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Cloning and Expression of *hpaA* Gene of Korean Strain *Helicobacter pylori* K51 in Oral Vaccine Delivery Vehicle *Lactococcus lactis* subsp. *lactis* MG1363

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Abstract In order to develop an oral vaccine to prevent *H. pylori* infection, we have expressed the *hpaA* gene of *H. pylori* K51 isolated from Korean patients, encoding 29-kDa HpaA that is known to be localized on the cell surface and flagella sheath, in a live delivery vector system, *Lactococcus lactis*. The *hpaA* gene, amplified by PCR using the genomic DNA of *H. pylori* K51, was cloned in the pGEX-2T vector, and the DNA sequence analysis revealed that the *hpaA* gene of *H. pylori* K51 had 99.7% and 94.8% identity with individual *hpaA* genes of the *H. pylori* 26695 strain (U.K) and the J99 strain (U.S.A). A polyclonal anti-HpaA antibody was raised in rats using GST-HpaA fusion protein as the antigen. The *hpaA* gene was inserted in an *E. coli-L. lactis*-shuttle vector (pMG36e) to express in *L. lactis*. Western blot analysis showed that the expression level of HpaA in the *L. lactis* transformant remained constant from the exponential phase to the stationary phase, without extracelluar secretion. These results indicate that the HpaA of *H. pylori* K51 was successfully expressed in *L. lactis*, and suggest that the recombinant *L. lactis* expressing HpaA may be applicable as an oral vaccine to induce a protective immune response against *H. pylori*.

Key words: *Helicobacter pylori*, HpaA, rat polyclonal anti-HpaA, oral vaccine vehicle, *Lactococcus lactis*

Helicobacter pylori infects gastric mucosa and often causes gastritis, peptic ulcers, duodenal ulcers, MALT lymphoma, and gastric adenocarcinoma in humans [3, 7, 32]. Once the infection of *H. pylori* is diagnosed, antibiotic therapy that combines a proton pump inhibitor with clarithromycin and either amoxicillin or metronidazole is generally employed to eradicate the infection. Unsatisfactory results of current therapeutic regimens due to bacterial antibiotic resistance as well as a high reinfection rate after successful eradication [19, 35], however, have led to a great interest in developing a vaccination method to prevent infection by *H. pylori*. Several cellular components of *H. pylori*, including the cytotoxin-associated antigen A (CagA) [23], adhesin [34], urease [15], vacuolating cytotoxin A (VacA) [6, 17], neutrophil-activating protein (NAP) [26], and catalase [4] have been selected as putative antigens for the vaccination of *H. pylori*. Among these putative antigens, cell surface adhesins are considered to be the most promising candidates, because the adhesin molecules are physically able to interact with various types of host cells in order to confer a communication system that facilitates bacterial colonization via cross-talk between *H. pylori* and the host cells [8]. *H. pyroli* adhesin A (HpaA) has been reported to fulfill several criteria that are important for a vaccine antigen [18], in that HpaA is detected in all strains of *H. pylori* both on the bacterial surface and on the flagellar sheath [10, 31] and it is known to be a lipoprotein that is highly immunogenic in humans [21].

Lactococcus lactis has a number of advantages regarding its use as a live delivery vector system for oral vaccination [20]. *L. lactis* is a Gram-positive, noninvasive, nonpathogenic, and food-grade bacterium [13, 33]. Although it lacks the ability to colonize *in vivo*, this bacterium has been shown to deliver heterologous antigens to the mucosal and systemic immune systems via mucosal routes [27]. *L. lactis* is

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approximately the same size as biodegradable microparticles that can be taken up by M cells in gut-associated immune tissues, thus supporting the capability of *L. lactis* to act as an effective oral vaccine vehicle. The phosphopolysaccharide produced by *L. lactis* is nontoxic, but can function as a B-cell mitogen [14]. 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 [33], *Brucella abortus* L7/L12 antigen [24], papillomavirus Type 16 E7 antigen [2], *Plasmodium falciparum* merozoite surface protein MSP3 antigen [28], *Erysipelothrix rhusiopathiae* SpaA antigen [5], SARS-coronavirus nucleocapsid antigen [22], and tetanus toxin fragment C [25].

In an attempt to develop an oral vaccine against *H. pylori* K51 that was isolated from Korean patients [16], we have cloned the *hpaA* gene of *H. pylori* K51 in the present study, and have expressed the *hpaA* gene in a live delivery vehicle *L. lactis* subsp. *lactis* MG1363. In order to investigate whether there is genetic diversity in the *hpaA* gene between the Korean strain K51 and the two previous isolates 26695 (U.K.) [29] and J99 (U.S.A.) [1], we compared the DNA sequence of the genes among these three *H. pylori* strains. In addition, a rat polyclonal anti-HpaA antibody was raised in order to evaluate the expression of HpaA in *L. lactis* by Western blot analysis.

To clone the *H. pylori hpaA* genes of strains *H. pylori* K51, 26695, and J99, the individual *hpaA* genes were amplified by a PCR with the genomic DNA of the three strains in the presence of the BamHI-forward primer 5'-CGCGGATCCATGAAAGCAAATAA-3' and EcoRI-reverse primer 5'-GCGGAATTCTTATCGGTTTCCTTT-3' (BamHI/EcoRI sites are underlined). The PCR products (801 bp) obtained (Fig. 1A) were then ligated with pGEX-2T vector plasmid. The ligation mixture was used for the transformation of *E. coli* BL21(DE3)pLysS. Subsequently, the recombinant plasmids were purified from individual transformants selected on Luria-Bertani (LB) plates containing ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml), and designated as pGEX-2T/*hpaA*-K51, pGEX-2T/*hpaA*-26695, and pGEX-2T/*hpaA*-J99. Since each recombinant plasmid appeared to contain the *hpaA* gene, as determined by restriction enzyme digestion (Fig. 1B), their DNA sequences were analyzed. As shown in Figs. 2A and 2B, the DNA sequence of the *hpaA* gene of *H. pylori* K51 showed 99.7% identity with that of 26695 and 94.8% identity with J99, whereas the amino acid sequence of HpaA of *H. pylori* K51 shared 99.2% and 94.6% identity with the 26695 and J99 counterparts, respectively. Since the nucleotide sequences of the *hpaA* genes obtained from both 26695 and J99 were exactly the same with the sequence information previously published in the GenBank databases, it was certain that the sequence difference between the *hpaA* gene of K51 and those of the two previous isolates was not due to the artifact that could be generated during DNA amplification by a PCR. Previously, a comparison of the genetic map of *H. pylori* K51 (1,679 kb) with those of 26695 (1,668 kb) and J99 (1,644 kb) indicated that the chromosome loci of the *hpaA* gene were conserved between 26695 and J91, whereas their loci were significantly different from those of K51 [16]. The current results, taken together with previous results, indicate that, although there is significant variation in the *hpaA* gene location in the chromosomal DNA between K51 and two previous isolates (26695 and J99), the DNA sequence of the *hpaA* gene is highly conserved among *H. pylori* strains. The nucleotide sequence data of the *hpaA* gene of *H. pylori* K51 have been submitted to the GeneBank Databases under the Accession No. DQ115385.

In order to examine the optimal conditions for the production of the GST-HpaA fusion protein using *E. coli* pGEX-2T/*hpaA*-K51, the effect of IPTG concentration ranging from 0.1 to 1.0 mM as well as the effect of temperature ranging between 20 and 40°C on the induction level of the GST-HpaA protein was investigated. The optimal conditions for the production of soluble GST-HpaA protein appeared to be the induction at 37°C for 4 h in the presence of 0.1 mM IPTG (data not shown). The soluble form of the GST-HpaA protein contained in the cell lysate of the *E. coli* transformant was purified using glutathione-sepharose 4B. As shown in Fig. 3A, the synthesis of the GST-HpaA fusion protein with a molecular mass of 55 kDa was detected on 9% SDS-polyacrylamide gel electrophoresis. Approximately 50 μg of GST-HpaA fusion protein were obtained from 20 ml of culture fluid under the

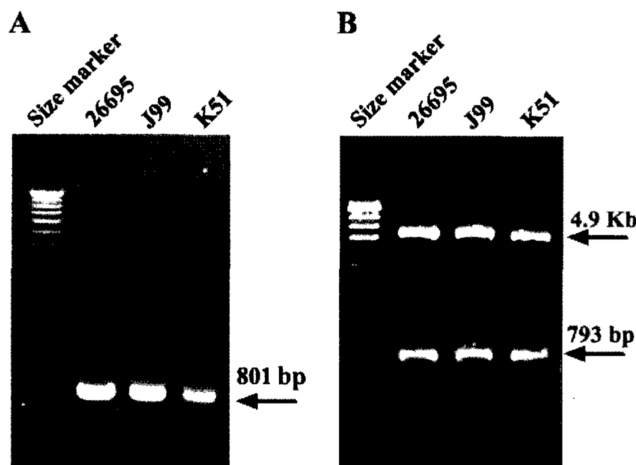

Fig. 1. Polymerase chain reaction (PCR)-based amplification of the *hpaA* gene with genomic DNA of *H. pylori* K51, 26695 (U.K), and J99 (U.S.A.) (A), and confirmation of their insertion into pGEX-2T plasmid by restriction enzyme digestion (B).
The chromosomal DNAs isolated from individual *H. pylori* strains were used as the template for amplification of the *hpaA* gene by a PCR. The products were subjected to 0.8% agarose gel electrophoresis in order to confirm the expected length of the gene fragment (801 bp). The *hpaA* gene (793 bp), inserted into pGEX-2T vector plasmid (4.9 kb), was identified by digestion with BamHI/EcoRI, followed by 0.8% agarose gel electrophoresis.

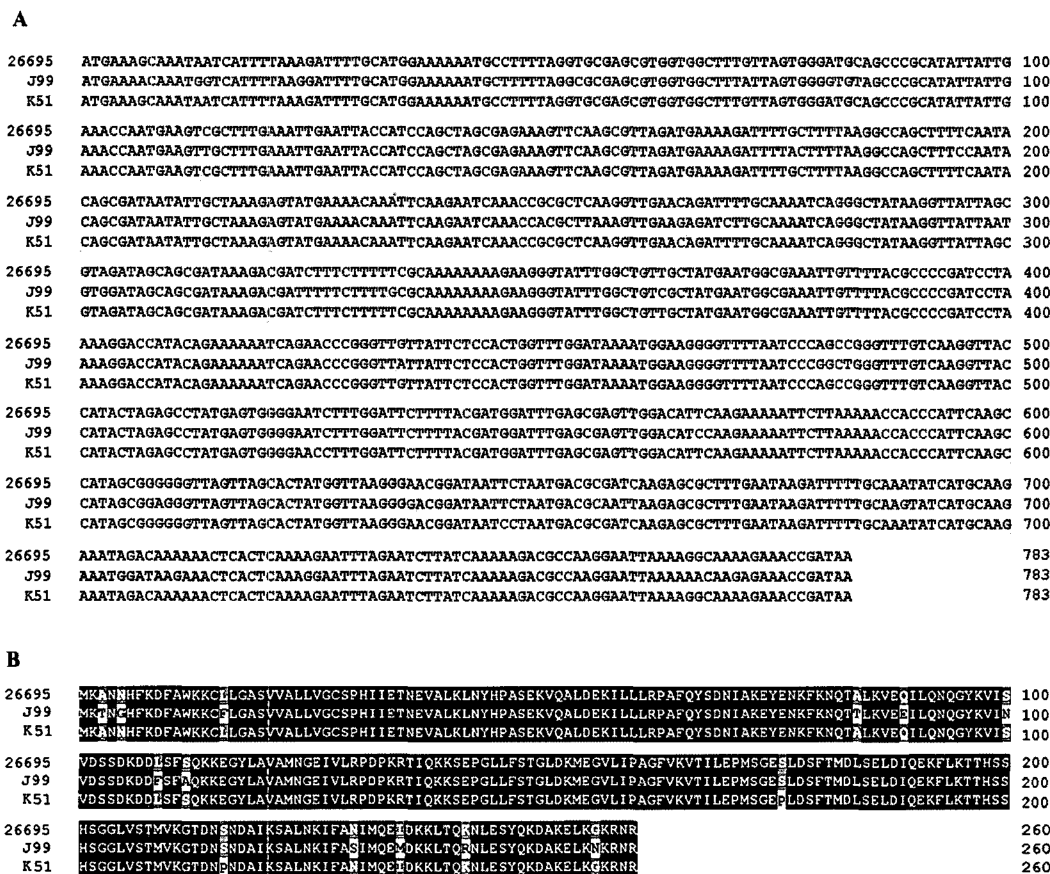

Fig. 2. Comparison of nucleotide sequences of the coding regions of the *hpaA* genes from *H. pylori* K51, 26695, and J99 (A), and their deduced amino acid sequences of HpaA proteins (B).

Each recombinant plasmid was purified from the *E. coli* transformants harboring pGEX-2T/*hpaA*-K51, pGEX-2T/*hpaA*-26695, or pGEX-2T/*hpaA*-J99, and the DNA sequences were analyzed. Amino acids are displayed in a single-letter abbreviation after alignment for maximal identity by the CLUSTALW program.

above purification conditions. To raise a rat polyclonal antibody against the HpaA protein of *H. pylori* K51, the purified GST-HpaA fusion protein (150 μg) was mixed with Freund's adjuvant and injected intramuscularly into the rats, as described elsewhere [11]. To evaluate the antibody titer by Western blot analysis, bleeding from the rat was conducted 7 days after each immunization. The antiserum, which was obtained 7 days after quaternary immunization in 4,000-fold dilution, was able to detect the GST-HpaA protein (55 kDa) in the cell lysate and the purified protein from the lysate using glutathione sepharose 4B (Fig. 3B). The antibody, however, was able to detect two additional protein bands with a molecular mass of 40 and 26 kDa, besides the GST-HpaA protein (55 kDa). These additional protein bands were predicted to be the GST protein (26 kDa), originating from the pGEX-2T empty vector, as well as protein fragments degraded from the intact GST-HpaA fusion protein *in vivo* or during the preparing of the cell lysate by sonication. The membrane was stripped and reprobed with anti-GST antibody in order to investigate this prediction. As shown in Fig. 3C, the protein bands detected by the rat anti-GST-HpaA fusion protein were also recognized by the anti-GST antibody, confirming that the 26-kDa protein band is GST, and that the 40-kDa protein band is derived from the degradation of the GST-HpaA fusion protein. These results indicate that the rat polyclonal antibody raised against the GST-HpaA fusion protein was able to specifically recognize both the GST and HpaA proteins.

In order to express the *H. pylori* HpaA protein in *L. lactis,* the *hpaA* gene was amplified by a PCR with the recombinant plasmid pGEX-2T/*hpaA*-K51, in the presence of both the SacI-forward primer 5'-TGAGCTCCAA-ATAATCATTTTAAAG-3' and the *Pst*I-reverse primer 5'-ATCTGCAGTTAGTTTCTTTTGCC-3' (SacI/PstI sites are underlined), and ligated with the *E. coli-L. lactis* shuttle vector pMG36e [30]. The ligation mixture was used for the transformation of *E. coli* DH5α, and the transformants were selected on an LB plate containing erythromycin (200 μg/ml). The recombinant plasmid pMG36e/*hpaA*-K51 was isolated from the transformant *E. coli* pMG36e/*hpaA*-K51, and was subjected to digestion with the restriction enzyme.

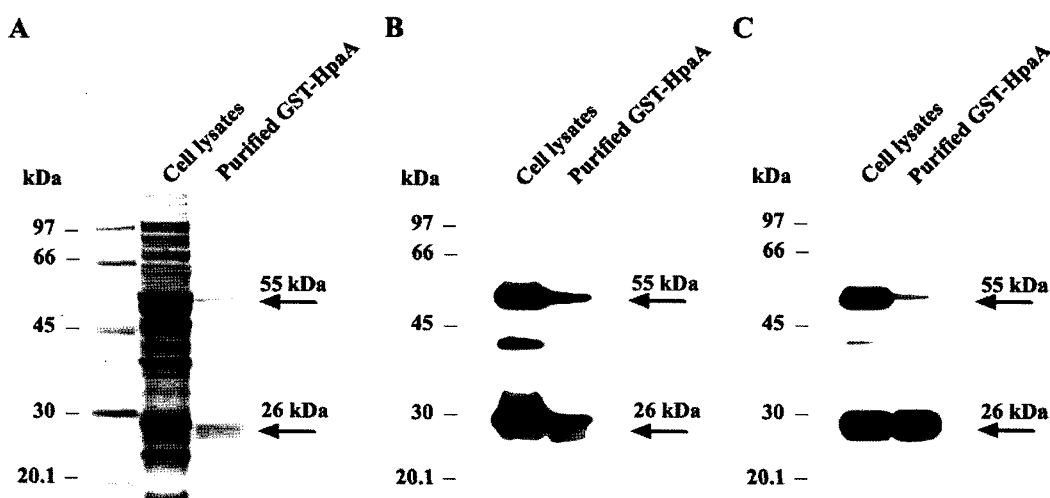

Fig. 3. Confirmation of the purified GST-HpaA fusion protein (55 kDa) on 9% SDS-PAGE (A), and Western blot analysis of the GST-HpaA by either rat polyclonal anti-HpaA (B) or mouse monoclonal anti-GST antibody (C).
The synthesis of the GST-HpaA fusion protein of the transformant, *E. coli* pGEX-2T/*hpaA*-K51, was induced under the optimal conditions. The cells were harvested, resuspended in PBS to disrupt by sonication, and then centrifuged to obtain the supernatant as the cell lysate. Purification of GST-HpaA from the cell lysate was carried out using glutathione sepharose 4B (Amersham Bioscience, Arlington Heights, U.S.A.). For Western blot analysis, 20 μg of the cell lysate or 2 μg of the purified GST-HpaA fusion protein were electrophoresed on 9% SDS-polyacrylamide gel and electrotransferred to an Immobilon-P membrane. The membrane was probed with a rat polyclonal anti-HpaA or mouse monoclonal anti-GST, and then with a horseradish peroxidase-conjugated secondary antibody. Detection of each protein was conducted using the ECL Western blotting detection system.

Since the restriction enzyme digestion showed the presence of inserted *hpaA* gene (776 bp) in the recombinant plasmid (Fig. 4A), the *hpaA* gene was further analyzed by DNA sequencing (data not shown). The results confirmed that the *hpaA* gene of *H. pylori* K51 was properly cloned in the *E. coli-L. lactis* shuttle vector pMG36e. Subsequently, the recombinant plasmid was transformed into *L. lactis* to obtain the transformant *L. lactis* pMG36e/*hpaA*-K51, by using an electroporation method as previously described [9]. In order to examine whether both *E. coli* and *L. lactis* transformants possessing the recombinant plasmid pMG36e/*hpaA*-K51 can produce the HpaA protein, Western blot analysis for the cell lysates of the *E. coli* and *L. lactis* transformants was performed using rat anti-HpaA antibody. The preparation of bacterial cell lysates and protein quantitation were performed as previously described [12].

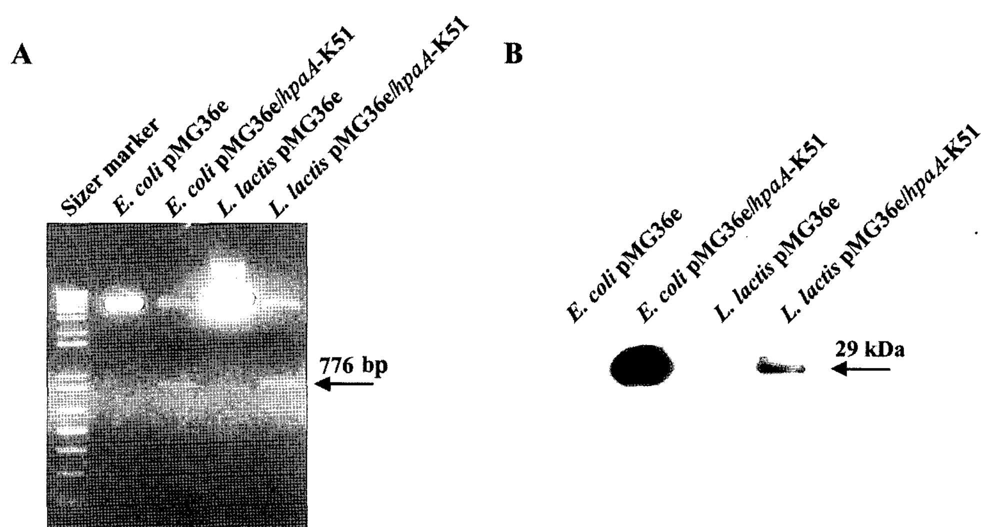

Fig. 4. Visualization of the recombinant plasmid pMG36e/*hpaA* and the empty vector plasmid pMG36e transformed into *E. coli* DH5α or *L. lactis* MG1363 after digestion with SacI/PstI followed by 0.8% agarose gel electrophoresis (A), and Western blot analysis of the expression of HpaA protein in each bacterial transformant (B).
For Western blot analysis, 20 μg of individual cell lysates were electrophoresed on 9% SDS-PAGE and electrotransferred to an Immobilon-P membrane. The membrane was probed with rat anti-HpaA, and then with a horseradish peroxidase-conjugated secondary antibody for detection of the HpaA band.

As shown in Fig. 4B, both *E. coli* and *L. lactis* transformants possessing the recombinant plasmid were able to synthesize the 29-kDa HpaA protein, but the expression level of the HpaA in *E. coli* was significantly higher than the expression level in *L. lactis*. Consequently, these results indicate that the *hpaA* gene of *H. pylori* K51 was expressed and could produce an easily detectable level of HpaA protein in *L. lactis*. Next, we investigated whether the HpaA could be secreted extracellularly from the *L. lactis* transformant into the culture fluid during cultivation. As shown in Fig. 5A, none of the HpaA was detected in the culture fluid, but they appeared to remain in a cellular fraction. Since *H. pylori* HpaA is known to be a lipoprotein localized on the cell surface and on a flagella sheath [31], the possibility of HpaA to be localized on the cell surface of *L. lactis* cannot be excluded. The exact localization of the HpaA protein expressed in *L. lactis*, however, needs to be further investigated. Time kinetics of the HpaA expression in *L. lactis* during cultivation for 72 h was also investigated by Western blot analysis. As shown in Fig. 5B, the expression level of 29-kDa HpaA was easily detectable as early as 5 h after cultivation, and the level remained constant by 72 h after cultivation, at which time the cell population began to decline slightly in the stationary phase of the growth phase (Fig. 5C). These results demonstrate that the expression level of HpaA was constant during the culture periods between 5 and 72 h, indicating that the HpaA level in *L. lactis* was not affected by the difference in growth phases from the exponential phase to stationary phase, in which the synthesis of total cellular proteins in *L. lactis* could significantly be changed in a growth-phase-dependent manner.

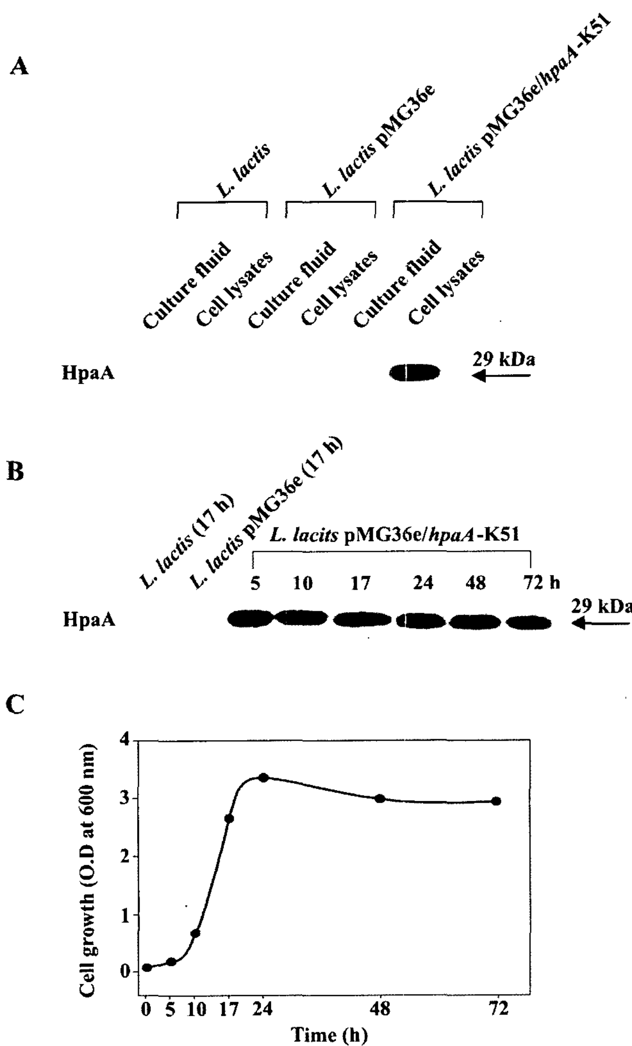

Fig. 5. Production of HpaA in *L. lactis* without extracellular secretion (A), and kinetic analysis of the production of HpaA (B) during cultivation of the *L. lactis* transformant for 72 h (C).

In order to confirm whether *L. lactis* pMG36e/*hpaA*-K51 can secrete the HpaA protein, both the culture fluids and the cell lysates, obtained from the *L. lactis* transformant harboring the pMG36e empty vector plasmid or the recombinant plasmid pMG36e/*hpaA*-K51, were analyzed by Western blotting. For the kinetic analysis of the expression level of HpaA in *L. lactis* pMG36e/*hpaA*-K51, the strain was cultured in M17 medium (Difco Laboratories, Detroit, U.S.A.) supplemented with 1% glucose, 40 mM DL-threonine, and 5 µg/ml of erythromycin for 72 h.

In summary, the current results demonstrate that the *hpaA* gene of *H. pyroli* is highly conserved among a Korean strain (K51) and two previous isolates (26695 and J99). The rat polyclonal antibody, raised against recombinant GST-HpaA fusion protein expressed in *E. coli*, was specific enough to detect the HpaA protein by Western blot analysis. The results also indicate that the *L. lactis* transformant harboring the recombinant plasmid pMG36e/*hpaA*-K51 was able to produce HpaA with no detectable extracellular secretion, and that the expression level of HpaA in the *L. lactis* remained constant throughout the population growth phases. This suggests that the recombinant *L. lactis* expressing HpaA may be applicable as an oral vaccine that can induce a protective immunity against *H. pylori*.

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