Natural Transformation of an Engineered *Helicobacter pylori* Strain Deficient in Type II Restriction Endonucleases

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Restriction-modification (RM) systems are important for bacteria to limit foreign DNA invasion. The naturally competent bacterium *Helicobacter pylori* has highly diverse strain-specific type II systems. To evaluate the roles of strain-specific restriction in *H. pylori* natural transformation, a markerless type II restriction endonuclease-deficient (REd) mutant was constructed. We deleted the genes encoding all four active type II restriction endonucleases in *H. pylori* strain 26695 using *sacB*-mediated counterselection. Transformation by donor DNA with exogenous cassettes methylated by *Escherichia coli* was substantially (1.7 and 2.0 $\log_{10}$ for *cat* and *aphA*, respectively) increased in the REd strain. There also was significantly increased transformation of the REd strain by donor DNA from other *H. pylori* strains, to an extent corresponding to their shared type II R-M system strain specificity with 26695. Comparison of the REd and wild-type strains indicates that restriction did not affect the length of DNA fragment integration during natural transformation. There also were no differentials in cell growth or susceptibility to DNA damage. In total, the data indicate that the type II REd mutant has enhanced competence with no loss of growth or repair facility compared to the wild type, facilitating *H. pylori* mutant construction and other genetic engineering.

*H*elicobacter pylori is a bacterium that persistently colonizes the human stomach and dominates gastric microbial populations (5, 12, 22). A striking characteristic of *H. pylori* is its remarkable genetic diversity (33, 50, 56), including strain-specific genes, mosaicism within some genes, and sequence diversity within conserved genes (36). *H. pylori* strains are naturally competent (7, 18, 29, 53), and analysis of the population structure provides evidence for extensive recombination, approaching pan-myxis (13, 51). Thus, intergenomic recombination has played an important role in the composition of present-day *H. pylori* strains (5, 7, 13, 51).

Comparison of the entire genomic sequences of two *H. pylori* strains 26695 (54) and J99 (1) showed that 6 to 7% of the genes are strain specific and that restriction-modification (R-M) genes are a major source of the differences (1). Recent analysis of the entire genomic sequences of *H. pylori* strains 98-10 (35), HPAG1 (39), B128 (21), G27 (6), and V225 (32), isolated from subjects from different geographic locales, reveals that a substantial proportion of the strain-specific genes are R-M genes (35). The type II R-M systems, consisting of at least two separate enzymes, a restriction endonuclease (RE) and one (type II) or two (type IIa) DNA methyltransferases (modification), are the major *H. pylori* R-M systems (3, 4, 31, 52, 59). Although many of the R-M system genes are shared, the enzymatically active R-M proteins in different *H. pylori* strains vary and are strain specific (27, 31, 52, 59). The REs create barriers against transformation (3, 4, 10) that limit genetic exchange *in vivo* and on the laboratory bench, which increases the difficulty to genetically manipulate *H. pylori*.

Strain 26695, the first *H. pylori* strain for which the whole genome sequence (WGS) was solved, has been extensively studied (43, 44, 54), and, in total, possesses 14 complete or partial type II R-M systems. However, only four are functional: HpyAII (encoded by HP1366), HpyAV (HP0053), HpyAIII (HP0091), and HpyAIV (HP1351) (31, 38). In the present study, we sequentially deleted each of the four active REs from 26695 using *sacB*-mediated counterselection (9), constructing a markerless strain deficient in type II RE function. We then characterized the effects of these mutations on *H. pylori* transformation and on growth, DNA repair, and spontaneous mutations *in vitro*. The constructed restriction endonuclease-deficient (REd) strain has a significantly enhanced natural transformation frequency while retaining similar growth, DNA repair, and spontaneous mutation phenotypes compared to wild-type 26695, indicating its utility for *H. pylori* mutant construction and other genetic engineering.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The *H. pylori* and *Escherichia coli* strains and plasmids used here are listed in Table S1 in the supplemental material. The *H. pylori* strains were grown at 37°C in 5% CO_2 on Trypticase soy agar (TSA) plates with 5% sheep blood (BBL Microbiology Systems, Cockeysville, MD) or brucella agar (BA) plates (Difco Laboratories, Detroit, MI) supplemented with 10% newborn calf serum (NBCS; Serologicals Corp., Norcross, GA) (30). Antibiotic-resistant *H. pylori* mutant strains were selected with kanamycin (Km; 10 μg/ml), chloramphenicol (Cm; 10 μg/ml), streptomycin (Str; 25 μg/ml), or rifampin (Rif; 7.5 μg/ml). For counterselection, we modified the Copass method (9), adding 6.5% (wt/vol) sucrose (Fisher Scientific, Gaithersburg, MD) to the BA plates to make sucrose-selective medium (SSM) plates. *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C (42). Ampicillin (Ap) (100 μg/ml), Km (50 μg/ml), and Cm (30 μg/ml) were used for selecting vector or constructed plasmids during cloning in *E. coli*.

Construction of *H. pylori* markerless restriction endonuclease-deficient mutants. The restriction endonuclease genes *hpyAII* (HP1366), *hpyAV* (HP0053), *hpyAIII* (HP0091), and *hpyAIV* (HP1351) of the active type II R-M systems were sequentially deleted from *H. pylori* 26695 using a modified *sacB*-mediated counterselection (9). The overall strategy was as follows: a *sacB-cat* cassette was first constructed in strain 26695 by
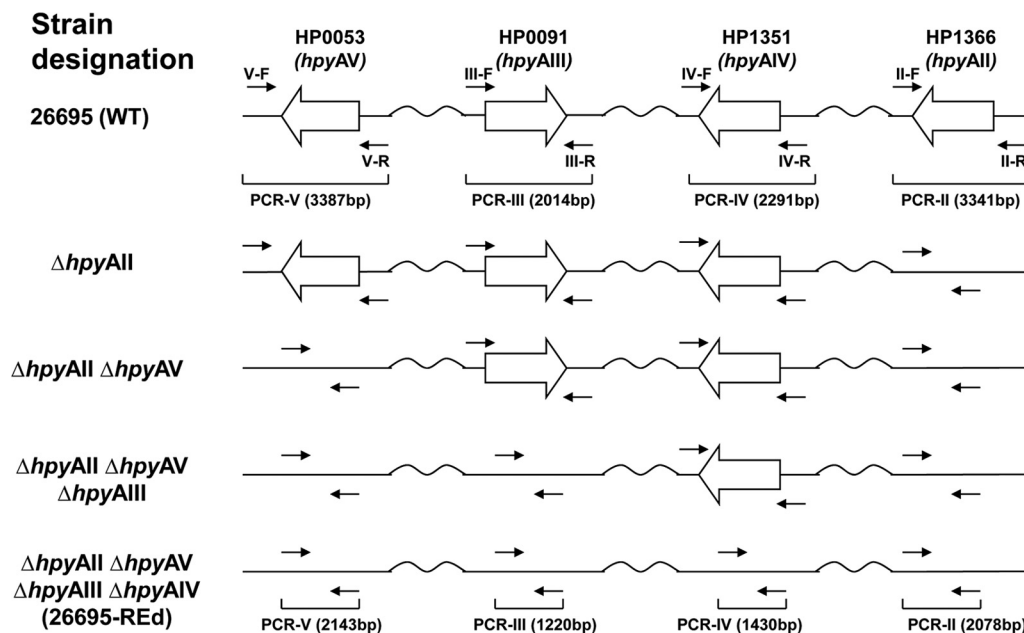
FIG 1 Construction of *H. pylori* 26695 mutants deficient in active type II restriction endonucleases. *H. pylori* wild-type 26695 strain has four active type II restriction endonuclease genes: *hpyAII* (HP1366), *hpyAV* (HP0053), *hpyAIII* (HP0091), and *hpyAIV* (HP1351) (31). With *sacB*-mediated counterselection, each of the genes encoding an active restriction endonuclease was sequentially deleted from the genome to finally construct a markerless KO-4 mutant (26695 Δ*hpyAII* Δ*hpyAV* Δ*hpyAIII* Δ*hpyAIV* [26695-REd]).

substituting *cat* for *kan* in the described *kan-sacB* cassette (9), based on our observations in 26695 that mutation construction with a *cat* cassette as a selective marker is more (>1 log$_{10}$-fold) efficient than using the *kan* cassette (data not shown). The first target gene (*hpyAII*) was then replaced by the *sacB-cat* cassette by homologous recombination (see Fig. S1 in the supplemental material). After confirming the correct replacement by PCR, using primers IIL-F-SacII and SC-R-XbaI (see Table S2 in the supplemental material), the *sacB-cat* cassette sequence then was deleted from the locus on SSM, by homologous recombination with a direct fusion of *hpyAII* upstream and downstream regions, leading to a markerless deletion mutant (KO-1; Δ*hpyAII*) (see Fig. S1 in the supplemental material). Each of the other target genes was deleted in the same way, leading to a series of multiple markerless mutations (KO-2 [Δ*hpyAII* Δ*hpyAV*], KO-3 [Δ*hpyAII* Δ*hpyAV* Δ*hpyAIII*], and KO-4 [Δ*hpyAII* Δ*hpyAV* Δ*hpyAIII* Δ*hpyAIV*]; REd mutant) in the 26695 background (Fig. 1).

These steps were accomplished by first obtaining a 1.7-kb *sacB* cassette by PCR using pKSF (9) as a template and the primers SC-F-XbaI and S-R-PstI (see Table S2 in the supplemental material) that add XbaI and PstI restriction sites to the ends of the PCR fragment. Then, a 0.8-kb *cat* cassette was obtained by PCR using pAD1-Cat (25) as a template and primers SC-R-XbaI and C-R-PstI (see Table S2 in the supplemental material) that add XbaI and PstI restriction sites to the ends of the PCR fragment. After digestion with PstI, the two fragments were ligated, and PCR performed with primers SC-F-XbaI and SC-R-XbaI to obtain a 2.5-kb *sacB-cat* cassette flanked by XbaI restriction sites. A 1.0-kb fragment downstream of *hpyAII* (IIdown) was obtained by PCR using genomic DNA from the wild-type *H. pylori* strain 26695 as a template and the primers IIL-F-SacII and IIL-R-SpeI (see Table S2 in the supplemental material) that add SacII and SpeI restriction sites to the ends of the fragment. Similarly, a 1.1-kb fragment upstream of *hpyAII* (IIup) was obtained by PCR using primers IIR-F-SpeI and IIR-R-PstI (see Table S2 in the supplemental material) that add the SpeI or PstI restriction sites to the ends of the fragment. The fragments IIdown and IIup were digested with SacII/SpeI and SpeI/PstI, respectively, and then ligated together into SacII/PstI-digested pGEM-T Easy (Promega, Madison, WI), creating pXZ017 (see Table S1 in the supplemental material). In the same way, a

1.1-kb fragment downstream of *hpyAV* (Vdown) was obtained by PCR with primers VL-F-SacII and VL-R-SpeI and a 1.0-kb fragment upstream of *hpyAV* (Vup) was obtained by PCR with the primers VR-F-SpeI and VR-R-PstI (see Table S2 in the supplemental material). The fragments Vdown and Vup were digested with SacII/SpeI and SpeI/PstI and then ligated together into SacII/PstI-digested pGEM-T Easy, creating pXZ016 (see Table S1 in the supplemental material). A 0.9-kb fragment upstream of *hpyAIII* (IIIup) was obtained by PCR with the primers IIIL-F-SacII and IIIL-R-SpeI, and a 1.1-kb fragment downstream of *hpyAIII* (IIIdown) was obtained by PCR with the primers IIIR-F-SpeI and IIIR-R-PstI (see Table S2 in the supplemental material). The fragments IIIup and IIIdown were digested with SacII/SpeI and SpeI/PstI and then ligated together into SacII/PstI-digested pGEM-T Easy, creating pXZ144 (see Table S1 in the supplemental material). A 0.7-kb fragment downstream of *hpyAIV* (IVdown) was obtained by PCR with the primers IVL-F-SacII and IVL-R-SpeI, and a 1.0-kb fragment upstream of *hpyAIV* (IVup) was obtained by PCR with the primers IVR-F-SpeI and IVR-R-PstI (see Table S2 in the supplemental material). The fragments IVdown and IVup were digested with SacII/SpeI and SpeI/PstI and then ligated together into SacII/PstI-digested pGEM-T Easy, creating pXZ145 (see Table S1 in the supplemental material). The 2.5-kb *sacB-cat* cassette was digested with XbaI and then ligated with SpeI-digested pXZ017, pXZ016, pXZ144, or pXZ145, creating pXZ033, pXZ032, pXZ146, and pXZ147, respectively (see Table S1 in the supplemental material).

The wild-type *H. pylori* strain 26695 was first transformed to Cm^r with pXZ033 using Cm-containing plates and then transformed to Cm^s with pXZ017 using SSM plates to create a markerless single (KO-1) mutant HPXZ285 (26695 Δ*hpyAII*) (see Table S1 in the supplemental material). In the same way, HPXZ285 was transformed to Cm^r with pXZ032 and then transformed to Cm^s with pXZ016 to create a markerless double (KO-2) mutant HPXZ490 (26695 Δ*hpyAII* Δ*hpyAV*) (see Table S1 in the supplemental material). HPXZ490 was then transformed to Cm^r with pXZ146 and then transformed to Cm^s with pXZ144 to create a markerless triple (KO-3) mutant HPXZ560 (26695 Δ*hpyAII* Δ*hpyAV* Δ*hpyIII*) (see Table S1 in the supplemental material). Finally, HPXZ560 was transformed to Cm^r with pXZ147 and then transformed to Cm^s with pXZ145

to create a markerless quadruple (KO-4) mutant HPXZ566 (26695 Δ*hpyAII* Δ*hpyAV* Δ*hpyIII* Δ*hpyIV*; called the REd [restriction-endonuclease deficient] mutant) (see Table S1 in the supplemental material).

Construction of mutants with Km^r, Cm^r, or Str^r genetic markers. An *aphA* (Km) cassette was introduced into the genomic *vacA* locus of *H. pylori* strains 26695, JP26, J99, or J166 by transformation with pCTB8:Km to create Km^r strains (see Table S1 in the supplemental material). PCR, using the primers VacA-F and VacA-R (see Table S2 in the supplemental material), was used to confirm the appropriate constructs in Km^r transformants. A *cat* cassette was introduced into the genomic *ureA* locus of 26695, HPXZ566, JP26, J99, or J166 by transformation with pAD1-Cat to create Cm^r strains (see Table S1 in the supplemental material), and results confirmed by PCR using Ure-F and Ure-R (see Table S2 in the supplemental material). An *rpsL*(A128G) point mutation was introduced into the genome of 26695, HPXZ566, JP26, J99, or J166, by transformation with p801R to create Str^r strains and similarly introduced into the genome of the Cm^r strains to create Cm^r/Str^r double-marked strains (see Table S1 in the supplemental material).

Natural transformation. The assay to evaluate natural transformation was performed as described previously (29), with modifications. In brief, recipient *H. pylori* cells were harvested from 24-h cultures on TSA plate into 1.0 ml of phosphate-buffered saline (PBS; pH 7.4) and centrifuged at $800 \times g$ for 5 min. The cell pellet was resuspended in PBS to a concentration of ~10^9 cell/ml. Each transformation mixture with recipient cells (100 μl) and donor DNA (1,000 ng for genomic DNA or plasmids with the *aphA* [pCTB8:Km] or *cat* [pAD1-Cat] cassette and 100 ng for the plasmid with the point mutation [p801R] in 10 μl of distilled water) was spotted onto a TSA plate. To detect transformation, we used much higher concentrations of donor DNA since the integration of long cassettes was required to encode selectable phenotypes compared to when point mutations were used for transformation, as described previously (29). Each plate was incubated for 24 h, as we (30) and others (34, 49, 57) have reported, at 37°C in 5% CO$_2$. The transformation mixture then was harvested and placed in 1.0 ml of PBS, and 100-μl 10-fold serial dilutions were plated onto either TSA plates or BA plates containing 10% NBCS and suitable antibiotics. The plates were incubated for 4 days at 37°C in 5% CO$_2$, and the total transformation frequency was determined by the number of colonies on selective plates divided by the total CFU on nonselective media. In each transformation experiment, *H. pylori* strains with no DNA added also were tested in parallel as negative controls; no colonies were seen in any case. Each experiment was repeated three to five times with independent cultures.

Horizontal DNA transfer during coculture. An assay was performed to evaluate the ability of *H. pylori* strains to obtain exogenous DNA during coculture with another strain. After 24 h of growth on TSA plates, cell cultures of a singly marked (Km^r) strain (as donor) and a doubly marked (Str^r/Cm^r) strain (as recipient) were collected, washed, and resuspended in BB medium. Aliquots of 50 μl containing ~2.5×10^8 cells of donor and recipient strains were mixed, spotted onto a TSA plate, and incubated for 24 h at 37°C in 5% CO$_2$. The cocultured cells then were collected in 1 ml of PBS and, after a series of suitable dilutions, the cells were plated and incubated onto BA plates containing 10% NBCS and Str/Km/Cm or onto BA/Str/Cm plates for 4 days. Colonies were counted and frequencies of doubly marked Str^r/Cm^r strains that obtained DNA from a singly marked (Km^r) strain were calculated as the CFU (BA/Str^r/Km^r/Cm^r)/CFU (BA/Str^r/Cm^r). As controls, a Km^r Rif^r strain (HPXZ660) and a Str^r Cm^r strain (HPXZ698 or HPXZ641) (see Table S1 in the supplemental material) were cocultured in the same way. No colonies grew on BA/Km^r/Rif^r/Str^r/Cm^r plates in any assay, indicating that no strain was able to obtain both selective markers from a donor strain during coculture. These results suggested that Str^r Km^r Cm^r colonies obtained during coculture of the Km^r and Str^r Cm^r strains were Str^r Cm^r strains (as recipients) that received donor DNA from the Km^r strain. As additional controls, the Km^r, or the Str^r Cm^r strains alone also were incubated and selected in the same way. No colonies grew on Str^r Km^r Cm^r plates, which confirmed that under the

test conditions, strains obtain the additional antibiotic marker due to DNA invasion during coculture rather than by spontaneous mutation. Coculture of a singly marked (Cm^r) strain (as donor) and a doubly marked (Str^r Km^r) strain (as recipient) was performed in a similar way, and the frequencies of doubly marked Str^r Km^r strains that obtained DNA from a singly marked (Cm^r) strain were calculated as the CFU (BA/Str^r/Km^r/Cm^r)/CFU (BA/Str^r/Km^r). All calculations were based on three independent experiments.

Mapping the transforming fragment. Mapping of the insertion sites was performed as described previously (30). For each of the recipient strains (26695 and 26695-REd), nine sets of transformants, each independently incubated with donor DNA (from strain HPXZ627), were examined. One Cm^r transformant from each independent transformation was picked, subcultured on a selective plate (containing Cm), and harvested (30). Chromosomal DNA was prepared using the Wizard genomic DNA purification kit (Promega). To analyze the region of DNA flanking the *cat* cassette, PCR was performed using isolated chromosomal DNA from each of the 18 (9 + 9) independent transformants, using primers designed to match the sequences in both 26695 and J99 (see Table S2 in the supplemental material). Sequencing was performed at Macrogen (Rockville, MD), and all sequence analysis was performed using Sequencher (Gene Codes, Ann Arbor, MI).
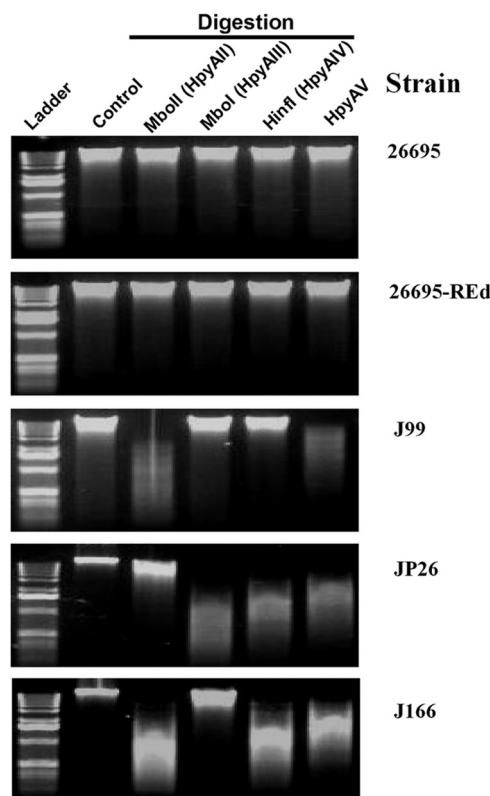
Susceptibility to DNA damage. To evaluate *H. pylori* strain recovery from DNA damage induced by UV irradiation, *H. pylori* cells from 48-h cultures on TSA plates were collected into 1.0 ml of brucella broth, and with suitable dilutions, equal suspensions were inoculated onto TSA plates. When dried, plates were exposed to 254-nm UV generated by USHIO G30T UV lamp (Fisher Scientific) at a distance of 78 cm for 0 to 60 s (resulting in doses of ~2.0 J/m^2/s) and then incubated at 37°C in 5% CO$_2$ for 4 days before the colonies were counted and the survival rates were determined. Each experiment was repeated three times with independent cultures.

Susceptibility to fluoroquinolones was evaluated using levofloxacin Etest strips (bioMérieux, Hazelwood, MO), according to the manufacturer's instructions. Briefly, cells from each *H. pylori* culture on a TSA plate after 48 h of growth were evenly spread onto a new TSA plate to cover the surface of the plate, and Etest strips were applied. Plates were incubated for 48 h at 37°C in 5% CO$_2$, and the MICs were determined, in at least three independent experiments.

Spontaneous mutation. The spontaneous mutation rate of *H. pylori* strains was evaluated as the rate of rifampin resistance, as reported (25), since a point mutation in *rpoB* converts rifampin-sensitive (Rif^s) *H. pylori* to rifampin-resistant (Rif^r) (17). Briefly, single colonies of Rif^s *H. pylori* strains on TSA plates after 48 h of growth were picked, streaked onto new TSA plates, and incubated for 48 h, allowing mutations to occur. The cells were harvested into PBS and serially diluted onto either BA plates containing 10% NBCS and rifampin or TSA alone. The plates were incubated at 37°C in 5% CO$_2$ for 96 h, the colonies were counted, and the spontaneous mutation frequencies were calculated. Each experiment was performed with nine independent single colonies for each strain.

RESULTS

Construction of restriction endonuclease-deficient *H. pylori* strains. Most wild-type *H. pylori* strains are naturally competent for uptake of exogenous DNA (20, 55) and simultaneously have strain-specific active type II R-M systems that limit the effects of natural competence (3, 10, 31, 59). To characterize the roles of R-M systems in *H. pylori* natural transformation, we sequentially deleted the RE genes within the four sets of 26695 active type II R-M systems (HpyAII, HpyAV, HpyAIII, and HpyAIV) using *sacB*-mediated counterselection, generating markerless mutants: Δ*hpyAII* (KO-1), Δ*hpyAII* Δ*hpyAV* (KO-2), Δ*hpyAII* Δ*hpyAV* Δ*hpyIII* (KO-3), and Δ*hpyAII* Δ*hpyAV* Δ*hpyAIII* Δ*hpyAIV* (KO-4; REd) (Fig. 1). Restriction analysis of genomic DNA

FIG 2 Restriction analysis of genomic DNA of *H. pylori* strains. Genomic DNA of *H. pylori* strains 26695, 26695-REd, J99, JP26, and J166 (from top panel to bottom panel) was digested with each of the four active 26695 strain-specific type II restriction endonucleases or their isoenzymes—HpyAII, HpyAIII, HpyAIV, and HpyAV (left to right)—or was not digested (control). The genomic DNA of 26695 and 26695-REd was not digested by any of the four restriction enzymes, indicating that all of the HpyAII, HpyAIII, HpyAIV, and HpyAV restriction sites were protected by methylation. Restriction endonucleases, HpyAII, and HpyAV digested the genomic DNA of strain J99, whereas the HpyAIII and HpyAIV restriction sites in J99 were protected by methylation. REs HpyAIII, HpyAIV, and HpyAV digested the genomic DNA of J99, while the HpyAII restriction sites were protected by methylation; HpyAII, HpyAIV, and HpyAV digested J166 genomic DNA, while the HpyAIII restriction sites were protected by methylation.

showed that the REd mutant, as with the 26695 parental strain, is resistant to digestion by each of the four REs (Fig. 2), indicating that the methylases belonging to each of the four affected RMs remained active. Moreover, all of the four RE-deficient mutants had similar growth rates to the wild type (see Fig. S2 in the supplemental material), similar abilities to repair UV-induced DNA damage (see Fig. S3 in the supplemental material) and fluoroquinolone (levofloxacin)-induced DNA damage (see Table S3 in the supplemental material), and similar spontaneous mutation rates (see Fig. S4 in the supplemental material). Thus, the mutant strains resembled the parental in important phenotypes related to growth, specific methylation, and DNA repair.

Relationship between size of the transforming DNA allele and transformation of RE-deficient strains. To evaluate the roles of REs in *H. pylori* natural transformation, we first incubated *H. pylori* wild-type 26695 and 26695-REd (KO-4) with p801R prepared in *E. coli*. Transformation by p801R, which carries an 801-bp *H. pylori* 26695 *rpsL* fragment with a single central point mutation (A128G), converts Str^s *H. pylori* to Str^r (29). A total of six type II RE recognition sites are located within the 801-bp *H. pylori* sequence that may be subject to cleavage by wild-type 26695 but not by 26695-REd. Under standard test conditions (29), the transformation frequencies (TFs) of the wild-type and 26695-REd strains with p801R were nearly identical (with values of 2.7 $\pm$ 0.9 $\times$ 10^{-3} versus 2.0 $\pm$ 0.7 $\times$ 10^{-3}) (Fig. 3A). These results indicate that, as anticipated by studies involving deletion of a single RE (4), the REs of recipient strains do not affect the transformation of this point mutation.

Next, we transformed *H. pylori* wild-type and RE-deficient mutant strains with pAD1-Cat, produced in *E. coli*. Transformation by pAD1-Cat, which carries a *ureAB* fragment from *H. pylori* strain 60190 with a central exogenous *cat* cassette (1,127 bp), converts Cm^s *H. pylori* to Cm^r (30). In pAD1-Cat, the *cat* cassette is flanked by 570 bp downstream and 339 bp upstream of the *ureAB* locus sequence. A total of six type II RE recognition sites are located within the 1127-bp *cat* cassette (two each for HpyAII and HpyAIV, and one each for HpyAIII and HpyAV) (Table 1), and 13 type II RE sites located outside the *cat* cassette but within *H. pylori* flanking sequence (six for HpyAII, three for HpyAIII, two each for HpyAIV and HpyAV). Under the standard test conditions, the TF of the wild-type strain was (3.0 $\pm$ 0.7) $\times$ 10^{-6}. In contrast, the TF
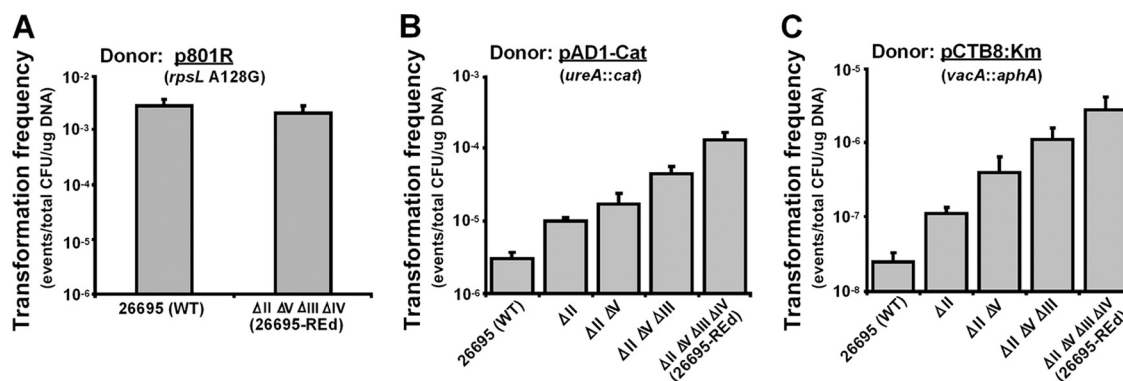
FIG 3 Transformation of *H. pylori* type II restriction endonuclease-deficient mutants with plasmid DNA. *H. pylori* 26695 and derivative strains were transformed with plasmids with selectable phenotypes cloned into pGEM-T Easy. (A) p801R (an 801-bp *H. pylori* *rpsL* fragment from a Str^r strain [*rpsL* A126G]). (B) pAD1-Cat (*H. pylori* DNA at *ureAB* locus flanking a 1.2-kb *cat* cassette conferring Cm^r). (C) pCTB8:Km (*H. pylori* DNA at *vacA* locus flanking a 1.3-kb *aphA* cassette conferring Km^r). Transformation frequency was expressed as transformation events/total CFU/μg of donor DNA.

TABLE 1 Restriction sites in selectable markers recognized by the four active type II restriction endonucleases in *H. pylori* strain 26695

Restriction endonuclease	Recognition sequence	No. of restriction sites	
		cat cassette (1,189 bp)	*aphA* cassette (1,288 bp)
HpyAII	GAAGA	2	4
HpyAV	CCTTC	2	3
HpyAIII	GATC	1	7
HpyAIV	GANTC	1	7
Total		6	21

of the $\Delta hpyAII$ single (KO-1) mutant was $(1.0 \pm 0.1) \times 10^{-5}$ ($\sim$0.5 $\log_{10}$-fold significantly increased compared to the wild type [WT], $P < 0.05$), the TF of the $\Delta hpyAII$ $\Delta hpyAV$ double (KO-2) mutant was $(1.7 \pm 0.7) \times 10^{-5}$ ($\sim$0.8 $\log_{10}$-fold significantly increased compared to the WT, $P < 0.05$), the TF of the $\Delta hpyAII$ $\Delta hpyAV$ $\Delta hpyAIII$ triple (KO-3) mutant was $(4.4 \pm 1.1) \times 10^{-5}$ ($\sim$1.2 $\log_{10}$-fold significantly increased compared to the WT, $P < 0.05$), and the TF of the 26695-REd mutant (KO-4) was $(1.3 \pm 0.3) \times 10^{-4}$ ($\sim$1.7 $\log_{10}$-fold significantly increased compared to the WT, $P < 0.05$) (Fig. 3B). These results indicated that REs of the recipient strains inhibit transformation by this exogenous 1127-bp *cat* cassette (synthesized in *E. coli*), which is consistent with functional restriction barriers in the wild-type strain (4, 48).

To confirm and extend this observation, we also transformed *H. pylori* wild-type and RE-deficient strains with pCTB8:Km (synthesized in *E. coli*), which carries an *H. pylori* 60190 *vacA* fragment with a central exogenous *aphA* cassette (1,288 bp) that converts Km^s *H. pylori* to Km^r (30). In pCTB8:Km, the *aphA* cassette is flanked by 672 bp downstream and 963 bp upstream of the *vacA* locus. A total of 21 type II RE sites are located within the 1,288-bp *aphA* cassette (four for HpyAII, seven each for HpyAIII and HpyAIV, and three for HpyAV) (Table 1), and 15 sites located outside the *aphA* cassette but within the *H. pylori* flanking sequence (four each for HpyAII and HpyAV, six for HpyAIII, and one for HpyAIV). Under the standard test conditions, the TF of the wild-type strain by pCTB8:Km was $(2.5 \pm 0.8) \times 10^{-8}$. The TF by pCTB8:Km of the KO-1 mutant was $(1.1 \pm 0.2) \times 10^{-7}$ ($\sim$0.6 $\log_{10}$-fold significantly increased compared to the WT, $P < 0.05$), the TF of the KO-2 mutant was $(3.9 \pm 2.5) \times 10^{-7}$ ($\sim$1.2 $\log_{10}$-fold significantly increased compared to the WT, $P < 0.05$), the TF of the KO-3 mutant was $(1.1 \pm 0.4) \times 10^{-6}$ ($\sim$1.7 $\log_{10}$-fold significantly increased compared to the WT, $P < 0.05$), and the TF of the 26695-REd mutant (KO-4) was $(2.7 \pm 1.3) \times 10^{-6}$ ($\sim$2.0 $\log_{10}$-fold significantly increased compared to the WT, $P < 0.05$) (Fig. 3C). Consistent with the results for transformation by the *cat* cassette at the *ureAB* locus, these results indicate that the active *H. pylori* REs inhibit transformation of the exogenous (synthesized in *E. coli*) 1.3-kb *aphA* cassette within the unrelated *vacA* locus.

Since *H. pylori* strain 26695 is not a good recipient for transformation by *E. coli*-*H. pylori* shuttle plasmids (3, 47), we also determined whether the 26695-REd strain can be transformed by shuttle plasmids through either natural transformation or electroporation. We found that no transformation of shuttle plasmid pHel3 (see Table S1 in the supplemental material) into 26695 or 26695-REd strains could be detected (data not shown), suggesting that factors other than type II REs affect shuttle plasmid transformation of *H. pylori*.

Strain-specific REs limit transformation between differing *H. pylori* source DNAs. To evaluate the roles of the strain-specific REs in horizontal gene transfer between different *H. pylori* strains in addition to transfer from *E. coli* to *H. pylori*, we transformed wild-type *H. pylori* strain 26695 and the four RE-deficient mutants with genomic DNA from *H. pylori* strains J99, JP26, and J166.

Restriction analysis indicates that J99 genomic DNA is digested by HpyAII and HpyAV but protected from digestion by HpyAIII and HpyAIV (Fig. 2), a result consistent with prior observations that J99 possesses the HpyAIII and HpyAIV methylases (3, 27, 31, 59). Genomic DNA from J99 with the *cat* cassette at the *ureAB* locus was used to transform 26695 and the RE-deficient mutants (Fig. 4B). Compared to the wild-type 26695, the KO-1 mutant ($\Delta hpyAII$) had a significantly increased TF ($\sim$0.7 $\log_{10}$-fold [$P < 0.05$]). The KO-2 mutant ($\Delta hpyAII$ $\Delta hpyAV$) had a further significantly increased TF ($\sim$0.4 $\log_{10}$-fold [$P < 0.05$] compared to the $\Delta hpyAII$ single mutant and $\sim$1.2 $\log_{10}$-fold [$P < 0.05$] compared to the WT). These data indicated that the active HpyAII and HpyAV REs form barriers that inhibit J99 genomic DNA transformation of the wild-type strain 26695. The KO-3 mutant and the REd mutant had similar TF values to the KO-2 mutant (Fig. 4B). Similar results were observed when J99 genomic DNA with the *aphA* cassette at the *vacA* locus was used to transform the wild-type and the RE-deficient 26695 mutants (Fig. 5A). Deletion of 26695 HpyAII and HpyAV significantly increased J99 genomic DNA transformation, but there were no further increase with deletion of HpyAIII and HpyAIV. These results indicate that HpyAIII and HpyAIV do not form a barrier inhibiting J99 genomic DNA, which is consistent with the known methylation of HpyAIII and HpyAIV chromosomal sites in strain J99 (31).

The RE analysis showed that JP26 genomic DNA was digested by HpyAIII, HpyAIV, and HpyAV but protected from HpyAII (Fig. 2), indicating that JP26 has methylases that modify HpyAII recognition sites, but not HpyAIII, HpyAIV, or HpyAV sites (3). Genomic DNA from JP26 with the *cat* cassette at the *ureAB* locus also was used to transform the wild-type and RE-deficient 26695 mutants (Fig. 4C). Compared to the WT strain, deletion of *hpyAII* did not significantly increase TF, while double deletion of *hpyAII* and *hpyAV* significantly increased TF $\sim$0.9 $\log_{10}$-fold (P < 0.05). These data indicate that active HpyAV forms a barrier to inhibit JP26 transformation of 26695. However, for JP26 DNA with the *cat* cassette, the KO-3 and the REd mutants slightly but consistently increased TF (Fig. 4C). Since the *aphA* cassette contains more sites recognized by the four REs than does the *cat* cassette, we also transformed the 26695 strains with genomic DNA from JP26 with the *aphA* cassette at the *vacA* locus (Fig. 5B). The results showed that *hpyAII* deletion did not increase JP26 transformation of 26695, whereas deletion of *hpyAV*, *hpyAIII*, and *hpyAIV* did. Compared to the WT, the KO-2, the KO-3, and the REd mutants increased TF $\sim$0.6, 1.1, and 1.5 $\log_{10}$-fold (P < 0.05), respectively, indicating that HpyAV, HpyAIII, and HpyAIV form barriers to inhibit JP26 transformation of 26695.

J166 genomic DNA was digested by HpyAII, HpyAIV, and HpyAV but protected from HpyAIII (Fig. 2), which indicates that J166 has methylases that modify HpyAIII, but not HpyAII, HpyAIV, or HpyAV recognition sites (3). The wild-type and RE-deficient mutants were transformed by genomic DNA from J166 with the *cat* cassette at the *ureAB* locus (Fig. 4D) or with the *aphA* cassette at the *vacA* locus (Fig. 5C). Consistent with the digestion analysis, the KO-1, KO-2, and the REd mutants showed signifi-
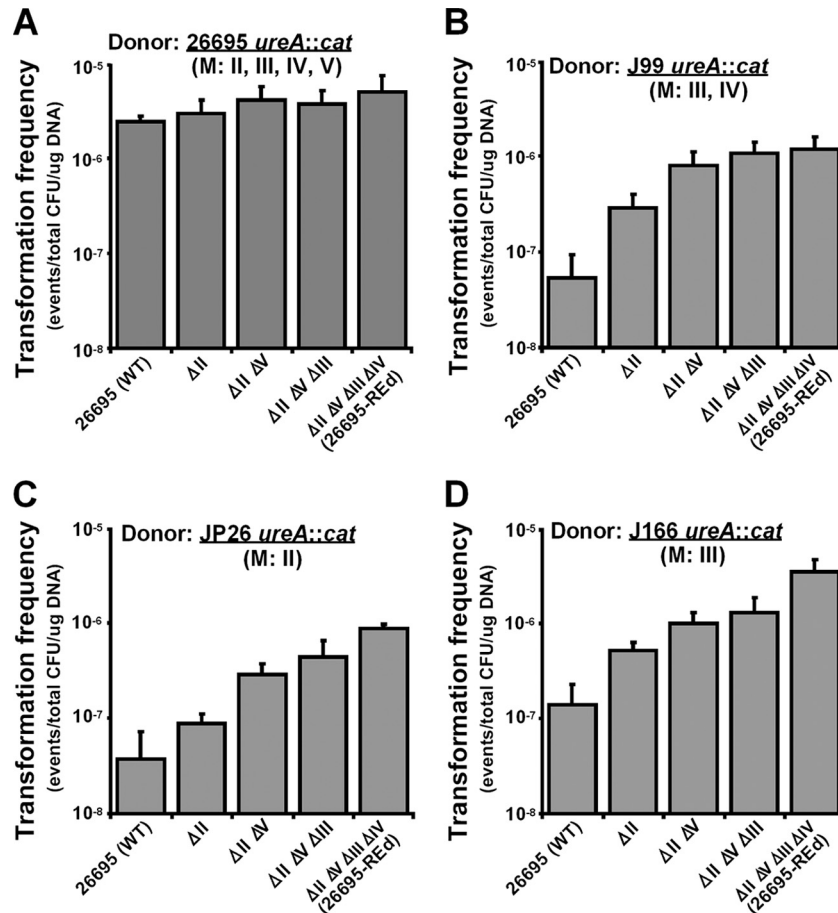
FIG 4 Transformation of *H. pylori* type II restriction endonuclease-deficient mutants with genomic DNA from Cm^r *H. pylori* strains with different methylation activities. *H. pylori* 26695 and derivative strains were transformed with genomic DNA from *H. pylori* strains that had been transformed with pAD1-Cat, yielding chromosomally encoded Cm^r (*ureA::cat*) strains. (A) Strain 26695 specifically methylates to protect at all four restriction sites (HpyAII, HpyAIII, HpyAIV and HpyAV). (B) Strain J99 specifically methylates HpyAIII and HpyAIV restriction sites. (C) Strain JP26 specifically methylates HpyAII restriction sites. (D) Strain J166 specifically methylates at HpyAIII restriction sites.

cantly increased TF, whereas deletion of *hpyAIII* did not significantly affect the TF by either J166 donor. Compared to the WT, the REd mutant had increased TF ~1.3 and ~1.4 $\log_{10}$-fold ($P < 0.05$) for the two J166 genomic donors, respectively.

In contrast, when the wild-type and RE-deficient 26695 mutant strains were transformed with 26695 DNA with the *cat* cassette at the *ureAB* locus, as expected, the TFs of each strain were similar (Fig. 4A). This served as a control for the above experi-
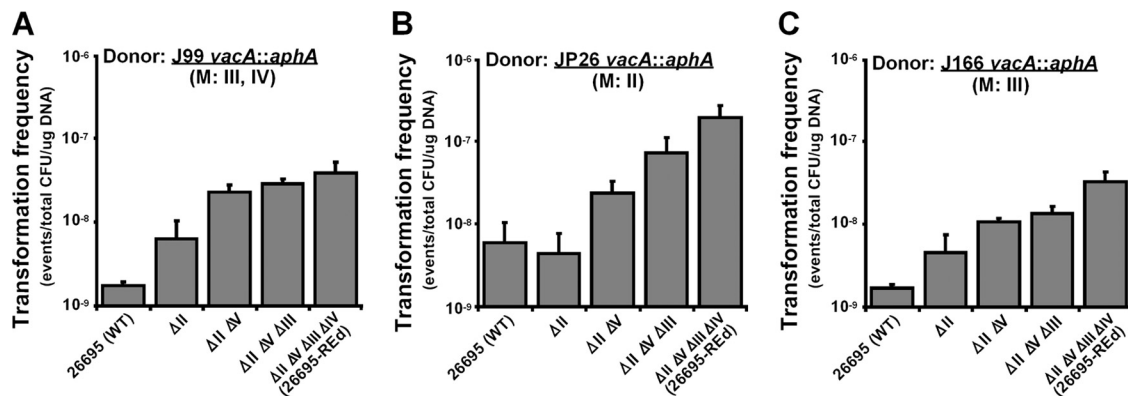

FIG 5 Transformation of *H. pylori* type II restriction endonuclease-deficient mutants with genomic DNA from Km^r *H. pylori* strains with different methylation activities. *H. pylori* 26695 and derivative strains were transformed with genomic DNA from *H. pylori* strains that had been transformed with pCTB8:Km yielding chromosomally encoded Km^r (*vacA::aphA*) strains. (A) Strain J99 methylates HpyAIII and HpyAIV restriction sites. (B) Strain JP26 methylates HpyAII restriction sites. (C) Strain J166 methylates HpyAIII restriction sites.
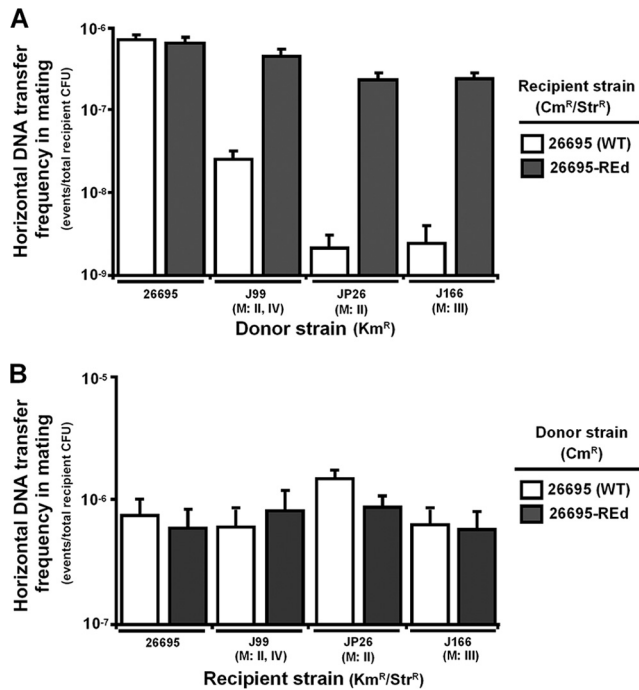
FIG 6 Frequency of horizontal DNA transfer in cell-to-cell mating. (A) Horizontal DNA transfer into *H. pylori* wild-type strain 26695 and 26695-REd in mating with *H. pylori* strains with different methylation activities. *H. pylori* wild-type strain 26695 or 26695-REd with double genetic markers (Cm^r Str^r) as recipients were mated with *H. pylori* strains 26695, J99, JP26, or J166, each containing a single genetic marker (Km^r) in the *vacA* locus, as donors. The numbers of transformants positive for all three phenotypes (Km^r, Cm^r, and Str^r) were determined, and the frequency of horizontal DNA transfer to the *H. pylori* wild-type and the REd strain was determined. (B) Horizontal DNA transfer from *H. pylori* wild-type strain 26695 and 26695-REd in mating with *H. pylori* strains. *H. pylori* wild-type strain 26695 or 26695-REd with a single genetic marker (Cm^r) in the *ureAB* locus as donors were mated with *H. pylori* strains 26695, J99, JP26, or J166, each containing double genetic markers (Km^r Str^r) as recipients. The numbers of transformants positive for all three phenotypes (Km^r, Cm^r, and Str^r) were determined, and the frequency of horizontal DNA transfer from the *H. pylori* wild type and the REd mutant to each recipient strain was determined.

ments. These results confirm that the RE mutations enhance transformation by reducing donor DNA digestion.

Effect of strain-specific REs on mating. To further evaluate the effects of the REs on the restriction barriers, we determined the frequency of horizontal DNA transfer into *H. pylori* wild-type strain 26695 or 26695-REd (KO-4) in mating with *H. pylori* strains of different methylation patterns (Fig. 6A). The wild-type and 26695-REd strains had similar frequencies of uptake of genetic markers in mating with a Km^r 26695 strain that methylates all four type II RE recognition sites. The TF of 26695-REd was ~1.2 log$_{10}$-fold ($P < 0.05$) significantly higher than the wild type in mating with a Km^r J99 strain, which methylates the recognition sites of only two of the four REs in 26695, and ~2.0 log$_{10}$-fold ($P < 0.05$) significantly higher than the wild type in mating with a Km^r JP26 strain or J166 strain, both of which methylate the recognition sites of only one of the four REs. These results indicate that REd mutation also enhances the 26695 transformation efficiency in cell-to-cell mating. As control experiments, the frequencies of horizontal DNA transfer from *H. pylori* wild-type strain 26695 and 26695-REd to the same *H. pylori* strains (26695, J99, JP26, or J166)

were evaluated. The lack of significant differences (Fig. 6B), indicates that, as expected, REd mutation does not affect the ability to serve as a DNA donor in cell-to-cell mating.

Strain-specific restriction endonucleases do not affect the import size of exogenous DNA during transformation. To evaluate whether RE mutations affect the pattern of DNA integration into the recipient genome, we performed independent transformations of wild-type 26695 or 26695-REd using genomic DNA from Cm^r strain J99. The length of DNA insert flanking the *cat* cassette at the *ureAB* locus was determined (Fig. 7). For nine independent transformants, a mean length of (3,221 ± 1,546)-bp donor (J99) genomic DNA was integrated into the wild-type 26695 chromosome, and similarly, a mean length of (3,202 ± 1,640)-bp donor (J99) genomic DNA into the 26695-REd chromosome (Table 2). These results indicate that restriction does not affect the DNA import size in *H. pylori* recipients, a finding consistent with previous observations of the transformation of wild-type 26695 by donor J99 genomic DNA with the same cassette, but at a different (*recG*) recipient locus [integration of (3,393 ± 1,013)-bp donor DNA] (30).

Since restriction analysis indicates that J99 genomic DNA is digested by HpyAII and HpyAV but protected from digestion by HpyAIII and HpyAIV (Fig. 2), we then analyzed the HpyAII and HpyAV restriction of the donor DNA at this locus (the *cat* cassette ± the 4-kb flanking region) (Fig. 7). In total, 16 restriction sites, with a high-density region (7 HpyAII sites within 156 bp), are present upstream of the *cat* cassette; somewhat more (6 versus 3) 26695-REd transformants have upstream insertions beyond this high-density region than do the wild-type transformants (Fig. 7). Although for the transformants of both the wild-type and 26695-REd strains, the endpoints in upstream flanking DNA appear random, endpoints of the downstream flanking DNA are not. There are only five patterns of insertion for the nine 26695-REd independent transformants and seven patterns of insertion of the nine 26695-independent transformants (Fig. 7). Similar endpoint clusters of import DNA also were observed in transformations involving the *rpsL* (30) and *rpoB* loci (28), raising the possibility that the uneven distribution of strain-specific restriction sites affects donor DNA import into particular loci.

DISCUSSION

In this study, we examined the effects of type II restriction on *H. pylori* transformation by sequentially deleting the genes of the four active type II REs from *H. pylori* strain 26695 and then introducing exogenous DNA in different forms to the strains. Our observation that the RE-ablating mutations increased transformation frequencies by donor DNA originating from *H. pylori* strains corresponding to the R-M strain specificities of the strains confirmed that the restriction barriers function to suppress *H. pylori* natural competence and to help maintain strain-specific genetic integrity. Our findings also support the previously proposed concept that *H. pylori* interstrain R-M diversity prevents genome subversion by chromosomal DNA from competing strains (4). For DNA of non-*H. pylori* origin (e.g., *E. coli*), the barriers to transformation are substantially higher.

The transformation frequency of the wild-type strain by the 1.3-kb *aphA* cassette was ~2.0 log$_{10}$-fold lower than the 1.1-kb *cat* cassette (Fig. 3C and B), a finding consistent with the previous observation of transformation of 26695 strains by donor DNA with *cat* or *aphA* cassette at different loci (16). This marked differ-
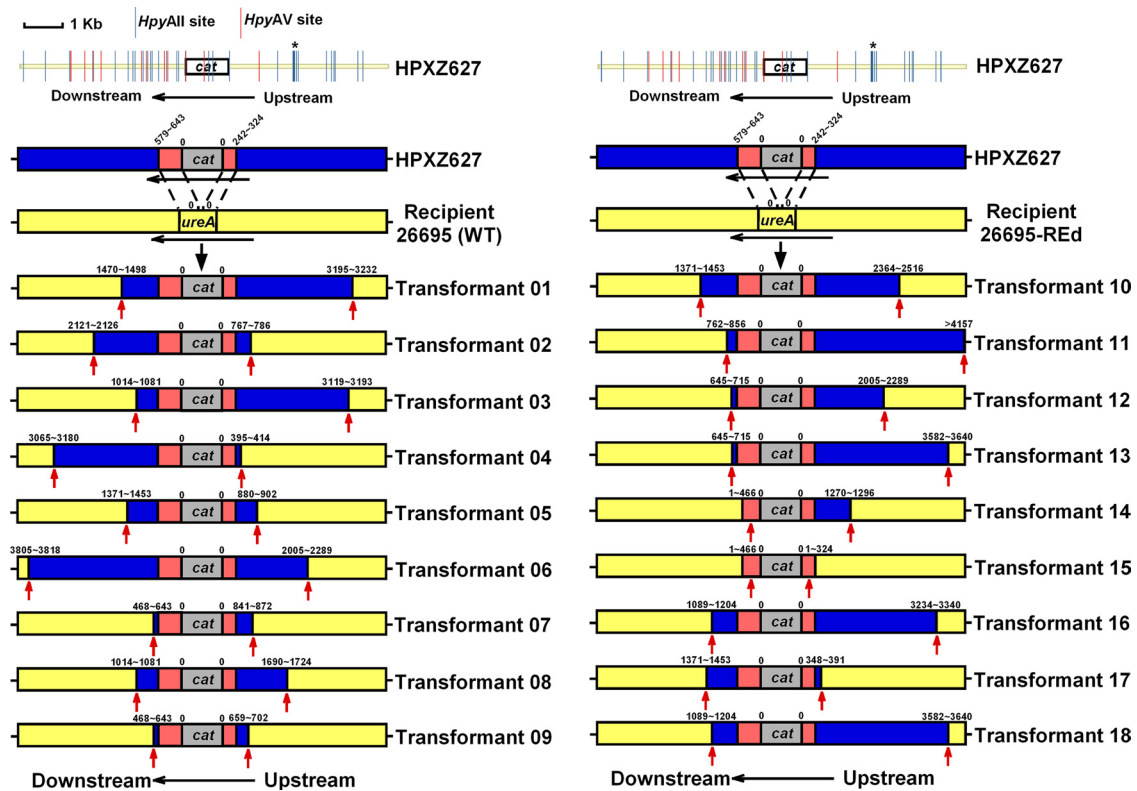
FIG 7 Length of imported DNA flanking the *cat* cassette following transformation with chromosomal DNA from donor *H. pylori* strains. The J99-derived donor HPXZ627 was used to transform *H. pylori* 26695 (A) or 26695-REd (B). The values above the bars indicate the distance (in bp) from the *cat* cassette to the recombination endpoints (red arrows), as determined by sequence analysis. The numbers reflect the distance from the nearest cassette boundary; the range reflects the locations of informative single-nucleotide polymorphisms (SNPs). Sequences corresponding to J99 are colored blue, those corresponding to 26695 are colored yellow, and those corresponding to 60190, which are from pAD1-Cat that flank the *cat* cassette, are colored red. All of the *H. pylori* 26695-strain specific HpyAII and HpyAV restriction sites on donor DNA are indicated. HpyAII cleavage sites (GAAGA $^{N8}/_{N7}$ ↓) are indicated by blue | lines, and HpyAV cleavage sites (CCTTC$^{N6}/_{N5}$ ↓) are indicated by red | lines. Within the ~4-kb flanking region on each side of the *cat* cassette, 26 restriction sites (17 HpyAII sites and 9 HpyAV sites) are present in the downstream flanking region, and 16 restriction sites (15 HpyAII sites and 1 HpyAV site) are present in the upstream flanking region. A high-density region of restriction sites (with 7 HpyAII sites within 156 bp) is present ca. 1,744 to 1,899 bp upstream of the *cat* cassette and is indicated by an asterisk (*).

ence may reflect that fewer sites recognized by the four active type II REs are present within the *cat* cassette (6 sites) than within the *aphA* cassette (21 sites) (Table 1). Another possibility is that other REs (type I and/or III) may differentially affect the exogenous cassettes. Identification of such alternative active endonucleases will further characterize *H. pylori* restriction. The results indicate that the constructed 26695-REd strain with

lessened barriers to transformation will be useful for *H. pylori* genetic engineering, especially for genomic integration of exogenous DNA cassettes.

DNA import during horizontal gene transfer between different strains was investigated to evaluate the effects of strain-specific restriction, in a manner that may be relevant to *in vivo* conditions, since multiple *H. pylori* strains may cocolonize an individual host (15, 24, 37, 50) and, in some cases, different strains may coexist at a single biopsy site (24, 37). Upon comparing independent transformations of the wild-type and 26695-REd strains, restriction did not affect donor DNA import sizes (Table 2). The mean DNA import sizes of the wild-type and REd strains were nearly identical (3.2 ± 1.5 kb and 3.2 ± 1.6 kb, respectively). Another group recently showed that mutation of two type II restriction enzyme genes (Hpy188III and HpyCH4V) in *H. pylori* strain NSH57 led to statistically significant increases in donor DNA import sizes at the *cagH* locus (5.7 ± 2.7 kb in the RE double mutant versus 4.1 ± 2.4 kb in the wild-type strain NSH57) (19), although with considerable variation in import size (standard deviations = 2.7 and 2.4) in the two strains. Comparison of those results (19) to ours may suggest that restriction effects on DNA import sizes during natural transformation may be strain dependent or locus dependent,

TABLE 2 Comparison of lengths of imported flanking DNAs in homologous transformation of *H. pylori* strains 26695 and 26695-REd with donor DNA from strain J99 *ureA*::*cat* (generated by transforming J99 with pAD1-Cat)

Category	Mean length (bp) ± SD for recipient strain[a]	
	26695	26695-REd
Length of flanking DNA upstream of *cat* cassette	1,537 ± 1,079	2,341 ± 1,467
Length of flanking DNA downstream of *cat* cassette	1,684 ± 1,132	861 ± 451
Total length of imported DNA	3,221 ± 1,546	3,202 ± 1,640

[a] Mean of nine independent transformants.

since other undefined factors involved appear to modify restriction effects.

Deletion of these four REs did not appear to enhance the growth characteristics of the *H. pylori* cells, confirming the utility of these engineered strains for future studies and indicating that the biological cost of carrying these genes is low in the presence of functioning methylases. That the deletions did not impair growth suggests *H. pylori* does not have alternative uses for DNA restriction other than for controlling incoming DNA. This contrasts with the subversion of methylases in introduced R-M systems that may provide regulatory functions for the host cells (2, 8, 41, 46). Considering that DNA methylation potentially affects bacterial cell cycle, DNA replication, repair, and mutation rates (23, 40, 41), the phenotypes we observed suggest that the targeted deletion of the RE genes most likely does not affect transcription of the adjacent DNA methylases. Although elements in upstream type II restriction genes (such as *hpyIR* [*iceA*]) also may affect *H. pylori* virulence *in vivo* through transcriptional regulation of the downstream DNA methylase (11, 14, 58), there is no evidence that the methylases of the four active type II R-M system REs affect *H. pylori* 26695 colonization. Similarly, the lack of effect on UV-induced or fluoroquinolone-induced DNA damage repair, or spontaneous mutation indicates that the endonuclease-induced double strand breaks are completely functionally suppressed by the paired endogenous methylases. These studies provide experimental verification of the hypothesized strong methylation phenotypes to control restriction (26, 45), and provide further rationale for the use of these engineered strains in future *H. pylori* research.

It should also be noted that all four active type II RE genes have immediate downstream genes with intergenic distances ranging from 86 to 432 bp. Our mRNA-based analysis indicated expression of these immediate downstream genes in the REd strain is not significantly different from that in the wild-type strain (<2-fold) when grown in nutrient-rich medium to mid-log phase (data not shown), which suggests deletion of the four RE genes may have no or only very limited polar effect on their downstream genes.

H. pylori strains (including strain 26695) not only have the active type II restriction-methylation systems but also contain restriction-methylation gene sequence remnants in their genome. Several display characteristics that indicate potential phase variation (31). It is possible that these phase-variable inactive restriction genes are in the process of disappearing, but they also may be reactivated and restore endonuclease activity. Whether abolishing active type II R-M systems would exert a selective pressure on those putative phase-variable inactive restriction genes awaits further investigation, using the REd strain as a tool. As such, our REd mutant strain also will be useful in R-M systems research in the perspective of *H. pylori* evolution.

ACKNOWLEDGMENTS

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