

Cloning of *nif* Gene Cluster from *Klebsiella pneumoniae* and Expression in *Escherichia coli*

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Klebsiella pneumoniae 의 *nif* Gene Cluster 의 Cloning 및 *Escherichia coli* 에서의 발현

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ABSTRACT: The chromosomal DNA of *Klebsiella pneumoniae* KNF1 was partially digested with HindIII. pBR322 was completely digested with same enzyme with additional alkaline phosphatase treatment. Both DNA samples were ligated and transformed into *E. coli* KO60. Single colonies of the transformed cells were isolated on N-free agar media. These colonies were ampicillin-resistant and tetracycline-sensitive. When the plasmids in transformants were cured, nitrogen-fixing activities were lost. Therefore, these transformants harbored recombinant plasmid including *nif* genes of *K. pneumoniae*. Nitrogenase activity of transformant was higher than or the same as that of *K. pneumoniae* KNF1.

KEY WORDS □ *nif* genes, cloning, transformation, expression

Biological nitrogen fixation which occurs through the agency of certain microorganism is the enzymatic reduction of dinitrogen(N_2) to ammonia and has an extremely important role in the biological nitrogen cycle. Thus, there is considerable interest in genetic study of nitrogen-fixing (*nif*) genes.

Nitrogen-fixing bacteria are divided into two broad categories: free living bacteria and symbiotic bacteria. The most extensively studied free living nitrogen-fixing bacteria is *Klebsiella pneumoniae*, which is physiologically and genetically similar to *E. coli*. The *his*-linked *nif* gene cluster of *K. pneumoniae* has been identified to 17 different genes divided into 7(or 8) transcriptional units (Dixon and Postgate, 1971; Dixon *et al.*, 1977; Kennedy, 1977; Merrick *et al.*, 1980; Beynon *et al.*, 1983). All nitrogen-fixing bacteria have single

enzyme complex called nitrogenase. This complex has remarkable similarity among different species and dissociates into two components: Fe protein (component 2) and MoFe protein (component 1) (Roberts *et al.*, 1978).

Recently, there have been many reports on genetic manipulation of *nif* genes in *Klebsiella pneumoniae*. Streicher *et al.* (1971) reported about the transduction of *nif* genes using bacteriophage P1. Dixon and Postgate (1971) transferred *nif* genes by R factor. Dixon *et al.* (1976) constructed a pRD1 carrying *nif* genes by conjugation. However, pRD1 was unsuitable for genetic experiments because of its large size and low copy number. As an attempt to overcome these problems, digested pRD1 was cloned on amplifiable plasmid, pMB9. This recombinant plasmid that carries part of the *nif* gene cluster was *nif*⁻ (Cannon *et al.*,

1977). Since then, pWK120 (*nif*⁺) carrying the entire of *nif* region was made by cloning HindIII fragments of pRD1 on multicopy plasmid, pWK 625 (Puhler *et al.*, 1979).

In this study, *nif* genes was directly prepared from chromosomal DNA of *K. pneumoniae* KNF1 independent of pRD1 or a certain recombinant plasmid and cloned on multicopy plasmid, pBR 322. The recombinant plasmid was transformed into *E. coli* KO60 and the expression of *nif* genes in *E. coli* was investigated.

MATERIALS AND METHODS

Bacterial strains and plasmid

As a donor, *K. pneumoniae* KNF1(*nif*) was isolated in our laboratory. *E. coli* KO60(Wang *et al.*, 1985) as a recipient was sensitive to ampicillin (30 $\mu\text{g}/\text{ml}$) and tetracycline (15 $\mu\text{g}/\text{ml}$). pBR322 had resistance to ampicillin and tetracycline.

Preparation of chromosomal DNA

The chromosomal DNA was prepared using the method of Berns and Thomas (1965).

Preparation of plasmid DNA

Large scale isolation of plasmid was performed by the method of Tanaka and Weisblum (1975). For small scale isolation of plasmid, procedure of Birnboim and Doly (1979) was used.

Agarose gel electrophoresis

DNA samples containing tracking dye were subjected to electrophoresis in 0.7% agarose gel dissolved in TBE buffer (45 mM Tris. HCl, 1.25 mM EDTA, pH 8.5). Electrophoresis was carried out at 80-120V by using horizontal gel chamber. The gel was then placed in solution of ethidium bromide (1 $\mu\text{g}/\text{ml}$ in water) and stained for 30 min. After destaining, the gel was photographed on UV-transilluminator.

Cloning of *nif* genes

As a preliminary step for ligation, chromosomal DNA of *K. pneumoniae* KNF1 and pBR322 were digested with HindIII partially and completely, respectively. Reaction mixtures were composed as follows: Mixture one was 20 μl of chromosomal DNA(0.3 $\mu\text{g}/\mu\text{l}$), 28 μl of H₂O, 6 μl of

MRB \times 10 (100 mM Tris. HCl, 100 mM MgCl₂, 50 mM NaCl, pH 7.4) and 3 μl HindIII (10 U/ μl), the other mixture was 5 μl of pBR322 (1 $\mu\text{g}/\mu\text{l}$), 19 μl of H₂O, 3 μl of MRB \times 10 and 1 μl of HindIII. Each of the two reaction mixtures was incubated at 37°C for 1 hr and heated at 70°C for 10 min to inactivate enzyme. For dephosphorylation of vector, 60 μl of LRB(10 mM Tris. HCl, 10 mM MgCl₂, pH 7.4) and 4 μl of bacterial alkaline phosphatase (0.25 U/ μl) were added to the tube containing digested pBR322 and incubated at 37°C for 1 hr. After that, phenol extraction was performed twice and ether extraction three times. DNA was precipitated with ethanol and resuspended 60 μl of LRB.

9 μl of 10 mM ATP, 9 μl of 40 mM DTT and 2 μl of T4 DNA ligase (100 U/84 μl) were mixed to solution consisted of 35 μl of digested chromosomal DNA and 28 μl of dephosphorylated pBR322. The mixture was incubated at 14°C overnight.

Overnight cultured *E. coli* KO60 was diluted 1:100 in fresh L-broth. The culture was incubated for an additional 2-2.5 hr with shaking (until A₅₅₀ = 0.28-0.32). Cells were harvested from 50 ml of the culture by 15 ml cold 0.1 M NaCl-5 mM MgCl₂-5 mM Tris. HCl, pH 7.6. After incubation for 25 min on ice in 12 ml 100 mM CaCl₂-250 mM KCl-5 mM MgCl₂-5 mM Tris. HCl, pH 7.6, cells were centrifuged for 10 min at 0°C and suspended in 0.4 ml 50 mM CaCl₂-10 mM Tris. HCl, pH 8.0. In order to increase efficiency of transformation, the cell suspension was incubated at 4°C for 12-24 hr.

Transformation was performed according to the method of Nogard *et al.* (1978). 0.4 ml of cell suspension was mixed with ligated DNA solution, and incubated on ice for 60 min with gentle mixing every 10 min. After transformation, the solution was added to 10 ml fresh L-broth and incubated for 1 hr at 37°C. The transformed cells were washed with N-free washing solution (13.9 g Na₂HPO₄, 1.7 g KH₂PO₄, 0.2 g MgSO₄·7H₂O, 0.008 g FeCl₃·7H₂O, 2.0 g NaCl, 0.001 g Na₂MoO₄·2H₂O, 0.001 g thiamine and 6.0 g glucose per liter) three times and suspended in N-free solution. The cell suspension was then plated on

N-free agar and the plated cells were incubated anaerobically with GasPack system. Colonies which grew on N-free media were selected and replica-plated on L-broth agar containing 30 $\mu\text{g}/\text{ml}$ ampicillin and 15 $\mu\text{g}/\text{ml}$ tetracycline to test antibiotic resistance.

Curing test

A typical curing procedure was used. Logarithmically grown culture was diluted to 10^3 - 10^4 cells/ml and then added to series of L-broth tubes containing several different concentration of acridine orange. The culture which showed a just detectable increase in turbidity was selected and plated on L-broth agar after dilutions. Colonies grown on L-broth agar were replica-plated on L-broth containing ampicillin. Colonies which lost ampicillin-resistance were picked up, inoculated on N-free agar and incubated anaerobically at 30°C for 5 days.

Acetylene reduction assay

Acetylene reduction test was performed to confirm the presence of nitrogenase. This test was carried out in 32 ml tube containing 5 ml N-free medium. The tube was injected with 15% acetylene and 85% argon gas. The strain for test was precultured in L-broth with shaking. One tenths ml of the preculture was inoculated into above-mentioned tube. The tube was incubated with shaking at 30°C for 48 hr and then 1 ml of gas sample from tube was injected into gas chromatograph. Amount of produced ethylene was measured.

RESULTS

Cloning of *nif* genes

Transformants were incubated on N-free media anaerobically. After that, single colonies which grew on N-free agar were selected. When these colonies were successively transferred to a second N-free agar, they grew well. These colonies were replica-plated on ampicillin- and tetracycline-containing agar media. All of them were ampicillin-resistance (Ap^r) and tetracycline-sensitive (Tc^s). Therefore, we confirmed that transformants harbored recombinant plasmids because they should be Tc^s on cutting of the Tc^r gene promoter

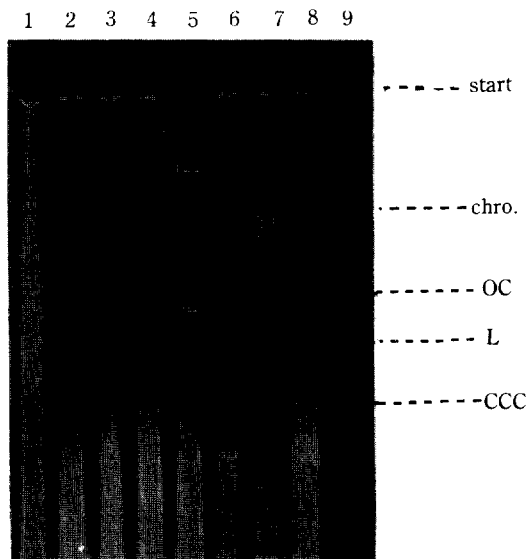


Fig. 1. Gel electrophoretic pattern of DNAs in transformants.

Lane 1: *K. pneumoniae* KNF1. 2: *E. coli* KO60. 3-5: Transformants. 6,7: Cured transformants. 8: Rp4(size marker; 56 Kb). 9: pBR322(size marker; 4.3 Kb). L, linear form; OC, open circular form; CCC, covalently closed circular form. Electrophoresis was carried out at 120V for 2.25 hr in 0.7% agarose gel. Gel dimension was 11 cm \times 8.8 cm \times 0.4 cm.

with HindIII.

Isolation of recombinant plasmids

The recombinant plasmids were isolated from transformants. Fig. 1 shows their gel electrophoretic pattern. All hybrid plasmids were classified into three types depending on their size. Their sizes were about 15 Kb, 24 Kb and 100 Kb, respectively. Three types of hybrid plasmids and cloning scheme are shown in Fig. 2.

Curing test

In order to confirm whether nitrogen-fixing activity was derived from recombinant plasmid or not, curing test was performed. Some of transformants grown in L-broth media containing acridine orange (850 $\mu\text{g}/\text{ml}$) lost ampicillin-resistance. Curing frequency was 0.2×10^{-2} . When these cured cells were incubated on N-free media anaerobically, all of them also lost their nitrogen-fixing activities (Fig. 3). And no recombinant plasmid from cured cells could be isolated (Fig. 1-lane 7,8).

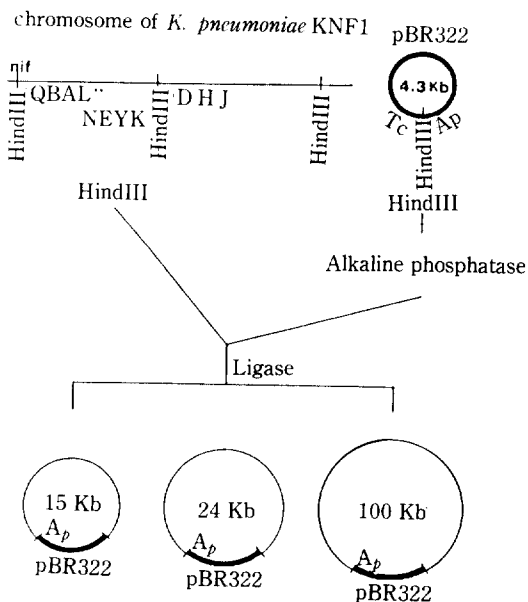


Fig. 2. The cloning scheme for recombinant plasmids in transformants.

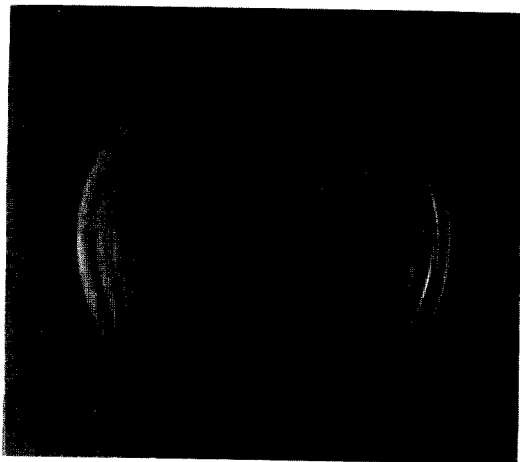


Fig. 3. Transformant and cured transformants on *N*-free agar medium.

1: *K. pneumoniae* KNF1 (donor). 2: *E. coli* KO60 (recipient). 3: Transformant. 4-8: Cured transformants.

From these results, we inferred that transformants might harbor recombinant plasmid including *nif* genes.

Nitrogenase activities

The enzyme nitrogenase reduce acetylene specifically to ethylene. Nitrogenase activities were measured using acetylene reduction test. Nitro-

Table 1. Nitrogenase activities of donor, recipient and transformants.

strains	n mole C_2H_4 /hr/tube
<i>K. pneumoniae</i> KNF1	284
<i>E. coli</i> KO60	0
Transformant 1	486
Transformant 2	283
Transformant 3	390
Cured transformant	0

genase activity of transformant was higher than or the same as that of the wild-type of *K. pneumoniae* KNF1 (Table 1). The former was 1.75 times, 1.02 times, 1.40 times of the latter, respectively. The cured transformants didn't have these activities at all. Meanwhile, a few transformants lost acetylene-reducing activities during the successive subcultures. The *nif*-plasmids didn't have considerably high stability.

DISCUSSION

If whole *nif* gene fragment on HindIII digestion (38.6 Kb) (Chung *et al.*, 1982) are inserted into pBR322 (4.3 Kb), the size of hybrid plasmid should be approximately 42.9 Kb. But we couldn't isolate recombinant plasmid of that size. We suppose that *K. pneumoniae* KNF1 might have different recognition site on HindIII from another strains.

It has been reported that colonies harbored plasmids with only *nif* Q-K fragment reduced dinitrogen (Chung *et al.*, 1982). Up to now, we couldn't confirm which regions of *nif* genes are contained in each recombinant plasmid. Further studies must be performed in order to solve these questions using methods such as *in situ* hybridization technique.

High nitrogenase activities of transformants seem to be derived from multicopy plasmid. Transformant 1 having three types of plasmid (15 Kb, 24 Kb, and 100 Kb) expressed highest nitrogenase activity. Transformant 2 had two types of plasmid (24 Kb, 100 Kb) and transformant 3 had only one plasmid (24 Kb). However, nitrogenase

activity of transformant 2 was lower than that of transformant 3 and the same as that of *K. pneumoniae* KNF1. We surmise from these results that 24 Kb plasmid of transformant 2 might be different from the same size plasmids in the other transformants.

It has been reported that the products of the gene of *glnG*(*ntrC*) or *glnF*(*ntrA*) were positive activators for *nif* genes (de Bruijn and Ausubel, 1981, 1983). On the other hand, unessential for extragenous gene product is supported by follow-

ing reports: The *nif* A gene product can substitute for the *glnG* gene product in its regulatory roles (Ow and Ausubel, 1983; Drummond *et al.*, 1983; Ow *et al.*, 1985) and whether *glnF* product plays a role in enhancing transcriptional activation of *nif* genes is less certain(Ow and Ausubel, 1983). Anyway, we obtained the result that *nif* genes cloned on a multicopy plasmid expressed high nitrogenase activity, though whether extragenous gene products were present or not in our transformants was uncertain.

적 요

한국산 *Klebsiella pneumoniae* KNF1의 chromosomal DNA를 HindIII로 부분소화하고 pBR322도 같은 효소로 완전소화했다. 소화된 pBR322의 5'-말단인산기를 제거하여 두 DNA 표품을 ligation한 후 *E. coli* KO60으로 transformation하였다. 이러한 transformants 중 N-free 한천배지에서 자라는 단일 colony들을 얻었으며, 이들은 모두 ampicillin에 대해 저항성을 가지고 있었고 tetracycline에 대해 저항성이 없었으며 curing 되었을 때는 질소고정능을 잃었다. 이런 사실로부터 이 transformants가 *K. pneumoniae*의 질소고정능 유전자를 포함하는 recombinant plasmid를 가지고 있음을 확인할 수 있었다. 이들 transformants의 nitrogenase 역가는 *K. pneumoniae* KNF1보다 더 높았다.

REFERENCES

- Berns, K.I., and C.A. Thomas, Jr., 1965. Isolation of high molecular weight DNA from *Haemophilus influenzae*. *J. Mol. Biol.* **11**: 476-490.
- Beynon, J., M. Cannon, V. Buchanan-Wollaston and F. Cannon, 1983. The *nif* promoters of *Klebsiella pneumoniae* have a characteristic primary structure. *Cell* **34**: 665-671.
- Birnboim, H.C., and J. Doly, 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**: 1513-1523.
- Cannon, F.C., G.E. Riedel and F.M. Ausubel, 1977. Recombinant plasmid that carries part of the nitrogen fixation (*nif*) gene cluster of *Klebsiella pneumoniae*. *Proc. Natl. Acad. Sci. U.S.A.* **74**: 2963-2967.
- Chung, K.S., T. Uozumi and T. Beppu, 1982. Cloning of *nif* genes from *Klebsiella oxytoca* NG13 on a multicopy plasmid in *Escherichia coli*. *Annul Reports of ICME.* **5**: 173-182.
- de Bruijn, F.J., and F.M. Ausubel, 1981. The cloning and transposon Tn5 mutagenesis of the *glnA* region of *Klebsiella pneumoniae*: Identification of *glnR*, a gene involved in the regulation of the *nif* and *hut* operons. *Mol. Gen. Genet.* **183**: 289-297.
- de Bruijn, F.J., and F.M. Ausubel, 1983. The cloning and characterization of the *glnF*(*ntrA*) gene of *Klebsiella pneumoniae*: Role of *glnF*(*ntrA*) in the regulation of nitrogen fixation (*nif*) and other nitrogen assimilation genes. *Mol. Gen. Genet.* **192**: 342-353.
- Dixon, R.A., C. Kenndy, A. Kondorosi, V. Krishnapillai and M. Merrick, 1977. Complementation analysis of *Klebsiella pneumoniae* mutants defective in nitrogen fixation. *Mol. Gen. Genet.* **157**: 189-198.
- Dixon, R., F. Cannon and A. Kondorosi, 1976. Construction of a plasmid carrying nitrogen fixation genes from *Klebsiella pneumoniae*. *Nature* **260**: 268-271.
- Dixon, R.A., and J.R. Postgate, 1971. Trans-

- fer of nitrogen fixation genes from *Klebsiella pneumoniae* to *E. coli*. *Nature* **237**: 102-103.
11. Drummond, M., J. Clements, M. Merrick and R. Dixon, 1983. Positive control and autogenous regulation of the *nif*LA promoter in *Klebsiella pneumoniae*. *Nature* **301**: 302-307.
 12. Kennedy, C., 1977. Linkage map of the nitrogen fixation(*nif*) genes in *Klebsiella pneumoniae*. *Mol. Gen. Genet.* **157**: 199-204.
 13. Merrick, M., M. Filser, R. Dixon, C. Elmerich, J. Sibold and J. Houmard, 1980. Use of translocatable genetic elements to construct a fine-structure map of the *Klebsiella pneumoniae* nitrogen fixation(*nif*) gene cluster. *J. Gen. Microbiol.* **177**: 509-520.
 14. Nogard, M.V., K. Keem and J.J. Monahau, 1978. Factors affecting the transformation of *Escherichia coli* strain X-1776 by pBR322 plasmid DNA. *Gene* **3**: 279-292.
 15. Ow, D.W., and F.M. Ausubel, 1983. Regulation of nitrogen metabolism genes by *nif*A gene product in *Klebsiella pneumoniae*. *Nature* **301**: 307-313.
 16. Ow, D.W., Y. Xiong, Q. Gu and S.C. Shen, 1985. Mutational analysis of the *Klebsiella pneumoniae* nitrogenase promoter: Sequences essential for positive control by *nif*A and *ntrC(glnG)* products. *J. Bacteriol.* **161**: 868-874.
 17. Pühler, A., H.J. Burkardt and W. Kupp, 1979. Cloning of the entire region for nitrogen fixation from *Klebsiella pneumoniae* on a multicopy plasmid vehicle in *Escherichia coli*. *Mol. Gen. Genet.* **176**: 17-24.
 18. Roberts, G.P., T. MacNeil, D. MacNeil and W.J. Brill, 1978. Regulation and characterization of protein products coded by the *nif* (nitrogen fixation) genes of *Klebsiella pneumoniae*. *J. Bacteriol.* **136**: 267-278.
 19. Streicher, S., E. Gurney and R.C. Valentine, 1971. Transduction of the nitrogen-fixation genes in *Klebsiella pneumoniae*. *Proc. Natl. Acad. Sci. U.S.A.* **68**: 1174-1177.
 20. Tanaka, T., and B. Weisblum, 1975. Construction of a colicin E1-R factor composite plasmid *in vitro*: Means for amplification of deoxyribonucleic acids. *J. Bacteriol.* **121**: 354-362.
 21. Wang, P.L., S.K. Koh, K.S. Chung, T. Uozumi and T. Beppu, 1985. Cloning and expression in *E. coli* of the whole *nif* genes of *Klebsiella oxytoca*, a nitrogen fixer in the rhizosphere of rice. *Agric. Biol. Chem.* **49**: 1469-1477.

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