* subsp. *polymorphum* 10953, and re-purification from this strain allowed transformation back into *F. nucleatum* subsp. *nucleatum* 23726. pHS30 from *F. nucleatum* subsp. *nucleatum* 25586 can be transformed into *F. nucleatum* subsp. *nucleatum* 23736, and re-purification from this strain allowed transformation back into *F. nucleatum* subsp. *polymorphum* 10953. (C) Heat map of the number of R-M systems in the five *F. nucleatum* strains analyzed. Colored dots below the strains correlate with the strains of the enzymes found in the phylogenetic tree in panel D. (D) Phylogenetic tree of 23 type II and III MTase genes from five *F. nucleatum* strains. Methylation sites are those predicted by REBASE.

F. nucleatum subsp. *polymorphum* 10953, but not *F. nucleatum* subsp. *nucleatum* 25586, *F. nucleatum* subsp. *animalis* 7_1, or *F. nucleatum* subsp. *animalis* 4_8. When plasmid was re-purified from *F. nucleatum* subsp. *polymorphum* 10953, this plasmid could only be retransformed back into *F. nucleatum* subsp. *nucleatum* 23726, revealing that the R-M systems in the other strains were not compatible with *F. nucleatum* subsp. *nucleatum* 23726 and *F. nucleatum* subsp. *polymorphum* 10953 (Fig. 4B). After methylating pHS30 with five MTases to allow transformation into *F. nucleatum* subsp. *nucleatum* 25586, re-purified plasmid was only able to be transformed into *F. nucleatum* subsp. *nucleatum* 23726. And once again, re-purification of the plasmid from *F. nucleatum* subsp.

nucleatum 23726 was only able to be transformed back into *F. nucleatum* subsp. *polymorphum* 10953.

To better understand why there was limited plasmid passaging between *F. nucleatum* subsp. *nucleatum* strains, we analyzed the type II and type III DMTases in the five *F. nucleatum* strains tested above. We first compared the number of genes present in the strains for all classes of MTases and noted that all strains contained a higher number of type II genes than the other classes (Fig. 4C). However, other than strain *F. nucleatum* subsp. *animalis* 7_1 having the highest number of type II R-M systems in strains analyzed, these data did not provide an obvious answer as to why the majority of these *Fusobacterium* strains were so genetically recalcitrant. To take a deeper look, we assembled a phylogenetic tree of the 23 type II and type III MTases from the five strains (Fig. 4D). Utilizing REBASE, we identified the predicted DNA recognition and methylation sites for all 23 type II and type III MTases in the five strains of *F. nucleatum* that we used in this study: *F. nucleatum* subsp. *nucleatum* 23726, *F. nucleatum* subsp. *nucleatum* 25586, *F. nucleatum* subsp. *animalis* 4_8, *F. nucleatum* subsp. *animalis* 7_1, and *F. nucleatum* subsp. *polymorphum* 10953 (Table S2). Nearly all MTases are predicted to be adenine DNA methyltransferases, where methylation occurs at the nitrogen at position 6 in the ring (N⁶) of the adenine (N⁶-mA or m⁶A), which is a common theme for AT-rich bacterial genomes (approximate GC concentration of 30%). Only one enzyme from *F. nucleatum* subsp. *polymorphum* 10953 was predicted to methylate the carbon at position 5 in the ring (C⁵) of the cytosine (C⁵-mC or m⁵C). We found clusters of enzymes with predicted MTase recognition sites that could be exploited to produce a library of enzymes that could be used for bypassing R-M systems in multiple strains. When analyzing the 23 MTases from these five *F. nucleatum* strains, it stands out that the enzymes are predicted to methylate only 10 recognition sites. These data also uncovered that of the nine enzymes in *F. nucleatum* subsp. *animalis* 7_1, which covered six predicted recognition sequences, only two of these sequences were predicted to be methylated by *F. nucleatum* subsp. *nucleatum* 23726 and *F. nucleatum* subsp. *nucleatum* 25586, leaving a large number of sequences unmethylated and the likely reason why plasmid was unable to be passed from these strains to *F. nucleatum* subsp. *animalis* 7_1.

***F. nucleatum* subsp. *nucleatum* 25586 and *F. nucleatum* subsp. *nucleatum* 23726 MTases allowed for the development of the first genetic system in *F. nucleatum* subsp. *nucleatum* 25586.** *F. nucleatum* subsp. *nucleatum* 25586 is a virulent and biomedically relevant strain that has been studied for more than 4 decades (69), yet molecular studies have not been possible because of the strain's inability to be transformed. Our goal was to use the same system we developed previously for gene knockouts in *F. nucleatum* subsp. *nucleatum* 23726 (66). As shown in Fig. 5, we used two MTases from *F. nucleatum* subsp. *nucleatum* 23726 (M.Fnn23.I and M.Fnn23.II; the same exact enzymes as M.Fnn25.II and M.Fnn25.III) (Fig. 1C) and three from *F. nucleatum* subsp. *nucleatum* 25586 (M.Fnn25.I, M.Fnn25.IV, and M.Fnn25.V) to bypass R-M systems in *F. nucleatum* subsp. *nucleatum* 25586. Purification of these recombinant enzymes was followed by methylation of pDJSVT13 and transformation by electroporation (Fig. 5A). Colonies that grew on thiamphenicol-containing plates indicated chromosomal integration by homologous recombination before (fragment A) or after (fragment B) the *galkT* operon (Fig. 5B). PCR and sequencing verification of chromosomal integration (A or B single crossover) (Fig. 5C and D) was followed by double-crossover events in nonselective medium and plating on medium containing deoxygalactose, which verified excision of the *galkT* operon because the presence of *galkT* makes 2-deoxy-D-galactose toxic (Fig. 5E and F) (66). *F. nucleatum* subsp. *nucleatum* 25586 Δ *galkT* grew with the same fitness as wild-type *F. nucleatum* subsp. *nucleatum* 25586, WT *F. nucleatum* subsp. *nucleatum* 23726, and *F. nucleatum* subsp. *nucleatum* 23726 Δ *galkT* (Fig. 5G).

Development of *F. nucleatum* subsp. *nucleatum* 25586 Δ *fap2* and Δ *fadA* strains.

As a proof of concept, we made clean chromosomal deletions in genes *fap2* and *fadA* in *F. nucleatum* subsp. *nucleatum* 25586 Δ *galkT* (Fig. 6). This approach followed the same system that we initially used to knock out the *galkT* operon (Fig. 5) to make a

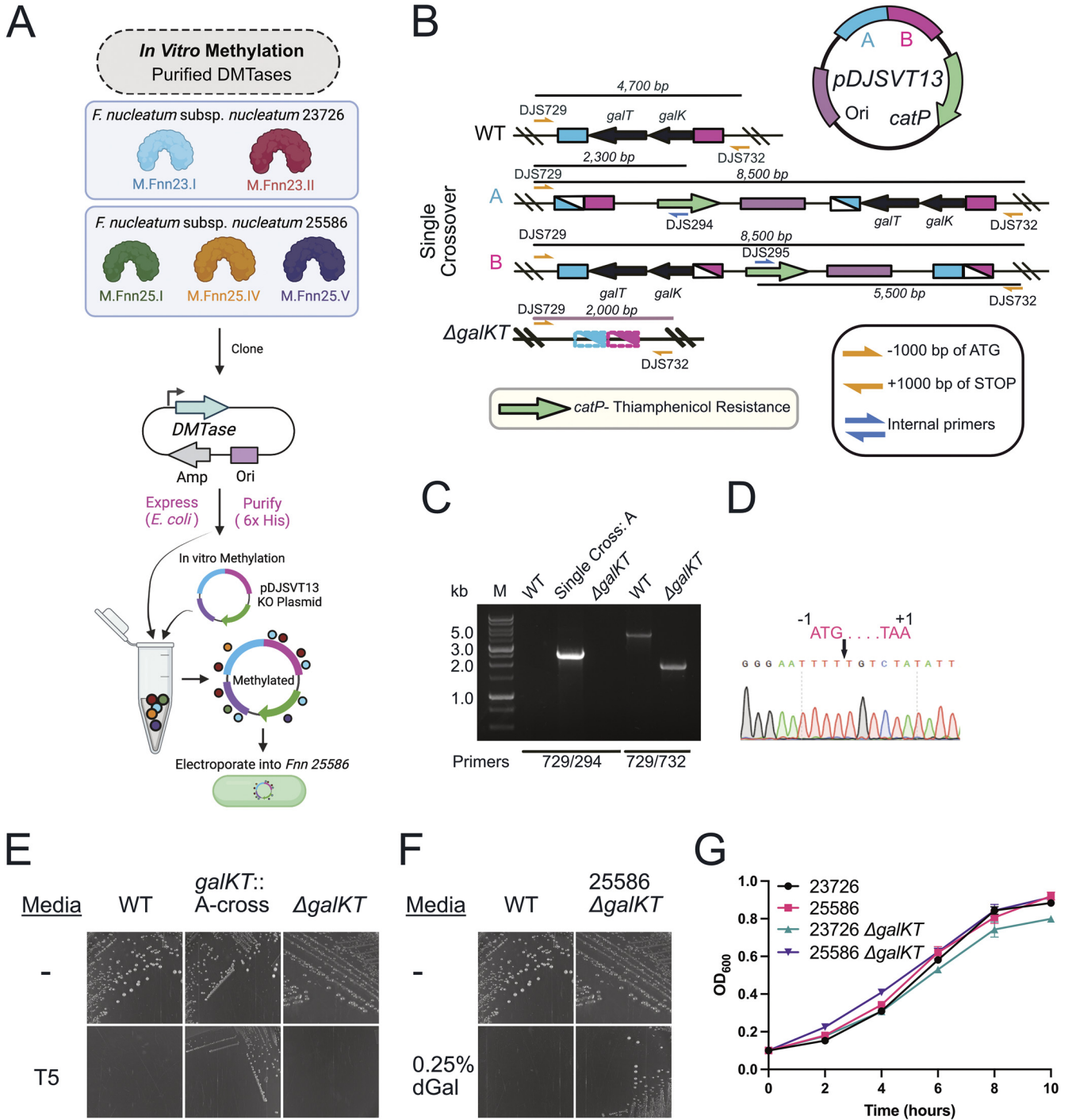


FIG 5 Development of a galactose-selectable genetic system in *F. nucleatum* subsp. *nucleatum* 25586. (A) Schematic of the strategy to use five purified *F. nucleatum* MTAse to methylate plasmid pDJSVT13 to transform into *F. nucleatum* 25586. (B) Schematic of single-crossover and *galKT* gene deletions using plasmid pDJSVT13, which was first homologously recombined with up- and downstream sequences of the *galKT* operon. Primers used for PCR verification of positive clones are denoted with yellow or blue arrows, and the labels DJSnnn and TNNnn refer to specific primers found in Table S5 in the supplemental material. (C) PCR verification of the initial chromosomal incorporation (a crossover) as well as the full operon deletion ($\Delta galKT$). (D) Sanger sequencing verification of a full, clean, deletion of the *galKT* operon. (E) Selection for A-crossover strains on thiamphenicol (T5) containing plates, and verification that the $\Delta galKT$ strain had removed the vector and antibiotic cassette and no longer grew on thiamphenicol. (F) Proof of survival of $\Delta galKT$ on plates containing deoxygalactose (dGal), which is toxic to wild-type *F. nucleatum* subsp. *nucleatum* 25586. (G) Growth curves showed no growth defect for *F. nucleatum* subsp. *nucleatum* 25586 $\Delta galKT$ compared to WT *F. nucleatum* subsp. *nucleatum* 25586, WT *F. nucleatum* subsp. *nucleatum* 23726, and *F. nucleatum* subsp. *nucleatum* 23726 $\Delta galKT$.

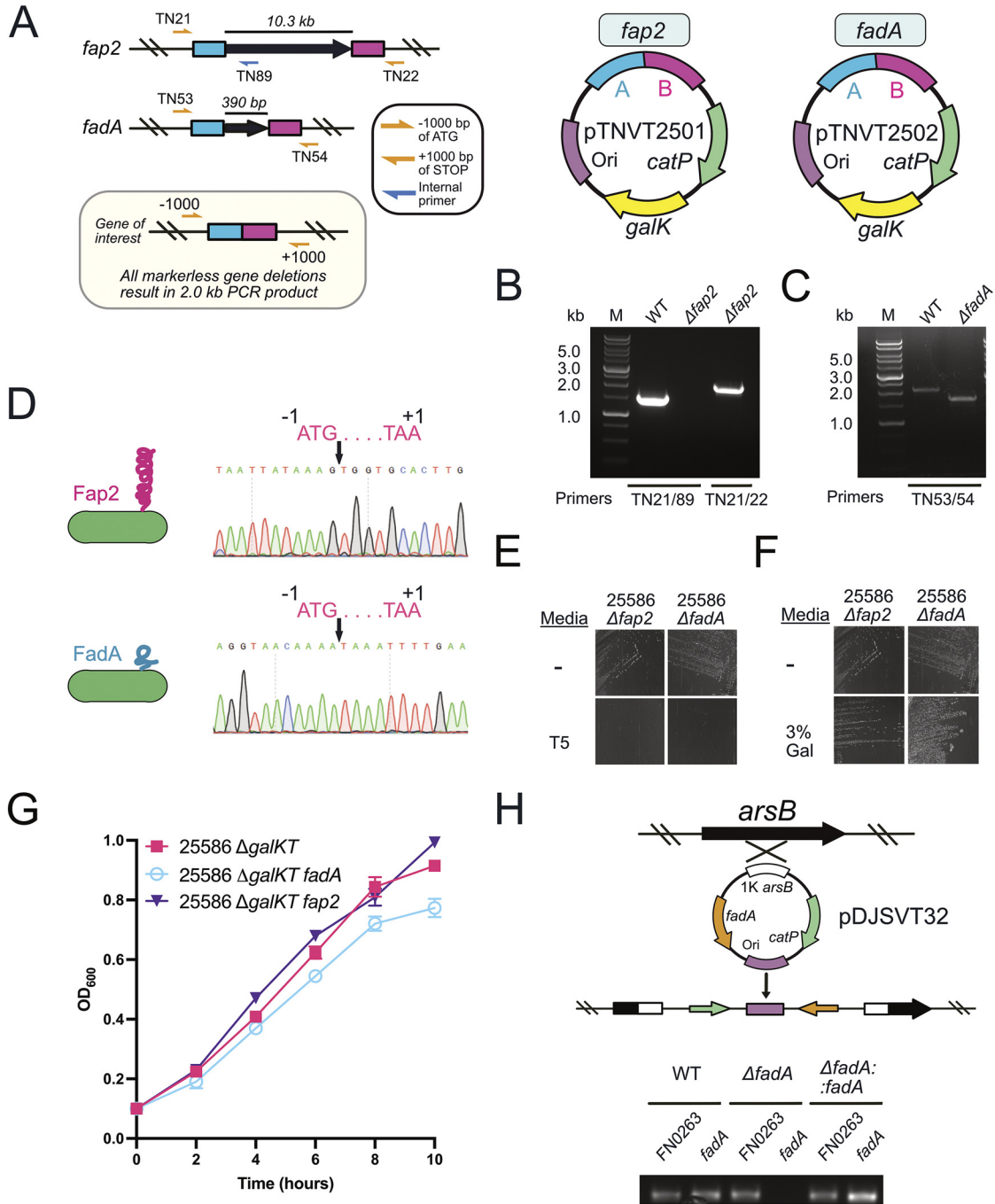


FIG 6 Gene deletions of *fap2* and *fadA*, as well as *fadA* complementation in *F. nucleatum* subsp. *nucleatum* 25586. (A) Schematic for deletion of the genes *fap2* (>10 kb) and *fadA* (390 bp). Primers used for PCR verification of positive clones are denoted with yellow or blue arrows, and the labels TNnnn refer to specific primers found in Table S5. Plasmids pTNVT01 and pTNVT02 correspond to plasmids created to delete *fap2* and *fadA*, respectively. (B) PCR verification of the $\Delta fap2$ mutant in *F. nucleatum* subsp. *nucleatum* 25586. (C) PCR verification of the $\Delta fadA$ mutant in *F. nucleatum* subsp. *nucleatum* 25586. (D) Sanger sequencing verification of a full, clean, deletion of the *fap2* and *fadA* genes. (E) Streaking of *F. nucleatum* subsp. *nucleatum* 25586 $\Delta fap2$ and $\Delta fadA$ on thiamphenicol-containing plates (T5) verified the chromosomally integrated plasmid had been excised by homologous recombination. (F) Streaking of *F. nucleatum* subsp. *nucleatum* 25586 $\Delta fap2$ and $\Delta fadA$ on galactose-containing plates (T5) verified the chromosomally integrated plasmid had been excised by homologous recombination. (G) Growth curves showed no fitness effects from the *fap2* and *fadA* gene deletions. (H) Complementation of the *fadA* gene ($\Delta fadA::fadA$) onto the chromosome at the *arsB* gene using a single-crossover homologous recombination plasmid and confirmation by PCR.

galactose-selectable system possible. Briefly, we cloned and fused 1,000 bp up- and downstream of the target gene before ligating into our gene knockout vector that worked by double-crossover homologous recombination of gene deletion. This method resulted in clean deletions of the large, outer membrane, autotransporter adhesin *fap2* (>10 kb) and the small, outer membrane adhesin *fadA* (390 bp), both of which have been studied extensively for their roles in *F. nucleatum* pathogenicity (Fig. 6A to F) (70–73). These gene deletions did not cause any adverse growth phenotypes compared to the parent strain *F. nucleatum* subsp. *nucleatum* 25586 Δ *galKT* (Fig. 6G). Our final experiment was to complement the *fadA* gene deletion back onto the chromosome of *F. nucleatum* subsp. *nucleatum* 25586 Δ *galKT fadA* at the *arsB* gene (66) (Fig. 6H), which confers arsenic resistance to bacteria but is not essential or necessary for *F. nucleatum* grown under laboratory conditions (74). In addition, we report that like the system for *F. nucleatum* subsp. *nucleatum* 23726, there appear to be no differences in efficiency when deleting large (*fap2*; 10 kb) or small (*fadA*; 390 bp) genes in *F. nucleatum* subsp. *nucleatum* 25586.

DISCUSSION

Bacterial restriction-modification systems are important in both protection of bacteria from invading foreign DNA as well as using methylation as an epigenetic switch to control gene regulation (75). Our hypothesis was that if we could bypass *Fusobacterium* restriction-modification systems, it would enhance genetic efficiency in currently tractable strains, as well as leap the hurdle of developing new systems in strains that are currently inaccessible to molecular methods. Here, we showed that using strain-specific MTases from *Fusobacterium nucleatum* to methylate custom gene deletion plasmids led to more efficient gene deletions and gene complementations on the chromosome, as well as the introduction of a multicopy plasmid that could be used for a range of tasks, including gene complementation and protein overexpression. Our results showed a multifold increase in the efficiency of transformations and subsequent chromosomal incorporation of gene deletion plasmids in the genetically tractable strain *F. nucleatum* subsp. *nucleatum* 23726. To enhance genetics in this strain, we cloned, expressed, and characterized two type II MTase enzymes, which we renamed M.Fnn23.I and M.Fnn23.II. Using both *in vitro* and *in vivo* analyses, we verified that methylation of plasmid DNA blocked cleavage by the enzymes NlaIII and SfiI, which cut at CATG and GATGC sites, respectively. We next showed that each enzyme individually increased the efficiency of plasmid introduction but that combining the two enzymes had a statistically significant effect. This synergistic effect could be attributed to blocking all of the potential restriction sites, where even one cut of the vector would likely stop it from being transformed. We chose to use the chromosomal integration plasmid pDJSVT13 for most of our studies, over the multicopy episomal plasmid pHS30, to focus on gene knockout improvement and not just introduction of a plasmid. Two areas that we noted that are variable between laboratories are (i) how competent bacteria are prepared and (ii) the plasmids used for transformation and subsequent generation of mutants and gene expression. Our analysis showed that the number of CATG and GATGC sites could be the differences in how other studies have shown variable transformation efficiencies, as we have not analyzed the sequences of these plasmids.

We next set our focus on creating the first gene knockouts in *F. nucleatum* subsp. *nucleatum* 25586, which had not been accomplished in over 40 years of studying the strain (20). To accomplish this, we produced five recombinantly expressed MTase enzymes to treat plasmid DNA *in vitro*, followed by transformation by electroporation. Through this method we were able to create the first clean gene deletions and complementations in *F. nucleatum* subsp. *nucleatum* 25586, with deletions in *galKT*, *fap2*, and *fadA* and complementation of *fadA* back onto the chromosome. This markerless gene deletion system can produce iterative gene deletions in a single strain, as the final gene deletions are free of antibiotic markers. We noted that all five enzymes were necessary to protect plasmid DNA for transformation into *F. nucleatum* subsp. *nucleatum* 25586; however, all MTases that we produced and used in these studies retained

enzymatic activity after at least one freeze-thaw cycle (data not quantitated), making it a robust solution for researchers to implement.

We acknowledge that there are still major limitations to genetically modifying most strains of *Fusobacterium* because of their unique R-M compositions. Therefore, we understand that using a core combination of MTase enzymes for universal protection across multiple *Fusobacterium* species may not be possible, as each strain frequently has unique MTases that create a broad range of methylation patterns between strains. This has been reported before, as type II and type III R-M systems vary significantly even in evolutionarily similar strains of bacteria. Future studies using DNA methylation analysis of MTase deletion strains to identify exact methylation sequences with specific enzymes will lead to the experimental determination of methylation sites by specific enzymes. To support this claim, previous studies have shown that use of PacBio SMRTseq technology to determine the methylome of a bacterium results in the identification of specific methylation sites, which can then be used to guide the creation of syngenic DNA plasmids devoid of methylation and REase cleavage sites that are not degraded by the host (15). An additional study that used SMRTseq technology identified all DNA recognition sites and methylation patterns in multiple species of bacteria, followed by placing these sequences in a methylation cassette within a plasmid that was then incubated with purified enzymes to identify specific methylation patterns (76). Using this technique for highly recalcitrant strains of *Fusobacterium* would allow for the first true matching of methylation sites with *F. nucleatum* MTases outside of bioinformatic predictions. Finally, one advantage we believe that use of recombinant MTases has over this approach is that DNA methylation analysis and synthetic DNA-based plasmids do not need to be made for each strain, which can keep down costs. Ultimately, we believe these methods are complementary and can be used in combination to enhance the chances of genetic modification in highly recalcitrant strains of *F. nucleatum*.

Potential future studies include an investigation into the role of the type I MTase systems in plasmid methylation. In this area, we briefly tried to recombinantly express HsdM and HsdS from *F. nucleatum* subsp. *nucleatum* 23726 but had difficulty achieving a pure, soluble protein complex. In addition, we reported that we tried to use a type I restriction modification inhibitor (Lucigen) in our transformations of *F. nucleatum* subsp. *nucleatum* 23726, but this did not change the transformation efficiency (data not shown). On a final note of the potential contribution of type I R-M systems, *F. nucleatum* subsp. *nucleatum* 25586 had no type I systems and was still genetically intractable until we used type II and type III enzymes. However, many transformable strains of bacteria have been made *hsdRMS* negative, which should be considered in the future as a method to potentially make more efficiently transformable strains of *Fusobacterium*.

Two additional strategies that could be used to increase transformation efficiency would be to delete the known REases in target *F. nucleatum* strains and creating *E. coli* strains with chromosomal integrations of *F. nucleatum* MTases. One disadvantage of deleting REase genes in *F. nucleatum* is the need to first transform and create a genetic system to be able to subsequently knock out these genes. But once accomplished, an REase-free strain could potentially bypass the need to treat entering plasmid DNA with MTases. However, many of the type II MTases do not have a paired REases, as shown in Fig. 1C, and therefore it is difficult to understand what could be cleaving the unmethylated DNA sequences that correspond to specific enzymes. *E. coli* strains with chromosomal integrations of *F. nucleatum* MTases would allow for efficient *in vivo* methylation of target plasmids while also not introducing plasmids that could be incompatible with the origin of replication or antibiotic resistance of the target plasmid to be methylated. In addition, expanding beyond the realm of *F. nucleatum* to other *Fusobacterium* species, including *F. necrophorum*, could be key to understanding the pathogenicity of this species in Lemierre's syndrome in humans (77), as well as serious organ infections in livestock (78).

In conclusion, we report that *F. nucleatum* MTases can be used to methylate plasmid DNA, which then allows for efficient transformation and gene deletion in the well-studied

strain *F. nucleatum* subsp. *nucleatum* 23726 as well as in the previously genetically intractable strain *F. nucleatum* subsp. *nucleatum* 25586. The broader implications of this work are the enhanced ability to study the role of specific genes and corresponding virulence factors expressed by *F. nucleatum* during infection and disease. The methods in this study can be directly applied to target strains of interest within the scientific community and therefore provide a roadmap for discovery biology that could lead to better understanding of how to inhibit the disease-driving mechanisms of this oral, opportunistic pathogen.

MATERIALS AND METHODS

Bacterial strains and plasmids. All *E. coli* strains utilized in these studies were grown aerobically overnight at 37°C on solid Luria-Bertani (LB) agar plates (10 g/liter NaCl, 5 g/liter tryptone, 10 g/liter yeast extract) or in liquid LB medium. *Fusobacterium* strains were grown on solid agar plates made with Columbia broth (Gibco), supplemented with hemin (5 µg/mL), menadione (0.5 µg/mL), and resazurin (1 µL/mL) under anaerobic conditions (90% N₂, 5% H₂, 5% CO₂) at 37°C (designated CBHK medium). Liquid growths were inoculated from single *F. nucleatum* colonies and grown in CBHK liquid medium under anaerobic conditions. Where necessary, antibiotics were supplemented at the suggested concentrations: gentamicin, 20 µg/mL; carbenicillin, 100 µg/mL; chloramphenicol, 10 or 25 µg/mL; thiamphenicol, 5 µg/mL (CBHK plates); or streptomycin 50 µg/mL (CBHK plates). The plasmids and bacterial strains utilized in these experiments are listed in Table S3 and Table S4 in the supplemental material, respectively.

Identification and classification of *F. nucleatum* DNA methyltransferases. REBASE, a curated database of restriction enzymes, was used to identify the DNA methyltransferases present in *F. nucleatum* subsp. *nucleatum* ATCC 23726 ([GCA_003019875.1](#)), *F. nucleatum* subsp. *nucleatum* ATCC 25586 ([GCA_003019295.1](#)), *F. nucleatum* subsp. *animalis* 7_1 ([GCA_000158275.2](#)), *F. nucleatum* subsp. *animalis* 4_8 ([GCA_000400875.1](#)), and *F. nucleatum* subsp. *polymorphum* 10953 ([GCA_000153625.1](#)) from the NCBI database. Type II and type III MTases were further bioinformatically characterized using NIH SMARTBLAST and pHMMER. SMARTBLAST and pHMMER provided conserved domains indicating functions of MTases. Phylogenetic analysis of *F. nucleatum* MTase genes identified in REBASE were downloaded from NCBI and the NCBI identification numbers are supplied in Table S2. The tree and analysis were done in Geneious Prime 2022.1.1 using the Geneious Tree Builder function.

Cloning, expression, and purification of MTases. The MTases M.Fnn23.I, M.Fnn23.II, M.Fnn25.I, M.Fnn25.IV, and M.Fnn25.V were cloned into pET16b under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-induced promoter for purification of the recombinant proteins using the C-terminal 6×His tag and benchtop metal affinity chromatography. In addition, M.Fnn23.I and M.Fnn23.II were cloned under the control of a constitutive promoter for continual expression in TOP10 *E. coli* cells to drive *in vivo* methylation of plasmids. All plasmids utilized and created in these studies are described in Table S3 along with the bacterial strains in Table S4 and primers in Table S5. The primers to clone the DNA methyltransferases were all ordered from Integrated DNA Technologies. For M.Fnn23.I and M.Fnn23.II, all constructs were made with *E. coli*, and codon-optimized synthetic DNA was used for PCR. For MTases from *F. nucleatum* subsp. *nucleatum* ATCC 25586, PCR was run with genomic DNA that was prepared with Wizard genomic DNA purification kits (Promega).

Genes were amplified by PCR, and products were purified utilizing a PCR purification kit (Biobasic) and digested for 2 h at 37°C along with pET16b, which was used as the expression vector and was obtained through EZ-10 spin column plasmid miniprep (Biobasic) with the restriction enzymes listed in Table S5 with their respective primers. The vector was then dephosphorylated with Antarctic phosphatase (FastAP; Thermo Fisher Scientific) for 1 h at 37°C. Digested products were purified utilizing a spin column and ligated by T4 DNA ligase (New England Biolabs) for 1 h at room temperature following the manufacturer's recommendations. Ligations were transformed into competent Mix&Go (Zymo Research) Top10 *E. coli* and plated on LB solid agar plates supplemented with 100 µg/mL carbenicillin (ampicillin). Confirmation of positive clones was performed by digestion and, if applicable, positive clones were then transformed into ARTIC(DE3) RIL or LOBSTR-BL21(DE3) RIL (79) for recombinant protein expression.

For protein expression, *E. coli* cells were grown in LB (15 g/liter NaCl, 15 g/liter tryptone, 10 g/liter yeast extract) medium at 37°C, 250 rpm shaking until the optical density (OD) reached 0.6. At an OD of 0.6, cells were induced with 50 µM IPTG (GoldBio). Expression was carried out at 8°C and cells were collected at 20 h after inoculation by centrifugation at 5,000 × *g* for 20 min at 4°C. Bacterial pellets were resuspended in a lysis buffer (20 mM Tris [pH 7.5], 400 mM NaCl, 20 mM imidazole). Bacteria were lysed with an EmulsiFlex-C3 homogenizer (Avestin) at 10,000 kPa. Unlysed cells and insoluble material were separated by centrifugation at 15,000 × *g* for 20 min at 4°C and then discarded. The supernatant containing the 6×His-tagged MTases was stirred with 6 mL of NiCl₂-charged chelating Sepharose beads (GE Healthcare) for 30 min at 4°C. The column was washed with 400 mL of wash buffer (20 mM Tris [pH 7.5], 400 mM NaCl, 40 mM imidazole). After washing, the methyltransferases were eluted in 10 mL of elution buffer (20 mM Tris [pH 7.5], 400 mM NaCl, 250 mM imidazole). The purified protein was then directly put into dialysis in a buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 10% glycerol). Protein concentrations were calculated using a Qubit fluorometer and bicinchoninic acid assays, followed by freezing at −80°C for long-term storage.

In vitro treatment of plasmid DNA with *Fusobacterium* DNA methyltransferases. Plasmid DNA (35 to 40 µg), prepared from *E. coli* TOP10 using the EZ-10 Spin column plasmid DNA miniprep from

Biobasic, was combined in a 30- μ L reaction mixture with 160 μ M S-adenosylmethionine (SAM; New England Biolabs), 1 \times Cutsmart buffer (New England Biolabs), and 1 μ M of one or more MTases. The reaction mixtures were incubated at 37°C for 2 h, and then plasmid was extracted by adding 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1; bioWORLD) and vortexed for 20 s. Mixtures were then centrifuged at 16,000 \times g for 5 min. Plasmid DNA was precipitated and washed with ethanol and dissolved in ultrapure water (bioWORLD), followed by further purification. Plasmid DNA purified from overnight expression or coexpression was isolated with an alkaline lysis and column purification technique using the EZ-10 spin column plasmid miniprep (Biobasic). Plasmid DNA was further purified for use in electroporation by precipitation overnight at -80°C in 75% ethanol with sodium acetate (pH 5.5) and 0.1 μ g/mL glycogen. After a 3-h minimum incubation at -80°C , the sample was spun at 4°C for 30 min at 16,000 \times g to pellet the DNA and washed five times with 70% ethanol carefully by spinning at 14,000 \times g for 3 min. Pellet was then dried at room temperature for 10 to 13 min. Finally, 15 μ L of ultrapure water was added and the mixtures were incubated at 37°C for 1 h to solubilize the pellet. DNA concentrations were determined using a NanoDrop spectrophotometer.

Coexpression of plasmid DNA with *F. nucleatum* MTases for *in vivo* methylation. Using the expression vector (constitutive activity) pET16b with the DNA methyltransferase under an Anderson medium promoter (described in Table S3), we methylated pDJSVT13 *in vivo*. Both pET16b (gene 622 and gene 635) and pDJSVT13 were transformed into *E. coli* TOP10 and grown in LB (15 g/liter NaCl, 15 g/liter tryptone, 10g/liter yeast extract) at 37°C with 250 rpm shaking for 24 h.

REase protection assays. Plasmid DNA (1 μ g), prepared from *E. coli* TOP10 cells using the Biobasic miniprep procedure, was combined with Cutsmart buffer (New England Biolabs; 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 100 μ g/mL bovine serum albumin [pH 7.9]), 160 μ M SAM (New England Biolabs), and 1 μ M of the correspondent MTases. As a control, plasmid DNA (1 μ g) was mock treated in reaction buffer without the methyltransferases. All samples were incubated 1 h at 37°C with the restriction enzymes, single cutters KpnI and MluI, or predicted restriction sites NlaIII and SfaNI (New England Biolabs [NEB]). For single-cut linearization, plasmid DNA was digested with restriction enzyme KpnI (NEB) following the manufacturer's instructions. After 2 h at 37°C, the ultrapure DNA underwent phenol-chloroform extraction and ethanol precipitation at -80°C , as described previously for ultrapure DNA purification. Samples were analyzed in a 1% agarose gel with ethidium bromide and imaged on a Syngene G:Box imager (Fig. 2C and D).

***F. nucleatum* transformation by electroporation.** All *F. nucleatum* strains were competently prepared by inoculating and growing a 100-mL anaerobic culture in CBHK medium to lag phase ($A_{600} = 0.1$) followed by centrifugation of bacteria at 3,200 \times g for 10 min. The supernatant was removed, and the resulting pellet was washed three successive times utilizing 1 mL of ice-cold 20% glycerol in deionized H₂O and 1 mM morpholinopropanesulfonic acid (MOPS) at 14,000 \times g for 3 min. Bacterial pellet was then resuspended in a final volume of 80 μ L of ice-cold 20% glycerol and 1 mM MOPS. Bacteria were transferred to cold, 1-mm electroporation cuvettes (Lonza), and 3 μ g (concentration of >300 ng/ μ L) of plasmid was added before electroporating at 2.5 kV/cm, 50 μ F, 360 Ω , using a BTX Electro Cell Manipulator 600 (Harvard Apparatus). The electroporated cells were promptly transferred by syringe into a sterile, anaerobic tube with 4 mL of recovery medium (CBHK; 1 mM MgCl₂) and incubated at 37°C for 20 h with no shaking in an anaerobic chamber. After the recovery outgrowth, cells were centrifuged at 14,000 \times g for 3 min, supernatant was removed, and pelleted cells were resuspended in 0.2 mL of recovery medium. Resuspension was plated on CBHK plates with 5 μ g/mL thiamphenicol and incubated in an anaerobic 37°C incubator for 2 days for colony growth. The transformation efficiency represented the number of thiamphenicol- or streptomycin-resistant colonies per microgram of DNA. Electroporation was conducted in triplicate as independent experiments.

Utilization of plasmid methylation to enable a galactose-selectable gene deletion system in *F. nucleatum* subsp. *nucleatum* 25586. A galactose-selectable gene deletion system for *F. nucleatum* subsp. *nucleatum* 23726 was previously developed in our lab and reported in detail by Casasanta et al. (66). As *F. nucleatum* subsp. *nucleatum* 23726 and *F. nucleatum* subsp. *nucleatum* 25586 are extremely similar at the DNA level, the plasmid pDJSVT13 that was previously used to delete the *galKT* operon in *F. nucleatum* subsp. *nucleatum* 23726 was also used on *F. nucleatum* subsp. *nucleatum* 25586 because of 100% nucleotide identity in the up- and downstream regions cloned for homologous recombination and gene deletion. pDJSVT13 was conditioned with five MTase enzymes (M.Fnn23.I, M.Fnn23.II, M.Fnn25.I, M.Fnn25.IV, and M.Fnn25.V) using the same conditions as describe above for the *in vitro* methylation protocol. Ultrapure DNA (3 μ g) was electroporated (2.5 kV, 50- μ F capacitance, 360- Ω resistance, 0.2-cm cuvette) into competent *F. nucleatum* subsp. *nucleatum* 25586, and single chromosomal crossovers of the pDJSVT13 plasmid were selected on thiamphenicol. Colonies were then inoculated into antibiotic-free CBHK medium overnight at 37°C to allow for a second crossover event, which effectively deleted the target gene and also the remaining plasmid that was integrated into the chromosome. Next, 100 μ L from this culture was streaked on solid medium containing 0.25% 2-deoxy-D-galactose to select for *galKT* gene deletions, as the absence of the *galT* gene makes 2-deoxy-D-galactose nontoxic to *F. nucleatum* subsp. *nucleatum*. *galKT* gene deletions were verified by PCR and Sanger sequencing. This new strain, *F. nucleatum* subsp. *nucleatum* 25586 Δ *galKT*, which we have named TNVT2501, is now the base strain used to create all future targeted gene deletions. Bacterial transformation of TNVT2501 allows for initial chromosomal integration and selection with thiamphenicol, followed by selection for double-crossover gene deletions on solid medium containing 3% galactose. We have shown that deletion of the *galKT* operon in *F. nucleatum* subsp. *nucleatum* 25586 does not result in altered fitness.

Creating *F. nucleatum* subsp. *nucleatum* 25586 Δ *fap2* and *F. nucleatum* subsp. *nucleatum* 25586 Δ *fadA*. As a proof of concept, we next generated targeted gene deletions in the *F. nucleatum* subsp. *nucleatum* 25586 Δ *galKT* background and in the two most well-studied *F. nucleatum* virulence

factors: *fap2* and *fadA*. The first step was to use the plasmid pDJSVT7, which contained a FLAG::*galk* gene under the control of an *F. necrophorum* promoter. Briefly, 750 bp directly upstream and downstream of the *fap2* and *fadA* genes was amplified by PCR and fused by overlap-extension-PCR. PCR product was digested with KpnI/MluI ligated into pDJSVT7 digested with the same enzymes, followed by transformation into TOP10 *E. coli* and selection on LB plates containing chloramphenicol. Positive clones were identified by restriction digest and Sanger sequencing to verify the new gene deletion plasmids pTNVT2501 (*fap2*) and pTNVT2502 (*fadA*) (Fig. 6A). pTNVT2501 and pTNVT2502 were next electroporated (3 μ g of DNA, 2.5 kV, 50- μ F capacitance, 360- Ω resistance, 0.2-cm cuvette) into competent *F. nucleatum* subsp. *nucleatum* 25586 Δ *galkT*, and chromosomal integration was selected for on thiamphenicol (single chromosomal crossover), followed by selection on solid medium containing 3% galactose, which produced either complete gene deletions or wild-type bacteria revertants. Gene deletions were verified by PCR and Sanger sequencing (Fig. 6). The new strain names are TNVT02 and TNVT03 for the Δ *fap2* and Δ *fadA* mutants of *F. nucleatum* subsp. *nucleatum* 25586. We showed that this system was accurate down to the single base level for creating clean genome excisions that therefore allowed for the deletion of an unlimited number of genes.

Complementation of a *fadA* gene deletion in *F. nucleatum* subsp. *nucleatum* 25586. We previously created the gene complementation vector pDJSVT11 to create single-copy chromosomal complementation at a chromosomal location within the *arsB* gene (66). Our previously developed plasmid, pDJSVT32, was used to complement *F. nucleatum* subsp. *nucleatum* 23726 Δ *galkT fadA* and was also used to complement *F. nucleatum* subsp. *nucleatum* 25586 Δ *galkT fadA* (TNVT03). Briefly, this plasmid contained a 1,000-bp central region of the *arsB* gene, driving homologous recombination, which resulted in chromosomal insertion of the thiamphenicol resistance plasmid. Complementation was selected for on CBHK plates containing thiamphenicol, followed by inoculation into liquid CHBK containing thiamphenicol. Complementation was further verified by PCR of the *fadA* gene as shown in Fig. 6H.

Statistical analysis. All statistical analyses were performed in GraphPad Prism version 8.2.1. For single analysis, an unpaired Student's *t* test was used. For grouped analyses, two-way analysis of variance (ANOVA) was used. In each case, the following *P* values correspond to symbols in figures: ns, *P* > 0.05; *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001. To obtain statistics, all studies were performed as three independent biological experiments. For all experiments in which statistical analysis was applied, an *N* of 3 independent experiments was used (see details in figure legends).

Data availability. Materials are available upon reasonable request with a material transfer agreement with Virginia Tech for bacterial strains or through the Addgene repository for plasmids. Plasmid sequences and maps can be accessed on our Open Science Framework repository at https://osf.io/yrbgj/?view_only=24a28a13c5194d22957f2fae1ce2f84b.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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A.U., T.T.D.N., and B.E.S. curated and analyzed the data, designed and optimized the methodology, and wrote, reviewed, and edited the manuscript. K.J.W. and B.W. curated the data and reviewed and edited the manuscript. D.J.S. helped conceptualize, supervise, and acquire funding for the study, performed data analysis, curated and analyzed the data, designed and optimized the methodology, and wrote, reviewed, and edited the manuscript.

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