Involvement of Adenosine 3':5'-Cyclic Monophosphate in the Yeast–Mycelium Transition of *Aureobasidium pullulans*

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The yeast–mycelium transition of *Aureobasidium pullulans* (IMI 45533) was induced by adenosine and adenosine 5'-monophosphate (AMP) in defined liquid medium. During the yeast–mycelium transition uptake of adenosine or AMP and transformation to adenosine 3':5'-cyclic monophosphate (cAMP) occurred; intracellular cAMP levels increased and maximum levels coincided with maximum germination. This, coupled with the findings that the cAMP phosphodiesterase inhibitors theophylline and caffeine also induced germination, indicated the involvement of cAMP in the regulation of the yeast–mycelium transition.

INTRODUCTION

Aureobasidium pullulans de Bary (Arnaud) is polymorphic and has a life-cycle which can involve yeast-like cells, mycelium and melanin-pigmented chlamydospores together with various transitory forms (Ramos & Garcia Acha, 1975; Pechak & Crang, 1977; Gadd, 1980; Cooper & Gadd, 1984). The yeast–mycelium transition has been the subject of a number of studies, particularly in connection with the influence of nutritional components like nitrogen source on the transition (Ramos & Garcia Acha, 1975; Park, 1982*a, b*), but clear demonstrations of nutritional control are lacking. This is partly due to the complexity of the organism, variations in culture conditions (Park, 1982*a*) and marked strain differences (Gadd & Cooper, 1984).

Previous work has identified suitable strains of *A. pullulans* for morphological studies (Gadd & Cooper, 1984) and a complete yeast–mycelium transition which was induced by the presence of yeast extract in liquid medium has been described (Cooper & Gadd, 1984). However, the specific factor(s) responsible for triggering mycelium formation were not identified. Subsequent work in our laboratory implicated the involvement of adenylates; adenosine and AMP were found to be significant components of yeast extract and could themselves induce a yeast–mycelium transition. This paper describes the effects of these adenine nucleotides and their involvement in the yeast–mycelium transition of *A. pullulans*.

METHODS

Organism, media and growth conditions. *Aureobasidium pullulans* (IMI 45533) was routinely maintained on MYGP agar of composition (g l⁻¹): malt extract (Oxoid), 3; yeast extract (Difco), 3; bacteriological peptone (Oxoid), 5; D-glucose, 10; agar (Lab-M, no. 2), 12. For liquid cultures, a defined medium, pH 6.8, was used of composition (g l⁻¹): glucose, 20; (NH₄)₂SO₄, 2; KH₂PO₄, 2.72; K₂HPO₄, 5.22; MgSO₄·7H₂O, 0.12; FeSO₄·7H₂O, 0.0022; ZnSO₄·7H₂O, 0.004; MnSO₄·4H₂O, 0.004; CuSO₄·5H₂O, 0.0004. When required, filter-sterilized adenosine or AMP was added to final concentrations of 1 mM and 5 mM respectively. Starter cultures were prepared by loop-inoculating 50 ml liquid medium which was then incubated for 36 h at 25 °C on a rotary shaker (100 cycles min⁻¹). For experiments, 100 ml medium was inoculated from exponentially growing starter cultures to an initial OD₅₅₀ of 0.2 (approximately 10⁶ cells ml⁻¹) measured in a Pye Unicam SP600 series 2 spectrophotometer.

Extraction of adenylates and thin layer chromatography. Cell samples (200 µl) were taken at intervals and separated from the medium by microcentrifugation (7000 g; 20 s) through 0.5 ml of a 'dinonyl' phthalate/silicone

fluid mixture (3:2, v/v) into 240 μ l 0.3M-perchloric acid. The perchloric acid layer was withdrawn and after the precipitate had been removed by centrifugation (7000 g, 20 s), 100 μ l of the supernatant was added to 40 μ l 0.5 M- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.2, and 4 μ l 5.0M- Na_2CO_3 for neutralization. Extracts were immediately frozen and maintained at -20°C . The adenylates, i.e. adenine, adenosine, cAMP and AMP, were separated using pre-coated, reverse phase TLC plates containing a fluorescent indicator (RP-8F_{254S}, Merck). Cell extract (20 μ l) was applied to the origin and eluted in an acetonitrile/water mixture (20:80, v/v) for 15 min. A standard solution containing the adenylates was used to identify the position of each compound on the plate and spots were detected under UV light (254 nm).

Cells were radiolabelled by growth in medium containing 0.02 μCi (0.74 kBq) [^{14}C]adenosine ml^{-1} or 0.1 μCi (3.7 kBq) [^{14}C]AMP ml^{-1} . Cell samples (200 μ l) were taken at intervals, separated from the medium, extracted and separated by TLC. Each application of extract contained approximately 2.5×10^{-5} μCi (9.25×10^{-4} kBq). The radioactivity of each fraction was determined by scraping the plate coating from the area corresponding to each adenylate into scintillation fluid based on toluene and Triton X-100 (2:1, v/v) and containing PPO (4 g l^{-1}) and POPOP (0.1 g l^{-1}) and counting with a Packard 300 CD scintillation counter.

cAMP assay. cAMP in extracts was assayed with a cAMP-binding assay kit (Amersham). cAMP in growth medium was estimated by centrifuging culture samples and assaying the supernatant.

Other methods. Cells were counted with a modified Fuchs-Rosenthal haemocytometer. Dry weights of washed cell pellets, obtained by centrifugation (2000 g, 5 min), were determined by means of tared aluminium foil cups dried to constant weight at 105°C .

Chemicals. All chemicals used were of analytical grade. Adenylates were obtained from Sigma. 'Dinonyl' phthalate[bis(3,5,5-trimethylhexyl)phthalate] (Fluka) was obtained from Fluorochem, Glossop, Derbyshire, UK, and Dow Corning 550 silicone fluid from Hopkin & Williams, Chadwell Heath, Essex, UK.

RESULTS

When *A. pullulans* was grown in the defined liquid medium, growth was predominantly yeast-like (95–98% of total cells), the only other types of cell present being swollen cells in low proportions (Gadd, 1980) (Fig. 1a). However, the addition of 1 mM-adenosine to the growth medium caused a change in cell morphology; after 2.5 h some of the yeast-like cells and swollen cells showed germ-tube formation. As growth proceeded the number of germinating cells and the length of germ-tubes increased and after 10 h, 97% of the cells had germinated (Fig. 1b). This was followed by mycelium formation, the mycelium itself giving rise to more yeast cells by budding. After approximately 20 h the mycelium fragmented into swollen cells and shorter filaments. The lowest concentration of adenosine which could induce germination was 0.05 mM. Concentrations in excess of 1 mM resulted in a weaker response. The presence of AMP in the medium induced a similar morphological change in the cells but to a lesser extent. At the optimum concentration for mycelial development (5 mM) a maximum of 56% (of total cells) showed germ-tube formation after 9 h (Fig. 1c). Subsequent mycelium production was reduced and fragmentation of mycelium into swollen cells and shorter filaments occurred after approximately 18 h. When adenine was present in the medium, a weak germination response was observed, a maximum of 35% cell germination occurring at 1 mM but with no subsequent mycelium formation.

To examine the intracellular fate of adenosine and AMP, the incorporation of [^{14}C]adenosine and [^{14}C]AMP was followed by TLC. The percentage (of total intracellular radioactivity) of ^{14}C label in each intracellular adenylate pool at various times through growth in [^{14}C]adenosine is shown in Fig. 2. After 1 h the ^{14}C was predominantly in the adenosine pool, with small amounts in the AMP and cAMP pools after 2 h. As growth proceeded the percentage of ^{14}C in the cAMP pool increased to a maximum of 86% at 10 h. It therefore appeared that the adenosine was taken up by the cells and converted to cAMP, via AMP.

Similar results were obtained when [^{14}C]AMP was supplied to the cells, the ^{14}C first appeared in the AMP pool and then moved to the cAMP pool (results not shown). However, the results were less pronounced than those obtained with [^{14}C]adenosine, which is not surprising in view of the fact that AMP was much less effective as an inducer of cell germination.

Exogenously supplied cAMP and dibutyryl cAMP failed to induce germination at all concentrations tested (1–100 μM and 0.5–50 μM respectively) although impermeability of cell to these compounds is well known (Uno & Ishikawa, 1973). In fact, when the cAMP

Fig. 1. Growth of *A. pullulans* in defined liquid medium. (a) Unsupplemented; (b) with 1 mM-adenosine; (c) with 5 mM-AMP. Typical results from one of five experiments are shown. ○, Yeast-like cells; ●, swollen cells; □, germ-tubes.

Fig. 2

Fig. 2. Proportions of ^{14}C in adenylate pools during the yeast-mycelium transition of *A. pullulans* in 1 mM- ^{14}C adenosine. Typical results from one of three experiments are shown. ○, Origin; ●, adenosine; □, AMP; ■, cAMP.

Fig. 3

Fig. 3. Effect of theophylline and caffeine on germination of *A. pullulans*. Bars indicate standard errors; ○, 0.1-1.0 mM-theophylline; ●, 0.5-1.0 mM-caffeine; □, 0.1 mM-caffeine.

concentration in the medium was measured throughout growth no uptake by the cells was detected. (Neither ATP nor ADP stimulated a yeast-mycelium transition at concentrations up to 5 mM.) However, the inhibitors of cAMP phosphodiesterase, theophylline and caffeine, which were added to the growth medium at concentrations which did not inhibit growth, did have some effects. Theophylline (0.1-1.0 mM) induced germination in a small number of cells (5%) but no mycelium formation occurred (Fig. 3). When 0.1 mM-caffeine was present in the medium, 35% of the cells had produced germ-tubes after 9 h but no further germination or mycelium production occurred. Higher concentrations of caffeine were less effective as inducers of germination.

Intracellular cAMP concentration was measured in yeast-like cells and in germinating cells after exposure to various inducers of germination (Fig. 4). In yeast-like cultures, cAMP showed

Fig. 4. Intracellular cAMP concentration during yeast-like growth and during the yeast-mycelium transition of *A. pullulans* in response to various germination inducers. ○, Unsupplemented medium; ●, 5 mM-AMP; □, 1 mM-adenosine; ■, 1.0% (w/v) yeast extract; △, 0.1 mM-caffeine.

a small decrease over 6 h growth from 32 pmol (mg dry wt)⁻¹ to 26 pmol (mg dry wt)⁻¹. In all cells growing in the presence of an inducer of germination there was an increase in cAMP, which appeared to be directly related to the extent of the yeast-mycelium response. This increase was greatest in cells growing in the presence of 1% (w/v) yeast extract. The cAMP concentration increased immediately and reached a maximum concentration of 79 pmol (mg dry wt)⁻¹ at 3 h, after which it decreased. In the presence of adenosine (1 mM) and AMP (5 mM) maximum cAMP concentrations were also seen at 3 h [69 and 59 pmol (mg dry wt)⁻¹ respectively]. A much smaller, but still obvious, increase occurred in the presence of 0.1 mM-caffeine.

DISCUSSION

Previous work has described a yeast-mycelium transition in *A. pullulans* that was induced by the presence of yeast extract in an otherwise defined liquid medium (Cooper & Gadd, 1984). While this was useful for morphological and kinetic observations, our finding that adenosine and AMP were significant components of the yeast extract and could induce a yeast-mycelium transition have enabled further evaluation of some of the intracellular changes that occur during the transition. Clearly, a defined medium is better for this purpose. Adenosine was better than AMP as an inducer of germination, but both were taken up by cells and metabolized to cAMP. Thus, intracellular cAMP concentrations increased during the yeast-mycelium transition and maximum levels coincided with maximum germination. This, coupled with the findings that inhibitors of cAMP phosphodiesterase also induced cell germination, suggested a possible role for cAMP in the regulation of germination.

The involvement of cAMP in the differentiation of some other fungi has been described. The yeast-mycelium transition of *Candida albicans*, induced by incubation at 40 °C, was accompanied by an increase in intracellular cAMP, and dibutyryl cAMP promoted germination at a non-inducing temperature (Niimi *et al.*, 1980; Chattaway *et al.*, 1981). In *Histoplasma capsulatum* the temperature-induced yeast-mycelium transition was accompanied by an increase in intracellular and extracellular cAMP levels, the reverse occurring during the mycelium-yeast transition (Medoff *et al.*, 1981). In contrast to these organisms, *Mucor* sp. showed a correlation between higher levels of cAMP and yeast-like growth with a decrease in endogenous cAMP during the yeast-mycelium transition (Orlowski, 1980).

However, the inference that a specific growth response is mediated by cAMP is fraught with difficulty. Although several of the criteria proposed by Pall (1981) are satisfied in this study, the use of mutants is not yet feasible with *A. pullulans* nor is the use of an appropriate cell-free system. A variety of roles are fulfilled by cAMP in cellular processes, including control of glycolysis (Pall, 1981), and whether cAMP involvement in germination is a direct effect or an indirect result of some other process cannot be discerned at this stage.

Nevertheless, our results are interesting in that they demonstrate that morphogenesis of *A. pullulans* can be manipulated under defined conditions. That this can be done in liquid medium facilitates harvesting of adequate biomass for biochemical analyses. As far as we know, stimulation of a yeast-mycelium transition in fungi by exogenous adenosine or AMP has not been reported and it would be of interest to know whether other polymorphic fungi respond similarly.

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