# Plastic cells and populations: DNA substrate characteristics in *Helicobacter pylori* transformation define a flexible but conservative system for genomic variation

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ABSTRACT **_Helicobacter pylori_, bacteria that colonize the human gastric mucosa, are naturally competent for transformation by exogenous DNA, and show a panmictic population structure. To understand the mechanisms involved in its horizontal gene transfer, we sought to define the interval required from exposure to substrate DNA until DNA uptake and expression of a selectable phenotype, as well as the relationship of transforming fragment length, concentration, homology, symmetry, and strandedness, to the transformation frequency. We provide evidence that natural transformation in _H. pylori_ differs in efficiency among wild-type strains but is saturable and varies with substrate DNA length, symmetry, strandedness, and species origin. We show that _H. pylori_ cells can be transformed within one minute of contact with DNA, by DNA fragments as small as 50 bp, and as few as 5 bp on one flank of a selectable single nucleotide mutation is sufficient substrate for recombination of a transforming fragment, and that double-stranded DNA is the preferred (1000-fold >single-stranded) substrate. The high efficiency of double-stranded DNA as transformation substrate, in conjunction with strain-specific restriction endonucleases suggests a model of short-fragment recombination favoring closest relatives, consistent with the observed _H. pylori_ population biology.—Levine, S. M., Lin, E. A., Emara, W., Kang, J., DiBenedetto, M., Ando, T., Falush, D., Blaser, M. J. Plastic cells and populations: DNA substrate characteristics in _Helicobacter pylori_ transformation define a flexible but conservative system for genomic variation. _FASEB J._ 21, 3458–3467 (2007)**

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MANY BACTERIAL SPECIES HAVE EVOLVED mechanisms for taking up exogenous DNA, thus using the advantage of sex for the generation of diversity (1, 2). Natural transformation of prokaryotes by free DNA involves release of DNA from donor cells, its dispersal and persistence in the environment, the development of competence for DNA uptake by adjacent recipient cells, and their interaction with DNA, leading to uptake, recombination, and potentially, the expression of an acquired trait (2). Host cell incorporation of exogenous DNA can be affected by expression of competence proteins, restriction endonucleases, and DNA repair and recombination enzymes, among other factors (1, 3–6).

*Helicobacter pylori* are Gram-negative curved bacteria that colonize the human gastric mucosa, leading to increased risk of peptic ulcer disease and gastric malignancies (7). The lifelong persistence of *H. pylori* in the human stomach may result from its ability to adapt to changes in its environmental niche (8, 9). *H. pylori* cells are naturally competent for DNA uptake (10–12), with specialized machinery (including reverse type IV secretion systems) for DNA uptake (13, 14) and horizontal gene transfer that generate substantial genetic diversity (15, 16). Studies of paired *H. pylori* isolates from the same host provide evidence that recombination is frequent and that the introduced fragments are short [417 bp, 95% confidence interval 259–732 bp] compared with *Streptococcus pneumoniae, Neisseria menigitidis*, *Bacillus subtilis*, and *Escherichia coli* (16). Moreover, *H. pylori* can proficiently take up both homologous and homeologous DNA, consistent with lack of a mismatch repair pathway, which in other organisms hinders incorporation of nonhomologous DNA (17, 18). Consideration of the mechanisms involved in genetic exchange may aid understanding of *H. pylori* adaptation to changing environments (19, 20).

Prior studies have examined the structural components of *H. pylori* required for or facilitating transformation (21–24). We now focus on substrate DNA

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requirements for *H. pylori* transformation. To accomplish this aim, we first defined the time required for DNA uptake after exposure to a suitable substrate and for expression of a selectable phenotype, as well as the relationship of transformation efficiency to transforming fragment length, concentration, homology, species origin, symmetry, and strandedness. This work provides evidence for the existence of a highly efficient and versatile *H. pylori* system for transformation and defines its limits and preferences, with findings consistent with the extensive intraspecies restriction barriers (4, 5, 25–27), and lack of *RecBCD* exonuclease activities (28). This work provides a model to explain plasticity of bacterial cells within a population, and potential solutions to the biological trade-offs between fidelity and diversification in a highly dynamic system (3).

## MATERIALS AND METHODS

### Strains and growth conditions

The primary *H. pylori* isolates used in this study were wild-type and streptomycin-resistant ($Str^R$) mutants of strains 26695 and HPK5 (29, 30) (Supplemental Table 1), as well as wild-type *H. pylori* strains 9999, HP1, 9886, HPK1, 8822, B128, J99, JP26, and J166, which have been the subject of prior studies (4, 5, 27, 31–35), and *Helicobacter cetorum* strain MIT 99–5656. *H. pylori* cells were grown on Trypticase soy agar (TSA) with 5% sheep blood (BBL Microbiology Systems, Cockeysville, MD, USA) or *Brucella*-serum (BS; BBL) agar with 10% newborn calf serum (Serologicals Corporation, Norcross, GA, USA) at 37°C in 5% $CO_2$ for 48 h. To avoid contamination by Gram-positive bacteria and fungi, 20 μg/ml of vancomycin, and 10 μg/ml of amphotericin B were added to all plates, and as expected, had no effect on *H. pylori* growth (36). Spontaneously occurring $Str^R$ mutants were selected by inoculating ~$10^{10}$ *H. pylori* or *H. cetorum* cells on TSA medium containing streptomycin (10 μg/ml), as described (29); the *H. pylori* $Str^R$ mutants have an A128G substitution in *rps12*, resulting in K43R that confers the resistance phenotype (10). $Str^R$ *H. pylori* transformants were selected on BS or BL plates with streptomycin (20 μg/ml) added.

### DNA techniques

Standard molecular techniques for DNA preparation, cloning, and PCR were used (37). Chromosomal DNA was prepared, as described (38), from *H. pylori* cells of each strain after 48 h growth on two agar plates. PCR was performed by standard methods with 30 cycles (see Supplemental Table 2 for primers) in a reaction volume of 50 μl containing 0.5 U of *Taq* (Qiagen, Valencia, CA), 1.5 mM $MgCl_2$ and 200 ng of each primer (Supplemental Table 2). All PCR products were electrophoresed at 120V for 60 min through agarose gels, with ethidium bromide. The desired band was extracted from the gel, the DNA was purified, using the Gel Extraction Kit (Qiagen), and then stored at –20° until ready for use.

### DNA Sequencing

For analysis of specific sequences surrounding the *rps12* loci in *H. pylori* and *H. cetorum*, PCR products were generated with relevant primers (Supplemental Table 2), then purified using the QIAquick gel extraction kit (Qiagen). These products then were examined using sequences obtained from both strands, utilizing an automated Applied Biosystems sequencer in the New York University Cancer Center Core Laboratory, and analyzed using Sequencer 3.1.1 (Gene Code Corp., Ann Arbor, MI, USA).

### Natural transformation

*H. pylori* cells, grown for 48 h on two TSA plates with 5% sheep blood agar, were harvested into 1 ml of phosphate-buffered saline (PBS), centrifuged at 8,500 *g* for 5 min, and the pellet resuspended in 300 μl PBS. In the standard procedure, each transformation mixture, consisting of ~$10^8$ *H. pylori* cells in 25 μl of elutant and 30 ng of donor DNA, was spotted onto a TSA plate. Preliminary studies showed that 20 ng of *H. pylori* chromosomal DNA per 25 μl of *H. pylori* cells is saturating (10). Plates were incubated for 24 h at 37°C in 5% $CO_2$; then the transformation mixture was harvested from the plate surface into 1 ml of PBS. These suspensions were subjected to serial 10-fold dilutions, and 100 μl aliquots were inoculated onto (nonselective) TSA plates and onto (selective) BS agar plates with 10% newborn calf serum and 20 μg/ml streptomycin, and plates were incubated for 5 days at 37°C in 5% $CO_2$. Transformation frequency was calculated as the number of $Str^R$ colonies per recipient cfu per microgram of donor DNA. Transformation frequencies were normalized for the number of $Str^R$-conferring mutations per 30 ng of donor DNA. To determine the time interval necessary for DNA to be secluded into a DNase-protected state, natural transformation experiments were performed as above, but included 3 μl DNase/buffer solution (Qiagen) added to the transformation mixture at differing time points. For control transformations, an equal volume of buffer solution was added to the mixture.

### Preparation of single-stranded and double-stranded transforming DNA

To compare the relative efficiencies of single-stranded (SS) and double-stranded (DS) DNA templates to transform *H. pylori*, an 800 bp PCR product encompassing *rpsL* from $Str^R$ strain 26695 was molecularly cloned into phagemid M13 in both orientations; DS (plasmid) and SS (phage) DNA was harvested from *E. coli* cells or their supernatants, according to standard M13 methods (37). A parallel M13 construct also was designed using the identical protocol, but including an 800-bp PCR product encompassing *rpsL* from $Str^S$ strain 26995, and both DS and SS DNA products were harvested for use as control DNA. Confirmation of the correct inserts and standardization of DNA concentrations was done by sequence analysis and by fluorometry, respectively.

### Competition inhibition assays

To characterize DNA concentration-dependent rate-limiting steps in natural transformation of *H. pylori* cells, we first determined whether transformation frequency is a saturable phenomenon in the experimental system employed. Increasing concentrations of 800 bp PCR products amplified from *H. pylori* strain 26695 $Str^R$ cells were added to wild-type 26695 cells to determine the quantity of DNA necessary to saturate the transformation process. In subsequent experiments, wild-type 26695 cells were transformed with mixtures of "hot" (containing $Str^R$ marker (A128G) *rpsL*) and "cold" (wild-type, without $Str^R$ marker) PCR products. The marked and unmarked DNA fragments were mixed together in equimolar ratios or at 10- or 50-fold excess, and then added simultaneously to the wild-type *H. pylori* cells. Calf thymus DNA (Sigma-Aldrich, St. Louis, MO, USA), and 800 bp PCR

products amplified from an irrelevant 26695 locus (*mutS*), and from *rpsL* from an *Escherichia coli* K12 strain, were used in other experiments to compete with the marked PCR product conferring $Str^R$ from 26695 *rpsL*.

## RESULTS

### Frequency of transformation of *H. pylori* strain 26695 by PCR products and chromosomal DNA

Since imported mosaics in *H. pylori* are much smaller than other bacteria (16), we sought to determine the smallest length of DNA that could be used to transform strain 26695. Cells from a $Str^R$ 26695 mutant (26695-$Str^R$) were used as a source of transforming chromosomal DNA, as well as a template to obtain 50 to 4400 bp PCR products that contained the A128G *rpsL* mutation (**Fig. 1*A***). Under the conditions used, each of the PCR products that carried the A128G *rpsL* transformed cells of wild-type strain 26695 to $Str^R$, whereas control PCR products induced no transformation as expected (data not shown). The transformation frequencies varied directly with the length of the transforming DNA (Fig. 1*B*); there was a nearly 2 $\log_{10}$ difference in frequency between the 4400 bp PCR product and the chromosomal DNA used as a positive control. Although products of 100 and 50 bp transformed the recipient strain, there was $>2$ $\log_{10}$ difference from a 224 bp product. The results of these experiments provide a standardized procedure in which *H. pylori* cells can be reproducibly transformed by PCR products of known length. These findings were consistent with observations involving other organisms (39, 40) that transformation efficiency for very short fragments was much reduced compared with longer fragments.

### Interval required for expression of transformed phenotype

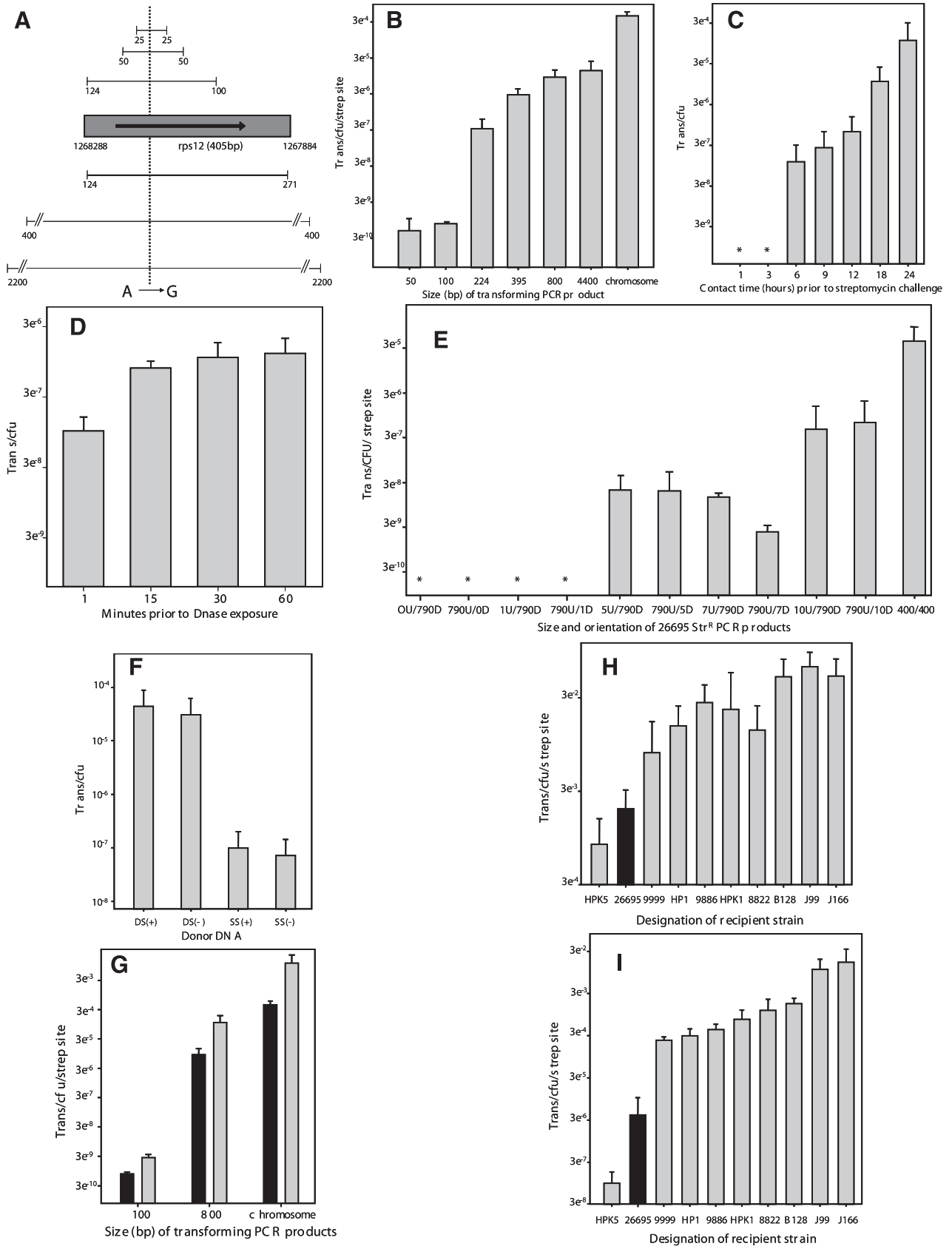
Transformation is an *in vivo* phenomenon, constrained by physical limitations; in the stomach, peristaltic gastric flow (41) limits contact time between free DNA and recipient *H. pylori* cells. To better understand *H. pylori* transformation dynamics, we sought to define the time required for transformed cells to express a detectable phenotype, in this case streptomycin-resistance. To address this question, we varied the duration of contact of recipient wild-type 26695 cells with the transforming DNA, an 800 bp rps12 PCR product amplified from $Str^R$ 26695 cells, prior to inoculation of the transformation mixture onto streptomycin-containing media. No transformants were observed from the mixtures with $\leq$3 h of contact before cells were exposed to the antibiotic selection (Fig. 1*C*). However, transformants were observed in all experiments with $\geq$6 h contact before the antibiotic challenge. Transformation efficiency increased by $\sim$1 $\log_{10}$ for each added 6 h of contact (through 24 h) before exposure to the antibiotic selection. These data, indicating $\sim$3 $\log_{10}$ increased transformation efficiency as *H. pylori* incubation on the nonselective plates lengthened, is consistent with cell replication under these conditions, permitting cycles of incorporation of the DNA substrate conferring antibiotic-resistance (12). On the basis of these studies, we used a 24-h period before exposure to the antibiotic to optimize phenotypic expression of the selected allele, with minimal contamination risk (data not shown).

### Interval required for DNA uptake

We now were prepared to determine the duration of contact necessary for DNA uptake in *H. pylori* 26695 cells. In these experiments, DNase was added at differing times

**Figure 1.** DNA substrate characteristics for *H. pylori* natural transformation. Chromosomal DNA or PCR products amplified from homologous $Str^R$ cells were used to transform cells of a wild-type $Str^S$ *H. pylori* strain to $Str^R$. Each transformation experiment included negative controls in which no DNA was added and in all cases yielded no detectable transformants ($6\times10^{-8}$). *A)* Representation of PCR products used as substrate in transformation experiments. The dashed line indicates the location of the A128G point mutation in codon 43 of *rps12*, resulting in a lysine arginine change conferring $Str^R$ (D. A. Israel *et al.*, unpublished results). The location of the PCR products in relation to the location of the point mutation (nucleotide 1268162 in the 26695 genome) is indicated by the hatched bar showing the genomic coordinates (30). Transformation used the following substrates: *B)* Transformation used chromosomal DNA and 50–4400 bp PCR products (see *A*). *C)* Contact time required for expression of selected ($Str^R$) phenotype after natural transformation of wild-type *H. pylori* strain 26695 with 800 bp (400/400) PCR products. The recipient cells were exposed to the transforming DNA for 1 to 24 h, and then the cells were plated to streptomycin-containing media. *D)* Duration of contact needed for DNA uptake event. After incubation of cells with the transforming 800 bp PCR product conferring $Str^R$, DNase was added at varying intervals (1–60 min), and the remainder of the transformation protocol was completed. *E)* PCR products of $\sim$800 bp with the $Str^R$ mutation with 400 bp upstream and downstream, or 790 bp downstream and 0, 1, 5, 7, or 10 bp upstream, and identical fragments with inverse orientation. *Experiment yielded no detectable transformants per $10^8$ cells. *F)* DNA from coding (+) and noncoding (−) strands (SS) of M13 containing an *rps12* 800bp PCR product, as well as double-stranded (DS) plasmid DNA containing the same PCR product; for the DS DNA, (+) and (−) to orientation with reference to the origin of replication. SS DNA from M13 with a PCR product from wild-type *rps12* ($Str^s$) yielded no transformants (not shown). *G)* GCR products (100 and 800 bp) amplified from isogenic $Str^R$ cells of strains 26695 (black columns) and HPK5 (gray columns), and isogenic chromosomal DNA. *H)* Chromosomal DNA from $Str^R$ cells of strain 26695 was transformed into 26695 (homologous) and nine other (homeologous) recipient strains. The black column indicates the homologous transformation. *I)* PCR products (800 bp) amplified from $Str^R$ cells of strain 26695 were transformed into 26695 (homologous) and into nine other (homeologous) recipient strains. The black column indicates the homologous transformation.

to the transformation mixture to remove free (noncell-associated) DNA. The transforming substrate again was the 800 bp rps12 PCR product amplified from $Str^R$ strain 26695. Each experiment included negative controls in which no transforming DNA was added, and in all cases no transformants were detected (frequency $<10^{-8}$). Transformation was essentially complete after DNA contact for 15 min before DNase addition (Fig. 1*D*). That



DNA uptake was already well underway within 1 min of contact is consistent with the presence of the specialized comH-dependent uptake system (23). For positive controls, in which no DNase was used, transformants were detected at the expected frequencies (data not shown).

### Transformation of *H. pylori* by fragments with asymmetric flanking sequence length

Since the prior studies (Fig. 1*B*) indicated that length of the sequence flanking the selectable marker is a determinant of transformation frequency, because of interstrain restriction barriers, we next examined the constraints related to symmetry around the transforming allele. Experiments were conducted using an 800 bp PCR product in which the point mutation (A128G) conferring $Str^R$ was equidistant (400/400 bp) from each end, or using ~800 bp asymmetric PCR products with the $Str^R$ mutation 790 bp, and 0, 1, 5, 7, or 10 bp from the downstream and upstream ends, respectively. Thus, in these experiments, the total length of the transforming fragment remained essentially constant, but the length of one of the flanking sequences was progressively shortened. To determine whether orientation in relation to the origin of replication affected recombination rates, we also examined fragments with the inverse asymmetries. Each experiment included negative controls in which no transforming DNA was added, and in all cases, no transformants were detected (frequency $<10^{-8}$). For the experiments using asymmetric PCR products with 0, 1, or 3 bp flanking DNA, no transformed 26695 cells were detected. The other asymmetrical PCR products with longer short arms transformed strain 26695, but at frequencies (>2 $\log_{10}$) lower than that of the symmetrical PCR product of similar size (Fig. 1*E*). These results indicate that with sufficient homology on the opposite flank, as few as 5 bp flanking the selectable alleles are sufficient for recombination of the transforming fragment, although at much reduced efficiencies. The same phenomena were observed when the asymmetry had the opposite orientation in relation to the chromosomal origin of replication, indicating independence from lagging or leading strand differences during DNA replication (Fig. 1*E*).

### Transformation of *H. pylori* by single-stranded or double-stranded DNA

*Haemophilus influenzae* and *Neisseria gonorrhoeae*, two other naturally competent bacteria, are transformable by both single-stranded (SS) and double-stranded (DS) DNA (42, 43). Although homologous recombination ultimately involves SS DNA invading the host cell double helix to form a D-loop, the relative efficiencies of DS and SS DNA for earlier events in *H. pylori* transformation had not been defined. Since *H. pylori* cells possess numerous active strain-specific restriction-modification (RM) systems that target DS DNA (4, 5, 25, 26, 30, 31), this question is of particular biological relevance. To address this question, we used the ability of the phagemid M13 to provide otherwise identical DS or SS DNA molecules (37). DNA from the 800 bp *rps12* PCR product conferring $Str^R$ was introduced into the coding or noncoding strand of M13, each version of the phage (SS) or plasmid (DS) DNA was purified, and then used to transform wild-type 26695 cells. The transformation efficiencies of both SS forms of DNA were similar ($\sim 6\times10^{-5}$ transformants), also indicating that strand invasion has no bias with relation to the leading or lagging strand (Fig. 1*F*). As expected, (control) SS DNA from M13 with a PCR product from wild-type ($Str^s$) *rps12* yielded no transformants (not shown). The DS plasmid DNA in both orientations transformed *H. pylori* cells at a nearly 3 $\log_{10}$ greater frequency than did the SS DNA. These data demonstrate that *H. pylori* cells can be transformed by both DS and SS DNA but that the pathway for DS DNA is highly (~1000-fold) facilitated.

### Homologous and homeologous transformation of wild-type *H. pylori* strains

Most, but not all, *H. pylori* isolates are competent (11), which requires activity of its specific type IV-like uptake system involving *comH* (13, 23, 44). We next sought to determine whether the ability to undergo transformation differs among *H. pylori* strains known to be competent. Chromosomal DNA from spontaneously $Str^R$ cells of strains HPK5 and 26695 (Supplementary Table 2) were used as sources of transforming DNA and as a template for 100 and 800 bp *rps12* PCR products. Whether homologous chromosomal DNA or PCR product DNA was used for transformation, HPK5 cells were consistently (0.7–1.3 $\log_{10}$) more efficiently transformed than 26695 cells (Fig. 1*G*). As expected, for both strains, transformation by homologous chromosomal DNA was the most highly efficient, but differences in transformation frequency between the two strains were similar for each of three types of DNA substrate. These experiments confirm the initial studies of strain 26695 in another strain.

We next examined homeologous transformation, since there is known to be interstrain variation of conserved genes in *H. pylori* (27, 31, 45). Examination of the 800-bp region flanking the *rpsL* A128G among the nine strains studied shows 94.7% to 98.3% (mean 96.4%±2.3) variation in pairwise comparisons, an extent typical for *H. pylori* (4). The 800 bp PCR product and chromosomal DNA from strain 26695 were used to transform eight other wild-type *H. pylori* strains (Supplemental Table 1). Transformation with strain 26695 donor DNA showed that the ability to undergo recombination varies among the isolates, ranging from nearly 2 $\log_{10}$ differences for chromosomal DNA (Fig. 1*H*) to over 5 $\log_{10}$ differences for PCR products (Fig. 1*I*). Cells of strain 26695 were less competent for transformation by its homologous chromosomal or PCR product DNA than were most of the other strains exposed to

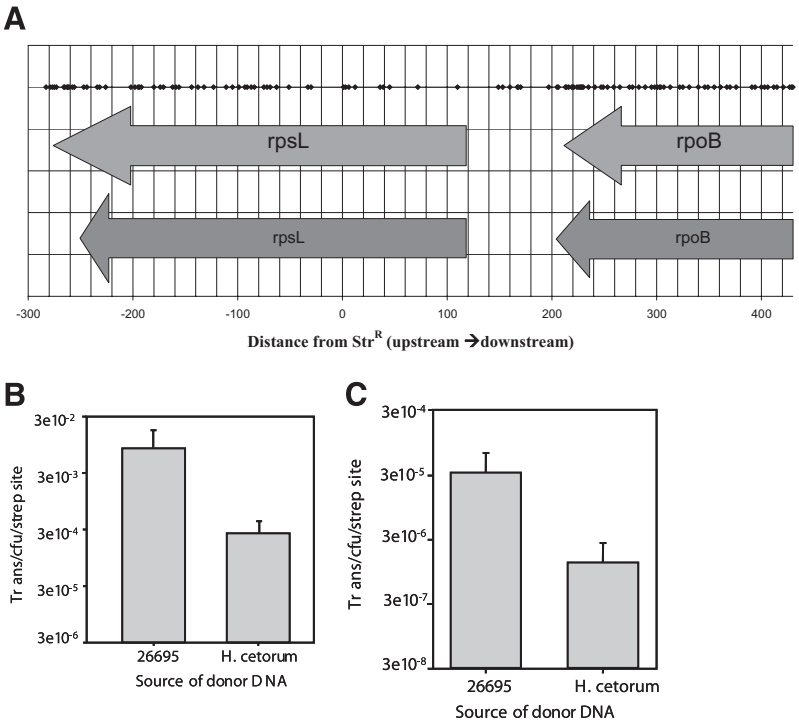



**Figure 2.** Interspecies transformation in *H. pylori*. Transformation of wild-type *H. pylori* 26695 cells to $Str^R$ with isogenic *H. pylori* and *H. cetorum* DNA. *A)* Location of single nucleotide polymorphisms (SNPs) between 26695 and *H. cetorum* within a 716-bp region surrounding the $Str^R$ mutation, indicated as 0 on the abscissa. Upstream and downstream of A128G are indicated by negative and positive signs, respectively, on the abscissa. In the top line, each point denotes the distance from the $Str^R$ mutation of a SNP between the two species. The *rpsL* and *rpoB* ORFs are shown as arrows, reflecting their orientation in *H. pylori* (middle line) and *H. cetorum* (bottom line). In *H. cetorum*, the stop codons of *rpsL* and *rpoB* occur 20 bp downstream and 7 bp upstream, respectively, of the corresponding codons in 26695. $Str^S$ *H. pylori* 26695 recipient cells were incubated with chromosomal DNA from strain 26695 (isogenic) and *H. cetorum* (heterologous) (*B*), or 800bp PCR products amplified from *rpsL* locus from $Str^R$ cells of strain 26695 and *H. cetorum* (*C*). Each transformation experiment included negative controls in which no DNA was added, and in all cases, it produced no detectable transformants.

homeologous DNA. The enhanced transformation by homeologous DNA among the strains provides evidence that the differences are mechanism based rather than sequence based and indicate a very broad range among these naturally competent cells. The competence of the strains (frequency of transformation) for these two markedly different forms (800 bp unmethylated PCR product and chromosome) of homeologous substrate DNA showed essentially the same rank order, indicating that fundamental strain-specific mechanistic differences exist, that are independent of transforming fragment size and of DNA methylation. In total, these results (Fig. 1*G–I*) show that even highly competent *H. pylori* cells differ in their ability to be transformed by homologous and homeologous DNA.

### Interspecies transformation in *H. pylori*

Since non-*H. pylori* bacteria, or at least their DNA, are present in or transiting the human stomach (46), we next sought to determine the susceptibility of *H. pylori* to non-*pylori* DNA. Since in other bacteria, a minimum extent of homology is necessary for the formation of the donor-recipient DNA complex, interspecies transfer is largely limited to members of the same genus (1). Because interspecies transformation may occur preferentially at conserved loci (47), we examined a 716-bp region surrounding the $Str^R$ mutation in *rpsL* from *Helicobacter cetorum*. This sequence was aligned with the published *rpsL* sequences of *H. pylori* strain 26695 (30) and *Campylobacter jejuni* strain 11168 (48) to determine the extent of conservation (**Fig. 2*A***). The mutation conferring the $Str^R$ mutation in *H. cetorum* was A128G in *rpsL*, exactly as determined for strain 26695. In the immediate region surrounding the $Str^R$ mutation, there is 89% (356/400) nucleotide identity between *H. pylori* strain 26695 and *H. cetorum*. For the remaining 316 bp flanking the $Str^R$ mutation that was examined, there was only 77% identity between the two species. Chromosomal DNA from spontaneously $Str^R$ forms of strains 26695 and *H. cetorum* (Supplemental Table 2) was used as a source of transforming DNA, as well as for templates for PCR amplification of 800-bp products. As expected, homologous chromosomal or PCR product DNA transformed strain 26695. Both the *H. cetorum* chromosomal DNA and the PCR product amplified from *H. cetorum* DNA also were capable of transforming the 26995 *H. pylori* cells, but the homologous DNA transformed at $\sim$1.5 $\log_{10}$ higher frequency (Fig. 2*B,C*). As expected, chromosomal DNA transformed at higher frequency than did the PCR products. *C. jejuni rpsL* has 76% identity to *H. pylori* strain 26695 *rpsL*. Attempts to transform *H. pylori* 26695 cells with either chromosomal or PCR product DNA from $Str^R$ *C. jejuni* cells yielded no transformants (per $10^8$ cells), even using DNA quantities >1000 ng for transformation. These data provide evidence that, as expected, *H. pylori* transformation is most efficient with *H. pylori* sequences but can function with sequences from other *Helicobacter* species.

### Saturability of *H. pylori* natural transformation

Since transformation is *Helicobacter* DNA-specific, understanding whether the ability of *H. pylori* to be transformed can be saturated with excess DNA will help define rate-limiting steps. In prior studies, 600 fg of chromosomal DNA was sufficient for the transformation of $10^8$ *H. pylori* cells to be detected, with 6 ng providing maximum efficiency ($\sim 10^{-3}$ transformants/cfu) (10). For *H. influenzae*, maximum transformation

efficiency ($7\times10^{-3}$ transformants/cfu) was similarly provided by 5–10 ng of chromosomal DNA (49–51). To more fully characterize the relationship between donor DNA concentration and transformation efficiency in *H. pylori*, wild-type ($Str^S$) 26695 cells were naturally transformed with 3 to 6,000 ng of the 800 bp *rps12* PCR product amplified from $Str^R$ 26695 cells. Transformation rates steeply increased between 3 and 300 ng of donor DNA and showed saturation at $\sim10^{-4}$ transformants/cfu with ≥ 30 ng of PCR product (**Fig. 3*A***). That the system could be reproducibility saturated using PCR product DNA permits examination of the specificity of uptake using competition studies.

### Competition-inhibition assay of natural transformation in *Helicobacter pylori*

Competition assays were used to determine whether *H. pylori* transformation is specific for *H. pylori* DNA. Wild-type cells of strain 26695 were incubated with a constant saturating amount (300 ng) of 800 bp *rps12* PCR product amplified from $Str^R$ cells of 26695 (Fig. 3). This transforming DNA was mixed with increasing amounts of "cold" DNA (with no detectable phenotype), from unrelated, or from $Str^S$ *H. pylori* strains, to determine whether transformation could be effectively competed. Neither calf thymus DNA nor *E. coli* (*rps12*) DNA showed any competition with the *H. pylori rpsL* DNA that encoded $Str^R$, even at 1000-fold excesses (Fig. 3*B, C*), indicating specificity of uptake in *H. pylori*. In contrast, "cold" PCR products of both the identical (*rpsL* from $Str^S$ cells) and unrelated (the *mutS* locus) *H. pylori* DNA both effectively competed with dose-related effects (Fig. 3*D, E*). These results confirm and extend our observation (Fig. 3*A*) that natural transformation in *H. pylori* is saturable. Since PCR products do not include *H. pylori*-specific methylation patterns, these results indicate that *H. pylori* and foreign DNA are differentiated at the level of primary DNA sequence.

## DISCUSSION

In other naturally competent bacteria, transformation frequency, even for point mutations, depends on the length of the donor DNA. *Neisseria gonorrhoeae* cells can be transformed by fragments ≥ 615 bp, encoding single point mutations (43). In *Bacillus subtilis*, shearing of donor DNA from (mean) 28.5 kb to 4.5 kb decreased transformation by 100-fold, and further shearing to 2 kb reduced transformation efficiency another 100-fold (40). In *Pseudomonas stutzeri*, transformation frequencies did not differ when donor chromosomal DNA fragments were between 10 and 60 kb, but decreased 10-fold for fragments between 1 and 10 kb (39). For *Acinetobacter calcoaceticus*, transformation frequency was directly related to transforming fragment (between 40

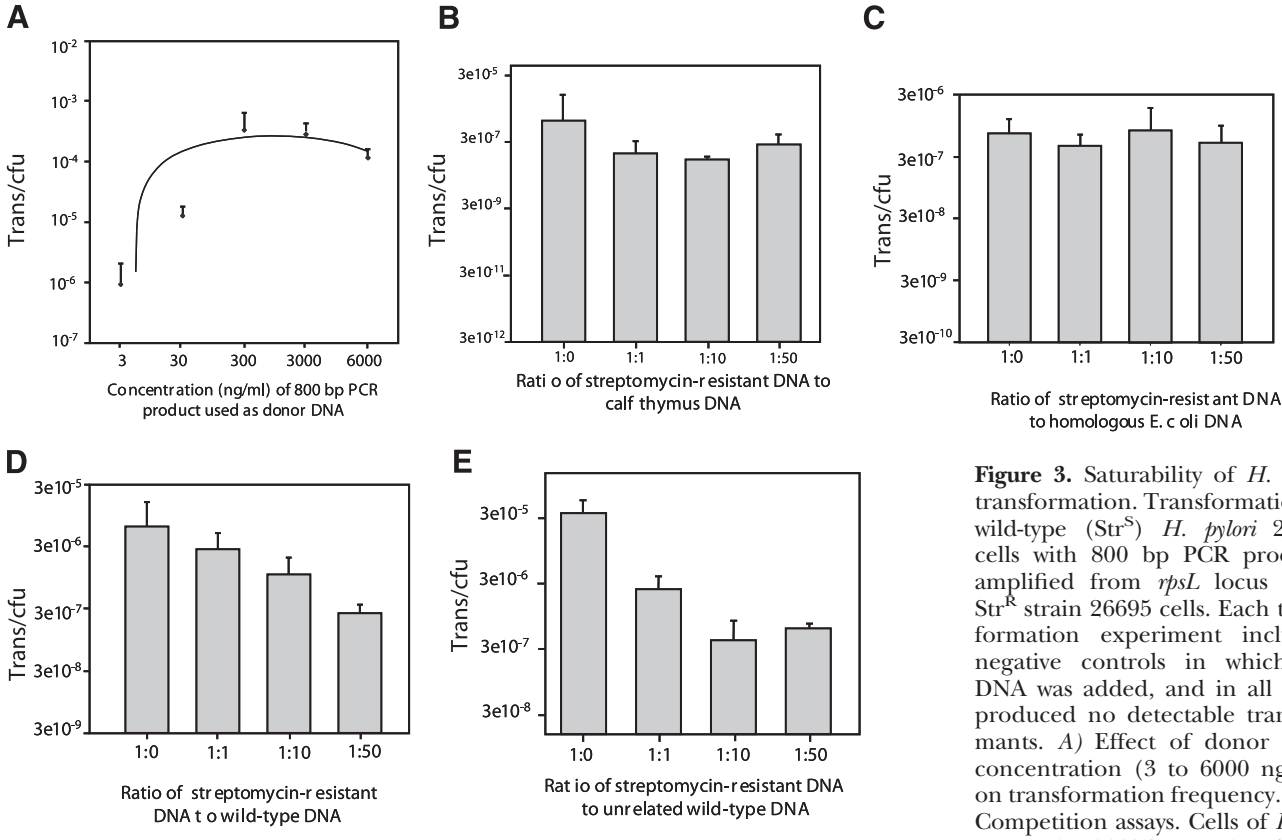



**Figure 3.** Saturability of *H. pylori* transformation. Transformation of wild-type ($Str^S$) *H. pylori* 26695 cells with 800 bp PCR products amplified from *rpsL* locus from $Str^R$ strain 26695 cells. Each transformation experiment included negative controls in which no DNA was added, and in all cases produced no detectable transformants. *A)* Effect of donor DNA concentration (3 to 6000 ng/μl) on transformation frequency. *B–E)* Competition assays. Cells of *H. pylori* strain 26695 were incubated with constant amounts (300 ng) of *rpsL* PCR product amplified from 26695 $Str^R$ cell templates, with varying amounts of competing DNA: calf thymus DNA (*B*); 800 bp PCR product of *rpsL* locus from *E. coli* (*C*); 800 bp PCR product of *rpsL* locus from wild-type 26695 cells (*D*); 800 bp PCR product from locus unrelated to *rpsL* from wild-type 26695 cells (*E*).

and 10 kb) length (2). Naturally occurring imported mosaics of *H. pylori* DNA are smaller (median 417 bp) than in other competent bacteria (16), suggesting that the transformation of *H. pylori* cells by short DNA segments may be more efficient; our finding that a 50-bp DNA fragment can reproducibly transform *H. pylori* is consistent with this hypothesis. Small DNA fragments generally are susceptible to exonucleases (28), but the absence of RecBCD in *H. pylori* (30) may permit transformation by short fragments.

The use of asymmetric donor DNA fragments showing that *H. pylori* requires minimal homology (≥5 bp) on one flank to enable recombination, indicates a highly efficient mechanism, reflecting the lack of mismatch repair (MMR) in *H. pylori* (52). In *E. coli* and other organisms, the MMR proteins MutS1, MutL, and MutH recognize single bp mismatches between donor and host DNA during chromosomal integration, which inhibits the integration event from reaching completion (53). Organisms with defective MMR require less homology between donor and host DNA for transformation (54, 55); the highly efficient recombination of homeologous *H. pylori* DNA (Fig. 1*B–I*), is consistent with its lack of MMR (30, 52). Our finding that *H. pylori* cells can be transformed by *H. cetorum* DNA indicates even greater heterology (89% homologous) than has been previously shown involving *H. acinonychis* DNA (56), which has 94% identity (57).

Although wild-type *H. pylori* strains generally are transformable (15, 21, 23, 24, 29), they each contain strain-specific restriction modification (R-M) systems (4, 5, 25, 26) that limit incoming DNA, based on the length of the transforming allele (4, 27). Restriction endonuclease (RE) recognition and cleavage of double-stranded (DS) DNA impedes transformation by DS but not SS substrates (4–6). Prior experiments showing that DNA methylated in an *H. pylori* strain-specific manner is more efficient than unmethylated DNA for transforming *H. pylori* cells (4, 27, 58) indicate that DS intermediates are at the least transiently present after DS donor DNA is internalized. Other competent bacteria, including *H. influenzae and N. gonorrhoeae*, are transformable by single-stranded DNA (42, 43), but DS DNA is substantially more efficient for *H. pylori* cells (Fig. 1*F*). Thus, *H. pylori* has evolved a system for maximal transformation efficiency by DS DNA substrates, which are subject to restriction. Although transformation ultimately requires substrate SS DNA to invade the host duplex, this adaptation provides *H. pylori* cells and populations (3) substantial surveillance over incoming DNA, favoring substrates from the most closely related cells in a gradient based on DNA methylation specificities. The low transformation efficiency of SS DNA substrate also is consistent with the lack of bacteriophage sequences in *H. pylori* genomes (59–61).

With DS DNA substrates, strain-specific restriction would be predicted to curtail the size of the transforming region flanking the selectable allele, consistent with the calculated small average size of recombining fragments from heterologous strains (16). The lack of differences in transformation frequency by the coding or noncoding M13 strands, or by the asymmetric PCR products of opposite orientation, indicates that *H. pylori* transformation is not polarity-specific, in contrast to that of *S. pneumoniae* (62, 63).

The generation of diversity through recombination may contribute to persistent *H. pylori* colonization of individual hosts, by creating a pool of variants from which best-fit organisms can be selected (3). With chromosomal DNA transforming at frequencies $>10^{-2}$ per cell, intergenomic recombination could play a major role in generation of diversity. Cocolonization of hosts with multiple *H. pylori* strains is apparently common (15, 64, 65), and the efficient mechanisms for horizontal gene transfer that have been characterized *in vitro* (10, 11, 21, 23, 66, 67); and this manuscript), if occurring *in vivo*, are sufficient to explain the observed high levels of homoplasy in *H. pylori* genes, and an essentially panmictic *H. pylori* population structure (15).

The ability to generate diversity through intergenomic recombination differs markedly among *H. pylori* isolates (Fig. 1*G–I*), reflecting interstrain differences in magnitude of DNA uptake, recombination protein activity, and/or restriction barriers (14, 15, 18, 27, 52). Expression of *dprA, recA, comB, comE3,* and *comH* all play important roles in *H. pylori* competence (21, 23, 66, 68, 69), but sequence differences in promoters, ORFs, or accessory genes that affect their function could explain the diverse transformation frequencies observed. Among the 10 *H. pylori* isolates tested, the relatively conserved rank-order of transformation frequencies by (methylated) chromosomal and (unmethylated) PCR product DNA (Fig. 1*H, I*), indicates that the transformation process is DNA methylation-(restriction-)independent, and is an intrinsic strain-specific property. Since the transforming allele is only a single nucleotide, and only short total and flanking lengths are required for successful transformation (Fig. 1), the effects of restriction are minimized, consistent with prior findings (27). This property permits a plastic system for rapidly increasing representation (*via* transformation) of a highly favored allele in a population when selective pressures change, if the DNA donors and recipients have a well-conserved genetic scaffold. Thus, transformation by DNA is maximized from the most closely related strains in a mixed population, representing a conservative strategy of sexual exchange.

Are *H. pylori* cells always maximally ready to take up DNA from other *H. pylori* cells? Particular times in the cell growth cycle appear particularly conducive for DNA uptake (12, 16). The present experiments, in which transformation rates increased with incubation of recipient cells with substrate DNA is consistent with these observations, suggesting that *H. pylori* natural transformation has saturable, rate-limiting steps, that with cell division, become available to participate in further transformation events.

We found that transformation of *H. pylori* by chromosomal DNA was clearly saturable, despite conflicting prior studies (70). That only a fraction of the *H. pylori*

cells were transformed, even with donor DNA in large excess, indicates the existence of other rate-limiting phenomena. The specificity of competition suggests the presence of an *H. pylori* DNA-specific rate-limiting step. A prior *in silico* study that used a frequent word analysis provided evidence that *H. pylori* DNA does not possess uptake sequences (71), but those data may not have been sufficient to reach that conclusion (72). Our observations are consistent with the presence of specific DNA sequences involved in enhancing the binding and uptake of homospecific DNA, similar to those present in other Gram-negative bacteria, such as *Haemophilus* and *Neisseria* spp (71), including *1)* the $>2$ $\log_{10}$ increase in transformation efficiency from the shortest (50 and 100 bp) to the longer (224 bp) transforming fragments; *2)* the absolute barriers to non-*Helicobacter* DNA as transformation substrate; *3)* the ability of the non-*rpsL H. pylori* DNA to compete as transformation substrate; and *4)* parallel competition differences involving chromosomal DNA and PCR products suggests that transformation substrate recognition is not related to methylation, but to primary sequence.

In summary, we provide evidence that natural transformation in *H. pylori* varies in efficiency between strains but is saturable and sensitive to the length, symmetry, and strandedness of the substrate DNA. The tractable experimental system of *H. pylori* competence provides a model to explore the tensions between recombination and genomic integrity (3, 4, 18) and to improve understanding of gene flow dynamics in natural bacterial populations. FJ

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