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Questionable Plasmid Involvement in *Streptomyces* ATP Nucleotide 3'-Pyrophosphokinase Formation

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A few actinomycetes produce ATP nucleotide 3′-pyrophosphokinase, a mechanistic stringent factor analog with broad acceptor spectrum. The plasmid-like DNA of an ATP nucleotide 3′-pyrophosphokinase producer Streptomyces *violascens* was more persistent than the enzyme production after acriflavin treatment. A 7.1Kbp long ATP nucleotide 3′-pyrophosphokinase gene-containing fragment, cloned from another producer *Streptomyces moro-okaensis,* was found to hybridize to both its *Xba* I- and *Spe* I-digested genomic DNA at about 400Kbp size, and also with the undigested DNA very close to or at the origin on pulsed field gel electrophoresis-Southern blotting analyses. The gene thus appears to reside not on a plasmid, but on the chromosome.

INTRODUCTION

We have shown that ATP nucleotide 3'-pyrophosphokinase (EC 2.7.6.4) produced by several actinomycetes has a novel mode of action, transferring 5′-β,γ-pyrophosphoryl group of either ATP or dATP to a variety of both purine and pyrimidine, and ribo- and deoxyribonucleotides and their derivatives including nucleotidic coenzymes and nucleic acids at their 3'-OH site (Mukai et al., 1978). The products, as exemplified by ppGpp (below), pppApp (sporulation factor) (Kameda et al., 1983) and 3′-pyrophospho CoA (2∼6-fold enhanced cofactor and allosteric effector activity) (Mukai and Noguchi, 1988b), exhibit various interesting actions (Mukai, 1988a). This broad acceptor spectrum contrasts those of *E. coli* and other bacterial stringent factors which catalyze the same pyrophosphate transfer with but a strict base specificity from ATP to only GD(T)P to synthesize (p)ppGpp, the pleiotropic signal molecule(s) for stringent control (Gallant, 1979). Nishino and Murao succeeded in curing *Streptomyces moro-okaensis* (IFO 13416) of pyrophosphokinase production with acriflavin, and consequently conjectured possible plasmid involvement therein (Nishino and Murao, 1981). They and we, interested in the possible regulatory function(s) of the enzyme, different from the stringent control as recently disclosed in our laboratory (Mukai *et al.*, 1989), searched for the plasmid that might possibly carry the gene encoding the enzyme, but its various attempts tried have failed thus far in detecting a plasmid-like DNA band distinct from chromosomal DNA.

MATERIALS AND METHODS

Bacterial strains and media

Streptomyces moro-okaensis (IFO 13416), S. *violascens* (IFO 12920), *Streptoverticillium septatum* (IFO 13471), *Streptoverticillium hachijoense* (IFO 12782), S. *aspergilloides* (IFO 13461) and S. *adephospholyticus* (IFO 13811) were purchased from IFO (Institute for Fermentation Osaka). GP medium consisted of 20 g glycerol, 20 g polypeptone, 1 g KH_2PO_4, 0.4 g $MgSO_47H_2O$, 10 mg $FeSO_47H_2O$ and 10 mg $MnSO_4nH_2O$ in 1liter of distilled water, and was adjusted to pH 7.2 with NaOH.

DNA preparation

The plasmid DNAs were extracted and fractionated by rapid alkaline SDS-phenol procedure (Kieser, 1984). The whole cellular DNAs were extracted and deproteinated with SDS and phenol, and then precipitated with 2-propanol (Hunter, 1985).

Acriflavin treatment

Sequential culture was curried out in initially 2.5 and then 5.0 μg/ml acriflavin-containing GP media essentially according to Nishino and Murao (Nishino and Murao, 1981).

Detection of ATP nucleotide 3'-pyrophosphokinase activity

The 3'-terminal phosphate of pppApp is very labile in acid, thus this nucleotide could be estimated as inorganic phosphate as follows. The reaction mixture contained 125 mM glycine-NaOH buffer, pH10, 6.25 mM ATP, 6.25 mM $MgCl_2$ and culture filtrate in a final volume of 255 μl. After incubation at 37°C for 20min, the reaction was terminated by addition of 250 μl of 5 % perchloric acid, and chilled on ice. Inorganic phosphate was determined with amidol reagent. The estimations of the enzyme production were based on the enzyme units (Sumichika and Mukai, 1989) per volume of the culture filtrate 48hrs after shaking in GP media at 30°C.

Pulsed field electrophoresis

The insert sample preparation for pulsed field electrophoresis was essentially the same as Kinashi (Kinashi *et al., 1987). S. moro-okaensis* ATP nucleotide 3'-pyrophosphokinase producer (wild) and non-producer (206B, unpublished data) cells were protoplasted with lysozyme and achromopeptidase, essentially according to Shirahama (Shirahama *et al.*, 1981). The protoplasts were embedded in agarose gel piece (3--6X 10^7 protoplasts), deproteinated with pronase and SDS, and then, after buffer replacement by 10 mM Tris HCI-1 mM EDTA, pH 8.0 (Kinashi *et al.*, 1987), treated with 20U each of *Xba* I and *Spe* I in the presence of 10 mM Mg^{2+} ions, separately for 18hrs at 37°C.

Pulsed field electrophoresis was performed in 1.5% agarose gel for 18hr at 10 V/cm with 25sec pulses in 45 mM Tris-45 mM boric acid-l mM EDTA, pH 8.0 (0.5 **x** TBE), with *Hind* III-digested lambda DNA and lambda ladder (New Englang Biolabs) as the markers, or in 0.8% agarose gel for 44hr at 11 V/cm with 25sec pulses in 0.5 × TBE, with *Saccharomyces cerevisiae* 334 chromosomal DNAs (Smithkline Beckman Co.) as the markers.

Southern blotting and radioactive labelling of DNA

After ethidium bromide staining and photography, the DNA was UV- and then HCl-depolymerized and blotted onto a nitrocellulose paper by a capillary transfer method. The 7.1 Kbp fragment containing the ATP nucleotide 3'-pyrophosphokinase gene was labelled by a random priming procedure (Feinberg and Vogelstein, 1984) with [α^{32}P]dCTP, mixed deoxyhexanucleotides and DNA polymerase Klenow fragment, and used as a hybridization probe (7 x 10^8 cpm/μg DNA).

RESULTS AND DISCUSSION

We conducted the plasmid search in all the five, the sole enzyme producers ever identified (interestingly they all belong to actinomycetes (Oki et *al., 1976)*) in the microbial kingdom including hundreds of actinomycetes, bacteria, molds, and yeasts. Of these five, only *S. violascens* was found to harbour distinct, plasmid-like DNA component(s) (Fig. 1), and so we continued the study further with this organism. The acriflavin treatment afforded 31 acriflavin-resistant strains, for each of which aerial mycelium, ATP nucleotide 3'-pyrophosphokinase formation (Nishino and Murao, 1974) and plasmid retainment were checked. The results (Table 1) indicate apparent causal independence among the three characteristics but rather their decreasing stability in order of plasmid, aerial mycelium and ATP nucleotide 3'-pyrophosphokinase production against the mutagen, in contrast with the intimate correlation formerly found between the ATP nucleotide 3'-pyrophosphokinase production and aerial mycelium formation in *S. moro-okaensis* (Nishino and Murao, 1981).

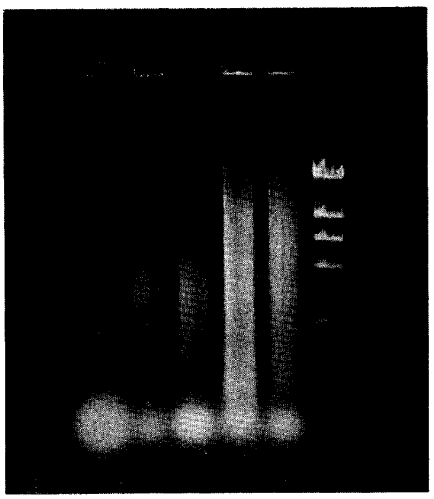

Fig. 1. Plasmid search in the ATP nucleotide 3'-pyrophosphokinase-producing five actinomycetes. Analyzed were *Streptomyces violascens, Streptoverticillium septatum, Streptoverticillium hachijoense, S. aspergilloides,* and S. *adephospholyticus* from left to right. Electrophoresis was performed in 0.7% agarose gel in 40 mM Tris acetate-2.5 mM EDTA, pH 8.0, with Hind III-digested lambda DNA as the markers.

Table 1. Mycelial growth, ATP nucleotide 3′-pyrophosphokinase production and plasmid maintenance of *Streptomyces violascens* wild and acriflavin-mutant strains

Strain No.	aerial mycelium	pyrophosphokinase	plasmid
wild	+	±	+
4	+		+
6		±	+
8	−	±	+
9		+	+
10	+		+
11			+
12	+		+
13	+		+
14	+		+
15	+		+
18	+		+
23	+	−	+
24			+
25	+	±	
29		+	+
31	+		+

Strains No. 1, 2, 3, 5 and so on omitted in the table are those which showed the same characteristics profiles as the wild strain. (+) and (−) in aerial mycelium column mean normal aerial and only substrate mycelium development, respectivly. The estimations of ATP nucleotide 3'-pyrophosphokinase production are expressed by (±) for the wild strain and shown gradationally (±, + or −) for the others. The plasmids detected all showed the same electrophoretic mobility.

Recently we succeeded in gene cloning and expression of *S. moro-okaensis* ATP nucleotide 3′-pyrophosphokinase using *S. Zividans* TK 24/pIJ 699 system (Sumichika and Mukai, 1989). A *Xba* I/*Xba* I restriction fragment of 7.1Kbp size, the gene-containing insert from the above recombinant plasmid pPPK1, was found to hybridize to *Bgl* II-fragmented total DNA at about 13Kbp from acriflavin untreated producer *S. moro-okaensis* but not to the DNA from acriflavin-cured, ATP nucleotide 3′-pyrophosphokinase non-producer(s), indicating the deletional nature of the ATP nucleotide 3'-pyrophosphokinase deficient mutation (Fig. 2). Further, the 7.1Kbp-long fragment was found to hybridize to the total DNA of the producer strain closely at the well, and at about 400Kbp size when digested with either a rare-cutter *Xba* I or *Spe* I, as analyzed by pulsed field gel electrophoresis in 0.8% agarose gel (Kinashi et *al.*, 1987) (Fig. 3). The hybridization data above nearly exclude involvement of the plasmid(s) of usual type and size in the ATP nucleotide 3'-pyrophosphokinase production, and also imply deletion of about 7Kbp or longer stretch of the gene-containing sequence caused by acriflavin treatment.

From these results together,we conclude that the ATP nucleotide 3′-pyrophosphokinase gene is somewhere chromosomally located while the physiological function(s) ·· ·· supposedly related to cellular differentiation or secondary metabolisms* ··· of the enzyme remains to be unveiled.

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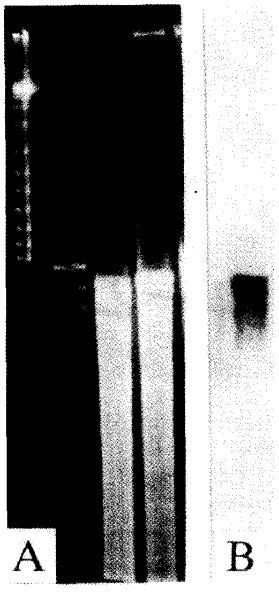

Fig. 2 A and B. Pulsed field electrophoretic (A) and Southern blotting analyses (B) of the *Bgl* II-digested whole cellular DNAs from S. *moro-okaensis* wild and non-producer 206B. From left to right are shown wild and non-producer 206B DNAs. Another non-producing strain (203C) also gave qualitatively the same result as the 206B (not shown). The lambda ladder and *Hind* III digested lambda DNA were used as the markers.

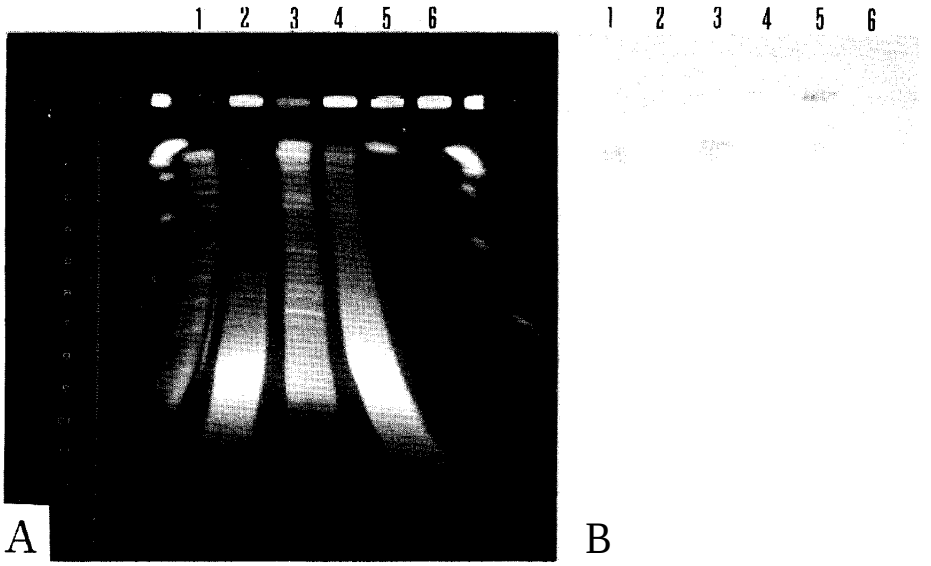

Fig. 3 A a n d Pulsed field electrophoretic (A) and Southern blotting analyses (B) of S. *moro-okaensis* genomic DNAs. From left to right are *Xba*I-digested (lane 1, 2), *Spe*I-digested (3, 4) and undigested (5, 6) wild and non-producer 206B DNA side by side, respectively. Markers were *Saccharomyces cerevisiae* **334** chromosomal DNAs among which 260, 290 and 370Kbp bands were seen whereas larger ones were not well separated under the conditions used.

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