

Helicobacter pylori acquisition of metronidazole resistance by natural transformation *in vitro*

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Subject headings *Helicobacter pylori*; metronidazole; drug resistance, microbial; transformation, bacterial

Abstract

AIM To study whether *Helicobacter pylori* is naturally transformable.

METHODS Transformation was performed in BHI broth supplemented with horse serum and yeast extract. Genomic DNA extracted from a metronidazole resistant *H. pylori* strain was added to *H. pylori* broth culture. The mixture was incubated at microaerophilic atmosphere. The DNA-treated cells were plated on blood agar containing 8mg/L metronidazole to select for transformants. Sterile distilled water was used as a negative DNA control. The DNA profiles of transformants were compared with that of their parent strains by randomly amplified polymorphic DNA (RAPD) fingerprinting.

RESULTS Transformation of *H. pylori* with DNA from a metronidazole resistant strain as a marker was demonstrated. Out of the 12 strains of *H. pylori* tested, 9 (75%) strains were found to be transformable. The transformation frequencies ranged from 3.4×10^{-6} to 2.4×10^{-4} . By RAPD, DNA fingerprints of the transformants and their parent strains showed no change in DNA profiles though transformants were all resistant to metronidazole as compared with their metronidazole-sensitive parent strains.

CONCLUSION *Helicobacter pylori* is naturally transformable which might be one of the ways that *H. pylori* develops resistance to metronidazole.

INTRODUCTION

Genetic transformation is a process by which a cell takes up naked DNA from the surrounding medium and incorporates it into its own genomic DNA to acquire an altered genotype. Natural transformation is widely distributed among bacteria. This process may enable bacteria to get advantageous mutations to escape and survive under unfavourable conditions. *Helicobacter pylori* is recognized as a major factor in the development of gastritis and peptic ulcer^[1]. This bacterium can be eradicated from stomach by antibiotics. However, effective treatment of *H. pylori* has proved difficult with the development of resistance to some antimicrobials. An increase in prevalence of metronidazole resistant *H. pylori* has been reported^[2]. In this study we test *in vitro* whether natural transformation could be one way for *H. pylori* to acquire metronidazole resistance.

MATERIALS AND METHODS

Strains

H. pylori isolates obtained from patients with gastroduodenal diseases were used in this study. The strain was isolated on chocolate blood agar No. 2 medium supplemented with 5% horse blood and incubated at 37°C under microaerophilic environment. The strain was identified by standard procedures as stated by Goodwin *et al*^[3].

DNA extraction

Plate culture of *H. pylori* was transferred into an Eppendorf tube and 1.5 mL volume of TE buffer (100 mM Tris-HCL and 1mM EDTA) was added. The suspension was centrifuged at $8000 \times g$ and washed once with TE buffer. The pellet was suspended in 800 µl TE buffer. The bacterial suspension was incubated in 100 µl of 10 g/L lysozyme (Sigma) at 37°C for 30 minutes, and then lysed with 100 µl of 10% sodium deodecyl sulfate for another 30 minutes at 37°C. Following the addition of 5 µl of 10 g/L proteinase K (Boehringer), the mixture was incubated for 1 hour at 56°C. DNA was purified by extracting twice with equal volume of phenol and once with equal volume of chloroform, DNA was then precipitated overnight with two volumes of absolute ethanol and 20 µl 3M sodium acetate at -20°C. The DNA precipitate was washed once with 70% ethanol. The pellet was vacuum-dried using speed-vac (Savant) and resuspended in

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200µl sterile distilled water. This served as target DNA for PCR-based RAPD. DNA concentration was measured at λ 260nm.

PCR-based RAPD fingerprinting

Universal primer for PCR-based RAPD was randomly chosen according to Akopyanz *et al* (1992)^[4] to allow for the fingerprinting of the whole DNA content of cells. The primer used in this study was 5-AAGAGCCCGT-3. PCR reaction was carried out in 25µl volume. Fifty ng of *H. pylori* genomic DNA, 2 mM MgCl₂, 20 pmol primer, 1 unit of Taq DNA polymerase and 250 mM each of dGTP, dCTP, dATP and dTTP were placed in standard PCR incubation buffer containing 10 mM Tris-HCl, 50 mM KCl, 2 mM MgCl₂ and 0.01% gelatin (Promega, USA). The reaction mixture was overlaid with a drop of mineral oil to prevent evaporation. PCR was performed with a thermal cycler (Amplifon, USA) consisting of an initial step of denaturation of target DNA at 94°C for 5 minutes. This was followed by 39 cycles of denaturation at 94°C for 1 minute, annealing at 36°C for 1 minute and extension at 72°C for 2 minutes. The microliters of the PCR products were electrophoresed in 1% horizontal agarose gels for 2 hours at 80 V in TBE buffer. The gels were stained with ethidium bromide (1 mg/L) and photographed with filtered UV illumination on Polaroid type 667 film.

Transformation experiment

H. pylori transformation was performed in BHI broth supplemented with horse serum and yeast extract. Briefly, DNA was extracted from a metronidazole-resistant *H. pylori* strain, H38. Twelve metronidazole-sensitive strains of *H. pylori* grown respectively in Brain-heart infusion broth supplemented with 10% horse serum and 0.4% yeast extract were incubated under microaerobic conditions at 37°C for 24 hours. Aliquots of 50 µg DNA of H 38 were added into 1mL test *H. pylori* broth culture. The mixture was incubated at 37°C for 6 hours. The DNA-treated cells were plated on blood agar containing 8 mg/L metronidazole to select transformants. The transformation frequencies were calculated by dividing the number of transformants from the total number of viable cells on chocolate blood agar. Sterile distilled water was used as a negative DNA control. The DNA profiles of transformants were compared with that of their parent strains by RAPD fingerprinting.

RESULTS

Transformation of *H. pylori* using metronidazole resistance as a marker was demonstrated. *H. pylori* H38 DNA (metronidazole resistance) was used as a

donor to test for natural transformation competence in broth cultures. To optimize conditions for transformation of *H. pylori*, DNA of H38 was added to NCTC 11637 broth at 6-36 hours after initial inoculation. Transformants were obtained at frequencies ranging from 2.8×10^{-6} to 5.9×10^{-5} (Figure 1 and Table 1). The highest number of transformants and frequency of transformation were found when DNA was added at 24 hours. It was interesting to note that the transformation frequency of NCTC 11637 increased with increasing donor DNA concentration (Figure 2).

Table 1 Effect of time on transformants of *H. pylori* NCTC 11637

	6 hours	12 hours	18 hours	24 hours	30 hours	36 hours
Total bacteria	7.1×10^6	8.1×10^6	2.8×10^7	5.4×10^7	6.3×10^7	9.8×10^7
Transformants	20	370	1300	3200	3100	290
Frequencies	2.8×10^6	4.6×10^5	4.6×10^5	5.9×10^5	4.9×10^5	3.0×10^6

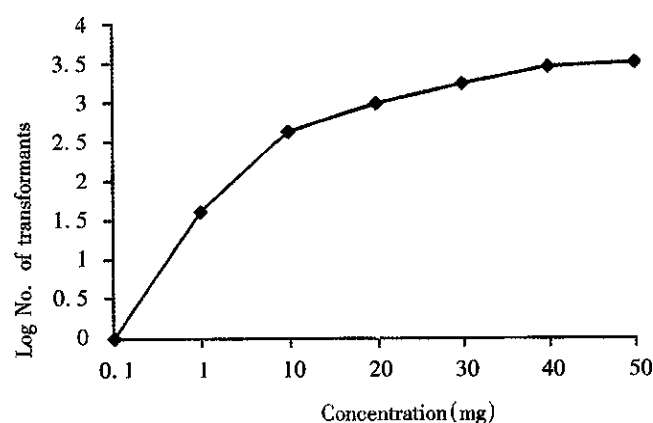


Figure 1 Effect of growth phase on the competence of natural transformation in *H. pylori*. H38 DNA (50mg/L) was added to NCTC 11637 at the indicated time intervals. Total cell number and transformants were enumerated.

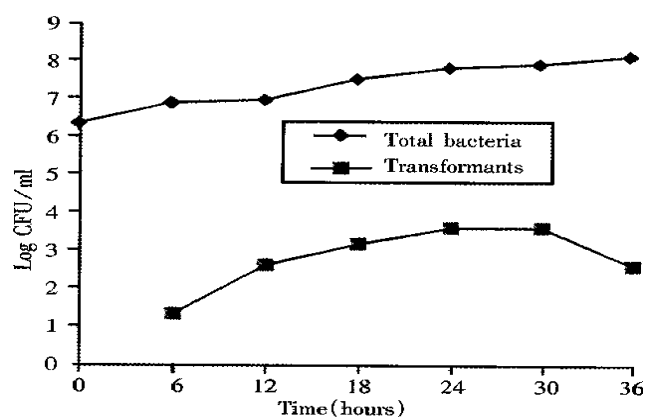


Figure 2 Dependence of transformation frequency on the concentration of donor H38 DNA. H38 DNA with different concentration was added to 108 NCTC 11637. Number of transformants was enumerated.

Using DNA of metronidazole resistant strain, H38, as a marker for transformation, 9 (75%) of 12 *H. pylori* strains tested were found to be transformable. The transformants were all resistant to metronidazole as compared with their metronidazole-sensitive parent cells. The transformation frequencies ranged from 3.4×10^{-6} to 2.4×10^{-4} (Table 2). By RAPD, the DNA fingerprints of the transformants and their parent strains showed no change in DNA profiles (Figure 3).

Table 2 Transformation of different strains of *H. pylori*

Recipient	Donor	Total bacteria	Transformants	Frequencies
H 1	H 38	1.8×10^8	43000	2.4×10^4
H 9		5.2×10^7	8900	1.7×10^4
H 11		9.3×10^8	No	
H 13		8.4×10^7	4100	4.9×10^5
H 29		5.6×10^6	No	
H 41		8.3×10^5	40	4.8×10^5
H 43		5.1×10^6	30	5.9×10^6
H 46		5.8×10^6	20	3.4×10^6
H 50		3.2×10^8	4500	1.4×10^5
H 53		3.5×10^7	320	9.1×10^6
H 62		3.9×10^5	No	
H 68		8.8×10^7	740	8.4×10^6

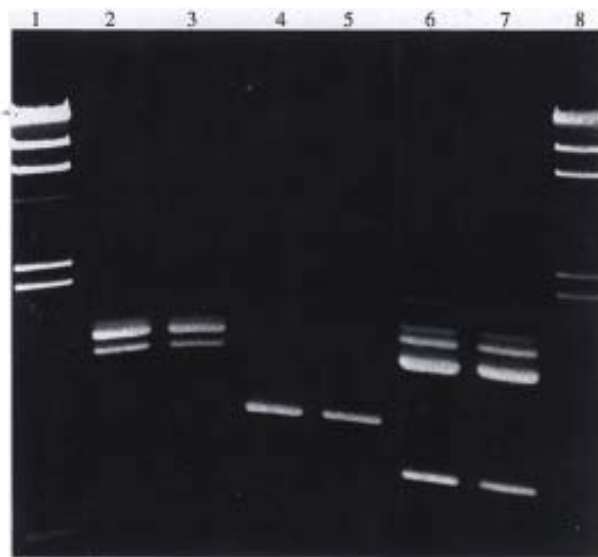


Figure 3 Comparison of DNA profiles of transformants and parent strains by RAPD. PCR products were run on 1% agarose gel. Strains H13, H41 and H43 were metronidazole sensitive. Lanes 1 & 8. λ DNA digested with Hind III. Lanes 2 & 3. H13 and its transformant. Lanes 4 & 5. H41 & its transformant. Lanes 6 & 7. H43 and its transformant.

DISCUSSION

Natural transformation competence was found among prokaryotes, such as *Streptococcus pneumoniae*^[5], *Haemophilus influenzae*^[6] and *Bacillus subtilis*^[7]. Stewart^[8] reported that competence was internally regulated and was in a

stable state once developed. Natural transformation in *H. pylori* has been demonstrated by Nedenskow-Srensen *et al*^[9] and Wang *et al*^[10]. This study has confirmed that *H. pylori* can acquire resistance to metronidazole by natural transformation. Nine (75%) of 12 strains were found to be transformable in this study. The transformation frequencies ranged from 3.4×10^{-6} to 2.4×10^{-4} . Furthermore, we examined the DNA fingerprints of recipient cell and its progeny by RAPD. DNA fingerprinting showed that no significant DNA profile change occurred. This is not unexpected. The size of metronidazole resistance gene may be insignificant compared with the entire chromosomal length. RAPD only can detect a small fraction of target DNA. It is possible that the universal primer used for RAPD in study could not recognise this slight difference especially if a point mutation is involved.

Ling *et al*^[11] found that *H. pylori* strains resistance to metronidazole increased from 29% in 1991 to 73% in 1995 in Hong Kong. We believe that the densely populated environment in Hong Kong and the increased use of metronidazole and other imidazoles in the population had contributed to this phenomenon. This study shows a 75% transformable frequency *in vitro*. The results indicate that natural transformation of metronidazole resistance may play an important role in the development of antibiotic resistance. Natural transformation might promote *H. pylori* in acquiring advantageous genes from other strains in order to adapt and survive in some particular environments.

In this study natural transformation of *H. pylori* was demonstrated *in vitro*. It might be one of the means by which *H. pylori* develops resistance to metronidazole.

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