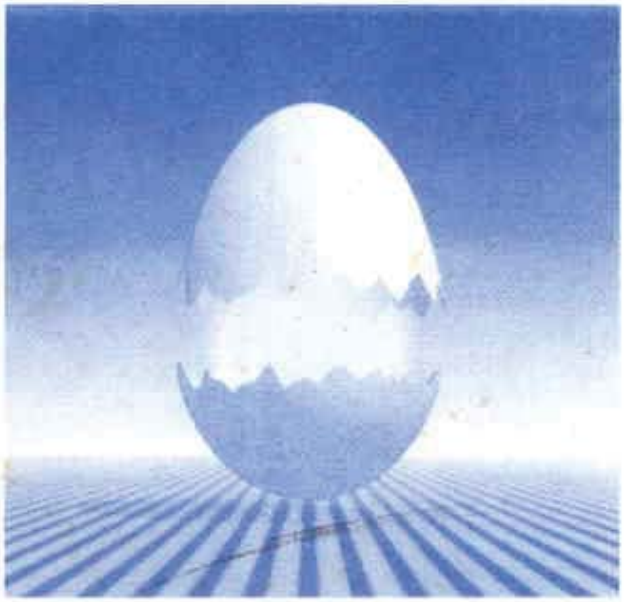
SESSION

C

ITSF SEMINAR
ON SCIENCE AND TECHNOLOGY

ITSF

Indonesia Toray Science Foundation



Jakarta, 3 February 2003

Indonesia Room
JAKARTA SHANGRI-LA HOTEL
Kota BNI, Jl. Jendral Sudirman Kav. 1, Jakarta 10220

ITSF SEMINAR

ON SCIENCE AND TECHNOLOGY SCHEDULE

SCIENCE AND TECHNOLOGY RESEARCH GRANT PROGRAM 2001
INDONESIA TORAY SCIENCE FOUNDATION

Monday, February 3, 2003
SHANGRI-LA HOTEL JAKARTA

SESSION C

08.30 - 09.00	**OPENING CEREMONY**		
	SESSION 1	**MODERATOR**	**: Dr. Ir. Suprihatin, Dipl-Ing**
09.00 - 09.45	I	SPEAKER	: Leenawati Limantara, M.Sc. Ph.D
09.45 - 10.30	II	SPEAKER	: Dr. Ir. Izarul Machdar, M.Eng
10.30 - 11.00	**BREAK**		
	SESSION 2	**MODERATOR**	**: Leenawati Limantara, M.Sc. Ph.D**
11.00 - 11.45	III	SPEAKER	: Dr.rer.nat. Karna Wijaya, M.Eng
11.45 - 12.30	IV	SPEAKER	: Dra. Mariana Wahyudi, M.Si
12.30 - 13.30	**LUNCH**		
	SESSION 3	**MODERATOR**	**: Dr.rer.nat. Karna Wijaya, M.Eng**
13.30 - 14.15	V	SPEAKER	: Wellyzar Sjamsuridzal, Ph.D
14.15 - 15.00	VI	SPEAKER	: Dra. Wega Trisunaryanti, MS, Ph.D
15.00 - 15.45	VII	SPEAKER	: Dr. Ir. Suprihatin, Dipl-Ing
15.45 - 16.15	**BREAK**		
16.15 - 16.45	**CLOSING**		

CLONING, CHARACTERIZATION AND OVEREXPRESSION OF THE *SALMONELLA TYPHI carB* GENE

Mariana Wahyudi[*+], E. Wonohadi[*+], A. Rudiretna[+].

Abstract

Salmonella typhi carAB operon is thought to be intimately involved in the pathogenesis mechanism of typhoid fever disease in human. The *carAB* operon consist of the *carA* gene and *carB* gene which encode two subunits of carbamoyl-phosphate synthetase, small and large subunits. The nucleotide sequence of *S. typhi carA* gene had been determined (Rudiretna et al, 1998) and its functional studies is still being done. But, information about the structural of the *S. typhi carB* gene is very little and there was not any information about functional properties of this gene. In order to obtain information related to the structure of this gene, Wahyudi, et. al (2001) had isolated the complete *S. typhi carB* gene and cloned this gene using the p-GemT vector into *E. coli* XL10. In this research attempts have been done to clone the *S. typhi carB* gene into the expression vector, characterize and overexpress the gene. Unfortunately, until now this work has not been succeded yet.

Introduction

Information regarding molecular pathogenesis and response to the bacterial host cell is very essential for efforts to combat an infection disease through diagnosis, immunization and therapy. Unfortunately, information regarding genes responsible for its pathogenesis had not been known completely, especially for *Salmonella typhi*. Until now the molecular pathogenesis of *S. typhi* has not been completely known. Genes which are responsible for virulence have not been identified. The information about the molecular pathogenesis mechanism of *S. typhi* and the response of its host cell is important for the prevention of typhoid fever disease.

S. typhi is a pathogenic bacteria that causes typhoid fever in man. According to statistical data, the morbidity of these patients in Indonesia is increasing. This is a serious health problem which needs attention.

Mekalanos *et al* (1993), using the IVET (In Vivo Expression Technology) system that he had developed, had successfully discovered the *S. typhimurium* LT2 genes that are induced and expressed only in the host cell in mice. Subsequently, the genes were named *ivi* (*in vivo induced*). The gene products are thought to play a role in causing typhoid fever in mice and it is considered that the genes are related to the virulence of *S. typhimurium* LT2. One of the *ivi* genes was the *ivi*I gene that indicates homology to the *E. coli* K12 *carAB* operon that encodes small and large subunits of carbamoyl-phosphate synthetase.

Previously, the complete nucleotide sequences of *carAB* operon of *E. coli* K12 and *S. typhimurium* LT2 were reported. *S. typhimurium carB* gene has sequence of 3,227 bp, whilst the *E. coli* K12 *carB* is 3,222 bp. The *S. typhimurium carAB* operon has been thought to have the similar control system as the *E. coli* K12 *carAB* operon. (Kilstrup, 1988). The Sanger Center was reported the entire chromosomal DNA of *S. typhi* CT18 (4,809 kb)

* Faculty of Pharmacy, University of Surabaya, Surabaya; + Center for Biotechnology Studies, University of Surabaya, Surabaya

(http://www.sanger.ac.uk.). Until now, the role of *S. typhi carAB* operon has not been fully understood.

Following the Mekalanos techniques, Salmonella team of IURC, Biotechnology, ITB had isolated the *S. typhi* 1499 bp fragment of the *carAB* operon, which is homologous to the *ivl* gene of *S. typhimurium* LT2, using PCR method. Analysis of the nucleotide sequence indicated that the 1499 bp DNA fragment consists of a 123 bp tandem promoter, a 1149 bp structural *carA* gene, a 15 bp spacer between the *carA* and *carB* genes, and a 212 bp of the upstream fragment of the *carB* gene. And now, the studies of the function of the gene is still being done [Rudiretna, *et al*, 1997 (a and b); Noer, *et al*, 1997; Rudiretna, 1998].

Carbamoyl phosphate synthetase (CPS; EC 6.3.5.5) catalyzes the production of carbamoyl phosphate from glutamine, bicarbonate, and two molecules of MgATP. The carbamoyl phosphate is used in the biosynthesis of pyrimidines, arginine, and urea. *E. coli* CPS is a key enzyme in the pyrimidine nucleotides and arginine biosynthetic pathways. In mammalia, the *de novo* synthesis of pyrimidine nucleotides is required for proliferating cells (Thoden, et al, 1997; Graves, et al, 2000). CPS from *Escherichia coli* has a total molecular weight of approximately 160K and composed of two polypeptide chains referred to as the small (40 kDa) and large (120 kDa) subunits. The small subunit, encoded by *carA*, is an amidotransferase (GLN subunit). The large subunit (CPS / synthetase subunit) is encoded by *carB*. The two subunits can be associated as separate subunits as in *E. coli* CPS or can be carried on a single polypeptide chain, sometimes in association with other enzymatic activities, as in the mammalian multifunctional protein, carbamoyl-phosphate synthetase — aspartate transcarbamoylase — dihydroorotase (CAD). Mammalian carbamoyl phosphate synthetase (CPS II) is part of a 240-kDa multifunctional protein, CAD, which also has the second (aspartate carbamoyltransferase, EC 2.1.3.2), and third (dihydroorotase, EC 3.5.2.3) activities of the de novo pyrimidine biosynthetic pathway (Hong, et al, 1995). The CPS component of CAD has a 40-kDa GLN domain connected to the amino-terminal end of a CPS domains via a 29-amino acid chain segment (GC linker) (Guy and Evans, 1997).

Glutamine is hydrolyzed on the small subunit (GLN domain) to glutamate and ammonia., and the ammonia is transferred to the large subunit (CPS domain), where it is used along with bicarbonate and ATP to make carbamoyl phosphate. (Guy and Evans, 1996). Large subunit contains a large tandem repeat for each of the nucleotides used in the overall synthesis of carbamoyl phosphate. (Stapleton, 1996; Thoden, et al, 1997). The two homologous halves of this subunit each contain a binding site for ATP (Potter, and Powers-Lee, 1992). The NH_2-terminal domain contains the portion of the large subunit that is primarily directed at the initial phosphorylation of bicarbonate by the first ATP to carboxy phosphate while the COOH-terminal domain contains the region of the enzyme that catalyzes the phosphorylation of carbamate to carbamoyl phosphate (Post, et al, 1990; Stapleton, 1996; Javid-Majd, et al, 1996). This mechanism, which is thought to be general for all members of this family of enzymes, was established for *Escherichia coli* CPS. Mitochondrial CPS I, which directly uses ammonia rather than glutamine, is the only known exception. (Guy and Evans, 1996)

The large subunit also have responsibilities for the binding of the allosteric effectors. The activity of the large subunit is feed-back inhibited by high concentrations of UMP but is activated ornithine, IMP, and phosphoribosyl-1-pyrophosphate (PRPP). The coupling of regulatory pathways occurs within the allosteric subunit of *E. coli* CPS. All effectors regulate enzyme activity by altering its affinity for ATP (Rubio, et al, 1991; Guy and Evan, 1996; Delannay, et al, 1999). Mammalian CPS is allosterically inhibited by UTP and activated by PRPP. The activation and subsequent down-regulation of the pathway can be attributed to altered allosteric regulation of the CPS activity of CAD (Sigoillot, et al, 2002). Thus, the rate-

limiting step in the de novo biosynthesis of pyrimidine is catalysed by carbamoyl phosphate synthetase (CPS II) (Graves, et al 2000).

Many CPS gene have been cloned and sequenced from a wide variety of sources, i.e from *E. coli*, rat, Syrian hamster (Simmer et al, 1990), yeast (Lusty, et al, 1983), *Squalus acanthias* (Hong et al, 1995), *Toxoplasma gondii* (Fox, and Bzik, 2002), *Helicobacter pylori* (Davids, et al, 2002) etc. The bacterial and mammalian CPS have a high degree of sequence similarity (Guy, and Evans, 1996). The CPS gene and its products from those organisms have been investigated. Those enzymes have similar activities and amino acid sequences, but their structural organization, sub-unit structure and mode of regulation can be different (Liu, et al, 1994, Eroglu, and Powers-Lee, 2002). Howeve, the information about functional properties of the *S. typhi carB* gene is very little. This research is a continuation of the previous *S. typhi carB* gene research that had been done by the previous researches [Wahyudi, et al, 2001; Noer, *et al*, 1997; Rudiretna, 1998].

The aim of the present research is to study of the functional aspects of this gene. Due to the limited amount of time, this research will be carried out to the overexpression of this gene and the validation of the overexpressed protein. We report here that until now we have not already succeded to clone the *S. typhi carB* gene into the expression vector nor expression host, the *E. coli* DH5α. We still do this work and hope we have this clone.

Materials and Methods

This proposed research has as its goal the cloning, characterization and overexpression of the *Salmonella typhi carB* gene. The objective of this research will be achieved through the following stages :

Isolation of the S. typhi carB gene from E. coli XL-10/pG-carB11-ST.

The *S. typhi carB* gene was isolated from *E. coli* XL-10/pG-carB11-ST. The isolated plasmid (pG-*carB*11-ST) was cut using restriction enzymes (*Nde*I and *Bam*HI) [Amersham Pharmacia Biotech], and then purified using gel agarose electrophoresis and GFX Kit [Promega].

Preparation of the Linearized pET-16b.

The pET16b-*carA*-ST plasmid was isolated from *E. coli* JM109/pET-*carA*-ST. Then, the plasmid was linearized using restriction enzymes (*Nde*I and *Bam*HI) [Amersham Pharmacia Biotech]. The pET-16b fragment was purified using gel agarose electrophoresis and GFX Kit [Promega].

Cloning of the *S. typhi carB* gene using an expression vector pET-16b into the non-expression host (*E. coli* DH5α) and characterization of the recombinant plasmid.

The *S. typhi carB* gene was ligated into the expression vector, pET-16b that have been linearized. The ligation mixture of the insert and pET-16b was used to transform *E. coli* DH5α cells (the non expression host) and plated out onto LB medium containing ampicillin. The cloning process was done according to the pET protocol [Novagen].

Several white colonies was screened for the presence of the appropiate recombinant plasmid by restriction enzymes and PCR using CB-1 and CB-2 primers. The recombinant plasmid of interest will be purified using Wizard plasmid purification kit (Promega) and the nucleotides will be sequenced. The plasmid of interest (pET16b-*carB*-ST) will be preserved and used for further work

Cloning the *S. typhi carB* gene into the expression host (*E. coli* BL21DE3).

The isolated plasmid of interest (pET16b-carB-ST) will be used to transform *E. coli* BL21DE3 strain containing a chromosomal copy of the gene for T7 RNA polymerase (expression host). The recombinant clone (BL21DE3/pET-16b-*car*B-ST) will be used to overexpress in further work.

Over expression of the *S. typhi carB* protein from *E. coli* BL21DE3/pET-16b-*carB*-ST) and characterization of the recombinant protein.

The overexpression procedure will be carried out according to the Novagen protocol. The overexpressed Car B protein of *S. typhi* will be analized by SDS-PAGE using Coomassie staining.

Purification of the *S. typhi* CarB Protein and characterization.

Prior to purification it is necessary to determine where in the cell the target protein has accumulated (inclusion bodies, media, periplasmic or cytoplasmic space). Purification will be carried out using His-Bind column according to the Novagen protocol kit (Novagen). The purified *carB* protein of *S. typhi* will then be sequenced at the N-terminal end. Sequencing of the amino acids will be carried out through collaboration with other institutions at cost.

Result and Discussion

Isolation of the S. typhi carB gene from E. coli XL-10/pG-carB11-ST.

The *S. typhi carB* gene was isolated from *E. coli* XL-10/pG-carB11-ST using restriction enzymes (*Nde*I and *Bam*HI). The gene was constructed with restriction sites, NdeI, in its 5`-end and BamHI in its 3`-end (Wahyudi, 2001). The purified gene has a molecular weight of 3.2 kb. (Fig.1)

Figure 1. Purification of *S. typhi carB* gene from *E. coli* XL-10 / pG-*car*B11-ST with restriction enzymes (*Nde*I and *Bam*HI) using 1% agarose gel electrophoresis. Lane **a**: DNA standard (ladder DNA fragments) in the sizes of 10.0, 8.0, 6.0, 5.0, 4.0, 3.0, 2.5, 2.0, 1.5, 1.0 and 0.5 kb. Lane **b**: uncut circular pGem-T. Lane **c**: uncut circular pG-*car*B11-ST. Lane **d**: pGem-T / *Nde*I. Lane **e**: pG-*car*B11-ST / *Nde*I. Lane **f**: pG-*car*B11-ST / *Nde*I / *Bam*HI and g: purified *car*B11-ST in the size of 3,2 kb. This analysis was carried on using 1% agarose gel electrophoresis in addition of ethidium bromide, voltage 70V for about two hours.

Preparation of the Linearized pET-16b.

Linearized pET-16b also have BamHI and NdeI restriction sites in its 5` and 3` ends, in order to enhanced the ligation process. The pET-16b is a 5.7 kb fragment (Fig. 2).

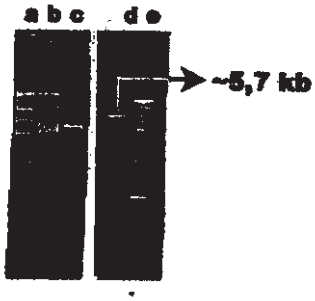


Figure 2. Linearization of pET-16b from pET-16b-*car*A-ST with restriction enzymes (*Nde*I and *Bam*HI) using 1% agarose gel electrophoresis
Lane **a**: DNA standard (λ/HindIII fragments) in the size of 23.13, 9.42, 6.56, 4.36, 2.32 and 2.03 kb. Lane **b**: pET-16b-*car*A-ST circuler. Lane **c**: pET-16b-*car*A-ST/*NdeI*/*Bam*HI. Lane **d**: linearized pET-16b and **e**: DNA standard (Ladder DNA fragments) in the sizes of 10.0, 8.0, 6.0, 5.0, 4.0, 3.0, 2.5, 2.0, 1.5, 1.0 and 0.5 kb. This analysis was carried on using 1% agarose gel electrophoresis in addition of ethidium bromide, voltage 70V for about two hours.

Cloning of the *S. typhi car*B gene using an expression vector pET-16b into the non-expression host (*E. coli* DH5α) and characterization of the recombinant plasmid.

Until now the cloning processes have been done many times. There were hundreds white colonies produced and screened for the presence of the appropiate recombinant plasmid, but the processes have been failed to produce any right clone. The molecular weight of the plasmids were analyzed using electrophoresis agarose gel. In Figure 3 we could see several example of the plasmids that have similar or different molecular weight as the circular pET-16b. Larger plasmids were analyzed further using restriction enzyme, *Bam*HI. The appropriate plasmid, which has *S. typhi car*B gene as its insert, will be linearized as a fragment in the size of ~ 8.9 kb. Several larger plasmid have been linearized but these have different size as the appropriate one. Their insert sizes are in average of ~1 kb to ~4,3 kb. There is one plasmid that is a contaminant, because its size is ~ 10 kb. Another contaminant plasmid has two fragments after digested using *Bam*HI. We effort a variety of condition to optimize the ligation process (i.e. ratio vector : insert and so on) and the cloning process (i.e. transformation efficiency and so on) and we still have a ligation problem.

The further works have not been done because there was not any right recombinant plasmid yet. We still do the cloning process and we will present the result at the seminar.

Fig. 3. Analysis molecular weight of several plasmid using 1% agarose gel electrophoresis. **Lane e and k: pET-16b circuler. Lane a-d, f-j and l-u : plasmids from white colonies. This analysis was carried on using 1% agarose gel electrophoresis in addition of ethidium bromide, voltage 70V for about two hours.**

IV. CONCLUSION

Cloning of the S. typhi carB gene using expression vector have not been success yet. Ligation process need to be enhanced.

V. ACKNOWLEDGEMENT

We would like to thank Indonesia Toray Science Foundation (ITSF) for financial support of this research and also to Silvia Kuncoro for conducting some of the laboratory work.

VI. REFERENCES

Davids W, Gamieldien J, Liberles DA and Hide W. (2002). J. Mol. Evol. 54(4):458-64

Delannay S., Charlier D., Tricot C., Villeret V., Pierard A., Stalon V. (1999). J.Mol.Biol. 286(4):1217-28.

Eroglu, B. and Powers-Lee, S.G. (2002). J. Biol. Chem. 277:45466-72

Fox, B.A. and Bzik, D.J. (2002). Nature. 415(6874):926-29.

Graves LM, Guy HI, Kozlowski P, Huang M, Lazarowski E, Pope RM, Collins MA, Dahlstrand EN, Earp HS 3rd, Evans DR. (2000). Nature 403(6767):328-32.

Guy, H.I. and D.R.Evans. (1996). JBC Online. 271(23):13762-69

Guy HI., A. Rotgeri, and DR. Evans. (1997). JBC Online. 272(32):19913-18

Hong, J, W.L. Salo, and P.M. Anderson. (1995). JBC 270:14130-39

Javid-Majd F, Stapleton MA, Harmon MF, Hanks BA, Mullins LS, Raushel FM.. (1996). Biochemistry. 35(45):14362-69.

Kilstrup, M., C.D. Lu, A.T. Abdelal and J. Neuhard. (1988). Eur.J.Biochem. 176:421 – 29.

Liu X, HI Guy and DR Evans. (1994) J. Biol. Chem. 269(44):27747-55.

Lusty CJ, EE Widgren, KE Broglie and H Nyunoya. (1983). J.Biol.Chem. 258(23):14466-77 .

Mahan, M.J., J.M. Slauch and J.J. Mekalanos. (1993). *Science*, 259 : 686 - 88.

Noer, A.S., A. Rudiretna, S. Kisman and Oei Ban Liang, Studi homologi fragmen DNA 618 pasang basa gen *carA Escherichia coli*, *S. typhimurium*, dan *S. typhi*, Prosiding Seminar Kimia UKM – ITB III. 1997.

Post LE, DJ Post and FM Raushel. (1990). J. Biol. Chem. 265(14):7742-47.

Potter, MD and SG Powers-Lee. (1992). J. Biol. Chem., 267(3):2023-31.

Rubio V, Cervera J, Lusty CJ, Bendala E, Britton HG. (1991). Biochemistry. 30(4):1068-75.

Rudiretna, A., A.S. Noer, S. Kisman and Oei Ban Liang. Sequencing A 903 Nucleotide *carA* Gene of *Salmonella typhi* Obtained Through PCR Employing CA-3 and CA-6 Primers. Proceding Third Asia – Pacific Symposium on Typhoid Fever and Other Salmonellosis, Indonesia. 1997 (a)

Rudiretna, A., A.S. Noer, S. Kisman and Oei Ban Liang. Amplification and Sequencing of a 0.6 kb *Salmonella typhi* DNA Fragmen Employing CA-1 and CA-2 Primers. Proceding Indonesian Biotechnology Conference, Indonesia. 1997 (b).

Rudiretna, A., Gen Mirip *carA* Pada *Salmonella typhi*. **Disertasi**, Program Pascasarjana, Institut Teknologi Bandung, Indonesia, 1998.

Sigoillot FD, Evans DR, Guy HI. (2002). J. Biol. Chem . 277(18):15745-51.

Simmer JP, RE Kelly, AG Rinker Jr, JL Scully and DR Evans. (1990). J. Biol. Chem. 265(18):10395-10402.

Stapleton MA, Javid-Majd F, Harmon MF, Hanks BA, Grahmann JL, Mullins LS, Raushel FM. (1996). Biochemistry. 35(45):14352-61.

Thoden JB, Holden HM, Wesenberg G, Raushel FM, Rayment I. (1997). Biochemistry. 36(21):6305-16.

Wahyudi, M., E. Wonohadi, A. Rudiretna, E. Purwantini, A.S. Noer, and Oei Ban Liang. 2001. Proceding Indonesian Biotechnology Conference, Indonesia.

CERTIFICATE

ITSF
Indonesia Toray Science Foundation

Indonesia Toray Science Foundation

Certifies that

Dra. Mariana Wahyudi, M.Si

from

Surabaya University

had attended the **ITSF Seminar** on Science and Technology
which was held at

Shangri - La Hotel Jakarta

as

SPEAKER

MENGESAHKAN
Salinan fotocopy sesuai dengan aslinya
Surabaya, _____
UNIVERSITAS SURABAYA
FAKULTAS TEKNOBIOLOGI
Dekan,

Dr. rer. nat. Maria Goretti M. Purwanto

The ITSF Selection Committee
on Science and Technology
Chairman,

Prof. Dr. Oei Ban Liang

Jakarta, 3 February 2003
The ITSF Board of Directors
Chairman,

Dr. H. Soefjan Tsauri, M.Sc, APU