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The Effects of Nitrogen Sources on the Expression of *Nif* Gene in *Klebsiella pneumoniae Nif-Lac* Fusants

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Klebsiella pneumoniae nif-lac 융합변이주의 질소고정 유전자 발현에 미치는 질소원의 효과

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The effects of various nitrogen sources on the expression of *nif* gene were investigated using *nif-lac* fusants of *Klebsiella pneumoniae*. *K. pneumoniae* UN 2979 was infected with Mudl lysate prepared by heat induction of *K. pneumoniae* UN 4482. About 80 *nif-lac* fusants were isolated and designated as LX series. In the prescence of NH_4^+, β-galactosidase activities on *nif-lac* fusants were greatly repressed. Amino acids, such as serine, glutamine and asparagine, were found to support the growth of *K. pneumoniae* M5al quite well, and showed a repressive effect on β-galactosidase activities of *nif-lac* fusants LX-9 and LX-22 in NFHM. Glutamic acid, histidine and arginine rendered poor growth but high activities of β-galactosidase. Good cell growth and high enzyme activity were observed when complex nitrogen sources, such as casitone, proteose pepone, were employed. β-Galactosidase activities of LX-9 and LX-22 in nitrogen free minimal medium increased sharply within first 4 hours.

Since *Klebsiella pneumoniae* is a member of Enterobacteriaceae, genetic techniques used for *Escherichia coli* system have been successfully applied to *nif* reaction of *K. penumoniae*.

Nif gene cluster of *K. pneumoniae* consists of sixteen gene organized into 5 polycitronic and 3 monocistronic transcription units (Dixon *et. al.*, 1977: MacNeil *et. al.*, 1978).

Regulatory studies have indicated that the nitrogenase structural genes are subject to the repression by ammonia (Roberts and Brill, 1980), oxygen (St. John *et. al.*, 1974) and other factors such as amino acids (Shanmugam and Morandi, 1976), temperature (Zhu and Brill 1981) and molybdenum (Shah and Brill 1977).

However, there still exist several obstacles for studies on the regulation of individual transcription units in *nif* gene cluster, since most of the gene products have not been conclusively identified and there is no simple assay techniqe for any of the individual gene products.

Gene fusion, especially the fusion of *lacZ* gene to other operons, has proved to be a valuable tool in studying the regulation of several systems (Casadaban, 1976; Casadaban and Cohen, 1979) and these methods were used to study the nitrogen fixation systems in *K. pneumoniae* (MacNeil *et. al.*, 1981, Dixon *et. al.*, 1980).

In this communication, we are reporting a result for gene fusions in which *E. coli lac* genes are fused to a promoter of interest. Such gene fusions have enabled us to monitor the response of each transcription unit to various effectors and determine the kinetics of derepression of *nif* operon.

MATERIALS AND METHODS

Bacterial strains

Wild-type *Klebsiella pneumoniae* M5al was kindly provided by Dr. S.L. Streicher (University of California, Berkeley, U.S.A.) and *K. pneumoniae* UN 2979 (*his D4226 lac Z4001 gal*^{r}), and UN 4482 (*hisD4226 lacZ4001 gal*^{r}/Mucts62*h*P15/Mud*l*) were obtained from Dr. W.J. Brill (Univ. of Wisconsin, U.S.A.).

Media and chemicals

Luria Broth (LB) is a rich medium containing tryptone 10g, yeast extract 5g, NaCl 0.5g and 0.2% glucose per liter and NFM is a nitrogen free minimal medium (MacNeil *et. al.*, 1978) for *K. pneumoniae* and NFHM is a NFM containing histdine (20μg/ml) and LN is a NFHM containing $(NH_4)_2SO_4$ 2mg/ml and SN is a NFHM containing $(NH_4)_2SO_4$ 0.5mg/ml. O-nitrophenyl-β-D-galactoside (ONPG) was purchased from Sigma Chemical Co., St. Lous, Mo. and 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) was from Cyclo Chemical, Los Angeles, Ca., U.S.A.

Isolation of *nif-lac* fusants

Mud*l* lysate was prepared from *K. pneumoniae* UN4482 by heat induction (Bachhuber *et. al*, 1976) and UN 2979 was infected with Mud*l* lysate and amp^r transductants were selected in LN broth containing ampicillin (60μg/ml). *Nif-lac* fusants were identified by blue color on NFHM plate containing serine (100μg/ml) and X-gal (40μg/ml) but they were white color on the same plate in the prescence of NH_4^+.

β-Galactosidase activity

After partial disruption of cell membrane using toluene, β-Galactosidase activities were assayed according to the procedure of Miller (1972).

Measurement of cell growth in the presence of nitrogen sources

Five ml of exponential growth cells of *K. pneumoniae* M5al cultivated in Luria Broth was harvested and washed with NFM broth once and resuspended in 1ml of NFM broth. Washed cells were inoculated at 2% level into each 5ml of NFM broth containing various nitrogen sources. After overnight cultivation at 30 °C in N_2, cell growth was determined by measuring the absorbance at 600nm.

Repression of *nif* gene in the prescence of various nitrogen sources

K. pnumoniae nif-lac fusants LX-9 and LX-22 cultures of exponential phase in LB were washed and inoculated at 2% level into 5ml of NFHM broth containing each indicated nitrogen source. After sparging with N_2 for 5 minutes and incubation for 18 hours at 30 °C with shaking, β-Galactosidase activities were assayed.

RESULTS

Isolation of *nif-lac* fusants

K. pneumoniae UN2979 was infected with Mud*l* lysate prepared by heat induction of UN4482 and about 4500 amp^r strains were selected and approximately 18% of them showed blue color on NFHM plate containing serine (100μg/ml) and X-gal (40μg/ml) after 2 days of incubation at 30 °C in N_2. Finally about 80 strains were observed to be repressed by NH_4^+ and designated as LX series. Among these *lac* fusants, LX-9 (*hisD4226 lacZ4001 gal*^{r} *nifD9*/Mud (*amp*^{r} *lac*)), LX-22 (*hisD4226*

Table 1. Comparison of β-galactosidase activity of *nif-lac* fusants with or without NH_4^+

Strains	β-Galactosidase activity (units)	
	$-NH_4^+$	$+NH_4^+$
LX-1	69	372
LX-6	13	228
LX-8	8	291
LX-9	7	189
LX-12	8	366
LX-14	15	240
LX-19	61	367
LX-22	10	337
LX-26	19	261
UN2979	0	3

Overnight culture of LX series in NFHM containing serine (100μg/ml) was collected and washed with NFM and each strain was inoculated into NFHM and LN and β-galactosidase activity was measured after 6 hrs in N_2.

lacZ4001, gal^r nif H22/Mud *(amp^r lac)*, of which their genetic characteristics were identified with reliability, were used in this experiment.

NH_4^+ effect on the expression of *nif* operons

β-Galactosidase activities of LX-9 and LX-22 were compared after incubation in NFHM with and without NH_4^+ under the condition discribed above. β - Galactosidase activities of *nif- lac* fusants were observed to decrease in the prescence of NH_4^+ (Table 1) and this result was consistent with the data by Dixon *et. al.* (1980) and Roberts and Brill (1980).

Response of *nif-lac* fusants to various nitrogen sources

As shown in Table 2 and 3, amino acids could be classified into two groups on the basis of the effects on cell growth and β-galactosidase activity. The first group, serine, asparagine, and glutamine supported cell growth well and repressed β-galactosidase activities like NH_4^+ and the other group, glutamic acid, histidine and arginine rendered poor cell growth but showed some effect of derepression on β-galactosidase, and MacNeil and Brill (1981) also reported similar results. Additionally, complex nitrogen sources promoted cell growth generally, repressing β-galactosidase differentially, which may be due to the different composition of amino acids of each nitrogen sources.

Derepression kinetics of *nif* operon

Nif-lac fusants LX-9 and LX-22 were transfered from NH_4^+ to nitrogen free medium and the change of β-galactosidase activity was observed (Fig. 1). β-Galactosidase activity of each strain showed a sharp increase within first 4 hrs of the cultivation in the derepression condition and then leveled off.

Table 2. Comparison of cell growth in various nitrogen sources with *K. pneumoniae* M5al.

N sources	Cell growth (Absorbance at 600nm)
Alanine	0.164
Serine	1.825
Arginine	0.826
Histidine	0.110
Glutamine	1.908
Proline	0.2
Glutamic acid	0.2
Lysine	0.2
Aspartic acid	1.306
Asparagine	>2.0
Isoleucine	0.2
Methionine	0.2
Peptone	1.620
Tryptone	1.933
Casitone	1.79
Soytone	1.923
Proteose peptone	1.727
Casamino acid	1.720
NH_4^+	>2.0
Urea	1.880
NO_3^-	>2.0

Amino acids, NH_4^+, NO_3^-, and urea were added at 20mM and remaining organic N sources were added at 2mg/ml into NFHM.

DISCUSSION

Two general methods were developed for the fusion of *lacZ* gene to other operons (Casadaban, 1976, Casadaban and Cohen, 1979) and *nif-lac* fusants here were isolated using a defective Mu prophage, Mud1 which carries the *lacZ* gene. Their genetic characteristics were identified by complementation analysis with *E. coli* UNF series harboring conjugative P plasmid (Dixon et. al., 1976) carrying *nif* genes derivatives (data not shown) obtained from Dr. R. Dixon (Univ. of Sussex, U.K.).

From the responses of *K. pneumoniae* to various nitrogen sources, it was confirmed that their effect on cell growth was closely related to the expression of *nif* operon and the previous data also supported this observation. (MacNeil *et. al.*, 1981).

On the other hand, complex nitrogen sources rendered good growth but different repression effects on *nif* operon, and this may be attributed to

Table 3. Repression effect of various nitrogen sources on the synthesis of β-galactosidase.

N sources	β-Galactosidase activity (units)	
	LX-9	LX-22
NH_4^+	24.1	22.6
Serine	21.3	25.2
Asparagine	11.6	11.3
Glutamine	12.46	11.86
Glutamic acid	122	104
Histidine	716	775
Arginine	669	696
Tryptone	8.6	4.7
Soytone	5.5	9.5
Casitone	767	678
Peptone	221	241
Proteose peptone	597	97.1

Amino acids and NH_4^+ were added at 20mM and complex N sources were at 2mg/ml.

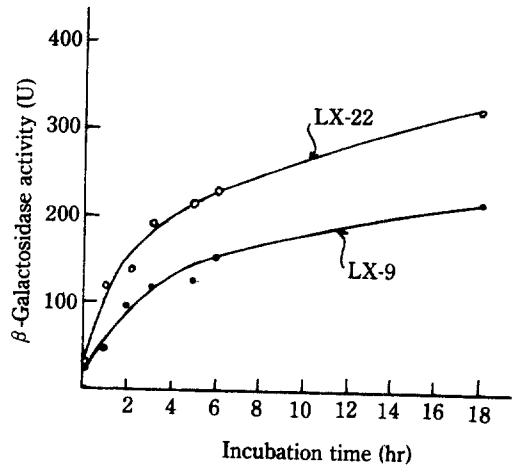

Fig. 1. Induction of β-galactosidase activity during incubation NFHM under N_2.

the fact that they carry complex amino acids and grwth factors necessary for cell growth, but concentrations of some amino acids with high repression effect on *nif* operon was limited (Shanmugam and Morandi, 1976).

This was confirmed further by the result that various amino acids tested here have different repression effect on *nif* gene expression. It has been already suggested that a certain amino acid plays an important role as the *nif* gene regulator (Shanmugam and Morandi, 1976).

All *nif-lac* fusants derived from insertion of Mud1 could synthesize a wild type β-galactosidase protein. The levels of β-galactosidase synthesized in the strains with *lac* fusions to different *nif* operon was different as shown in Table 1, and this could be an indication on the relative transcription of each *nif* operon.

In this experiment, *K. pneumoniae nif-lac* fusants isolated were proved to be very useful for the study of *nif* gene regulation and further investigations using these fusants are now being carried out.

적 요

*Klebsiella pneumoniae*의 *nif-lac*융합변이주를 사용하여 질소고정 유전자 발현에 미치는 질소원의 효과를 검토하였다. *Klebsiella pneumoniae* UN4482를 heat induction하여 만든 Mud1 lysate를 *K. pneumoniae* UN2979에 접종시켜 *nif-lac*융합체 약 80여주를 분리하고 이들을 LX series로 명하였다. 이들의 β-galactosidase 활성은 NH_4^+의 존재하에 크게 억제되었다. Serine, glutamine, asparagine 같은 아미노산을 질소원으로 사용했을때 *K. pneumoniae*의 성장은 양호하였으며, NFHM배지에서 *nif-lac* 융합주 LX-9, LX-22의 β-galactosidase의 활성은 억제효과도 높았으나, glutamine, histidine, arginine 같은 amino acid는 위와 반대의 효과를 나타냈다. Casitone, proteose peptone같은 유기질소원(2mg/ml)의 균성장에 대한 효과는 전반적으로 양호했으나LX-9, LX-22 의 β-galactosidase활성에 대한 억제효과는 각 질소원에 따라 다르게 나타났다. 한편, 질소원이 없는 최소배지에서의 LX-9 와 LX-22의 β-galactosidase의 활성은 초기 4시간내에 급격히 증가하였다.

REFERENCES

1. Bachhuber M., W.J. Brill, and M.M. Howe, 1976. Use of bacteriophage Mu to isolate deletions in the *his-nif* region of *K. pneumoniae*. *J. Bacteriol.*, 128:749-753.

2. Casadaban M.J., 1976. Transposition and fusion of the *lac* genes to selected promoters in *E. coli* using bacteriophages λ and Mu. *J. Mol. Biol.*, 104: 541-555.
3. Casadaban M.J., and S.N.Cohen, 1979. Lactose gene fused to exogenous promoters in one step using a Mu-*lac* bacteriophage *In vivo* probe for transcriptional sequences. *Proc. Natl. Acad. Sci.*, 76:4530-4533.
4. Dixon R., F. Cannon, and A. Kondorosi, 1976 Construction of a P plasmid carrying nitrogen fixation genes from *K. pneumoniae. Nature,* 260:268-272.
5. Dixon R., C. Kennedy, A. Kondorish, V. Krishnapilai and M. Merrick, 1977. Complementation analysis of *K. penumoniae* mutants defective in nitrogen fixation. *Mol. Gen. Genet.*, 157:189-198.
6. Dixon R., R.R. Eady, G. Espin, S. Hill, M. Jaccarino, D. Kahn, and M, Merrick, 1980. Analysis of regulation of *K. pneumoniae* nitrogen fixation (*nif*) gene cluster with gene fusions . *Nature,* 286:128-132.
7. MacNeil T., D. Macneil, G.P. Roberts, M.A. Supiano, and W.J. Brill, 1978. Fine structure mapping and complementation analysis of *nif* gene in *K. pneumoniae. J. Bacteriol.*, 136:253-266.
8. MacNeil D., J. Zhu, and W.J. Brill, 1981. Regulation of nitrogen fixation in *K. pneumoniae:* Isolation and characterization of strain with *nif-lac* fusions. *J. Bacteriol.*, 145:348-357.
9. Miller J.H., 1972. Experiments in molecular genetics, p.352-356, Cold Spring Habor Lab.
10. Roberts G.P., and W.J. Brill, 1980. Gene product relationships of the *nif* regulon of *K. pneumoniae. J. Bacteriol.*, 144:210-216.
11. Shah V.K., and W.J. Brill, 1977. Isolation of an ironmolybdenum cofactor from nitrogenase. *Proc. Natl. Acad. Sci.*, 74:3249-3253.
12. Shanmugam K.T., and C. Morandi, 1976. Amino acids as repressors of nitrogenase biosynthesis in *K. pneumoniae. Biochem. Biophys. Acta.*, 437:322-332.
13. St. John, R.T., V.K. Shah, and W.J. Brill, 1974. Regulation of nitrogenase synthesis by oxygen in *K. pneumoniae. J. Bacteriol.*, 119: 266-269.
14. Zhu J., and W.J. Brill, 1981. Temperature sensitivity of the regulation of nitrogenase synthesis by *K. pneumoniae. J. Bacteriol.*, 145:1116-1118.

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