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**A simple method to isolate mutants in repressible cyclic phosphodiesterase**

in *Neurospora crassa*.

A simple isolation and detection method for mutants in repressible cyclic phosphodiesterase (cPDase) using a filtration-enrichment method with cyclic 3',5'-AMP as a sole source of phosphorus is described. The cPDase mutants showed no or limited growth in orthophosphate-free cyclic 3',5'-AMP media. Mutants in cPDase showed banding or reduced growth of submerged mycelia and produced no or limited amount of aerial hyphae in low phosphate (0.5 mM  $\text{KH}_2\text{PO}_4$ ) liquid media.

Orthophosphate repressible cyclic phosphodiesterase (cPDase) has been detected in the culture filtrate of wild-type (74A) mycelia grown in low phosphate media. It has hydrolyzing activities for cyclic 2',3'- and cyclic 3',5'-nucleotides and for p-nitrophenyl phosphate. cPDase was originally identified as a repressible acid phosphatase (Hasunuma 1933 J. Bacteriol. 156: 291-300). The activity of cPDase was also detected in extracts of mycelia and the enzyme was suggested to be involved in the formation of aerial hyphae and conidia in low phosphate liquid media (Hasunuma, submitted), since wild-type mycelia grown in low phosphate liquid media produced luxuriant aerial hyphae, and conidia, whereas, nuc-1 and nuc-2 mutants, which cannot derepress cPDase produced no or limited amounts of them

To explore the in vivo function of cPDase, mutants in cPDase were required. As a progenitor strain pho-2 (FGSC #3062 a) was used. Conidia from a 5 day-old culture on solid glycerol complete medium were suspended in sterilized water and the suspension was filtered with double clothed gauze. The cell concentration was adjusted to  $1 \times 10^7$  cells/ml (O.D. 660 nm = 1.0) and it was irradiated with UV with stirring in a petri dish for 5 min to a survival of 10%. Ten milliliters of the irradiated cell suspension was inoculated into 100 ml of orthophosphate-free cyclic 3',5'-AMP medium in a Sakaguchi flask (500 ml). Orthophosphate-free Fries minimal sucrose (1.5%) medium contained KCl in place of  $\text{KH}_2\text{PO}_4$  (Hasunuma 1983 J. Bacteriol. 156: 291-300) and to it 5 ml of filter sterilized 10mM cyclic 3',5'-AMP was added (final 0.5 mM). The pH of the media was adjusted to 6.8 with 0.1 M KOH to avoid hydrolysis of cyclic 3',5'-AMP by cPDase excreted into the culture medium (cPDase has no ability to hydrolyze cyclic 3',5'-AMP at pH 6.8 and is unstable at neutral to alkaline pH) At pH greater than 7.0, *N. crassa* shows a drastic reduction in growth rate. The culture ( $1 \times 10^6$  cells/ml) was shaken at 37° C and filtered every 12 h through double clothed gauze. This procedure was repeated for three days, then 5 ml of filter sterilized 10 mM cyclic 3',5'-AMP was added and shaking and filtration of the culture was continued for two additional days. At this stage, cell number was  $1 \times 10^8$  conidia/ml and the cells were plated in low phosphate (0.1 mM  $\text{KH}_2\text{PO}_4$ ) Fries minimal sorbose agar medium. The plates were incubated for 3 days at 25° C. Only small colonies should be isolated since filtration of the culture with double clothed gauze was insufficient to remove aggregated cells with long germ tubes.

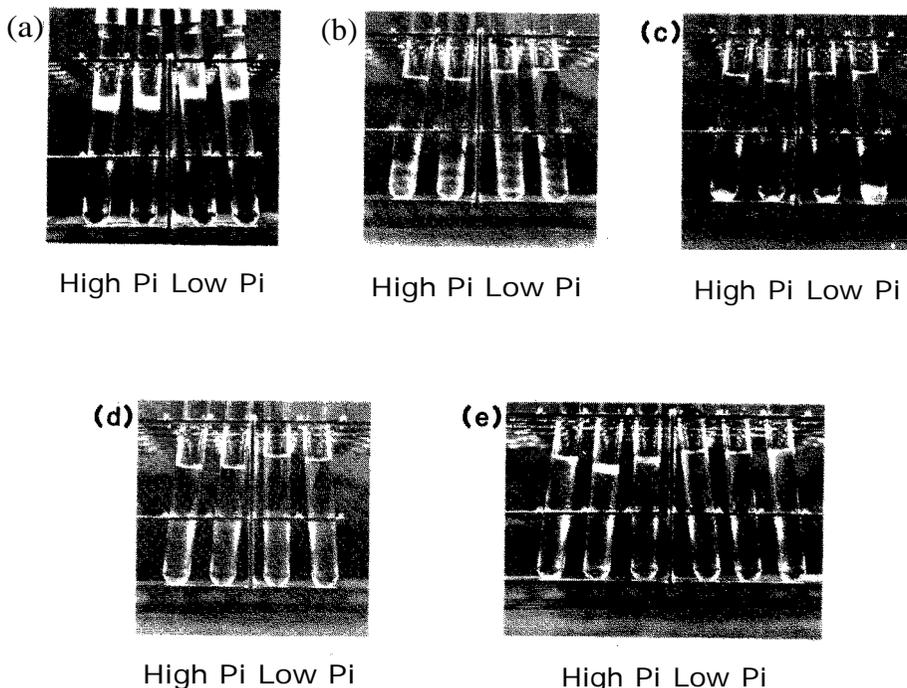


Figure 1. -- Typical growth patterns of cPDase mutants in high phosphate (7.35 mM  $\text{KH}_2\text{PO}_4$ ) and in low phosphate (0.5 mM  $\text{KH}_2\text{PO}_4$ ) liquid media. Conidial suspensions from various mutants were plated in sorbose agar medium and a single colony isolated with a pasteur pipette was inoculated into the bottom of high phosphate and low phosphate medium (5 ml) in test tubes (12x105 mm) and incubated at 25° C for 4 days. (a). Progenitor strain pho-2 (FGSC #3062 a) (b), (c), (d) and (e). cPDase mutants from various typical groups.

Colonies in agar blocks isolated by Pasteur pipettes were inoculated to the bottom of low phosphate (0.5 mM  $\text{KH}_2\text{PO}_4$ ) Fries minimal liquid medium and the isolates were incubated at 25° C for four days. Submerged mycelia of wild type grew up rapidly to the surface of liquid media and formed thick pads of surface mycelia and aerial hyphae (Fig. 1a). About 9% of the isolates showed banding growth of submerged mycelia as shown in Fig. 1b. All of these isolates were found to be mutant in cPDase and constitute at least two complementation groups, A and B. Most of these isolated showed rhythmic conidiation on glycerol complete medium. About 2% of the isolates showed very slow growth of submerged mycelia and formed a spherical mycelial pad at the bottom of the test tubes (Fig. 1c). About half of them were found to be mutants in cPDase and constituted a single complementation group (complementation group C). Other mutants (about 1%) in cPDase showed slow growth of submerged mycelia (Fig. 1d) and did not form aerial hyphae (Fig. 1e). These mutants were screened by direct assay of cPDase activity using p-nitrophenyl phosphate in the culture filtrate. The number of complementation groups has not yet been determined. All of the isolated mutants in cPDase (about 500 strains) formed no or limited amounts of aerial hyphae in low-phosphate liquid media. The growth characteristics of representative mutants and nuc-1, nuc-2 and pho-3 mutants in various ortho-phosphate-free nucleotide media are summarized in Table I. pho-3; al-2 (FGSC #3052 a) is a mutant in repressible acid phosphatase.

TABLE I

Growth characteristics and morphology of mycelia of typical mutants in cPDase.<sup>1)</sup>

Strain	Growth (mg fresh weight*) in media containing 0.25 mM					Morphology of mycelia <sup>3)</sup>
	$\text{KH}_2\text{PO}_4$	3'-AMP	5'-AMP	cyclic 2',3'-AMP	cyclic 3',5'-AMP	
FGSC#3062(pho-2)	142±16	111±5	105±5	140±9	75±5	(a)
74A	79±13	82±1	65±3	94±8	50±2	(a)
A1 ( <u>nuc-1</u> )	71±13	16±0	24±4	0.4±0	0	(e)
B1 ( <u>nuc-2</u> )	54±5	26±3	40±1	8±3	tr	(e)
FGSC#3052( <u>pho-3; al-2</u> )	111±7	110±3	84±1	115±9	7±1	(e)
<u>cpd</u> (1-3; A) <sup>4)</sup>	44±1	49±7	43±3	46±4	3±0	(b)
<u>cpd</u> (4-27; B)	79±1	67±4	57±2	77±6	7±2	(b)
<u>cpd</u> (3-2; C)	88±14	80±4	62±18	75±5	7±0	(c)
<u>cpd</u> (4-14)	75±7	60±6	50±4	67±1	6±1	(d)
<u>cpd</u> (4-4)	73±3	52±1	59±3	45±1	0	(e)

1) Conidial suspension (OD 660 nm = 0.1, 0.2 ml) was inoculated to 20 ml of culture media in 100 ml Erlenmeyer flask and incubated at 25° C for 5 days.

2) Mycelial pads were push dried with filter paper.

3) Morphology of mycelia in low phosphate liquid media from (a) to (e) corresponds to those presented in Figure 1, (a) to (e).

4) Mutants in cPDase (cpd) was isolated as double mutants, pho-2; cpd. Number in parentheses indicates isolation number. A, B and C indicate complementation groups.

Although cPDase hydrolyzes both cyclic 2', 3'-AMP and cyclic 3',5'-AMP, the cPDase mutants showed good growth in orthophosphate-free cyclic-2',3'-AMP media. This was explained by the finding that there existed repressible 2',3'-cyclic nucleotide phosphodiesterase activity in the culture media of wild-type mycelia grown in low-phosphate media with bubbling aeration (Shinohara and Hasunuma unpublished). These cPDase mutants are very unstable and readily produce suppressor mutations; therefore, they should be kept in a frozen-state. (This research was supported by Grant-in-Aid for Special Project Research from the Ministry of Education, Science and Culture of Japan (No. 58119002)). - - - National Institute for Basic Biology, 38 Nishigonaka, Myodaijicho Dkatakai, 444 Japan.