Journal of Biochemistry and Molecular Biology, Vol. 31, No. 3, May 1998, pp. 240–244

JOURNAL OF
Biochemistry
and
Molecular Biology

Polyclonal Antibody Against the Active Recombinant *Helicobacter pylori* Urease Expressed in *Escherichia coli*

Yu Mi Lim, Jae Young Sung, and Mann Hyung Lee*

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

Received 13 January 1998

***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 in a potent immunogen. In order to express the recombinant urease at a higher level, a DNA fragment containing the minimal *H. pylori* urease gene cluster was subcloned into a high copy-number vector. The recombinant *H. pylori* urease expressed in an *E. coli* strain that was grown in a rich medium supplemented with added nickel was purified to near homogeneity by using DEAE-Sepharose, Superdex HR200, and Mono-Q (FPLC) columns and the purified enzyme possessed the specific activity of 1255 U/mg. Polyclonal antibodies raised against the purified recombinant *H. pylori* urease were shown to be very specific when subjected to Western blot analysis, in which crude extracts from the *H. pylori* ATCC strain and the recombinant *E. coli* strains expressing various bacterial ureases were examined for cross-reactivity.**

Keywords: Antibody, *Helicobacter pylori*, Nickel, Urease.

Introduction

Helicobacter pylori is a microaerophilic, spiral-shaped, Gram-negative bacterium which colonizes gastric mucosa of humans, nonhuman primates, and pigs. *H. pylori* produces bacterial urease of high activity of up to 6% of the soluble cell protein (Hu and Mobley, 1990) and this surface-presented, two-subunit enzyme (Dunn *et al.*, 1990) is distinct from other bacterial ureases which are made up of three subunits and localized in the cytoplasm. *H. pylori* urease serves as a major surface immunogen (Newell, 1987; Perez-Perez and Blaser, 1987) and as an important survival factor for the bacterium in the acidic environment of the gastric lumen (Eaton *et al.*, 1991). As in the cases of other bacterial enzymes, *H. pylori* urease requires nickel ions as an essential cofactor for the enzyme activity. Because of the fastidious culture conditions of *H. pylori* and its slow growth rate, growing large number of cells for various biochemical characterizations and analyses is difficult. There have been many attempts to express recombinant *H. pylori* urease in the recombinant *E. coli* strains which harbors a recombinant plasmid containing the entire urease gene cluster. However, recombinant *E. coli* cells grown in rich media such as LB broth used to produce functionally inactive apo-urease instead of the active holoenzyme (Cussac *et al.*, 1992; Lee *et al.*, 1995). This seemed to be due to chelation of the minute amount of nickel ions by certain components present in the medium since it was shown that catalytically active recombinant urease could be expressed only when the host cells were grown in minimal medium that contains no Ni-chelating agents (Hu and Mobley, 1993). Recently, Mobley *et al.* (1995) cloned and sequenced the *nixA* gene which encodes a specific nickel-transport membrane protein and found that when the *nixA* was co-expressed in *E. coli* with the *H. pylori* urease gene cluster, it potentiated the expression of catalytically active urease even when cultured in a complex medium.

In this report, we subcloned a minimal urease gene cluster into a high copy-number plasmid vector and used it to express the recombinant *H. pylori* urease at high levels by supplementing nickel ions in milimolar quantities in the complex medium without co-expressing the nickel-transporter gene, *nixA*, *of H. pylori.* Nearly homogeneous urease was purified and used to raise a polyclonal antibody specific for *H. pylori* urease.

Materials and Methods

Plasmid construction The previously described plasmid pHP808 (Hu and Mobley, 1993; a pACYC184 derivative, kindly

* To whom correspondence should be addressed.
Tel: 82-53-850-3611; Fax: 82-53-850-3602
E-mail: mhlee@cuth.cataegu.ac.kr

provided by Dr. H. L. T. Mobley of University of Maryland, USA) includes an 11 kb insert which contains additional genes such as *ureC* and *lspA* (formerly *ureD*) in addition to the whole urease gene cluster of *H. pylori*. Similarly, pKAU17 (Mulrooney *et al.*, 1989) and pBU11 (Kim and Spizizen, 1985) contain the urease gene clusters of *Klebsiella aerogenes* and *Bacillus pasteurii*, respectively. A *Mlu*NI–*Eco*RI fragment from pHP808 was isolated by using the Gene Clean II kit (Bio101 Inc., USA), and then inserted into the *Sma*I–*Eco*RI digested, and calf intestinal phosphatase-dephosphorylated pBlueScript KSII(+) vector to produce pHU1013 (Fig. 1). Recombinant colonies were selected on X-Gal-containing LB agar plates and Christensen urea agar plates.

Bacterial strains and growth conditions *E. coli* BL21(DE3)/pHU1013, DH5α/pKAU17, and HB101/pBU11 were grown in LB media containing 1 mM nickel chloride and 100 μg/ml ampicillin. *Helicobacter pylori* ATCC43504 (kindly provided by Dr. Jong-Baik Park of KRICT, Yoosung, Korea) was plated on Brucella agar plates containing 7% horse serum and routinely grown at 37°C in a Brucella broth containing 10% fetal bovine serum in an anaerobic jar equipped with CampyPak Plus (BBL, MD, USA) for 3 days.

Urease purification Cultures (2 L) of *E. coli* BL21/pHU1013 were grown to late exponential phase in LB media containing 1 mM nickel chloride and 100 μg/ml ampicillin. Cells were harvested by centrifugation, washed twice with ice-cold 20 mM potassium phosphate (pH 7.2), 1 mM EDTA, 1 mM 2-mercaptoethanol (PEB) buffer, resuspended in an equal volume of PEB containing 1 mM phenylmethylsulfonyl fluoride, disrupted by three passages through a French pressure cell (SLM Instruments, Inc., Urbana, USA) at 18,000 lb/in^2, and centrifuged at 100,000 $\times$ *g* for 90 min at 4°C.

The extracts were chromatographed on a DEAE-Sepharose CL-6B column (2.5 $\times$ 15 cm) in the same buffer and eluted with a 400 ml linear gradient to 1 M KCl. The pooled sample was concentrated to 3 ml by using an Amicon pressure filtration stirred-cell with a YM30 ultrafiltration membrane and subjected to Superdex HR200 (1.6 $\times$ 60 cm) gel filtration chromatography in PEB buffer supplemented with 0.15 M KCl. The pooled sample was dialyzed for 18 h against PEB buffer at 4°C, and then

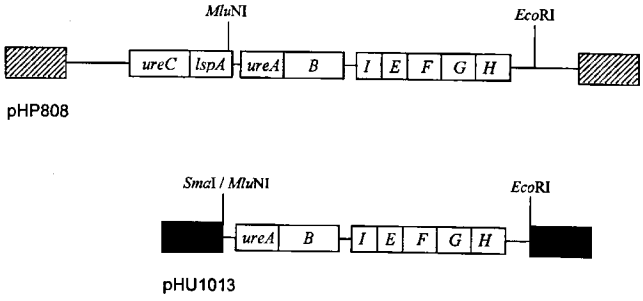

Fig. 1. Construction of the recombinant plasmid pHU1013. A *Mlu*NI–*Eco*RI fragment from the plasmid pHP808 was inserted into the *Sma*I–*Eco*RI site of the vector pBlueScript KSII(+) yielding the recombinant plasmid pHU1013 containing *ureA–ureH* genes. The hatched bars represent pACYC184 vector regions and the solid bars represent pBlueScript KSII(+).

applied to a Mono-Q HR10/10 column and eluted with a multistep gradient of increasing KCl in the same buffer. All resins and columns were purchased from Pharmacia Inc. (Uppsala, Sweden). The presence of urease protein in the column fractions was assessed by the one-time-point urease assay method based on indophenol production.

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 (Weatherburn, 1967). The assay buffer consisted of 25 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 50 mM urea, and 0.5 mM EDTA (pH 7.75). The reactions were initiated by the addition of enzyme, the concentration of released ammonia was measured in timed aliquots, and 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°C under the assay conditions described above. Protein concentration was measured by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard.

Polyacrylamide gel electrophoresis SDS-polyacrylamide gel electrophoresis was carried out by using buffers of Laemmli (Laemmli, 1970) and included either a 12% polyacrylamide running gel or a 10–15% polyacrylamide gradient running gel with a 4.5% polyacrylamide stacking gel. Gels were stained with Coomassie brilliant blue R250.

Preparation of polyclonal antibodies and purification of the IgG fraction Antibodies directed against the recombinant *H. pylori* urease were generated in a white, New Zealand rabbit by subcutaneous injection with 400 μl (1.2 mg/ml) of homogeneous protein in PBS emulsified with the same volume of Freund's complete adjuvant (Sigma Inc.). The rabbit was boosted after 28 d, and after an additional 14 d, whole blood was drawn and the serum was separated by centrifugation after 16 h at 4°C. The IgG fraction was purified from the serum by using the caprylic acid precipitation method (McKinney and Parkinson, 1987) followed by HiTrap Protein A-affinity column (Pharmacia) chromatography. Briefly, the serum was diluted 4-fold with 40 mM acetate buffer (pH 4) and the pH was adjusted to 4.8 with 5 N NaOH. Caprylic acid (25 μl/ml sample) was slowly added to the solution while mixing and the resulting precipitate was removed by centrifugation at 10,000 $\times$ *g* for 30 min. The supernatant was dialyzed against 4 L of phosphate-buffered saline, and then loaded onto a Protein A-Sepharose column (5 ml) pre-equilibrated with 20 mM sodium phosphate buffer (pH 7.0). Bound IgG fractions were eluted with 100 mM citrate buffer (pH 4). Purified IgG was concentrated by using an Amicon pressure filtration stirred-cell with a YM10 ultrafiltration membrane. Antibodies in the samples were titrated by using standard ELISA methods (Engvall and Permann, 1972).

Western blot analysis Crude extracts of *E. coli* DH5α/pKAU17 and *E. coli* HB101/pBU11 were obtained by growing cells in 100 ml LB broth containing 1 mM nickel chloride and 100 μg/ml ampicillin, followed by ultrasonication and centrifugation. *Helicobacter pylori* ATCC43504 cells which were grown at the conditions described above, were also sonicated and centrifuged to prepare the supernatant as described above. These

crude extracts, together with the recombinant *H. pylori* urease samples from each purification step, were loaded onto a 10–15% gradient SDS-polyacrylamide gel. After electrophoresis, the peptide bands were electrically transferred onto a nitrocellulose membrane in CAPS buffer (3-(cylcohexylamino)-1-propane sulfonic acid, 10% methanol, pH 10) and the membrane was stained with 0.1% Fast Green dye (Sigma) to visualize the peptide bands. The nitrocellulose membrane was blocked with Blotto/Tween solution, and then incubated with diluted (1:100,000) anti-urease polyclonal IgG, and finally with goat anti-rabbit-alkaline phosphatase conjugate (1:30,000), respectively. All incubations were carried out at room temperature. Peptide bands which specifically cross-reacted with the IgG molecules were visualized by applying 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) solution onto the membrane.

Results and Discussion

Subcloning of *H. pylori* urease gene cluster Previously described pHP808, which was a low copy-number pACYC184 derivative, contains the entire *H. pylori* urease gene cluster and additional flanking regions on both sides. In this study, we constructed a new recombinant plasmid by sucloning the minimal DNA region for expression of functional recombinant urease into a high copy-number vector, pBlueScript KSII(+), producing the pHU1013 plasmid. In this construct, the urease genes were inserted into the *Sma*I–*Eco*RI direction of the vector and appeared to have been expressed in a large quantity under the influence of the strong T7 promoter in *E. coli* BL21 (Fig. 2, lane 2). The overexpressed urease subunit peptides were clearly visible even in the cell extracts from *E. coli* BL21/pHU1013.

Urease purification The recombinant urease was purified to near homogeneity by using combinations of anion-exchange and gel filtration chromatography (Table 1). The recombinant urease eluted at approximately 0.15 M KCl from both the DEAE-Sepharose and Mono-Q columns. The enzyme was already practically pure after the second purification step, Superdex HR200 chromatography. The purified urease consisted of the characteristic two peptide subunits (apparent molecular masses of 66 and 31.5 kDa, respectively) as shown on a 12% SDS-polyacrylamide gel (Fig. 2, lane 5) and possessed the specific activity of 1255 U/mg.

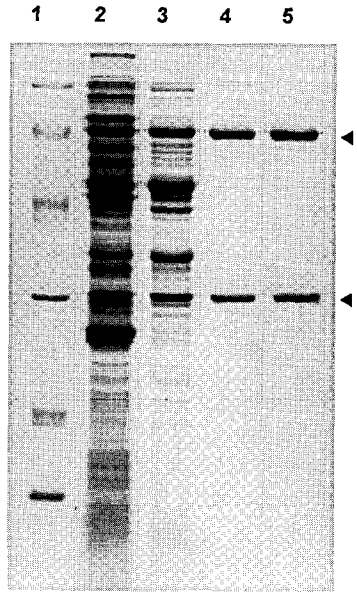

Fig. 2. SDS-polyacrylamide gel electrophoresis of the *H. pylori* urease at each purification step. Cell extracts from *E. coli* BL21 (pHU1013) (lane 2), DEAE-Sepharose pool (lane 3), Superdex HR200 pool (lane 4), and Mono-Q HR10/10 pool (lane 5) were subjected to 12% SDS-polyacrylamide gel electrophoresis, followed by Coomassie blue staining. Molecular weight markers (lane 1) 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. Arrowheads indicate the locations of the two urease subunits.

Purification of polyclonal antibody directed against the recombinant *H. pylori* urease The IgG fraction was purified by using caprylic acid precipitation and protein A-affinity chromatography from the rabbit serum containing the polyclonal antibody directed against the purified recombinant *H. pylori* urease. The recovery of IgG from the rabbit serum was more than 80% when determined by standard ELISA methods. A 12% SDS-polyacrylamide gel showed highly purified IgG peptide bands (Fig. 3, lane 4). The light chain of the purified IgG did not migrate as a discrete band and appeared as a diffuse area between the two molecular weight markers (M_r = 21,000–31,000), as observed in the previous studies (McKinney and Parkinson, 1987).

Table 1. Purification of the recombinant *H. pylori* urease.

Purification steps	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	24,261	40.3	1.0	100
DEAE-SR	22,563	134.6	3.3	93
Superdex HR200	17,953	992.5	24.6	74
Mono-Q	9,947	1,254.8	31.1	41

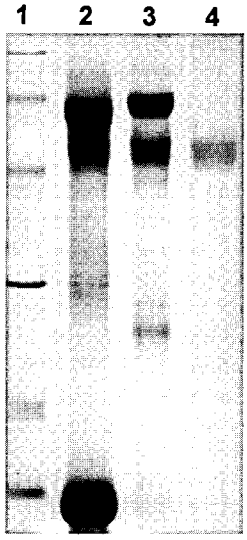

Fig. 3. SDS-polyacrylamide gel electrophoresis of the rabbit anti-*H. pylori* urease IgG at each purification step. Rabbit serum (lane 2), supernatant of the caprylic acid precipitation (lane 3), and the protein A-Sepharose pool (lane 4) were subjected to 12% SDS-polyacrylamide gel electrophoresis. The molecular weight markers (lane 1) used were identical to that in Fig. 2.

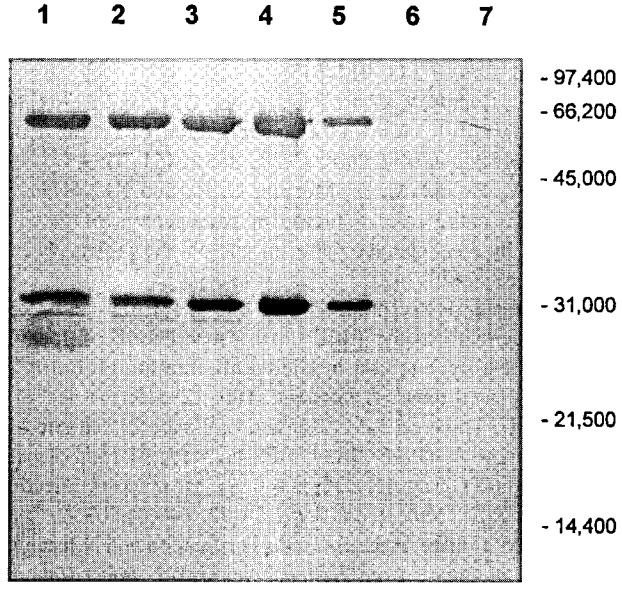

Fig. 4. Western blot analysis of the urease samples from different bacterial strains with the purified rabbit anti-*H. pylori* urease IgG. Cell extracts from *E. coli* BL21(pHU1013) (lane 1), DEAE-Sepharose pool (lane 2), Superdex HR200 pool (lane 3), Mono-Q HR10/10 pool (lane 4), cell extracts from *H. pylori* ATCC43504 (lane 5), from *E. coli* HB101(pBU101), and from *E. coli* DH5α (pKAU17) were subjected to SDS-polyacrylamide gel electrophoresis by using a 10–15% gradient gel, followed by Western blot analysis, as described in Materials and Methods.

Specificity of the anti-urease IgG As shown in Fig. 4, the purified polyclonal antibody specifically cross-reacted with the UreA and UreB subunits of *H. pylori* ureases of either the recombinant strains (lanes 1–4) and the wild-type *H. pylori* strain (lane 5). In contrast, neither *Klebsiella aerogenes* nor *Bacillus pasteurii* urease subunits were recognized (lanes 6, 7) which suggests the high specificity of the antibody. These results were a little surprising considering the fact that bacterial ureases from different genera were known to share extensive sequence homology and similarity (Mobley *et al.*, 1995). Since these polyclonal antibodies were directed against the soluble form of urease, and not the denatured one, it is possible that these antibodies may recognize only the unique sequence-specific spatial epitope of *H. pylori* urease. Further studies, including immunoprecipitation analyses, will show whether these antibodies recognize native ureases from different bacterial species.

H. pylori urease can be used as an excellent antigen for the specific and sensitive detection of *H. pylori*-specific serum immunoglobulin by ELISA. A large-scale culture of this pathogen, however, is more difficult than one of *E. coli* because of its fastidious growth requirements. Therefore, the active recombinant urease purified in a large quantity from *E. coli* can be used for the production of a standardized ELISA system. Our data indicate that the recombinant enzyme is antigenically equivalent to the native enzyme and would be suitable for the production of antigen for standardized diagnostic tests. *H. pylori* urease is known to be surface-presented and serves as an important survival factor in the acidic gastric lumen, so the recombinant urease could also be used to induce protective immunity in humans, a possibility which was demonstrated in mouse model studies (Ferrero *et al.*, 1995; Marchetti *et al.*, 1995) and nonhuman primate model studies (Shuto *et al.*, 1993; Stadtländer and Stutzenberger, 1996). Also, more importantly, there has been a report in which an orally administered recombinant vaccine actually cured existing infection, raising the possibility of therapeutic immunization (Doidge *et al.*, 1994). In these vaccination experiments, however, catalytically inactive apoenzyme or urease subunits were used instead of active holo-urease.

The expression and purification of a highly active recombinant urease in large amounts, established in our study, could also be used in screening for urease inhibitors as therapeutic agents.

Acknowledgments The first two authors contributed equally to this work. This work was supported by a non-directed research fund (Grant No. 02-F-0275), Korea Research Foundation, 1996.

References

Cussac, V., Ferrero, R. L., and Labigne, A. (1992) Expression of *Helicobacter pylori* urease genes in *E. coli* grown under nitrogen-limiting conditions. *J. Bacteriol.* **174**, 2466–2473.

Doidge, C., Gust, I., Lee, A., Buck, F., Hazell, S., and Manne, U. (1994) Therapeutic immunization against *Helicobacter* infection. *Lancet* **343**, 914–915.

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.

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.

Engvall, E. and Perlmann, P. J. (1972) Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. *J. Immunol.* **109**, 129–135.

Ferrero, R. L., Thiberge, J. M., Kansau, I., Wusher N., Huerre M., and Labigne A. (1995) The GroES homolog of *Helicobacter pylori* confers protective immunity against mucosal infection in mice. *Proc. Natl. Acad. Sci. USA* **92**, 6499–6503.

Hu, L. T. and Mobley, H. L. T. (1990) Purification and N-terminal analysis of urease from *Helicobacter pylori*. *Infect. Immun.* **58**, 992–998.

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.

Kim, S. D. and Spizizen, J. (1985) Molecular cloning and expression of *Bacillus pasteurii* urease genes in *Escherichia coli*. *Kor. J. Appl. Microbiol. Bioeng.* **13**, 297–302.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227**, 680–685.

Lee, C. K., Weltzin, R., Thomas, W. D. Jr., Kleanthous, H., Ermak T. H., Soman G., Hill, J. E., Ackerman, S. K., and Monath, T. P. (1995) Oral immunization with recombinant *Helicobacter pylori* urease induces secretory IgA antibodies and protects mice from challenge with *Helicobacter felis*. *J. Infect. Dis.* **172**, 161–172.

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.

Marchetti, M., Arico, B., Burroni, D., Figura, N., Rappuoli, R., and Ghiara, P. (1995) Development of a mouse model of *Helicobacter pylori* infection that mimics human disease. *Science* **267**, 1655–1658.

McKinney, M. M. and Parkinson, A. (1987) A simple, non-chromatic procedure to purify immunoglobulins from serum and ascites fluid. *J. Immunol. Methods* **96**, 271–278.

Mobley, H. L. T., Garner, R. M., and Bauerfeind, P. (1995) *Helicobacter pylori* nickel-transport gene *nixA*: synthesis of catalytically active urease in *E. coli* independent of growth conditions. *Mol. Microbiol.* **16**, 97–109.

Mobley, H. L. T., Island, M. D., and Hausinger, R. P. (1995) Molecular biology of microbial ureases. *Microbiol. Rev.* **59**, 451–480.

Mulrooney, S. B., Pankratz, H. S., and Hausinger, R. P. (1989) Regulation of gene expression and cellular localization of cloned *Klebsiella aerogenes* (*K. pneumoniae*) urease. *J. Gen. Microbiol.* **135**, 1769–1776.

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.

Perez-Perez, G. I. and Blaser, M. J. (1987) Conservation and diversity of *Campylobacter pyloridis* major antigens. *Infect. Immun.* **55**, 1256–1263.

Shuto, R., Fujioka, T., Kubota, T., and Nasu, M. (1993) Experimental gastritis induced by *Helicobacter pylori* in Japanese monkeys. *Infect. Immun.* **61**, 933–939.

Stadtländer, C. T. K.-H. and Stutzenberger, F. J. (1996) Adaptation of the [^{13}C]-urea breath test as a non-invasive method for detection of *Helicobacter pylori* infection in squirrel monkeys (*Saimiri* spp.). *Lab. Anim. Sci.* **45**, 239–243.

Weatherburn, M. W. (1967) Phenol-hypochlorite reaction for determination of ammonia. *Anal. Chem.* **39**, 971–974.