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Optimization of Culture Conditions for Production of *Helicobacter pylori* Adhesin Protein Genetically Linked to Cholera Toxin A2B in *Escherichia coli* JM101

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Abstract – *Helicobacter pylori* is a major cause of gastric-associated diseases. In our previous study, the Adhesin/CTXA2B was expressed as insoluble recombinant chimeric protein derived from the *H. pylori* adhesin genetically coupled to CTXA2B subunit in *Escherichia coli*. Since it is very important to optimize IPTG concentration, culture temperature and composition of medium to maximize cell growth and productivity, these conditional growth factors were determined for increasing the productivity of the expressed Adhesin/CTXA2B chimeric protein in *Escherichia coli* JM101 carrying pTEDhpa/ctxa2b. Our data demonstrate that optimal medium for increased production of chimeric protein was a YCP/Glu medium composed of 2% yeast extract, 1% casamino acid, phosphate solution [0.3% KH_2PO_4, 0.4% Na_2HPO_4, 0.25% $(NH_4)_2HPO_4$], and 0.5% glucose. In addition, optimal concentration of IPTG was 1 mM and culture temperature, 37°C.

Key words □ *H. pylori* adhesin, CTXA2B, Culture conditions

Helicobacter pylori infection is responsible for chronic active gastritis, peptic ulcers, and gastric cancers (Telford *et al.*, 1994; Ghiara *et al.*, 1995; Andersen *et al.*, 1991; Parkin *et al.*, 1988; Marchetti *et al.*, 1995). An attractive approach to eradicate this infection has been suggested by the therapeutic use of vaccines. The mouse model of persistent infection by mouse-adapted *H. pylori* strains allowed assessment of the potential of vaccine candidates such as VacA and CagA (Ghiara *et al.*, 1997) Our previous study showed the expression of Adhesin/CTXA2B chimeric protein in *E. coli* JM101 carrying pTEDhpa/ctxa2b that is *H. pylori* adhesin molecule genetically coupled to cholera toxin A2B subunits (Kim *et al.*, 2000). In addition, it has been known that the serine-rich *Entamoeba histolytica* protein (SREHP)/cholera toxin B chimeric protein expressed in *E. coli* JM101 has a high G_{M1}-ELISA binding activity (Zhang *et al.*, 1995).

Methods for expressing large amounts of protein from a cloned gene introduced into *E. coli* have proven invaluable in functional analysis of proteins. In addition, irrespective of the expression system used, dramatic differences in expression levels are often realized in different growth conditions (Weikert *et al.*, 1998). Therefore, one can approach to various strategies to produce enough protein in *E. coli*.

Inasmuch as it is hoped that the Adhesin/CTXA2B chimeric protein can be highly expressed by optimization of various cultures conditions such as culture medium, IPTG concentration and culture temperature, the present study was undertaken to optimize culture condition for production of the chimeric protein by the microorganism. Here, our results demonstrate the development of medium components and the effect of cultivation temperature and IPTG concentration on the protein production by the microorganism. In addition, the kinetic relationship among cell growth, glucose consumption, IPTG induction, and the protein production are analyzed and determined.

MATERIALS AND METHODS

Chemicals and bacterial strains

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (St Louis, MO). *E. coli* strain XL-1 Blue was used as the host for cloning and *E. coli* strain JM101 was used for expression of cloned DNA.

Genetic construction

Hpa/ctxa2b fused gene was constructed as described previ-

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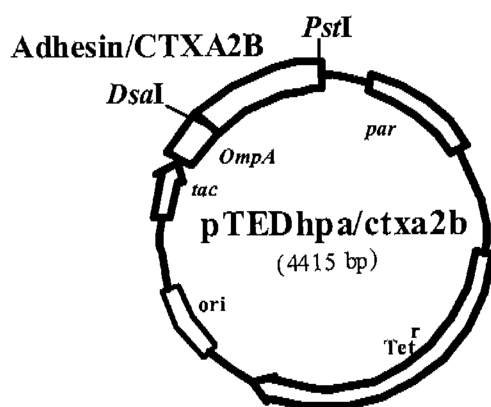

Fig. 1. Structure of the pTEDhpa/ctxa2b expression vector. Abbreviation: ColE1 ori, origin of replication; tac, tac promoter; OmpA, signal peptides of secretion; Tet, tetracycline.

ously by Kim *et. al* (2000). Briefly, amplified *H.pylori* adhesin gene (hpa) and *V. cholera* ctxa2b genes were cloned into the pBluescript II SK (+) (Stratagene Ltd., Cambridge, UK), respectively. The correct nucleotide sequence and in-frame sequence were verified by nucleic acid sequencing [Sequenase version 2.0 DNA sequencing kit (USB^{TM})]. The plasmid constructed by insertion of the hpa gene genetically coupled to ctxa2b gene into pTED was used to transform the *E.coli* JM101 expression host (Fig. 1).

Growth Condition for increased expression of Adhesin/CTXA2B chimeric protein

Escherichia coli JM101 was grown from 1 ml seed culture (1×10^6 CFU/ml) in 50 ml YCP or LB (Luria-Bertani) with various carbon sources, IPTG concentrations and culture temperatures. YCP medium was consisted of yeast extract (20 g/L), casamino acid (10 g/L), $MgSO_4$ (0.224 g/L), $CaCl_2$ (0.01 g/L), KH_2PO_4 (3 g/L), Na_2HPO_4 (4 g/L), $(NH_4)_2HPO_4$ (2.5 g/L), glucose or glycerol (5 g/L) and antifoam-289 (0.5 g/L). LB medium was consisted of bacto peptone (10 g/L), yeast extract (5 g/L), sodium chloride (5 g/L), glucose or glycerol (5 g/L) and antifoam-289 (0.5 g/L). Tetracycline stock solution (12.5 mg/ml) was also added to both media and final concentration was 12.5 μg/ml. IPTG concentration was determined by serial concentrations (0.05, 0.1, 0.2, 0.5, and 1 mM). Bacterial cultures were shaken for 24 hrs at 250 rpm and different culture temperatures such as 25, 30 and 37°C. Cell growth was determined by reading OD at 600 nm using 10×15 mm culture tube as a cuvette directly.

In fermentor experiments, 80 ml seed suspension was transferred into a 5-liter jar fermentor containing 4 liters of the culture medium. The culture was carried out for 4 hrs after various concentration of IPTG induction at various temperatures with an air flow rate of 4.0l/min. As the majority of G_{M1}-ELISA binding activity of Adhesin/CTXA2B protein occurred in cell pellet, especially cytoplasm fraction, cytoplasm fraction was separated from cell pellet by modification of the technique described previously (George *et al.*, 1995). The resulting lysate was then centrifuged at $12,000\times g$ for 15 min at 4°C. The resulting pellet containing insoluble recombinant chimeric protein as inclusion bodies was isolated according to previously described methods (Philippe *et al.*, 1994; Sanchez *et al.*, 1989). The denatured chimeric protein by 8 M urea and 0.1 M DTT was renatured by dialysis using a glutathione redox buffer (5 mM GSH; glutathione reduced form and 0.5 mM GSSH; glutathione oxidized form) for 16 hours at 4°C (Alberto *et al.*, 1994). The supernatant containing the renatured chimeric protein was subjected to size-exclusion chromatography on an FPLC TSK-GEL G-3000 SW column (21.5×300 mm; TosoHaas, Montgomeryville, PA), equilibrated with working solution. The protein yield was estimated by the Bradford assay method using BSA (bovine serum albumin) as the standard.

Characterization of adhesin/CTX2B Chimeric protein

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (pre-cast 10~20% gradient Tris-Tricine gel; Novex) and Western blotting were used to examine the expressed Adhesin/CTXA2B chimeric protein from recombinant *Escherichia coli* JM101 carrying pTEDhpa/ctxa2b. Growth of recombinant *E. coli* JM101 was conducted in various media containing tetracycline 12.5 μg/ml at various temperatures. Target gene expression was induced at midlog phase by various concentrations of isopropyl-β-D-thiogalactoside (IPTG). The cells were harvested by centrifugation ($6000\times g$ for 10 min) and subsequently lysed by sonication.

Synthesis of the peptide for the receptor-binding motif of Adhesin and the production of antiserum

The peptide corresponding to the putative sialic acid binding motif of Adhesin (Leu-Arg-Pro-Asp-Pro-Lys-Arg-Thr-Ile-Gln-Lys-Lys) was synthesized by Multiple Peptide Systems (TAKARA SHUZO Co., Ltd. Japan). Rabbits were injected with 100 ug of protein carrier (keyhole limpet hemocyanin)-conjugated peptide and the antibody was isolated by the method of Evans and coworkers (1993).

Modified G_{M1}-ganglioside ELISA

To determine of chimeric protein with GM1 ganglioside binding activity and adhesin antigenicity, binding to GM1 ganglioside (from Bovine brain, Sigma) in ELISA plate was performed (Hajishengallis *et al.*, 1995). Wells were coated overnight at room temperature with 100 μl/well 1.5 μM GM1 ganglioside in PBS and then washed with PBS. Purified chimeric protein was diluted with an assay buffer (PBS containing 0.05% Tween-20 and 0.5% BSA) and transferred to the coated wells of the plates. The plates were incubated at 37°C for 120 min. After the wells were washed three times, the plates were incubated with rabbit antibody to peptide containing the receptor binding motif of Adhesin at 37°C for 120 min and then with alkaline phosphatase-conjugated goat antiserum to rabbit IgG. After incubation for 120 min at 37°C, the plates were developed with pNPP (para-nitrophenyl phosphate) solution and the absorbance read at 405 nm in a Molecular device microplate reader (Menlo Park, CA).

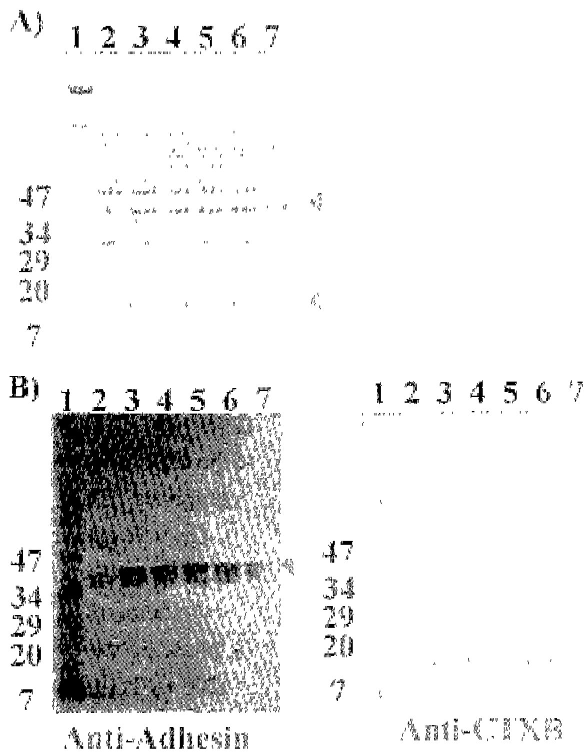

Fig. 2. A) SDS-PAGE analysis of crude extract from a recombinant *E. coli* JM101 carrying pTEDhpa/ctxa2b. B) Western blots with polyclonal antibody (1:1000 dilutions) to peptide containing the receptor-binding motif of Adhesin and to CTXB. Cell lysate proteins from *E. coli* JM101 carrying pTEDhpa/ctxa2b after induction with 1mM IPTG were resolved by electrophoresis on pre-cast 10-20% gradient polyacrylamide gel and immunoblotted on a PVDF membrane. Lane 1, prestained molecular weight marker; lane 2, 0 hr cultured; lane 3, 1 hr cultured; lane 4, 2 hr cultured; lane 5, 4 hr cultured; lane 6, 6 hr cultured; lane 7, 24 hr cultured. In all cases, samples that were applied to the gel were equivalent to 5ml of cell lysates. Arrowhead indicates Adhesin/CTXA2 (upper band) and CTXB (lower band).

RESULTS AND DISCUSSION

The proteins of *E. coli* JM101 producing chimeric protein was separated by SDS-PAGE (Fig. 2A). The Adhesin/CTXA2B chimeric protein was specifically detected by Western blot analysis using antibody to peptide containing the receptor-binding motif of *H. pylori* Adhesin and to CTXB. As shown in Fig. 2B, the exprssion of Adhesin protein increased several-fold following the induction with 1 mM IPTG after 1 hour. The expression of CTXB protein also was increased compared to uninduced condition. Additionally, SDS-PAGE and Western blots were used to analyze the purified fractions by size-exclusion chromatography (Fig. 3).

To determine the optimal growth medium, 4 different culture media (YCP/Glu, YCP/Gly and LB/Glu, and LB/Gly) were examined. YCP/Glu media gave rise to the highest G_{M1}-ELISA binding activity of Adhesin/CTXA2B protein (1.2 units/ml) and increased cell growth (Fig. 4). Glucose (5 g/L) as a carbon source has been known to result in better cell growth and protein production than glycerol (Sambrook and Russell, 2001). YCP media has also been known to result in better cell growth and protein production than LB (Sambrook and Russell, 2001). When the microorganism was grown in

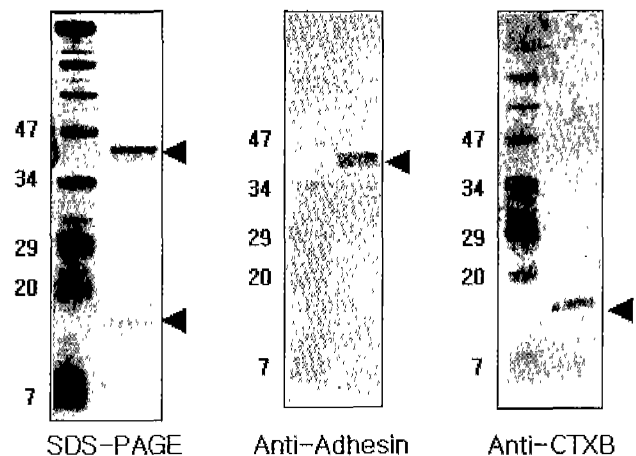

Fig. 3. SDS-PAGE and Western blots to analyze the purified fractions. Purified Adhesin/CTXA2B were resolved by electrophoresis on precast 10~20% gradient polyacrylamide gel and immunoblotted on PVDF membrane. Arrowhead indicates Adhesin /CTXA2 (upper band) and CTXB (lower band).

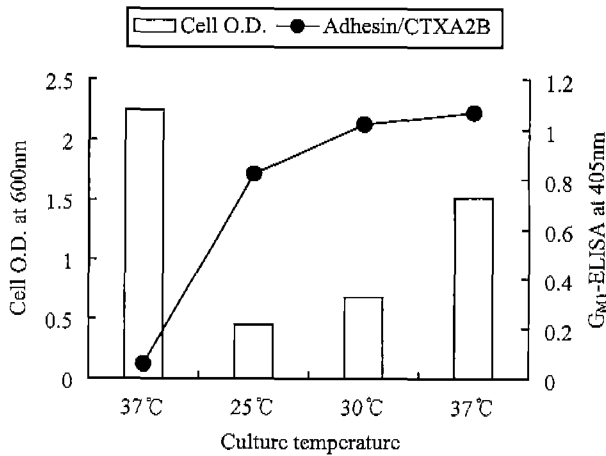

Fig. 4. The effect of culture media on changes of cell density, products of Adhesin/CTXA2B of *E. coli* carrying pTEDhpa/ctxa2b after 4 hours induction with 1 mM IPTG at 37°C. Growth was determined by reading OD at 600 nm and products of Adhesin/CTXA2B were measured by the G_{M1}-ganglioside ELISA using Adhesin antibody at 405 nm.

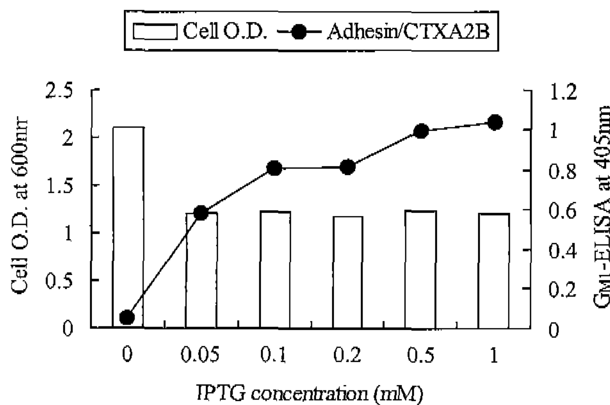

Fig. 5. The effect of IPTG concentration on changes of cell density, products of Adhesin/CTXA2B of *E. coli* carrying pTEDhpa/ctxa2b after 4 hours induction with IPTG at 37°C. Growth was determined by reading OD at 600 nm and products of Adhesin/CTXA2B were measured by the G_{M1}-ganglioside ELISA using Adhesin antibody at 405 nm.

YCP/Glu medium in a 5-liter jar fermentor, the maximum protein production was increased up to 10-folds (data not shown). This increase was due to the improvement in the O_2 transfer rate in the fermentor. Effects of IPTG concentration on cell growth and protein production in shaky culture were plotted (Fig. 5). With increased IPTG concentration, the protein production was increased as well but cell growth was decreased. As cell growth was inhibited by IPTG, the suitable condition for IPTG concentration was determined on 1 mM. Fig. 6 also shows the effect of culture temperature on cell growth and protein production. The higher the culture temperature was, the more cell growth and the protein production was increased by IPTG induction. The highest protein production was obtained at 37°C. Finally, Fig. 7 shows the change of cell density, production of Adhesin/CTXA2B, plasmid stability and glucose consumption rate of recombinant *E. coli* JM101 carrying pTEDhpa/ctxa2b after 4 hrs induction with 1 mM IPTG in YCP/Glu media at 37°C culture in fermentation. After 4 hrs incubation, cell growth curve was at the center of log phase and glucose concentration was less than 30% of the original concentration. However, the plasmid

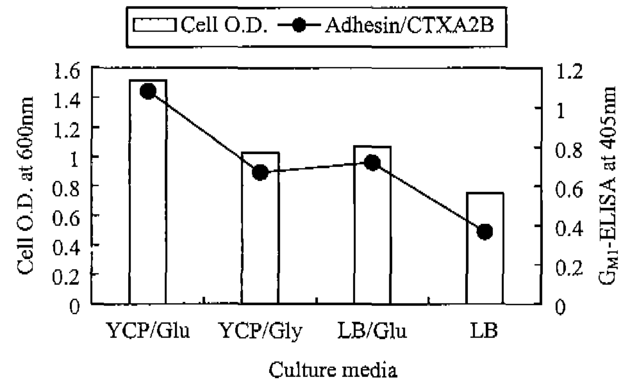

Fig. 6. The effect of culture temperature on changes of cell density, products of Adhesin/CTXA2B of *E. coli* carrying pTEDhpa/ctxa2b after 4 hours induction with 1mM IPTG at 37°C. Growth was determined by reading OD at 600 nm and products of Adhesin/CTXA2B were measured by the G_{M1}-ganglioside ELISA using Adhesin antibody at 405 nm.

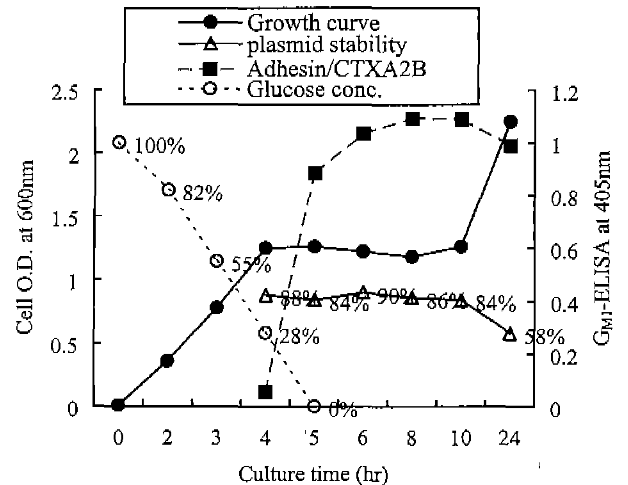

Fig. 7. The changes of cell density, production of Adhesin/CTXA2B, plasmid stability and glucose consumption rate of *E. coli* carrying pTEDhpa/ctxa2b after 4 hours induction with 1mM IPTG in YCP/Glu media at 37°C. Growth was determined by reading OD at 600 nm and products of Adhesin/CTXA2B were measured by the G_{M1}-ganglioside ELISA using Adhesin antibody at 405 nm.

was continously stabilized except 24 hrs cultivation. Because glucose acts as a competitive inhibitor of IPTG, 1 mM IPTG was added for the protein production at point that reached the maximum after 4 hrs. Thus our data indicated that the addition of 1 mM IPTG results in the highest protein production in YCP/Glu media at 37°C.

In conclusion, our data demonstrate that optimal medium is selected as YCP/Glu medium composed of 2% yeast extract, 1% casamino acid, phosphate solution [0.3% KH_2PO_4, 0.4% Na_2HPO_4, 0.25% $(NH_4)_2HPO_4$], and 0.5% glucose. In addition, optimal IPTG concentration was 1 mM and culture temperature, 37°C.

REFERENCES

Alberto, P. and Hiram, F. G. (1994) Protein disulfide isomerase exhibits chaperone and anti-chaperone activity in the oxidative refolding of lysozyme. *J. Biol. Chem.* **269**, 7764-7771

Andersen, L. P., S. Holck, L. E., and T. Justesen. (1991) The Helicobacter (Campylobacter) pylori-colonized duodenal mucosa and gastric metaplasia The Helicobacter (Campylobacter) pylori-colonized duodenal mucosa and gastric metaplasia. *APMIS* **99**, 244-248.

Evans, D. G., Karjalainen, T. K., Evans, Jr., Graham, D. Y. and Lee, C. H. (1993) Cloning, nucleotide sequence, and expression of a gene encoding an adhesin subunit protein of *Helicobacter pylori. J. Bacteriol.* **175**, 674-683.

George, C. P., Lira-DeVito, L. M., Wampler, S. L. and Kadonaga, J. T. (1995) A spectrum of mechanisms for the assembly of the RNA polymerase II transcription preinitiation complex. *Mol. Cell. Biol.* **15**(2), 1049-59.

Ghiara, P., Marchetti, M., Blaser, M. J., Tummuru, M. K. R., Cover, T. L., Segal, E. D., Tompkins, L. S., and Rappuoli, R. (1995) Role of the Helicobacter pylori virulence factors vacuolating cytotoxin, CagA, and urease in a mouse model of disease. *Infect. Immun.* **63**, 4154-4160.

Ghiara, P., Rossi, M., Marchetti, M., Tommaso, AD, Vin digni, C., Ciampolini, F., Covacci, A., Telford, J. L., Margistris, M. T., Pizza, M., Rappuoli, R., and Giudice, G. D. (1997) Therapeutic intragastric vaccination against Helicobacter pylori in mice eradicates an otherwise chronic infection and confers protection against reinfection. *Infect. Immun.* **65**, 4996-5002.

Hajishengallis, G., Hollingshead, S. K., Koga, T., and Russell, M. W. (1995) Mucosal immunization with a bacterial protein antigen genetically coupled to cholera toxin A2/B subunits. *J. Immunol.* **154**, 4322-4332.

Kim, B. O., Shin, S.S., Yoo, Y. H., and Pyo, S. (2000) Expression and Characterization of *Helicobacter pylori* Adhesin Protein Linked to Cholera Toxin A2/B Subunits in *Escherichia coli. J. Microbiol. Biotechnol.* **10**, 54-61.

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.

Parkin, D. M., Laara, E., and Muir, C. S. (1988) The international incidence of childhood cancer. *Int. J. Cancer* **41**, 184-197.

Philippe, S. D., Accart, S. N., Kolbe, H. V. J., Schubnel, D., Bernadette, B., Bischoff, R. and Marie-Paule, K. (1994) Recombinant cholera toxin B subunit in *Escherichia coli*: High-level secretion, purification, and characterization. *Protein Expres. Purif.* **5**, 518-526.

Sanchez, J. and Holmgren, J. (1989) Recombinant system for overexpression of cholera toxin B subunit in *Vibrio cholerae* as a basis for vaccine development. *Proc. Natl. Acad. Sci. USA* **86**, 1481-1485.

Sambrook, J. and Russell, D. W. (2001) *Molecular cloning* (a laboratory manual). Cold Spring Harbor Laboratory Press, New York.

Telford, J. L., Covacci, A., Ghiara, P., Montecucco, C. and Rappuoli, R. (1994) Unravelling the pathogenic role of Helicobacter pylori in peptic ulcer: potential new therapies and vaccines. *Trends. Biotechnol.* **12**, 420-426.

Weikert, C., Saver, U., and Bailey, J. E. (1998) An *Escherichia coli* host strain useful for efficient everprodution of secreted recombinant protein. *Biotechnol. Bioeng.* **59**, 386-391.

Zhang, T., Li, E. and Stanley, S. L., Jr. (1995) Oral immunization with the dodecapeptide repeat of the serine-rich Entamoeba histolytica protein (SREHP) fused to the cholera toxin B subunit induces a mucosal and systemic anti-SREHP antibody response. *Infect. Immun.* **63**, 1349-1355.