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Cloning and Expression of Recombinant *Helicobacter pylori* Urease A and B Subunits as a Putative Vaccine

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ABSTRACT

Helicobacter pylori **infection is among the most prevalent infections in the world involving more than half the adult population. *H. pylori* infection results in active chronic gastritis, peptic ulcers and enhances the risk of gastric malignancies. It is of utmost importance to prevent *H. pylori* infection particularly in highly prevalent countries including Iran. The urease holoenzyme produced by the entire *Helicobacter* species is essential for their virulence such that urease-negative mutant strains are unable to colonize the gastric lumen of various animal models. Furthermore, urease has shown to be an effective immunogen. Despite the fact that urease is considered among the very conserved genes of this pathogen, our molecular studies have shown that *H. pylori* strains obtained from Iranian patients vary considerably from those of other populations particularly the Western strains. Therefore, in order to develop a putative vaccine against *H. pylori* infection for the Iranian population, we have PCR-amplified and cloned the A and B subunits of this gene from a local *H. pylori* strain. Following identity confirmation, it was subcloned into a pET expression vector under the control of T7 promoter. The resulting plasmid was transformed into *E. coli* BL21-DE3 strain. Laboratory scale culture of the resulting transformants was analyzed by SDS-PAGE and Western blotting techniques. This analysis confirmed the expression of the A and B subunit of *H. pylori* urease protein up to 25% of the total cellular protein.** *Iran. Biomed. 1 5 (4): 107-111, 2001*

Keywords: Urease, *H. pylori*, Recombinant Protein, *E. coli*

INTRODUCTION

Helicobacter pylori organisms are spiral, microaerophilic, Gram-negative bacteria found in the gastric biopsy specimens [1]. This bacterium infects the gastric tissue of humans worldwide. In the developing countries, 70-90% of the population carries *H. pylori* but in the developed countries, this prevalence is approximately 50%. Most cases of the infections occur in childhood [2]. The infection is transmitted through oral-oral or fecal-oral contacts and via endoscope [3]. All *H* pylori-infected patients develop chronic gastric inflammation, but most of them are asymptomatic [4]. *H pylori* infection is the cause of nearly all of the idiopathic peptic ulcer diseases in adults [5]. *H pylori* is also strongly associated with the risk of atrophic gastritis which is considered a precursor to gastric cancer [4]. *H pylori* has been associated with gastric non-Hodgkin's lymphoma [6] and gastric mucosa-associated lymphoid tissue (MALT) lymphoma [7].

Urease is an important colonization factor of *H pylori* such that urease negative mutants are unable to colonize the stomachs of mice [8]. Urease activity is required for the production of a neutral micro-environment for the organisms within the gastric lumen. Urease activity is also toxic to human gastric epithelial cells and stimulates phagocy tic activity and cytokine production. Therefore, urease appears to function dually as a colonization (maintenance) factor and a virulence one [3]. Davin *et al.* [9] induced protective immunity in mice against *Helicobacter felis* by oral administration of *H. pylori* urease. Michetti *et al.* [10] used urease A and B subunits separately and demonstrated that the B subunit is more effective in protection although both subunits were found immunogenic. Lee *et al.* [11] achieved a 60-100% protection in mice using recombinant urease

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apoenzyme, they also reconfirmed that the the most important protective factor is secretory IgA. Orthesy-Theulaz *et al.* [12] induced 60% protective immunity in mice using recombinant salmonella host to deliver urease A and B subunits.

Dubois *et al.* [13] tested recombinant urease in Rhesus monkeys. This antigen was not protective in this model but the immunized animals had less inflammatory lesions in comparison to the non-immunized controls.

Due to the essential role of urease in *H pylori* infection, it has been considered as a suitable vaccine candidate against infection. Several groups have studied urease for its protective capacity in experimental mice [14]. Furthermore, due to the existing vast heterogeneity among H. *pylori* strains it is essential to develop candidate vaccine for strains infecting the target population. In this paper, we report the cloning and expression of urease A and B subunit from a local *H. pylori* strain.

MATERIALS AND METHODS

Cloning of urease gene. Two primers were designed according to urease sequence reported by Clayton *el at* [15]. A 23-bp oligonucleotide containing Ndel site was used as the forward primer. This primer was complementary to the starting sequence of the *ureA* gene (UreA23: ATC GAT CAT ATG AAA CTC ACC CC). A 26-bp oligonucleotide containing an EcoR1 site was designed as the reverse primer. The primer was complementary to the ending sequence of the *ureB* gene (Ure-End: AAA GAA TTC-TAG AAA ATG CTA AAG AG). This primer pair amplifies both *ureA* and *ureB* genes that are located sequentially on the genome. Genomic DNA of a local *H. pylori* strain, was extracted using Qiagene DNA extraction kit (Santa Clarita, USA) according to the instruction provided by the manufacturer. PCR amplification of the urease A and B genes was performed according to the standard protocol under the following conditions; 94°C (5 min) plus 30 cycles of 94˚C (1 min), 58˚C (I min), 72˚C (1.5 min) and a final extension cycle of 72˚C (10 min). The PCR product was cloned in pCR2.1 TA cloning vector (Invitrogen Inc., Groningen, Netherlands) according to manufacturer's instructions. The cloned genes were confirmed by sequencing.

Subtyping of the cloned genes. PCR amplification and subtyping of the *ureB* gene was performed according to report by Colding *et al.* (submitted for publication). A 933-bp fragment of the *ureB* gene was amplified by PCR. This fragment was digested by Sau3al and analyzed by 2% agarose gel electrophoresis. An American Western *H. pylori* strain (P17) was used for comparison.

Expression of urease A and B subunits. The wet(and *ureB* genes were cloned in the pET23a expression vector (Novagen Inc., Darmstadt, Germany) using Ndel and EcoRI restriction sites created by synthetic primers. The recombinant plasmid (pET-urease) was transformed into *E. coli* expression host (BL2 I -DE3). The transformants were then inoculated in 5 ml LB-ampicillin culture tubes and grown at 37˚C on a shaker incubator until an optical density at 600 nm of 0.7 was reached following induction with IPTG (400 µM) for 4 hours. Aliquots (1 ml) were taken before and after the induction. The apparent mass of the constituent proteins of each sample was analyzed by SDS-PAGE and Coomassie Blue staining [16].

Western blotting. To confirm the identity of the expressed proteins as urease A and B subunits of *H. pylori*, Western blotting was used according to standard protocols [17]. Two Western blots were performed. In the first blot, a patient serum containing high titer of anti- *H. pylori* antibodies was used at 1:1000 dilutions. A second immunoblot was performed· using rabbit polyclonal antibodies raised against urease B subunit (a gift from Pasteur Institute of Paris). Mouse anti-human IgG and goat anti-rabbit IgG HRP-conjugates (DAKO, Glostrup, Denmark) were used as secondary antibodies, respectively.

RESULTS AND DISCUSSION

Cloning of urease genes. The 2444-bp fragment corresponding to urease A and B coding sequences was amplified using ureA23 and ureEnd primers (Fig. 1). This fragment was cloned into pCR2. 1 cloning vector and partially sequenced at both ends. In comparison to urease sequences reported by Clayton *et al.* [15], there were only two different nucleotides among the total number of bases sequenced. This nucleotide changes were T(45)→C and G(2283)→A. Both changes were nonsense and caused no amino acid change in the expressed protein.

Subtype analysis. In order to determine the homogeneity of the cloned urease regarding to native strains, we compared the cloned gene with that of native Iranian and American strains. The comparison was performed by PCR amplification of a 933-bp fragment of the ureB gene followed by restriction digestion of the PCR product via the enzyme Sau3AI. PCR-RFLP profiles were then compared on agarose gel electrophoresis (Fig. 2). Subtype analysis demonstrated that the cloned *ureB* gene possessed identical profile as the majority of the Iranian strains and distinct from that of the American strain (Fig. 2). In other relevant studies performed by Mohammadi *et al.* (unpublished data) 60% of the Iranian *H. pylori* strains possessed this digestion profile.

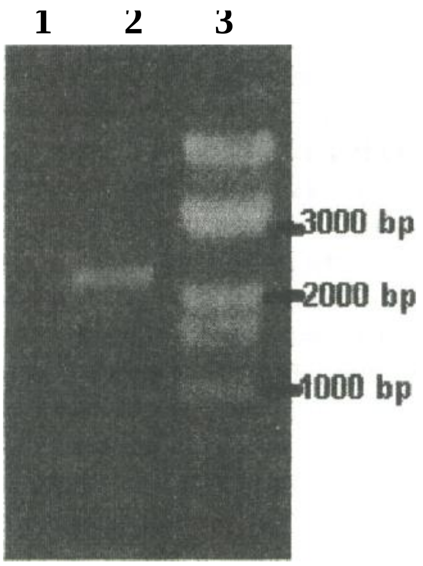

Fig. 1. PCR product containing *ureA* and ureB*:* Lane I, Molecular weight marker; Lane 2, Urease PCR product; Lane 3, Negative control.

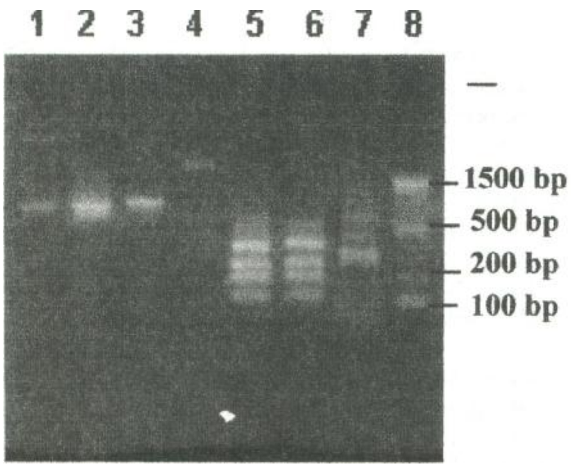

Fig. 2. Urease Subtyping via PCR-RFLP. Stated lanes view PCR products and digestion profiles, respectively. Lanes 1 & 7, Western strain; Lanes 2 & 6, Iranian strain; Lanes 3 & 5, pT-Urease; Lane 4; 50 bp; Lane 8, 100 bp molecular weight markers.

Subcloning and transformation. The cloned genes were subcloned in pET-23a using Ndel/EcoRI digestion sites. The resulting plasmid was named pET-urease (Fig. 3). In this construct, the genes for urease A and B subunits are located under the control of T7 promoter. PET-urease was transformed into *E. coli* BL21-DE3 cells. These cells contain the gene for T7 polymerase under the control of the Lac promoter. Thus, by addition of IPTG, T7 polymerase is expressed which results in the transcription of T7 promoter and the downstream genes. Samples before and after induction were analyzed on SDS-PAGE (Fig. 4A). Two bands with molecular weights of 30 kDa and 60 kDa corresponding to the urease A and B subunit respectively were intensified in the samples after induction [15]. The level of expression of urease A and B was determined by densitometry to be 10% and 15%, respectively (data not shown). In order to confirm the identity of the expressed proteins, the resulting protein bands on the SDS-PAGE underwent Western blotting using patient serum. Despite the appearance of our desired bands, additional bands were also observed which may be due to heat shock protein-reactive antibodies (Fig. 4B). Therefore, a rabbit polyclonal serum raised against the B subunit of *H. pylori* urease was used in the Western blot and the presence of the B subunit was confirmed (Fig. 4C).

In comparison with previous reports, the system we have used possesses a highly significant efficiency. In 1990, Hu and Molbey [18] reported expression of *H. pylori* urease in *E. coli* using its own promoter, whilst its low level of expression

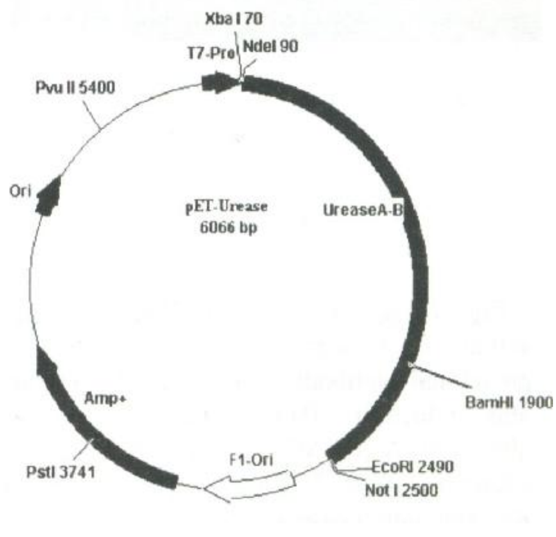

Fig. 3. Map of the pET-Urease plasmid.

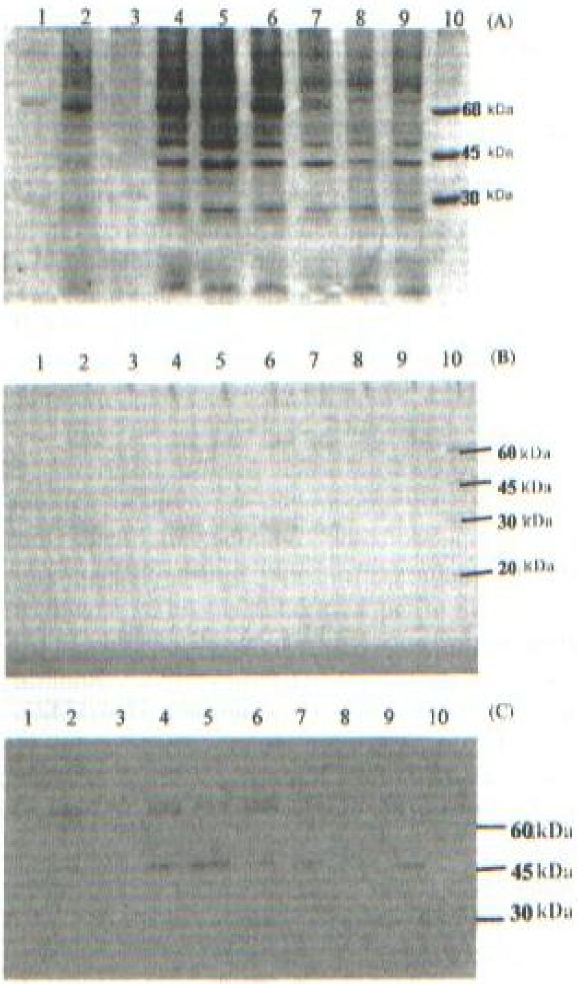

Fig. 4. A, SDS-PAGE; B, Western blotting by patient serum; C, Western blotting by Rabbit anti Urease B polyclonal antibodies; of the cellular proteins before and after induction. Paired lanes view samples before and after induction 1, MWM; 2 & 3, Negative control; 4 & 5, Clone #1; 6 & 7, Clone #2; 8 & 9, Clone # 3; 10, Recombinant urease as positive control.

was only visible via Western blotting. Michetti *et al.* [10] expressed urease A and B subunits separately in *E. coli* and were able to achieve considerable levels of expression. Ferrero *et al.* [19] produced the urease subunits as a fusion to MalE protein, in order to enhance protein expression. The expression levels were high but the proteins lacked native conformation. A system very similar to ours was reported by Lee *et al.* [11], who used pET24+ expression vector and cloned urease A-B coding region and were able to obtain high yields with near native conformation observed by electron microscopy.

Present study has been able to achieve the following: 1) Urease A and B subunits from a local *H. pylori* strain have been cloned and expressed; 2) The subunits were expressed with a significantly high yield and thus can be used for further studies. 3) It has provided local access to a potential vaccine and other laboratory applications.

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