Restriction of DNA encoding selectable markers decreases the transformation efficiency of Helicobacter pylori

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Abstract

Helicobacter pylori populations recovered from the human stomach display extensive recombination and quasispecies development, and this suggests frequent exchange of DNA between different strains in vivo. In vitro, however, most H. pylori strains display restriction to the uptake of non-self DNA, as measured using selectable markers, regardless of their competency for transformation with self DNA. We have examined the effect of different selectable markers on double-crossover recombination efficiencies in three reference strains (1061, 26695 & SS1) and one clinical isolate (CHP1) of H. pylori. All strains were efficiently transformable to kanamycin or chloramphenicol resistance by using self-genomic DNA from isogenic mutants bearing the aphA3 or cat cassettes, respectively. However, strains 26695 and CHP1 showed a 3–5-log reduction in transformation efficiency by non-self recombinant DNA containing aphA3, when compared to cat. Strain 1061 readily accepted either cassette, and strain SS1 was poorly tolerant of any non-self DNA. Genome-wide random mutagenesis of these strains was only achievable with a selectable marker that allowed high transformation efficiency. Digestion of 32P-labelled cassettes by H. pylori lysates mirrored the transformation results and indicated that in some strains these cassettes are the targets of enzymatic restriction.

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1. Introduction

Helicobacter pylori is a Gram-negative pathogenic bacterium which is highly adapted to survival in the human stomach. H. pylori populations are genetically capricious in vivo and colonizing strains may develop over time into an accumulation of quasispecies due to failed cleansing by inefficient selective sweeps or sequential bottle necks [1]. In addition to a high mutation frequency [2], the natural competency and ensuing panmictic nature of this bacterium allows rapid evolutionary responses in a harsh and variable milieu such as the stomach [3,4]. Accordingly, H. pylori has evolved strong protective barriers against the negative consequences that may arise from continuous DNA uptake. The numerous restriction-modification (R–M) systems of H. pylori provide potent protection from deleterious DNA and each strain bears its own combination of functional systems [5]. These systems also appear to limit the success in H. pylori of genome-wide mutagenesis
approaches frequently used for the study of pathogenic bacteria [6].

Although H. pylori efficiently exchanges DNA in vivo, there is no evidence of plasmid-borne or cassette-based antibiotic resistance genes unlike other naturally competent pathogenic bacterial genera such as Neisseria [7], Haemophilus [8] or the closely related Campylobacter [9]. Antibiotic-resistant H. pylori isolates are increasingly common, however all mechanisms of resistance identified to date have been attributed to substitutions or deletions within chromosomally encoded genes [10–13]. Therefore the antibiotic resistance cassettes used as selectable markers for H. pylori mutagenesis are not native to any Helicobacter species, and potentially may contain sequences that are particularly susceptible to attack by H. pylori defenses. Whilst recombination is readily observed in H. pylori strains [6] and other Helicobacter species [14] using genomic DNA from various spontaneously antibiotic resistant Helicobacter strains, this is not the case when resistance cassettes are used as markers of successful double-crossover recombination [6].

In this study, we used various recombinant DNA species to investigate if the antibiotic resistance cassettes commonly used for H. pylori mutagenesis, namely aphA3 and cat from Campylobacter coli, could influence the transformation efficiency of different H. pylori strains. In addition, we examined the susceptibility of these cassettes to restriction by these strains.

2. Materials and methods

2.1. Bacterial strains and culture

Helicobacter pylori wild-type strains used in this study were 26695 [15], 1061 [16], SS1 [17] and a low passaged duodenal ulcer-associated clinical isolate, designated CHP1, isolated from a patient in Melbourne. All H. pylori culture was performed at 37 °C under microaerophilic conditions (Microaer/Genbox system; BioMerieux, Marcy l’Etoile, France). H. pylori wild-type strains were routinely cultured on Columbia agar (Oxoid, Basingstoke, UK) containing 7% saponin-lysed horse blood and Dent selective supplement (Oxoid), referred to as Dent plates. H. pylori mutant strains were grown on Dent plates supplemented with kanamycin sulphate (Km; 20 μg ml⁻¹; Roche Diagnostics, Mannheim, Germany) or chloramphenicol (Cm; 20 μg ml⁻¹; Sigma–Aldrich, St. Louis, MO). Brain heart infusion broth (BHIB, Oxoid) containing 5% heat inactivated fetal bovine serum and Dent supplement was used routinely for broth culture of H. pylori strains. Escherichia coli strain DH5α was grown on Luria Agar supplemented with Km (50 μg ml⁻¹) or Cm (20 μg ml⁻¹) as required.

2.2. Recombinant DNA techniques

Standard DNA manipulations and heat shock transformation of E. coli were performed as described [18]. All cloning were performed using the pGEM-T easy cloning vector (Promega, Madison, WI). Plasmid DNA was prepared using Wizard Plus SV miniprep kit (Promega) and PCR amplicons were purified using Qiaquick Gel Extraction or PCR purification kits (Qiagen Pty Ltd, Clifton Hill, Aus). All enzymes were purchased from Promega unless otherwise stated.

2.3. Donor DNA

Campylobacter coli antibiotic resistance cassettes conferring resistance to Km (aphA3) or Cm (cat) were obtained from pJMK30 and pAV35, respectively [19]. These cassettes were amplified by PCR using primers listed in Table 1 to introduce the same restriction site sequences flanking each cassette, thus allowing the cassettes to be used interchangeably. The modified resistance cassettes were cloned and expressed in E. coli DH5α to confirm maintenance of the resistance phenotypes in vivo.

The H. pylori genes targeted for mutagenesis and subsequently used for comparisons of transformation efficiency are listed in Table 2. When appropriate restriction sites were present in the target genes, these were used as sites for the introduction of the resistance cassettes. Where no suitable restriction sites were present, we used an overlap extension PCR protocol for integration of the resistance cassettes (Fig. 1). PCR was conducted using Vent DNA polymerase (New England Biolabs, Beverly, USA) and proper primer design directed orientation of the cassette. Target genes were

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Oligonucleotide primers used in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer</td>
<td>Sequence (5’ → 3’)</td>
</tr>
<tr>
<td>KanREfor</td>
<td>C GGATCCb GAATTCb AGATCT</td>
</tr>
<tr>
<td>KanRErev</td>
<td>G GGATCCb TGATCAb GAATCb</td>
</tr>
<tr>
<td>CatREfor</td>
<td>C GGATCCb GAATCc AGATCt</td>
</tr>
<tr>
<td>CatRErev</td>
<td>G GGATCCb TGATCda GAATC</td>
</tr>
<tr>
<td>Hpl[gene]AF</td>
<td>GGGCTTTAGTCCCTAAnGGG</td>
</tr>
<tr>
<td>Hpl[gene]RF</td>
<td>AGATCTb GAATCb GGATCc G</td>
</tr>
</tbody>
</table>

Restriction sites are underlined.

a BamHI site.
b EcoRI site.
c BglII site.
d BstI site.

Primer sequences included a 12-bp variable region specific for each target gene of H. pylori.
amplified with gene-specific primers (HP[\text{gene}]F and HP[\text{gene}]R). These primers were combined with primers HP[\text{gene}]AR or HP[\text{gene}]AF (Table 1), respectively, in nested PCR to delete a portion of the target gene, and introduce adaptor sequences homologous to each restriction site region flanking the resistance cassettes. The two resulting amplicons were combined with resistance cassette DNA and two rounds of amplification were used to generate recombinant DNA. Firstly, the template DNA primed the extension to generate full-length products spanning the three template species (25 \mu l reaction volume, 5 cycles). Secondly, primers HP[\text{gene}]F and HP[\text{gene}]R were added in an additional 25 \mu l reaction mix for amplification of the interrupted gene by conventional PCR.

Donor isogenic and heterogenic mutant genomic DNA (gDNA) was purified from mutant strains derived using the recombinant DNA. Preparation of donor DNA for generation of random insertional mutant libraries was performed as described previously [20]. Briefly, intact gDNA from each wild-type strain was digested using Bst 98I or HindIII and ligated under conditions conducive to circularization. The DNA loops were fragmented further using Sau3AI and ligated with plasmid-derived \textit{aphA3} or \textit{cat} excised with BamHI to generate gDNA fragments containing randomly integrated resistance cassettes (random gDNA).

### 2.4. Natural transformation of \textit{H. pylori}

Routine transformation of \textit{H. pylori} patches on Dent plates was conducted as described elsewhere [21] using 100–200 ng PCR product or plasmid DNA, and 1–2 \mu g intact or random gDNA. For determination of transformation efficiencies, BHIB (20 ml) was inoculated with \(5 \times 10^6\) colony forming units (cfu) of plate-grown \textit{H. pylori} and incubated microaerobically for 7 h. Cultures were transferred into 24-well plates (1 ml per well) and donor DNA was added to each well. Cultures were incubated overnight before concentrating or diluting the broths as required for quantitative culture on selective and non-selective agar plates.

### 2.5. In vivo stability of antibiotic cassettes

\(^{32}\text{P}\)-labelled KanR\textit{Erev} and Cat\textit{REfor} primers were used with cold Kan\textit{REfor} and Cat\textit{RErev}, respectively, to generate end-labelled resistance cassettes. Plate-grown \textit{H. pylori} was harvested into ice-cold 10 mM Tris–HCl [pH 8.0] (T8 buffer), washed twice and resuspended in T8 buffer to give \(10^6\) cfu ml\(^{-1}\). Cells were
sonicated (1 min on ice) and the lysates were used immediately without any centrifugation. Reactions containing 32P-labelled DNA (3000 counts per min), 5 μg herring sperm DNA and H. pylori sonicate (equivalent to 1.5 × 10^6 cfu) in One-Phor-All buffer (Amersham Biosciences, Buckinghamshire, UK) were incubated for 5 or 30 min at 37 °C. Control reactions containing T8 buffer instead of H. pylori sonicates were incubated for 30 min. Reactions were stopped with EDTA (50 mM final concentration) followed by phenol/chloroform extraction before separation of DNA fragments using a 5% polyacrylamide, non-denaturing gel.

3. Results

3.1. Transformation efficiencies of H. pylori strains using self and foreign donor DNA differ depending on the selectable marker

When conducting mutagenesis of various H. pylori genes by allelic exchange using plasmid- or PCR-derived recombinant DNA, we observed reproducible differences in transformation efficiencies, depending on the recipient H. pylori strain and the selectable marker embedded within the donor DNA (Table 3). Three types of foreign-DNA tolerance were observed in the H. pylori strains used: strain 1061 readily accepted any recombinant DNA containing the cat gene but were poorly tolerant of foreign DNA containing the aphA3 gene; and SS1 was poorly tolerant of any recombinant DNA species. These differences were not influenced by the target gene, or degree of non-H. pylori methylation of the donor DNA: E. coli-methylated plasmid DNA and unmethylated PCR amplicons elicited the same cassette preferences. When gDNA from isogenic mutants was used to re-derive a selection of mutants from fresh wild-type cultures, these discrepancies were not observed and transformation was highly efficient for all strains using either selectable marker. This confirmed that both aphA3 and cat are expressed and the gene products were fully functional in all strains. In addition, these results eliminated codon bias and the difference in size between the aphA3 (1351 bp) and cat (835 bp) cassettes as possible explanations for differences in transformation efficiency.

When strains were transformed with gDNA from mutants made in other H. pylori strains, the differences were again evident. Moreover, random mutant libraries of the 26695 and CHP1 strains could only be achieved using cat as the selectable marker, and no transformants were obtained using SS1 as the recipient strain with either cat or aphA3 as the selectable marker (random gDNA, Table 3). As the protocol used for mutant library construction utilized fragments of self-gDNA flanking selectable markers excised from E. coli-derived plasmid DNA, the only non-self region of the donor DNA susceptible to attack by R–M systems of the host strain was the antibiotic-resistance cassette.

3.2. Resistance cassette bias is independent of DNA uptake and recombination efficiencies of individual strains

To investigate whether the embedded selectable marker alone influenced transformation of the 26695 and CHP1 strains, we compared transformation efficiency using standardized, PCR-derived donor DNA. These samples differed only in the selectable marker (hopZ::aphA3 or hopZ::cat). In addition to individual transformations, the PCR products were also co-transformed to correct for any differences in DNA uptake and recombination efficiencies between cell populations. The results showed a 3–5-log reduction in the transformation efficiency of strains 26695 and CHP1 when aphA3 was used as the selectable marker compared to the cat gene (Table 4). This difference was

<table>
<thead>
<tr>
<th>Donor DNA type</th>
<th>H. pylori strains by various donor DNA species containing aphA3 or cat cassettes</th>
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</thead>
<tbody>
<tr>
<td></td>
<td><strong>H. pylori</strong> recipient strain <strong>cat</strong></td>
</tr>
<tr>
<td></td>
<td>1061 26695 CHP1 SS1</td>
</tr>
<tr>
<td>PCR product</td>
<td>++ +++++ ++ +++++ + ++ +</td>
</tr>
<tr>
<td>Plasmid DNA</td>
<td>+++ ++ +++ +++++ + ++</td>
</tr>
<tr>
<td>Isogenic gDNA</td>
<td>+++ +++ +++ +++++ +++++ +</td>
</tr>
<tr>
<td>Heterogenic gDNA</td>
<td>+++ +++ + +++</td>
</tr>
<tr>
<td>Random gDNA</td>
<td>+++ +nd + ++</td>
</tr>
</tbody>
</table>

a Isogenic gDNA: from a mutant derived in the recipient strain; Heterogenic gDNA: from a mutant made in a strain other than the recipient strain; Random gDNA: gDNA from the recipient strain containing randomly inserted plasmid-derived resistance cassette.

b This data is compiled from a series of non-concurrent experiment and represent most, but not all, combinations of the recipient strain and donor DNA species (Table 2). The results are semi-quantitative and represent the typical magnitude of antibiotic resistant colonies achieved by multiple samples of each donor DNA type. Antibiotic-resistant cfu per transformation: -, zero cfu; +, 1–10^2 cfu; ++, 10^2–10^3 cfu; ++++, 10^3–10^4 cfu; +++++, 10^4–10^5 cfu; ++++++, 10^5–10^6 cfu; nd, not determined.
not dependent on the amount of DNA added although the transformation efficiency with hopZ::cat was slightly higher when added to the cells alone compared to when in direct competition with hopZ::aphA3. Additionally, co-transformations spread on double selective plates containing both Km and Cm yielded no transformants for any of the strains indicating that the recombination of hopZ::cat was site-specific. In contrast, strain 1061 co-transformed with hopZ::aphA3 and sabB::cat yielded numerous double mutants (data not shown). No antibiotic resistant colonies grew on any control plates indicating the absence of spontaneous resistance or exhaustion of the bacteriostatic Cm as explanations for the discrepancy.

### Table 4
Transformation efficiencies of *H. pylori* strains to Km\(^R\) or Cm\(^R\) by identical PCR derived DNA, differing only in the selectable marker (hopZ::aphA3 and hopZ::cat), co-transformed at varying ratios

<table>
<thead>
<tr>
<th>Ratios of hopZ::aphA3 to hopZ::cat DNA(^a)</th>
<th><em>H. pylori</em> strain 1061</th>
<th><em>H. pylori</em> strain 26695</th>
<th><em>H. pylori</em> strain CHP1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Km(^R) Efficiency</td>
<td>Km(^R) Efficiency</td>
<td>Km(^R) Efficiency</td>
</tr>
<tr>
<td></td>
<td>cfu(^b)</td>
<td>cfu</td>
<td>cfu</td>
</tr>
<tr>
<td>120 ng:120 ng</td>
<td>4890 1.5 × 10(^{-3})</td>
<td>170,000 3.1 × 10(^{-3})</td>
<td>390 2.3 × 10(^{-5})</td>
</tr>
<tr>
<td>120 ng:40 ng</td>
<td>4560 9.7 × 10(^{-4})</td>
<td>69,800 3.9 × 10(^{-3})</td>
<td>110 2.0 × 10(^{-5})</td>
</tr>
<tr>
<td>120 ng:10 ng</td>
<td>3570 7.6 × 10(^{-4})</td>
<td>15,000 3.3 × 10(^{-3})</td>
<td>22 1.6 × 10(^{-5})</td>
</tr>
<tr>
<td>120 ng:1 ng</td>
<td>4880 1.0 × 10(^{-3})</td>
<td>1880 4.2 × 10(^{-3})</td>
<td>2 1.4 × 10(^{-5})</td>
</tr>
<tr>
<td>hopZ::aphA3 only (120 ng)</td>
<td>7750 1.7 × 10(^{-3})</td>
<td>1 1.9 × 10(^{-6})</td>
<td>0 NA</td>
</tr>
<tr>
<td>hopZ::cat only (1 ng)</td>
<td>0 NA</td>
<td>4480 9.9 × 10(^{-3})</td>
<td>12 8.6 × 10(^{-5})</td>
</tr>
</tbody>
</table>

\(^a\) hopZ::aphA3 – 2497 bp; hopZ::cat – 1891 bp.

\(^b\) Antibiotic-resistant cfu per ml transformation (Km\(^R\), kanamycin resistant; Cm\(^R\), chloramphenicol resistant).

\(^c\) Transformants (viable cell) \(^{-1}\) (µg donor DNA) \(^{-1}\); NA, not applicable.

#### 3.3. Resistance cassettes are a direct target of some *H. pylori* R–M systems

The in vivo stability of PCR-derived aphA3 and cat cassettes following uptake by *H. pylori* was demonstrated by incubating \(^3\)P-labelled resistance cassettes with sonicated *H. pylori* (Fig. 2). After incubation for 30 min, strain 26695 had efficiently cleaved aphA3 at numerous sites but left detectable amounts of intact cat, and SS1 had rapidly fragmented both cassettes leaving no obvious intact product. With strain 1061, intact forms of both cassettes were still detectable after 30 min incubation reflecting the high tolerance of this strain for foreign DNA. These results emulated the transformation data and confirmed that in some *H. pylori* strains the resistance cassette provides a target for endonucleases of the recipient strain.

### 4. Discussion

The minimum requirements for double-crossover homologous recombination to succeed in a detectable form are adequate specific sequence for target recognition and a fully functional selectable marker. Therefore, whilst digestion within sequences flanking the selectable marker is inconsequential if the remaining region is sufficient to facilitate recombination, fragmentation within the selectable marker immediately renders the donor DNA useless for the purpose of allelic exchange. This barrier has little impact on site-directed mutagenesis of most *H. pylori* strains, which is generally achievable even with relatively low transformation efficiency. However, genome-wide mutagenesis approaches require highly efficient uptake and recombination of donor DNA for success. In addition, whilst allelic exchange is achievable with linear DNA, insertional mutagenesis mediated by single-crossover recombination is totally dependent on plasmid integrity for success. Similarly,
complementation of mutants using plasmid-born copies of functional genes also requires plasmid DNA to be intact for replication and persistence [6]. However, recircularization of plasmid DNA following natural transformation becomes less achievable with each additional specific recognition site present in the construct. Thus, the findings of this study provide an explanation for the failure of many standard molecular approaches dependent on the use of selectable markers to derive and characterize mutants of *Helicobacter* species.

Although the mechanism of natural transformation in *H. pylori* is yet to be fully elucidated, the requirement of a type IV secretion system homologue containing a comB locus-encoded core complemented by several additional putative components or accessory proteins appears essential. A model of the transformation machinery proposed previously [22] has been largely supported by topological mapping of the comB-encoded proteins [23] and includes as yet unidentified nucleases involved in fragmentation and processing of the DNA before uptake. In the absence of identified *H. pylori* specific DNA uptake sequences [24], these nucleases may also function as a mechanism for restricting incoming DNA before entry to the cell and subsequent exposure to intracellular R–M components [25]. The lysates used in this study for examining digestion of aphA3 and cat contained both the cytoplasmic and membrane fractions. Consequently, digestion may have been mediated by membrane-associated, periplasmic or cytoplasmic nucleases. No attempt was made to delineate at which stage digestion might occur during natural transformation.

Numerous studies investigating *H. pylori* R–M systems have provided important insights into gene regulation [26], evolution [27] and virulence [28] of this diverse pathogen. Additional studies have proposed roles for these systems in restricting the length of DNA transmissible by horizontal transfer [5] and protocols for overcoming these systems during mutagenesis of *H. pylori* strains [29]. However, these latter studies have used only the aphA3 cassette as a marker of transformation efficiency, which is unfortunate given the rapid destruction of this gene by several *H. pylori* strains. Although the present study included only four strains, it is likely that many other *H. pylori* strains also specifically and rapidly digest the aphA3 cassette. This could directly influence the results of studies using this cassette to clarify the biological importance of the R–M and natural competency systems of *Helicobacter* species. Such efforts may be better served by using counterselectable markers (such as sacB) to introduce deletions of varying lengths, and measuring successful transformation by determining the reversion to wild-type following the introduction of *H. pylori*-only sequence.

In conclusion, we have shown that for some *H. pylori* strains, the use of cat for selection of transformants in preference to aphA3 may enhance the transformation efficiency to a level sufficient for successful genome-wide random mutagenesis in strains previously considered unsuitable for such an approach. However, certain strains, such as SS1, whilst being highly naturally competent are intolerant of non-self forms of both the aphA3 and cat cassettes.

Acknowledgements

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References


