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Studies on Differentiation of *Aspergillus nidulans*

—Ⅰ. Characterization of temperature-sensitive mutants defective in differentiation of *Aspergillus nidulans*—

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*Aspergillus nidulans*의 분화에 있어 온도 감수성 돌연변이주의 특성

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ABSTRACT

From FGSC 159 strain of *Aspergillus nidulans*, temperature sensitive mutants that are defective in growth and differentiation have been isolated by N-methyl-N'-nitro-N—nitrosoguanidine (NTG) treatment. The optimum concentration of NTG and incubation timec to get the highest mutation frequency was 100 μg per m*l* and 1 hour, respectively. The survival frequency was 1%.

Among the isolated mutants, five strains that were affected in early steps of differentiation were selected for further studies and named smK, smY, smB, smF, and smZ.

The execution point of each mutant was determined and the growing pattern of each mutant at the restrictive temperature was observed under the microscope. Growth of mutant was arrested near at the execution point.

From genetic analysis, each temperature-sensitive mutants was thought to have a single recessive gene. The genes of smK, smY, smB, smF, and smZ are linked to the chromosome Ⅶ, Ⅳ, Ⅷ, Ⅰ, and Ⅵ, respectively. It can be concluded that the genes controlling the differentiation are widely dispersed in the genome. From the results of mutant, smK, it is considered that a single gene can affect a function (functions) which act(s) at two different steps during differentiation.

INTRODUCTION

The studies on the dfferentiation of microorganism have been concentrated on the bacteria, such as *Bacillus*, because of their genetic simplicity. However, active investigations are being carried out recently on the relatively higher microorganisms, such as *Neurospora* and *Aspergillus*.

Halvorson *et al.*(1966) reported that the spore of *Bacillus* and *Clostridium*, while developing, synthesized different kinds of proteins compared with the vegetative cell. He also described that sequential enzyme synthesis was occured when they developed.

In *Bacillus subtillus*, Doi(1977) observed the sequential synthesis of new mRNA which were not seen during the vegetative cell growth. He proposed the gene selection by RNA polymerase hypothesis that accesory regulatory factors, which were acting on the genes that were masked during the vegetative cell growth, induced the expression of the development-specific genes by changing the affinity of RNA polymerase to the relevant promoters.

Vold(1978) described the post-transcriptional modification mechanism, observing the difference of the tRNA modification between vegetative and developmental growth in *B. subtilis*.

In *Neurospora*, Brody *et al.*(1967) isolated a mutant whose glucose-6-p dehydrogenase activity was repressed about 90%. This mutant showed normal cell cycle, but the spore formation was inhibited. He concluded that single enzyme change made morphological change.

Gamjobst *et al.*(1967), investigating the morphological mutants of *Neurospora*, reported that the genes affecting spore formation were located in the relatively defined area.

In *Aspergillus nidulans*, Zimmermann *et al.*, (1980) extracted the poly(A) RNA and classified it into three groups, that is, somatic hyphae-specific, conidiation-specific, and solely conidia-specific RNA. They then carried out the hybridization experiment between the conidiation-specific poly(A) RNA and the cloned cDNA from the conidia. They concluded that the conidiation-specific genes are relatively clustered on the genome by observing the fact that only 1.4% of the genome hybridized to the conidiation-specific poly(A) RNA.

By analyzing the mutants that were defective in the formation of spore in *A. nidulans*, Clutterbuck(1969) reported that only several locus were concerned with this event.

As shown in Fig. 1, *Aspergillus nidulans* germinates from the uninucleated conidia, forms the multinucleated hyphae, produces branches, from which it forms conidiophores and vesicles which are uninucleated. Then it rises sterigmatae and finally forms conidia (Smith *et al.*, 1977).

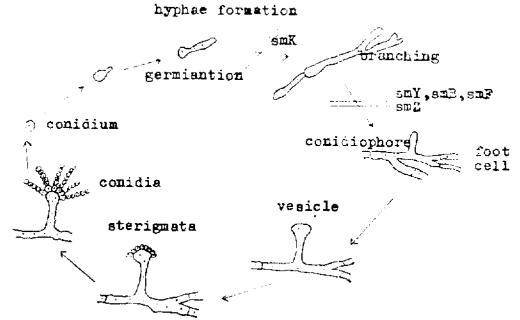

Fig. 1. sexual cycle of *Aspergillus nidulans* and defective sites of mutants.

In case of *A. nidulans*, little study on differentiation has been carried out. Moreover, study was concentrated on the conidia rather than the somatic cell.

In this paper, some temperature-sensitive mutants that could not show normal somatic cell growth were isolated, and their morphological, biochemical and genetic characters were analyzed.

MATERIALS AND METHODS

1. Strain

Temperature-sensitive mutants were derived from FGSC(Fungal Genetic Stock Center) 159 by treating N-methyl-N'nitro-N-nitrosoguanidine(NTG). The genotype of FGSC 159 was shown in Table 1. This strain has yellow conidia and its genetic markers are all recessive, except acriflavin-resistance marker(Acr A).

The isolated mutants were named smK, sm B, smY, smF and smZ.

2. Media and buffer solutions

1) Complete medium

MM stock solution 20ml, D-glucose 10g,

Table 1. Genotypes of FGSC strains(Clutterbuck et al., 1974)

Strain	Genotype*
FGSC 159	suAladE 20(Ⅰ)** paba A1(Ⅰ) yA2(Ⅰ) Acr A1(Ⅱ)*** phen A2 (Ⅲ) pyro A4(Ⅳ) lys B5(Ⅴ) sB3(Ⅵ) nic B8(Ⅶ) ribo B2(Ⅷ)
FGSC 154	adE 20(Ⅰ) biA1(Ⅰ) wA2(Ⅱ) sC12(Ⅲ) meth G1(Ⅳ) nic A2(Ⅴ) lac A1(Ⅵ) cho A1(Ⅶ) cha A1 (Ⅷ)
FGSC 515	su A1 adE 20(Ⅰ) ribo A1(Ⅰ) yA2(Ⅰ) adE 20(Ⅰ) pyro A4(Ⅳ) cha A1(Ⅷ)

* Requirement…ad, adenine; bi, biotin; met, methionine; nic, nicotinic acid; cho, choline; phe, phenylalanine; paba, p-aminobenzoic acid; pyro, pyridoxine; lys, lysine; ribo, riboflavin
Conidial color…w, white; y, yellow; fw, fawn; cha, chartreuse.
Inability to use…lac, lactose; s, sulfate
Resistant…Acr, acriflavin
Suppression…suAladE 20, adenine suppressor
** Chromosome number in ( )
*** Acr is temperature-sensitive.

vitamin solution 10m*l*, adenine solution 2m*l*, yeast extract 1.5g, casamino acid 1.5g, D.W. 1000m*l*

2) MM(Minimal medium) stock solution

$NaNO_3$ 76g, $MgSO_4 \cdot 7H_2O$ 13g, KH_2PO_4 76g, KCl 13g, ammonium molybdate 2g, $ZnCl_2$ 0.2 g, $MnCl_2 \cdot 4H_2O$ 4mg, $CuSO_4 \cdot 5H_2O$ 16mg, $FeCl_3 \cdot 6H_2O$ 80mg, D.W. 500m*l*

3) Adenine solution

One gram of adenine was dissolved in 100m*l* of D.W., and conc. HCl(0.62m*l*) was added to it.

4) Vitamine solution

riboflavin 50mg, nicotinic acid 50mg, p-amino benzoic 250mg, pyridoxin 250mg, thiamin 250mg, biotin 1mg, folic acid 250mg, ascorbic acid 250mg, D.W. 500m*l*

5) Minimal medium

MM stock solution 20m*l*, D-glucose or lactose 10g, D.W. 1000m*l*

6) Supplement(per 1,000m*l* medium)

p-amino benzoic acid 0.1mg, adenine 20mg, phenylalanine 80mg, methionine 250mg, pyridoxine 0.5mg, lysine 70mg, nicotinic acid 0.25mg, riboflavin 1mg, sulfite 400mg, choline 0.25mg biotin 0.05mg

7) Citrate buffer(pH5.5)

citric acid 10.5g, NaOH 4.4g, D.W. 500m*l*

8) Cold lysis buffer

spermidine 5mM, KCl 10mM, sucrose 0.2 M, Tris-HCl(pH 7.4) 10mM

0.005%(w/v) acriflavin was added to the medium to select the acriflavin-resistant strain. (Roper *et al.*, 1957) 0.08%(w/v) sodium desoxycholate was added to solid medium to minimize the colony size.

3. Mutagenesis

The conidial suspension (10^5/m*l*) of FGSC 159 was cultured in the complete broth at 37°C for 2hrs. The cells were centrifuged(10,000rpm, Hitachi, RPR 20-2-720, 15min) and washed with citrate buffer. These were treated with NTG and incubated at 37°C. The concentration of NTG varied from 50μg/m*l* to 200μg/m*l* at the interval of 50μg/m*l*. The incubation time varied from 30 min. to 2hrs. at the interval of 30 min.

After NTG was washed by centrifugation the NTG-treated cells were expressed on the complete medium for 3 days. Among the expressed colonies, temperature-sensitive mutants that grew normally at 37°C but didn't show normal differentiation at 42°C were selected. The selected mutants were once again tested and stocked for further study(Pringle, 1975).

4. Determination of execution point

To determine the execution point of each mutant, the mutant was subjected to temperature-shift experiment. That is, the mutant

was incubated at the permissive temperature at first, then shifted to the restrictive temperature. Conversely, the mutant was incubated at the restrictive temperature at first, and then shifted to the permissive temperature. The temperature shift was done every 4hrs.

5. Cytological observation

Each mutant was incubated at the restrictive temperature and their growth pattern was observed under the microscope at the interval of appropriate time. The method of Harsanyi *et al.*,(1977) was adopted. The method was as follows. Squares of cellophane dialyzer membrane, 2×2cm, previously sterilized in an autoclave, was placed aseptically on the complete medium. A few drops of conidial suspension (10^4/m*l*) were spread on the surface of the membranes. After appropriate incubation, the membrane was immersed for 10 min. in a modified Helly's fixative solution. The fixative consisted of 5%(w/v) mercuric chloride and 3% (w/v) potassium dichromate in distilled water. Immediately before use one part of 40% formaldehyde was added to nine parts of the fixative. After fixation the membrane was rinsed with 70% ethanol until the yellow color was no longer appeared. The membrane was then stored in 70% ethanol until staining. The membrane was rinsed several times with distilled water. The membrane was then immersed in 2m*l* of 0.005% acid fuchsin solution in 1% acetic acid for 2.5 min. After this period the membrane was rinsed with 1% acetic acid until it no longer appeared red, placed on a slide in a drop of 1% acetic acid, and examined under the microscope.

6. Genetic analysis

Heterokaryon and diploid were formed between each mutant and FGSC 154(Table 1), and incubated at the restrictive temperature to determine the dominance or recessiveness of the allele.

For the purpose of investigating the linkage group, meiotic recombination between each mutant and FGSC 515 as well as mitotic recombination between each mutant and FGSC 154 was exploited(Prichard, 1968). Benlate was added to the medium at the concentration of 3.5ppm for the segregation of the diploid (Upshall *et al.*, 1977). Recombinant ascospores were obtained on the selective media using the three-week-old heterokaryon.

RESULTS AND DISCUSSION

1. Mutagenesis

About 20 temperature-sensitive mutants defective in differentiation were isolated by the procedure described in Materials and Methods.

The mutation rate was highest when the cells were treated with NTG at the concentration of 100μg/m*l* for 1hr(Table 2). The survival rate was 1%.

2. Determination of execution point

Execution point is the very time when the mutational character is expressed during the life cycle of a mutant(Orr *et al.*, 1967). Execution point has important meaning since the gene expression of differentiation in *A. nidulans* is thought to occur sequentially. The differentiation-specific genes can be ordered by determining the execution points of mutants that

Table 2. Mutant frequency by NTG treatment

NTG concentration	50μg/m*l*	100μg/m*l*		200μg/m*l*
Incubation time	60min.	30min.	60min.	30min.
Survival frequency	10%	50%	1%	35%
Mutant frequency	0.9%	0.6%	2.4%	0.3%

Table 3. Determination of execution points

Strain	37°C→42°C		42°C→37°C	
	No growth	Growth	Growth	No growth
sm K	24hr. (20hr*)	28hr.	44hr.	48hr.
sm Y	24hr.	28hr.	40hr.	44hr.
sm B	28hr.	32hr.	40hr.	44hr.
sm Z	28hr.	32hr.	28hr.	32hr.
sm F	32hr.	36hr.	36hr.	40hr.

* When smK is shifted to restrictive temperature between 20hr. and 24hr., it grcws cnly to fail to produce conidia.

Other mutants (smC, smX) also show such phenomenon, that is, growth is inhibited at different periods.

are unable to differentiate.

The methcd to determine the execution point is described in the Materials and Methods, and the result is illustrated in Table 3.

The executicn point of smK, smY, smB, smZ and smF is 24∼28hr, 24∼28hr, 28∼32hr, 28∼32hr and 32∼36hr. respectively.

The temperature-sensitive mutants can be divided into 2 groups. One group is the mutants for synthesis. This group cannot synthesize gene product at the restrictive temperature. The other group is the mutants for function. This group can synthesize gene product but the gene product can not function at the restrictive temperature. The former group shows similar periods for growth when this group is subjected to the temperature-shift experiment, from restricitive temperature to permissive temperature and from permissive temperature to restrictive temperature. On the contrary the latter group shows different periods for growth in the same experiment (Pringle, 1975).

It can be said that smK, smB and smY are mutants for function and that smF and smZ are mutants for synthesis.

3. Cytological analysis

The growth patterns of each mutant at the restrictive temperature are shown in Fig. 2. It is impossible to distinguish smF and smZ from the wild type strain(FGSC 159) under the microscope.

As shown in Fig. 2B. smK could not branch and its hyphae became thick as time passed. It was interesting that this strain showed hyphae lysis after about 55hrs. In case of smY, it could branch but its hyphae became thick, tco(Fig. 2C). As time flew, it could not grow any longer and its hyphae became aggregated. Strain smB grew somewhat faster than smK and smY. However, its hypal tip growth was blocked at a certain time(Fig. 2D).

If growth patterns of each mutant at the restrictive temperature and the execution points of each mutant were compared together, it is easy to find that the growth is inhibited around the execution point.

5. Genetic analysis

The heterokaryon and diploid from the cross of FGSC 154 and the mutants were made and let to grow at the restrictive temperatare to determine the dominance or recessiveness of the alleles. They all showed normal growth, thus indicating that all alleles of the mutants are recessive.

To determine the linkage group of each

Fig. 2A. Growth of FGSC 159 at restrictive temperature.
a. 9hr(×600) b. 12hr(×600) c. 16hr(×150) d. 20hr(×150)

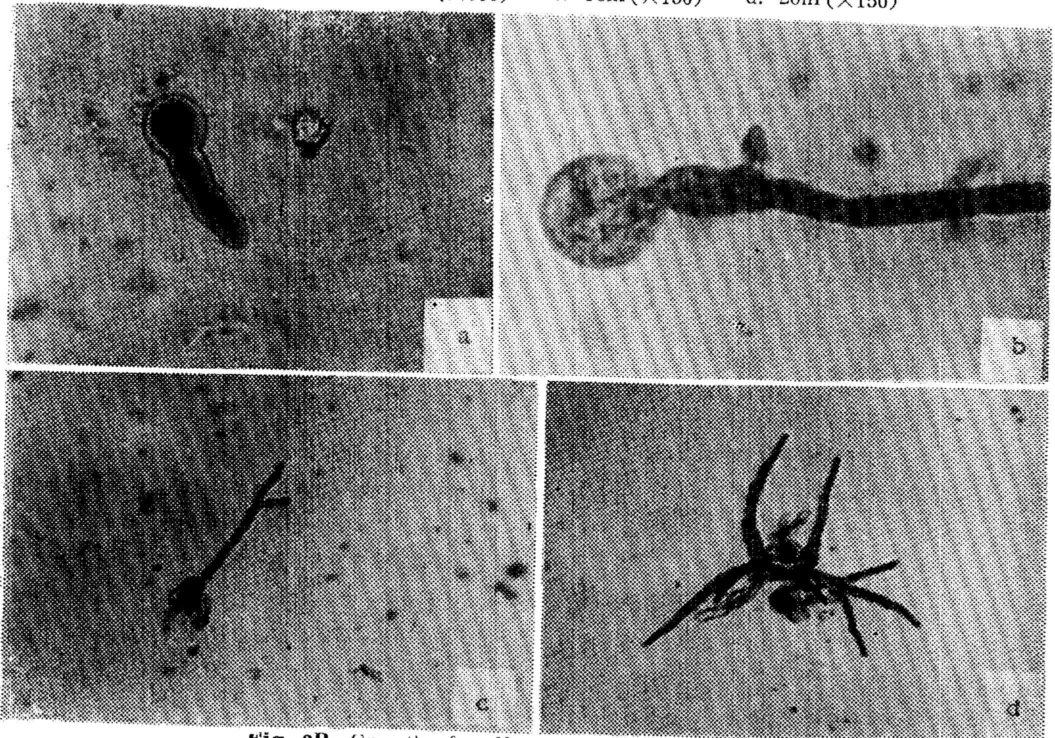

Fig. 2B. Growth of smK at restrictive temperature
a. 12hr(×600) b. 20hr(×600) c. 33hr(×150) d. 55hr(×150)

Fig. 2C. Growth of smY at restrictive temperature
a. 12hr(×600) b. 20hr(×150) c. 33hr(×150) d. 55hr(×150)

Fig. 2D. Growth of smB at restrictive temperature.
a. 12hr(×600) b. 20hr(×150) c. 33hr(×150) d. 55hr(×150)

Table 4. Survival frequency of segregants from the diploid(mutant×FGSC 154) at restrictive temperature

Diploid	Segregated haploid	Number of colony		Survival frequency
		37°C	42°C	
smK×FGSC 154	nic⁺(Ⅶ)*	35	32	91.4%
	lys⁺(Ⅴ)	33	18	54.5%
smB×FGSC 154	ribo⁺(Ⅷ)	23	23	100.0%
	nic⁺(Ⅶ)	10	5	50.0%
smY×FGSC 154	pyro⁺(Ⅳ)	20	14	70.0%
	met⁺(Ⅳ)	15	6	40.0%
	lys⁺(Ⅴ)	33	19	57.6%
	white(Ⅱ)	48	27	56.3%
smF×FGSC 154	yellow(Ⅰ)	23	2	8.7%
	white(Ⅱ)	19	8	42.1%

* Chromosome number

Table 5. Survival frequency of recombinant ascospore from the cross(mutant×FGSC515) at restrictive temperature.

Cross	Selected ascospore	Number of colony		Survival frequency
		37°C	42°C	
smK×FGSC515	ribo⁺, nic⁺(Ⅶ)*	42	41	97.6%
	ribo⁺, lys⁺(Ⅴ)	79	38	48.1%
smB×FGSC515	ribo⁺(Ⅷ)	165	163	98.8%
smY×FGSC515			Approximately	50%**
smZ×FGSC515	ribo⁺, S⁺(Ⅵ)	33	32	97.0%

( ) * Chromosome number

** See text.

mutant gene, mitotic recombination were used. In mitotic recombination, segregants from the cross of FGSC 154 and mutant were analyzed by inoculating them on the selective medium. In meiotic recombination, recombinant ascospores from the cross of FGSC 515 and mutant were analyzed by spreading them on the selective medium. The result of mitotic recombination is shown in Table 4, and the result of meiotic recombination is shown in Table 5.

For making the fine chromosome map it is not accurate to use mitotic recombination method, since the yield of segregants was relatively small and the segregants segregated spontaneously. However, mitotic recombination was of great help to determine the linkage group.

According to the genetic theory, when one uses unliked genetic marker, the survival frequency of the recombinant is theoretically 50%. But in case of mitotic recombination, it is more than 50% survival frequency because of the disomy resulted from nondisjunction. If one uses linked genetic marker of the objective mutant strain, the survival frequency is 50~100% conversely, if ones use linked genetic marker of the test-crossed strain, the survival frequency is 0~50%. As shown in Table 4, the allele of smK is located on the linkage group Ⅶ. And the alleles of smB, smF and

Table 6. Linkage group detrmination and morphological defect of each mutant.

Strain	Linkage group	Linked genotype	Morphological defect
smK	Ⅶ	nic B8	no branching
smY	Ⅳ	pyro A4	hyphae swelling
smB	Ⅷ	ribo B2	hyphae tip growth inhibition
smF	Ⅰ	yA2	no conidiophore formation
smZ	Ⅵ	sB3	no conidiophore formation

smY are located on the linkage group Ⅷ, linkage Ⅰ and linkage group Ⅳ, respectively.

As shown in Table 5, the genes of smK, smB, smZ are linked to the linkage group Ⅶ, linkage group Ⅷ and linkage group Ⅵ, respectively. It was difficult to determine the linkage group of the gene of smY because this strain showed approximately 50% survival frequency on the selective media of all possible genetic markers(ribo, nic, s, phe, lys). Nevertheless, from the data of Table 4, this mutant is thought to be linked to chromosome Ⅳ. It is impossible to determine the linkage group of the gene, if smY is linked to the chromosome Ⅳ. The reason is that there is no selective marker on the chromosome Ⅳ between smY(derived from FGSC 159) and FGSC 515, as shown in Table 1.

It appeared that the result by meiotic recombination is more reliable than the result by mitotic recombination. It is because the meiotic recombination is processed more precisely than the mitotic recombination.

The overall data on linkage group and morphological defect of the five mutants are shown in Table 6.

It can be concluded that the genes of differentiation are dispersed widely on the genome.

Strain smK is considered a mutant of single gene on the chromosome Ⅶ. However, the gene affects two steps of differentiation, as shown in Table 3. This result means that a single gene may function at different periods during differentiation.

It will be of help to know the mechanism of differentiation if accumulated substances or altered enzymes of the mutants at the restrictive temperature are analyzed.

Furthermore, should more mutants in differentiation be collected and studied on their natures and sequence of their gene expression, the more will be known for the control mechanism of sequential functions in differentiation.

摘　　要

Aspergilus nidulans FGSC 159로 부터 성장과 분화에 관계하는 온도감수성 돌연변이주를 NTG를 사용하여 분리하였다. 돌연변이 물질인 NTG의 농도 100μg/ml에서 1시간 배양(생존율 1%)하였을 때 변이율이 가장 높았다. 분리된 돌연변이주 중에서 비교적 분화초기 단계에 관여하여 유전자를 지닌 5개의 변이주를 선정하여 각각 smK, smY, smB, smF, smZ라 명명하였다.

각 돌연변이주의 변이점을 결정하고 세포학적 관찰을 실시하였는데, 변이점 부근에서 세포성장이 저해되는 것을 볼 수 있었다. 각 돌연변이주의 유전인자는 모두 열성이었으며, 체세포 재조합(mitotic recombination)과 감수분열재조합(meiotic recombination) 방법으로 유전자의 위치를 결정한 결과, smK는 염색체 Ⅶ에, 그리고 smY는 염색체 Ⅳ에, smB는 염색체 Ⅷ에, smF는 염색체 Ⅰ에, 그리고 smZ는 염색체 Ⅵ에 각각 연관되어 있어 분화에 관계하는 유전자는 여러 염색체에 흩어져 있음을 알 수 있었다. 또한 sm K의 경우를 보아 단일돌연변이가 분화과정의 두 예에 영향을 미치는 것으로 사료된다.

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