Intracellular ATP Levels Affect Secondary Metabolite Production in Streptomyces spp.

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The addition of extracellular ATP (exATP) to four Streptomyces strains had similar effects: low exATP levels stimulated antibiotic production and high levels reduced it. Compared with antibiotic production, the concentrations of intracellular ATP (inATP) in the tested strains were opposite, which suggests a role of inATP in regulating secondary metabolite production. Under inactivation of the polyphosphate kinase gene (ppk) in Streptomyces lividans, we observed the same results: when the inATP level in the mutant strain was lower than in the parent strain, more antibiotic was produced. Combining all the results, a strong inverse relationship between [inATP] and the secondary metabolite production is suggested by this study.

Key words: intracellular ATP; secondary metabolites; Streptomyces; polyphosphate kinase

Streptomyces are Gram-positive filamentous soil bacteria with a complex life cycle. They produce a wide range of secondary metabolites, including antibiotics, anti-fungals, anti-parasites, and immunosuppressants. The regulation of secondary metabolite production in Streptomyces sp. involves a complex network that responds to environmental and nutritional factors. Many intracellular compounds play an important regulatory role in these networks, including phosphorylated guanosine nucleotide (pppGpp), S-adenosyl-L-methionine (SAM), and cyclic AMP (cAMP).

As the molecular currency of intracellular energy, ATP exists in all living organisms. It provides energy for chemical reactions and participates in phosphorylation of regulatory proteins directly. Over the past 10 years, evidence has suggested that ATP is an important molecule in controlling plant physiology, but the importance of ATP in cell physiology in microorganisms has not been demonstrated clearly. In a previous study, we discovered that the addition of extracellular ATP (exATP) to the culture media affected the morphological differentiation and antibiotic production of Streptomyces coelicolor. Enhanced and reduced production of actinorhodin is related to low and high intracellular ATP (inATP) levels. Recently, a study of Streptomyces cacaoi revealed that ATP and ADP bind to the ATPase domain of PolY and significantly affect its activity in regulating polyoxin production. (3) On the basis of this research, it can be suggested that ATP, like its derivatives SAM or cAMP, also works as a regulatory molecule in Streptomyces.

To test the hypothesis that ATP works as a regulatory molecule in Streptomyces, we investigated several Streptomyces strains, including S. lividans, S. avermitilis, S. antibioticus, and S. griseus. Both antibiotic production and [inATP] levels were measured for each strain. We also provided some genetic evidence to support this hypothesis by inactivating [inATP] related gene — ppk (polyphosphate kinase) in S. lividans.

Materials and Methods

Bacterial strains, plasmids, and culture conditions. Escherichia coli DH5α was used as a host for routine subcloning, and S. lividans TK23 was the parent strain for ppk mutation and overexpression. The pre-cultures were inoculated and grown for 24 h, and then inoculated with a 1% inoculum into the main culture. All Streptomyces sp. were cultivated in liquid media at 28°C with shaking at 220 rpm. The ppk mutant was cultivated in the presence of 50 μg/mL apramycin. Strains containing pWHM3 and its derivatives were cultured with thiostrepton at 50 μg/mL for solid agar media and 5 μg/mL for liquid media. Protoplast transformation was done according to a documented procedure. Dry cell weight (DCW) was measured as cell growth, and was used in the calculation of specific secondary metabolite production. Cells were harvested by centrifugation and were dried at 70°C overnight for DCW. exATP was added at the beginning of the main cultures with various concentrations ranging from 2 μM to 100 μM. Antibiotic production and [inATP] were measured every 24 h. All chemicals were purchased from Sigma (Sigma-Aldrich Korea, Seoul, Korea).

Determination of secondary metabolite production. The production of undecylprodigiosin by S. lividans was measured by the method described by Shim et al. (2006). Undecylprodigiosin was extracted with methanol from mycelium grown in R2YE media and was acidified with HCl. The concentration of undecylprodigiosin was measured at 530 nm absorbance and was converted using a molar extinction coefficient of 41000 M⁻¹ cm⁻¹.

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Abbreviations: exATP, extracellular ATP; inATP, intracellular ATP; SAM, S-adenosyl-L-methionine; cAMP, cyclic AMP
coefficient value of 100,500 [\times 10^3]. To analyze the avermectin produced by S. avermitilis, cells were grown in YEME medium, washed with methanol, and extracted with dichloromethane. The quantities of avermectin were determined by high-pressure liquid chromatography (HPLC) with a C18 column (3.9 mm x 150 mm) from Waters (Milford, MA) using methanol-water (85:15, v/v) as the mobile phase. To analyze the oleandomycin from S. antibioticus, cells were inoculated in YEME medium. The oleandomycin content in the supernatants was estimated by bioassay using oleandomycin-sensitive Micrococcus luteus, as described previously. To measure the streptomycin produced by S. griseus, an agar diffusion method was used, with Bacillus subtilis ATCC 6633 as indicator organisms. Following incubation of the cells in YMPD media, equivalent volumes of culture broth were applied onto 8-mm paper disks and placed on 1% nutrient agar plates containing a layer of B. subtilis. The diameter of the inhibition zone was measured. Relative antibiotic production was calculated as the value of the antibiotic production of the exATP-treated cells divided by that for the untreated ones.

**Measurement of [inATP]**. To measure [inATP] in Streptomyces, 1 mL of cells was harvested by centrifugation for 2 min at 7,000 rpm. After washing with PBS buffer (0.8% NaCl, 0.02% KCl, 0.15% Na2HPO4, and 0.024% KH2PO4 at pH 7.4), the harvested cells were resuspended in 1 mL of 0.2% trichloroacetic acid (TCA). The cellular debris was removed by centrifugation for 10 min at 13,000 rpm. The ATP concentration in the supernatant was measured by luciferase reaction following the manufacturer’s instructions using the ENLITEN® ATP Assay System (Promega, Madison, WI) and a GLOMAX™ 96 microplate luminometer (Promega).

**Construction for ppk mutation and overexpression**. For the ppk mutant, a 787-bp fragment of the ppk open reading frame from +786bp to +1,517bp was amplified by PCR from genomic DNA of S. lividans using two primers: ppkF (CAAGGCACGGTGCTGTCCGGC/GGCGCAACC, introduced BamHI site underlined) and ppkR (CAGGCTTCGACACCCGGGACTCC, introduced HindIII site underlined). The PCR product was digested with BamHI and HindIII and the purified fragment was subcloned into the same restriction enzyme sites of pOJ260 to generate pJWS-PPKM in E. coli. The pJWS-PPKM was introduced into S. lividans TK23 via protoplast transformation, and candidate colonies were screened on R2YE agar plates containing 50 μg/mL of ampicillin. Disruption of the ppk gene was confirmed by PCR. The genomic DNA of the candidate colonies was purified, and PCR was performed using a primer pair, one from pOJ260 located outside the cloning site, and the other from chromosomal DNA located outside the cloned region.

For complementation and overexpression of ppk, the total open reading frame of ppk and its own promoter region was amplified by PCR from genomic DNA of S. lividans TK23 by a primer pair of ppk2385F (CAAGCTTACGGTCTGCACCCGGCC/GGCGCAACC, introduced BamHI site underlined) and ppk2385R (CAATCTCGCAACCCGGGACCAACCCGGCCCCGACTCC, introduced HindIII site underlined). The PCR product was digested with BamHI and HindIII, subcloned into the corresponding sites of pWH3 to generate pJWS-PPPKO in E. coli. Construction of pJWS-PPKKO was confirmed by sequencing of ppk. The pJWS-PPKKO was transformed into the ppk mutant for complementation and into S. lividans TK23 for overexpression. Candidate colonies were screened on R2YE agar plates containing 50 μg/mL of thiostrepton. The constructed strains were confirmed by plasmid purification using a Qiagen Plasmid Mini Kit (Qiagen Korea, Seoul, Korea).

**Quantitative analysis of ppk expression by reverse transcription PCR**. Strains of S. lividans were grown in R2YE, harvested from the first through the 4th day with RNAlater (Ambion, Austin, TX), and frozen at −80°C. RNA was isolated using an RNeasy Mini kit following the manufacturer’s instructions (Qiagen Korea, Seoul, Korea). Residual genomic DNA was removed by DNase digestion (Qiagen Korea, Seoul, Korea). Following the RNA clean-up procedure, RNA concentrations were determined by measuring A260. CDNA was synthesized with a randomized oligonucleotide primer (Bioneer, Daejeon, Korea) using Omniscript following the manufacturer’s instructions (Qiagen). Expression of hrdB, the gene for the major vegetative sigma factor, was chosen as control. The expression levels of ppk and hrdB were measured with two primer pairs, ppkRT-S (CACCACTGCTCT-CCGGTCTCC)/ppkRT-AS (CGGGTGAGCCGCGAAGGTGGT), and hrdBF (GAAGACACCGCAGAAAGG)/hrdBR (GTCGCTCCTGTC-TCCTGC) respectively. Reverse transcript PCR (RT-PCR) was performed in a 25 μL reaction volume containing 1 μL cDNA, 1 μL the gene-specific primers (10 μM), and 12.5-μL the SYBR PCR master mix (Applied Biosystems, Seoul, Korea). The amplified products were detected with the iCyclerIQ Multi-Color RT-PCR detection system (Bio-Rad, Hercules, CA) with the following cycle profile: 1 cycle at 95°C for 3 min, followed by 40 cycles at 95°C for 15 s, 58°C for 15 s, and 72°C for 15 s. The quality of the amplified product was verified by melting with a final step of 40 cycles at 0.5°C increments every 10 s from 58°C to 95°C. Loss of fluorescence was observed at the denaturing/melting temperature of the product. For each condition, quadruple assays were tested. The analysis gave a threshold cycle (Ct) value for each sample, defined as the cycle at which a significant increase in the amplification product occurs. The Ct value was calculated and subtracted from the mean Ct value of the hrdB reference gene. Data were transformed from an exponential to a linear scale using the formula x = 2^(-ΔCt).

**Results**

**Effects of exATP on antibiotic production in Streptomyces**

In order to investigate changes in antibiotic production, ATP was added to the culture media of Streptomyces strains; S. lividans, S. avermitilis, S. antibioticus, and S. griseus. The details of these Streptomyces strains and the methods of determining antibiotic production are given in Table 1. The final concentrations of ATP in the media ranged from 2 μM to 500 μM. Undecylprodigiosin production in S. lividans increased in the presence of 2 μM to 50 μM exATP, and decreased when [exATP] was in excess of 80 μM (Fig. 1A). Avermectin production in S. avermitilis also increased in the presence of 2 μM to 200 μM exATP, and decreased when [exATP] was at 500 μM (Fig. 1B). Oleanodinycin production in S. antibioticus was measured, the level was markedly enhanced by 10 μM to 100 μM exATP, and decreased when [exATP] was 500 μM (Fig. 1C). Streptomycin produced in S. griseus was enhanced slightly by the addition of 2 μM to 50 μM exATP, and decreased when [exATP] was in excess of 100 μM (Fig. 1D). These results indicated that secondary metabolite production in the four tested streptomycetes was enhanced, more or less by a relatively low concentration exATP treatment, whereas relatively high concentration exATP treatment decreased the antibiotic production of Streptomyces.

**Effects of exATP on [inATP] in Streptomyces**

Changes in [inATP] were measured in response to exATP treatment using a luciferase reaction (Fig. 2). Two exATP concentrations representing low and high [exATP] were selected to test changes in [inATP] in the four strains (Fig. 2). Cells treated with a low concentration exATP had lower [inATP] than non-treated cells especially on the second culture day. In contrast, the cells treated with a high concentration exATP had higher [inATP] than the non-treated cells (Fig. 2A to D). These results were consistent in all four tested streptomycetes, indicating that [inATP] was affected by [exATP] in a concentration-specific manner.
Changes in [inATP] due to mutation and overexpression of ppk in S. lividans TK23

To test the correlation between [inATP] and secondary metabolite production genetically, we tested several genes which might be involved in the control of [inATP]. We found a mutation of the polyphosphate kinase gene (ppk) of S. lividans changed [inATP] without any apparent change in growth (data not shown). The amount of mRNA was measured by quantitative polymerase chain reaction showed that the overexpression strain had 2-fold more ppk mRNA than its parent strain (Fig. 3A). The mutant strain had almost no increased ppk mRNA during the culture time but complementation with the ppk restored its level to that of the parent strain (Fig. 3A). Using these constructed strains, the change in [inATP] was measured. The ppk mutant exhibited less [inATP] than the parent strain, and complementation restored [inATP] to the parent level (Fig. 3B). On the other hand, cells overexpressing ppk showed increased [inATP] (Fig. 3B). These observations confirmed that we successfully constructed the strains, which has a changed [inATP] by genetic modifications of ppk.

Morphology changes by mutation and overexpression of ppk in S. lividans TK23

Morphological and antibiotic production with different ppk expression levels were investigated (Fig. 4). The ppk mutant produced more undecylprodigiosin than the parent strain (Fig. 4A). The dark red color of the ppk
mutant on the plates confirmed enhanced production of undecylprodigiosin (Δppk in Fig. 4B). The complemented strain of the ppk mutation was indistinguishable from the parent strain (Fig. 4A and B). ppk overexpression repressed undecylprodigiosin production (Fig. 4C), and the light red color of the cells overexpressing ppk on the plates confirmed this (TK23+ppk in Fig. 4D). The addition of pWHM3 did not change the physiology of the cells (Δppk+V in Fig. 4B and TK23+V in Fig. D). These results were consistent with our hypothesis that the strain with lower [inATP] was related to higher antibiotic production in Streptomyces.

Discussion

[inATP] in E. coli declines rapidly when cells enter the nutritionally limited stationary phase of growth.22) The stationary phase is the phase at which streptomycetes start to produce secondary metabolites, including antibiotics. Rather than energy charge, [inATP] is known to correlate with candicidin production in S. griseus, accompanying changes in inorganic phosphate and guanosine-5′-monophosphate.23) However, to date, only a few studies have focused on [inATP] effects on antibiotic production in Streptomyces, although its derivates, including S-adenosy-methionine and cyclic-AMP, have been studied.6,8) Our previous results for S. coelicolor gave the first insight into the possible regulatory role of [inATP] in antibiotic biosynthesis.12) We investigated four further Streptomyces strains in this study. The results confirmed that the [inATP] change related specifically to secondary metabolite production in all four tested strains. This suggests that relationship between [inATP] and secondary metabolism is common, at least in streptomycetes. Treatment with ‘low concentration exATP’ enhanced antibiotic production, accompanying a reduction [inATP] in all the tested strains. On the contrary, treatment with ‘high concentration exATP’ repressed antibiotic production, accompanying an increase [inATP]. So we hypothesized that antibiotic production was related to low concentration inATP.

To confirm our hypothesis, one antibiotic producing strain with a low [inATP] level is needed. A previous study has revealed that disruption of ppk in S. lividans
there is a transport system, exATP treatment should result in a dose-dependent increase in [inATP], but in this study we observed that low concentration exATP treatment decreased [inATP] slightly, indicating that low concentration exATP serves as a stimulus that alters the innate homeostatic balance of [inATP].

In this study, we found a strong inverse relationship between [inATP] and secondary metabolite production in *Streptomyces*. However, the possibility that changes in [inATP] and antibiotic production are independent processes are eliminated, even though we have tried many different experimental methods.

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**References**


