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## Expression of *Helicobacter pylori* *cag12* gene in *Lactococcus lactis* MG1363 and its oral administration to induce systemic anti-Cag12 immune response in mice

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**Abstract** To develop an oral vaccine against *Helicobacter pylori* infection, we have expressed the *H. pylori* *cag12* (HP0532) gene, encoding the outer membrane protein Cag12 (31 kDa), in a live delivery vehicle *Lactococcus lactis*. The *cag12* gene was amplified by polymerase chain reaction (PCR) using the genomic DNA of *H. pylori* K51 isolated from Korean patients. DNA sequence analysis revealed that the *cag12* gene of *H. pylori* K51 has 98.1 and 97.4% identity with individual *cag12* genes of the *H. pylori* 26695 and J99, respectively. The GST–Cag12 fusion protein, produced using the *Escherichia coli* expression system, was used to raise a rat polyclonal anti-Cag12 antibody. The PCR-amplified *cag12* gene of *H. pylori* K51 was cloned in the *E. coli*–*L. lactis* shuttle vector (pMG36e) and transformed into *L. lactis*. Western blot analysis demonstrated that the Cag12 protein was expressed in the *L. lactis* transformant, with a maximum level at the log phase without extracellular secretion. The oral administration of the transformant into mice resulted in the generation of the anti-Cag12 antibody in serum in two out of five

cases. These results suggest that the recombinant *L. lactis*, which expresses Cag12, may be applicable as an oral vaccine to induce protective immunity against *H. pylori*.

### Introduction

*Helicobacter pylori*, which is a spiral and motile Gram-negative bacterium, causes gastritis, gastric and duodenal ulcer, mucosal-associated lymphoid tissue (MALT) lymphoma, and finally the development of gastric adenocarcinoma in humans (Czinn 2004; Crespo and Suh 2001). During the last decade, the pathogenic mechanisms of *H. pylori* have been intensively investigated. In particular, a significant advance in our understanding of the pathogenicity of *H. pylori* has been achieved by the investigation of the *cag* pathogenicity island (*cag*-PAI) that is ~40 kb in length. Severe forms of gastric diseases are mainly associated with infection by type I *H. pylori* strains, which can be distinguished from type II strains by the presence of *cag*-PAI (Rohde et al. 2003). *Cag*-PAI consists of 27–31 genes and encodes the type IV secretion system (T4SS) that builds a needlelike structure on the *H. pylori* surface. *H. pylori* is known to use T4SS to inject the cytotoxin-associated gene A (CagA) protein and possibly other factors into gastric epithelial cells, in a similar manner to T4SS of *Agrobacterium tumefaciens* that transfers oncogenic DNA and effector proteins to host cells during infection (Tanaka et al. 2003; Llosa and O’Callaghan 2004). Although the protein components of *H. pylori* T4SS, as well as the precise mechanisms by which T4SS mediate its diverse effects on host cells, remain obscure, it has been elucidated by microscopic techniques that the VirB7-homologous protein HP0532 (Cag12) is the T4SS component located in the outer membrane to form the base for the T4SS-associated needlelike structure (Rohde et al. 2003). A possible role of Cag12 could be as a secretin or a lipoprotein associated with a secretin that allows the transit of HP0527 (Cag7 or CagY) and/or the needlelike pilus structure. So far, several cellular components of *H. pylori*, including the CagA (Prinz et al. 2003), adhesin (Xu et al.

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2005), urease (Lee et al. 1999), vacuolating cytotoxin A (VacA) (Liu et al. 2004), neutrophil-activating protein (NAP) (Satin et al. 2000), and catalase (Chen et al. 2003), have been selected as putative antigens for the vaccination of *H. pylori*. However, none of the protein components of the T4SS, except for CagA, has been tested as a vaccine antigen that induces protective immunity against *H. pylori*, although these protein components are regarded as having the potential to be used as vaccines.

*Lactococcus lactis* is a Gram-positive, noninvasive, nonpathogenic, and food-grade bacterium (Xin et al. 2003). *L. lactis* can be used as a live delivery vector system of heterologous proteins for oral vaccination, irrespective of their cellular localization (cytoplasmic, cell surface, or secreted) (Nouaille et al. 2003). Although *L. lactis* is known to lack the ability to colonize in vivo, this bacterium has been shown to deliver heterologous antigens to induce mucosal and systemic immune responses via mucosal routes (Steidler et al. 2000). Recently, a number of research groups have reported that *L. lactis* can be genetically engineered to express bacterial or viral antigens, including the HIV Env antigen (Xin et al. 2003), *Brucella abortus* L7/L12 antigen (Ribeiro et al. 2002), papillomavirus Type 16 E7 antigen (Bermudez-Humaran et al. 2002), *Plasmodium falciparum* merozoite surface protein MSP3 antigen (Theisen et al. 2004), *Erysipelothrix rhusiopathiae* SpaA antigen (Cheun et al. 2004), SARS coronavirus nucleocapsid antigen (Pei et al. 2005), and tetanus toxin fragment C (Robinson et al. 2004). In an attempt to develop an oral vaccine against *H. pylori* in the present study, we have cloned the *cag12* gene from three different strains of *H. pylori*, namely, K51 (Korea), 26695 (UK) (Tomb et al. 1997), and J99 (USA) (Alm et al. 1999), and we analyzed the DNA sequence of the individual genes. The *cag12* gene of *H. pylori* K51 was expressed in a live delivery vehicle *L. lactis* by using the *Escherichia coli*-*L. lactis* shuttle vector (pMG36e). The results demonstrate that the *cag12* gene is highly conserved among the *H. pylori* isolates, and the successful expression of Cag12 (31 kDa) in *L. lactis* was detected by Western blot analysis. The results also indicate that two out of five mice were able to generate the anti-Cag12 antibody in serum by the oral administration of the *L. lactis* expressing Cag12.

## Materials and methods

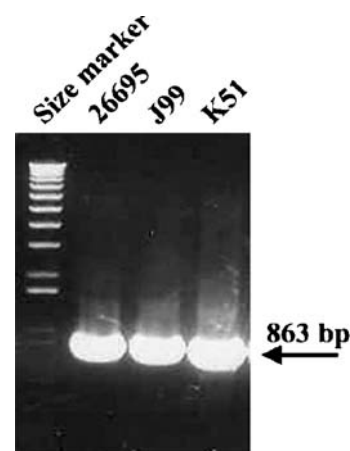
### Microorganism, vector plasmid, and growth conditions

The genomic DNAs purified from *H. pylori* K51 (Lee et al. 1999), *H. pylori* 26695, and *H. pylori* J99 were obtained from Dr. Kwang Ho Lee (Department of Microbiology, College of Medicine, Gyeongsang National University, Jinju, South Korea). *L. lactis* subsp. MG1363 and the *Escherichia coli*-*L. lactis* shuttle vector pMG36e plasmid were provided by Dr. Jeong Hwan Kim (Department of Food Technology, College of Agriculture, Gyeongsang

National University). *E. coli* BL21(DE3)pLysS and the protein expression vector pGEX-2T were purchased from Amersham (Arlington Heights, IL, USA). *L. lactis* was grown in M17 Medium (Difco, Detroit, MI, USA) supplemented with 1% glucose (M17G) at 30°C, and *E. coli* BL21(DE3)pLysS was grown in Luria-Bertani (LB) broth (Difco) at 37°C. When necessary, antibiotics were added to the culture medium at the following concentrations: ampicillin, 50 µg ml<sup>-1</sup>, and erythromycin, 200 µg ml<sup>-1</sup> for *E. coli* and 5 µg ml<sup>-1</sup> for *L. lactis*. The recombinant plasmid pGEX-2T harboring *H. pylori cag12* gene was designated as pGEX-2T/*cag12*, and the recombinant plasmid pMG36e inserted with the *H. pylori cag12* gene was designated as pMG36e/*cag12*.

### Polymerase chain reaction procedure and cloning of *cag12* gene in pGEX-2T vector

To insert the *H. pylori cag12* gene into the *Bam*HI site of the pGEX-2T vector, the *cag12* gene was amplified by polymerase chain reaction (PCR) in the presence of *H. pylori* genomic DNA and both *Bam*HI-forward primer 5'-AGGGATCCATGAAACTGAGA-GCA-3' and *Bam*HI-reverse primer 5'-AGGGATCCCAATCACTTACCACTG-3'. The target DNA was amplified in 50 µl of reaction mixture containing 5 µl of 10× buffer (100 mM Tris-HCl, 15 mM KCl, 1 mg ml<sup>-1</sup> gelatin, pH 8.3), 4 µl of 1.25 mM dNTP, 1 µl of target DNA (100 ng), 50 pmol of each forward and reverse primer, and 1 U of Taq polymerase (Promega, Madison, WI, USA). PCR was carried out under the following conditions: 30 cycles of 30 s at 94°C, 45 s at 55°C, and 1 min at 72°C. The amplified *cag12* gene was purified using an Elutip-D column (Schleicher & Schuell, Keene, NH, USA) and was cloned in the *Bam*HI site of



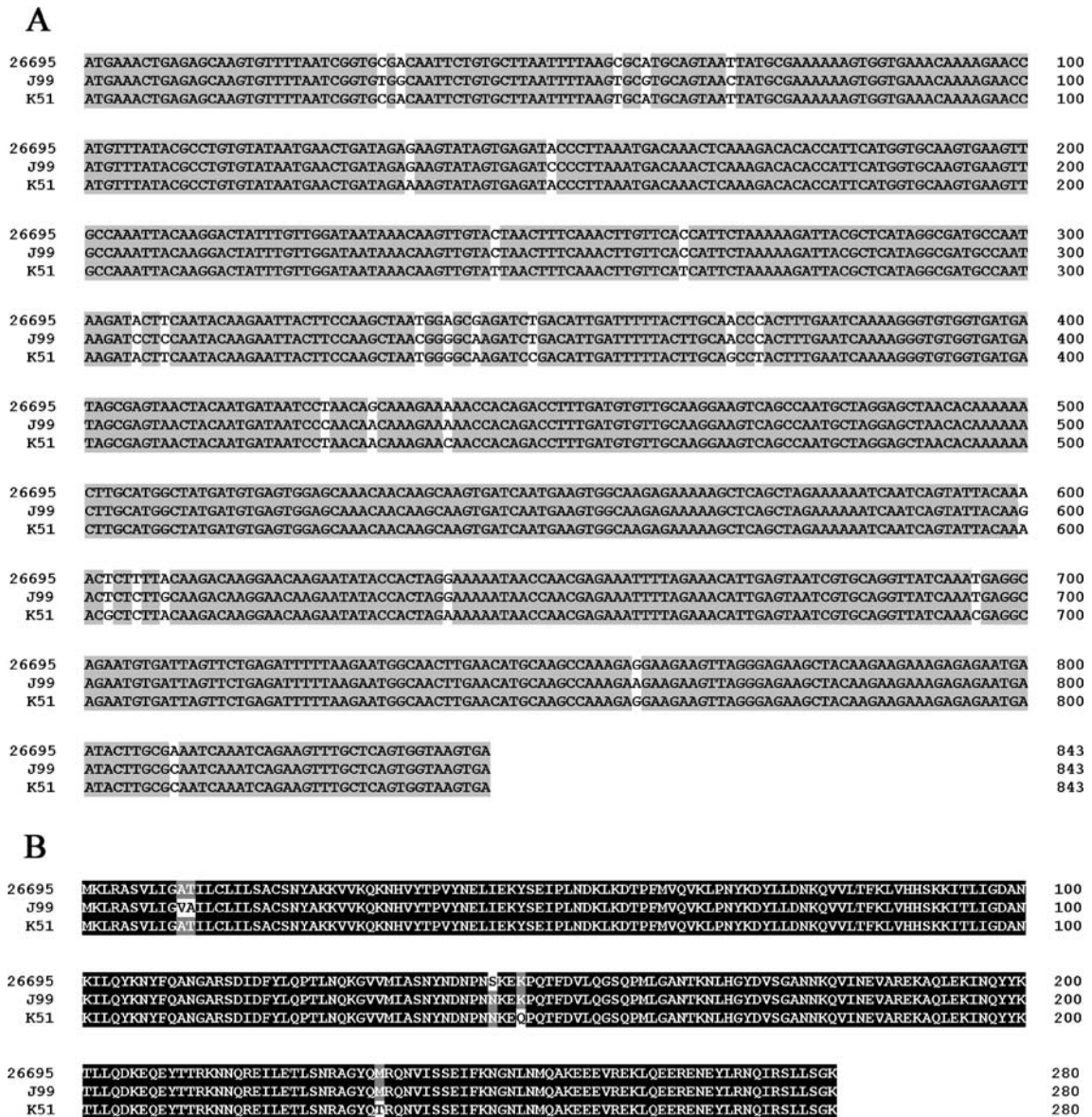
**Fig. 1** PCR-based amplification of the *cag12* gene with genomic DNA of *H. pylori* K51, 26695 (UK), and J99 (USA). The chromosomal DNAs isolated from individual strains of *H. pylori* were used as a template for the amplification of the *cag12* gene by PCR. The products were subjected to 0.8% agarose gel electrophoresis to confirm the expected length of the gene fragment (863 bp)

pGEX-2T. The plasmid was then introduced into *E. coli* BL21(DE3)pLysS and the transformants were selected on LB plates containing ampicillin (50 µg ml<sup>-1</sup>) and chloramphenicol (34 µg ml<sup>-1</sup>). The synthesis of the GST-Cag12 fusion protein in the *E. coli* transformant was induced by isopropyl-1-thio-β-D-galacto-pyranoside (IPTG), as previously described (Jun et al. 2003).

Construction of recombinant plasmid pMG36e/cag12

To insert the *cag12* gene into the *XbaI/PstI* site of the pMG36e vector, PCR was performed in the presence of *H. pylori* genomic DNA and both *XbaI*-forward primer 5'-CGTCTAGAAATGAACTGAGAGCA-3' and *PstI*-re-

verse primer 5'-ACTGCAGCAATCACTTACCACTG-3' (*XbaI/PstI* sites are underlined). PCR conditions are as follows: 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min. The amplified *cag12* gene was cloned in the *XbaI/PstI* site of the pMG36e, thus resulting in pMG36e/*cag12*, in which the *cag12* gene was placed under a strong tac promoter in sense orientation. The plasmid was then transformed into *E. coli* DH5α or *L. lactis*. For the transformation of *L. lactis*, electroporation was performed as previously described (Holo and Nes 1989). Briefly, to prepare competent cells, *L. lactis* cultured in a M17 medium until the optical density (OD) at 600 nm reached 0.5-0.7 was washed in ice-cold water and resuspended in 1/200 volume of 0.5 mol l<sup>-1</sup> sucrose containing 10% glycerol. After the



**Fig. 2** Comparison of nucleotide sequences of the coding regions of the *cag12* genes from *H. pylori* K51, 26695, and J99 (a) and their deduced amino acid sequences of the Cag12 proteins (b). Amino acids are displayed in a single-letter abbreviation after alignment for maximal identity by the CLUSTALW program

mixture of competent cells and plasmid DNA was treated with the Gene Pulser Apparatus (Bio-Rad, Richmond, CA, USA) according to the manufacturer's instructions, it was immediately diluted with a M17G broth and incubated for 2 h at 30°C, and then plated onto M17G plates containing 0.5 M sucrose and erythromycin ( $5 \mu\text{g ml}^{-1}$ ). The transformant was obtained after incubation for 48 h at 30°C.

#### DNA sequence analysis

Individual recombinant plasmid DNA constructs purified from the transformants of *E. coli* or *L. lactis* were subjected to DNA sequencing. The DNA sequence analysis was performed by the cycle sequencing method with ABI BigDye terminator, with an ABI Prism 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

#### Immunization of rat with GST-Cag12 fusion protein

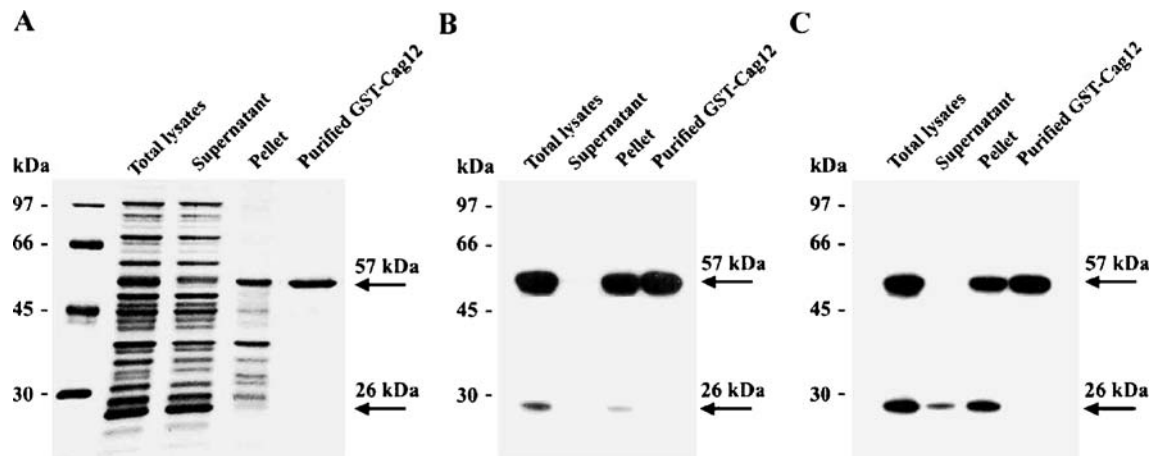
*E. coli* pGEX-2T/cag12-K51 was cultivated with shaking at 37°C and, when the growth OD at 600 nm reached to 0.4, 1 mM IPTG was added and cultivation was continued for an additional 3 h. Three hundred microliters of the GST-Cag12 (150  $\mu\text{g}$ ) purified by electroelution after Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the inclusion body fraction were mixed with an equal volume of Complete Freund's Adjuvant, and were injected into the thigh muscle of the rear legs of rats. For secondary and tertiary immunization, protein mixed with Incomplete Freund's adjuvant was injected into the rats in the same manner every 2 weeks. Bleeding was carried out 7 days after each immunization to test the antibody titer.

#### Cell lysate, protein quantitation, and Western blot analysis

The preparation of bacterial cell lysates were performed as described elsewhere (Jun et al. 2003). Briefly, the bacterial cells of *E. coli* or *L. lactis* were suspended in phosphate buffered saline (PBS), disrupted by sonication, and then incubated at 4°C for 30 min. After centrifugation at 14,000 rpm for 20 min, the supernatant was obtained as the soluble fraction of cell lysates and pellets were obtained as the inclusion body fraction of the cell lysate. Protein quantitation was carried out using Micro BCA kit (PIERCE, Rockford, IL, USA). Equivalent amounts of cell extracts (15  $\mu\text{g}$ ) were electrophoresed on a 9% sodium dodecyl sulfate(SDS)-polyacrylamide gel and electrotransferred to the Immobilon-P membrane (Millipore, Bedford, MA, USA). The membrane was probed with a primary antibody and then with a horseradish peroxidase-linked secondary antibody. Detection of each protein was visualized using the ECL Western blotting detection system, according to the manufacturer's instructions (Amersham).

#### Oral administration of recombinant *L. lactis* into mice

Either recombinant *L. lactis* pMG36e/cag12 or *L. lactis* pMG36e was cultured at 30°C for 18 h and harvested by centrifugation at 3,000 rpm for 5 min. The cell pellets were washed twice with cold PBS, and then resuspended in 300  $\mu\text{l}$  of PBS to a concentration of  $1 \times 10^{11}$  CFU/ml. For oral immunization, *L. lactis* pMG36e/cag12 or *L. lactis* pMG36e was administered into 8-week-old C57BL/6 male mice via the orogastric route using an oral sonde at weeks 0, 1, 2, 3, and 4. Mice were not provided with food and water for 1 h before the challenge, and food and water



**Fig. 3** Confirmation of the GST-Cag12 fusion protein (57 kDa) on 9% SDS-PAGE (a), and by Western blot analysis using either rat polyclonal anti-Cag12 (b) or mouse monoclonal anti-GST antibody (c). For the production of GST-Cag12 fusion protein from *E. coli* pGEX-2T/cag12-K51, the strain was cultivated with shaking at 37°C and, when the growth O.D at 600 nm reached 0.4, 1 mmol l<sup>-1</sup> IPTG was added and cultivation continued for an additional 3 h. Purification of GST-Cag12 from the inclusion body fraction was

carried out by electroelution. For Western blot analysis, 20  $\mu\text{g}$  of the cell lysate or 2  $\mu\text{g}$  of the purified GST-Cag12 fusion protein were electrophoresed on 9% SDS-polyacrylamide gel, and electrotransferred to the Immobilon-P membrane. The membrane was probed with rat polyclonal anti-Cag12 or mouse monoclonal anti-GST and then with a horseradish peroxidase-conjugated secondary antibody. Detection of each protein was performed using the ECL Western blotting detection system

were given 2 h after the challenge. Bleeding was carried out 4 days after the fifth oral administration.

## Results

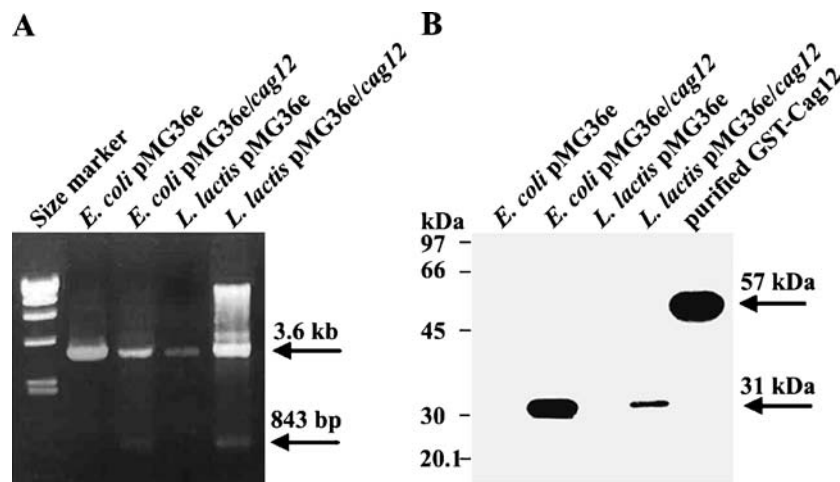
Sequence analysis of the *cag12* gene of *H. pylori* K51 and generation of polyclonal anti-Cag12 using GST-Cag12 fusion protein as the antigen

To investigate whether there is genetic diversity in the *cag12* gene between the Korean strain *H. pylori* K51 and two previous isolates, 26695 (UK) and J99 (USA), the DNA sequences of the *cag12* gene among these three strains were compared. For the cloning of the *cag12* genes of the *H. pylori* K51, 26695, and J99, the 863-bp of the PCR product (Fig. 1) amplified by PCR was ligated with the pGEX-2T vector plasmid, and then the ligation mixture was used for the transformation of *E. coli* BL21(DE3) pLysS. The recombinant plasmids (pGEX-2T/*cag12*-K51, pGEX-2T/*cag12*-26695, and pGEX-2T/*cag12*-J99) were purified from the transformants, and their DNA sequences were analyzed. As shown in Fig. 2a,b, the DNA sequence of the *cag12* gene of *H. pylori* K51 showed 98.1 and 97.4% identity with individual *cag12* genes of the *H. pylori* 26695 and J99, respectively, whereas the deduced amino acid sequence of Cag12 of *H. pylori* K51 appeared to share 98.9 and 98.6% identity with its 26695 and J99 counterparts, respectively. It is noteworthy that three independent recombinant plasmids for the individual *cag12* genes from *H. pylori* K51, 26695, and J99 were sequenced to exclude a possible occurrence of sequence errors due to the poor fidelity of the Taq polymerase and that there were no sequence differences among three independent clones of each *cag12* gene. The DNA sequences of the PCR-amplified *cag12* genes of both 26695 and J99 were

identical to the DNA sequence information that was previously published in the GenBank Databases. The DNA sequence data of the *cag12* gene of *H. pylori* K51 was submitted to the GenBank Databases under Accession No. DQ115386. These results demonstrate that the DNA sequence of the *cag12* gene is highly conserved among these three strains.

The optimal conditions for the production of the GST-Cag12 fusion protein using *E. coli* pGEX-2T/*cag12*-K51 were determined to be the following; 1 mM IPTG, 37°C, and 3-h induction period. These conditions brought out the maximum level production of GST-Cag12 fusion protein, although it was localized in the insoluble inclusion body fraction. To prepare this protein, the inclusion body fraction, which could be easily isolated from other cellular soluble proteins, was harvested from the cell lysate of *E. coli* pGEX-2T/*cag12*-K51 and was resolved on a SDS-PAGE, from which the protein was purified further by electroelution. As shown in Fig. 3a, the synthesis of the GST-Cag12 protein with a molecular mass of 57 kDa, which was induced as an insoluble form, could be detected on 9% SDS-PAGE. Approximately, 50 µg of GST-Cag12 fusion protein were obtained from 20 ml of culture fluid under the aforementioned purification conditions.

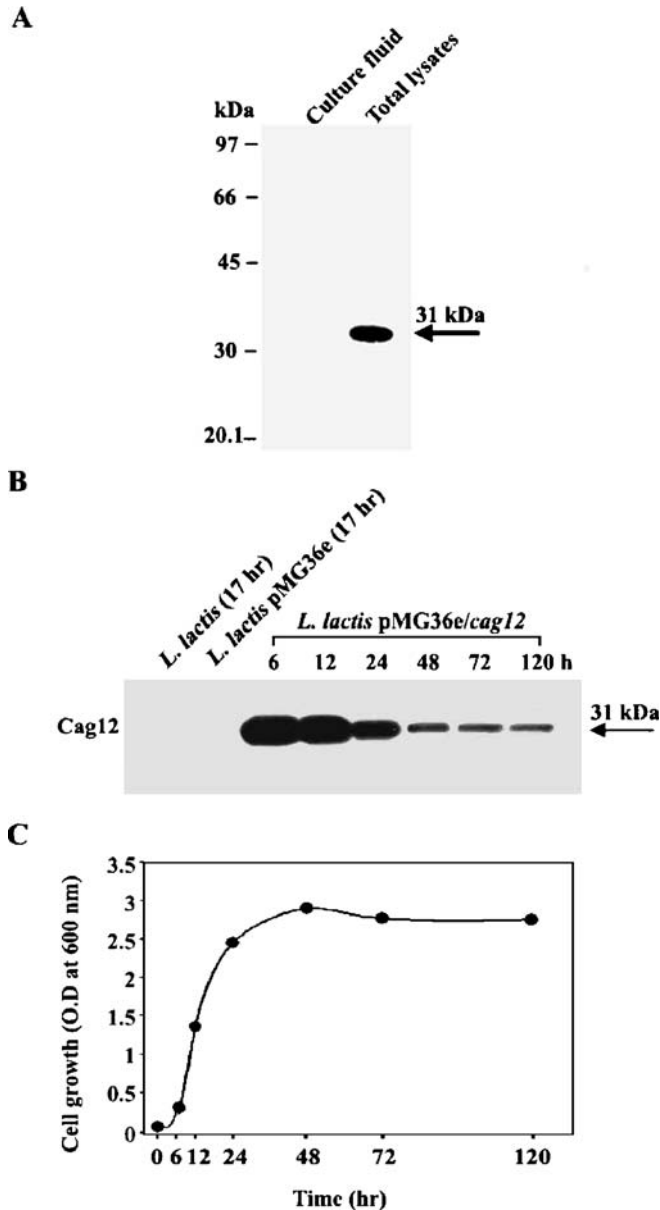
To produce a rat polyclonal antibody against the Cag12 protein of *H. pylori* K51, rats were immunized with the purified GST-Cag12 protein. The antiserum obtained 7 days after quaternary immunization in a 10,000-fold dilution was specifically able to detect the purified GST-Cag12 protein (57 kDa) by electroelution and the GST-Cag12 protein that was contained in the insoluble fraction or total cell lysate of *E. coli* pGEX-2T/*cag12*-K51 (Fig. 3b). It is noteworthy that the antiserum was able to detect an additional protein band with a molecular mass of 26 kDa. Because this additional protein band was predicted to be the GST protein (26 kDa) that originated from the



**Fig. 4** Visualization of recombinant plasmid pMG36e/*cag12*, introduced in *E. coli* DH5 $\alpha$  and *L. lactis* subsp. *lactis* MG1363, on agarose gel electrophoresis (a), and Western blot analysis of the expression of the Cag12 protein in each bacterial strain (b). The plasmid DNA, purified from individual bacterial strains, was electrophoresed on 0.8% agarose gel. For Western blot analysis,

20 µg of individual cell lysates was electrophoresed on 9% SDS-PAGE, and electrotransferred to Immobilon-P membrane. The membrane was probed with rat anti-Cag12 antibody, and then with a horseradish peroxidase-conjugated secondary antibody for detection of the Cag12 protein band using the ECL Western blotting detection system

pGEX-2T empty vector, the membrane was stripped and reprobed with anti-GST antibody to investigate this prediction. As shown in Fig. 3c, the 26-kDa protein band detected by the rat antiserum was also recognized by anti-GST antibody, confirming that it was GST protein. These results indicate that the rat polyclonal antibody, raised against the GST-Cag12 fusion protein, was able to specifically recognize the Cag12 protein.



**Fig. 5** Production of the Cag12 protein in *L. lactis* without extracellular secretion (a), and kinetic analysis of the production of Cag12 (b) during cultivation of the *L. lactis* transformant for 120 h (c). To confirm whether *L. lactis* pMG36e/cag12 can secrete the Cag12 protein, both the culture fluid and the cell lysate obtained from the *L. lactis* transformant harboring the pMG36e empty vector plasmid or the recombinant plasmid pMG36e/cag12 were analyzed by Western blotting. For the kinetic analysis of the expression level of Cag12 in *L. lactis* pMG36e/cag12 during the culture period, the strain was cultured in a M17 medium (Difco) supplemented with 1% glucose, 40 mmol l<sup>-1</sup> DL-threonine, and 5 µg ml<sup>-1</sup> of erythromycin

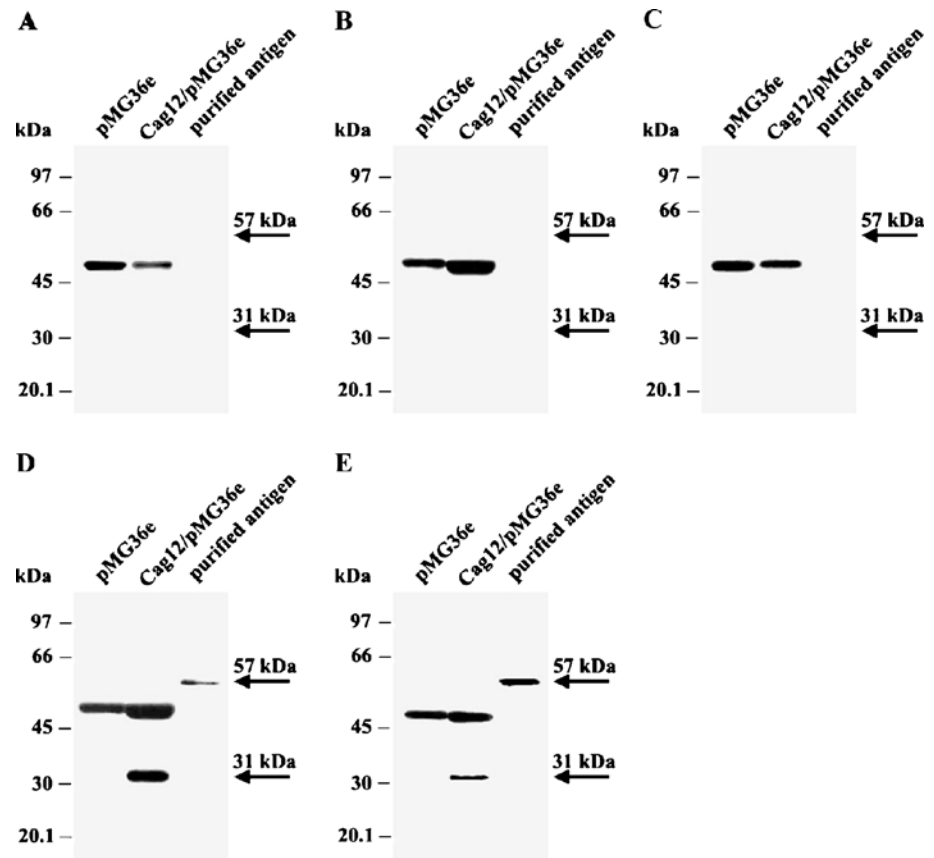
Cloning and expression of *cag12* gene of *H. pylori* K51 in *E. coli* and *L. lactis* using the shuttle vector plasmid pMG36e

To express the Cag12 protein of *H. pylori* K51 in the oral vaccine delivery vehicle *L. lactis*, the *cag12* gene was inserted into the *E. coli*-*L. lactis* shuttle vector pMG36e and the recombinant plasmid was then transformed into *E. coli* DH5 $\alpha$ . When the recombinant plasmid pMG36e/cag12-K51, isolated from the *E. coli* transformant, was subjected to digestion with the restriction enzyme, the presence of the inserted *cag12* gene (843 bp) was detected (Fig. 4a). The recombinant plasmid was further analyzed by DNA sequencing to confirm that the *cag12* gene of *H. pylori* K51 was properly cloned in the *E. coli*-*L. lactis* shuttle vector pMG36e (data not shown). Subsequently, the plasmid pMG36e/cag12-K51 was transformed into *L. lactis* to obtain the *L. lactis* transformant. The presence of the *cag12* gene in the *L. lactis* transformant was also confirmed by restriction enzyme digestion (Fig. 4a). To examine whether both *E. coli* and *L. lactis* transformants possessing the plasmid pMG36e/cag12-K51 can successfully produce the Cag12 protein, Western blot analysis for the cell lysates of the transformants was performed using the rat polyclonal anti-Cag12 antibody. As shown in Fig. 4b, the *E. coli* and *L. lactis* transformants were able to produce the 31-kDa Cag12 protein as a soluble form, but the expression level in *E. coli* was significantly higher than the level in *L. lactis*. These results indicate that the *cag12* gene of *H. pylori* K51 successfully induced the expression of the Cag12 protein in *E. coli* and in *L. lactis*. In addition, we have investigated whether the Cag12 protein could be secreted extracellularly from the *L. lactis* transformant into the culture fluid during cultivation. The Cag12 protein was detected in the cellular fraction, whereas it was not detected in the culture fluid (Fig. 5a). Time kinetics of the Cag12 protein expression in the *L. lactis* transformant during cultivation for 120 h was also investigated by Western blot analysis. As shown in Fig. 5b,c, the expression level of the Cag12 protein reached a maximum during an early log phase. It then gradually decreased as the population growth moved to the stationary phase. These results demonstrate that the expression level of Cag12 in the *L. lactis* was affected by the difference in growth phases from the log phase to the stationary phase, with a maximum level at the early log phase.

Antibody response against orally immunized recombinant *L. lactis* expressing Cag12

To examine whether orally delivered *L. lactis* expressing the Cag12 protein can induce a systemic humoral immune response against the Cag12 protein, two groups of C57BL/6 male mice (five mice in each group) were intragastrically inoculated with *L. lactis* pMG36e/cag12-K51 or *L. lactis* pMG36e. Then, the immune response against the Cag12 protein was evaluated by Western blot analysis using the individual mice sera. As shown in Fig. 6,

**Fig. 6** Western blot analysis of the Cag12 protein by using the antiserum obtained from the individual mice (A~E) that were orally immunized by the recombinant *L. lactis* expressing Cag12. To investigate whether a systemic humoral immune response against the Cag12 protein was induced in mice after oral administration of the recombinant *L. lactis* that expressed Cag12, equivalent amounts of cell extracts (15  $\mu$ g) prepared from the *L. lactis* transformant harboring empty vector pMG36e or recombinant plasmid *cag12*/pMG36e and 0.2  $\mu$ g of the purified GST-Cag12 fusion protein were electrophoresed on a 9% SDS-polyacrylamide gel, and then electrotransferred to the Immobilon-P membrane. The membrane was probed with each antiserum at 1,000 dilution and then with a horseradish peroxidase-linked secondary antibody. Detection of the Cag12 protein was visualized using the ECL Western blotting detection system



the serum of two out of five mice at a 1,000-fold dilution was able to detect the Cag12 (31 kDa) protein expressed in the *L. lactis* transformant and the purified GST-Cag12 fusion protein (57 kDa). At the same time, each serum could commonly recognize a protein band with a molecular mass of 49 kDa in the cell lysate. Because the sera of control mice immunized with *L. lactis* pMG36e also detected the 48-kDa protein band (data not shown), it was likely that the 48-kDa protein being expressed in the *L. lactis* was able to induce the humoral immune response after the *L. lactis* is orally administered into mice. However, none of the sera from the control group mice inoculated with *L. lactis* pMG36e were able to recognize the Cag12 protein. Consequently, these results demonstrate that the oral administration of the *L. lactis* expressing Cag12 protein resulted in the induction of a systemic humoral immune response against Cag12 in two out of five mice. This suggests that the recombinant *L. lactis* expressing Cag12 has the potential as an oral vaccine candidate that can induce protective immune response against *H. pylori*.

## Discussion

Infection with *H. pylori* is the most frequent gastroduodenal human disease in the world caused by bacteria. Once infection with *H. pylori* is diagnosed, antibiotic therapy combining a proton pump inhibitor with clarithromycin

and either amoxicillin or metronidazole is generally employed to eradicate the infection (Nakayama and Graham 2004). However, unsatisfactory results of current therapeutic regimens, due to bacterial antibiotic resistance, and the high reinfection rate after successful eradication have led to great interest in developing a vaccination method to prevent infection by *H. pylori* (Lee et al. 2001; Zendehdel et al. 2005). In the present study, the *H. pylori* Cag12 protein has been selected as the putative vaccine antigen and is expressed in *L. lactis*, which can deliver heterologous proteins to mucosal and systemic immune systems via mucosal routes. The Cag12 protein employed in this study is thought to fulfill the criteria that are important for a vaccine antigen because it is located in the outer membrane as a component of the T4SS by which *H. pylori* injects virulent factors, including CagA protein, into gastric epithelial cells (Tanaka et al. 2003; Llosa and O'Callaghan 2004). In addition, it shows no sequence homology with other known proteins in the GenBank Databases. Although a recent report that compared the genetic map of *H. pylori* K51 (1,679 kb) with those of *H. pylori* 26695 (1,668 kb) and *H. pylori* J99 (1,644 kb) indicated that there can be some variations in the genetic information between K51 and two previous isolates (26695 and J99) (Lee et al. 1999), the current results demonstrate that the DNA sequence of the *cag12* gene has 98.1 and 97.4% identity with those of 26695 and J99 counterparts, respectively, whereas the sequence identity between 26695 and J99 was 97.9%. This highly conserved character in the

DNA sequence of the *cag12* genes among different *H. pylori* isolates also supports the potential of the Cag12 protein as a vaccine antigen against *H. pylori*. To express the *H. pylori* Cag12 protein in an oral vaccine delivery vehicle, *L. lactis*, the PCR-amplified *cag12* gene was cloned in the *E. coli*-*L. lactis* shuttle vector pMG36e. The vector plasmid pMG36e contains a promoter, a ribosomal binding site, the start of an open reading frame, and a transcriptional terminator, and it is known to be a constitutive expression vector for the inserted gene in both *E. coli* and *L. lactis* (Van De Guchte et al. 1989). When the expression level of the Cag12 protein was compared between the *E. coli* and *L. lactis* transformant, the expression level of the 31-kDa Cag12 protein in *E. coli* was significantly higher than that in *L. lactis*, as determined by Western blot analysis using the rat polyclonal anti-Cag12 antibody. In the *L. lactis* transformant, the Cag12 was detected only in cellular fraction, indicating that Cag12 accumulates in intracellular fraction or on the cell surface without secretion. The expression level of the Cag12 protein appeared to vary during the growth phases in the *L. lactis* transformant so that it reached a maximum level at early log phase and decreased to the basal level at the stationary phase. Because the vector plasmid pMG36e is a constitutive expression vector, these results suggest that the Cag12 protein produced in *L. lactis* may be unstable during the stationary phase. It is worthy to note that the expression level of the *H. pylori* HpaA protein by the vector plasmid pMG36e in the *L. lactis* transformant remained constant regardless of the population growth phases (Kim et al. 2006). The oral administration of the *L. lactis* transformant expressing the Cag12 protein into mice generated the anti-Cag12 antibody in the serum of two out of five mice, indicating that the systemic humoral immune response to Cag12 delivered by *L. lactis* via mucosal route was induced.

In summary, the current results demonstrate that the *cag12* gene of *H. pylori* is highly conserved among the Korean strain (K51) and two previous isolates (26695 and J99). Also these results show that the *L. lactis* transformant possessing the recombinant plasmid pMG36e/*cag12* produced the Cag12 protein without extracellular secretion. The oral administration of the recombinant *L. lactis* expressing Cag12 into mice was able to generate the anti-Cag12 antibody in serum, thus suggesting that it may be used as an oral vaccine to induce protective immunity against *H. pylori*.

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