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Application of methylation in improving plasmid transformation into *Helicobacter pylori*

Huilin Zhao[a,1], Linlin Xu[b,1], Qianyu Rong[a], Zheng Xu[a], Yunfei Ding[a], Ying Zhang[a], Yulong Wu[a], Boqing Li[a,*], Xiaofei Ji[a,*]

[a] Department of Pathogenic Biology, School of Basic Medical Sciences, Binzhou Medical University, Yantai, China
[b] Department of Neurology, Second Hospital, Shandong University, Jinan, China

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ABSTRACT

Helicobacter pylori is an important gastrointestinal pathogen. Its strains possess different levels of powerful restriction modification systems, which are significant barriers to genetic tools used for studying the role of functional genes in its pathogenesis. Methylating vectors *in vitro* was reported as an alternative to overcome this barrier in several bacteria. In this study we used two *H. pylori-E. coli* shuttle plasmids and several single/double-crossover homologous recombination gene-targeting plasmids, to test the role of methylation in *H. pylori* transformation. According to our results, transformants could be obtained only after shuttle plasmids were methylated before transformation. It is helpful in gene complementation and over-expression although at a low frequency. The frequency of gene-targeting transformation was also increased after methylation, especially for the single-crossover recombination plasmids, the transformants of which could only be obtained after methylation. For the double-crossover recombination targeting plasmids, the initial yield of transformants was 0.3–0.8 × 10^2 CFUs per microgram plasmid DNA. With the help of methylation, the yield was increased to 0.4–1.3 × 10^2 CFUs per microgram plasmid DNA. These results suggest that *in vitro* methylation can improve *H. pylori* transformation by different plasmids, which will benefit the pathogenic mechanism research.

1. Introduction

Helicobacter pylori is a Gram-negative microaerophilic bacterium, which can colonize human stomach. Infection by this bacterium has been associated with peptic ulcer disease and stomach cancer (Blaser, 1999). *H. pylori* produces numerous virulence factors (Gressmann et al., 2005) contributing to bacterial colonization and pathogenesis in the gastrointestinal environment (Blaser and Atherton, 2004; Bauer et al., 2011). For example, sialic acid-binding adhesin (SabA) (Aspholm et al., 2006), blood group antigen binding adhesin (BabA) (Ilver et al., 1998), adherence-associated lipoproteins A and B (AlpA and AlpB) (Odenbreit et al., 1999) and several membrane proteins have been shown to be involved in colonization. Vacuolating cytotoxin A (VacA), cytotoxin associated gene A (CagA) and type IV secretion system (T4SS) also strongly correlate with serious *H. pylori*-induced pathologies (Blaser and Atherton, 2004; Ogura et al., 2000; Huang et al., 2003).

Although extensive research has been done on *H. pylori*, presently we cannot completely understand its pathogenic mechanism of action. More potential virulence factors need to be identified to fully dissect its pathogenesis. In order to achieve this, genetic manipulations including transposon mutagenesis (Haas et al., 1993; Kahrs et al., 1995), site-directed mutation (Ferrero et al., 1992; Bauerfeind et al., 1996; Yuan et al., 2003) and unmarked deletion (Copass et al., 1997) have been established in *H. pylori*. Several *H. pylori-Escherichia coli* shuttle plasmids have also been constructed (Kleanthous et al., 1991; Lee et al., 1997; Heuermann and Haas, 1998). However, the effective utilization of these genetic engineering tools depends on their effective transfer into *H. pylori*.

Bacteria have evolved many defenses to protect their own genomes against foreign DNA, among which the most well known method is restriction modification system (RM). RM system essentially comprises of two components, the DNA methyltransferases (MTase) and the restriction endonucleases (REase). REase can specifically identify foreign DNA inside bacteria and degrade it. Meanwhile, MTase can modify its own DNA through methylation, to differentiate it from foreign DNA and prevent degradation by REase (Meselson and Yuan, 1968; Arber and Linn, 1969; Wilson and Murray, 1991). Genome analysis of *H. pylori* 26,695 and J99 indicated that both contained multiple putative RM

* Corresponding authors.
E-mail addresses: sdboqingli@163.com (B. Li), jxf_email@126.com (X. Ji).
[1] These authors contributed equally to the paper.

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systems including type I, II, IIS or III (Tomb et al., 1997; Alm and Trust, 1999), which provide a powerful defense against invasion by foreign DNA. Additional reports have shown that specific components in different *H. pylori* strains make the RM systems strain-dependent (Xu et al., 2000; Ando et al., 2000) and result in some *H. pylori* strains more resistant to transformed plasmid DNA than others (Lee et al., 1997; Tsuda et al., 1993; Wang et al., 1993). For example, the widely studied pathogenic strains, 26,695 and SS1, are not easily transformed by plasmids propagated in *E. coli* (Donahue et al., 2000), which is a significant barrier for studying functional genes in *H. pylori* pathogenesis.

To protect the exogenous DNA from digestion by host's REase, one feasible way is to make the foreign DNA acquire the methylation pattern of the host. Methylating foreign DNA has been successfully used to improve transformation frequency in different bacteria. *Campylobacter jejuni* hardly accepts foreign DNA propagated in *E. coli* or produced with PCR. However, these plasmids or PCR products can efficiently be transferred into *C. jejuni* after they are methylated *in vitro* (Beauchamp et al., 2017). In *Bacillus* species, plasmids isolated from *E. coli* MC1061 (methylation strain) have higher transformation frequency than those isolated from *E. coli* JM110 (methylation-deficient strain) (Yi and Kuipers, 2017). Methylation also helped the transformation of *H. pylori* by *H. pylori-E. coli* shuttle plasmids, such as pHel3, pVU1017, pILL2150 and pILL2157 (Donahue et al., 2000; Boneca et al., 2008). However, the efficacy of this method in transformation of *H. pylori* by other plasmids has not yet been fully explored. In this study, we constructed several vectors including shuttle plasmids and gene-targeting plasmids, which were used to transform *H. pylori* with or without *in vitro* methylation. The effect of plasmid methylation on *H. pylori*-transformation was analyzed by a statistical analysis of the transformants.

2. Materials and methods

2.1. Bacterial strains and growth conditions

H. pylori strains 26695, SS1 and 11637, provided by Chinese Center for Disease Control and Prevention, were used as recipient cells in this study. Chocolate agar supplemented with 10% sheep blood was used to cultivate *H. pylori* strains. Culture conditions were set at 37 °C under microaerobic environment (85% N_2, 10% CO_2 and 5% O_2). The *E. coli* strains required for construction of plasmids were routinely cultured in Luria-Bertani medium at 37 °C. Antibiotics were used when needed at the following concentrations: kanamycin (Km) - 15 μg/ml or chloramphenicol (Cm) - 10 μg/ml for *H. pylori*; ampicillin (Ap) - 100 μg/ml or kanamycin (Km) - 30 μg/ml for *E. coli*. Bacterial strains and plasmids used in this study are listed in Table 1, and primers are listed in Table 2.

2.2. Construction of H. pylori-E. coli shuttle plasmids pCHHP and pCHHPK

The plasmid pCHF-p (Ji et al., 2017) containing a *H. pylori*-specific replicon was used as the backbone to construct *H. pylori-E. coli* shuttle plasmids. The chloramphenicol-resistance gene (*catGC*) from plasmid pTnMax9 was amplified with primers cm-1 and cm-2 to replace the original invalid chloramphenicol-resistance gene and FLP recombinase gene, generating plasmid pCHHP. Kanamycin-resistance plasmid pCHHPK was constructed using the same method, where the kanamycin-resistance gene (*aphA*) was amplified from pHimarEm1 using primers km-1 and km-2.

2.3. Construction of gene-targeting plasmids

Plasmid pSJHK constructed previously (Ji et al., 2016) was used as template to generate gene-targeting plasmids. Single-crossover recombination plasmid targeting gene *hp0169* was constructed as follows: a 662 bp fragment within *hp0169*, amplified with primers 0169-F and 0169-R, was used as the homologous arm. This fragment was inserted into the multiple cloning site of pSJHK to generate plasmid pSJHK-s0169. Single-crossover recombination plasmids pSJHK-s0535, pSJHK-s0547, pSJHK-s0607 and pSJHK-s0788 were constructed in the same way, where the homologous arms were amplified with primers 0535-F/0535-R, 0547-F/0547-R, 0607-F/0607-R and 0788-F/0788-R, respectively.

Double-crossover recombination gene-targeting plasmids (pSJHK-0169, pSJHK-0535, pSJHK-0547, pSJHK-0607 and pSJHC-0788) were constructed according to the procedure described previously (Ji et al., 2016). Primers used in amplification of homologous arms are listed in Table 2.

2.4. Methylation of plasmids in vitro

Lysates of *H. pylori* cells were used to methylate plasmid DNA *in vitro* and lysis was performed as described in a previous study (Boneca et al., 2008) with some modifications. Methylation buffer comprised of the following components: 20 mM Tris-acetate (pH 7.9), 40 mM potassium acetate, 15 mM EDTA and 1 mM dithiothreitol. *H. pylori* cells on blood agar plates were harvested and washed twice with ice-cold phosphate buffered saline (PBS). The cell pellet was resuspended in ice-cold methylation buffer and subjected to sonication. The remaining intact cells were removed by centrifugation at 20,000 ×*g* for 10 min. After freshly prepared *S*-adenosyl-L-methionine (0.5 mM) was added into the collected supernatant solution, the mixture was immediately used to methylate plasmid DNA with the mass ratio of 20:1 (20 μg of protein extracts mixed with 1 μg of plasmid DNA) for 1 h at 37 °C. Finally, the methylated plasmids were extracted from the mixture using the plasmid miniprep kit (TianGen). Protein concentration was measured using Bradford method (Bradford, 1976).

2.5. Electro-transformation of H. pylori cells

Preparation of competent *H. pylori* cells and electroporation were performed as described previously (Ji et al., 2016). After electroporation, the recipient cells were first plated on nonselective blood agar plates for 24 h, and then on selective medium with 15 μg/ml kanamycin or 10 μg/ml chloramphenicol.

PCR was used to confirm the homologous recombination as previously described (Ji et al., 2016). For the single-crossover recombination gene-targeting, diagnostic PCRs were performed with 0169-F, 0535-F, 0547-F, 0607-F and 0788-F as upstream primers and km-r (inside of *aphA*) as downstream primer. If homologous recombination had occurred in *H. pylori* transformants, an expected amplicon would exist for the mutant. For the double-crossover recombination gene-targeting, primers 0169-1 and 0169-4, 0535-1 and 0535-4, 0547-1 and 0547-4, 0607-1 and 0607-4, 0788-1 and 0788-4 were used respectively in diagnostic PCRs to analyze if the target genes were replaced by the kanamycin resistance gene (*aphA*) in *H. pylori* transformants.

3. Results

3.1. Construction of H. pylori-E. coli shuttle plasmids

Plasmid pCHF-p containing a *H. pylori*-specific replicon from pHel1 was constructed in a previous study (Ji et al., 2017). The fragment of invalid chloramphenicol-resistance gene and FLP recombinase gene on pCHF-p was replaced by chloramphenicol-resistance gene (*catGC*) or kanamycin-resistance gene (*aphA*) to generate *H. pylori-E. coli* shuttle plasmids pCHHP or pCHHPK. The constructed process was illustrated in Fig. 1A and B.

3.2. Construction of gene-targeting plasmids

Five genes (*hp0169*, *hp0535*, *hp0547*, *hp0607* and *hp0788*) were selected as targets, and gene-targeting plasmids were constructed based