ELSEVIER

FEMS Immunology and Medical Microbiology 44 (2005) 213–219

FEMS Immunology and Medical Microbiology

www.fems-microbiology.org

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

Rebecca J. Gorrell [a,b,*], Ji Yang [a], Johannes G. Kusters [c], Arnoud H.M. van Vliet [c], Roy M. Robins-Browne [a,b]

[a] *Department of Microbiology & Immunology, University of Melbourne, Parkville Vic. 3010, Australia*
[b] *Microbiological Research Unit, Murdoch Childrens Research Institute, Parkville Vic. 3052, Australia*
[c] *Department of Gastroenterology & Hepatology, Erasmus MC-University Medical Center, Rotterdam, The Netherlands*

Received 20 August 2004; received in revised form 19 October 2004; accepted 20 October 2004

First published online 8 December 2004

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 ^{32}P-labelled cassettes by *H. pylori* lysates mirrored the transformation results and indicated that in some strains these cassettes are the targets of enzymatic restriction.

Keywords: *Helicobacter pylori* mutagenesis; Restriction endonuclease; Natural transformation; Antibiotic resistance

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

* Corresponding author. Tel.: +61 3 8344 9926; fax: +61 3 8344 8276.
E-mail address: rebecca.gorrell@mcri.edu.au (R.J. Gorrell).

0928-8244/$22.00
doi:10.1016/j.femsim.2004.10.019

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 passage, duodenal ulcer-associated clinical isolate, designated CHP1, isolated from a patient in Melbourne. All *H. pylori* culture was performed at 37 °C under microaerophillic 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 clonings 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 1
Oligonucleotide primers used in this study

Primer	Sequence (5′ → 3′)
KanREfor	C GGATCC[a] GAATTC[b] AGATCT[c] AAGCTACCAAGACGAAGAG
KanRErev	G GGATCC[a] TGATCA[d] GAATTC[b] AGACATCTAAATCTAGGTAC
CatREfor	C GGATCC[a] GAATTC[b] AGATCT[c] TCGGCGGTGTTCCTTTCCAAG
CatRErev	G GGATCC[a] TGATCA[d] GAATTC[b] GCCCTTTAGTTCCTAAAGGG
Hp[gene]AF	GAATTC[b] TGATCA[d] GGATCC[a] C (Hp gene specific sequence)$_{12mer}$[e]
Hp[gene]RF	AGATCT[c] GAATTC[b] GGATCC[a] G (Hp gene specific sequence)$_{12mer}$[e]

Restriction sites are underlined.
[a] *Bam*HI site.
[b] *Eco*RI site.
[c] *Bgl*II site.
[d] *Bcl*I site.
[e] Primers included a 12-bp variable region specific for each target gene of *H. pylori*.