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Short Communication

Demonstration of stage-specific nucleolytic activity in cell-free extracts of *Phycomyces* and inhibition of this activity by EDTA and G-actin

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Stage-specific nucleolytic activity was identified in cell-free extracts (CFEs) of *Phycomyces*. Such activity was not detected in spore germlings or mycelia for the first 36 h after the start of cultivation. However, it was detected in mycelia more than 48 h after the start of cultivation, as well as sporangiophores and sporangia. The nucleolytic activity was completely inhibited by the addition of EDTA or G-actin to the CFE, and the various results together suggest that the activity was due to deoxyribonuclease I (DNase I).

Key Words——DNase I; EDTA; G-actin; *Phycomyces*; stage-specific nucleolytic activity.

Transformation is one of the most important techniques for the analysis of gene expression. There are many reports about the development of transformation systems for filamentous fungi, and such transformation systems have been effectively used for analysis of gene expression and its regulation (Lemke and Peng, 1994). There are four reports of the development of systems for the transformation of *Phycomyces blakesleeanus* Burgeff (Revuelta and Jayaram, 1986; Suarez and Eslava, 1988; Arnau et al., 1988; Ootaki et al., 1991). However, the efficiency of transformation and the stability of transformants in each case were low, and attempts to complement and disrupt genes using these systems have not yet succeeded. To clarify the reasons for such failure to transform *P. blakesleeanus*, it is necessary to determine whether or not the transferred exogenous DNA is retained constitutively and whether or not the transferred genes can function normally. In this study, we examined the former issue, attempting to determine the probable fate of exogenous transferred DNA.

As fungal materials, we used a standard wild-type (−) strain of *Phycomyces blakesleeanus*, IGE1101 (=NRRL1555), and a wild-type strain of *Neurospora crassa* Shear & Dodge, IFO 6966. Initially, 2.0×10^6 spores of *P. blakesleeanus* were activated by heating at 48°C for 10 min (Rudolph, 1960). The activated spores were inoculated onto agar-solidified SIVYC medium (Sutter, 1975), covered with a dialysis membrane (Sanko Junyaku, Tokyo, Japan) and cultured at 20°C under white fluorescent light (20 mW/cm^2). In the case of *N. crassa*, 2.0×10^6 conidia were inoculated onto agar-solidified Fries complete medium (Ryan et al., 1943), covered with a dialysis membrane and cultured at 30°C under white fluorescent light (20 mW/cm^2).

As experimental materials (see Fig. 1), we used spore germlings collected 16 h after the start of cultivation, 1-cm segments of mycelial fronts excised 36 h and 48 h after the start of cultivation, sporangiophores at stage I and sporangia at stages II and III that were collected 72 h after the start of cultivation. We also used 1-cm segments of mycelial fronts excised from cultures of *N. crassa* 48 h and 96 h after the start of cultivation. To prepare each cell-free extract (CFE), we homogenized 150 mg of materials, collected as described above, with 100 μl of extraction buffer [10 mM Tris-HCl, 1% (w/v) NaCl, pH 7.4, referred to as TBS] and a small amount of quartz sand, using a pestle and mortar that had been chilled in an ice-bath. Each homogenate was transferred to a 1.5-ml microcentrifuge tube and centrifuged in a refrigerated centrifuge ($15{,}000\times g$, 20 min, and 4°C). Each supernatant was recovered and transferred to another 1.5-ml microcentrifuge tube. The transferred supernatants were used as CFEs. The CFE from *N. crassa* was designated NcCFE. The concentrations of protein in each CFE and NcCFE were determined by Bradford's method (Bradford, 1976), with bovine serum albumin (F-V, Sigma Chemical, St. Louis, MO, USA) as the standard.

To examine the nucleolytic activities of CFEs, we

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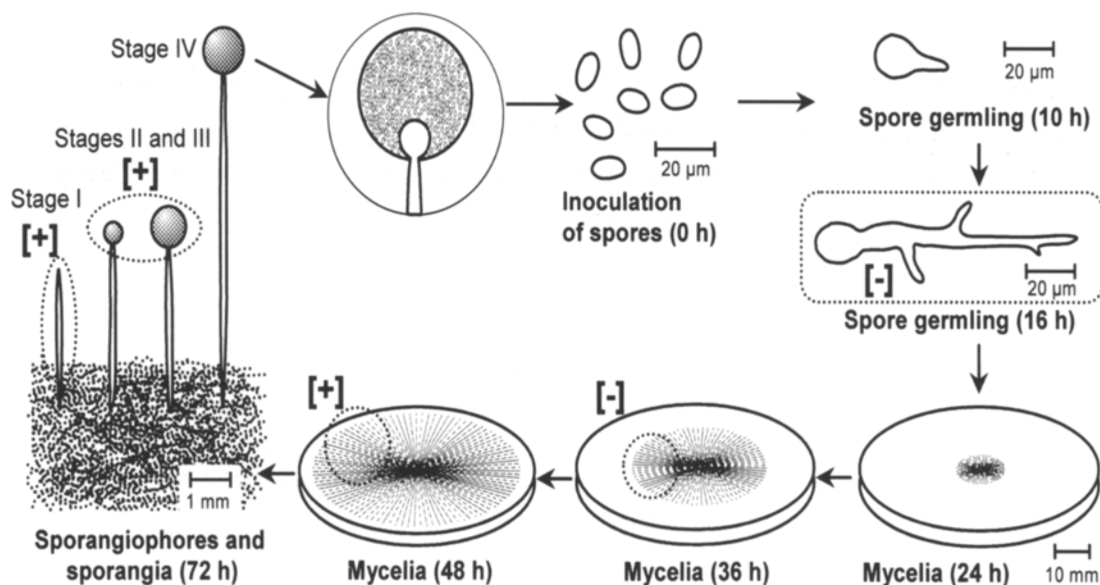

Fig. 1. The asexual life cycle of *P. blakesleeanus* and the presence of nucleolytic activity in the CFEs.
Plus signs indicate nucleolytic activity; minus signs indicate the absence of detectable nucleolytic activity. "0 h" is the time at which spores were inoculated on agar-solidified plate SIVYC medium and dotted lines indicate the stages at which each CEF was prepared.

used plasmid pJL2 (Revuelta and Jayaram, 1986) as the DNA substrate. Solutions of pJL2 were treated with CFE as follows. Mixtures of 40 μl of TBS with 5 μl of pJL2 (0.2 μg/ μl) and 10 μl of CFE (=25 μg of protein) were incubated at 37°C for a few seconds, 0.5 h, 1 h, 2 h, or 4 h. After each incubation, DNA was recovered by extraction twice with phenol-chloroform and precipitation with ethanol (Sambrook et al., 1989). The recovered DNA was dissolved in 10 μl of 10 mM Tris-HCl buffer (pH 8.0) with 1 mM EDTA. The DNA (5 μl) was then analyzed by electrophoresis on a 0.8% (w/v) agarose gel (AGE) and staining with 0.5 μg/ml ethidium bromide.

We also used the polymerase chain reaction (PCR) to detect small amounts of plasmid DNA. To detect pJL2 specifically, primers (5′-ATG AGC CAT ATT CAA CGG GAA-3′ and 5′-ACT CAT CGA GCA TCA ATT GAA-3′) were prepared by reference to the total nucleotide sequence of kanamycin-resistance gene (Grindley and Joyce, 1980) in pJL2. PCR was performed in a thermal cycler (PC-700; ASTEC, Tokyo, Japan). Each 50-μl reaction mixture contained 5 μl of 10× PCR buffer (TOYOBO, Tokyo, Japan), 5 μl of 2 mM dNTP mixture (TOYOBO), 4 μl of 25 mM $MgCl_2$, 0.2 μl of Taq DNA polymerase (5 U/μl, TOYOBO), 5 μl of a 10 μM solution of each primer and 50 ng (when without CFE treatment) of template DNA. PCR was performed with 30 cycles of incubation at 93°C for 1 min, 55°C for 1 min and 72°C for 2 min, and a final extension at 72°C for 10 min. Products of PCR (5 μl) were analyzed by AGE.

To analyze the effects of EDTA, we used CFEs prepared from mycelia harvested 48 h after the start of cultivation and a mixture of sporangiophores at stage I and sporangia at stages II to III collected 72 h after the start of cultivation. Reaction mixtures of 50 μl were prepared that containing 5 μl of pJL2 (0.2 μg/μl) and 10 μl of CFE (=25 μg protein) with final concentrations of EDTA of 0.5, 1, 2 and 5 mM. Each mixture was mixed thoroughly before the addition of pJL2 solution, and incubated at 37°C for 30 min following the addition. Reactions were stopped by addition of 50 μl of phenol-chloroform. Finally, the DNA was recovered as described above.

In the case of G-actin, a 2% (w/v) solution of G-actin was prepared by dissolving 5 mg of actin (from chicken muscle; product No. A 0398, Sigma Chemical) in 250 μl of G-actin buffer (Macanovic and Lachmann, 1997), which contained 10 mM Tris-HCl (pH 8.0), 0.5 mM dithiothreitol, 0.2 mM $CaCl_2$ and 0.2 mM ATP. A CFE was prepared from mycelia collected 48 h after the start of cultivation. Reaction mixtures of 50 μl were prepared that containing 5 μl of pJL2 (0.2 μg/μl) and 10 μl of CFE (=0.4 μg protein) with final concentrations of G-actin of 0.063%, 0.125%, 0.25%, 0.5% and 1.0% (w/v). All other details were as described above for incubation with EDTA.

Plasmid DNA was incubated with CFEs prepared from: (A) spore germlings collected 16 h after the start of cultivation; (B) mycelial front collected 36 h after the start of cultivation; (C) mycelial front collected 48 h after the start of cultivation; (D) sporangiophores at stage I collected 72 h after the start of cultivation; and (E) sporangia at stages II to III, collected 72 h after the start of cultivation. The results are shown in Fig. 2A. According to Revuelta and Jayaram (1986), pJL2 is 6.2 kbp long. However, the electrophoretic mobility of pJL2 suggested a length of 4.0 kbp (lane 1, Fig. 2A). This observation indicates that pJL2 was in a covalently closed circular (CCC) form (Johnson and Grossman, 1977). Lane 2 in Fig. 2A indicates that the mobility of pJL2 after brief treatment with CFE prepared from (A) corresponded to a length of 4.0 kbp. Lane 3 in Fig. 2A indi-

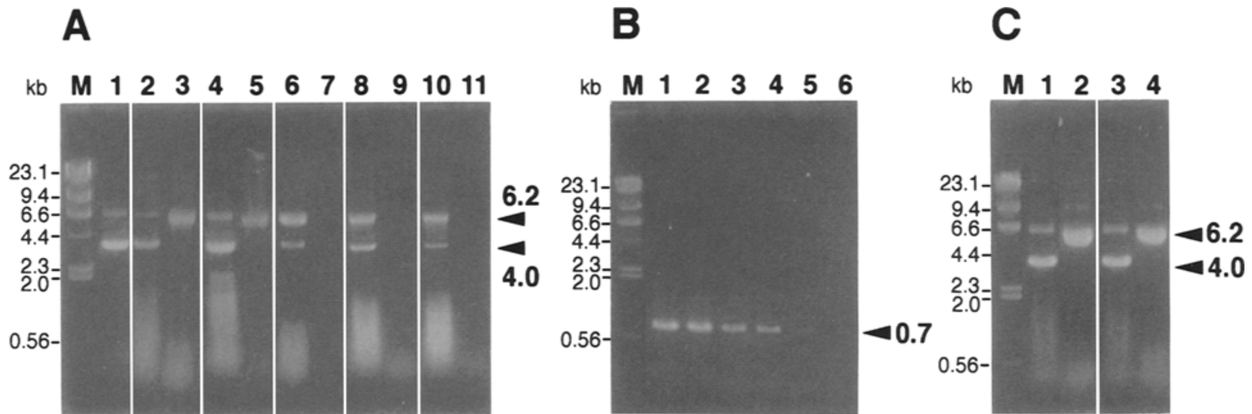

Fig. 2. Detection of nucleolytic activities.
A. Detection of nucleolytic activities in CFEs prepared at various developmental stages. Lane 1 shows 0.25 μg of pJL2 (negative control). pJL2 treated for a few seconds (lanes 2, 4, 6, 8 and 10) or 30 min (lanes 3, 5, 7, 9 and 11) with CFE prepared from 16-h spore germlings (lanes 2 and 3), 36-h mycelia (lanes 4 and 5), 48-h mycelia (lanes 6 and 7), stage I sporangiophores (lanes 8 and 9) and stage II-III sporangia (lanes 10 and 11) was visualized with ethidium bromide. Smeared signals represent RNA in CFE (lanes 2, 4, 6, 8 and 10). B. Detection by PCR of pJL2 treated with CFE prepared from mycelia harvested 48 h after the start of cultivation. Lane 1 shows PCR-amplified product of pJL2 without treatment with CFE (positive control). PCR-amplified product of pJL2 that had been treated with CFE for a few seconds (lane 2), 30 min (lane 3), 60 min (lane 4), 120 min (lane 5), and 240 min (lane 6) was visualized with ethidium bromide. C. Detection of nucleolytic activity in NcCFEs prepared from cells at two different stages. pJL2 treated for a few seconds (lanes 1 and 3) or 30 min (lanes 2 and 4) with NcCFE prepared from 48-h mycelia (lanes 1 and 2) and 96-h mycelia including conidiophores and conidia (lanes 3 and 4) is visualized with ethidium bromide. Smeared signals represent RNA in NcCFEs (lanes 1 and 3). Lane M shows DNA size markers (0.5 μg of *Hin*dIII-digested DNA).

cates that pJL2 that had been treated with CFE prepared from (A) for 30 min was 6.2 kbp long. These results indicate that the CCC form of pJL2 treated with CFE prepared from (A) became linear, and moreover, that the CFE prepared from (A) had no degradative activity. Lanes 4 and 5 in Fig. 2A indicate that the CFE prepared from (B) produced almost the same result as the CFE prepared from (A). However, lane 6 in Fig. 2A shows that CFE prepared from (C) immediately converted pJL2 from the CCC form to the linear form. Lane 7 in Fig. 2A shows that CFE prepared from (C) degraded pJL2 completely. Lanes 8, 9, 10 and 11 in Fig. 2A show that CFEs prepared from (D) and (E) had the same activity as the CFE prepared from (C). These results indicate the absence of nucleolytic activity in the early mycelial stages and the presence of the activity in the late mycelial phases including sporangiophores and sporangia, more than 48 h after the start of cultivation in *P. blakesleeanus*. This nucleolytic activity is indicated in Fig. 1 by plus signs. The appearance of the nucleolytic activity appears to be associated with the developmental stage of *P. blakesleeanus*.

Lanes 7, 9 and 11 in Fig. 2A indicate that pJL2 had been digested completely, but to confirm that digestion was complete, we used PCR, which is a more sensitive method of detection than AGE. Our results are shown in Fig. 2B. From lane 1, it is clear that PCR specifically amplified a 0.7-kb fragment of the *kan*^{r} gene in pJL2. We treated pJL2 with the CFE prepared from (C) for various times and performed PCR to detect amplified 0.7-kb fragments. From lanes 2 to 6, it is clear that the amount of pJL2 decreased with time and pJL2 was completely digested within 120 min.

As a control experiment, we used NcCFE, prepared from mycelia of *N. crassa* that had been collected 48 h after the start of cultivation. The results are shown in Fig. 2C. In lanes 1 and 2, DNAs with electrophoretic mobilities corresponding to 4.0 and 6.2 kbp are visible. When pJL2 was treated with NcCFE prepared from mycelia including conidiophores and conidia harvested 96 h after the start of cultivation, the electrophoretic pattern of pJL2 DNA was the same as that of pJL2 treated with NcCFE prepared from mycelia harvested 48 h after the start of cultivation (Fig. 2C; lanes 3 and 4). Thus, the nucleolytic activity of NcCFE with the same protein concentration as that of CFEs from *P. blakesleeanus* was very weak at all developmental stages examined. Chow and Fraser (1979) and Fraser et al. (1989) reported that cell extracts prepared from mycelia for 7 d after the start of cultivation had no nucleolytic activity against double-stranded DNA without activation by trypsin. These observations might explain why *N. crassa* is a filamentous fungus that can easily be transformed, as reported by Grant et al. (1984).

We next attempted to determine whether EDTA (Hori et al., 1979) and G-actin (Lazarides and Lindberg, 1974) might inhibit the nucleolytic activity in CFEs of *P. blakesleeanus*. The results are shown in Fig. 3. Lanes 1, 2, and 3 in Fig. 3A-1 show that pJL2 treated with CFE prepared from mycelia harvested 48 h after the start of cultivation was well digested at concentrations of EDTA below 1 mM. pJL2 treated with CFE was partially and incompletely digested when the concentration of EDTA was around 2 mM (lane 4). pJL2 treated with CFE was barely digested at all at concentrations of EDTA above 5 mM (lane 5). These results suggest that the CFE of *P.*

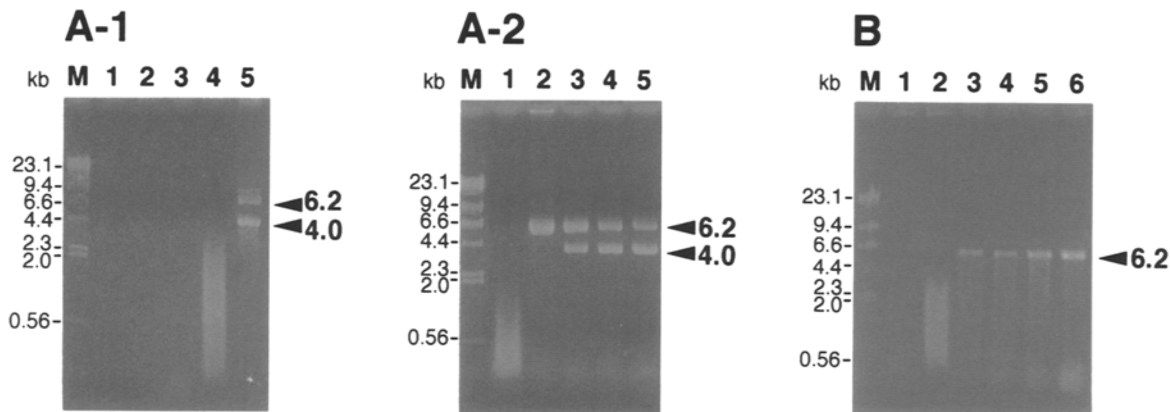

Fig. 3. Inhibition of nucleolytic activities.
A-1. Inhibition by EDTA of nucleolytic activities in CFE prepared from mycelia collected 48 h after the start of cultivation. pJL2 treated with CFE for 30 min without EDTA (lane 1) and with 0.5 mM (lane 2), 1 mM (lane 3), 2 mM (lane 4) and 5 mM (lane 5) EDTA was visualized with ethidium bromide. A-2. Inhibition by EDTA of nucleolytic activity in CFE prepared from a mixture of sporangiophores at stage I and sporangia at stage II-III, collected 72 h after the start of cultivation. pJL2 treated with CFE for 30 min without EDTA (lane 1) and with 0.5 mM (lane 2), 1 mM (lane 3), 2 mM (lane 4) and 5 mM (lane 5) EDTA was visualized with ethidium bromide. B. Inhibition by G-actin of nucleolytic activity in the same CFE as in A-1. pJL2 treated with CFE for 30 min without G-actin (lane 1) and with 0.063% (w/v) (lane 2), 0.125% (lane 3), 0.25% (lane 4), 0.5% (lane 5), and 1% (lane 6) of G-actin was visualized with ethidium bromide. Lane M shows DNA size markers (0.5 μg of *Hin*dIII-digested DNA).

blakesleeanus contains active deoxyribonuclease(s) (DNase).

We examined a mixture of sporangiophores at stage I and sporangia at stages II to III, collected 72 h after the start of cultivation. Fig. 3A-2 (lanes 2 through 5) shows that fragmentation of pJL2 was inhibited at concentrations of EDTA above 0.5 mM. Moreover, lane 3, 4 and 5, indicate that, as the concentration of EDTA increased, the amount of the linear form of pJL2 decreased while that of the CCC form increased. The inhibition by EDTA of the activity in the CFE prepared from the mixture of (D) and (E) was stronger than that of the activity in the CFE prepared from (C). Thus, the DNase activity in CFE prepared from mycelia was higher than that in CFE prepared from sporangiophores and sporangia.

G-actin is known as a specific inhibitor of DNase I (Lazarides and Lindberg, 1974). In preliminary experiments, we could not demonstrate dose-dependent inhibition by G-actin like that described above for EDTA. To identify the most suitable concentration of CFE on a protein basis to demonstrate such an effect, we incubated pJL2 with 0.125% (w/v) G-actin and various concentrations of CFE prepared from mycelia that had been collected 48 h after the start of cultivation. We determined that the most suitable concentration of CFE was 4 μg protein/ml (data not shown), and then examined the effect of various concentrations of G-actin. The results are shown in Fig. 3B. Specific inhibition by G-actin was observed at concentrations above 0.125% (w/v) G-actin (lanes 3 through 6) and the effect was dose-dependent, suggesting the presence of a DNase I in the CFE of *P. blakesleeanus*.

The nucleolytic activity, probably due to DNase I in the cytoplasm of *P. blakesleeanus*, might explain why, even if external DNA can be artificially inserted into cells, it might disappear within several generations. Our results might explain why it is very difficult to transform *P. blakesleeanus* efficiently. This is the first report, to our knowledge, of detection of DNase I in *P. blakesleeanus*. We are now trying to find ways to circumvent its probable effect and to transform *P. blakesleeanus*.

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