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World J Gastroenterol 2005;11(21):3255-3259
World Journal of Gastroenterology ISSN 1007-9327

Deletion of *cagA* gene of *Helicobacter pylori* by PCR products

Xun Zeng, Li-Hua He, Yan Yin, Mao-Jun Zhang, Jian-Zhong Zhang

Xun Zeng, Li-Hua He, Yan Yin, Mao-Jun Zhang, Jian-Zhong Zhang, Department of Diagnostics, National Institute of Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China
Supported by the National Natural Science Foundation, No. 30370078
Correspondence to: Dr. Jian-Zhong Zhang, Department of Diagnostics, National Institute of Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, PO Box 5, Changping, Beijing 102206, China. zhangjianzhong@icdc.cn
Telephone: +86-10-6173-9456 Fax: +86-10-6173-9439
Received: 2004-12-28 Accepted: 2005-02-18

Abstract

AIM: Cytotoxin-associated protein (antigen) A (CagA) plays an important role in *Helicobacter pylori* (*H pylori*) pathogenesis. Our aim was to obtain *cagA*⁻ mutant strains by a new mutation method so as to better understand the mechanism of CagA in epithelial cells.

METHODS: In contrast with the traditional method using suicide plasmid, we constructed *cagA*⁻ mutant strains directly with PCR products. The constructed mutant clones grew on selective media and allelic exchange was confirmed by Southern blot. Furthermore, two different transformation methods, electroporation, and natural transformation, were compared with regard to the efficiency of recombination.

RESULTS: The mutation by PCR products could be completed within 3-5 d, and the recombination rate by electroporation and natural transformation was 4.02×10^{-8} and 1.03×10^{-9} respectively. Mutation rate by electroporation (4.02×10^{-8}) was far higher than by natural transformation (1.03×10^{-9}) ($P = 0.000<0.005$).

CONCLUSION: *cagA*⁻ mutant strains have been constructed, which is important for further study on the function of CagA in epithelial cells. A mutation method by directly using PCR products has been proved successful with a much higher mutation rate, and is easier, especially when in combination with electroporation. This method could be widely used in gene deletion of *H pylori*.

Key words: *H pylori*; CagA; Deletion

Zeng X, He LH, Yin Y, Zhang MJ, Zhang JZ. Deletion of *cagA* gene of *Helicobacter pylori* by PCR products. *World J Gastroenterol* 2005; 11(21): 3255-3259
http://www.wjgnet.com/1007-9327/11/3255.asp

INTRODUCTION

Helicobacter pylori (*H pylori*), a Gram-negative bacterial pathogen, is highly successful in that it has colonized the human stomach in at least half of the world population. Epidemiological studies suggest that *H pylori* have close relationship with chronic gastritis, peptic ulcer, gastric cancer, and gastric mucosa-associated lymphoid tissue lymphomas and some of them have been confirmed by animal experiments[1-4]. *H pylori* strains are divided into two categories, type I and type II[5]. Clinical isolates of *H pylori* from gastric diseases indicate that the pathogenesis of types I and II strains is different[6]. Compared with type II strains, type I strains are more closely associated with duodenitis, duodenal ulcers, and gastric cancer[7]. Type I strains express CagA protein which is encoded by a gene located in the cag pathogenicity island (*cag PAI*). The *cag PAI* is a 40-kb DNA segment which encodes for type IV secretion apparatus for delivering virulent proteins[8]. In contrast, type II strains lack the entire *cag PAI* and do not possess the CagA and have no cytotoxin *in vitro*[1,9]. It is clear that CagA is transferred into gastric epithelial cells through type IV secretion system and phosphorylated by src family protein tyrosine kinases of host cells. Then, the phosphorylated CagA may lead to cell skeleton rearrangement and hummingbird phenotype of epithelial cells[10-15]. Recent studies have shown that the main difference of CagA of clinical isolates between different gastric diseases is the amount of EPIYA motif that can be phosphorylated at C-terminals of CagA[16,17]. Other researches showed that the sequences near the EPIYA motif also had important functions, which might affect the function of the EPIYA motif[17,18]. Moreover, some research found that EPIYA motif of CagA had its own functions[19].

However, the function of CagA is very complicated and the roles of CagA in host cells are still unclear. To better understand the mechanisms involved in the induction of host cell responses to CagA and the signal transduction, it is important to construct *cagA* mutated strains in an easier way. Here, we report a simple mutation method by PCR products to directly construct *cagA* knockout strains with a high recombination rate.

MATERIALS AND METHODS

Bacterial strains

H pylori 26695 was grown on Columbia agar plates, which contain sheep blood (5%) supplemented with vancomycin (10 μg/mL), nystatin (1 μg/mL), and trimethoprim (5 μg/mL) for 2 d at 37 ℃ in an anaerobic jar consisting of 5% O_2, 100 mL/L CO_2, and 85% N_2. Chloramphenicol (20 μg/mL) was added for the mutated strains selection.

Polymerase chain reaction (PCR)

The method of one-step deletion of *H pylori* gene was as described previously with slight modifications[20]. Briefly, the strategy for constructing fragments with chloramphenicol resistance gene and gene allelic replacement by PCR is shown in Figure 1. *H pylori* genomic DNA was extracted by using BioDev genomic DNA extraction kits (BioDev, China) as per the manufacturer's instructions. By use of primers designated as C1 and C2, chloramphenicol resistance gene was amplified from plasmid pBSC103, which contained chloramphenicol resistance gene cassette (Cm^r). Two pairs of primers specific for *cagA* gene were designed, P1-P2 and P3-P4, which amplified the up- and downstream regions of *cagA* gene respectively. Primers P2 and P3 were designed with leaders at 5' ends which were complemented with C1 and C2, respectively. In addition, primers B1 and B2 were designed to detect whether *cagA* had been deleted. A pair of primers of *cagE* gene, E1 and E2, was selected as positive control. All the primers are shown in Table 1.

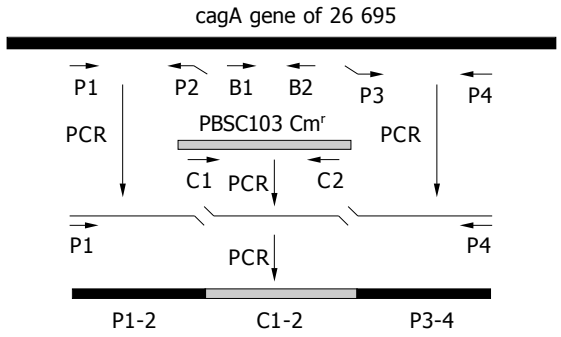

Figure 1 Strategy for constructing fragments with chloramphenicol resistance gene and gene allelic replacement by PCR. Primers P1-P2, P3-P4 and C1-C2 were amplified and each PCR products were purified. Each three PCR products were mixed and amplified by primer P1 and P4. Then, the PCR product P1-4 was acquired containing three fragments.

Table 1 Primers for amplifying Cm^r and up- and downstream regions of *cagA*

Primer	Sequence (5'–3')	Expected product size
C1	GAT ATA GAT TGA AAA GTG GAT[20]	742
C2	TTA TCA GTG CGA CAA ACT GGG[20]	
P1	GCC ACT ACT ACC ACC GAC AT	364
P2	ATC CAC TTT TCA ATC TAT ATC TAT GAC T AA GCC ACT GCC GT	
P3	CCC AGT TTG TCG CAC TGA TAA TCA AAT A CA CCA ACG CCT	484
P4	GCA TCC CTA TTA GCC TCT T	
B1	ATT AGA CAA CTT GAG CGA G	276
B2	ACA AAC ATC ACG CCA TC	
E1	ATG CGA GCC TAT AAT GAG AAG C	841
E2	GAA GCG TGA TAA AAG AGC AAT GTT	

Lineated sequences are complementary to C1 and C2. E1, E2 are positive controls for *cagE* gene.

Each PCR product was purified by DNA purification system (Promega, USA). Templates mixture containing 1 μg each of three purified PCR products (P1-2, P3-4, C1-2) were then amplified using primers P1 and P4 in a single reaction to generate the linear product with Cm^r. The hot-start PCR conditions are as follows: the templates mixture were amplified five cycles without primers P1 and P4, at 94 ℃ for 1 min, 45 ℃ for 1 min, and 72 ℃ for 5 min, followed by 40 cycles with primers P1 and P4, at 94 ℃ for 45 s, 40 ℃ for 1 min, and 72 ℃ for 3 min. Then, the PCR products were extracted by Qiagen gel extraction kits (Qiagen, Germany).

H pylori DNA transformation by electroporation and natural transformation

H pylori were harvested after 2 d' cultivation and washed thrice by deionized water with 12% glycerol. They were then suspended in 0.1 mL deionized water with 12% glycerol and 0.5 μg PCR product was added and mixed well. To calculate the transformation rate, 9.7×10^9 *H pylori* were used. For electroporation, the mixture was added into electroporation cuvette (Equibio, USA) and after that subjected to two-pulse electroporation and scraped onto Columbia agar plates. The condition is as follows: first pulse: 2500 V, 25 μF, delay 10 s; second pulse: 120 V, 150 μF. For natural transformation, the mixture was spotted onto Columbia agar plates directly. After incubated for 24 h, cells were scraped onto selective Columbia agar plates containing 20 μg/mL chloramphenicol and incubated for 3-5 d.

Southern blot

The transformants were harvested from selective Columbia agar plates and genomic DNA was extracted. In order to confirm that the correct chromosome rearrangement occurred, transformants were initially screened by diagnostic PCR using primers B1 and B2. To prepare the blot probes, the PCR products of C1-2 were purified and labeled using DIG DNA labeling and detection kits (Roche, Germany). The genomic DNA of transformants was digested by *Eco*RI for 4 h. The protocol of Southern blot was performed following instructions of DIG DNA labeling and detection kits.

Statistical analysis

χ^2 test was used to calculate the difference of two mutation methods. $P<0.05$ was taken as significant.

RESULTS

PCR products with chloramphenicol resistance gene

PCR products C1-2, P1-2, P3-4 and the mixture products P1-4 are shown in Figure 2. The 1.6-kb PCR products suggested that the three parts of PCR products C1-2, P1-2, P3-4 were linked by primers P1 and P4.

Construction and identification of transformants

H pylori transformed by the 1.6-kb PCR products P1-4 were cultivated on selective Columbia agar plates. After 3-5 d, single clones were isolated. In comparison between the two different mutation methods, 39 clones were isolated by electroporation and one clone was isolated by natural transformation from 9.7×10^9 bacteria. The mutation rates by

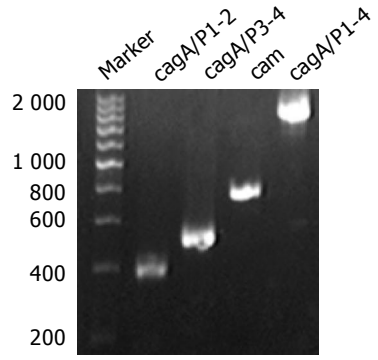

Figure 2 PCR amplification for constructing fragments with chloramphenicol resistance gene and gene allelic replacements. The length of *cagA*/P1-4, a 1.7-kb fragment, is equal to the sum of the length of *cagA*/P1-2, *cagA*/P3-4, and cam.

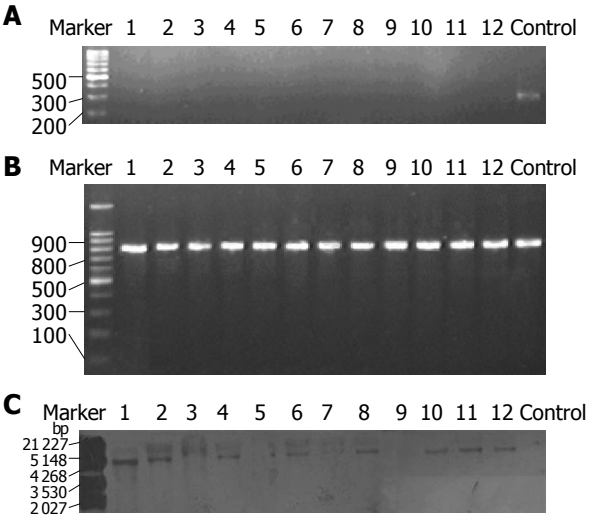

Figure 3 **A**: Result of PCR amplified for diagnosing homologous recombination strains of *cagA* gene. Lanes 1-12 are 12 clones selected from chloramphenicol-resistant plates. Control is 26695 wild type. Twelve clones were all negative for diagnostic primers B1-2 while control is positive, suggesting that the flanking region of *cagA* gene of all the selected clones was deleted; **B**: Result of PCR amplified for positive control of homologous recombination strains of cagE gene. Lanes 1-12 are 12 clones selected from chloramphenicol-resistant plates. Control is 26695 wild type. Twelve clones were all positive of cagE gene; **C**: Result of Southern blot for diagnosing homologous recombination strains of *cagA* gene. Lanes 1-12 are 12 clones selected from chloramphenicol-resistant plates. Control is 26695 wild type. All the 12 clones except 3, 5, and 9 were positive hybridization with probes of chloramphenicol-resistant gene while control was negative.

electroporation and natural transformation were 4.02×10^{-8} and 1.03×10^{-9}, respectively (χ^2 test: $P = 0.000<0.005$). All clones (26695△*cagA*) were diagnosed by primers B1, B2 and E1, E2 as positive control (Figures 3A and B). The results suggested that the flanking region of *cagA* gene, we designed to delete in all the clones, had been replaced. Southern blot using probes labeled with chloramphenicol resistance gene further confirmed that the chloramphenicol resistance gene had only one copy in the genomic DNA of *H pylori* except clones 3, 5, and 9 (Figure 3C). Consequently, the allelic exchange occurred only in the *cagA* gene just as we designed in all clones except clones 3, 5, and 9.

DISCUSSION

The correlation between the expression of CagA and *H pylori* virulence was described long time ago[21]. Early studies found that a 145-ku host protein was phosphorylated in epithelial cells after being infected by *H pylori*[22]. Recent investigations demonstrated that the phosphorylated 145-ku protein was CagA which was delivered by type IV secretion system[23,24]. Then, the phosphorylated CagA, binding with SHP-2, led to rearrangements of the actin cytoskeleton and hummingbird phenotype[25,26]. The further consequence of phosphorylated CagA is tyrosine dephosphorylation of several cell proteins of 120 and 85 ku[23,27-29]. However, the precise function of this protein is still not understood well. To study the mechanism of CagA, we constructed CagA mutant strains.

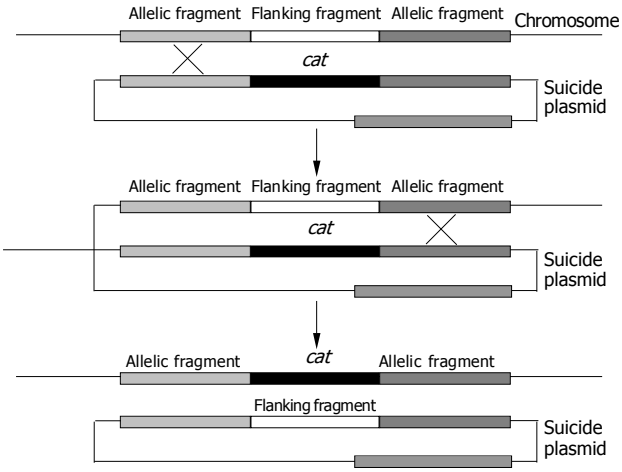

Figure 4 Mechanism of allelic fragments recombination used by suicide plasmid. The recombination consists of two steps. In the first step, suicide plasmid with PCR products to be exchanged recombined with one allelic fragment and formed a loop. Then, the suicide plasmid recombined with another allelic fragment.

Because *H pylori* has higher mutation ratio than other bacteria, it is suitable to use PCR products as the allelic fragments to disrupt genes. The normal method for gene mutation is by suicide plasmid[29] or PCR products with plasmid expressing recombinase[29]. Compared with these techniques, our method was much more simple and efficient. Suicide plasmid has potential disadvantage in that it may have many false clones without mutation. The mechanism is as shown in Figure 4. The suicide plasmid with PCR products to be exchanged firstly recombines with one allelic fragment and then recombines with another allelic fragment. If the second recombination does not occur, it would form a loop without flanking regions deletion. Thus, some investigations disrupting genes with suicide plasmid normally use two screening markers to confirm the flanking regions deletion. Another method, by recombination of PCR products with plasmid expressing recombinase, requires the phage λ Red recombinase, which is synthesized under the control of an inducible promoter of an easily curable, low copy number plasmid. In this way, allelic fragments of PCR products are very short, less than 50 bp. However, phage λ Red recombinase may lead to other unwanted recombination of events which can hardly be under control. Moreover, phage λ Red recombinase system is mainly used in *E.coli*. Its application in other bacteria still needs to be proved. Allelic exchange directly by PCR products has no such

problems because it is linear DNA and cannot form the loop. In our study, we successfully deleted *virB4*, *virB10*, *virB11*, and *cag PAI* (data not shown). To confirm whether the flanking regions were replaced, the detective PCR was used and results proved that the flanking fragment we wanted to replace by resistant gene was disrupted. On the other hand, PCR for detecting chloramphenicol resistance gene indicated that all the mutated strains contained the resistant gene except the control. In addition, Southern blot using probes for resistant gene further confirmed that no other gene replacement occurred except in our designed region. However, the results of Southern blot suggested that gene replacement of clones 3, 5, and 9 did not happen in our designed region. This could suggest that unwanted replacement also occurred.

Disrupting *H pylori* chromosome gene by PCR products was firstly described by Chalker *et al*[20]. However, details were improved by which electroporation was used in this study. Normally, bacteria can take up free DNA directly from their environment. This process is called transformation. However, natural transformation rate is far lower. One factor that affects the efficiency of transformation rate is the size of DNA to be transformed. Natural transformation is more sensitive to the size of DNA. Compared with natural transformation, electroporation can increase the transformation rate by high electric shock opening the cell membrane, through which DNA can move into cells. During this process, the size of DNA has minor effects on its movement. Moreover, another advantage of electroporation is that it can transform not only the linear DNA such as PCR products, but also circular plasmids or phage DNA. Natural transformation cannot transform circular DNA efficiently because natural transformation requires breakage of the double-stranded DNA and degradation of one of the two strands so that a linear single strand can enter the cells. So, natural transformation is more suitable for uptaking PCR products which are linear. Our data indicated that, in *H pylori*, at least in 26695, the efficiency of transformation rate of electroporation was 4.02×10^{-8}, far higher than natural transformation, 1.03×10^{-9} (χ^2 test: $P = 0.000<0.005$).

In conclusion, we have successfully constructed the 26695 *cagA* strain which is very important for investigation of mechanism of CagA. Moreover, we have developed a new mutation method which can be wildly used in gene mutation of *H pylori*.

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