Kor. J. Appl. Microbiol. Bioeng.
Vol. 13, No. 3, 297 – 302 (1985)

Molecular Cloning and Expression of *Bacillus pasteurii* Urease Gene in *Escherichia coli*

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(Received August 17, 1985)

B. pasteurii Urease遺伝因子의 *E. coli* 內의 復製와 発現

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(1985년 8월 17일 수리)

The 7.1 Mdal XbaI fragment of *Bacillus pasteurii* ATCC 11859 containing gene for urease was inserted into the XbaI site of bifunctional plasmid pGR71, and its urease gene was cloned and expressed in *E. coil* RR1. But the cloned gene was not expressed in *Bacillus subtilis* BR151 in consequence of deletion of inserted DNA fragment. The recombinant plasmid thus formed was named pGU66. The restriction map of the plasmid pGU66 was determined, and the size of the plasmid was estimated to be 12.6 Mdal by double digestion of restriction enzymes of the plasmid. The urease of the cloned strain was accumulated in periplasmic space and very similiar to that of donor strains in their enzymatic properties.

Urease (EC 3.5.1.5) is an important enzyme in the clinical field with immobilized urease[1,3] and in the agricultural view for foliar spray of urea[4] and nitrogen uptake of plants[5]. The ureas of *Bacillus pasteurii* was purified at first and its highest enzymatic activity was determined among the bacterial ureases[6]. But *B pasteurii* require biotin, nicotinic acid, casein hydrolysate, urea and alkaline pH for its growth[7,8]. Furthmore growth of the strain is inhibited by the increase of medium pH as cultural time go by. Because of these disadvantages, we tried to transfer urease gene of *B. pasteurii* into other bacterial strains which can mass-culture easily in the general media.

We report here the cloning of the urease gene from *B. pasteurii* using the plasmid pUB110 and pGR71 and the expression in *E. coli*. The inserted plasmid containing the urease gene has been partially mapped and the properties of the urease enzyme have been described.

Materials and Methods

Bacterial strains

Bacillus pasteurii ATCC 11859 was chosen as the donor strain of urease gene among 13 kinds of *Bacillus* strain producing urease. *Bacillus subtilis* IE32 BR151 (*try*C2, *lys*3, *met*B10) and *Escherichia coli* RRI (*leu, pro, thi*) were used as host strains. Two host strains did not show the activity of urease even after disruption of the cells.

Plasmid vector

Plasmid pUB110 and pGR71 were used as cloning vectors. The plasmid pUB110 (Km^R, Neo^R) of 3.0 Mdal was purified from *B. subtilis* BD366 (pUB110) and bifunctional shuttle plasmid pGR71 (Km^R, Neo^R, Cm^R) of 5.5 Mdal[9] which can be replicated both in *E. coli* and *B. subtilis* was purified from *E. coli* DEC148 (pGR71). *E. coli* DEC148 (pGR71) was obtained from Dr. R.H. Doi (University of Califor-

nia, Davis).

Media

B. pasteurii ATCC 11859 was grown in nutrient broth (pH 9.0) containing 5% filtered urea, 0.05% casein hydrolysate, 0.1% yeast extract, 1μg/ml of biotin (called NB/urea-CYB). L-Broth supplemented with 5ug/ml of neomycin and that with 10μg/ml of neomycin were used for *B. subtilis* BD366 (pUB110) and *E. coli* DEC148 (pGR71) respectively.

Isolation of DNA

Chromosomal DNA of *B. pasteurii* was prepared from cells grown in NB/urea-CYB at 30°C and by the method of Dot[10]. Plasmid pUB110 was isolated according to large scale plasmid isolation method of Lovett[11] and CsCl-EtBr equilibrium gradient centrifugation (40K-40hrs-20°C, SW56). Plasmid pGR71 was purified from *E. coli* DEC148 amplified with Cm by the preparative alkaline extraction of Birnboim[12] and the glass powder adsorption of Marko et al[13]. Plasmid cloned with urease gene (named pGU66) was purified from amplified transformant cell by the preparative alkaline extraction and banding in CsCl density gradients. For screening of recombinant DNA in transformants, the rapid alkaline extraction[12] was used.

Cloning procedures

Plasmid pUB110 and pGR71 were linearized with EcoRI and Hind III respectively and then treated with alkaline phosphatase of calf intestine. Digestion was terminated by heat treatment and phenol extraction. Chromosomal DNA was completely digested with EcoRI and fractionated with sucrose density gradient centrifugation by their molecular weight for cloning of *B. subtilis*. On the other hand, the DNA was partially digested with Hind III for cloning of *E. coli* by pGR71.

The ligation was achieved with DNA T_4 ligase for 18hrs at 17°C in 50mM Tris-HCl (pH 7.6), 10mM $MgCl_2$, 10mM Dithiothreitol, 1mM ATP and 50mg/ml BSA.

Protoplast transformation of *B. subtilis* was carried out according to the mannitol regeneration method[14]. Transformation of *E. coli* was accomplished by $CaCl_2$/RbCl method of Kirshner[15].

Analysis of restriction fragments

The size of restricted fragments of DNA was estimated by agarose gel electrophoresis after single or double digestion with restriction enzymes of Bethesda Research Laboratories. ϕ29 DNA cleaved with EcoRI was used as a molecular weight reference.

Gel electrophoresis

DNA molecules were separated on a 0.8% vertical agarose gel in Tris-PO_4 buffer (pH 7.8) and DNA band were visualized by staining with ethidium bromide.

Polyacrylamide gel electrophoresis for urease protein separation was accomplished by the method of Davis. Gel was equilibrated with cresol red and colored with urea by the method of Shaik-M et al[16]

Screening of Urease-positive transformants

Transformant colonies which were grown on the mannitol regeneration agar containing 5μg/ml neomycin or LB plate containing 10μg/ml neomycin were smeared heavily on the Christensen urea plate[17] containing 10 μg/ml of neomycin. Also, the urea R broth composed with 0.1% yeast extract, 0.045% KH_2PO_4, 0.048% Na_2HPO_4, 1 mg% phenol red and neomycin was used for detection of urease-positive colonies. Urease-positive colony changes color of the media from yellow to pink.

Urease activity determination

Urease activity was measured by Nesslerization method[18].

Localization of urease in *E. coli*

E. coli RRI (pGU66) was cultured in L-broth containing 10μg/ml of neomycin for 17 hrs at 37°C. Extracellular, periplasmic and cellular ureases of the strain were prepared by the method of Tsukagoshi et al[19].

Results and Discussion

Cloning of urease gene in *B. subtilis*

The DNA fragments which were fractionated into 4 size groups by sucrose density gradient from *B. pasteurii* chromosomal DNA digested with EcoRI, were ligated with the pUB110 cleaved by EcoRI at the ratios of 2, 5 and 10 to 1. The ligated DNAs transformed protoplast of *B. subtilis* BR151 in the frequency range of 5.4×10^{-4} to 1.2×10^{-3}.

Although 16,000 transformants of neomycin resistance on the mannitol regeneration agar containing neomycin were tested, any colony did not express urease gene on Christensen urea plate containing neomycin. The plasmids from transformants of neomycin resistance were isolated by the rapid alkaline extraction and their sizes were measured by agarose gel electrophoresis. Though the molecular size of ligated DNA before transformation was larger than that of the original vector, the size of replicated plasmid was found to be reduced to the same size of the vector plasmid.

We suppose the *B. subtilis* host deleted the inserted foreign DNA fragment by their restriction system, or excised and incorporated homologous region into the chromosome

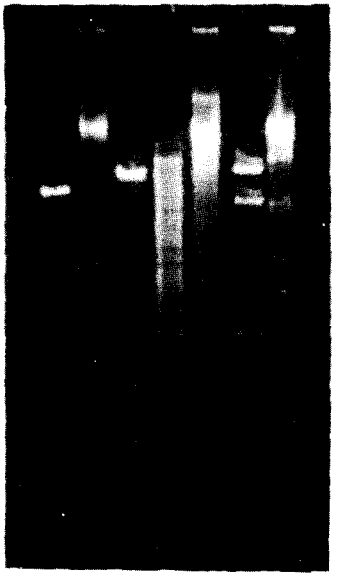

Fig. 1. Agarose gel electrophoresis of digestion and ligation.

Lane 1:undigested pGR71, 2: undigested chromosomal DNA of *B. pasteurii*, 3:pGR71 digested with Hind III, 4:chromosomal DNA of *B. pasteurii* digested with Hind III, 5 :ligated DNA (pGR71 / Hind III : chromosomal DNA/Hind III = 1:4), 6: ϕ29/EcoRI, 7:plasmids of *E. coli* V517.

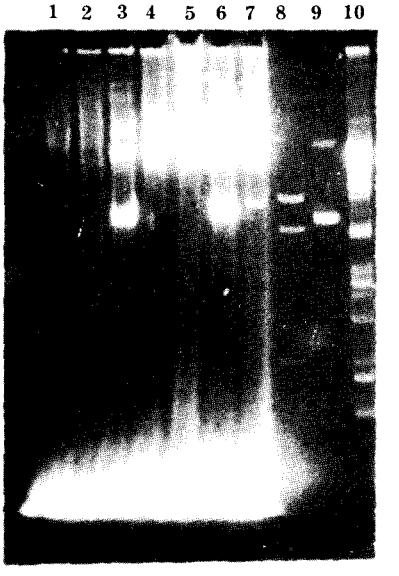

Fig. 2. Size of plasmids isolated from transformants of urease-positive.

Lane 1:*B. pasteurii*, 2: *E. coli* RR1, 3 : *E. coli* RR1 transformed by pGR71, 4, 5 : plasmid isolated from transformant of urease-positive, 7:transformant producing weak urease, 8:ϕ29/EcoRI, 9:pGR71 purified from *E. coli* DEC148, 10: *E. coli* V517.

by the recombination process.

Cloning of urease gene in *E. coli*

Because of the impossibility of cloning of urease gene with *B. subtilis*, we tried to do cloning first into *E. coli* by pGR71 of *B. subtilis-E. coli* bifunctional plasmid, and to do subsequent cloning into *B. subtilis* by cloned plasmid of *E. coli*. The DNA fragments partially digested with Hind III were ligated with the pGR71 cleaved by Hind III at the ratio of 4 to 1 (Fig. 1).

The ligated recombinant plasmids transformed *E. coli* RRI with an efficiency of 4×10^2 Neo^R transformants per 5μg of total DNA (frequency of 1.1×10^{-5}). Among 6,500 Neo^R transformants, only 2 transformants were positive for production of urease. One transformant was ommitted for poor productivity of urease. The hybrid plasmid of *E. coli* RRI (pGU66) cloned with urease gene was confirmed by rapid alkaline extraction method. Fig. 2 shows the replicated plasmid (pGU66) carring urease gene is much larger than vector pGR71, but the replicated plasmid producing poor urease is very little larger than vector size.

Besides transformants of urease-positive, several hundreds of Neo^R transformants replicated larger plasmid than vetor. This means *E. coli* RRI has genetic property which can replicate foreign DNA thoughtlessly same as common knowledge.

We chose the transformant of *E. coli* RRI cloned with urease gene and named as *E. coli* RRI (pGU66).

Transformantion of *B. subtilis* by pGU66

Plasmid pGU66 which was isolated from *E. coli* RRI (pGU66) cloned with urease gene of *B. pasteurii* was retransformed in *B. subtilis* BR151. But all Neo^R transformants did not express urease gene in the urea R broth containing neomycin. Moreover, according to electrophoretic results, we found out the molecular size of pGU66 was greatly reduced during replication, but it was slight larger than that of pGR71.

As these results, we guessed the large part of insertion DNA from *B. pasteurii* was deleted by restriction system of *B. subtilis* or incorporated into the chromosomal DNA for homologous sequences. Therefore we could not success the cloning into *B. subtilis* via *E. coli* by any means.

Subcloning with Hind III fragment of insertional DNA

To reduce the size of the insert, half fragments of the in-

Fig. 3. Plasmids of *E. coli* subcloned with Hind III fragment of 7.1 Mdal insert.

Lane 1: *E. coli* RR1, 2: pGR71 of transformed *E. coli* RR1, 3, 4, 5: plasmid of *E. coli* RR1 subcloned with Hind III fragment of insert, 6: pGU66 of cloned strain, 7: purified pGR71 of *E. coli* DEC148, 9: ϕ29/EcoRI.

sert which were isolated by low melting point agarose gel electrophoresis from pGU66 digested completely with Hind III, were religated with pGR71 digested by Hind III. Religated new recombinant plasmids transformed *E. coli* RRI on L-broth plate containing neomycin. But all of a thousand transformants could not express urease gene in urea R broth containing neomycin. Among the transformants, the size of replicated plasmid of a hundred transformants were tested (Fig. 3). This electrophoretic result indicates that the half fragment DNA were cloned with pGR71 in *E. coli* RRI.

As this results of urease-negative and cloning of half insertional fragment, we can suppose indirectly that Hind III restriction site locate in the urease gene sequence. Attempts to reduce the size of the insert will be continued by the other restriction enzymes.

Physical map of plasmid pGU66

Restriction physical map of pGU66 which was constructed with pGR71 and urease gene of *B. pasteurii* was made out by the double digestion method[20] with 4 kinds of restriction enzymes that can recognize hexanucleotide sequences. (Fig. 4).

Restriction endonuclease clevage map of 12.6 Mdalton was built up from restriction fragments patterns (Fig. 5). In

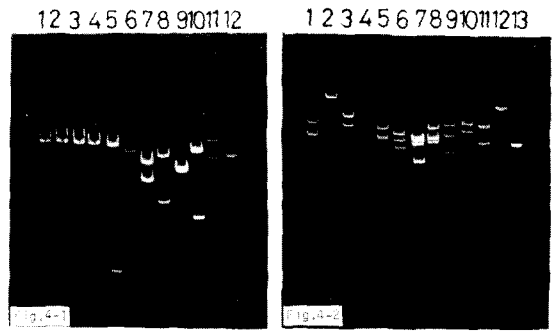

Fig. 4. Double digestion patterns of pGR71 (Fig. 4-1) and pGU66 (Fig. 4-2).

Lane 1: digested with Hind III, 2: Sal I, 3: Xba I, 4: Bgl II, 5: Hind III + Sal I, 6: Hind III + Xba I, 7: Hind III + Bgl II, 8: Sal I + Xba I, 9: Sal I + Bgl II, 10: Xba I + Bgl II, 11: ϕ29/EcoRI, 12 (Fig. 4-1), 13 (Fig. 4-2): undigested pGR71, 12 (Fig. 4-2): undigested pGU66.

addition to indication of the map, two Pvu II restriction sites, two Bam HI sites, five Hinc II sites and eight EcoRI sites could be found out in insertional fragment of 7.1 Mdal (not shown).

Localization of urease in *E. coli* transformant

The localization of urease in *E. coli* RRI (pGU66) are shown in Table 1. Although the total urease activity of *B. pasteurii* is little higher than that of *E. coli* transformant in base on the same amount of protein, the activity of *E. coli* is

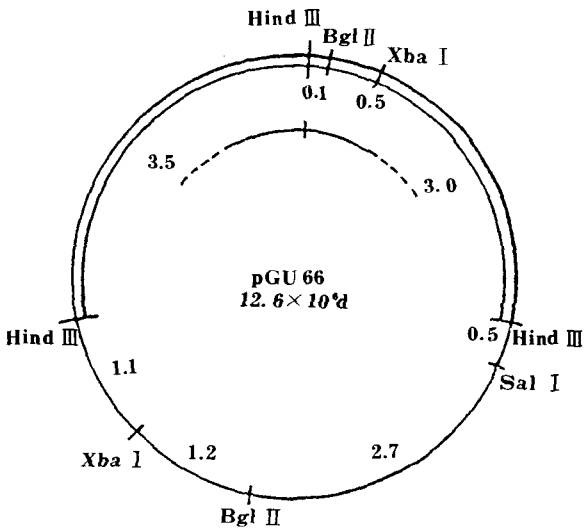

Fig. 5. Physical restriction map of plasmid pGU 66 carring urease gene.

single line: vector (pGR71) 5.5 Mdal
double line: insertion fragment 7.1 Mdal.

Table 1. Localization of urease in *E. coli*.

	Extra	Periplasm	Cellular	Total
E. coli				
8hrs	2.04*	33.04	26.32	61.40
24hrs	2.72	38.03	28.19	68.94
B. pasteurii				
24hrs	34.78			84.24

*μg as NH_4 per mg of protein.

2.5 times as much as that of *B. pasteurii* in base on the same volume of culture. The urease, which is an extracellular enzyme in *B. pasteurii*, is located in the periplasmic membrane in *E. coli*. More than 55% of the total activity of the urease was found in the periplasmic space.

Cornelis et al[21] and Tsukagoshi et al[19] cloned α-amylase gene of *B. coagulans* and *B. stearothermophilus* in *E. coli*, and found large portion of α-amylase was secreted into the periplasmic fraction in *E. coli*, suggeseting that the signal sequence of α-amylase of the gram-positive bacterium was correctly processed in *E. coli* cells. On the other hand, xylanase of *B. pumilus* was located in cytoplasmic region of *E. coli* at 89-99% of total enzyme activity[22].

Enzymatic properties of urease produded in *E. coli*

Properties of urease produced by either *E. coli* RRl (pGU66) or the donor strain of *B. pasteurii* were determined. Optimal pH of the two ureases were same as pH 7.4. Also the activities of two urease showed maximum at 60°C. These data show that the properties of urease produced by *E. coli* (pGU66) are very similiar to those of the donor stain.

The electrophoretic mobilities of the enzyme protein produced by both strains were compared by the method of Shaik-M. The mobility of the urease protein produce by *E. coli* (pGU66), was shorter than that of donor strain. From the results (Fig. 6), we guess indirectly the protein size of urease produced by cloned strain is bigger than that of *B. pasteurii*.

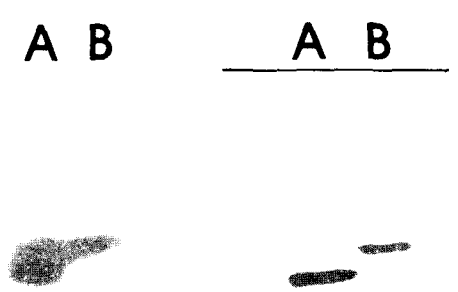

Fig. 6. Polyacrylamide gel electrophoresis of urease protein.

Lane A: urease of *B. pasterii*,
Lane B: urease of *E. coli* RR1 (pGU66)

요 약

비생물중 urease 생성능이 아주 강한 *B. pasteurii* 의 Hind Ⅲ partial digest된 chromosomal DNA 를 *E. coli-B. subtilis* bifunctional plasmid vector pGR 71으로 *E. coli* RR1 균주에 cloning하므로써 그 urease gene을 expression시킬 수 있었다. 그러나 *B. subtilis*에서는 insertion DNA fragment의 deletion으로 expression되지 않았다. Cloning된 *E. coli* RR1 균주로부터 분리 정제한 urease gene함유 plasmid (pGU66)의 restriction map을 작성하여 본 결과 7.1Mdal의 insertion fragment가 삽입된 12.6 Mdal의 plasmid에 Hind Ⅲ, Bgl Ⅱ, Xba Ⅰ, Sal Ⅰ 등 몇개의 cleavage site위치를 찾을 수 있었다. Cloning된 *E. coli*의 urease는 periplasmic space에 많은 비율로 축적되며, 그 효소학적 성질은 donor인 *B. pasteurii* 의 그것과 매우 유사하였다.

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