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An Easy Way for the Rapid Purification of Recombinant Proteins from *Helicobacter pylori* Using a Newly Designed Expression Vector

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We constructed a *H. pylori* expression vector which consisted of both a His-tag and a GST tag as purification tools for recombinant protein and a chloramphenicol resistant *cat* gene as a reporter. The backbone of the vector pBK contained an ColEI origin of replication and a kanamycin resistant gene. A set of oligos for the His-tag and the PCR product of *gst* (glutathione S-transferase) gene were inserted sequentially in frame in the multi-cloning site of pBK. The orf of *cat* was inserted downstream of the *gst* to generate pBKHGC. The 3′ part of *H. pylori clpB* and *flaA* were cloned into the vector which was introduced into *H. pylori*. Recombinant proteins were purified by GSH affinity column, digested with thrombin and were analyzed by western blotting. The final recombinant proteins were successfully purified.

Keywords: *Helicobacter pylori*, pBKHGC, vector

Introduction

Helicobacter pylori, a capnophilic, Gram-negative and spiral-shaped bacterium infects at least half of the world population. In Korea, the infection rate reaches up to 50% at 5–6 years of age and 80–90% at 7 years of age and then maintains this approximate percentage throughout all ages of adulthood (Baik *et al*., 1990; Rhee *et al*., 1990). *H. pylori* can causes (not 70% of the population suffers from these ailments) gastric disease such as chronic gastritis, peptic ulceration, and plays important roles in the development of gastric cancer (Marshall 1983; Marshall and Warren *et al*., 1984; Baik *et al*., 1996; Youn *et al*., 1996). This microorganism has been categorized as a class I carcinogen by the World Health Organization (World Health Organization, 1994). Although, the treatment for *H. pylori* infection is very important for these reasons, the pathogenic mechanisms caused by this bacteria has not yet been unveiled. It is difficult to manage disease caused by *H. pylori* by treatment with antimicrobial drugs as they live in the gastric mucosa and furthermore immune mechanisms would not work in this habitat. To date, there have been about 1,600 ORFs revealed in *H. pylori* strains about 30% of which were annotated as hypothetical proteins (Tomb *et al*., 1997).

Therefore, to carry out biological research on *H. pylori*, and to study potential mechanisms for its eradication, it is essential to purify and characterize its proteins. However, producing soluble protein from *H. pylori* in an *Escherichia coli* system is very difficult (Baik *et al*., 2005; Song *et al*., 2011). More than half of the *H. pylori* genes are not expressed and only about 20% are soluble when expressed in *E. coli*. To solve this problem, we have constructed an expression vector that operates efficiently in *H. pylori*.

Materials and Methods

Bacterial strains, media, plasmids, and antibodies

H. pylori was obtained from the *H. pylori* Korean Type Culture Collection (HpKTCC, http://hpktcc.knrrc.or.kr). Cells were cultured as described previously (Joo *et al*., 2010). Frozen cells were thawed and streaked onto *Brucella* agar (BA) containing vancomycin (10 μg/ml), nalidixic acid (25 μg/ml), amphotericin B (5 μg/ml), and 10% bovine serum, and were then incubated at 37°C, under 5% O_2, 10% CO_2, and 100% relative humidity. One loop of overnight culture was used to inoculate *Brucella* agar plates enriched with 10% bovine serum, which were then grown overnight under the conditions described above. The plasmid pBK that contained the replication origin of ColEI and a kanamycin resistant marker was also obtained from the HpKTCC. The bacterial strains, plasmids and primers used in this study are shown in Tables 1 and 2. Anti-*H. pylori* FlaA monoclonal antibody was obtained from the HpKTCC and anti-GST antibody was purchased from Santa Cruz (USA).

Construction of a vector containing His-tag sequence

The plasmid pBK was digested with *Sma*I and *Eco*RI (Song *et al*., 2003). To insert the HIS-tag, a set of complementary oligos (Histag1 and Histag2) which contained 5′ *Sma*I end, thrombin recognition sequence, six histidine codons (CAT and CAC) and 3′ *Eco*RI cohesive end was designed (Table 1) (Narayana *et al*., 1997). The oligomer mix (5 pmole each)

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Table 1. Strains and plasmids used in this study

Plasmid and strains	Genotype and phenotype	Reference/source
Strains		
H. pylori 26695	wild type	Tomb *et al.* (1997)
H. pylori 1061	wild type	Bijlsma *et al.* (2002)
E. coli DH10B	*F⁻ endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lacZΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ⁻*	Durfee *et al.* (2008)
Plasmids		
pBK	ColEI ori; Km^r	Song *et al.* (2003)
pBKH	pBK; His-tag	this study
pBKHG	pBK; His-tag; GST-tag	this study
pBKHGC	pBK; His-tag; GST-tag; CM^r	this study
pBKHGC-flaA	pBK; His-tag; GST-tag; CM^r; *flaA*	this study
pBKHGC-clpB	pBK; His-tag; GST-tag; CM^r; *clpB*	this study

was boiled for 5 min and was chilled on ice for 5 min, inserted into the pBK that were digested *Eco*RI and *Sma*I to generate pBKH (Table 1).

Insertion of the GST-tag

Two sets of PCR primers were synthesized for the GST tag from pGEX-4T-1 (GE Healthcare, USA). Two fragments of the *gst* gene were amplified by using the primer set GST1 and GST2, GST3, and GST4, respectively (Table 1). Both PCR products were purified, mixed together and full-length *gst* was amplified with GST1 and GST4. The final product was digested with *Eco*RI and was cloned into the *Eco*RI site of pBKH to generate pBKHG (Fig. 1). Direction and reading frame of the both tags on the plasmid were confirmed by sequencing.

Insertion of the *cat*

The orf of chloramphenicol resistant marker *cat* was PCR-amplified from pBC SK(+) with two sets of oligos (CAT1 and CAT2, CAT3, and CAT4), digested with *Hin*dIII and *Nhe*I, and inserted into pBKHG which was digested with *Hin*dIII and *Nhe*I to generate the final construct pBKHGC (Table 1 and Fig. 1).

Construction of an expression vector containing gene fragment

About 500 bp of 3′ end fragments of *clpB* and *flaA* were PCR-amplified form *H. pylori* 26695, digested with *Sma*I and *Xba*I, and inserted into the pBHGC that digested with the same enzymes. Ligated DNA was introduced into *E. coli* DH10B by chemical transformation. *H. pylori* 1061 was transformed by the final construct as described previously (Lee *et al.*, 1997).

Rapid screening of the expression of the fusion protein

One loop of well-grown *H. pylori* 1061 and recombinant clone on the plate were resuspended in 0.2 ml of PBS. 0.2 ml of 2X loading buffer (20% Glycerol, 120 mM Tris/HCl; pH 6.8, 4% SDS, 0.02% bromophenol blue, 2.5% beta-mercaptoethanol) were added and mixed. Cells were boiled for 5 min and were analyzed on 12% SDS-PAGE.

Purification of the recombinant proteins

About 30 g of *H. pylori* cells were resuspended in 10 ml of PBS buffer and were lysed by sonication (Sonic & Materials, USA). The cleared lysate was obtained by centrifugation at

Table 2. List of the names and sequences of the oligos

Name	Sequence
Histag1[a]	GGGATGCTTGTTCCGCGTGGATCTAGC**CATCATCATCATCATCAC**AGCAGCGGCG
Histag2[a]	AATTCGCCGCTGC**TGTGATGATGATGATGATG**GCTAGATCCACGCGGAACAAGCATCCC
GST1	AAACAGAATTCATGTCCCCTATACTAG
GST2	AGCaATATAtCtaATGATGGCCATAGACTG
GST3	CATCATtaGaTATATtGCTGACAAGCACAAC
GST4	GCGACTGAATTCAAGCTTCAACGCGGAACCAAATCCGATTTTGG
CAT1	ATGCAAGCTTGATGCAATTCACAAAGATTG
CAT2	TTAAAGCCTTCAAAGCTgGTCCACGGTATCATAGA
CAT3	TCTATGATACCGTGGACcAGCTTTGAAGGCTTTAA
CAT4	AAACTGCTAGCTTATTTATTCAGCAAGTCT
clpB-F	TCTAGACAACATCGCTGAGATCGTGAGC
clpB-R	CCCGGGCTTAATCTTAGGCACAATCT
flaA-F	TCTAGACCGCTTCTGGAGATATTAGCT
flaA-R	CCCGGGAGTTAAAAGCCTTAAGATATTG

[a] underline, thrombin-recognition sequence; bold letters, histidine 6-mer. Small letters in the sequences are mutant bases for correction of the rare codons.

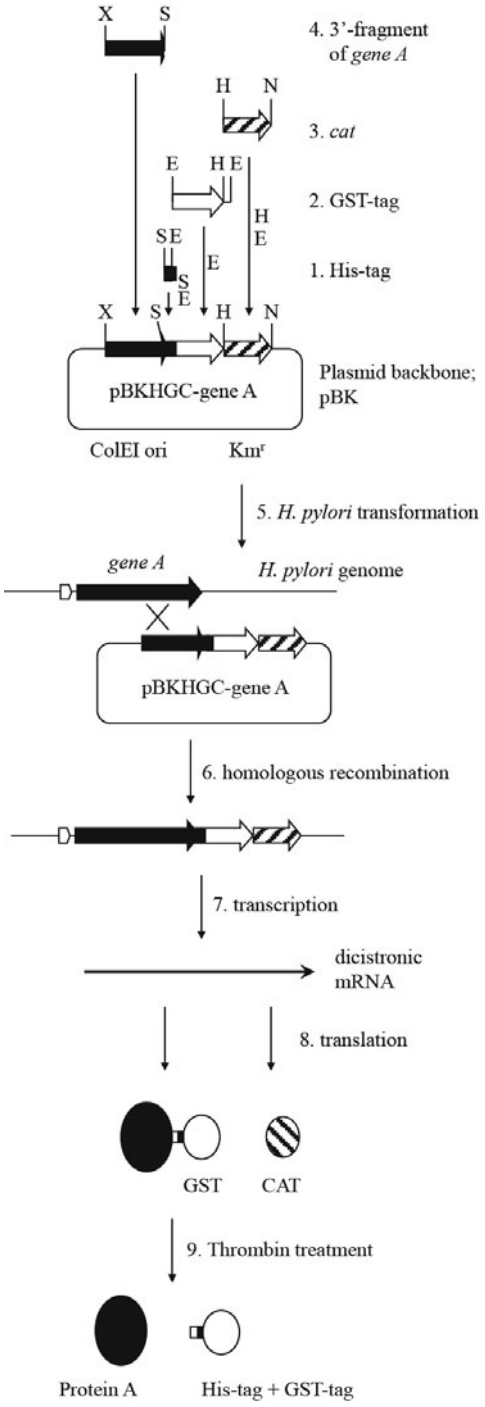

Fig. 1. Strategy for expression of the recombinant gene on the genome of *H. pylori* and purification of the gene product. Four DNA fragments were inserted into the pBK vector sequentially as follows; 1) the annealed oligo set for His-tag was inserted into the multicloning site (MCS) of pBK; 2) the PCR product of *gst* was inserted into the downstream of the His-tag; 3) the PCR product of *cat* was inserted into the downstream of the GST-tag; 4) 3′-terminal part of *H. pylori* gene without stop codon was inserted into the upstream of the His-tag. Thus, *H. pylori* gene, His-tag, GST-tag were aligned in same direction in one reading frame. At the downstream of the termination codon of GST-tag, cat was located as a separated cistron that would be transcribed as a part of a dicistronic mRNA. The fusion protein would be digested by thrombin protease to separate tags from the *H. pylori* protein.

13,000 rpm for 15 min at 4°C, and was subjected to the GSH (glutathione) affinity column according to the manual. The resin was washed with 50 ml of PBS solution and the GST-fusion protein was eluted in 2 ml of elution buffer (50 mM Tris-HCl; pH 8.0, 5 mM glutathione). The glutathione in the solution was eliminated by using a spun column with Sephadex-G50 (Sigma Aldrich, USA). 1 unit of thrombin (Roche, Swiss) was added and was incubated at 18°C for 17 h. After reaction, the GST protein was removed by using a GSH-affinity chromatography.

Results and Discussion

Construction of the vector containing tags and *cat* gene

The orf of *gst* on the pGEX-4T-1 vector contained 4 ATA and 4 CGT codons which encoded isoleucine and arginine, respectively (Smith and Johnson, 1988). These rare codons were expected to reduce the expression level of the GST fusion protein in *H. pylori* (Del Tito *et al.*, 1995; Sayers *et al.*, 1995; Kurladn and Gallant, 1996). To solve this problem, two sets of PCR primers were synthesized. The primer set GST1 and GST2 amplified 229 bp of GST which contained an *Eco*RI site at the 5′-end and mutated codons (ATA to ATT, and CGT to AGA). The primer set GST3 and GST4 synthesized 570 bp of 3′ end of *gst* which contained mutated codons and an *Eco*RI site at the 3′-end. The overlapped parts of both fragments contained same mutated codons. Full length GST in which rare codons were changed to the non-rare codons of *H. pylori* was obtained by PCR amplification with GST1 and GST4.

The orf of *cat* was inserted at downstream of the GST. Because this *cat* in the vector didn't contain its own promoter, *cat* would express only if *gst* were transcribed. The *cat* gene also contained the rare codon ACA for threonine in *H. pylori*. To change this codon, two sets of PCR primers were synthesized. The primer set CAT1 and CAT2 synthesized 459 bp fragment and CAT3 and CAT4 synthesized 226 bp of 3′-part of *cat*. 35 bp of each fragment were overlapped. Full-length *cat* was amplified with CAT1 and CAT4 from the mixture of both fragments with mutated threonine codon (ACA to ACC).

Expression of the fusion proteins

Some *H. pylori* proteins such as FlaA, a flagellin and ClpB, an ATP-dependent protease were not expressed in *E. coli* (data not shown) (Suerbaum *et al.*, 1993). We have cloned and expressed more than 40 *H. pylori* genes in several *E. coli* strains. About half of them were expressed but formed an insoluble inclusion body. The rest were not expressed at all because of possible codon bias. Although all the rare codons were changed by site-directed mutagenesis, most of them were not expressed. Some mutated genes were expressed at a very low level (data not shown) which is essentially why this *H. pylori* expression vector was designed.

About 500 bp of 3′-terminal fragments without the stop codon of both *clpB* and *flaA* were PCR-amplified form *H. pylori* 26695, and were inserted into the expression vector. Final constructs containing either the *flaA* fragment or the

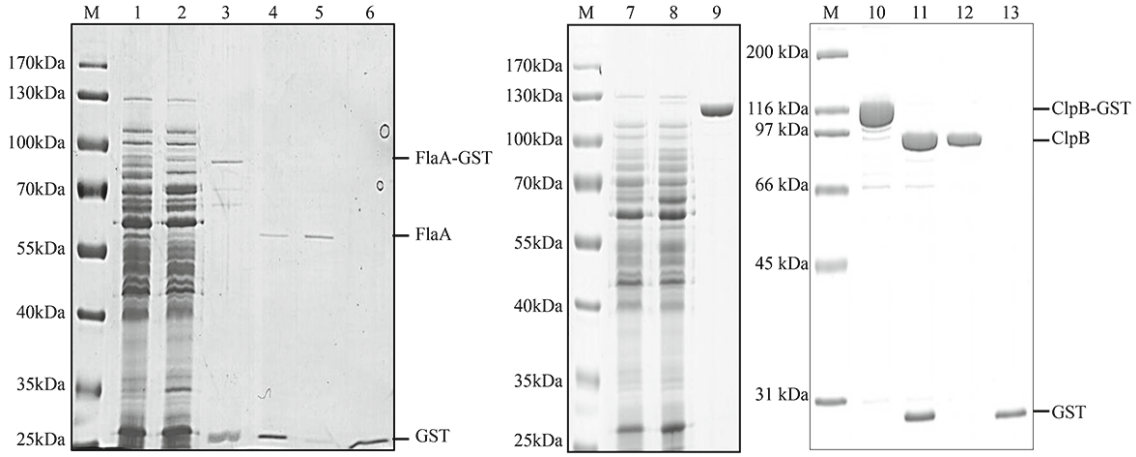

Fig. 2. Digestion of GST fusion protein of FlaA (A) and ClpB (B). GST was separated by thrombin protease. Lanes: M, protein size marker; 1, whole cell lysate of *H. pylori* 1061; 2, whole cell lysate of the recombinant *H. pylori*; 3, purified recombinant FlaA-GST; 4, thrombin-digested FlaA-GST; 5, purified FlaA using GSH-affinity column; 6, Eluted GST from the column; 7, whole cell lysate of *H. pylori* 1061; 8, whole cell lysate of the recombinant *H. pylori*; 9, 10, purified recombinant ClpB-GST; 11, thrombin-digested ClpB-GST; 12, purified ClpB using GSH-affinity column; 13, Eluted GST from the column.

clpB fragment, were introduced into *H. pylori* 1061. Transformants of *H. pylori* were cultured on the agar plate containing kanamycin and well grown colonies were picked and transferred to the plate containing chloramphenicol. The promoterless *cat*, which was located at the 6-nt downstream of GST tag would be transcribed only when the GST fusion gene was transcribed as a polycistronic mRNA. Therefore, it was expected that any chloramphenicol resistant *H. pylori* transformant expressed GST fusion protein using its own promoter.

Purification of the fusion proteins

The plasmid that contained target gene fragments was inserted into the *H. pylori* 1061 genome by homologous recombination, like other *H. pylori* integration vectors (de Vries *et al.*, 2001; Vannini *et al.*, 2012). In this vector system, the expression level of the fusion protein was regulated by its own promoter. Therefore, it is expected that the fusion proteins that were expressed in this system would not over-express in *H. pylori*. The same amount of *H. pylori* 1061 and recombinant clones were analyzed on 12% SDS-PAGE. A new protein band with the desired size was shown on the SDS-PA gel (data not shown).

After the affinity column, the recombinant fusion protein with the expected size was isolated. Thrombin digested the fusion protein into two protein fragments, one of which was the desired target protein and the other was GST protein (28 kDa). GST protein was removed successfully by GSH affinity chromatography after spin column removal of the GSH. Finally the target protein was purified and it alongside the digested protein FlaA were identified by western blotting with monoclonal antibody against *H. pylori* FlaA protein (Fig. 3). Although non-specifically generated GST protein was consistently co-purified by GSH-affinity chromatography, it was finally removed after thrombin digestion. 4 g and 8.3 g of wet weight of *H. pylori* cells for FlaA-GST and ClpB-GST were collected, respectively. The final purified recombinant proteins without tag were 38.8 μg of FlaA and 120 μg of ClpB. Therefore, the purification yields of the recombinant protein FlaA and ClpB from wet *H. pylori* cells were 0.001% and 0.0015%, respectively

This system does not contain *Helicobacter*-specific factors. Therefore, this vector can be integrated into any bacterial genome and can produce the GST-fusion proteins. We expect that this vector would be helpful for isolation of the pure protein for functional work or for the structural study of any proteins.

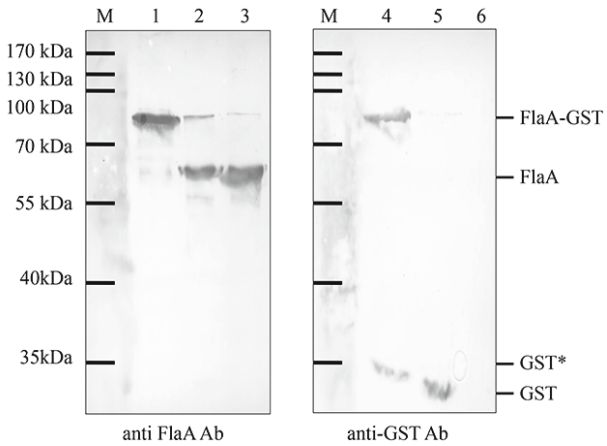

Fig. 3. Western blot analysis of the recombinant protein and GST using anti-FlaA antibody (A) and anti-GST antibody (B). Lanes: M, marker; 1,4, purified fusion proteins; 2,5, digested protein using thrombin; 3,6, eluted FlaA. A small amount of nonspecifically degraded GST fragment was detected on lane 4 by the anti-GST antibody (This fragment was also seen on lane 1 of Fig. 2).

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