J. Life Science. (1999), **9**(1), 5~8

JOURNAL OF
Life Science

Deletion Mutageneses of the *Helicobacter pylori* Urease Accessory Genes

Jae Young Sung and Mann Hyung Lee†

College of Pharmacy, Catholic University of Taegu-Hyosung, Hayang, Kyongsan, Kyongbook 712-702, Korea

Received: April 9, 1999

Abstract *Helicobacter pylori* is the etiologic agent of human gastritis and peptic ulceration and produces urease as the major protein component on its surface. *H. pylori* urease is known to serve as a major virulence factor and a potent immunogen. Deletion mutageneses were performed in the *H. pylori* urease accessory genes by using combinations of restriction enzymes and other DNA modifying enzymes in order to assess the function of these accessory gene products in the expression of the active urease. Selective disruptions in the accessory gene regions resulted in complete abolishment of the urease activity, which is consistent with other bacterial ureases. Interestingly, deletions in *ureE*-containing regions caused reduced expression of the structural enzyme subunits.

Key words: *Helicobacter pylori*, urease, nickel, deletion mutagenesis, accessory genes

Introduction

Helicobacter pylori is a microaerophilic, spiral-shaped, Gram-negative bacterium which colonizes gastric mucosa of humans, non-human primates and pigs. *H. pylori* produces bacterial urease of high activity up to 6% of the soluble cell protein [1] and this surface-presented, two-subunit enzyme [2] is distinct from other bacterial ureases which are made of three subunits and localized in the cytoplasm. *H. pylori* urease serves as a major surface immunogen [3,4] and as an important survival factor for the bacterium in the acidic environment of the gastric lumen [5]. As in the cases of other bacterial enzymes, *H. pylori* urease requires nickel ions as an essential cofactor for the enzyme activity. Sequence analysis revealed the presence of two structural genes (*ureA* and *ureB*) followed by the additional accessory genes (*ureI, E, F, G* and *H*). These non-subunit auxiliary genes are thought to be involved in nickel processing into the apo-urease since the corresponding accessory genes in the *K. aerogenes* urease gene cluster were previously shown to be required for urease metallocenter assembly [6]. For example, deletion analysis of the *Klebsiella aerogenes* urease gene cluster revealed that one gene (*ureD*) located directly upstream and three genes (*ureE*, *ureF*, and *ureG*) found immediately downstream of the three structural subunit-encoding genes (*ureA*, *ureB*, and *ureC*) are involved in the functional assembly of the urease metallocenter [6,7]. Although detailed and precise function of these accessory proteins has not been established, UreE has been shown to be a nickel-binding protein [8], and other accessory proteins are thought to function as complexes with the urease subunits [9,10].

In this report, we generated deletion mutants which include selective gene disruptions in each or combinations of the accessory genes and the urease activities of these mutants were compared with the clones containing parental recombinant plasmids. Effects of the absence of these accessory gene products on the expression of urease-related proteins were also examined.

Materials and Methods

Construction of plasmids and deletion mutageneses of the accessory genes

Previously described plasmid pHU1013 [11], a pBluscript KSII(+) derivative, containing the whole urease gene cluster of *H. pylori* was isolated and purified from the *E. coli* BL21 (pHU1013) culture by using standard alkaline-lysis methods and Gene Clean (II) kit (Bio101 Inc., CA, USA). Separately, pHU1012 was constructed by subcloning a *Mlu*I-*Eco*RI fragment (6.3 kb) from the plasmid pHP808 [12] into a *Eco*RI-*Sma*I digested, calf intestinal phosphatase-dephorylated pUC19 vector.

Selective deletions within *ureE, ureIEF, ureGH, ureH* regions were obtained by using a combination of restriction digestion and *Bal*31 digestion. For *ureE* deletion, pHU1013 was digested with *Bsi*WI then the ends were bidirectionally digested with *Bal*31 and made blunt-ended by treating with T4 DNA polymerase and then with Klenow fragment for 15 min at 12°C, respectively. The linearized DNA was extracted with phenol-chloroform and self-ligated with T4 DNA ligase.

†Corresponding author
Phone: 82-53-850-3611, Fax: 82-53-850-3602
E-mail: mhlee@cuth.cataegu.ac.kr

This clone, designated Δ*ureE*, contained about 250 bp deletion. For *ureH* deletion, pHU1013 was digested with *Mun*I and the larger fragment was isolated from an agarose gel by using Gene Clean (II) kit then self-ligated and designated Δ*ureH*. For *ureIEF* deletion, pHU1013 was digested with *Nco*I and self-ligated as above and designated Δ*ureIEF*. For *ureGH* deletion, pHU1012 was digested with *Nsi*I and self-ligated after removing smaller fragment and designated Δ *ureGH*. These deletion-mutagenized plasmids were transformed into *E. coli* BL21 or DH5α. The extent of deletions was confirmed by 1.2 or 1.5% agarose gel electrophoresis by using 100 bp-, 500 bp- and 1 kb-ladders as standards after restriction pattern analyses with appropriate restriction enzymes.

Cell Growth and Disruption

E. coli BL21 (pHU1013), *E. coli* DH5α (pHU1012), and cells carrying deletion mutant plasmids were grown to late exponential phase in LB media containing 1 mM nickel chloride and 100 μg/ml ampicillin. *Helicobacter pylori* ATCC43504 and NCTC11638 cells were incubated in brain heart infusion agar plates and brucella broth in an anaerobic jar containing a CampyPak Plus (BBL, USA) gas generator pouch.

Cells were harvested by centrifugation, washed twice with ice-cold 20 mM potassium phosphate (pH 7.0)-1 mM EDTA-1 mM 2-mercaptoethanol (PEB) buffer, resuspended in an equal volume of PEB containing 1 mM phenylmethylsulfonyl fluoride, disrupted by using a sonic dismebrator (Fisher Scientific Co., PA, USA) with a maximum output of 70 W for 1 min/ml, and centrifuged at 50,000 xg for 30 min.

Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out by using buffers of Laemmli [13] and included a 12% polyacrylamide running gel with a 4.5% polyacrylamide stacking gel. Gels were stained with Commassie brilliant blue R250.

Urease Assay

Urease activity was measured by quantitating the rate of ammonia released from urea by formation of indophenol, which was monitored at 625 nm as previously described [14]. The assay buffer consisted of 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 50 mM urea, and 0.5 mM EDTA (pH 7.5). The reactions were initiated by the addition of enzyme, the concentration of released ammonia was measured in time aliquots, an the rates were determined by linear regression analysis. One unit of urease activity is defined as the amount of enzyme required to hydrolyze 1 μmol of urea per min at 37℃ under the assay conditions described above. Protein was measured by the method of Lowry *et al.* [15], with the bovine serum albumin as the standard.

Results And Discussion

Extracts of *H. pylori* ATCC43504, NCTC11638, *E. coli* BL21(pHU1013) and *E. coli* DH5 α (pHU1012) cells were examined for urease activity as shown in Table 1. *E. coli* BL21(pHU1013) produced the recombinant urease of highest specific activity, whereas *E. coli* DH5α (pHU1012) produced the enzyme of relatively low specific activity compared to wild-type *H. pylori* strains. It is as expected since in *E. coli* BL21(pHU1013) expression of the urease structural genes were apparently directed by a strong T7 phage promoter, while in *E. coli* DH5α (pHU1012) RNA transcription was under the influence of its own *H. pylori*-specific promoter. Deletion mutagenesis, however, were performed with both pHU1013 and pHU1012 for different availability of restriction sites.

A map of the *H. pylori* urease gene cluster indicating deletions within the *ureE, ureH, ureIEF,* and *ureGH* genes is provided in Fig. 1. Extracts of recombinant *E. coli* cells from control cultures and from each of the deletion mutants were examined for urease activity, as shown in Table 2. Ureases from all the deletion mutants were inactive. The Δ*ureE* mutant also showed completely inactivation of the urease, which is in contrast with the *Klebsiella aerogenes* Δ*ureE* mutants that retained partial activity [6].

The extracts from the deletion mutants were subjected to SDS-polyacrylamide gel electrophoresis, as shown in Fig. 2. The urease subunits (UreA and UreB) were present at high levels except in the Δ*ureE* and Δ*ureIEF* clones. This is also in contrast with the *K. aerogenes* studies in which the mutations in the accessory genes did not affect urease expression. Lee *et al.* [8] observed that *K. aerogenes* UreE

Table 1. Urease specific activities of cell extracts from *Helicobacter pylori* standard strains and the recombinant *E. coli* cells

Strains	Specific activity (U/mg)
H. pylori ATCC43504	20.98
H. pylori NCTC11638	13.71
E. coli BL21(pHU1013)	39.90
E. coli DH5 α (pHU1012)	7.70

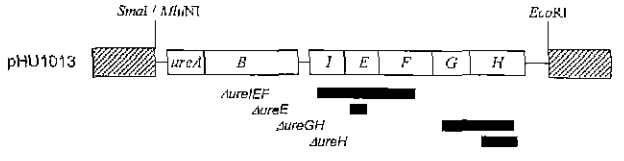

Fig. 1. *H. pylori* urease gene cluster contained in the plasmid pHU1013 and location of deletions in mutant derivatives. Sites of deletions are indicated by solid bars. The hatched bars indicate pBluscriptKSII(+) sequences. The map of pHU1012 is identical except the pUC19 vector sequences at both ends of the urease gene cluster.

Table 2. Urease specific activities of cell extracts from the recombinant *E. coli* cells carrying control plasmids or deletion mutants

Plasmids	Specific activity (U/mg)	% Activity
pHU1013	37.45	100
pHU1013Δ*ureE*	0	0
pHU1013Δ*ureH*	0	0
pHU1013Δ*ureGH*	0	0
pHU1012	8.26	22
pHU1012*ureE*	0	0

protein exists as a dimer binding nickel ions during equilibrium dialysis and thus suggested that it may act as a nickel donor in the nickel metallocenter assembly of urease. Predicted peptide sequence of UreE contained multiple histidine residues clustered at the carboxyl terminus [7], and it was implied that these His residues were participating in nickel ligation. The predicted amino acid sequence of the *H. pylori* UreE protein is homologous to that of *K. aerogenes* UreE peptide, but unlike *K. aerogenes*, *H. pylori* UreE lacks poly-histidine tail at the carboxyl terminus.

Absence of the urease subunits in these deletion mutants (Δ*ureE* and Δ*ureIEF*) is intriguing and it might have been caused by two different ways. First, absence of UreE peptide caused failure in the assembly of the functional accessory protein complex that might include UreE, thus could not assist the proper folding of and/or nickel incorporation into the active urease subunits. Alternatively, deletion in *ureE* gene region might have caused a kind of polarity effects in transcription and/or translation, thus prevented the expression of the urease structural genes altogether. Either way, *H. pylori* UreE peptide appears to play a different role from that of *K. aerogenes* protein.

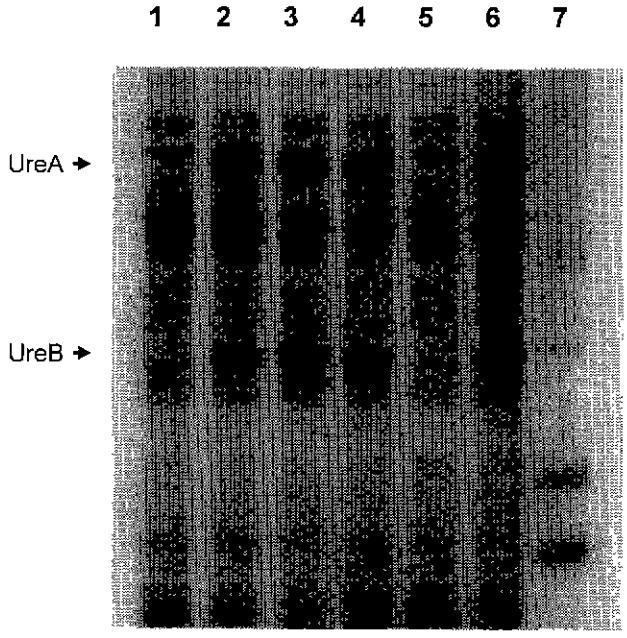

Fig. 2. 12% SDS-polyacrylamide gel electrophoresis of the cell extracts of *E. coli* (pHU1012Δ*ureIEF*)(lane 1), *E. coli* (pHU1012) (lane 2), *E. coli* (pHU1013Δ*ureGH*)(lane 3), *E. coli* (pHU1013Δ *ureH*)(lane 4), *E. coli* (pHU1013Δ*ureE*)(lane 5) and *E. coli* (pHU 1013)(lane 6). Molecular weight markers(lane 7) were phosphorylase b, M_r = 97,400; bovine serum albumin, M_r = 66,200; ovalbumin, M_r = 45,000; carbonic anhydrase, M_r = 31,000; soybean trypsin inhibitor, M_r = 21,500; and lysozyme M_r = 14,400.

References

1. Hu, L. T. and Mobley, H. L. T. 1990. Purification and N-terminal analysis of urease from *Helicobacter pylori*. *Infect. Immun.* **58**, 992-998.
2. Dunn, B. E., Campbell, G. P., Perez-Perez, G. I. and Blaser, M. J. 1990. Purification and characterization of urease from *Helicobacter pylori*. *J. Biol. Chem.* **265**, 9464-9469.
3. Newell, D. G. 1987. Identification of the outer membrane proteins of *Campylobacter pyloridis* and antigenic cross-reactivity between *C. pyloridis* and *C. jejuni*. *J. Gen. Microbiol.* **133**, 163-170.
4. Perez-Perez, G. I. and Blaser, M. J. 1987. Conservation and diversity of *Campylobacter pyloridis* major antigens. *Infect. Immun.* **55**, 1256-1263.
5. Eaton, K. A., Brooks, C. L., Morgan, D. R. and Krakowka, S. 1991. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. *Infect. Immun.* **59**, 2470-2475.
6. Lee, M. H., Mulrooney, S. B., Renner, M. J., Markowicz, Y., and Hausinger, R. P. 1992. *Klebsiella aerogenes* urease gene cluster : sequence of *ureD* and demonstration that four accessory genes (*ureD, ureE, ureF and ureG*) are involved in nickel metallocenter biosynthesis. *J. Bacteriol.* **174**, 4324-4330.
7. Mulrooney, S. B., and Hausinger, R. P. 1990. Sequence of the *Klebsiella aerogenes* urease genes and evidence for accessory proteins facilitating nickel incorporation. *J. Bacteriol.* **172**, 5837-5843.
8. Lee, M. H., Parkratz, H. S., Wang, S., Scott, R. A., Finnegan, M. G., Johnson, M. K., Ippolito, J. A., Christianson, D. W., and Hausinger, R.P. 1992. Purification, characterization of *Klebsiella aerogenes* UreE protein: a nickel-binding protien that functions in urease metallocenter assembly. *Protein Sci.* **2**, 1042-1052.
9. Moncrief, M. C. and Hausinger, R. P. 1996. Purification and activation of UreD-UreF-urease apoprotein complexes. *J. Bacteriol.* **178**, 5417-5421.
10. Park I S., and Hausinger R. P. 1995. Evidence for the presence of urease apoprotein complexes containing *ureD, ureF,* and *ureG* in cells that are competent for *in vivo* enzyme activation. *J. Bacteriol.* **177**, 1947-1951.
11. Lim, Y. M., Sung, J. Y., and Lee M. H. 1998. Polyclonal antibody against the active recombinant *Helicobacter pylori* urease expressed in *Escherichia coli*. J. Biochem. Mol. Biol. **31**, 240-244.
12. Hu, L. T. and Mobley, H. L. T. 1993. Expression of catalytically active recombinant *Helicobacter pylori* urease at wild-type levels in *Escherichia coli*. *Infect. Immun.* **61**, 2563-2569.
13. Laemmli, U. K. 1970. Cleavage of structural proteins during

the assembly of the head of the bacteriophage T4. *Nature* (London) **227**, 680-685.
14. Weatherburn, M. W. 1967. Phenol-hypochlorite reaction for determination of ammonia. *Anal. Chem.* **39**, 971-974.
15. Lowry, O. H., Rosebrough, N. J., Farr, A. J. and Randall, R. J. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.