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Helicobacter pylori interstrain restriction-modification diversity prevents genome subversion by chromosomal DNA from competing strains

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ABSTRACT

***Helicobacter pylori*, bacteria that colonize the human gastric mucosa, possess a large number of genes for restriction-modification (R-M) systems, and essentially, every strain possesses a unique complement of functional and partial R-M systems. Nearly half of the *H.pylori* strains studied possess an active type IIs R-M system, *Hpy*II, with the recognition sequence GAAGA. Recombination between direct repeats that flank the R-M cassette allows for its deletion whereas strains lacking *hpyIIRM* can acquire this cassette through natural transformation. We asked whether strains lacking *Hpy*II R-M activity can acquire an active *hpyIIRM* cassette [containing a 1.4 kb kanamycin resistance (*aphA*) marker], whether such acquisition is DNase sensitive or resistant and whether restriction barriers limit acquisition of chromosomal DNA. Our results indicate that natural transformation and conjugation-like mechanisms may contribute to the transfer of large (4.8 kb) insertions of chromosomal DNA between *H.pylori* strains, that inactive or partial R-M systems can be reactivated upon recombination with a functional allele, consistent with their being contingency genes, and that *H.pylori* R-M diversity limits acquisition of chromosomal DNA fragments of ⩾1 kb.**

INTRODUCTION

Restriction-modification (R-M) systems defend bacteria against invasion by foreign DNA such as conjugative plasmids and bacteriophages (1). The type II family of R-M systems consists of paired enzymes that recognize identical DNA sequences but have contrasting enzymatic functions. The restriction endonuclease (ENase) cleaves DNA within the recognition site while the modification enzyme (MTase) methylates adenosyl or cytosyl residues within the recognition sequence, thereby protecting the host chromosome from cognate restriction activity (1).

Helicobacter pylori are Gram-negative curved bacteria that colonize the human stomach and increase the risk of development of peptic ulcer disease and gastric adenocarcinoma (the major form of stomach cancer in the world) (2). For *H.pylori*, several studies have shown significant interstrain variation in R-M system activity (3–7), a diversity that influences strain transformability (8,9). Lack of conservation in R-M activity could be due to the absence or partial absence of, or point mutations in, the R-M systems, and comparison of the genome sequences of strain 26695 and J99 provide evidence for all three phenomena (3,4,10–12).

Previous work in our laboratory has shown that repetitive DNA sequences flanking the *H.pylori hpyIIRM* system facilitate deletion of the R-M cassette from the chromosome (13). The full *hpyIIRM* cassette can be re-acquired through natural transformation using chromosomal DNA from the parental strain (Fig. 1) (13). Comparison of the two available *H.pylori* genome sequences identified long repeat sequences flanking many strain-specific R-M systems (14) suggesting that the deletion/re-acquisition model might represent a general mechanism through which *H.pylori* strains may vary R-M content and that *H.pylori* R-M systems may act as 'transposon-like' mobile genetic elements (14).

Strain-specific R-M system activity in *H.pylori* presents interstrain barriers to the transfer of plasmid DNA (8); however, the role of R-M diversity in restricting chromosomal DNA uptake and transformation has not been defined. For a naturally competent organism such as *H.pylori* (15), the ability to restrict incoming chromosomal DNA is an efficient means of preventing competing strains from subverting the genome of a co-colonizing strain through natural transformation.

To test the hypothesis that *H.pylori* interstrain R-M diversity restricts transformation by chromosomal DNA from other *H.pylori* strains, we conducted interstrain recombination experiments between isogenic or non-isogenic strains using chromosomal DNA, containing markers ranging in size from 1 to 4.8 kb. Further, we examined the role of R-M activity in restricting chromosomal DNA acquired through natural transformation or via conjugation-like mechanisms. Our findings support the hypothesis that, although both natural transformation and conjugation-like mechanisms contribute to the transfer of large (4.8 kb) fragments of chromosomal DNA, diversity in functional R-M systems represents a barrier to the

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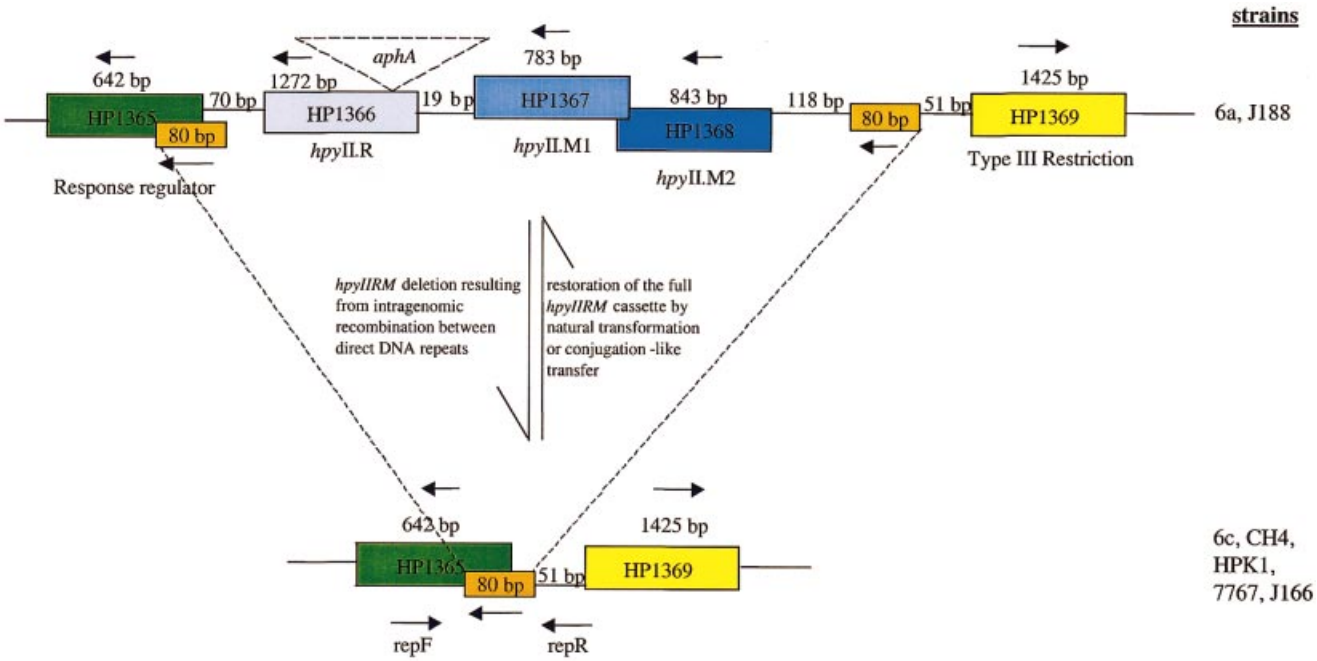

Figure 1. Spontaneous deletion and horizontal re-acquisition of the *hpyIIRM* system. Sequence analysis of the *hpyIIRM* system in *H.pylori* strain 26695 identified 80 bp repeats flanking the R-M system. The presence of direct repeat sequences facilitates generation of deletions through mechanisms involving sister-strand exchange or strand slippage. These deletions result in excision of the intervening region and one copy of the repeat. *Helicobacter pylori* strains lacking the *hpyIIRM* cassette, but containing one copy of the repeat unit, may re-acquire a functional cassette through transformation. Primers repF and repR flanking the 80 bp repeats were used in PCR to confirm acquisition of *hpyIIRM::aphA* cassettes by recipient strains. An *aphA* cassette was inserted into *hpyIIR* of strain 6a to create strain RA1.

acquisition of chromosomal DNA fragments of at least 1.0 kb. These results suggest that there is a double-stranded DNA intermediate in the *H.pylori* chromosomal transformation process.

MATERIALS AND METHODS

Bacterial strains

Helicobacter pylori strains used in this study (Table 1) were obtained from the NYU *Helicobacter*/*Campylobacter* reference strain collection. To select for spontaneous streptomycin- or spectinomycin-resistant *H.pylori* strains, ~10^{10} cells were incubated on brucella agar (BA) plates containing streptomycin (10 µg/ml) or spectinomycin (10 µg/ml), respectively. The plates were incubated at 37°C (5% CO_2) for 6–7 days and antibiotic-resistant colonies were harvested.

Helicobacter pylori interstrain recombination

Each interstrain recombination experiment involved two strains (A and B) with mutually exclusive antibiotic resistance properties, as described previously (13). After 48 h of growth on appropriate selective BA plates, cells of each strain were harvested and suspended in 1 ml of saline. The cells were centrifuged, the supernatant discarded, the cells resuspended in 175 µl of saline and then 25 µl aliquots spotted onto trypticase soy agar (TSA) plates as follows: strain A alone, strain B alone, strain A + B, strain A + B + DNase I (250 µg/µl). The plates were incubated overnight at 37°C in an atmosphere with 5% CO_2, after which bacteria were harvested in 1 ml of saline, centrifuged for 5 min at 6000 *g*, supernatant discarded and cells resuspended in 1 ml of saline. The suspensions from strain A alone and strain B alone were serially diluted, and 100 µl of 10^{-5}, 10^{-6} and 10^{-7} dilutions was inoculated on TSA plates without antibiotics. For all suspensions, 50 and 250 µl were inoculated onto BA plates containing 10% newborn calf serum and either kanamycin (25 µg/ml), streptomycin (20 µg/ml) and spectinomycin (20 µg/ml) (BA-KStSp plates); or kanamycin, streptomycin and rifampin (5 µg/ml) (BA-KStR plates). All plates were incubated for 96 h, then colonies counted and transformation frequencies calculated. Calculations were based on at least three independent experiments.

Natural transformation

Transformation of *H.pylori* was performed as described previously (9). In short, *H.pylori* cells were scraped from 36 h cultures grown on TSA with 5% sheep blood and resuspended in 175 µl saline. Then, 25 µl aliquots of cell suspension were spotted onto a TSA plate and 100 ng of the transforming chromosomal DNA or PCR product added. After incubating the mixture at 37°C (5% CO_2) for 12 h, cell spots were transferred to BA plates containing 5% fetal calf serum and appropriate selective antibiotics, and incubated at 37°C (5% CO_2) for 4–5 days.

PCR and restriction endonuclease digestion

To confirm acquisition of the *H.pylori hpyIIRM* cassette, PCR, using primers repF (nt 1427401–1427424; www.tigr.org/hpylori) and repR (nt 1430845–1430867) that flank *hpyIIRM*, was performed using 100 ng of chromosomal

Table 1. *Helicobacter pylori* strains used in this study

Strain designation	Relevant genotype	*hpyIIRM* phenotype	Antibiotic resistance marker[a]	Reference
6a	*hpyIIRM*$^+$, *cagA*$^+$, *vacA* s1m1	R$^+$M$^+$	None	(13)
6c	*hpyIIRM*$^-$, *cagA*$^+$, *vacA* s1m1	R$^-$M$^-$	None	(13)
CH4	*hpyIIRM*$^-$, *cagA*$^+$, *vacA* s1am1	R$^-$M$^-$	None	(5)
HPK1	*hpyIIRM*$^-$, *cagA*$^+$, *vacA* s1m1	R$^-$M$^-$	None	(8)
7767	*hpyIIRM*$^-$, *cagA*$^+$, *vacA* s2m2	R$^-$M$^-$	None	(8)
J166	*hpyIIRM*$^-$, *cagA*$^+$, *vacA* s1bm1	R$^-$M$^-$	None	(8)
J188	*hpyIIRM*$^+$, *cagA*$^-$, *vacA* s1m1	R$^-$M^{-b}	None	(8)
RA1 (fomerly 6aRK)	6a (*hpyIIR::aphA*)	R$^-$M^{+c}	Kan	(13)
RA2	6a (*hpyIIR::aphA*, *rpoB15*)	R$^-$M^{+c}	Kan, Rif	This study
RA3	6a (*vacA::aphA*)	R$^+$M$^+$	Kan	This study
RA4	6a (*rpsL1*, *rrn16S1*)	R$^+$M$^+$	Strep, Spec	This study
RA5	6c (*rpsL1*, *rrn16S1*)	R$^-$M$^-$	Strep, Spec	This study
RA6	J188 (*rpsL1*, *rrn16S1*)	R$^-$M^{-b}	Strep, Spec	This study
RA7	J166 (*rpsL1*, *rrn16S1*)	R$^-$M$^-$	Strep, Spec	This study

[a]Resistance to rifampin, streptomycin and spectinomycin due to point mutationsin *rpoB* (30), *rpsL* (31) and *rrn16S* (32), respectively. Resistance to kanamycin based on insertion of *aphA* in specified gene.
[b]Contains complete but inactive *hpyIIRM* cassette.
[c]*hpyIIR*$^-$ due to interruption by *aphA*.

DNA from the recipient strain as template, as described previously (13). PCR, using primers vacAF (5′-GTGAAAGCGAAAAACAAGAAATTG-3′) and vacR (5′-CGTGCCATCGGCTTTAGTGTTG-3′) were performed to amplify, from plasmid pCTB8 (13), *vacA::aphA* cassettes used in transformation experiments described above. Unless otherwise noted, all reactions were run for 35 cycles in mixtures containing 100 ng template DNA, 200 ng of each primer, 0.5 U *Taq* polymerase (Qiagen, Valencia, CA) in a 50 µl volume. DNA was digested with 10 U of *Mbo*II overnight and bands were resolved in 1% agarose gels. To assess M.*Hpy*II activity amongst *H.pylori* strains, 1 µg of chromosomal DNA was digested with 5 U of *Mbo*II (NEB) at 37°C for 2 h and products visualized by agarose gel electrophoresis.

RESULTS

Effect of DNase I on natural transformation of *H.pylori* cells by chromosomal DNA

To examine mechanisms for chromosomal DNA transfer in *H.pylori*, we conducted transformation experiments between strains containing mutually exclusive antibiotic resistance markers (Table 1), in the presence and absence of DNase I. *Helicobacter pylori* strain RA4 was incubated with chromosomal DNA from strain RA2 and transformants were selected on BA plates containing kanamycin, streptomycin and spectinomycin (BA-KStSp). In the absence of DNase I, strain RA4 acquired a functional *aphA* cassette at a frequency of $(1.4 \times 10^{-4}) \pm (2.0 \times 10^{-4})$, however, when DNase I was mixed with RA2 DNA, no transformants were observed in ~10^9 recipient cells. These experiments confirm, with these strains and the *aphA* cassette, that DNase treatment abrogates natural transformation (16).

Horizontal transfer of a chromosomal point mutation between isogenic *H.pylori* strains

Next, we sought to measure the frequency of transfer of a point mutation by natural transformation or via conjugation-like mechanisms. The recipient strain was strain RA2 and strain RA4 or RA5, which have the same clonal origin but differ in the presence of the *hpyIIRM* locus (13), was used as the donor of the Strep^r-conferring point mutation. In interstrain recombination experiments, strain RA2 cells acquired the Strep^r point mutation from RA4 cells at a frequency of $(2.6 \times 10^{-5}) \pm (1.4 \times 10^{-5})$, but the addition of DNase I decreased this rate by 95.4% (Table 2). Similarly, the point mutation conferring Strep^r in RA5 cells was transferred to RA2 cells at a frequency of $(5.0 \times 10^{-5}) \pm (3.5 \times 10^{-5})$ with a 94.5% reduction in the number of recombinant cells in the presence of DNase I (Table 2). That transformation experiments using RA4 DNA incubated with DNase yielded no RA2 transformants suggests that Strep^r transformants observed during interstrain recombination experiments did not result from natural transformation of recipient cells with DNA that escaped DNase activity, consistent with earlier studies (16). All KRSt-resistant colonies also were spectinomycin sensitive, indicating that the observed transformants did not result from concomitant transfer of kanamycin and rifampin resistance from strain RA2 to strain RA4 or RA5. That incubation of strain RA2 on streptomycin-containing plates yielded no Strep^r transformant indicated that spontaneous mutation was not responsible for the observed transformants. These results support the earlier finding that although natural transformation accounts for most of the observed genetic exchange between *H.pylori* strains, a Strep^r-conferring point mutation can be transferred between *H.pylori* strains via a DNase-resistant mechanism (16).

Horizontal transfer of the *hpyIIRM* system between isogenic *H.pylori* strains

Next, to determine whether larger DNA fragments could be mobilized by conjugation-like mechanisms between isogenic strains, we examined horizontal transfer of a 1.3 kb *aphA* cassette within the *hpyIIRM* locus (from strain RA2 to RA4) or the entire 4.8 kb *hpyIIRM*::*aphA* cassette (which contains *aphA* in *hpyIIR*) from strain RA2 to RA5, in the presence or absence of DNase I. Co-culture of strains RA2 and RA4 and selection for transformants on BA-StSpK plates indicated that strain RA4 acquired the *aphA* cassette at a frequency of $(7.9 \times 10^{-6}) \pm (2.0 \times 10^{-6})$, and that addition of DNase I

Table 2. *Helicobacter pylori* matings to compare transfer of detectable alleles

H.pylori strain A	*H.pylori* strain B[a]	Treatment with DNase (250 μg/μl)	Recombination frequency ($\times 10^{-7}$)[b] Transfer of a point mutation to strain A[c]	Transfer of an altered *hpyIIM* allele to strain B[d]
RA2[e]	RA4	–	262 ± 144	79 ± 20
		+	12 ± 6	9 ± 2
RA2	RA5	–	496 ± 354	88 ± 72
		+	27 ± 23	4 ± 4
RA2	RA6	–	513 ± 262	3 ± 2
		+	29 ± 22	<0.66
RA2	RA7	–	319 ± 225	<0.18
		+	16 ± 5	<0.19

[a]Each of these strains have point mutations in *rpsL* and *rrn16S* resulting in Strep^r, Spec^r phenotypes.
[b]Each result shown represents the mean ± SD of three independent matings.
[c]Horizontal transfer of a point mutation was determined by the ability of strain A to acquire Strep^r from strain B producing a Kan^{r}Rif^{r}Strep^r strain. No spontaneous mutations to Strep^r were detected.
[d]Transfer of the *hpyIIRM* system was determined by the ability of strain B to acquire Kan^r from strain A (*hpyIIR::aphA*) producing a Kan^{r}Strep^{r}Spec^r strain.
[e]RA2 is Kan^r (*aphA* in *hpyIIRM)* and Rif^r.

decreased this frequency by 88.9% (Table 2). Similarly, co-culture of strains RA2 and RA5 and selection for transformants on BA-StSpK plates resulted in the transfer of the entire 4.8 kb *hpyIIRM*::*aphA* cassette from RA2 cells to RA5 cells at a frequency of (8.8×10^{-6}) ± (7.2×10^{-6}) (Table 2), confirming that a strain lacking *hpyIIRM* (6c) can re-acquire an R-M system through horizontal gene transfer (13). Addition of DNase I decreased this recombination frequency by 96.0%. All observed transformants were spectinomcyin sensitive, indicating that they did not result from the concomitant transfer of streptomycin and spectinomycin resistance from strain RA4 or RA5 to strain RA2. Similarly, that strain RA4 or RA5 incubated on kanamycin-containing plates yielded no kanamycin-resistant transformants indicated that the observed transformants did not result from spontaneous kanamycin resistance. For strains RA2, RA4 and RA5, there were no significant differences in the frequency of transfer of the Strep^r point mutation (1 bp), the *aphA* cassette (~1.3 kb) or the *hpyIIRM*::*aphA* cassette (~4.8 kb) between clonal variants. These results indicate that chromosomal DNA fragments of ⩽4.8 kb can be transferred without significant barriers between strains of the same clonal origin through both natural transformation and DNase-resistant methods (13).

Genetic exchange of chromosomal DNA between non-isogenic *H.pylori* strains

Previous studies have shown that plasmid DNA uptake by *H.pylori* from non-isogenic strains is limited by restriction barriers (8,9). To examine now whether barriers against transfer of chromosomal DNA exist between non-isogenic *H.pylori* strains, we conducted interstrain recombination experiments between strain RA2 and the *hpyIIRM*$^+$ strain RA6 or the *hpyIIRM*$^–$ strain RA7. Co-culture of strains RA2 and RA6 and selection on BA-KRSt plates indicated that strain RA2 acquired Strep^r at a frequency of (5.1×10^{-5}) ± (2.6×10^{-5}) with a 94.3% decrease in transformation frequency in the presence of DNase I (Table 2). That all transformants were spectinomycin sensitive, and that strain RA2 incubated alone yielded no Strep^r transformants, indicated that strain RA2 had acquired the Strep^r-conferring point mutation from a non-isogenic *H.pylori* strain (RA6) at a frequency similar to that of strains of the same clonal origin (RA4, RA5). Parallel results were obtained from co-culture of strain RA2 with strain RA7 and selection on BA-KRSt plates, confirming that there are no significant barriers against DNase-sensitive or -resistant transfer of a chromosomal point mutation between non-isogenic *H.pylori* strains.

Transfer of the *aphA* cassette between non-isogenic strains was measured by co-culture of strains RA2 and RA6 and selection on BA-StSpK plates. Strain RA6 acquired the *aphA* cassette at a frequency of (3.4×10^{-7}) ± (2.1×10^{-7}) and no transformants were observed in the presence of DNase I (Table 2). That strain RA6 acquired the *aphA* cassette from strain RA2 at a significantly lower frequency ($P = 0.02$) than either strain RA4 or RA5 indicates that barriers between non-isogenic *H.pylori* strains affect the frequency of insertion of the 1.3 kb cassette. Co-culture of strain RA2 and RA7 and selection on BA-StSpK plates yielded no transformants in the presence or absence of DNase I (Table 2). Failure of the 4.8 kb *hpyIIRM*::*aphA* cassette to be transferred from RA2 to RA7 at a detectable frequency further defines the extent of the barriers existing between non-isogenic *H.pylori* strains that limit integration of larger chromosomal DNA insertions.

Acquisition of M.*Hpy*II function

Next, we sought to determine whether the kanamycin-resistant RA5 and RA6 transformants acquired a functional M.*Hpy*II in the original locus. Chromosomal DNA from these transformants was subjected to PCR using primers that flank the *hpyIIRM* cassette in strain RA2, and was also digested with *Mbo*II, an isoschizomer of *Hpy*II. That strain RA5 DNA yielded only a 0.3 kb empty-site PCR product, and was sensitive to *Mbo*II digestion, whereas all kanamycin-resistant transformants yielded 4.8 kb PCR products consistent with the presence of the entire *hpyIIRM*::*aphA* cassette and were resistant to *Mbo*II digestion (Fig. 2), confirms that RA5 transformants acquired the full R-M cassette containing a functional *hpyIIM* in its original locus. The presence of empty-site products reflects the relative ease of amplifying shorter PCR products, and the existence of mixed populations (13). For strain RA6, which contains a full-length *hpyIIRM* cassette but lacks M.*Hpy*II activity (13), PCR indicated that all 10

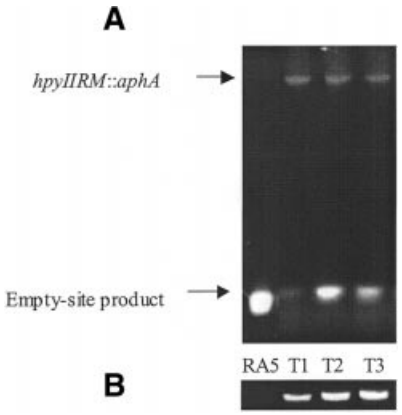

Figure 2. Acquisition of *hpyIIM* function in kanamycin-resistant RA5 transformants. (**A**) To determine whether kanamycin-resistant RA5 transformants acquired the *hpyIIRM::aphA* cassette in its original locus, PCR was performed using primers that flank the R-M cassette in strain RA2, with template chromosomal DNA from strain RA5 (control) and three transformants. (**B**) To determine whether kanamycin-resistant RA5 transformants acquired M.*Hpy*II function, chromosomal DNA from strain RA5 (control) and the three transformants was digested with *Mbo*II, an isoschizomer of *Hpy*II.

transformants examined acquired the *aphA* cassette in the original *hpyIIR* locus as expected; however, *Mbo*II digestion indicated that only seven (70%) of these 10 transformants acquired the functional *hpyIIM* allele. These results indicate that the recombination events in RA6 that yielded a selectable marker (*aphA* in *hpyIIR*) involved exchanges that varied in the extent of replacement of the flanking DNA containing *hpyIIM*.

Restriction barriers limit horizontal transfer of chromosomal DNA between non-isogenic *H.pylori* strains

Next, we sought to determine whether the *H.pylori* barriers that limit acquisition of chromosomal DNA from non-isogenic strains reflect differences in natural competence. Experiments were conducted to examine the frequency of transforming the Strep^r point mutation (1 bp), the *aphA* cassette (1.3 kb) or the *hpyIIRM::aphA* cassette (4.8 kb) originating from strain RA4, RA3 or RA1, respectively, into the *hpyIIRM*$^-$ strain 6c, which shares the same clonal origin or non-isogenic *hpyIIRM*$^-$ strains J166, CH4, HPK1, 7767 (Table 3). All five strains were transformed to Strep^r to essentially the same extent, indicating that there were no significant differences in the competence of these strains. For control recipient strain 6c, there were also no significant differences in transformation by chromosomal DNA when selecting for the Strep^r point mutation, the *aphA* cassette or *hpyIIRM::aphA*. However, for all four non-isogenic strains studied, the Strep^r point mutation was acquired at significantly higher frequencies ($P < 0.05$) than was the 1.3 kb *aphA* or 4.8 kb *hpyIIRM::aphA* cassette. These data confirm that the limited acquisition of chromosomal DNA >1 kb from non-isogenic *H.pylori* strains reflects barriers rather than a lack of competence or competence signals.

To determine specifically whether restriction affects *H.pylori* transformation frequencies of chromosomal DNA fragments, PCR products containing the Strep^r point mutation, the *aphA* cassette or the *hpyIIRM::aphA* cassette from the strain 6a derivatives were used to transform strain 6c (Table 4). There was no significant difference in Strep^r transformation frequency of 6c between chromosomal DNA and the unmethylated PCR products. However, there was significantly less transformation ($P < 0.05$) by the *aphA* or the *hpyIIRM::aphA* cassette when the PCR product was used compared with chromosomal DNA. That <50 bp homology is needed for successful transformation of *H.pylori* (S.Levine and M.J.Blaser, manuscript in preparation), suggests that the lengths of the PCR product-flanking regions (Table 4) were sufficient, and thus were not responsible for the significant reduction in transformation frequency. To determine whether the difference in transformation frequency was due to the lack of DNA methylation of the PCR products in the pattern specific for strain 6a, all of the products were premethylated using cell extracts from strain 6a, as described previously (9), and then used to transform strain 6c. For the product containing Strep^r, there was little difference between the three forms of donor DNA. For both cassettes >1 kb, transformation frequency increased significantly ($P < 0.05$) compared with the unmethylated PCR products. To confirm that differences in transformation frequency resulted from modification of chromosomal DNA (e.g. methylation), strain 6c was transformed with donor strain (RA4, RA3 or RA1) DNA, the PCR products or the 6c-transformant DNA (Table 4) and transformation frequencies for all three markers were calculated. As expected, for the *aphA* and *hpyIIRM::aphA* markers, 6c transformant DNA transformed strain 6c at a

Table 3. Transformation of isogenic and non-isogenic *H.pylori* strains by chromosomal DNA containing selectable markers of different sizes

Recipient *H.pylori* strain	Transformation frequency ($\times 10^{-7}$) Transfer of a point mutation (1 bp)[a] from RA4	Transfer of *aphA* cassette (1.3 kb)[b] from RA3	Transfer of *hpyIIRM::aphA* cassette (4.8 kb) from RA1
6c[c]	141 ± 32	100 ± 19	49 ± 40
J166[d]	98 ± 7[e]	4 ± 0.9	<0.4
CH4[d]	106 ± 38[e]	3 ± 2	<0.4
HPK1[d]	222 ± 160[e]	1 ± 0.7	<0.5
7767[d]	113 ± 67[e]	3 ± 2	<0.4

[a]Strep^r point mutation.
[b]*aphA* present in *vacA*.
[c]Strain 6c has the same clonal origin as strain 6a.
[d]Strain has different origin to strain 6a.
[e]Strain was transformed by the point mutation in *rpsL* (A128G) conferring Strep^r at a significantly higher frequency ($P < 0.05$) than by the *aphA* cassette or by the *hpyIIRM::aphA* cassette.

Table 4. Effect of DNA methylation status on transformation frequency of strain 6c

Donor[a] DNA	Transformation frequency ($\times 10^{-7}$) Point mutation from RA4	*aph*A cassette from RA3[b]	Altered *hpyIIRM* cassette from RA1[c]
Chromosome	138 ± 32	102 ± 20	81 ± 30
PCR product[d]	193 ± 81	10 ± 8	<0.4
Methylated PCR product[e]	94 ± 50	35 ± 12	12 ± 10

[a]Strep[r] point mutation, *aphA* cassette or *hpyIIRM::aphA* cassette, respectively, which were selected for during transformation experiments.
[b]The PCR product contains 1577 bp flanking the *aphA* cassette that have homology to *vacA*.
[c]The PCR product contains 367 bp flanking the *aphA* cassette that have homology to *hpyIIR*.
[d]PCR products for the Strep[r] point mutation, *aphA* cassette and *hpyIIRM::aphA* cassette were amplified from strains RA4, RA3 and RA1 chromosomal DNA, respectively.
[e]PCR products were methylated to the host strain specificities, as described previously (9).

significantly higher frequency ($P < 0.05$) than did PCR products, but not at a significantly different frequency than donor strain DNA (data not shown). These results provide evidence that *H.pylori* R-M systems represent barriers to acquisition of chromosomal DNA >1 kb from non-isogenic strains. These barriers may result from differing distributions of relevant restriction sites or differing methylation patterns in the donor DNA.

DISCUSSION

Although *H.pylori* R-M systems exhibit significant interstrain variation (5–7), and R-M diversity influences plasmid transformation frequencies (8,9), its effect on transformation by *H.pylori* chromosomal DNA transformation was not established. Here, we show that *H.pylori* can transfer 4.8 kb chromosomal DNA inserts through natural transformation or conjugation-like mechanisms between clonal variants, but that restriction barriers between non-isogenic strains limit transfer of DNA fragments ⩾1 kb. These findings are consistent with the observation that horizontally transferred DNA fragments in *H.pylori* have a median size of 417 bp, much smaller than for other bacteria studied (17). For the naturally competent *H.pylori*, the presence of barriers that limit incoming chromosomal DNA from other *H.pylori* strains may be an effective means to prevent one strain from completely transforming the genome of another strain during co-colonization of a host (8). The acquisition of relatively smaller DNA fragments is also consistent with the presence of mosaic loci (17,18) within the *H.pylori* chromosome.

It has been suggested that R-M systems represent 'selfish' mobile genetic elements that, once established within the genome, would lead to adverse consequences for their host cell if eliminated (19). That the full 4.8 kb *hpyIIRM::aphA* cassette cannot be transferred between non-isogenic *H.pylori* strains indicates that R-M systems within cells are barriers to acquisition of other R-M systems, since the resident restriction endonuclease activity attacks improperly methylated incoming DNA. Since elimination (or inactivation) of resident R-M systems may increase host-cell susceptibility to subversion by competing *H.pylori* DNA, the resident R-Ms may be considered to be 'selfishly' (19) improving their own security by retarding novel R-Ms from entering the chromosome. The strong avoidance of cognate R-M recognition sequences within the *H.pylori* genome is indicative of the strong selective pressures exerted by the competing R-M systems (20). Conversely, cognate sequence avoidance may contribute to improved acquisition of DNA fragments of *H.pylori*, but not of non-*H.pylori* origin, thereby deepening the differential between species-homologous and -heterologous DNA.

In that context, the presence of partial or inactive R-M systems (3,4,10) may serve as a new class of 'contingency locus' (21) that can be reactivated upon recombination with a functional allele, as illustrated by the acquisition of M.*Hpy*II activity by strain RA6 through natural transformation using donor DNA from RA2. Such findings provide evidence for the 'fluidity' of the *H.pylori* R-M systems with the potential to move both vertically and horizontally throughout the host cell population. Recent studies of *ice*A1 (*hpy*IR) indicate four potential states: functional enzyme, single point mutations that can be fixed by endogenous repair (i.e. frameshifts, reversion point mutations), multiple mutations and complete absence of the gene; all three of the latter states can be repaired by recombination with the exogenous wild-type gene (22). That in each strain the cognate methylase (*hpy*IM) is fully functional (6,23,24), indicates that any *H.pylori* strain is capable of acquiring R.*Hpy*I function regardless of the initial *hpyIR* status. That *H.pylori* genomes are diverse in sequence and gene content (10) suggests that the reactivation of inactive 'contingency genes' through acquisition of fully functional alleles may be a general paradigm for gene regulation.

Naturally competent organisms are capable of ingesting environmental DNA with genomic integration through homologous recombination (25). For *Haemophilus influenzae* (26) and *Neisseria gonorrheae* (27), but not *H.pylori* (28), DNA uptake sequences have been identified that permit donor DNA to bind to recipient cells before transport across outer and inner membranes and integration into the chromosome. For Gram-negative prokaryotes such as *H.influenzae*, transformation results in single-stranded integration (29); however, single-stranded DNA of donor origin has not been detected within *H.influenzae* cells. Type II ENases, including several carried by *H.pylori,* preferentially cleave double-stranded DNA (1,3–5,8). That pre-methylated *hpyIIRM::aphA* PCR products preferentially transformed *H.pylori* cells suggests that, after internalization by recipient cells, double-stranded donor DNA intermediates are present and thus subject to restriction. Consistent with this hypothesis are recent experimental findings that indicate that double-stranded DNA is substantially more efficient at transforming *H.pylori* cells

than is identical single-stranded DNA (S. M. Levine and M. J. Blaser, unpublished results).

In conclusion, our findings demonstrate that *H.pylori* R-M diversity presents barriers to interstrain transfer of chromosomal DNA fragments of >1 kb, and extend the theory that R-M systems are 'selfish genes' by providing evidence that acquisition of novel R-M systems is limited by the presence of resident R-M systems. Further exploration of this highly tractable experimental system encompassing both *H.pylori* competence and restriction may broaden our understanding of the dynamics of gene flow in natural bacterial populations.

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