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Adenylyl cyclase activity of the fission yeast *Schizosaccharomyces pombe* is not regulated by guanyl nucleotides

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The adenylyl cyclase activity of the fission yeast *Schizosaccharomyces pombe* is localized to the plasma membrane of the cell. The enzyme utilizes Mn^{2+}/ATP as substrate and free Mn^{2+} ions as an effector. Unlike the baker yeast *Saccharomyces cerevisiae*, *S. pombe* adenylyl cyclase does not utilize Mg^{2+}/ATP as substrate and the activity is not stimulated by guanyl nucleotides. The optimal pH for the *S. pombe* adenylyl cyclase activity is 6.0. The activity dependence on ATP is cooperative with a Hill coefficient of 1.68 ± 0.14.

Adenylyl cyclase; *Schizosaccharomyces pombe*; Yeast

1. INTRODUCTION

cAMP plays a regulatory role in eucaryotes as well as in procaryotes and in both cases its intracellular level is determined by extracellular signalling molecules. In mammalian cells these signalling molecules are hormones and neurotransmitters interacting with specific receptors which activate or inhibit adenylyl cyclase (EC 4.6.1.1) through heterotrimeric G_s and G_i, respectively [1,2].

In bacteria, adenylyl cyclase activity is regulated by sugars via the sugar transport system and the cAMP receptor protein [3–5]. There is no evidence for the involvement of a G-protein in the regulation of the enzyme. Little is known on adenylyl cyclase in lower eucaryotes. It has been reported that adenylyl cyclase activity of the fungi *Neurospora crassa* [6,7], *Mucor rouxii* [8] and of the pathogenic protozoon *Trypanosoma cruzi* [9,10] utilizes Mn^{2+}/ATP as substrate and free Mn^{2+} ions as an effector. Some reports, however, suggest that guanyl nucleotide-sensitive adenylyl cyclase which utilizes Mg^{2+}/ATP, does exist in fungi, but is unstable [11]. A low activity level of such adenylyl cyclase could be measured in *Neurospora crassa* when special care was taken in lysate preparation and assay conditions [11].

Measuring in vitro the adenylyl cyclase activity in the social amoeba *Dictyostelium discoideum* is problematic technically because cell lysates lose activity shortly after preparation probably due to enzyme sequestration [12]. Carefully prepared cell lysates show adenylyl cyclase activity which is stimulated by GTP or its analogs [13–15]. It seems that G-proteins are involved in signal transduction in *Dictyostelium discoideum* by regulating adenylyl cyclase and phospholipase C [16].

Several features of the adenylyl cyclase system in the baker yeast *Saccharomyces cerevisiae* are analogous to those of avian and mammalian systems: (i) *S. cerevisiae* adenylyl cyclase seems to be regulated by an external glucose signal [17]; (ii) the activity is regulated by a monomeric G-protein of the RAS family [18], where the GDP to GTP exchange is catalyzed by the CDC25 protein [19–22]; and (iii) activation of the enzyme exhibits first-order kinetics similar to the pattern of activation of the avian and mammalian enzymes [22]. Yet, there are major differences between the avian and mammalian systems and the *S. cerevisiae* system: (i) the *S. cerevisiae* adenylyl cyclase is not activated by agents that activate avian and mammalian systems such as fluoride ions and forskolin [23]; (ii) the G-protein which regulates the *S. cerevisiae* adenylyl cyclase activation is a monomer highly homologous to the mammalian protooncogene *ras* [18] which is completely different from the heterotrimeric G-proteins; (iii) no inhibitory G-protein has been found yet in *S. cerevisiae*; and (iv) this enzyme system is not sensitive to cholera toxin or pertussis toxin [23].

The gene coding for bovine brain adenylyl cyclase shows no significant homology to bacteria or *S. cerevisiae* adenylyl cyclases [24].

In this communication we describe the adenylyl cyclase activity of the yeast *Schizosaccharomyces pombe*, an organism that can be easily manipulated genetically and studied biochemically. *S. pombe* serves as a major model organism in the study of cell cycle [25,26], gene expression [27] and meiosis [28]. *S.*

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Table 1

Adenylyl cyclase activity in *S. pombe* strains

Strain	Growth phase	Cell fraction[a]	Specific activity[b] (pmol cAMP/mg prot./min)	Total activity (pmol cAMP/min)
972 leu1-32	logarithmic	lysate	6.0 ± 1.3	246 ± 23
		membranes	15.0 ± 2.0	154 ± 11
		soluble fraction	0.3 ± 0.1	9.7 ± 1.5
975h$^+$	logarithmic	lysate	5.0 ± 0.8	300 ± 25
		membranes	32.0 ± 1.3	512 ± 62
		soluble fraction	1.0 ± 0.3	43 ± 10
975h$^+$	stationary	lysate	0.5 ± 0.1	30 ± 2
		membranes	4.0 ± 0.65	32 ± 2.7
CBS356	logarithmic	lysate	0.6 ± 0.2	25 ± 4
		membranes	6.0 ± 0.75	11 ± 3.6
0202	logarithmic	membranes	8.0 ± 1.0	48 ± 5
0209	logarithmic	membranes	7.0 ± 0.07	22 ± 3.5
CSIRY 467	logarithmic	membranes	10.0 ± 2.0	61 ± 8

[a] Cell lysate refers to the supernatant of 1000 × *g* centrifugation at 4°C for 10 min of crude extract preparation. Membrane fraction refers to the pellet of 100000 × *g* centrifugation at 4°C for 30 min of cell lysate and soluble fraction refers to the supernatant of the 100000 × *g* centrifugation

[b] Lysates and membranes were assayed in the presence of 1.3 mM ATP and 4.0 mM $Mn(acetate)_2$. The results are shown as the mean ± SE of at least 3 independent experiments. These fractions were also assayed in the presence of $Mg(acetate)_2$ (1–15 mM), or $Mg(acetate)_2$ plus GPPNHP or GTPγS (50–250 μM), or $CaCl_2$ (10–150 μM plus or minus EGTA), or forskolin (50–150 μM), or NaF (0.2–10 mM). No activity was measured under any of these conditions

pombe proteins involved in these processes are highly homologous to genes involved in the same processes in higher eucaryotes including human [29,30]. Furthermore, the structure of unprocessed mRNA molecules [31], the U2 small nuclear RNA [32] and chromosome replication [33] in *S. pombe* resemble these properties in higher eucaryotes. On the other hand certain genes such as *URA3/ura4*$^+$ [34] were found to be functional interchangeable between *S. cerevisiae* and *S. pombe* and the cell cycle, control gene of *S. cerevisiae, CDC28*, is active in *S. pombe* [35].

The gene coding for *S. pombe* adenylyl cyclase has been cloned recently [36,37] and found to be 60% homologous to the *S. cerevisiae* adenylyl cyclase gene (CDC35; CYR1). However, 620 residues in the NH_2-terminus and 140 residues in the COOH-terminus of the *S. cerevisiae* CDC35 gene are not found in the *S. pombe* adenylyl cyclase gene. It was shown that the *S. pombe* adenylyl cyclase is not activated by RAS2 as is the *S. cerevisiae* adenylyl cyclase when its COOH-terminus is deleted [36]. It was therefore of interest to further examine to which group of cyclases the enzyme of *S. pombe* belongs: to fungi such as *Neurospora crassa* and *Mucor rouxii*, *S. cerevisiae*, or higher eucaryotes.

2. MATERIALS AND METHODS

2.1. *Yeast strains and growing conditions*

S. pombe strains used were isolated independently from each other and are described in detail by Zimmer et al. [38]. Briefly, strain 972 leu1-32h$^-$ was isolated from grape juice in Switzerland and together with strain 975h$^+$ is a parent of mutants comprising the 'Leupold-collection'. Strain 0209 was isolated from red currant jelly, strain CSIRY 467 was isolated from young wine in South Africa and strain CBS356 was isolated in Czechoslovakia.

All strains were grown on YEPD media (1% yeast extract, 2% peptone and 2% glucose). Cultures were grown under vigorous shaking at 30°C.

2.2. *Lysates and membrane preparations*

Yeast cultures were grown in a volume of 1 l to a concentration of 1–2 × 10^7 cells/ml (or to 3 × 10^8 cells/ml as stationary phase) and then crude extracts were prepared as described by Casperson et al.

Table 2

Adenylyl cyclase activity in *S. pombe* permeabilized cells[a]

Assay conditions	Activity (pmol cAMP/10^8 cells/min)
0.8 mM $Mn(acetate)_2$	0.3
2.5 mM $Mn(acetate)_2$	4.0
5.0 mM $Mn(acetate)_2$	4.5
3.0 mM $Mg(acetate)_2$	0
6.0 mM $Mg(acetate)_2$	0
18.0 mM $Mg(acetate)_2$	0
7.0 mM $Mg(acetate)_2$ plus:	
65 μM GPPNHP	0
100 μM GPPNHP	0
270 μM GPPNHP	0
20 μM $CaCl_2$	0
40 μM $CaCl_2$	0
80 μM $CaCl_2$	0

[a] See section 2 for permeabilization procedure. Strain used was 975h$^+$

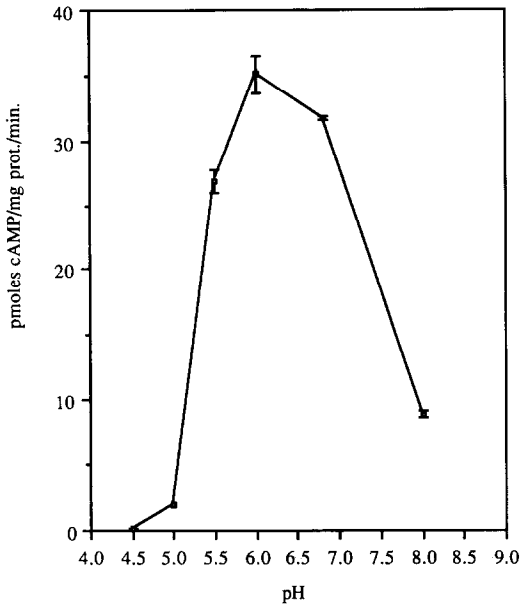

Fig.1. pH profile of *S. pombe* adenylyl cyclase activity. Activity was measured as described in section 2 in the presence of 1.3 mM ATP and 4.0 mM Mn(acetate)$_2$. The experiment was performed in triplicate on membranes of strain 975h$^+$ and the graph shows the experimental points as the mean ± SD.

[23] for the preparation of crude extracts from *S. cerevisiae* cells. Cells were collected by centrifugation and washed with double-distilled water and then with 0.9 M sorbitol (Merck). Cells were incubated in 20 ml 0.9 M sorbitol plus 0.5 ml Glusulase (purchased from New England Nuclear; Glusulase is prepared from the intestinal juice of the snail *Helix pomatia* and contains a mixture of enzymes including β-glucuronidase, sulfatase and a cellulase) at 30°C for 1 h to digest the cell wall. Spheroplasts were washed with 0.8 M sorbitol, 1 mM $CaCl_2$, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM $MnCl_2$ and 50 mM Hepes, pH 7.0 (buffer B) and resuspended in 5.0 ml buffer B plus 15 ml of 50 mM Mes, pH 6, 0.1 mM EDTA, 0.1 mM $MgCl_2$ and 1 mM PMSF (buffer C). Spheroplasts were then broken usually with a dounce homogenizer (5 strokes, tight fit). We also tried to break the spheroplasts using cavitation bomb (30 s under 500 p.s.i. nitrogen) and found no differences in the adenylyl cyclase activity obtained by the two methods. The crude extracts were stored at −70°C until use. The frozen lysates were found to be stable for at least 3 months. The lysate used was the supernatant of 200 × *g* centrifugation for 10 min at 4°C of the crude preparation. Membranes used were obtained by centrifuging the lysates at 100000 × *g* for 30 min at 4°C and were then resuspended in buffer C to a final concentration of 2–3 mg/ml.

2.3. *Cell permeabilization procedure*

Yeast cultures were grown, collected and washed as described for lysate preparation. Cells were resuspended in 0.9 M sorbitol plus 0.5 ml Glusulase to digest the cell wall and incubated at 30°C for 15 min. The reaction was stopped by transferring the cells to 0°C. Spheroplasts were collected by centrifugation (1000 × *g* for 5 min at 4°C), resuspended in ice-cold 0.9 M sorbitol and added without further treatments to adenylyl cyclase reaction mixture. After this treatment cell viability was found to be 80–100% even after exposing the spheroplasts to the adenylyl cyclase assay conditions (see below).

2.4. *Adenylyl cyclase assay*

Adenylyl cyclase activity was assayed as described by Casperson et al. [23] and [^{32}P]cAMP produced was determined as described by Salomon et al. [39]. The reaction mixture contained 50–70 μg protein (or 2 × 10^7 permeabilized spheroplasts) in 50 mM Mes, pH 6.0, 0.1 mM EGTA, 20 mM creatine phosphate, 20 units/ml of creatine phosphokinase, 2.0 mM 2-mercaptoethanol, 1.3 mM [α-^{32}P]ATP (15–25 cpm/pmol) (Amersham) and 1.0 mM [^{3}H]cAMP (12000 cpm) (Amersham) in a final volume of 150 μl. Ions, nucleotides and hormones were added as described in the figure legends and in the table footnotes. Reaction time was 60 min. The reaction was stopped by addition of 10 × stopping solution (2% SDS, 1 mM cAMP and 12 mM ATP), boiling for 3 min and addition of

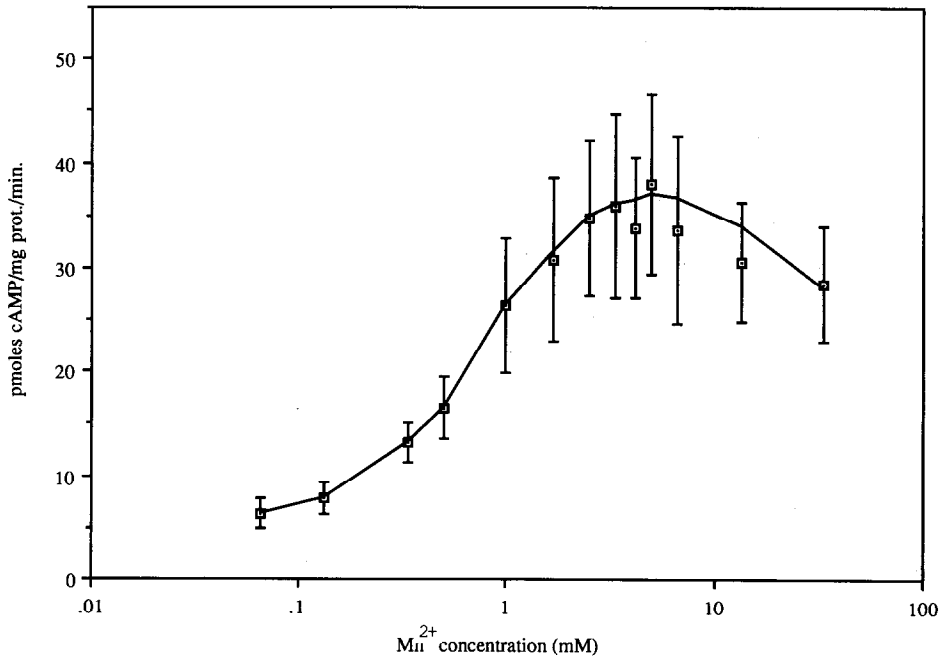

Fig.2. Mn^{2+} dependence of adenylyl cyclase activity. Activity was measured in the presence of 1.3 mM ATP and 1.3 mM Mn(acetate)$_2$. The abscissa depicts the level of extra Mn(acetate)$_2$ over the 1.3 mM MnATP in the assay. The graph shows the experimental points as the mean ± SE of 3 independent experiments performed on membranes of strain 975h$^+$.

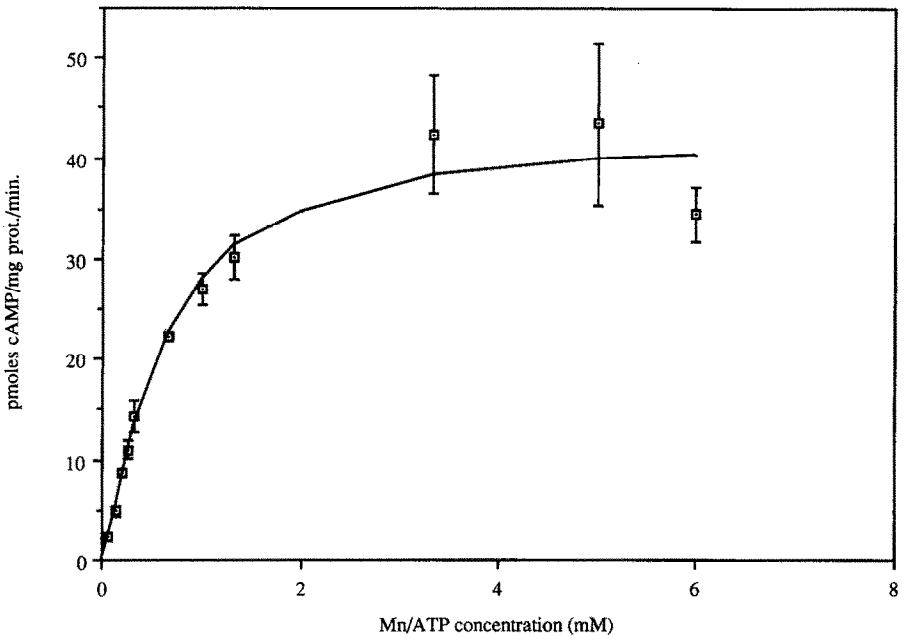

Fig.3. ATP concentration dependence of adenylyl cyclase activity. Activity was measured in the presence of 4.0 mM Mn(acetate)$_2$ as a function of ATP added with stoichiometric amounts of Mn^{2+}. The graph shows the experimental points as the mean ± SE of 3 independent experiments performed on membranes of strain 975h$^+$. The line shows computer calculated curve that best fit the experimental results and found to show cooperative behaviour (Hill coefficient n_H = 1.68 ± 0.14).

1 ml ice-cold double-distilled water. To measure the activity at different pH values the buffers used were as follows: sodium acetate pH 4.5–5.0, Mes pH 5.5–6.5 and Tris-HCl pH 6.8–8.

3. RESULTS

Adenylyl cyclase activity was measured in whole cell lysates and in membranes prepared from several, independently isolated, *S. pombe* strains. Table 1 shows the results obtained when free Mn^{2+} ions were present in the reaction mixture. In the presence of either free Mg^{2+} ions, Ca^{2+} ions, fluoride ions, forskolin or EGTA, at various combinations and concentrations no activity was detectable in cell lysates or membranes. Mg^{2+}/ATP could not serve as a substrate for the *S. pombe* enzyme. The nucleotide GTP or its analogs GPPNHP and GTPγS do not stimulate the activity. Table 1 also shows that most of the activity measured in whole cell lysates is localized to the membrane fraction and only 5–10% remains in the 100000 × *g* supernatant.

The absence of guanyl nucleotide sensitivity of the cyclase activity could be due to an uncoupling event caused by the preparation procedure. In order to test this possibility we measured adenylyl cyclase activity in permeabilized *S. pombe* cells. We found that mild treatment with Glusulase permeabilizes the cells, enabling molecules such as ATP and GPPNHP to enter and the cells which remain fully viable (see section 2). In permeabilized cells as in lysates and membrane preparations, adenylyl cyclase activity was measurable only in the presence of free Mn^{2+} ions (table 2) and no activity was detected with Mg^{2+}, Mg^{2+}/GPPNHP, or added Ca^{2+}. The total Mn^{2+}-dependent activity measured in permeabilized cells was 28% of the activity measured in membranes prepared from the same number of cells. It is possible that only 28% of the cells undergo full permeabilization by our Glusulase treatment.

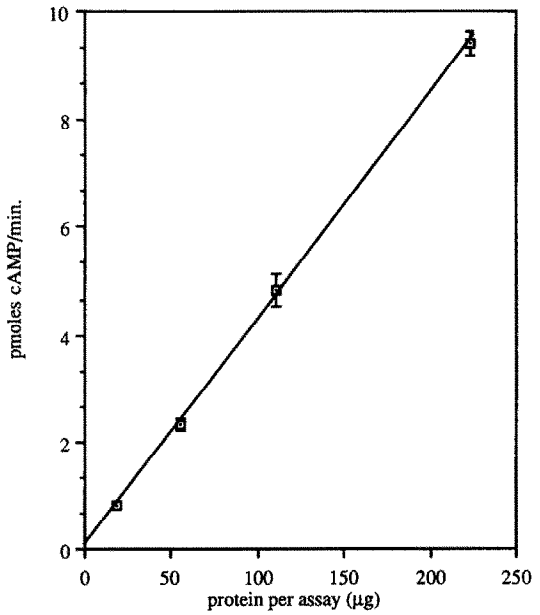

Fig.4. Adenylyl cyclase activity as a function of protein concentration. The experiment was performed in triplicates and the experimental points are shown as the mean ± SD.

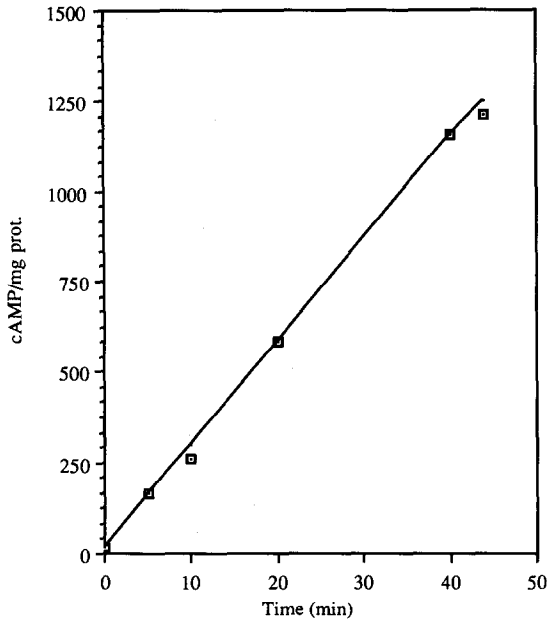

Fig.5. Time dependence of adenylyl cyclase activity. Assay was performed in a volume of 1.2 ml and at the time points shown in the graph, aliquots of 150 μl were removed into 100 μl of 10 × stop solution and further treated normally as described in section 2.

The pH-activity profile is shown in fig.1 and Mn^{2+} dependence of enzyme activity in fig.2. The ATP concentration dependence exhibits a cooperative pattern (fig.3) with a Hill coefficient of 1.68 ± 0.14. The activity is linear with respect to protein concentration from 18 μg to 225 μg protein per assay (fig.4) and with respect to time up to at least 45 min (fig.5).

4. DISCUSSION

Adenylyl cyclase activity of *S. pombe* is localized to the membrane fraction of the cell and utilizes only Mn^{2+}/ATP as substrate when assayed in vitro. Free Mn^{2+} ions are the only effector we could use to activate the enzyme. In light of the reports that GTP-sensitive adenylyl cyclase activity in *Neurospora crassa* and *Dictyostelium discoideum* were measured only after certain precautions were taken during lysate preparations [11,13], we tried to prepare our lysates under different conditions and yet only when free Mn^{2+} ions were present, was activity measurable. Furthermore, the results obtained with cells which were permeabilized under mild conditions reduce the likelihood that a coupling protein factor was lost during the preparation of cell lysates. It is, however, possible that some mediator molecules are present or active only during a specific stage of the cell cycle or the cell life. Since we have studied adenylyl cyclase activity in membranes prepared from cells in either logarithmic or stationary phase of the culture (table 1) and since the logarithmic culture contains cells in all stages of the cell cycle this possibility also seems remote. The role of cAMP in *S. pombe* is not known and its level does not change drastically during cell cycle or starvation [40].

The properties of adenylyl cyclase activity of *S. pombe* reported here resemble the properties of adenylyl cyclase from other fungi such as *Neurospora crassa* [6,7] and *Mucor rouxii* [8] and the protozoon *Trypanosoma cruzi* [9], but are totally different from adenylyl cyclase of another yeast, namely the *S. cerevisiae*. These results are in accordance with those described by Yamawaki-Kataoka et al. [36] who showed that purified RAS2 protein was not able to activate *S. pombe* adenylyl cyclase. *S. cerevisiae* is thus far the only unicellular organism whose adenylyl cyclase was shown to be regulated by a G-protein that was fully characterized and was found to be a member of the RAS family [18]. In some aspects *S. pombe* is closer to mammalian cells than to *S. cerevisiae* [29–33], but it seems that its adenylyl cyclase system is unique and resembles neither.

Although from the point of view of classical systematics *S. cerevisiae* and *S. pombe* belong to the same yeast family (the *Saccharomyceteae* [41]), molecular studies suggest that they are not related at all. Their 5 S RNA genes, for example, are very different and suggest a divergence 1200 million years ago [42,43].

Another puzzle is what is the biochemical meaning of Mn^{2+} activation of the enzyme since intracellular Mn^{2+} concentrations are well below those found to activate adenylyl cyclase.

Evidently, a great deal of study is necessary to reveal the mechanism of the regulation of *S. pombe* adenylyl cyclase and the role of cAMP in this important organism.

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