Transformation of *Helicobacter pylori* by chromosomal metronidazole resistance and by a plasmid with a selectable chloramphenicol resistance marker

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Most strains of *Helicobacter pylori* are naturally competent for uptake of chromosomal DNA. Transformation frequencies for streptomycin resistance or rifampicin resistance markers ranged from 1×10^{-4} to 1×10^{-3} per viable cell using a plate transformation procedure. Transformation of a metronidazole resistance marker (Mtr^R) was demonstrated when either a laboratory-derived mutant or a Mtr^R clinical isolate were used as the source of donor DNA. Mtr^R was transformed at a frequency of 3×10^{-5} per viable cell. All *H. pylori* strains tested produce large amounts of DNAase, which may reduce DNA available for transformation. Four *H. pylori* plasmids were isolated. DNA fragments from *H. pylori* plasmids were deleted or rearranged when cloned in pUC19 and propagated in *Escherichia coli* DH5 α . An *H. pylori* plasmid, pUOA26 which contained a chloramphenicol resistance determinant from *Campylobacter coli*, was constructed in *H. pylori*. This plasmid could be successfully introduced by natural transformation only into *H. pylori* recipients which contained a homologous resident plasmid. Transformation of pUOA26 into plasmid-free cells of *H. pylori* was achieved by electroporation. Transformation frequencies were 1×10^{-4} transformants per viable cell when plasmid DNA was isolated from the same strain; however, introduction of pUOA26 DNA derived from *H. pylori* 8091 into a different *H. pylori* strain, NCTC 11639, resulted in transformation at much lower frequencies ($\leq 1 \times 10^{-7}$ per viable cell). Changes in plasmid restriction patterns were also noted when pUOA26 was isolated from *H. pylori* NCTC 11639, suggesting the presence of at least two different DNA restriction and modification systems in *H. pylori* which may interfere with uptake of plasmid DNA.

Introduction

Helicobacter pylori, a Gram-negative microaerophilic bacterium, has been recognized as a major factor in the development of gastritis and ulcer disease in man (Dick, 1990). Little information is available concerning the genetics of *H. pylori*, and only a few genes have been cloned and sequenced (for a review, see Taylor, 1992). Nedenskov-Sorensen *et al.* (1990), however, observed transformation to streptomycin resistance in 22 out of 25 *H. pylori* strains tested. Kleanthous *et al.* (1991) characterized a 1.5 kb plasmid, pHPK255, from *H. pylori*. Nucleotide sequence analysis revealed that ORF1 of pHPK255 has significant similarities to Rep proteins of some plasmids from Gram-positive bacteria which replicate via the 'rolling-circle' mechanism. However, as

yet no gene transfer systems have been developed for genetic analysis and *in vitro* DNA manipulation in *H. pylori*.

In this study, we confirmed that most *H. pylori* strains are naturally transformable and constructed an *H. pylori* plasmid with a chloramphenicol resistance (Cm^R) marker, which is expressed in *H. pylori*. We also obtained evidence for restriction and modification of DNA in *H. pylori*.

Methods

Strains, plasmids and culture conditions. The strains of *Helicobacter* species used in this study are listed in Table 1. Most of these strains were clinical isolates from the University of Alberta hospital (Taylor *et al.*, 1987). *Campylobacter coli* UA585 (Wang & Taylor, 1990a) and *Escherichia coli* DH5 α (Sambrook *et al.*, 1989) were also used. Plasmids used and constructed in this study are shown in Table 1. Plasmids employed were pUC19 derivatives (Yanisch-Perron *et al.*, 1985). Plasmid pHPK255 was kindly provided by H. Kleanthous (Kleanthous *et al.*, 1991). Plasmid pUOA23 was constructed by inserting a 920 bp *Bss*HII–*Eco*RI DNA fragment from pUOA20, containing the *C. coli* Cm^R gene (Wang & Taylor, 1990b) which had been blunt-ended with

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Table 1. *Helicobacter* strains and plasmids used in this study

Strain or plasmid	Resistance phenotype*	Plasmid content (size, kb)†	Source or reference
<i>H. pylori</i>			
UA759	—	ND	H. Lior, Canada
UA802	—	ND	Canada
UA831‡	—	ND	Canada
UA841	—	pUA841 (2.7)	Canada
UA875	—	pUA875A (5.5)	Canada
		pUA875B (ca 15)	
UA877	—	ND	Canada
UA883	Mtr ^R	pUA883 (ca 12)	Canada
UA889	—	ND	Canada
C2R	Mtr ^R	ND	J. A. Washington, USA (Knapp <i>et al.</i> 1991)
NCTC 11639	—	ND	UK
JAH60	—	ND	Canada
JAH60 (Mtr ^R)	Mtr ^R	ND	This study
8091‡	Mtr ^R	ND	Glaxo, UK
8091R‡	Mtr ^R Rif ^R Str ^R	ND	This study
<i>H. mustelae</i>			
F251	NT	NT	Glaxo, UK
F251 (Rif ^R)	Rif ^R	NT	Glaxo, UK
Plasmid			
pUC19	Ap ^R	(2.7)	Yanisch-Perron <i>et al.</i> (1985)
pHPK255	—	(1.5)	Kleanthous <i>et al.</i> (1991)
pUOA20	Cm ^R	(4.8)	Wang & Taylor (1990a, b)
pUOA23	Ap ^R Cm ^R	(3.6)	This study
pUOA26	Cm ^R	(4.5)	This study

* Abbreviations: Ap^R, ampicillin resistance; Cm^R, chloramphenicol resistance; Mtr^R, metronidazole resistance; Rif^R, rifampicin resistance; Str^R, streptomycin resistance. Most of the *H. pylori* strains were also tested for chloramphenicol, kanamycin and tetracycline sensitivity, and none were resistant. —, Sensitive to all antibiotics tested; NT, not tested.

† ND, Not detected by the modified alkaline lysis method; NT, not tested.

‡ These strains are able to undergo limited growth in a 7% CO₂ incubator.

DNA polymerase Klenow fragment, into the blunt-ended *Aat*II site of pUC19. Plasmid pUOA26, which consisted of an *H. pylori* plasmid containing the *C. coli* Cm^R gene, was also constructed in this study (Fig. 3).

H. pylori and *H. mustelae* strains were cultured in BHI-YE (3.5%, v/v, Brain Heart Infusion, 0.2% yeast extract, 5%, w/v, horse serum) at 36 °C in anaerobic jars containing gas generating kits (Oxoid) which provide a residual concentration of about 6% (v/v) O₂ and 10% (v/v) CO₂. Cold plates or broth were inoculated with *H. pylori* and placed immediately in jars. Although a few *H. pylori* strains were able to grow in a 37 °C incubator containing 7% CO₂, cells became non-viable in approximately 4 d. *C. coli* was grown in Mueller–Hinton medium at 37 °C with 7% CO₂, whereas *E. coli* was grown in LB (Luria–Bertani) broth (Sambrook *et al.*, 1989) at 37 °C. When necessary, the medium was supplemented with ampicillin (100 µg ml⁻¹), chloramphenicol (10–15 µg ml⁻¹), metronidazole (16 µg ml⁻¹), rifampicin (20 µg ml⁻¹) or streptomycin (10–20 µg ml⁻¹).

Plasmid and chromosomal DNA isolation. Chromosomal DNA and *E. coli* plasmid DNA were prepared by conventional methods (Sambrook *et al.*, 1989). *H. pylori* plasmid DNA extractions were carried out by method of Birnboim & Doly (1979) with the following

modifications. Cells from plate cultures were washed once in TES (10 mM-Tris, pH 8.0, 1 mM-EDTA, 100 mM-NaCl) and were suspended in solution I (40 mM-Tris, pH 7.9, 2.5 mM-EDTA, 15%, w/v, sucrose) without the addition of lysozyme. Plasmid DNA was purified by the 'Magic miniprep' (Promega) procedure, or by caesium chloride-ethidium bromide density gradient centrifugation. *H. pylori* cells collected from plate cultures gave higher yields of plasmid DNA than those from broth cultures.

Transformation by natural competence. *H. pylori* transformation was performed on an agar surface unless otherwise specified. Briefly, fresh recipient cells (30 h growth on BHI-YE agar) were plated at high density on BHI-YE agar on areas of 8–10 mm in diameter, and incubated for 5 h. Aliquots of DNA [0.3–2 µg in 8 µl BHI-YE broth or TE buffer (10 mM-Tris, 1 mM-EDTA, pH 8.0)] were spotted directly onto the inoculated agar, and incubation was continued for 12 h. The DNA-treated cells were scraped up with a loop in a small volume of TE buffer and were then spread on BHI-YE agar containing appropriate antibiotics with or without dilution, to select transformants. Transformation frequencies were calculated from the number of transformants divided by the number of viable cells at the time of plating on selective agar. Some transformation experiments were performed in

broth using the procedure described by Nedenskov-Sorensen *et al.* (1990). In these experiments 5–10 µg DNA ml⁻¹ was added. Transformation frequencies were calculated as described above. *C. coli* transformation was performed on Mueller–Hinton agar as described previously (Wang & Taylor, 1990a).

Detection of DNA hydrolysis. The production of DNAase by *H. pylori* cells was detected using toluidine blue-DNA agar as described by Lior & Patel (1987).

DNAase activity was also assayed using ³²P-labelled DNA. λ DNA (1 µg) was digested with *Hind*III, end-labelled with [³²P]dCTP, and added to 0.3 ml *H. pylori* UA831 cells in BHI-YE broth (ca 10⁸ cells ml⁻¹). The culture was incubated at 37 °C in 7% CO₂. Samples were withdrawn and transferred to ice, and EDTA was added to a concentration of 25 mM. The samples were extracted with phenol/chloroform. Five microlitres of each supernatant was subjected to electrophoresis in a 1.2% agarose gel.

DNA labelling, Southern hybridization and detection of single-stranded plasmid DNA. Restricted plasmid DNA was excised from low-melting-point agarose gel and radiolabelled with [³²P]dCTP by the standard random primer labelling method (Feinberg & Vogelstein, 1983). Southern transfer (Sambrook *et al.*, 1989) was carried out with pure nitrocellulose membrane (Bio-Rad). Hybridization was carried out in 5 × SSC (1 × SSC is 0.15 M-NaCl, 0.015 M-sodium citrate, pH 7.0), 25% (w/v) formamide, 50 µg denatured salmon sperm DNA ml⁻¹, 5 × Denhardt's solution and ³²P-labelled DNA at 37 °C. The filter was washed under low-stringency conditions: four times in 2 × SSC, 0.1% SDS at 45 °C for 30 min each, and exposed to X-ray film for 4 h. The same filter was then washed under high-stringency conditions: two times in 1 × SSC, 0.1% SDS at 50 °C for 15 min each, and two times in 0.1 × SSC, 0.5% SDS at 63 °C for 2 h and 30 min, respectively, and exposed overnight.

The method used to detect single-stranded plasmid DNA by Southern hybridization was described by te Riele *et al.* (1986). Total DNA was extracted from exponential phase bacteria. The DNA was then either denatured or left non-denatured before transfer to nitrocellulose and hybridization to pUOA26 as described above.

Electroporation of *H. pylori*. Electroporation of *H. pylori* was performed using the Gene Pulser electroporation apparatus (Bio-Rad) by a method similar to that used for transformation of *C. jejuni* (Miller *et al.*, 1988). Plate cultures were used to inoculate BHI-YE plates. After 18–24 h incubation, cells were collected in 30 ml cold double-distilled water and washed again in 20 ml cold 10% (v/v) glycerol. The pellet was resuspended in 1 or 2 ml 10% glycerol. Plasmid DNA (0.5–2 µg in 5–10 µl TE buffer) was mixed with 0.2 ml cell suspension. The mixture was transferred into a prechilled 0.2 cm cuvette, and subjected to single-pulse electroporation of initial voltage 2.5 kV (12.5 kV cm⁻¹), 25 µF and 600 Ω (τ = 12.5 ms) in parallel. A sample of 10 µl was diluted in cold BHI-YE broth and plated on BHI-YE agar to determine the number of cells that survived electroporation. The suspension was transferred onto a cold plate and incubated for 12 h at 36 °C. The cells were then transferred onto selective media and incubated for 5 d to allow the growth of transformants.

For long-term storage, competent cells in 10% glycerol were frozen in dry ice and stored at -90 °C until needed. Frozen cells were thawed on ice and used immediately for electroporation.

Results

Isolation of antibiotic-resistant mutants

Spontaneous Str^R mutants of *H. pylori* 8091 were isolated by plating fresh 8091 cells onto BHI-YE agar containing streptomycin (10 µg ml⁻¹). The MIC of streptomycin for

these mutants was greater than 1000 µg ml⁻¹. A Str^RRif^R double mutant (designated 8091R) was isolated from one of these Str^R mutants. The MIC of rifampicin for this mutant was greater than 100 µg ml⁻¹. Mutation frequencies for both Str^R and Rif^R in this strain were about 10⁻⁸ per viable cell. Except for 8091, Str^R or Rif^R spontaneous mutants were not obtained using any of eight other *H. pylori* strains. Although several strains were tested for metronidazole resistance, no Mtr^R mutants were obtained, except from *H. pylori* JAH60. The mutation frequency for Mtr^R in this strain was approximately 10⁻³ per cell when plated on BHI-YE agar containing metronidazole (16 µg ml⁻¹). The MIC of this Mtr^R mutant was 60 µg ml⁻¹.

Natural transformation of *H. pylori* by chromosomal markers

Using *H. pylori* 8091R DNA as donor to test for natural competence in broth cultures, we found some *H. pylori* strains which were transformable. The maximum transformation frequency for the chromosomal markers (Str^R or Rif^R) was 1 × 10⁻⁶ per viable cell when broth cultures were used. However, when tested on plate cultures, natural transformation was demonstrated for 10 out of 12 strains. Transformation frequencies for Str^R or Rif^R markers ranged from 1 × 10⁻⁴ to 1 × 10⁻³ per viable cell (Table 2).

Transformation of *H. pylori* by a metronidazole resistance marker was also demonstrated. Both a laboratory-derived mutant of *H. pylori* JAH60 (Mtr^R) and a Mtr^R clinical isolate C2R (Knapp *et al.*, 1991) were used as the source of donor DNA. Transformation frequencies for the Mtr^R marker were about one-tenth those for either Rif^R or Str^R markers (Table 2).

To optimize conditions for transformation of *H. pylori* using 8091R DNA as the donor and JAH60 as the recipient, DNA was added to a JAH60 plate culture at 0–16 h after initial inoculation. Transformants were obtained at frequencies ranging from 4 × 10⁻⁵ to 3 × 10⁻⁴ per viable cell. Highest transformation frequencies were found when DNA was added at 5 and 10 h.

In some naturally transformable bacteria, such as *Haemophilus influenzae*, competence develops when cell division is blocked under conditions that allow continued protein synthesis (Stewart & Carlson, 1986). Therefore, transformation experiments were performed using three different media, BHI-YE agar, Mueller–Hinton agar and supplemented minimal medium agar (M9CA, Sambrook *et al.*, 1989). The latter two media do not support growth of *H. pylori* JAH60. Transformation efficiencies decreased dramatically using Mueller–Hinton and M9CA agar from approximately 3 × 10⁻⁴ to 1.4 × 10⁻⁵ and 8 × 10⁻⁶ transformants per viable cell, respectively. These

Table 2. Natural transformation of *H. pylori* with homologous chromosomal DNA

Donor	Recipient	Transformation frequency per viable cell*		
		Rif ^R	Str ^R	Mtr ^R
<i>H. pylori</i>				
8091R	UA841†	4.0×10^{-4}	4.0×10^{-4}	—
JAH60 (Mtr ^R)	UA841 or NCTC 11639	—	—	3.0×10^{-5}
C2R	UA841 or NCTC 11639	—	—	3.0×10^{-5}

* Transformation was performed three times on BHI-YE agar. Transformation frequencies were determined by spotting 1 µg DNA on to agar inoculated with *H. pylori* cells. The plate was incubated for 12 h, then about 1×10^7 cells were scraped off the agar and plated onto selective medium. Cell number was determined from plate counts in several representative experiments. —, Not applicable.

† Transformation frequencies of Rif^R and Str^R markers were similar to UA841 when using UA759, UA802, UA831, UA875, UA877, UA883, JAH60, NCTC 11639 and 8091 as hosts. Similar transformation frequencies were obtained, although transformation frequencies for the Mtr^R marker were not tested in these strains.

Fig. 1. Degradation of DNA by *H. pylori*. *H. pylori* UA831 cells were incubated with *Hind*III-cut, ³²P-labelled λ phage DNA. Samples were withdrawn at the indicated time intervals (min) and run in a 1.2% (w/v) agarose gel.

Fig. 2. Dependence of transformation frequency on the concentration of donor DNA. The recipient was UA831, and the donor DNA was from 8091R. Transformation was performed in BHI-YE broth. DNA was added at the indicated concentrations. Str^R transformants were selected after 12 h incubation.

results demonstrate that active metabolism in the recipient cell is required for transformation. Transformation efficiencies were decreased by one-half to one-third when cultures were incubated with DNA in a 7% CO₂ incubator instead of in a jar containing 6% O₂ and 10% CO₂ (data not shown). Therefore, actively growing cells appeared more competent than slow growing or static cultures.

The *H. mustelae* strain F251 was also transformed to Rif^R at a frequency of approximately 1×10^{-6} transformants per viable cell using F251 (Rif^R) DNA as

the donor. No interspecies transformation was observed between an *H. mustelae* F251 recipient and Str^R *H. pylori* DNA, nor between a *Campylobacter coli* recipient and Str^R *H. pylori* DNA.

DNAase activity of *H. pylori*

All *H. pylori* strains listed in Table 1 produced large amounts of DNA-hydrolysing activity on toluidine blue-DNA agar. This activity of *H. pylori* was examined using λ phage DNA (Fig. 1). Most of the ³²P-labelled DNA disappeared within 2 min. Hydrolysis of plasmid DNA was also examined. Five micrograms of *E. coli* plasmid pUC19 or *H. pylori* plasmid pUOA26 were added to 100 µl *H. pylori* 8091 broth culture (ca 5×10^7 cells ml⁻¹),

and incubated at 37 °C. Both plasmids were completely linearized in 3 min, and most of the DNA was digested and disappeared from the agarose gel in 20 min. Plasmids pUC19 or pUOA26, which had been cut with *EcoRI*, were digested a little faster than their supercoiled forms.

The transformation frequencies of *H. pylori* UA831 to Str^R by 8091R DNA increased linearly with the donor DNA concentration. The linear plot demonstrates that the DNA concentration was always below the saturation level (Fig. 2). In another experiment, the number of Rif^R transformants obtained with 5 µg DNA was 12-fold higher than that obtained using 0.5 µg DNA which suggests a similar conclusion regarding the saturation level. However, Fig. 2 cannot be regarded as a true dose response curve (i.e. transformation frequency versus DNA concentration) but is rather a measurement of the DNA that escaped the DNA-hydrolysing activity of *H. pylori*.

DNA uptake and competition

DNA from *H. pylori* JAH60 and 8091, *C. jejuni* UA466 and *E. coli* was labelled with ³²P as described previously (Wang & Taylor, 1990a), and used to examine DNA uptake by *H. pylori* 8091 and UA831 either in broth cultures or on plate cultures (approximately 0.1 µg DNA per 10⁸ cells). Less than 0.5 % of the input radioactivity was found to be associated with the cells 20 min after the addition of DNA. Similar quantities of radioactivity from both homologous and heterologous DNA were found to be associated with *H. pylori* cells.

Transformation of UA831 with 0.4 µg 8091R DNA was performed in the presence of five times the concentration of competing DNA from *H. pylori* JAH60 and 8091, *C. jejuni* and *E. coli*. The competing DNA of all three species reduced the transformation frequency by five- to sixfold. Therefore, heterologous DNA competed at almost the same extent with *H. pylori* DNA for uptake.

Construction of *H. pylori* plasmid pUOA26

A cryptic plasmid, pUA841, was isolated from *H. pylori* strain UA841, and purified using Magic minipreps (Promega). The plasmid was digested by *HindIII* to produce a single fragment of 2.7 kb or was partially digested by *EcoRI*. The restricted plasmid was ligated to *EcoRI*- or *HindIII*-cut pUC19, and used to transform *E. coli* DH5α. Six recombinant plasmids were obtained which contained insertions of various sizes. DNA rearrangements were also observed when cloning pUA883 in *E. coli*.

A recombinant plasmid containing 3 kb of pUA841-derived DNA inserted at the *EcoRI* site of pUC19 was

Fig. 3. Physical map of *H. pylori* plasmid pUOA26 isolated from *H. pylori* 8091. Values are in kb.

digested with *EcoRI* and ligated to *EcoRI*-cut pUOA23 which contains a chloramphenicol resistance marker (*cat*) originating from *C. coli* (Wang & Taylor, 1990b). The mixture was used to transform UA841(pUA841) and Cm^R transformants were obtained (180 transformants per 0.3 µg DNA). Plasmids from four transformants were isolated, all contained both pUA841 and a recombinant plasmid containing the *cat* gene. pUA841 was apparently a stable high-copy-number plasmid, whereas the recombinant Cm^R plasmids were relatively unstable. All four transformants became susceptible to chloramphenicol after growth on non-selective media. A small recombinant plasmid (4.5 kb) was chosen to transform *H. pylori* 8091 by electroporation using the Gene Pulser (Bio-Rad) in a 0.4 cm cuvette at 2.5 kV (6.25 kV cm⁻¹) and 25 µF. Twelve Cm^R transformants were obtained (ca 0.5 µg DNA and 1 × 10⁷ recipient cells). Each contained a single plasmid which was named pUOA26.

Plasmid pUOA26 isolated from *H. pylori* 8091 could be cut with *AvaI*, *DraI*, *HindIII*, *MboI* and *Sau3AI* (Fig. 3). The plasmid was cleaved by *EcoRI* into two fragments. The DNA was not cut by the following 22 restriction enzymes tested: *AccI*, *ApaI*, *BamHI*, *BglII*, *BssHII*, *ClaI*, *EcoRV*, *HincII*, *HpaI*, *KpnI*, *MspI*, *NcoI*, *NdeI*, *PstI*, *SalI*, *ScaI*, *SmaI*, *SphI*, *SspI*, *SstI*, *XbaI*, *XhoI*. This list includes *ClaI* which has a recognition site within the *cat* ORF (Wang & Taylor, 1990b). The chromosomal DNAs from *H. pylori* 8091, JAH60 and UA883 were also found to be resistant to *ClaI* digestion. However, the restriction sites shown in Fig. 3 and the results described above applied only to experiments in which pUOA26 was isolated from *H. pylori* 8091. Isolation of the pUOA26 plasmid from *H. pylori* strain NCTC 11639 resulted in acquisition of an *EcoRI* site at approximately 2.9 kb (Fig. 3) and loss of two *HindIII* sites. Additional sites for both *SmaI* and *SstI* were also acquired. Since the relative positions of all original sites remaining was not altered and the plasmid size was unchanged, we concluded that these changes were due to

Table 3. Natural transformation of *H. pylori* by plasmid DNA

Donor	Recipient	Transformation frequency per viable cell*
pUA841:pUA23 (EcoRI)†	UA841(pUA841)	2.0×10^{-5}
	UA831, 8091 or JAH60	$< 1.0 \times 10^{-8}$
pUOA26	UA841(pUA841)	1.0×10^{-4}
	UA831, 8091 or JAH60	$< 1.0 \times 10^{-8}$
	UA875(pUA875A)(pUA875B)	4.0×10^{-7}
	UA883(pUA883)	1.2×10^{-4}
pUA883:pUA23 (SspI:SmaI)‡	UA833(pUA883)	2.5×10^{-5}
	UA875(pUA875A)(pUA875B)	3.0×10^{-7}
	UA831 or 8091	$< 1.0 \times 10^{-8}$

* Experiments were performed twice on BHI-YE agar. Transformation frequencies were determined by spotting 0.5 µg plasmid DNA onto various recipient strains. Transformation frequencies were determined as described in Table 2.

† The ligation mixture of EcoRI-cut pUA841 and pUOA23 was used as donor DNA.

‡ The ligation mixture of SspI-cut pUA883 and SmaI-cut pUOA23 was used as donor DNA.

changes in modification of DNA in this strain rather than being due to deletion and/or rearrangements.

Transformation with plasmid DNA

Natural transformation of *H. pylori* plasmid DNA is summarized in Table 3. Plasmid transformants were never obtained with plasmid-free recipients using any of the ligation mixtures, purified DNA, miniprep DNA or crude cell lysates. When plasmid-containing strains, namely UA841(pUA841) and UA883(pUA883), were used as recipients, transformants were obtained at frequencies of approximately 1×10^{-4} except for UA875(pUA875A)(pUA875B), which was a poor recipient for pUOA26 (Table 3). The results indicate that plasmid transformation in *H. pylori* can only occur when the recipient contains a plasmid with which the incoming plasmid can recombine. Therefore, it is likely that plasmid pUOA26 is more homologous to pUA883 than to pUA875A or pUA875B.

Transformation of pUOA26 into plasmid-free *H. pylori* was achieved by electroporation (Table 4). When the plasmid was transformed into the same strain from which it was isolated, the transformation frequency was approximately 1×10^{-4} per viable cell. In contrast, when the plasmid was transformed into a different strain, the transformation frequency was reduced by more than 1000-fold.

Interspecies plasmid transformation was also tested. Plasmid pUOA26 could transform neither *E. coli* DH5α nor *C. coli* UA585 to Cm^R. Plasmid pUOA23, which

Table 4. Electroporation of pUOA26 plasmid DNA in *H. pylori*

Recipient	Origin of plasmid DNA	Transformation frequency per viable cell*
8091	8091	$1.5 \times 10^{-4}†$
NCTC 11639	NCTC 11639	$1.5 \times 10^{-4}†$
NCTC 11639	8091	1.0×10^{-7}
UA802	NCTC 11639	$< 1.0 \times 10^{-8}$

* All experiments were performed using a voltage of 2.5 kV and 25 µF, resistance in parallel corresponded to 600 Ω with a time constant of 12.5 ms in 0.2 mm cuvettes. Each experiment was performed four times.

† This transformation frequency corresponds to 2×10^3 transformants (µg DNA)⁻¹.

Fig. 4. Hybridization of pHPK255 to other *H. pylori* plasmids. (a) Membrane washed under low stringency conditions. (b) Same membrane washed under high stringency conditions. Lanes: 1, pUOA26; 2, pUA841; 3, pUA875A and pUA875B; 4, pUA883:pUA23; 5, pIHPK255; 6, HindIII-cut pIHPK255; 7, HindIII-cut λ DNA.

consists of an *E. coli* replication origin and a *cat* gene, and plasmid pUOA20, which contains a *Campylobacter* replication origin and the *cat* gene, also did not transform

Fig. 5. Hybridization of pUOA26 to denatured (a) or non-denatured (b) DNA. Lanes: 1, pUA841 inserted in pUC19 at *Eco*RI site isolated from *E. coli* DH5 α ; 2, pUA841 inserted in pUC19 at *Hind*III site isolated from *E. coli* DH5 α ; 3, pUOA841 isolated from *H. pylori* UA841; 4, pUOA26 isolated from *H. pylori* 8091; 5, pUA883 isolated from *H. pylori* UA883; 6, *Hind*III-cut λ DNA.

H. pylori 8091 or NCTC 11639 to Cm^R, either by natural transformation or by electroporation.

Characterization of *H. pylori* plasmids

The *Hind*III-cut 1.5 kb *H. pylori* plasmid pHPK255 (Kleanthous *et al.*, 1991) was isolated from an agarose gel and hybridized to the *H. pylori* plasmids used in this study under low stringency conditions. All *H. pylori* plasmids, but not λ DNA, hybridized to the probe (Fig. 4a). When the same membrane was washed under high stringency conditions, the probe remained hybridized only with itself (Fig. 4b). The results indicate that these plasmids share a certain degree of homology with pHPK255.

The deduced Rep protein of pHPK255 has been shown to have similarities to the Rep proteins from a group of plasmids which replicate by the rolling-circle mechanism such as pADB201 from *Mycoplasma mycoides* subsp. *mycoides* and pLB4 from *Lactobacillus plantarum* (Kleanthous *et al.*, 1991). The replication of these plasmids frequently involves single-stranded intermediates which can be detected by hybridization after Southern transfer under non-denaturing conditions (te Riele *et al.*, 1986). No single-stranded DNA was detected in *H. pylori* cells harbouring either pUA883 or pUA841 and its derivatives (Fig. 5).

Discussion

This study demonstrates that *H. pylori* can acquire resistance to rifampicin and streptomycin, as well as to metronidazole, by natural transformation. Almost all *H.*

pylori strains were naturally competent. Transformation of metronidazole resistance may have some clinical relevance, since it is one component of 'triple therapy'. This is the most effective regime for eradication of *H. pylori* from the gastric mucosa and consists of bismuth, metronidazole, and either amoxicillin and/or tetracycline (Borsch *et al.*, 1988). Metronidazole resistance was found to develop rapidly in some patients who receive triple therapy (Rautelin *et al.*, 1991). Natural transformation might contribute to development of antibiotic resistance by promoting acquisition by *H. pylori* of advantageous genes from other strains or from other species. However, transformation of a rifampicin resistance marker between *H. pylori* and *H. mustelae*, an organism isolated from the gastric mucosa and faeces of ferrets (Fox *et al.*, 1992), was not demonstrated.

H. pylori produces large amounts of DNAase as reported by McNulty & Dent (1987). It is not yet clear if this DNAase is extracellular or cell-membrane-associated and it may play a role in natural transformation, perhaps by processing DNA prior to uptake. However, the low percentage of radioactively labelled input DNA associated with transformed cells also appears to be a consequence of DNAase activity.

No DNAase⁻ mutants of *H. pylori* have been reported to our knowledge. Therefore, this trait is likely to be advantageous to the host cells. The DNAase may be important in nutrition and it could also play a role in pathogenesis, since the DNAase could degrade any available DNA present in mucus covering the gastric epithelial cells, and thereby facilitate colonization. Although natural transformation of chromosomal DNA markers occurred in most *H. pylori* strains, natural transformation of plasmids was not demonstrated. This could be due to DNAase activity on plasmid DNA and/or restriction of incoming plasmid DNA.

Genomic DNA from *H. pylori* isolates displays considerable diversity in electrophoretic analysis in both pulsed field (Taylor *et al.*, 1992) and conventional gels (Majewski & Goodwin, 1988). Among the hypotheses put forward to explain this unusual genomic diversity is the production of various endogenous methylases with associated restriction endonucleases in different *H. pylori* strains (Taylor *et al.*, 1992). These enzymes would methylate nucleotides within the recognition sequences for specific restriction endonucleases.

We found that *H. pylori* chromosomal DNA was resistant to digestion with *Cla*I and partially resistant to *Eco*RI which indicates that DNA modification is occurring in *H. pylori*. Chromosomal DNA from *H. pylori* UA765, NCTC 11639, JAH60 and 8091 was also found to be resistant to digestion with *Eco*47111, *Hpa*II, *Msp*I and *Xho*I (N. Chang & Y. Wang, unpublished results). Furthermore, the methylation systems are likely

to be different among *H. pylori* strains, for example, DNA from *H. pylori* NCTC 11639 and JAH60 was resistant to *Bcl*I digestion, whereas DNA from *H. pylori* 8091 was completely digested by the same enzyme (N. Chang & Y. Wang, unpublished results).

Additional evidence for the presence of unrelated restriction/modification systems in different *H. pylori* strains comes from our studies with pUOA26. This plasmid acquired some restriction sites but lost others after propagation in a different *H. pylori* host strain. Also, electroporation into the strain from which the plasmid was originally isolated was 1000 times more efficient than electroporation into a new host (Table 4).

The *H. pylori* plasmid pHPK255 contains DNA sequence found in plasmids which replicate through a rolling-circle intermediate (Kleanthous *et al.*, 1991). Several *H. pylori* plasmids examined in this study (pUOA26, pUA841, pUA875A, pUA875B and pUA883) hybridized with pHPK255. Although no evidence of single-stranded DNA was seen on gels containing these plasmids, the notion that these plasmids also replicate via an asymmetric rolling-circle mechanism, cannot be ruled out. Plasmids, such as pUB110, which replicate via a rolling-circle mechanism but contain a minus origin, produce almost no detectable single-stranded DNA in either *Staphylococcus aureus*, its natural host, or in *Bacillus subtilis* (te Riele *et al.*, 1986). Therefore, it is likely that the *H. pylori* plasmids we investigated also belong to the rolling-circle group of plasmids.

Only a few recombinant plasmids were obtained after *H. pylori* plasmid pUA841 was transformed into *E. coli* DH α . DNA from pUA841 experienced rearrangements or deletions. Similar observations were reported by Kleanthous *et al.* (1991) in cloning the *H. pylori* plasmid pHPK255 using pUC18 and *E. coli* JM105; however, in that case a single stable recombinant was obtained. Plasmid pHPK255, and probably also pUA841, replicates via the rolling-circle method and uses a single-stranded intermediate. When used as cloning vectors such plasmids sometimes display physical instability as the inserted fragments often carry fortuitous minus origins of replication and are prone to deletions and recombinational rearrangements (Gruss & Ehrlich, 1989). Alternatively restriction/methylation systems discussed above could inhibit the stable inheritance of *H. pylori* DNA in some *E. coli* strains.

Plasmid-mediated antibiotic resistance had not been observed previously in *H. pylori* (Taylor, 1992). In this study, we demonstrated that a *C. coli* *cat* gene can be expressed in *H. pylori*. Plasmid pUOA26 containing the *cat* gene replicates stably in *H. pylori* 8091, even in the absence of chloramphenicol selection, and no plasmid-free variants have been isolated after many generations of growth (K. P. Roos & D. E. Taylor, unpublished

data). This plasmid should be useful for further studies of restriction systems in *H. pylori*, and should provide important preliminary data for the construction of *H. pylori* vectors and for the introduction of *H. pylori* DNA for investigations of pathogenicity.

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